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Ribosomal Proteins L5 and L11 Cooperatively Inactivate c-Myc via RNA-induced Silencing Complex

Jun-Ming Liao¹, Xiang Zhou¹, Anne Gatignol², and Hua Lu^{1,*}

¹Department of Biochemistry & Molecular Biology and Cancer Center, Tulane University School of Medicine, 1430, Louisiana, LA 70112

²McGill University AIDS Centre, Lady Davis Institute for Medical Research, McGill University, 3755 Côte Ste Catherine, Montréal, Québec H3T 1E2, Canada

Abstract

Oncogene MYC is highly expressed in many human cancers and functions as a global regulator of ribosome biogenesis. Previously, we reported that ribosomal protein (RP) L11 binds to c-Myc and inhibits its transcriptional activity in response to ribosomal stress. Here, we show that another ribosomal protein L5, cooperatively with RPL11, guides the RISC complex to c-Myc mRNA and mediates the degradation of the mRNA, consequently leading to inhibition of c-Myc activity. Knocking down of RPL5 induced c-Myc expression at both mRNA and protein levels, while overexpression of RPL5 suppressed c-Myc expression and activity. Immunoprecipitation revealed that RPL5 binds to 3'UTR of c-Myc mRNA and two subunits of RISC complex, TRBP and Ago2, mediating the targeting of c-Myc mRNA by miRNAs. Interestingly, RPL5 and RPL11 co-resided on c-Myc mRNA and suppressed c-Myc expression cooperatively. These findings uncover a mechanism by which these two RPs can cooperatively suppress c-Myc expression, allowing a tightly controlled ribosome biogenesis in cells.

Keywords

c-Myc; TRBP2; microRNA; RPL11; RPL5; ribosomal stress

Introduction

The c-Myc oncoprotein is a globally regulatory factor of cell growth and proliferation^{1, 2}. Deregulation of c-Myc is highly associated with a wide range of cancers^{3–9}. In cancer cells, constant activation of c-Myc plays a vital role in cancer development by transcriptionally regulating a number of genes that are involved in cell division, metabolic adaptation, cell survival, and ribosome biogenesis^{1, 10}. A number of studies have shown that amplification

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*Corresponding Author: Hua Lu, Department of Biochemistry & Molecular Biology and Cancer Center, Tulane University School of Medicine, 1430, Louisiana, LA 70112, Telephone: (504)-988-0394; Fax: (504)-988-1611; hlu2@tulane.edu.

Conflict of interest:

The authors declare no conflict of interest.

of MYC, the c-Myc-encoding gene, through multiple mechanisms, including chromosomal translocation and mutations^{11–14}, is correlated with poor clinical outcome and tumor aggression^{15, 16}. Proliferation of tumor cells with high levels of c-Myc is no longer dependent on growth-factor stimulation, whereas in normal cells, growth-factor stimulation is required for c-Myc-dependent proliferation, metabolic pathways, cell adhesion, and ribosome biogenesis^{13, 14, 17–20}. Thus, the role of c-Myc in controlling ribosome biogenesis and translation is crucial for the development and progression of tumors¹⁷.

Ribosome biogenesis is essential for fast-growing cancer cells and tightly regulated in normal growing cells. In prokaryotes, free ribosomal proteins resulted from deregulation of ribosome biogenesis can bind to their own mRNAs and inhibit their transcription to avoid unneeded synthesis of ribosomal proteins²¹. This simple mode of autoregulation of ribosomal protein expression, though not yet found in eukaryotes, has evolved to a more complicated mechanism involving c-Myc in eukaryotic cells. Previously, we discovered that ribosomal protein (RP) L11 binds to c-Myc and inhibits its activity²². Later on, RPL11 was shown to bind to c-Myc mRNA and promote its degradation via miRNAs²³. Because c-Myc stimulates the transcription of ribosomal proteins-encoding mRNAs^{17, 22}, RPL11 can negate c-Myc activity via a negative-feedback mechanism and consequently lead to a tightly monitored ribosome biogenesis in mammalian cells. A “byproduct” of this negative regulation of c-Myc by free RPL11 is to prevent cell transformation, as ribosome biogenesis is usually elevated in cancer cells¹⁷. Indeed, free forms of several ribosomal proteins, generated by unexpected ribosome biogenesis or in response to ribosomal stresses, have been identified to play important roles in preventing tumorigenesis. A number of ribosomal proteins, including RPL11^{24–27}, RPL5²⁸, RPL23^{29, 30}, RPL26³¹, RPS3³², RPS7^{33, 34}, RPS14³⁵, RPS25³⁶, RPS27³⁷ or RPS27a³⁸, have been shown to suppress tumor cell growth by activating p53 in response to ribosomal stress. Although RPL11 has been shown to negatively regulate c-Myc activity independently of the MDM2-p53 pathway²², and also, RPL11 and RPL5 have been shown to work together to activate p53³⁹, it remains largely unclear whether these p53-activating ribosomal proteins could also exert their extra-ribosomal function individually or cooperatively toward c-Myc in a sub-ribosomal complex. Thus, this prompted us to determine whether RPL5 also plays a role in regulating c-Myc level or activity, and if true, whether this regulation is executed by cooperating with RPL11.

In this study, we intended to address these two questions. First, we found out that RPL5 binds to c-Myc mRNA and inhibits its expression by mediating the binding of the RISC complex to c-Myc mRNA. Also, we showed that RPL5 co-resides with RPL11 on c-Myc mRNA and inhibits the expression of c-Myc cooperatively with the latter. Hence, our studies as detailed below demonstrate the cooperative action of these two ribosomal proteins on inactivation of c-Myc by guiding the RISC complex to its mRNA and inhibiting its expression.

Results

RPL5 inhibits c-Myc-dependent proliferation by suppressing c-Myc expression

To investigate whether c-Myc expression is affected by RPL5, we first used siRNA to knock down endogenous RPL5 in H1299 cells. As shown in Figure 1A, knockdown of RPL5 led to

the increase of c-Myc mRNA level as determined by RT real-time quantitative PCR (RT-qPCR), while overexpression of RPL5 by transfection of a plasmid that expressed a flag-RPL5 fusion protein only caused a mild reduction of c-Myc mRNA (left, Figure 1A). However, interestingly, after stimulating cells by 20% serum to induce c-Myc expression, overexpression of RPL5 led to the reduction of c-Myc mRNA level dramatically (right, Figure 1A). The inhibitory effect of RPL5 on c-Myc level was also confirmed at its protein level by Western blot (WB) analysis. RPL5 knockdown resulted in the elevation of c-Myc protein level, similar to that of RPL11 knockdown (Figure 1B, upper panel); Consistently, RPL5 overexpression led to the reduction of c-Myc protein level (Figure 1B, lower panel). Because of the importance of c-Myc for cell proliferation^{18, 40, 41}, we next checked if increased RPL5 expression could affect cell proliferation by regulating c-Myc level. As shown in Figure 1C, ectopic RPL5 led to a marked decrease in BrdU (5-bromodeoxyuridine) incorporation in serum-re-stimulated H1299 cells, compared to cells without exogenous RPL5. Interestingly, the inhibitory effect of RPL5 on proliferation could be observed only in serum-re-stimulated cells, suggesting that c-Myc induction might be required for this regulation (Figure 1C). To further determine the effect of RPL5 on c-Myc-enhanced proliferation, we performed a BrdU cell proliferation assay with c-Myc-transfected cells. As expected, c-Myc induced cell proliferation as indicated by the increase in BrdU incorporation. Again, co-expression of Flag-RPL5 in c-Myc-expressing cells resulted in the significant decline of BrdU incorporation in a dose-dependent manner (Figure 1D). Together, these results indicate that RPL5 suppresses c-Myc expression and inhibits its activity, consequently retarding c-Myc promoted cell proliferation.

RPL5 inhibits induction of c-Myc by serum stimulation

c-Myc is rapidly induced in response to serum stimulation. To determine if this serum responsive induction could be affected by RPL5, we compared c-Myc levels in H1299 cells transfected with RPL5, RPL11 or pcDNA vector upon serum stimulation. H1299 cells were serum-starved for 24h and then cultured in medium containing 20% bovine serum. Cells were then harvested at different time points after serum stimulation. As expected²³, the induction of c-Myc expression was drastically reduced in cells that overexpressed RPL11 at each time point after serum stimulation (Figure 2A). Similarly and interestingly, overexpression of RPL5 also markedly decreased the level of endogenous c-Myc, in comparison with control cells (Figure 2A). Consistent with this result, knockdown of either of these two endogenous RPs in H1299 cells led to the induction of c-Myc mRNA levels, compared to control cells (Figure 2B). These results indicate that RPL5 also regulates c-Myc expression after serum stimulation.

Because RPL5 suppresses c-Myc mRNA expression (Figures 1A and 2B) similarly to what RPL11 does²³, and RPL11 was previously shown to target c-Myc mRNA via miRNAs²³, we suspected that RPL5 might employ the same mechanism to inhibit c-Myc expression. First, we investigated whether disruption of the miRNA machinery would affect the regulation of c-Myc by RPL5 by knocking down TRBP (HIV-1 TAR RNA-binding protein), an important subunit of RISC^{42, 43}, in H1299 cells, as TRBP knockdown was previously shown to impair the miRNA processing and silencing steps^{44, 45}. Indeed, disruption of miRNA functions by TRBP knockdown rescued the inhibitory effect of RPL5 on c-Myc induction by serum-

stimulation, at least partially (Figure 2C), suggesting that miRNAs might be involved in the regulation of c-Myc by this ribosomal protein. Because H1299 cells are p53-deficient, this effect is p53-independent.

RPL5 targets c-Myc 3'UTR

We assumed that RPL5 might bind to c-Myc 3'UTR and thus suppress c-Myc expression by utilizing a mechanism similar to that for RPL11²³. To test this idea, we first checked the binding of RPL5 with c-Myc mRNA in HEK 293 cells by ribonucleoprotein immunoprecipitation (RNP IP) assay^{23, 46}. As shown in Figure 3A, c-Myc mRNA, but not gapdh mRNA, was enriched more than six-fold in Flag-L5 IP samples than that in IgG IP samples. Also, this enrichment of c-Myc mRNA depended on RPL5 expression, for c-Myc mRNA was not enriched by the anti-flag antibody in cells without overexpression of flag-RPL5. This association between RPL5 and c-Myc mRNA was further confirmed using an HEK293 cell line that stably expresses flag-RPL5, as c-Myc mRNA was only pulled down by the flag antibody in flag-RPL5-HEK293 stable cells, but not in parental HEK293 cells (Figure 3B). Thus, it is likely that RPL5 can form a complex with c-Myc mRNA in cells.

Next, we tested if RPL5 could directly target c-Myc 3'UTR by conducting a luciferase assay with the pMIR-REPORT vector that contains c-Myc 3'UTR at the 3' end of the luciferase ORF. As shown in Figures 3C–3D, when HEK293 cells were cotransfected with flag-RPL5 and pMIR-REPORT containing the intact c-Myc 3'UTR, the luciferase reporter expression was significantly reduced, similar to the reduction of luciferase activity by RPL11, compared to cells transfected with pMIR-REPORT-c-Myc 3'UTR and pcDNA3. Conversely, knockdown of either RPL5 or RPL11 in HEK293 cells led to a marked induction of the luciferase activity (Figures 3E–F). These results suggest that RPL5, similarly to RPL11, may regulate c-Myc expression by interacting with the 3'UTR region of its mRNA.

RPL5 binds to the TRBP2 and Ago2 subunits of the RISC complex

Our finding that RPL5 associates with c-Myc mRNA and targets its 3'UTR suggests that this RP might, like RPL11²³, regulate c-Myc mRNA level via miRNA as well. To begin to test this possibility, we first determined if RPL5 could bind to the RNA-induced silencing complex (RISC). As one of the RISC subunits, TRBP has been shown to be an essential component of this complex and play a vital role in facilitating the binding of the other two subunits of RISC, Dicer and Ago2^{42, 43}. Therefore, we tested the association of RPL5 with TRBP2 by transfecting HEK293 cells with myc-TRBP2 and flag-RPL5, followed by carrying out immunoprecipitation-WB (IP-WB) analysis. Interestingly, myc-TRBP2 was pulled down by anti-flag antibodies in cells expressing both flag-RPL5 and myc-TRBP2, but not in cells without flag-RPL5, indicating that RPL5 binds to TRBP2 in cells (Figure 4A). We also tested whether other ribosomal proteins might bind to TRBP2 by performing a set of co-IP-WB experiments. As shown in Figures 4B and 4C, myc-TRBP2 was co-immunoprecipitated with flag-RPL11 as well, but not flag-RPL23 and flag-RPL26, suggesting that the interaction with TRBP2 is specific to RPL5 and RPL11, but not other RPs as tested here. Also, this specific interaction was not due to the different expression levels of these proteins, as WB analysis of HEK293 cell extracts confirmed that their steady

state levels were equivalent in all of the transfected cells (Figure 4B and C). The interaction of TRBP2 with RPL5 and RPL11 was also verified using endogenous proteins, as anti-TRBP2, but not non-specific IgG, antibodies could pull down endogenous RPL5 and RPL11 in HEK293 cells as well (Figures 4D and 4E). These results indicate that RPL5 and RPL11 can bind to TRBP2 in cells. As RPL11 was previously reported to bind to another RISC subunit Ago2²³, we also tested if this is true to RPL5. Indeed, RPL5 was co-immunoprecipitated with Ago2 as shown in Figure 4F. Taken together, these results demonstrate that RPL5, similar to RPL11, can bind to the RISC complex in cells.

RPL5 mediates the binding between RISC and c-Myc 3'UTR

Our findings that RPL5 binds to RISC subunits and c-Myc mRNA via its 3'UTR suggest that RPL5 might mediate the association of RISC with c-Myc 3'UTR. To test this idea, we performed an RNP-IP assay by introducing myc-TRBP2 with flag-L5 or pcDNA3 control plasmid into HEK293 cells. Then, ribonucleoprotein complexes were immunoprecipitated from cell lysates using an antibody against myc-TRBP2; Subsequently, RNA was extracted, reverse-transcribed, and subjected to qRT-PCR with primers for c-Myc mRNA. As shown in Figure 5A, the c-Myc transcript level as immunoprecipitated in the complex was significantly increased in the presence of flag-RPL5 compared to the pcDNA3 control, suggesting that RPL5 may promote the binding of TRBP2 to c-Myc mRNA.

Since TRBP2 was previously reported to be essential for miRNA functions^{44, 45}, we examined whether TRBP2 is required for RPL5 regulation of c-Myc expression in cells. Indeed, this was the case as siRNA-mediated silencing of TRBP2 expression in H1299 cells abrogated the inhibitory effect of RPL5 on c-Myc expression (Figure 5B). To check if RPL5 is required for the binding of TRBP2 to c-Myc mRNA, we carried an RNP-IP assay after knocking down endogenous RPL5 by siRNA. As expected, RPL5 knockdown impaired the binding of TRBP2 to c-Myc mRNA (Figure 5C). Consistent with this result, knockdown of RPL5 by siRNA abrogated the inhibitory effect of overexpressed miR-145 on c-Myc expression, as in the absence of RPL5 siRNA, ectopically expressed miR-145 led to the marked decrease of endogenous c-Myc in H1299 cells as determined by WB (Figure 5D). Taken together, these results suggest that RPL5 can act as a mediator for the targeting of c-Myc mRNA by miR-145-guided RISC.

RPL5 and RPL11 inactivate c-Myc cooperatively

Since both RPL5 and RPL11 had been shown to protect each other from degradation⁴⁷ and to stimulate p53 cooperatively³⁹, we would like to test if RPL5 might also cooperate with RPL11 to inhibit c-Myc mRNA expression via miRNAs. To this end, we first carried sequential RNP-IP to examine whether these two RPs could simultaneously interact with c-Myc mRNA. Flag-RPL5, GFP-RPL11, and c-Myc 3'UTR were co-expressed in HEK293 cells and their expression was confirmed by WB (Right, Figure 6A). Sequential RNP-IP analysis was then performed with anti-flag antibodies for the first IP to pull down flag-RPL5 followed by secondary IP with anti-GFP antibodies to pull down GFP-RPL11 after eluting the RPL5-associated complexes with flag peptides. Then the captured GFP-RPL11- and Flag-RPL5-containing complexes were used for RNA extraction, which was used for Q-RT-PCR assays. As shown in Figure 6A, a ~7-fold enrichment in c-Myc 3'UTR, but not gapdh

mRNA, was clearly evident in the complexes, demonstrating that RPL5 and RPL11 do co-interact with c-Myc mRNA.

Next, we tested whether RPL5 and RPL11 act cooperatively to inhibit c-Myc expression via miRNAs. To determine if RPL11 is required for the inhibitory effect of RPL5 on c-Myc expression, RPL11 gene expression in H1299 cells was silenced using siRNA methodology. As expected, RPL11 siRNA transfection decreased the level of endogenous RPL11; interestingly, this decrease of RPL11 level impaired the inhibitory effect of RPL5 on c-Myc expression, as determined by WB (Figure 6B). Similarly, RPL5 knockdown also impaired the declining of c-Myc level by RPL11 (Figure 6C). These findings suggest RPL5 and RPL11 need each other to regulate c-Myc level in cells. To further determine if they could cooperatively help each other, we introduced different amounts of flag-RPL5 or flag-RPL11 expression vectors in H1299 cells and monitored the c-Myc level by WB. Indeed, although c-Myc protein level was not obviously affected by each of these RPs alone at lower concentrations (lane 2 and 4, Figure 6D), co-expression of RPL5 and RPL11 at the same levels significantly reduced the expression of c-Myc (lane 5, Figure 6D), indicating the two RPs could cooperate with each other in inhibiting the expression of c-Myc. Because RPL5 and RPL11 can form a complex via 5S rRNA, we tested whether this association is essential for their cooperative effect on c-Myc expression by employing a mutant RPL11, R75Q, as this mutant was defective in binding to 5S rRNA and RPL5³⁹. As expected, GFP-RPL11 bound to flag-RPL5 in an RNase-dependent fashion (Figure 6E); Also, flag-RPL11 bound to endogenous RPL5, whereas flag-RPL11 R75Q failed to do so (Figure 6F). To determine whether the binding between RPL5 and RPL11 is essential for the regulation of c-Myc expression, we knocked down RPL11 and investigated whether RPL5 or RPL11 R75Q mutant can prevent the c-Myc induction by knocking down endogenous RPL11. As shown in Figure 6G, overexpression of ectopic RPL5 failed to do so (lane 1 to 4); This result, consistent with the above results, again indicates that RPL11 is required for the targeting of c-Myc by RPL5. Interestingly, co-overexpressing ectopic wild-type RPL11, but not its mutant R75Q, with RPL5 reversed the RPL11 depletion-induced c-Myc expression (Figure 6G). These results confirm that the binding between RPL5 and RPL11 is essential for this regulation. In summary, these results indicate that RPL5 and RPL11 cooperatively suppress the expression of c-Myc via RISC.

Discussion

c-Myc overexpression is highly associated with tumorigenesis, which is at least partially due to the global deregulation of ribosomal biogenesis¹⁷. Previously we showed that RPL11 inhibits c-Myc activity through direct binding to c-Myc protein²². Later on, this protein was found to also suppress c-Myc activity by recruiting miRNAs to c-Myc mRNAs²³. These findings indicate the existence of the autoregulation of c-Myc by some of its downstream transcriptional target genes that encode ribosomal proteins. Our study, as presented here identified RPL5 as another regulator of c-Myc. Moreover, RPL5 can cooperate with RPL11 in suppressing the expression of c-Myc through a RISC-mediated miRNA targeting mechanism. First, we showed that RPL5 suppresses the expression of c-Myc and consequently inhibits proliferation induced by this oncoprotein (Figure 1). Interestingly, we found that TRBP, one of the RISC components, is critical for this inhibition by RPL5, as

knocking down TRBP partially rescued the inhibitory effect of RPL5 on c-Myc induction in response to serum-stimulation (Figure 2). Also, RPL5 facilitated the targeting of c-Myc mRNA by miRNAs, such as miR145, by binding to c-Myc mRNA and two RISC subunits, TRBP and Ago2 (Figure 3, 4, and 5). This binding to RISC appears specific to these two RPs, as neither RPL23 nor RPL26 could bind to this complex (Figure 4C). Finally, we uncovered that RPL5 and RPL11 form a complex with c-Myc mRNA, acting together to repress c-Myc expression in a cooperative fashion (Figure 6). These results demonstrate that RPL5 and RPL11 are the two partners that act as critical feedback regulators of c-Myc during ribosomal biogenesis. These two ribosomal proteins have also been previously shown to work together in activating p53 by cooperatively inhibiting the activity of the MDM2 oncoprotein³⁹. Our study that recapitulated their cooperative effect on another oncoprotein c-Myc further validates the notion that RPL5 and RPL11 can indeed work as one complex in cells to control cell proliferation and growth (Figures 1–6), although it remains unclear why eukaryotic cells utilize two, instead of one, RPs to control c-Myc activity.

Interestingly, our finding also presents an evolutionarily conserved molecular mechanism underlying the feedback regulation of ribosomal biogenesis. In prokaryotes, in response to ribosomal stress or shortage of complementary rRNA, ribosomal proteins could bind to their own mRNA and repress ribosomal protein synthesis²¹. This negative feedback loop of ribosomal proteins and their mRNAs plays a vital role in fine-tuning of ribosome biogenesis in prokaryotic cells. Although this simple autoregulatory feedback circuit of ribosomal biogenesis has not yet been reported thus far in eukaryotes, this mechanism is resembled by the feedback regulation of c-Myc level and activity by RPL11 and RPL5, as the genes encoding both of the RPs are the transcriptional targets of c-Myc. Different from the prokaryotic mechanism, these eukaryotic RPs target c-Myc mRNA, instead of their own mRNAs, to execute their inhibitory action of c-Myc through a more complicated and RISC-mediated mechanism. Through this unique and sophisticated mechanism, ribosomal biogenesis is finely tuned so that no excess amounts of ribosomal proteins would be produced under normal growth conditions, as extra amounts of these proteins would also be toxic to cells by activating the p53 pathway^{48–51}.

A number of ribosomal proteins have been shown to activate p53 by inhibiting MDM2-induced polyubiquitination of p53 in response to ribosomal stresses^{24–31, 33–35, 37, 38}. However, how free ribosomal proteins act in p53-null cells is still largely unknown. Here, we show that RPL5 can suppress c-Myc expression by targeting its mRNA via RISC and miRNA independently of the p53 pathway (Figures 1–5). Together with RPL11, RPL5 among all of the MDM2-interacting RPs^{22, 23} might play a more important role in preventing neoplasia by acting on both of the p53 and c-Myc pathways important in tumor development and progression. Indeed, both of RPL11 and RPL5 have been shown to negate tumor growth in genetic studies^{48–51}. The negative action of RPL5 and RPL11 on c-Myc and MDM2 could be utilized as a strategy for the development of anti-cancer therapy in the future.

Materials and Methods

Cell lines, plasmids, and antibodies

Human HEK293 and H1299 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% CO₂ humidified atmosphere. RPL5, RPL11, RPL23, RPL26, TRBP and c-Myc expression plasmids were described previously^{28, 29, 43, 52}. RPL11 mutant R75Q plasmids was a gift from Dr. Vousden³⁹. c-Myc 3'UTR was amplified from HCT116 cells and inserted into pMIR Reporter vector. The primers used were: P1: 5'-GGACTAGTTCTCAGAGGCTTGGCGGGAAAAAGA-3' and P2: 5'-CCCAAGCTTGGCTCAATGATATATTTGCCAGTTA-3'. Anti-L5²⁸ and anti-L11²² have been previously described. Anti-Flag (Sigma), anti-c-Myc (N262, Santa Cruz Biotechnology), anti-actin (Sigma), anti-BrdU (IIB5, Santa Cruz Biotechnology), anti-TRBP2 (1D9, LifeSpan BioSciences, Inc), anti-HA (F-7, Santa Cruz Biotechnology), anti-myc (9E10, Santa Cruz Biotechnology) were commercially purchased.

Transient transfection, Western blot and co-immunoprecipitation analyses

As described previously⁵³, briefly, cells were transfected with indicated plasmids as shown in each figure by using TransFectin (Bio-Rad), following the company's instruction. Unless specifically mentioned, forty eight hrs post transfection, cells were harvested and lysed in lysis buffer. The total protein concentration for each sample was determined and equal amount of total proteins were then subjected to SDS-PAGE, followed by Western blot (WB). Immunoprecipitation (IP) was conducted by using antibodies as indicated in the figure legends and described previously⁵⁴. Beads were washed three times with lysis buffer. Bound proteins were detected by IB with antibodies as indicated in the figure legends.

Reverse transcriptase-polymerase chain reaction and quantitative real-time PCR analysis

RT and Q-PCR for mRNAs were done by using the methods described previously^{28, 55}. Briefly, quantitative real-time PCR was performed on an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR Green Mix (Applied Biosystems). Relative gene expression was calculated using the C_T method, following the manufacturer's instruction. All reactions were carried out in triplicate.

Luciferase reporter assays

Cells were transfected with pMIR-c-Myc-3'UTR and indicated plasmids (total plasmid DNA 1 µg/well) as indicated in figures. Luciferase activity was determined and normalized by a factor of β-gal activity in the same assay as described previously⁵⁶.

Ribonucleoprotein immunoprecipitation (RNP IP) assay

The RNP-IP assay was performed as described previously²³ using anti-flag, anti-myc, or anti-GFP for ectopic proteins. Immunoprecipitated RNAs were extracted and analyzed by real-time PCR amplification using primers for c-Myc.

BrdU incorporation assays

BrdU incorporation assays were carried out by basically following the previously described protocol⁵⁵. Cells were incubated in the presence of 10 μ M of BrdU for 5 h. Cells were then fixed with 95% of ethanol and 5% of acetic acid, treated with 2 M HCl containing 1% Triton X-100, and stained with the monoclonal anti-BrdU antibody, followed by staining with Alexa Fluor 546 (red) goat anti-mouse antibodies and DAPI. Stained cells were analyzed under a Zeiss Axiovert 25 fluorescent microscope.

Knockdown of the endogenous mRNAs

siRNAs for RPL5 and RPL11 were described previously^{22, 28}. siRNA for TRBP was purchased from Santa Cruz Biotechnology. Transfection of siRNAs were performed the same as that of normal siRNA as described previously⁵⁷ by using siLentFect™ Lipid (Bio-Rad), following the manufacturer's protocol.

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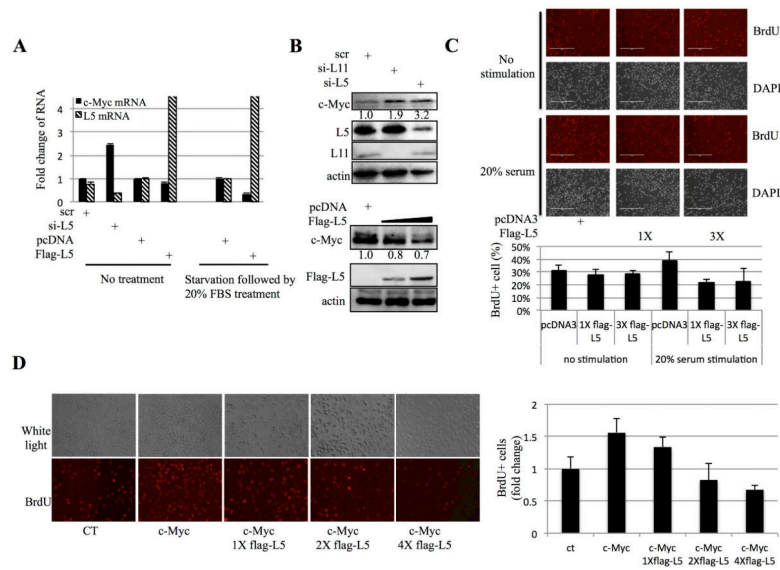


Figure 1. RPL5 suppresses c-Myc expression and inhibits cell proliferation

(A) Knocking down RPL5 increases, but overexpression of RPL5 decreases, c-Myc mRNA level in H1299 cells. Left panel: cells were transfected with indicated plasmids or siRNAs and the expression of c-Myc mRNA was measured by q-RT-PCR. Right panel: cells were starved in 0.2% serum for 36 hours and stimulated with 20% serum for 1.5 h before harvesting. Data are presented as mean \pm standard error, $n = 3$. (B) Knocking down RPL5 increases, but overexpression of RPL5 decreases, c-Myc protein level in H1299 cells. Upper panel: cells were transfected with indicated siRNAs and harvested for IB with antibodies as indicated. Lower panel: after transfected with indicated plasmids, cells were starved in 0.2% serum for 36 hours and stimulated with 20% serum for 1.5 h before harvesting for WB. (C) RPL5 inhibits cell proliferation. H1299 cells transfected with an increasing amount of flag-RPL5 plasmid were treated with starvation followed by stimulation as described in panel A. Cell then were labeled with BrdU and subjected to anti-BrdU staining (red) and counter-stained with DAPI. Percentage of BrdU-positive cells is shown in right panel. (D) Serum stimulation of c-Myc is dispensable for the inhibitory effect of RPL5 on proliferation. The assay as that in panel C was conducted except the indicated cells were not treated with serum-stimulation.

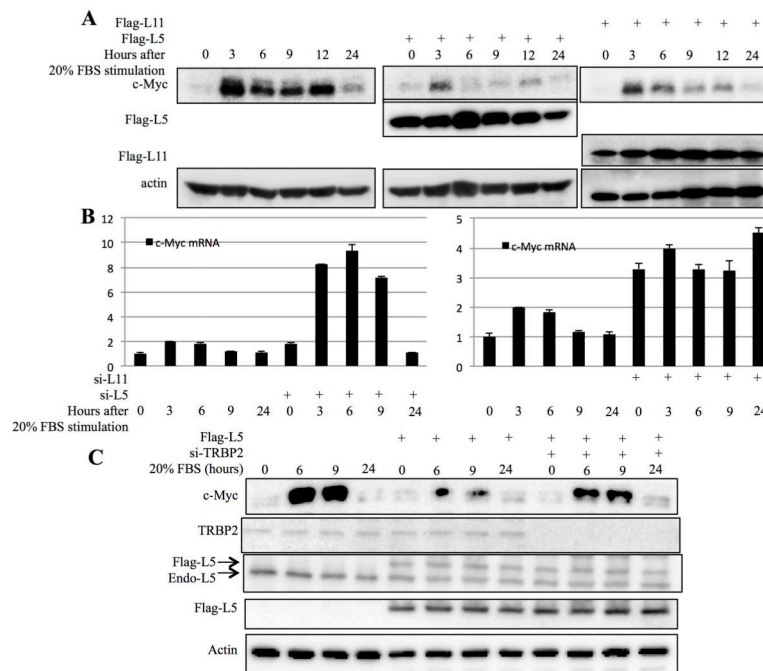


Figure 2. RPL5 and RPL11 suppress c-Myc expression in response to serum stimulation (A) and (B) RPL5 and RPL11 suppress c-Myc expression in response to serum stimulation. H1299 cells transfected with indicated plasmids or siRNAs were starved in 0.2% serum for 36 hours and stimulated with 20% serum for indicated time. c-Myc protein and mRNA were measured by WB (A) and q-RT-PCR (B) respectively. (C) Knocking down TRBP2 impairs the inhibitory effect of RPL5 on c-Myc expression. H1299 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested at indicated time points. Cell lysates were subjected to WB with indicated antibodies.

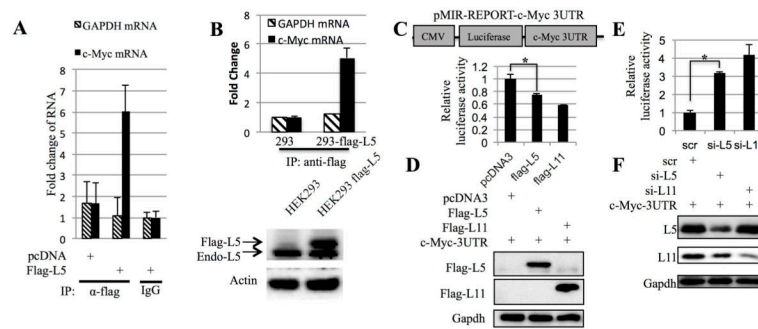


Figure 3. RPL5 binds to c-Myc mRNA and targets its 3UTR

(A) RPL5 binds to endogenous c-Myc mRNA. HEK 293 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for immunoprecipitation assay with anti-flag or mouse IgG. c-Myc and GAPDH mRNAs pulled down by indicated antibody were determined by q-RT-PCR. (B) RPL5 binds to endogenous c-Myc mRNA. Flag-RPL5-293 stable cells or HEK293 cells were treated with serum starvation and stimulation, and harvested for immunoprecipitation (IP) assays with anti-flag. c-Myc and GAPDH mRNAs pulled down by indicated antibodies were determined by q-RT-PCR. (C) and (E) RPL5 and RPL11 target c-Myc 3UTR. H1299 cells were cotransfected with pMIR-c-Myc 3UTR and indicated plasmids. Forty-eight hours after transfection, luciferase activities were measured. Luciferase activity was normalized to beta-gal expression. RPL5 and RPL11 protein expressions were measured by WB as shown in panel (D) and (F). Schematic representation of the pMIR-REPORT-c-Myc 3UTR expression vector is shown in the top part of panel C.

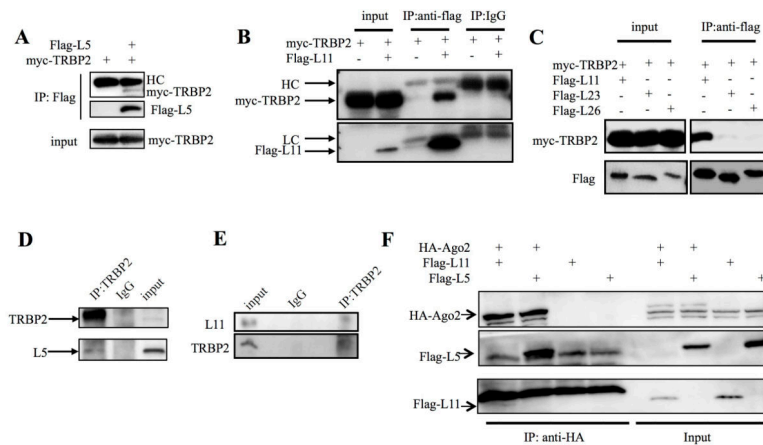


Figure 4. RPL5 and RPL11 interact with TRBP2 in cells

(A) RPL11 binds to TRBP2. HEK 293 Cells were transfected with myc-TRBP2 and Flag-RPL11 as indicated in the figure and harvested for IP (0.5 mg total proteins) using anti-Flag or mouse IgG, followed by IB with the antibodies as indicated. (B) RPL5 binds to TRBP2. HEK 293 Cells were transfected with myc-TRBP2 and Flag-RPL5 as indicated in the figure and harvested for IP (0.5 mg total proteins) using anti-Flag, followed by IB with the antibodies as indicated. (C) RPL23 and RPL26 cannot bind to TRBP2. HEK 293 Cells were transfected with indicated plasmids and harvested for IP (0.5 mg total proteins) using anti-Flag, followed by IB with the antibodies as indicated. (D) Endogenous RPL11 and RPL5 interact with endogenous TRBP2 in HEK 293 cells. Cells lysates (0.6 mg) were prepared from HEK 293 cells after treatment with Act D for 8 h. IP was conducted with anti-TRBP2 or rabbit IgG followed by IB with anti-TRBP2 or anti-ribosomal proteins. All the experiments were repeated more than 2 times and true to the rest of data.

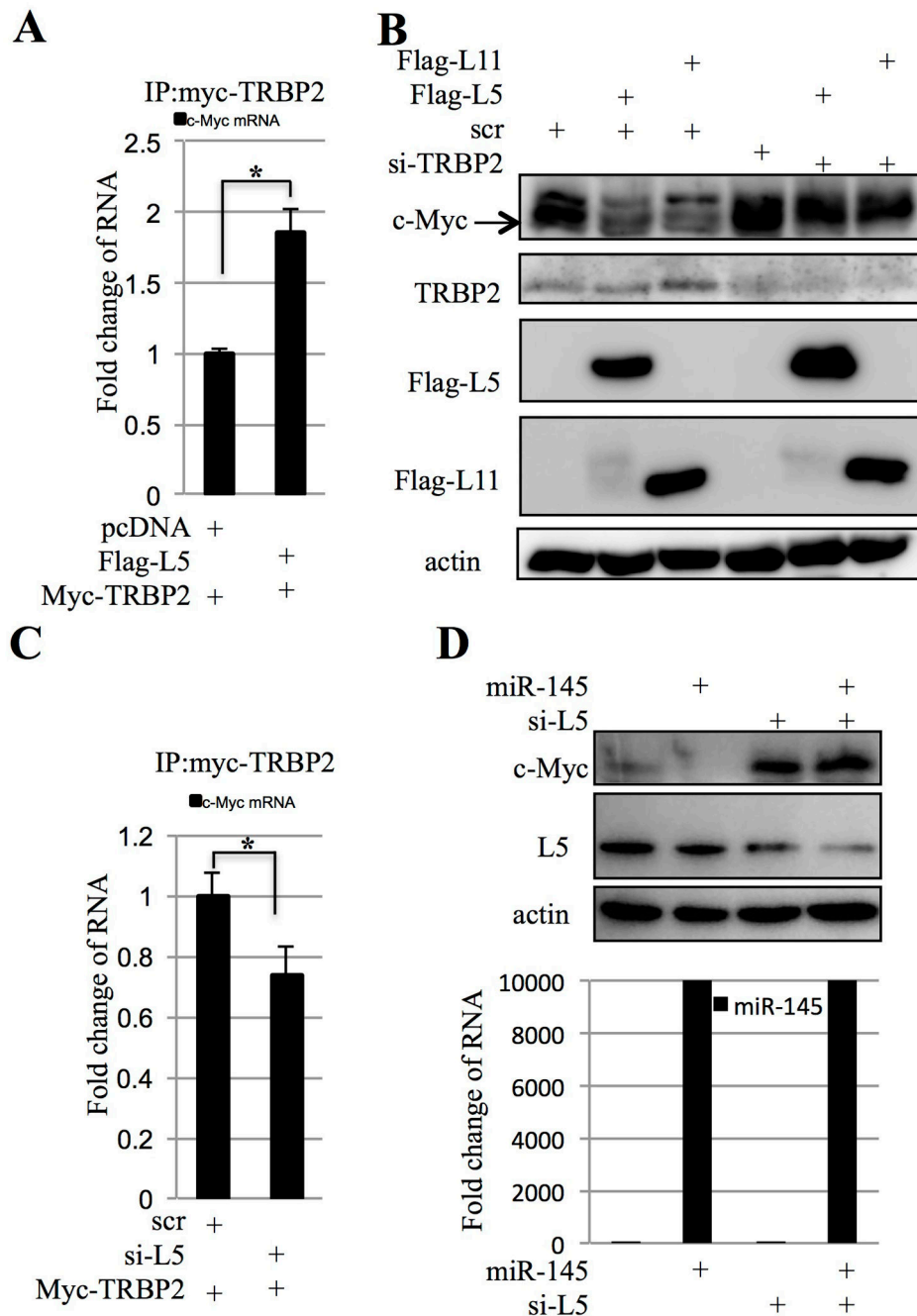


Figure 5. RPL5 mediates the binding of RISC to c-Myc mRNA

(A) Overexpression of RPL5 promotes TRBP2 and c-Myc mRNA binding. HEK 293 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for immunoprecipitation assay with anti-myc. c-Myc mRNAs pulled down by myc-TRBP2 were determined by q-RT-PCR. (B) Knocking down TRBP2 abrogates the inhibitory effect of RPL5 on c-Myc expression. H1299 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for WB with indicated antibodies. (C) Knocking down RPL5 suppresses TRBP2 and c-Myc mRNA

binding. HEK 293 cells transfected with indicated plasmids were harvested for immunoprecipitation assay with anti-myc. c-Myc mRNAs pulled down by myc-TRBP2 were determined by q-RT-PCR. **(D)** RPL5 is required for the targeting of c-Myc by miR-145. H1299 cells transfected with indicated plasmids were harvested for WB with indicated antibodies. The expression of miR-145 was determined by q-RT-PCR as shown in the lower part of this panel.

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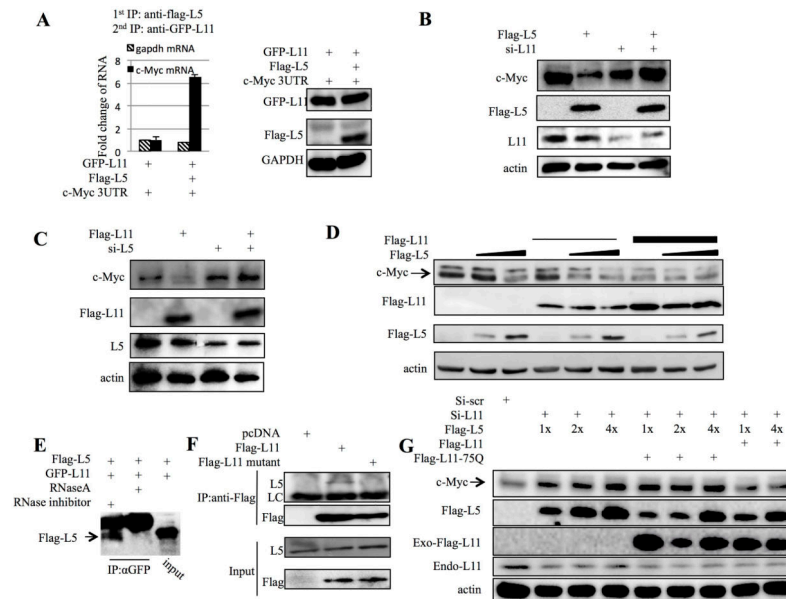


Figure 6. RPL5 and RPL11 cooperatively inactivate c-Myc

(A) RPL5 and RPL11 co-bind to c-Myc mRNA. HEK 293 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for immunoprecipitation assay with anti-flag antibody. The IPed complex were then washed off by flag peptide and subjected to a second immunoprecipitation assay with anti-GFP antibody. c-Myc and GAPDH mRNAs were determined by q-RT-PCR. The expressions of RPL11 and RPL5 were shown in the right part of this panel. (B) Knocking down RPL11 abrogates the inhibitory effect of RPL5 on c-Myc expression. H1299 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for WB with indicated antibodies. (C) Knocking down RPL5 abrogates the inhibitory effect of RPL11 on c-Myc expression. H1299 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for WB with indicated antibodies. (D) RPL5 and RPL11 inactivate c-Myc cooperatively. H1299 cells transfected with indicated plasmids were treated with serum starvation and stimulation and harvested for WB with indicated antibodies. (E) RPL5 binds to RPL11 in an RNase dependent fashion. HEK 293 cells were transfected with flag-RPL5 and GFP-RPL11 plasmids. Cells were harvested for IP-WB with the anti-GFP antibodies as indicated. RNaseA or RNase inhibitor was added into indicated samples. (F) RPL11 R75Q mutant fails to pull down endogenous RPL5. HEK 293 cells were transfected with pcDNA3, flag-RPL11, or flag-RPL11 R75Q. Cells were harvested for immunoprecipitation with anti-flag antibody. LC: lighter chain of IgG. (G) RPL11 R75Q fails to reduce c-Myc level cooperatively with RPL5. H1299 cells transfected with indicated plasmids were harvested for WB with indicated antibodies.