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CDK11p46 and RPS8 associate with each other and suppress translation in a synergistic manner

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ABSTRACT

CDK11p46, a 46 kDa isoform of the PITSLRE kinase family, is a key mediator of cell apoptosis, while the precise mechanism remains to be elucidated. By using His pull-down and mass spectrometry analysis, we identified the ribosomal protein S8 (RPS8), a member of the small subunit ribosome, as an interacting partner of CDK11p46. Further analysis confirmed the association of CDK11p46 and RPS8 *in vitro* and *in vivo*, and revealed that RPS8 was not a substrate of CDK11p46. Moreover, RPS8 and CDK11p46 synergize to inhibit the translation process both in cap- and internal ribosomal entry site (IRES)-dependent way, and sensitize cells to Fas ligand-induced apoptosis. Taken together, our results provide evidence for the novel role of CDK11p46 in the regulation of translation and cell apoptosis.

1. Introduction

Cyclin-dependent kinase 11 (CDK11) isoforms are members of the cyclin-dependent kinase superfamily and closely related to cell cycle regulation, oncogenesis, and apoptosis [1,2]. To date, at least 10 CDK11 isoforms have been cloned from eukaryotic cells, with their molecular masses varying from 46 to 110 kDa [3]. The full-length isoform CDK11p110 is present at a constant level during the cell cycle and involved in pre-mRNA splicing and regulation of transcription [4–6]. During the G2/M phase of the cell cycle, CDK11p58 is translated from the same mRNA as CDK11p110 through targeting IRES element contained in open reading frame (ORF) [7]. Overexpression of CDK11p58 in eukaryotic cells can result in prolonged late telophase [8]. We previously have identified that CDK11p58 interacts with cyclin D3 to regulate G2/M phase cell cycle progression [9], and also represses androgen receptor (AR)-mediated transactivation [10]. During apoptosis, CDK11p110 and CDK11p58 are cleaved by caspases to generate a smaller 46–50 kDa protein which contains the catalytic kinase domain in the C-terminus of CDK11 [3]. CDK11p46 isoform can be induced by Fas ligand (FasL), staurosporine and tumor necrosis factor A [3,11] and its function is linked with apoptotic regulation [12,13]. Our previous studies have shown that CDK11p46 interacts with p21-activated kinase (PAK1) [12] and inhibits its activity which leads to BAD translocation [14]. Other studies also have showed that CDK11p46 may interact and phosphorylate the Mov34 domain of eukaryotic initiation factor 3f (eIF3f) [13,15]. These results suggest that CDK11p46, the final caspase-processed isoform, may be a downstream effector protein in apoptotic signaling. However, the mechanism of apoptotic regulation about CDK11p46 has not been fully elucidated.

In this study, we identified RPS8,which was found in a mRNP granule complex containing untranslated mRNAs [16], as a novel interacting partner of CDK11p46 by His pull-down and mass spectrometry. The interaction between CDK11p46 and RPS8 occurred *in vitro* and *in vivo*. In addition, overexpression of CDK11p46 and RPS8 inhibited the translation process both in cap- and IRES-dependent way, and exhibited a positive effect on apoptotic induction. Taken together, these results suggest a novel role of CDK11p46 in translational regulation during apoptosis.

2. Materials and methods

2.1. Antibodies and reagents

Mouse anti-myc antibody was purchased from Invitrogen. Mouse anti-HA and anti-GAPDH antibodies were from Santa Cruz Biotechnology. Anti-Fas antibody was obtained from Millipore. Cycloheximide (CHX) and Hoechst 33258 were purchased from Sigma. Dual-luciferase reporter assay system was purchased from Promega.

2.2. Cell culture, transfection and induction of apoptosis

HEK293T cells and HeLa cells were obtained from the Institute of Cell Biology Academic Sinica and cultured in Dulbecco's modi-

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fied Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified CO_2 incubator (5% CO_2, 95% air). H1299 cells were cultured in RPMI 1640 (Sigma) medium containing 10% fetal bovine serum, 100 μg/ml penicillin and 50 μg/ml streptomycin. Transient transfection of cells was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. To induce apoptosis, HEK293T cells and H1299 cells were treated with anti-Fas monoclonal antibody at a final concentration of 50 ng/ml for 18 h. Analysis of nuclear morphology by fluorescence staining was performed as previously described [17].

2.3. Plasmids constructions

CDK11p46 coding sequence corresponding to nucleotides 1282–2465 of PITSLRE p110 (GenBank ID: U04824) was amplified by PCR from HA-CDK11p58 and inserted into pcDNA3.0-HA vector. RPS8 coding sequence was amplified by PCR from human thymus cDNA library and inserted into pEGFP-N1, pcDNA3.1-myc and pGEX-4T-1 vectors. pcDNA-Fluc-ECMV/XIAP IRES-Rluc constructs were generated by cloning the PCR product of Firely and Renilla luciferase into pcDNA3.0 vectors, with IRES sequence of ECMV or XIAP between these luciferase genes. Sequencing was performed for all constructs to verify that the constructs were correctly generated and that the reading frames were maintained.

2.4. Recombinant protein purification

GST fusion proteins were expressed in *Escherichia coli* and purified using immobilized glutathione-Sepharose 4B beads (GE Healthcare Life Sciences) according to the manufacturer's protocols. His fusion proteins were purified using Ni Sepharose high performance (GE Healthcare Life Sciences). Purified proteins were stored at −70 °C. The purity of proteins was confirmed by SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining.

2.5. His pull-down assay and mass spectrometry

Purified His-CDK11p46 protein was mixed with extract of HEK293T cells in binding buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 50 mM Imidazole, 10% Glycerol) at 4 °C overnight. Pre-equilibrated Ni Sepharose high performance beads were added and rotated for 30 min at room temperature. Beads were gently washed three times with the lysis buffer and boiled for 5 min, electrophoresed on a 10% SDS–polyacrylamide gel, and analyzed by Coomassie blue stain. The protein band of interest was trypsin-digested as described previously [18] and protein identification was performed by mass spectrometry.

2.6. Co-immunoprecipitation

HEK293T cells were transfected with indicated plasmids. Approximately 48 h after transfection, cells were washed with ice-cold phosphate-buffered saline (PBS) and solubilized with co-immunoprecipitation (co-IP) buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Cell lysates were rotated with 2 μg relevant antibody at 4 °C for 2 h. Pre-equilibrated protein G-agarose beads were added and collected by centrifugation after incubation overnight and then gently washed three times with the lysis buffer. The bound proteins were eluted and analyzed using western blot.

2.7. GST pull-down assay

GST pull-down assay was performed as described [19]. Briefly, purified GST or GST-RPS8 protein was incubated with equal amount of His-CDK11p46 protein at 4 °C overnight, and subsequently glutathione-Sepharose 4B beads were added to the mixture. After rotation for 30 min at room temperature, beads were washed and boiled for western blot analysis.

2.8. Confocal microscopy

HeLa cells grown to 60% were transiently co-transfected with GFP-RPS8 and HA-CDK11p46 vectors. 24 h after transfection, cells were washed with PBS, fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100 and blocked in 1% bovine serum albumin (BSA) for 1 h at room temperature. Cells were stained with anti-HA antibody for 2 h at room temperature followed by incubation with rhodamine-conjugated goat anti-mouse secondary antibody for 1 h at room temperature. Cells were washed three times with PBS, inverted, mounted on slides, and examined in Leica TCS SP5 confocal microscope.

2.9. In vitro kinase assay

The CDK11 kinase assay was performed as described previously [20]. In brief, wild type CDK11p46 or CDK11p46D149N mutant (a kinase-dead mutant) [3] was transfected into HEK293T cells. Exactly 48 h post-transfection, cells were solubilized in lysis buffer, and totally 200 μg of cell extracts were precipitated using HA antibody. Protein kinase activity of the immunoprecipitates was assayed in kinase buffer (50 mM Tris–HCl, pH 7.5, 15 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM ATP, 1 μCi of [γ-^{32}P]ATP) containing1 μg of purified GST-RPS8, GST or His-PAK1 as substrates. After incubation at 30 °C for 30 min, the samples were boiled with 5xSDS loading buffer and separated by 10% SDS–PAGE. The gel was then fixed, dried and analyzed by FLA-5100 phosphoimaging system (Fujifilm).

2.10. Dual-luciferase reporter gene assay

HEK293T cells were co-transfected with indicated plasmid. After 48 h of transfection, dual-luciferase reporter gene assay (Promega) was performed according to the manufacturer's instructions using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

2.11. Reverse transcription (RT)-PCR

Total RNA was isolated from HEK293T cell with Trizol Reagent (Invitrogen) according to manufacturer's instructions. RNA was reverse-transcribed by using RNA PCR Kit AMV (Takara). Amplification was carried out by initial denaturation at 94 °C for 2 min, followed by 26 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a further extension of 72 °C for 10 min. After amplification, the PCR product was analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide (EB) staining on an image system (Pharmacia Biotech). The intensity of the DNA bands was analyzed with Totallab 2.01. The following primers were used: β-actin, 5′-AGCACAGAGCCTCGCCTTT-3′ and 5′-GGAATCCTTCTGACCCATGC-3′; PRL, 5′-GGCCATGATTGGGGTGCTTGTT-3′ and 5′-GGCCATTCATCCCATGATTCAATC-3′; RPS8 5′-AATGGGCATCTCTCGGGACAAC-3′ and 5′-TTTATTTGCCTTTGCGGGCCTTG-3′.

3. Results

3.1. The association between CDK11p46 and RPS8 in vitro and in vivo

To better understand the character of CDK11p46, His pull-down assay was performed to explore the interacting protein of CDK11p46. Coomassie Blue staining of the gel revealed the appearance of a specific band about 32 kDa (Fig. 1A). RPS8 was identified by mass spectrometry analysis as a potentialCDK11p46 binding protein with one unique peptide of 13 consecutive amino acids corresponding to aa158–170 of RPS8 (Fig. 1B).

To further verify the interaction between CDK11p46 and RPS8, recombinant His-CDK11p46 and GST-RPS8 proteins were purified from sf9 cells and *E. coli* respectively and GST pull-down assay was conducted *in vitro*. Results revealed that CDK11p46 specifically bound to GST-RPS8, but not GST (Fig. 1C). Co-IPassay also confirmed the interaction between CDK11p46 and RPS8 *in vivo* (Fig. 1D). Furthermore, immunofluorescence analysis showed nucleoplasmic and cytoplasmic distribution of RPS8, which co-localized with CDK11p46 mainly in the nucleoplasm (Fig. 1E).

3.2. RPS8 is not a substrate of CDK11p46

Since several potential phosphorylation sites of RPS8 have been identified through phosphoproteome analysis [21,22], we next performed the kinase assay to test whether CDK11p46 could phosphorylate RPS8. *In vitro* kinase assay was performed using purified RPS8 or PAK1 (positive control) as substrates. Results demonstrated that CDK11p46 kinase phosphorylated PAK1, but not RPS8 (Fig. 2A). Moreover, overexpression of CDK11p46 showed no effect on phosphorylation of RPS8 (Fig. 2B). Taken together, our results suggest RPS8 is not a substrate of CDK11p46.

3.3. CDK11p46 synergizes with RPS8 to inhibit both cap- and IRES-mediated translation

RPS8 is a component of ribosome, and identified in a mRNP granule complex containing untranslated mRNAs [16]. We next tested the role of RPS8 in translational regulation by luciferase reporter assay. Results showed that either overexpression or interference of RPS8 decreased the luciferase activity, suggesting RPS8 is a rate-limiting factor in translational regulation (Fig. S1).

Previous studies showed that CDK11p46 suppressed translation *in vitro* and *in vivo* by luciferase reporter analysis [13,15]. Therefore, we took advantage of a dual luciferase assay system using the pcDNA-Fluc-ECMV IRES-Rluc reporter, which expresses Firefly luciferase in cap-dependent way and Renilla luciferase in ECMV IRES-dependent manner, to investigate the role of CDK11p46 and RPS8in cap-dependent and IRES-mediated translation (Fig. 3A). Results demonstrated that transfection of CDK11p46 and RPS8 synergistically repressed both Firefly and Renilla luciferase activities, compared with CDK11p46 and RPS8 alone (Fig. 3B). We also tested the synergistic effect of CDK11p46 and RPS8 on the activity of XIAP IRES, and similar results were obtained (Fig. 3C). Transfection of CDK11p46 and/or RPS8 showed no effect on the mRNA level of

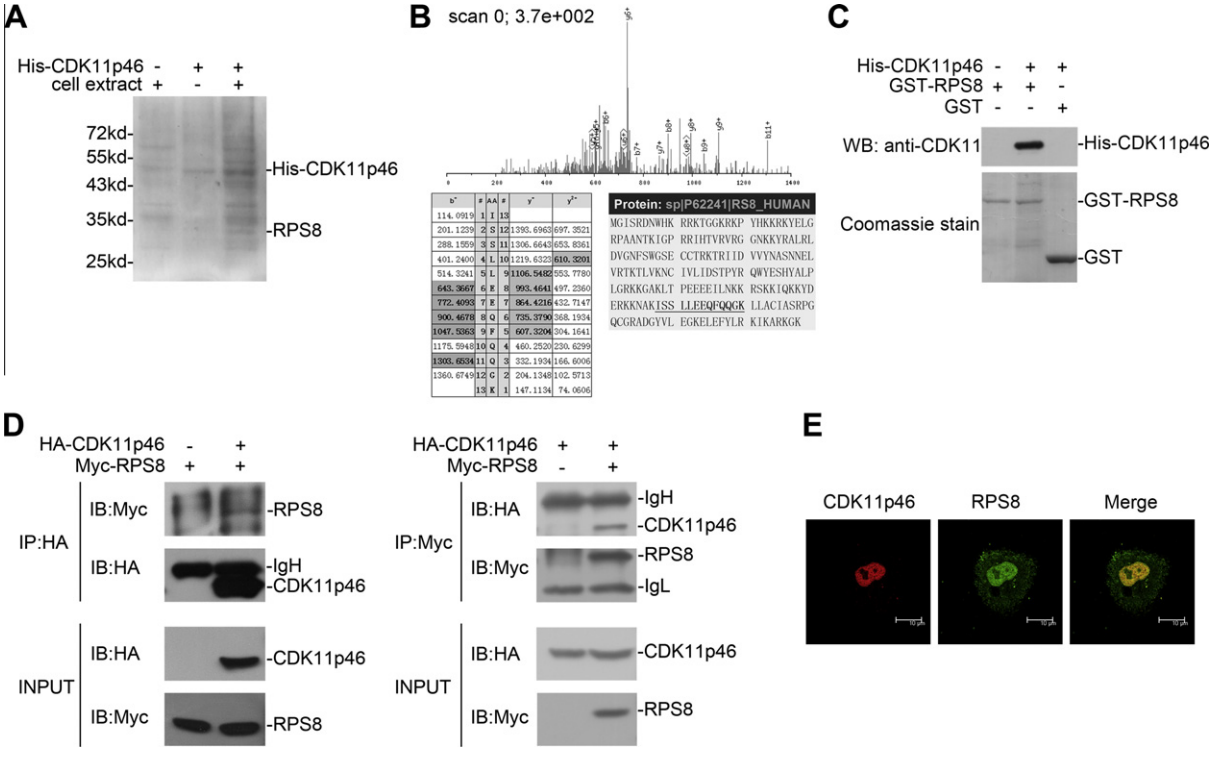

Fig. 1. Identification of RPS8 as a CDK11p46 interacting protein. (A) His Pull-down assay was performed as described in Section 2. Coomassie Blue staining of the SDS–PAGE gel revealed the appearance of a ~32 kDa band in the His-CDK11p46 and HEK293T cell extract coexistent condition (lane 3) as found to bind to CDK11p46 (lane 3). (B) The 32 kDa band was subjected to mass spectrometric analysis and identified as a unique peptide of RPS8. (C) *In vitro* interaction between His-CDK11p46 andGST-RPS8. Purified GST or GST-RPS8 protein was incubated with His-CDK11p46 protein. GST pull-down assay was performed to detect the interaction between CDK11p46 and RPS8. Low panel shows the Coomassie stain of purified proteins. (D) Interaction between CDK11p46and RPS8 *in vivo*. HEK293T cells were transfected with plasmid expressing HA-CDK11p46 and/or Myc-RPS8 as indicated. 48 h after transfection, cell lysates were subjected to co-IP with anti-HA or anti-Myc antibodies, followed by western bolt with related antibodies. IgG light chains (IgL) and heavy chains (IgH) cross-reacting with antibodies are also indicated. 10% of whole cell lysates was probed for the expressions of CDK11p46 and RPS8. (E) Confocal microscopy revealed co-localization of CDK11p46 and RPS8 in Hela cells. Cells were transfected with HA-CDK11p46 and GFP-RPS8, and fixed 48 h later, confocal immunofluorescence microscopy assay was performed. The subcellular localization of CDK11p46 (red), RPS8 (green) and their co-localization (yellow) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

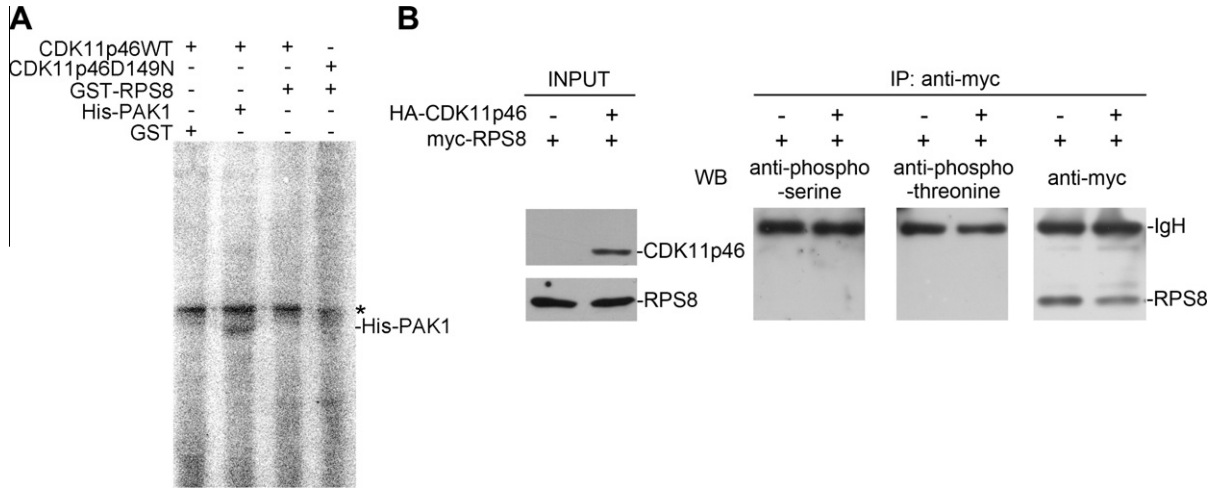

Fig. 2. RPS8 is not a substrate of CDK11p46. (A) HEK293T cells were transfected with HA-CDK11p46 or HA-CDK11p46D149N, then the cells were harvested and lysed. CDK11p46 or CDK11p46D149N was immunoprecipitated using anti-HA antibody. The precipitated proteins were then incubated with 1 μg of the substrates GST, GST-RPS8, or His-PAK1 proteins and [γ-^{32}P] ATP in kinase buffer followed by autoradiography. Asterisk denotes nonspecific bands. (B) HEK293T cells were transfected with pcDNA3/myc-RPS8 and HA-CDK11p46/myc-RPS8. 48 h after transfection, cells were immunoprecipitated with 2 μg of anti-HA monoclonal antibody. The proteins were subjected to western blot and probed with anti-phospho serine/threonine antibody.

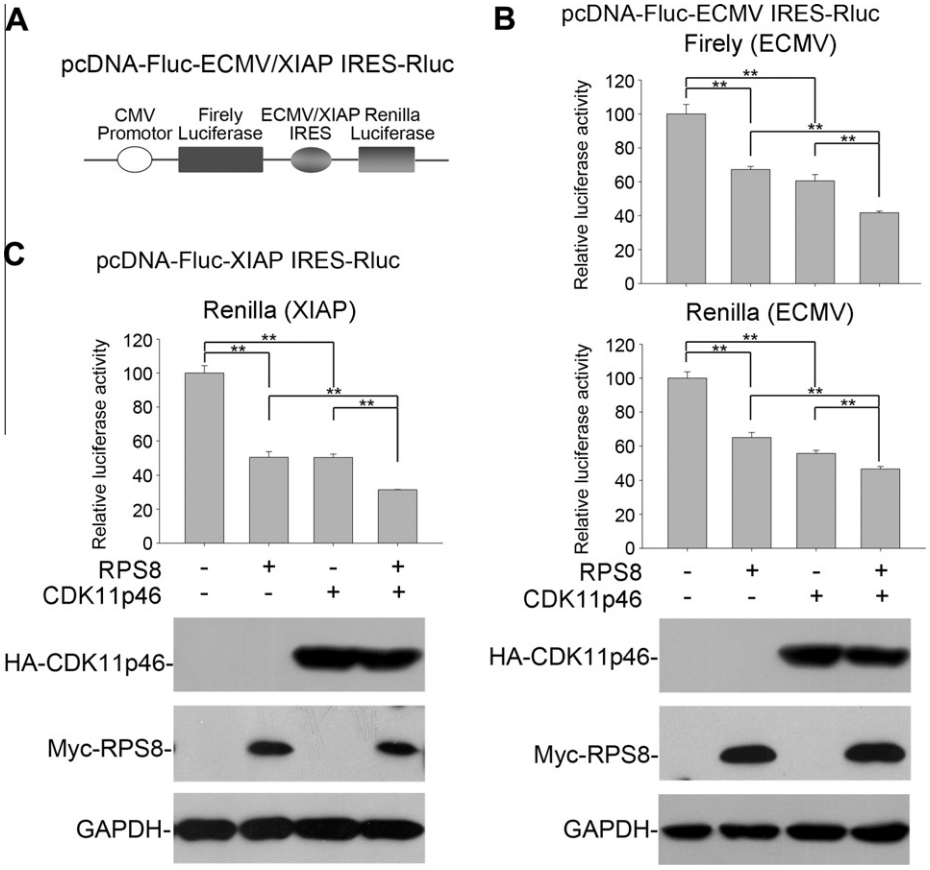

Fig. 3. CDK11p46 synergizes with RPS8 to inhibit both cap- and IRES-mediated translation. (A) Schematic diagram of the pcDNA-Fluc-ECMV/XIAP IRES-Rluc construct. (B) The bicistronic reporter pcDNA-Fluc-ECMV IRES-Rluc construct was transfected into HEK293T cells along with pcDNA3-HA-CDK11p46 and/or pcDNA3.1-myc-RPS8.48 h after transfection, cells were collected, lysed and Firely and Renilla luciferase activities were measured. Shown are the means ± SD from three separate experiments with equivalent results. Bars indicate the SD (**$P < 0.01$). The expression of CDK11p46 and RPS8 was detected by western blot (bottom panel). GAPDH was served as a quantitative control. (C) HEK293T cells were transfected with pcDNA3-HA-CDK11p46 and/or pcDNA3. 1-myc-RPS8 plasmid, and treated as described in (B) except that pcDNA-Fluc-XIAP IRES-Rluc was used as the reporter plasmid. The lower panel shows the expression levels of several proteins indicated.

luciferase, which excludes the transcriptional effect of CDK11p46 and RPS8 (Fig. S2). Thereby, our data suggest that CDK11p46 and RPS8 synergistically suppress translation in both cap- and IRES-dependent way.

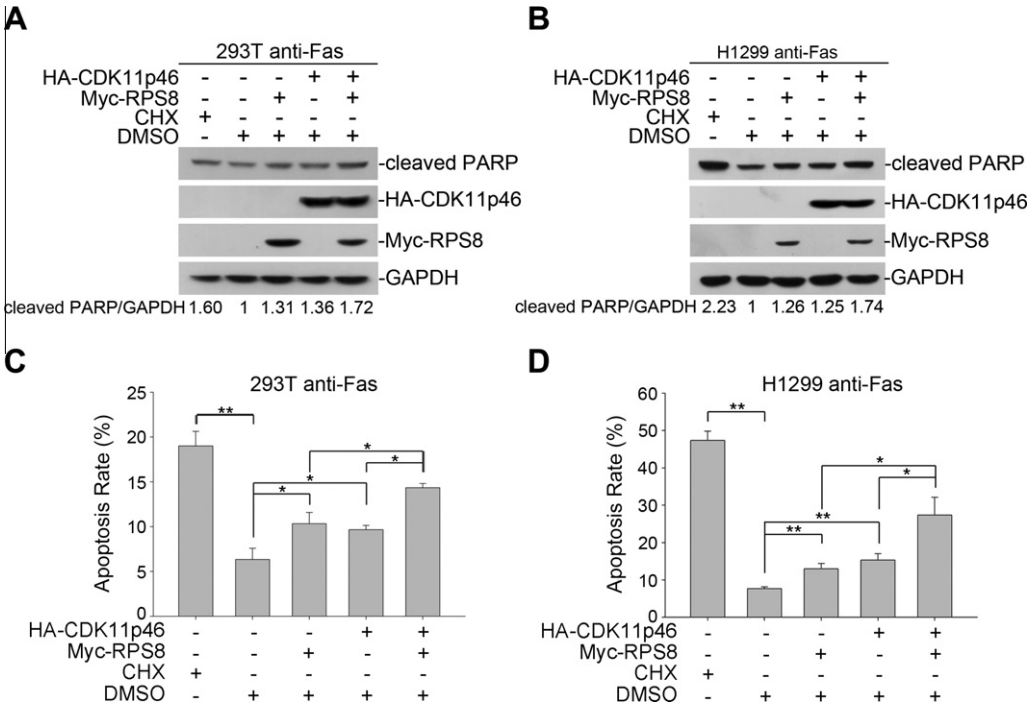

Fig. 4. RPS8 is functionally involved in CDK11p46 apoptotic regulation and promotes FasL-induced apoptosis. HEK293T and H1299 cells were transiently transfected with HA-CDK11p46, Myc-RPS8 plasmids as indicated. About 24 h later, cells were treated with or without CHX (50 μg/ml) for 4 h. Subsequently, cells were treated with anti-Fas antibody for 18 h. (A and B) PARP cleavages were analyzed with anti-cleaved PARP antibody. GAPDH was used for a loading control and the ratio of cleaved PARP/GAPDH measured for each sample was indicated at the bottom. (C and D) The cells were stained with Hoechst 33258 and examined under a fluorescence microscope. Apoptotic cells were counted among at least 300 cells randomly. The apoptotic index was the mean of three independent experiments. The standard deviations are shown (**$P < 0.01$ and *$P < 0.05$).

3.4. RPS8 is functionally involved in CDK11p46 apoptotic regulation and promotes FasL-induced apoptosis

Translational control plays a pivotal role in apoptosis regulation [23–25]. CDK11p46 appears to be the final caspase-processed product of CDK11 and overexpression of CDK11p46 inhibits overall protein synthesis and induces apoptosis [12,13]. We next examined the synergistic effect of CDK11p46 and RPS8 on the regulation of apoptosis, and CHX treatment served as the positive control. Similar to CDK11p46, transfection of RPS8 alone increased cleavage of PARP and cellular apoptosis induced by FasL. Furthermore, a synergistic effect was observed in CDK11p46 and RPS8 co-transfected cells (Fig. 4). Together, these observations suggest that RPS8 may be involved in CDK11p46-mediated regulation of apoptosis.

4. Discussion

CDK11 appears to be downstream of caspase-3 in apoptotic signaling [3,11]. Under apoptotic stimulation, CDK11p110 and CDK11p58 isoforms are cleaved by caspases to generate CDK11p46, a CDK11 isoform containing C-terminal kinase domain [11]. In the present study, we identified that RPS8 associated with CDK11p46 *in vitro* and *in vivo*. CDK11p46 and RPS8 synergistically repressed protein synthesis and sensitized cells to FasL-induced apoptosis. However, CDK11p46 showed no effect on the phosphorylation of RPS8 *in vitro* and *in vivo*. Previous research has indicated that CDK11p46 interacts with but not phosphorylates NOT2 during apoptosis *in vitro* [26]. Moreover, cyclin-dependent kinase 6 (CDK6), another member of cyclin-dependent kinase family, associates with AR and stimulates its transcription independent of the kinase activity [27]. These results suggest that cyclin-dependent kinases may function beyond the kinase activity. However, recent studies also demonstrated that CDK11p46 phosphorylates eIF3f, leading to increased association of eIF3f with eIF3 core complex and inhibition of translation [13,15]. In combination, these data indicate that CDK11p46 may regulate translation through multiple mechanisms. Our results showed that RPS8 co-localized with CDK11p46 mainly in the nucleoplasm where pre-ribosomal subunits were formed, suggesting a functional role of CDK11p46 in the maturation of ribosomal subunits (Fig. 1E).

As a component of 40S ribosome, RPS8 is in a mRNP granule complex containing untranslated mRNAs [16], while the precise function of RPS8 is still not clear. In our study, we found either overexpression or knockdown of RPS8 decreased luciferase activity through suppressing translation, suggesting RPS8 as a rate-limiting factor in translational regulation. Previous reports show that though eIF6 is essentially required for 60S subunit biogenesis [28,29], overexpression of which inhibits assembly of 80S ribosome [30]. The role of RPS8 in controlling translation needs to be clarified in the future.

Translational control has emerged as a central player in cell growth, differentiation and apoptosis, whereas aberrant translation often contributes to tumor transformation [31,32]. Initiation of translation in eukaryotic cells can occur by two distinct mechanisms, cap-and IRES-dependent. Previous studies have demonstrated that CDK11p46 inhibits cap-dependent translation and decreases overall protein synthesis during apoptosis [13,15]. Here, we provide evidence that CDK11p46 is also involved in IRES-dependent translation. Recently much more attention has been paid to IRES-dependent translation involved in cell growth, division and cell survival which are relevant for tumorigenesis [33]. It has been reported that region of chromosome 1 band p36, which contains *Cdc2L* gene locus encoding for CDK11, is frequently deleted or translocated in human tumors [34]. Moreover, *cdc2L* +/− mice show more sensitivity to DMBA/TPA-induced skin cancer

[35]. Our data show that CDK11p46, a member of CDK11 family, depresses translation during apoptosis and suggest that CDK11 family may link protein synthesis, including growth and survival factors, with cell cycle progression and tumorigenesis.

In summary, the data presented here suggest that CDK11p46 is involved in translation control during apoptosis. For amount of ribosomal proteins are phosphorylated *in vivo* [21], other ribosomal proteins or substrates that associate with CDK11p46 may exist. More importantly, since cell cycle progression couples with translation control [36], whether other members of CDK11 family interact with ribosomal proteins and participate in translational regulation needs further investigation. Our data suggest that CDK11p46 is involved in the control of protein synthesis through multiple ways, and provide the basis for the understanding of translational regulation during cell cycle progression and apoptosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.132.

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