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Corresponding Author: Joon Kim

Authors: Tae-Sung Kim[1,#], Hag Dong Kim[1,2,#], Yong Jun Park[1], EunBin Kong[1], Hee Woong Yang[1], Youjin Jung[1], YongJoong Kim[1], Joon Kim[1,2,*]

Institution: [1]Laboratory of Biochemistry, Division of Life Sciences and [2]HAEL Lab, Korea University,

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JNK activation induced by ribotoxic stress is initiated from 80S monosomes but not polysomes

Tae-Sung Kim[1, #], *Hag Dong Kim*[1,2,#], *Yong Jun Park*[1], *EunBin Kong*[1], *Hee Woong Yang*[1], *Youjin Jung*[1], *YongJoong Kim*[1] *and Joon Kim*[1,2,*]

[1]Laboratory of Biochemistry, Division of Life Sciences, Korea University, Seoul 02841, Republic of Korea

[2]HAEL Lab, TechnoComplex Building, Korea University, Seoul 02841, Republic of Korea

Running Title: JNK activation by ribotoxin in 80S monosome

[#] These authors contributed equally to this work.

[*] To whom correspondence should be addressed: Joon Kim, PhD

Laboratory of Biochemistry, Hana Science Hall #601, Korea University, Seoul 02841, Republic of Korea, Tel: 82-2-3290-3442, Fax: 82-2-927-9028, E-mail: joonkim@korea.ac.kr

ABSTRACT

Translation is a costly, but inevitable, cell maintenance process. To reduce unnecessary ATP consumption in cells, a fine-tuning mechanism is needed for both ribosome biogenesis and translation. Previous studies have suggested that the ribosome functions as a hub for many cellular signals such as ribotoxic stress response, mammalian target of rapamycin (mTOR), and ribosomal S6 kinase (RSK) signaling. Therefore, we investigated the relationship between ribosomes and mitogen-activated protein kinase (MAPK) activation under ribotoxic stress conditions and found that the activation of c-Jun N-terminal kinases (JNKs) was suppressed by ribosomal protein knockdown but that of p38 was not. In addition, we found that JNK activation is driven by the association of inactive JNK in the 80S monosomes rather than the polysomes. Overall, these data suggest that the activation of JNKs by ribotoxic stress is attributable to 80S monosomes. These 80S monosomes are active ribosomes that are ready to initiate protein translation, rather than polysomes that are already acting ribosomes involved in translation elongation.

Keywords: 80S monosome, Deoxynivalenol, Emetine, JNK, Ribotoxic stress

INTRODUCTION

Recently, ribosomes have received a lot of attention as hubs for cellular signals such as ribotoxins and growth factors (1-3). When the Human Genome Project was established, the number of proteins in human beings was considered to be insufficient for performing all body functions. Many biologists have suggested that multifunctional proteins and various signaling pathways emerge from an identical complex. Therefore, other than its inherent translational function, a ribosome complex acts as a scaffold protein for various kinase signaling pathways. Ribosomal protein S6 (rpS6), a component of the 40S ribosomal small subunit, accepts the signal from ribosomal S6 kinase (RSK). The phosphorylated rpS6 transmits the signal to the ribosome in response to different mitogens such as growth factors and insulin (2, 4). S6 kinase is also a downstream effector of the mammalian target of rapamycin (mTor), which is a key regulator of mammalian cell growth (5). Ribosomal protein S3 (rpS3) is located on a beak portion of the ribosomal small subunit and plays a role in translation initiation (6). Recently, rpS3 was demonstrated to have several extra-ribosomal functions in inflammation (7), metastasis (8), NF-κB signaling (9), and DNA repair (10, 11). Furthermore, phosphorylation of rpS3 is controlled by various signaling proteins such as extracellular signal-regulated kinase (Erk), protein kinase C delta (PKCδ), protein kinase B (PKB/Akt), and protein phosphatase 2A (PP2A) (12-15). The receptor for activated protein C kinase-1 (RACK1), a component of the 40S ribosomal subunit, is a scaffold protein in many signaling pathways such as protein kinase C (PKC) and focal adhesion kinase (FAK) (14-17). Thus, RACK1 is considered a receptor for extracellular signaling in the ribosomal complex.

Ribotoxins cause sequence-specific damage to the α-sarcin/ricin loop of 28S rRNA (16, 17). The damage blocks peptidyl transferase activity and inhibits the translation activity of the

ribosome (17, 18). Previous studies have demonstrated that the ribosome acts as a scaffold for mitogen-activated protein kinases (MAPKs) such as p38 mitogen-activated protein kinase (p38) and c-Jun N-terminal kinases (JNK) under ribotoxic stress (18, 19). Bae *et al* reported that the translocation of p38 to the ribosome increases when the macrophages are treated with deoxynivalenol (DON) (20).

The activation of JNK and p38 induced by ribotoxins is essential for active ribosomes. Many researchers have reported that translation inhibition suppresses the activation of JNK and p38 under ribotoxic stress (21). Here, we have demonstrated that the 80S monosome is important for JNK activation under ribotoxic stress.

RESULTS

Knockdown of ribosomal proteins decreased JNK activation induced by ribotoxic stress

A previous study has shown that JNK activation induced by a low dose of ultraviolet C (UVC; 45 J/m^2) is attenuated by RACK1 knockdown (22). To apply this phenomenon to ribotoxic stress, RACK1 knockdown HT1080 cells were treated with various ribotoxins such as a high dose of UV (Fig. 1A), DON (Fig. 1B), and anisomycin (Fig. 1C). Then, activation of the stress-activated protein kinases JNK and p38 was examined in a time-dependent manner. In all the cases, JNK activation was significantly reduced in the RACK1 knockdown cells when compared with the scramble (SC) knockdown cells (Fig. 1). In contrast, activation of p38 did not change significantly in all the cases. Next, we investigated whether attenuated JNK activation occurred under the decreased state of other 40S ribosomal proteins due to rpS3 and rpS6 knockdown. The activation of JNK was attenuated by a decrease in ribosomal proteins, as in the case of RACK1 knockdown. (Fig. 1D). Therefore, we suggest that knockdown of most ribosomal proteins confer cells with similar properties that inhibit JNK activation under ribotoxic stress.

Knockdown of ribosomal proteins caused a serious decrease of 80S monosomes in the ribosomal profile

To characterize the effects of ribotoxic stress on the ribosomes, we performed translational profiling of the ribosomes under various stresses. We performed ultracentrifugation analyses with a 5% to 45% (w/v) linear sucrose gradient (Fig. 2A). Cells with various stresses exhibited

different abnormal ribosome profiles when compared with the unstressed cells (Fig. 2B). DON and UV showed a distinct ribosome profile with a significant increase in the 60S ribosomal subunit and 80S monosome peak, whereas anisomycin showed a slight decrease in 80S monosome and increase in the polysome. According to previous studies (23, 24), anisomycin and DON can slightly increase polysome fractions by inducing ribosomal stalling in the mRNA templates. In contrast, a translation inhibitor, emetine, induced a significant decrease in 80S monosomes and increase in the polysomes (Fig. 2C). Interestingly, emetine inhibits ribosomal peptidyl transferase activity but does not induce the activation of JNK. Emetine has previously been reported to disrupt JNK activation induced by ribotoxins, such as anisomycin (16, 25) and UV (26). These results suggest that JNK activation induced by ribotoxic stress requires a normal number of 80S monosomes but not polysomes, and JNK activation subsequently is blocked by a decrease in 80S monosomes. This indicates that the 80S monosome is "an active ribosome," which is active to translate associated mRNAs. However, once 80S monosomes are initiated for elongation, they become "acting ribosomes" and JNK cannot be activated. To confirm this suggestion, we next examined the ribosome profiles of rpS3, ribosomal protein L13 (rpL13), and RACK1 siRNA transfected cells. The rpS3 and rpS6 knockdown cells exhibited an abnormal ribosome profile and significant decrease in 80S monosomes and mild increase in 60S ribosomal subunits (Fig. 2C), which is consistent with the results of our previous study (27). Additionally, the rpL13 siRNA transfected cells showed a striking decrease in 80S monosomes (Fig. 3A). The 80S monosomes were reduced in the RACK1 knockdown cells, but the rest of the ribosome profiles did not change significantly when compared with the control cells. (Fig. 2C). Furthermore, we confirmed that the UV-induced JNK activation was inhibited by knockdown of two 60S ribosomal proteins, rpL13 and ribosomal protein L30 (rpL30) (Fig. 3B and C). Thus, we concluded that a normal number of active 80S monosomes

are required for ribotoxic stress-induced JNK activation, probably because active 80S monosomes remain ready for the next action.

JNK activation by ribotoxic stress occurred in the monosomes but not the polysomes

Our findings suggested that the ribotoxic stress-induced JNK activation is initiated in the 80S monosomes. To support this hypothesis, we first examined the presence of JNK in each separate ribosomal fraction by using a sucrose cushion. The mRNA-associated ribosomes, such as polysomes and 80S monosomes, can be easily pelleted by sedimentation of lysates through 20% sucrose cushion. The mRNA-free ribosomes, such as the 40S and 60S ribosomal subunits, were predominantly located in the middle fractions of the 20% sucrose cushion. JNK was detected in the middle fraction, but not in the ribosome pellet, and mostly disappeared because of RACK1 knockdown (Fig. 4A). However, the location pattern of p38 in the middle fraction was similar in both scramble (SC) and RACK1 knockdown cells. In addition, we confirmed a reduction in UV-induced JNK activation under RACK1 knockdown conditions by performing *in vitro* kinase analysis with the middle fraction of the sucrose cushion (Fig. 4B). Next, we investigated the existence of JNK in the ribosomal fractions separated by linear sucrose gradient centrifugation. For comparison of ribosome distribution in the normal and UV-irradiated cells, we quantified the polysomes, 80S monosomes, and 60S and 40S ribosomal subunits. UV irradiation significantly increased the number of monosomes and decreased the number of polysomes (Fig. 4C), which is consistent with our previous results. Under UV irradiation, unphosphorylated JNK disappeared in the 80S monosome fractions, and phosphorylated JNK began to appear in the non-ribosomal fractions (Fig. 4D and E). Therefore,

we concluded that the activated JNK may have been released from the active ribosome, which is ready to participate in the process of translational elongation.

Next, although emetine, an inhibitor of translation, decreased ribotoxic stress-induced JNK activation, it is unclear whether the inhibition of all translation steps had the same effect as emetine. Therefore, we investigated UV-induced JNK activation by using various protein synthesis inhibitors. NSC119889 inhibits eIF2 ternary complex (eIF2-GTP-Met-$tRNA_i^{Met}$) formation in the translation initiation step. Emetine inhibits protein synthesis by binding to the 40S ribosomal subunit, but the exact mechanism has not yet been elucidated. Cycloheximide inhibits eEF2-mediated tRNA translocation by binding to the 60S ribosomal subunit (28). As shown in Figure 4F, NSC119889, and not cycloheximide, had the same negative effect on UV-induced JNK activation as emetine. Therefore, we propose that blocking translation initiation results in the inhibition of ribotoxic stress-induced JNK activation.

DISCUSSION

Recently, the ribosome, a translation machinery for protein biosynthesis, was reported to act as a scaffold for various kinase signaling pathways. Eukaryotic cells respond to ribotoxic stimuli in two ways: inhibition of protein translation or activation of MAPK signaling (16). Translation inhibition impairs the peptidyl transferase activity of the ribosomes by cleavage of the 3′-end of 28S rRNA, the binding region of aminoacyl tRNA. Then, activation of JNK and p38 occurs in active ribosomes. However, it has not been determined whether ribotoxin-sensitive active ribosomes are polysomes or 80S monosomes. The former undergoes mRNA translation, and the latter is present on the mRNA ready for translation. We propose the polysome is an acting ribosome, and the 80S monosome is an active ribosome.

Here, we have confirmed that knockdown of ribosomal proteins inhibits ribotoxic stress-induced JNK activation, as after treatment with emetine (Fig. 1D). Then, we confirmed that the knockdown of ribosomal proteins, rpS3, rpS6, and rpL13, resulted in a common decrease in the 80S monosome and polysome fractions by using ribosome profile analysis. In accordance with our previous data (27), 80S monosomes decreased because of ribosomal protein knockdown, but no significant changes were observed in the polysome fractions (Fig. 2). Emetine causes a serious decrease in 80S monosomes. Thus, we suggest that the decrease in 80S monosomes is responsible for the inhibition of ribotoxic stress-induced JNK activation.

We were interested in the correlation between the ribosomal peptidyl transferase activity and UV-induced JNK activation because various translational inhibitors have different effects on the inhibition of UV-induced JNK activation (Fig. 2B). We considered the ribonucleolytic activity of ribotoxin in the process of peptide bond formation in ribosomes. Most ribotoxins,

such as anisomycin, DON, and UV, induce a malfunction in the peptidyl transferase activity of the ribosome. Thus, ribotoxin can inhibit the formation of peptide bonds between P-position loaded amino acids and A-position loaded amino acids. Indeed, anisomycin has been reported to bind to the A-site and inhibit the formation of peptide bonds. The translation inhibition mechanism of cycloheximde inhibits eEF2-mediated translocation from A-site to P-site through the binding of E-sites in the 60S ribosomal subunit (28). Emetine can bind to 40S and 60S ribosomal subunits and 80S monosomes and can inhibit peptide bond formation at high concentrations. Therefore, it seems that the 80S monosome immediately before the initiation of polypeptide chain elongation is important for ribotoxin-induced JNK activation (Fig. 4D).

Many kinases bind to ribosomes and regulate ribosomal functions through phosphorylation of the ribosomal proteins or ribosomal components (29-32). Although the upstream kinases involved in JNK and p38 activation induced by ribotoxic stress are not fully understood, several studies provide clues. The mixed lineage kinase (ZAK) plays a pivotal role in ribotoxic stress responses (33). Under ribotoxic stress conditions induced by doxorubicin, activation of JNK and p38 was suppressed by ZAK inhibition. In addition, double-stranded RNA-dependent protein (PKR) (34) and hematopoietic cell kinase (Hck) (35) have been suggested as upstream kinases for MAPK activation under ribotoxic stress. These kinases have been reported to activate MAPK signaling pathways involved in JNK and p38 activation in response to a variety of ribotoxins (16, 36). Finally, PKC, the RACK1 binding protein, can stimulate the MAPK signaling pathway in response to diverse stimuli and is recruited into ribosomes through RACK1 interaction (22). However, further studies are required to understand the mechanism underlying ribotoxic stress-induced MAPK activation.

Here, we tried to understand the relationship between ribotoxic stress-induced MAPK

activation and active ribosomes. Both JNK and p38 were activated under ribotoxic stress, which is consistent with the findings of previous studies; however, interestingly, JNK activation was significantly affected by impaired ribosome biogenesis. In addition, we found that 80S monosomes are essential for JNK activation under ribotoxic stress, indicating 80S monosomes with associated mRNAs are active ribosomes.

MATERIALS AND METHODS

Ribosomal fractionation and pelleting

For ribosomal fractionation and profiling, HT1080 cells were treated with 100 μg/ml cycloheximide for 30 min and lysed in 1 ml of hypotonic lysis buffer (1.5 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), 0.5% sodium deoxycholate, 1% Triton X-100, 1.25 mM DTT, and 40 Units/ml RNasin). After centrifugation at 12,000 × *g* for 15 min to remove the mitochondria and cell debris, the supernatants were layered on top of a 5% to 45% (w/v) sucrose gradient set in dilution buffer (80 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), and 1 mM DTT) and centrifuged with a Beckman SW41Ti rotor at 149,000 × *g* for 2.5 h at 4 °C. The absorbance of the sucrose gradients was measured, and each fraction was collected, as described previously (37). For ribosomal pelleting, the HT1080 cells were lysed in 2 ml of hypotonic lysis buffer. After centrifugation, the supernatant was layered over a sucrose (20% (w/v)) cushion and centrifuged at 149,000 × *g* for 2.5 h. The ribosome-containing pellet and middle fraction and non-ribosomal supernatant were collected separately.

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CONFLICT OF INTEREST

The authors declare that they do not have any commercial or financial relationships that could be construed as a potential conflict of interest.

FIGURE LEGENDS

Fig. 1. Ribotoxic stress-induced JNK activation is decreased by RACK1 depletion.

HT1080 cells transfected with scramble (SC) or RACK1 siRNA were treated with 150 J/m^2 UV (A), 2 μg/ml DON (B), or 2 μg/ml anisomycin (C) and incubated for the indicated times. The densitometric ratio of pJNK/JNK is shown below the blots (n = 3). Error bars, standard deviation; *, $P < 0.05$; **, $P < 0.01$; NS, not significant. (D) HT1080 cells transfected with the indicated siRNAs were irradiated with 150 J/m^2 UV, followed by harvesting at the indicated times. The cell lysates were analyzed with immunoblotting.

Fig. 2. Comparison of ribosomal profiles under various stress conditions and after the knockdown of ribosomal proteins.

(A) Scheme of a ribosome profiling method using a linear sucrose gradient. (B) HT1080 cells were treated with 2 μg/ml DON, 2 μg/ml anisomycin, or 150 J/m^2 UV. The untreated HT1080 cells were used as the control, and the profile was designated “Mock.” (C) Scramble, each ribosomal protein siRNA, and emetine-treated HT1080 cells were fractionated in a linear sucrose gradient, as described in the Materials and Methods. The polysome, monosome (80S), and ribosomal subunit (40S, 60S) regions are marked in the ribosome profile.

Fig. 3. Knockdown of 60S ribosomal protein decreases the activation of JNK by UV irradiation.

(A) Cell lysates from HT1080 cells transfected with scramble or rpL13 siRNA were fractionated in a linear sucrose gradient, as described in the Materials and Methods. The regions of the polysome, monosome (80S), and ribosome subunits (40S, 60S) are indicated in the ribosome profile. (B, C) HT1080 cells transfected with rpL13 (B) or rpL30 (C) siRNA were treated with 150 J/m^2 UV and incubated for 1 h. These cell extracts were resolved using SDS-

PAGE and analyzed with immunoblotting.

Fig. 4. UV-induced JNK activation by the 80S monosome is attenuated by translation initiation inhibitors.

(A) HT1080 cells were transfected with scramble or RACK1 siRNA and treated with different ribotoxins (2 μg/ml DON, 2 μg/ml anisomycin, or 150 J/m^2 UV) for the indicated times. The cell extracts were subjected to ultracentrifugation by using a 20% sucrose cushion. The ribosome-containing pellet, middle fraction, and non-ribosomal supernatant were collected separately. For immunoblot analysis, the ribosome pellets were resuspended in SDS-PAGE sample buffer, and the middle fractions were precipitated with TCA/acetone and mixed with the SDS-PAGE sample buffer. (B) HT1080 cells transfected with scramble or RACK1 siRNA were irradiated with 150 J/m^2 UV. After 1 h, non-ribosomal and middle fractions were isolated by ultracentrifugation. Kinase assays were performed by mixing immunoprecipitated JNK of each fraction with GST-cJun in the presence of γ-^{32}P. (C) Normal or UV-irradiated HT1080 cells were fractionated in a linear sucrose gradient, as described in the Materials and Methods. Distribution (%) of ribosome content (right) in the ribosomal fractions was calculated by measuring the area in each fraction on the basis of the ribosome profile (left). Error bars, standard deviation; ***, $P < 0.001$; NS, not significant (n = 3). (D, E) Each fraction was resolved using 10% SDS-PAGE and subjected to immunoblot analysis with the indicated antibodies (D). The relative amount of JNK in the 80S monosome was obtained by measuring the signal intensities of fractions 5 and 6. Error bars, standard deviation; *, $P < 0.05$ (n = 3) (E). (F) HT1080 cells were pre-treated with 25 μg/ml cycloheximide (CHX), 20 μM emetine (Eme), and 5 μM NSC119889 (NSC) for 30 min and

then irradiated with 150 J/m^2 of UV. After 1 h, the cell lysates were subjected to immunoblot analysis by using the indicated antibodies.

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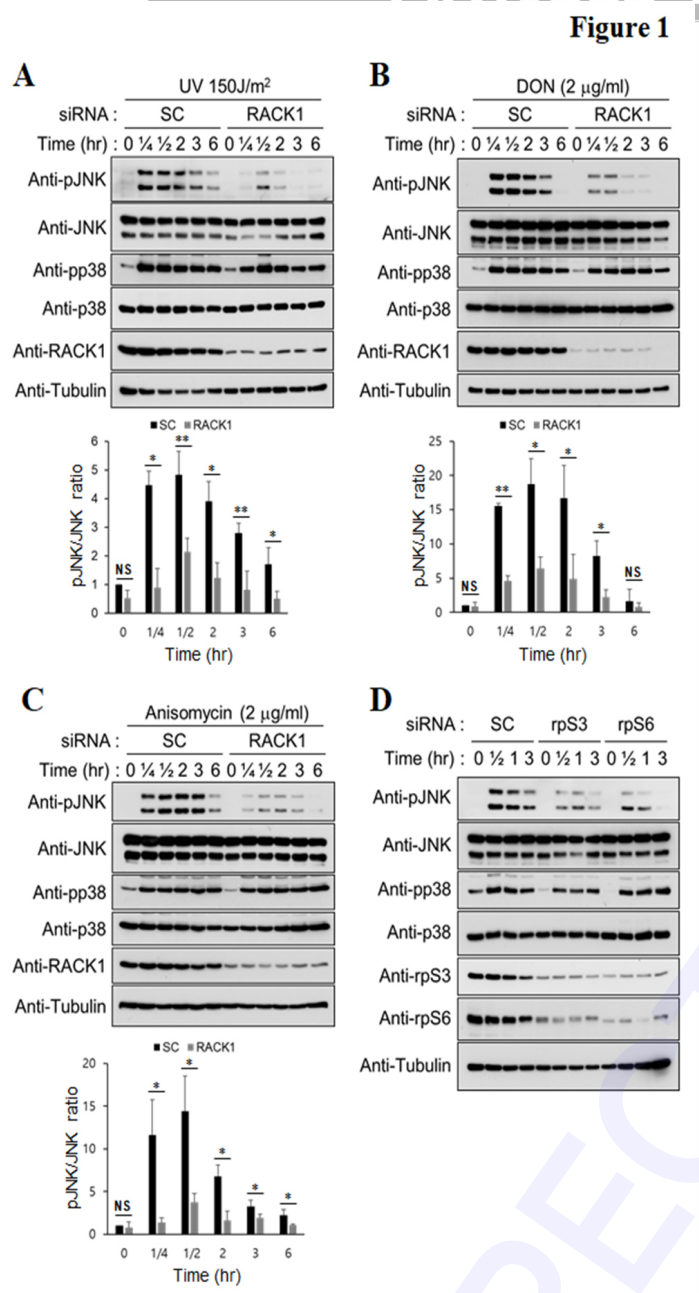

Fig. 1. Figure 1

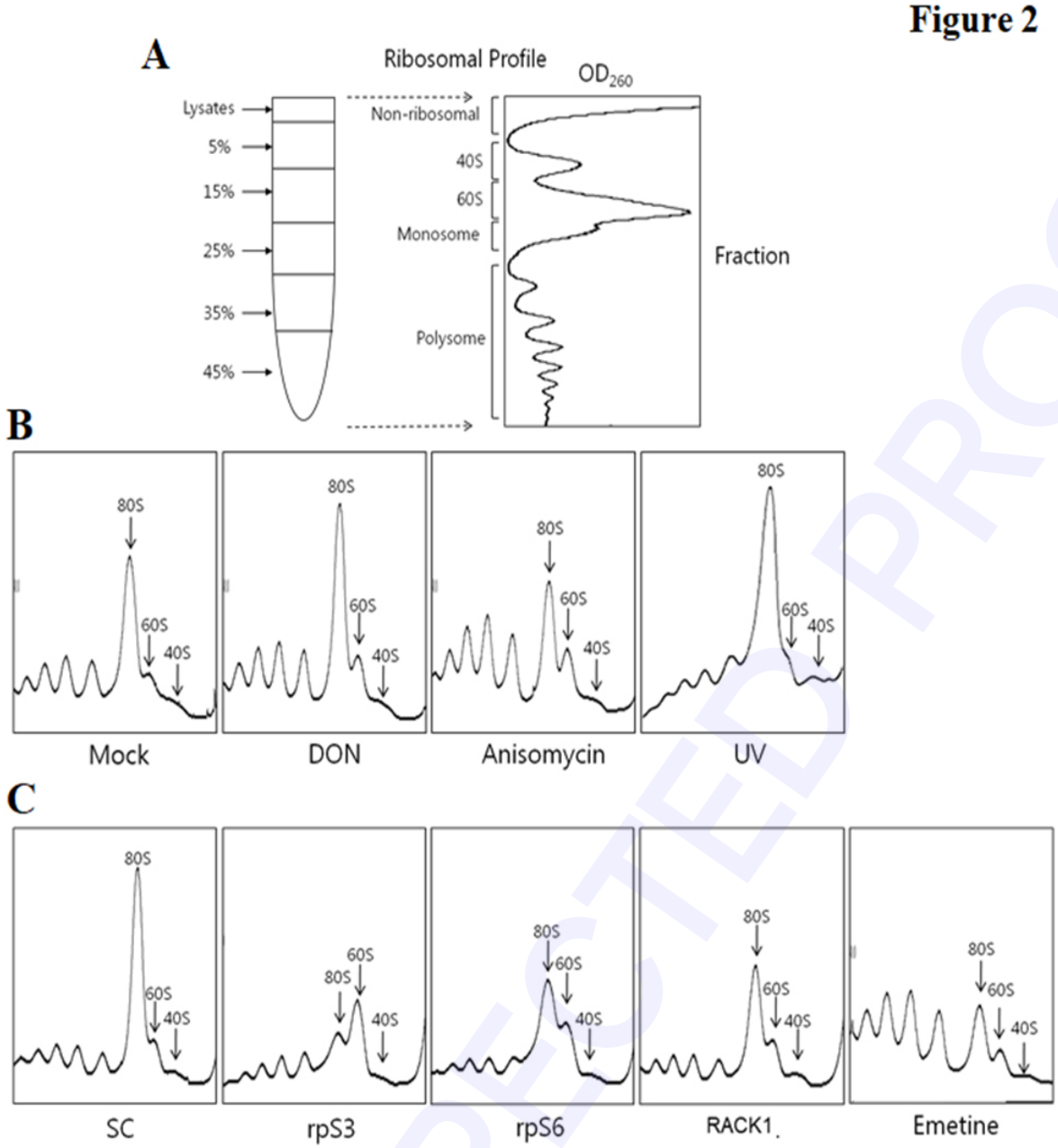

Fig. 2. Figure 2

Figure 3

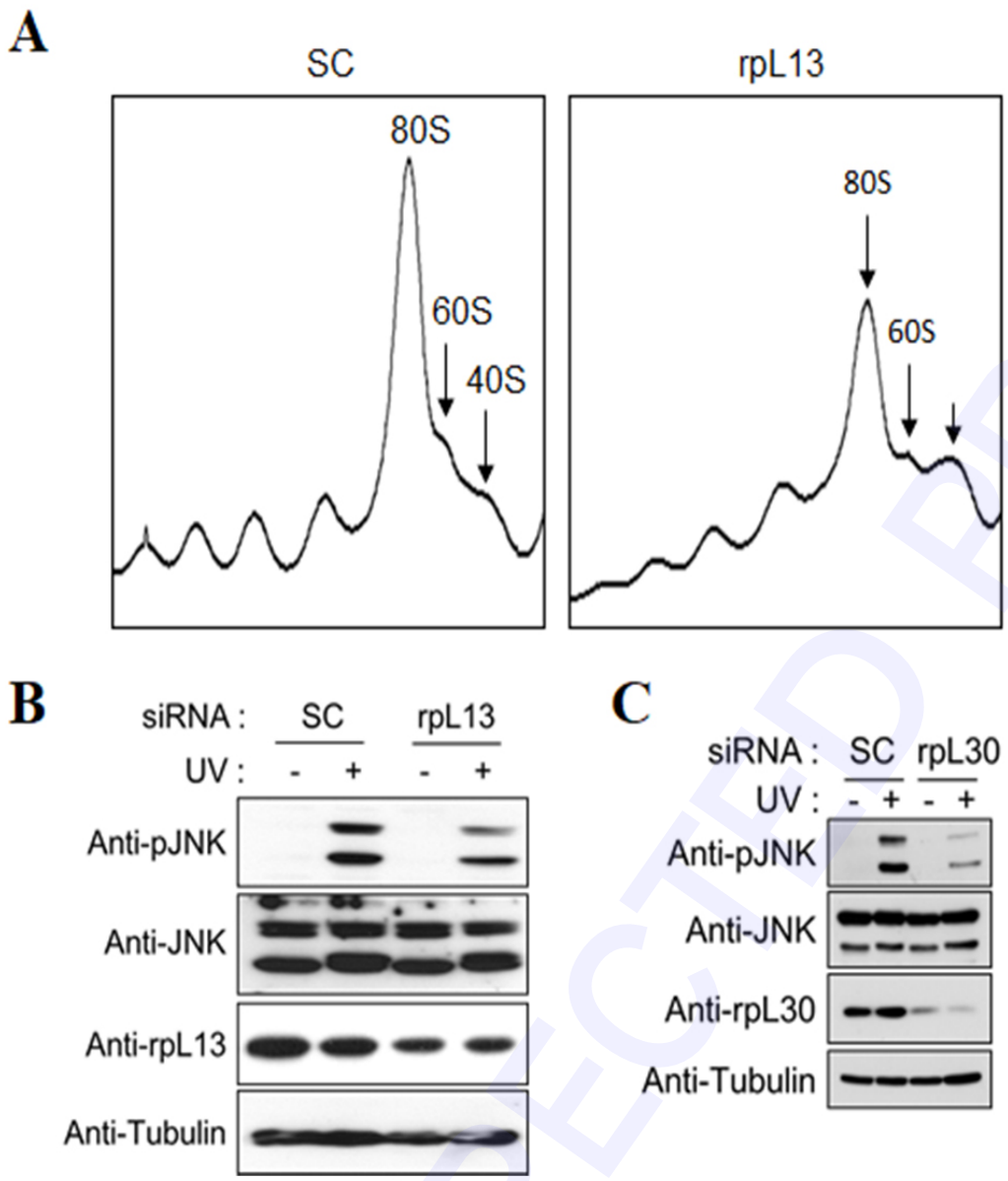

Fig. 3. Figure 3

Figure 4

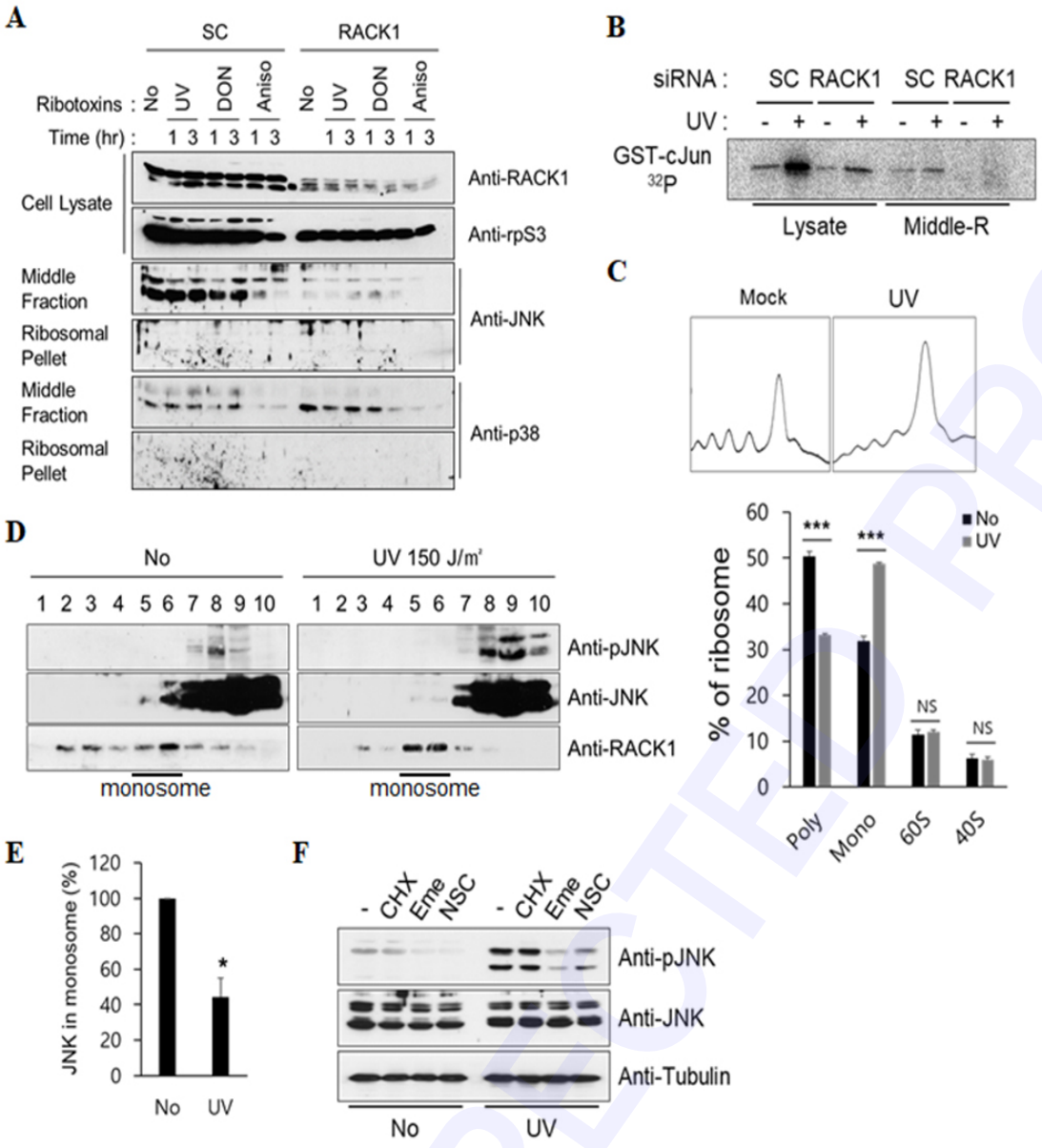

Fig. 4. Figure 4

SUPPLEMENTARY MATERIAL

Reagents

Deoxynivalenol (DON) and ATP were purchased from Sigma-Aldrich (USA). Anisomycin, emetine, and NSC119889 were purchased from Merck Millipore (USA).

Cell culture

Human fibrosarcoma HT1080 cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. These cells were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2.

siRNA and transfection

We purchased siRNA, including scramble and rpS6, from Bioneer (Korea). RACK1, rpL13, and rpL30 siRNA oligos were purchased from Santa Cruz Biotechnology (USA), and rpS3 siRNA was obtained from HAEL Lab (Korea). Each siRNAs transfection was performed with Lipofectamine™ RNAiMAX reagent (Invitrogen, USA) as instructed by the manufacturer. DNA transfection was also performed using Lipofectamine™ 2000 (Invitrogen, USA).

Antibodies and immunoblotting

Antibodies against JNK, p38, RACK1, rpL13, and rpL30 were purchased from Santa Cruz Biotechnology (USA). Antibodies against pJNK, pp38, and rpS6 were obtained from Cell Signaling Technology (USA) and anti-α-tubulin from Calbiochem (USA). Anti-rpS3 antibody

was obtained from HAEL Lab (Korea). The cells were lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.05% sodium deoxycholate, 50 mM NaF, 200 μM Na_3VO_4, 2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 30 min on ice. The supernatants were collected by centrifugation at 12,000 × *g* for 10 min at 4 ℃, and protein concentrations were determined using the Bradford protein assay. The lysates were boiled in SDS-PAGE sample buffer and separated with SDS-PAGE gel, transferred to PVDF membranes, probed with the antibodies, and illuminated with the enhanced chemiluminescence (ECL) system (Roche, Switzerland).

Protein precipitation

Proteins in the ribosomal fractions were precipitated with TCA/acetone. Briefly, 100% TCA solution was added to adjust to 20% final concentration in each fraction, and these samples were incubated for 1 h on ice. After centrifugation, the pellets were washed five times with ice-cold acetone and then dried in a heat block for 5 min to remove the residual acetone.

Immunoprecipitation

The HT1080 cells were lysed with cold lysis buffer for 30 min on ice. After centrifugation at 12,000 × *g* for 10 min at 4 ℃, the supernatant was collected, pre-cleared by adding protein A agarose, and then incubated at 4 ℃ for 2 h with specific antibodies. Then, 30 μl of protein A agarose was added, and the solution was incubated for 16 h at 4 ℃. After extensive washing, the immunoprecipitates were ready for use in other experiments.

JNK kinase assay

The cell lysates were subjected to immunoprecipitation with the anti-JNK antibody. The immunoprecipitates were washed three times with lysis buffer, twice with tyrosine kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM sodium vanadate), and then resuspended in 20 μl of kinase buffer containing 2 μCi of [γ-^{32}P] ATP and GST-cJun protein. The mixture was incubated for 30 min at 30 °C, and the reaction mixture was resolved using 11% SDS-PAGE and analyzed with autoradiography.

Statistical analysis

All the experiments were performed at least three times independently. The quantitative data are presented as mean ± standard error of the mean (SEM) values. *P* values were calculated with the unpaired two-tailed *t*-test from the mean data of each group.