SUPPORTING MATERIAL

INVENTORY OF SUPPORTING MATERIAL

1. Supplementary materials and methods.
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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids. Expression vectors for His_6-tagged eIFs 1, 1A, 3j, 4A, 4B, 5, eRF1, eRF3 as well as transcription plasmids for MVHL-STOP mRNA, GUSmod mRNA, $tRNA_i^{Met}$, $tRNA^{His}$ and $tRNA^{Val}$ have been described (15,16). Vector for the expression of human full-length SLFN14 in *Escherichia coli* cells was prepared by cloning of protein coding region followed by His_6-tag into *NcoI/BamHI* sites of pET28a (GenScript Corp.). 95 kDa (amino acids 1-833), 85 kDa (amino acids 1-745), 65 kDa (amino acids 1-571) and 45 kDa (amino acids 1-392) C-terminal truncated His_6-tagged forms of human SLFN14 were constructed by PCR amplification from mentioned above full-length form and cloning into *NcoI/BamHI* sites of pET28a. Vector for the expression in *Escherichia coli* of murine version of rabbit SLFN14-65kDa was constructed by cloning of corresponding murine protein coding region (amino acids 1-562) followed by His_6-tag into *NcoI/BamHI* sites of pET28a (GenScript Corp.). SLFN14-45kDa mutants D115A, D248A, D249A, and D265A were prepared by the substitution of Asp for Ala employing Q5 Site-Directed Mutagenesis Kit (NEB). Transcription vector for HBBmod mRNA was made by inserting DNA flanked by a T7 promoter and an *EcoRI* restriction site into pUC57 (GenScript Corp.). mRNAs and tRNAs were transcribed using T7 RNA polymerase. 5'-end 32P-labelling of GUSmod mRNA, HBBmod mRNA, $tRNA^{His}$ and $tRNA^{Val}$ was performed with Vaccinia virus capping enzyme (NEB) and [α-32P]GTP according to the manufacturer's protocol. Vector containing *Xenopus laevis* 18S rDNA (43) was used to generate sequence ladders for the identification of sites of SLFN14-mediated cleavages in rabbit 18S rRNA.

Antibodies. Rabbit polyclonal anti-SLFN14, anti-GAPDH, anti-eIF2α and rabbit monoclonal anti-HDAC2 antibodies for immunoblotting were purchased from Abcam.

Cell Extracts. The experiment on intracellular localization of SLFN14 with the following detection of 80S ribosome to SLFN14 ratio was performed with Flexi RRL (Promega). To prepare whole cell, nuclear and cytoplasmic extracts, HEK293T and MCF7 cells were cultured in DMEM (GIBCO) supplemented with 10 % fetal bovine serum, harvested using trypsin/EDTA solution (GIBCO) according to the manufacturer's instructions and washed 3 times with 1 x PBS pH 7.4. For the whole cell extract, 8 x 10^6 HEK293T cells were resuspended in an equal volume of pre-chilled HEPES-based hypotonic lysis buffer (10 mM HEPES-KOH pH 7.6, 10 mM potassium acetate, 0.5 mM magnesium acetate, 5 mM DTT, HALT protease inhibitor cocktail EDTA-free from Thermo Scientific). The cells were allowed to swell for 30 minutes on ice and were subsequently disrupted with 15 strokes of a 1 ml syringe with a 27 $G^{3/4}$ needle. After lysis, cell lysate was centrifuged at 13,000 g for 1 min at 4°C. For nuclear and cytoplasmic extracts, 2 x 10^6 HEK293T or MCF7 cells were processed with NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific) according to the manufacturer's protocol. Rabbit brain, lung and liver tissue lysates were purchased from ProSci incorporated.

Purification of Translation Factors and Ribosomal Subunits. Native rabbit 40S and 60S subunits, DHX29, ABCE1, eIFs 2, 3, 4F, 5B, eEFs 1H, 2, and recombinant His_6-tagged eIFs 1, 1A, 3j, 4A, 4B, 5, eRFs 1, 3 were purified as described (15,16). To isolate cleaved and intact (control) ribosomal subunits, 200 µl RRL after incubation in a 400 µl reaction mixture containing buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.25 mM spermidine) with 0.2 mM ATP at 37°C for 12 h and without incubation, respectively, was treated with 1mM puromycin for 10 min at 37°C, supplemented with KCl until 0.5 M final concentration and subjected to centrifugation through 10-30% SDG prepared in buffer A + 0.4 M KCl in a Beckman SW55 rotor at 53,000 rpm for 75 min. Peaks of ribosomal subunits were collected and frozen at -80°C. The integrity of rRNA in ribosomal subunits was assayed by DAFGE.

Aminoacylation of tRNA. Rabbit aminoacyl-tRNA synthetases were purified, and *in vitro* transcribed $tRNA_i^{Met}$, $tRNA^{Val}$ and rabbit native total tRNAs (Promega) were aminoacylated with Met, Val, His, Leu, Phe and Cys, as appropriate, as described (16). For methionyl-puromycin assay, *in vitro* transcribed $tRNA^{Met}{}_i$ was aminoacylated with 35S-labelled Met (5 x 10^5 cpm/pmol).

SLFN14 Purification. Native rabbit SLFN14 was purified from the 0-40% ammonium sulfate (AS) precipitation fraction of the 0.5 M KCl RSW that was prepared from 450 ml RRL. The fraction was dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 10 % glycerol) + 100 mM KCl and applied to a diethylaminoethyl cellulose (DEAE) column equilibrated with buffer B + 100 mM KCl. SLFN14 was eluted in the flow-through fraction with buffer B + 100 mM KCl. This fraction was applied to a phosphocellulose (P-11) column equilibrated with buffer B + 100 mM KCl. SLFN14 was eluted at 400-600 mM KCl. SLFN14-containing fractions were dialyzed against buffer C (20 mM HEPES-KOH, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 10 % glycerol) + 100 mM KCl and applied to a FPLC MonoS 5/50 GL column. Fractions were collected across a 100-500 mM KCl gradient. SLFN14 eluted at ~420 mM KCl. SLFN14-containing fractions were diluted 6.7-fold with 20 mM phosphate buffer, pH 7.5 with 10 % glycerol and applied to a hydroxyapatite column pre-equilibrated with the same phosphate buffer. Fractions were collected across a 20-600 mM phosphate buffer gradient. SLFN14 was eluted at ~500 mM phosphate buffer. SLFN14-containing fractions were dialyzed against buffer B + 100 mM KCl and applied to a FPLC MonoQ 5/50 GL column. Fractions were collected across a 100-500 mM KCl gradient. SLFN14 eluted at ~280 mM KCl. SLFN14-containing fractions were concentrated and transferred into buffer B + 100 mM KCl on Amicon Ultra 10K (Millipore). The identity of SLFN14 was confirmed by LC-nanospray tandem mass spectrometry of peptides derived by in-gel tryptic digestion at the Rockefeller University Proteomics Resource Center.

Native murine SLFN14 was purified from the 40-50% AS precipitation fraction of the 0.5 M KCl RSW, obtained from 100 ml Krebs ascites cell lysate, according to the same strategy as for the rabbit form and only the order of ion-exchange columns was different. The protein eluted at 100 mM KCl after DEAE column, 350-1000 mM KCl after P-11 column, ~480 mM KCl after hydroxyapatite column and ~250 mM KCl after MiniS 4.6/50 PE column. The identity of SLFN14 was confirmed by immunoblotting.

Recombinant rabbit and murine SLFN14-65kDa were expressed in 4 L of *E. coli* BL21(DE3) after induction by 0.1 mM IPTG for 16 h at 16°C and isolated by affinity chromatography on Ni-NTA agarose followed by affinity purification on 50 pmol 80S ribosomes, reconstituted from 40S and 60S subunits, by means of centrifugation through 10-30 % SDG prepared in buffer A in

a Beckman SW55 rotor at 53,000 rpm for 75 minutes. Attempts to express recombinant full-length, 95 kDa and 85 kDa forms of rabbit SLFN14 did not yield soluble protein.

Recombinant rabbit SLFN14-45kDa and its mutants were expressed in 1 L of *E. coli* BL21(DE3) each after induction by 0.1 mM IPTG for 16 h at 16°C and isolated by affinity chromatography on Ni-NTA agarose followed by FPLC on MonoS column with the elution at 280 mM KCl.

ATPase Assay. 1 pmol SLFN14 or 1 pmol DHX29 were incubated in a 10 µl reaction mixture containing buffer A supplemented with 5 µM ATP and 0.33 µM [γ-32P]ATP in the presence/absence of 1 pmol 40S subunits, 1 pmol 80S ribosomes or 20 pmol $(CU)_{17}$ RNA for 40 min at 37°C and subjected to PEI cellulose chromatography as described (16).

Analysis of SLFN14's Sssociation with Ribosome and Ribosomal Subunits. To investigate ribosomal binding of different forms of SLFN14, 100 pmol SLFN14 was incubated with 50 pmol 40S subunits, 50 pmol 60S subunits or 50 pmol 80S ribosomes in the presence/absence of 1 mM AMPPNP or ADP, as indicated, for 5 min at 37°C in a 400 µl reaction mixture containing buffer A and subjected to centrifugation through 10-30 % SDG prepared in buffer A in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. Due to the limited availability, 25 pmol native murine SLFN14 was used in the same reaction. Fractions that corresponded to ribosomal complexes were resolved in NuPAGE 4-12 % Bis-Tris SDS-PAGE (Invitrogen) and stained with SimplyBlue SafeStain (Invitrogen) or assayed by immunoblotting.

rRNA Degradation. To study rRNA degradation in RRL and HEK293T cell extract, 5 µl RRL or HEK293T cell extract were incubated in a 40 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP and 0.2 mM GTP for periods ranging from 3 h to 12 h at 37°C. To compare rRNA degradation in RRL and 80S/SLFN14 binary system, 1.5 pmol 80S ribosomes were incubated with 1.5 pmol SLFN14 in a 40 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP and 0.2 mM GTP for periods ranging from 3 h to 12 h at 37°C. To investigate rRNA degradation of purified 40S and 60S subunits in the presence of SLFN14, 1.5 pmol 40S or 60S subunits were incubated with 30 pmol SLFN14 in a 40 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP and 0.2 mM GTP for periods ranging from 1 min to 40 min at 37°C. To test the endonucleolytic activity of SLFN14 in the ribosome-bound state, 1.5 pmol mentioned above SDG-purified 80S/SLFN14 complexes or 80S ribosomes were incubated in a 80 µl reaction mixture containing buffer A for 4 h at 37°C. To compare the endonucleolytic activity of rabbit and murine native forms of SLFN14 in the degradation of 18S rRNA, 2 pmol 40S subunit was incubated with 1.25-10 pmol protein in a 20 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP for 1 h at 37°C. After incubation, RNA was isolated with TRIzol reagent (Invitrogen) according to manufacturer's protocol and analyzed by DAFGE or 6 % denaturing PAGE, as appropriate.

Denaturing Agarose/Formaldehyde Gel Electrophoresis. Technique was performed essentially as described (44). RNA samples were loaded in a loading buffer (5x = 4 mM EDTA, 0.9 M formaldehyde, 20 % glycerol, 30.1 % formamide, 4x FA buffer, 0.4 µg/ml bromphenol blue) onto 1.2 % denaturing agarose/formaldehyde gel (3 % formaldehyde) prepared in FA buffer (20 mM MOPS, pH 7.0, 5 mM NaAc, 1 mM EDTA, 0.1 µg/ml ethidium bromide) and were resolved in FA buffer for 22 min at 4°C at 200V. After electrophoresis, gel was stained 2 times for 8 min each with 0.5 µg/ml ethidium bromide solution in water, washed 3 times for 5 min each with water and analyzed using shortwave UV (254 nm).

mRNA and tRNA Degradation. To detect the endonucleolytic activity in RRL, 0.1 pmol SDG-purified stalled EC was incubated with 10 µl RRL or RSW in a 40 µl reaction mixture

containing buffer A supplemented with 0.2 mM ATP and 0.2 mM GTP for 20 min at 30°C. To test mRNA and tRNA cleavage by SLFN14, 0.1 µg 5'-end 32P-labelled HBBmod mRNA, GUSmod mRNA, $tRNA^{His}$ or $tRNA^{Val}$ were incubated with 1 pmol SLFN14 in a 20 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP for periods ranging from 1 min to 30 min at 37°C. To study the dependence of SLFN14 on metal ions in the cleavage activity, 3 pmol SLFN14 was incubated with 50 ng 5'-end 32P-labelled GUSmod mRNA in a 20 µl reaction mixture containing Mg^{2+}-free buffer A supplemented with 1 mM metal ion and 0.2 mM ATP, as appropriate, for 25 min at 37°C. After incubation, RNA was isolated by phenol/chloroform extraction and resolved in 6% denaturing PAGE. Gels were dried and autoradiographed.

Analysis of Ribosome and Ribosomal Subunits Integrity after rRNA Degradation. To test the integrity of ribosome and ribosomal subunits after rRNA cleavage in the binary system, 35 pmol 80S ribosomes, 40S or 60S subunits were incubated in a 400 µl reaction mixture containing buffer A in the presence/absence of 160 pmol SLFN14 for 4 h at 37°C and subjected to centrifugation through 10-30 % SDG prepared in buffer A in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. To study the integrity of 80S monosomes and polysomes after RNA cleavage in RRL, 150 µl RRL after incubation in a 400 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP, 0.2 mM GTP and 0.1 mg/ml CHX for 12 h at 37°C or without incubation (control) was subjected to centrifugation through 10-50 % SDG prepared in buffer A in a Beckman SW41 rotor at 35,000 rpm for 150 minutes. After centrifugation, rRNA appearance and protein content of 80S-complex fractions were assayed by DAFGE and SDS-PAGE, respectively.

Functional Activity of rRNA-cleaved Ribosomes in Translation Cycle. To compare the activity of cleaved and intact ribosomes in the formation of 80S initiation complex, Met-Puro assay was employed essentially as described (17). 1 pmol MVHL-STOP mRNA, 5 pmol each eIFs 1, 1A, 2, 3, 4A, 4B, 4F, 1 pmol 35S-labelled Met-$tRNA_i^{Met}$ and 1 pmol intact or cleaved 40S subunits were incubated in a 20 µl reaction mixture containing buffer A with 0.2 mM ATP and 0.2 mM GTP for 10 min at 37°C, supplemented with 5 pmol eIF5, 5 pmol eIF5B, 1 pmol intact or cleaved 60S subunits, as appropriate, and additionally incubated for 10 min at 37°C. After assembly, ribosomal complexes were treated with 1 mM puromycin and extracted with ethyl acetate. 35S-labelled methionyl-puromycin formation was measured by scintillation counting of ethyl acetate extract. To compare the activity of cleaved and intact ribosomes in the entire translation cycle, different stages of translation- initiation, elongation, termination and ribosomal recycling- were reconstituted *in vitro* from individual purified components, and the formation of 80S initiation complex (as a result of initiation stage), pretermination complex (as a result of elongation stage) or mRNA release (as a result of termination and ribosomal recycling stages) were assayed by toeprint essentially as described (16).

RRL Ribosomal Profile Preparation. 50-200 µl Flexi RRL supplemented with 70 mM KCl and 1 mM GMPPNP was incubated for 5 min at 37°C and subjected to centrifugation through 10-30 % SDG prepared in buffer A in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. 200 µl fractions were collected and stored at -80°C. Ribosomes and ribosomal subunits-containing fractions were determined by the analysis of their rRNA content in DAFGE.

SLFN14 Protein Level and Localization in Cells. To test SLFN14 distribution in RRL ribosomal profile, 20 µl of each SDG fraction was subjected to SDS-PAGE and assayed by immunoblotting. To calculate SLFN14 to 80S ribosome ratio in 80S ribosome-containing fractions after RRL ribosomal profile preparation, 1 pmol 80S-complex and different amounts of

purified SLFN14 were subjected to SDS-PAGE and assayed by immunoblotting. To estimate SLFN14 prevalence among 80S ribosome-associated proteins in RRL, 20 µl 80S-complex fraction after RRL ribosomal profile preparation was subjected to SDS-PAGE and stained with SimplyBlue SafeStain. To compare SLFN14 protein level in RRL and different rabbit tissue lysates, 40 µg total protein of RRL from two different sources (Promega and Green Hectares) and brain, lung and liver rabbit tissue lysates were subjected to SDS-PAGE and assayed by immunoblotting with anti-SLFN14 and anti-eIF2α (loading control) antibodies. To determine SLFN14 distribution between nucleus and cytoplasm in cells, 40 µg total protein of nuclear or cytoplasmic extracts of HEK293T or MCF7 cells were subjected to SDS-PAGE and assayed by immunoblotting with anti-SLFN14, anti-HDAC2 (nuclear marker) and anti-GAPDH (cytoplasmic marker) antibodies.

Endonucleolytic Activity of Different Recombinant Forms of SLFN14. After affinity purification of SLFN14-65kDa on 80S ribosomes by centrifugation through SDG, 50µl aliquots of SDG fractions containing 80S/SLFN14-65kDa complex, 80S/empty vector expression complex and empty 80S ribosome were incubated in a 100 µl reaction mixture in buffer A supplemented with 0.2 mM ATP for 12 h at 37°C, and rRNA degradation was assayed by DAFGE.

To test the endonucleolytic activity of SLFN14-45kDa and its mutant forms, 50 ng 5'-end 32P-labelled HBBmod mRNA was incubated with 0.9µM protein in the presence/absence 1 mM Mg^{2+}-ions in a 20 µl reaction mixture containing buffer A supplemented with 0.2 mM ATP for 15 min at 37°C. After incubation, RNA was isolated by phenol/chloroform extraction and resolved in 6% denaturing PAGE. Gel was dried and autoradiographed.

Sequence and Structure Specificity of SLFN14 in Cleavage of Free and Ribosome-Associated mRNA. To test the sequence and structure specificity of SLFN14 in the cleavage of free mRNA, 1.5 pmol SLFN14 was incubated with 60 ng GUSmod mRNA or HBBmod mRNA in a 20 µl reaction mixture containing buffer A for 10 min at 37°C, mRNA was isolated, and cleavage sites were determined by AMV reverse transcriptase-mediated extension of 32P-labelled primer complimentary to mRNA. cDNA products were resolved in 6% denaturing PAGE. To compare the site specificity of SLFN14 in the cleavage of free and ribosome-associated mRNA, 80S initiation and elongation complexes were assembled on HBBmod mRNA *in vitro* from individual purified components, purified by SDG centrifugation and analyzed by toeprint as described (16). 0.15 pmol SDG-purified complexes were incubated with 1.5 pmol SLFN14 in a 20 µl reaction mixture containing buffer A for 10 min at 37°C, mRNA was isolated and assayed by primer extension.

Ribosomal Position of SLFN14. 4 pmol 40S subunits were incubated with 30 pmol SLFN14 in a 40 µl reaction mixture containing buffer A for 1 h at 37°C. Cleavage sites in 18S rRNA were identified by the extension of primers 1, 2 and 3 complimentary to 341-358 nt, 886-903 nt and 1825-1842 nt, respectively, of rabbit 18S rRNA.

SUPPLEMENTARY REFERENCES

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