Supporting Information

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SI Materials and Methods

Plasmids. pcDNA3.1-TPIp-Firefly (FLuc+i) and pcDNA3.1-TPIm-Firefly (FLucΔi) were created by cloning the human triose phosphate isomerase (TPI) exons 6 and 7 (85 and 38 nt, respectively) plus or minus intron 6 in frame with the Firefly luciferase gene in the pcDNA3.1+ vector (Life Technologies) downstream of the CMV promoter. The plasmid corresponding to the isolated *Renilla* luciferase (RLuc) cDNA was cloned in the same vector to create pcDNA3.1-Renilla (1). HA-Flag (HF)–tagged metastatic lymph node 51 plasmid (p-MLN51-HF) and HF-tagged eukaryotic initiation factor 4A3 plasmid (p-eIF4A3-HF) were cloned by inserting human MLN51 and eIF4A3 cDNAs in phase with the HF tag in the pcDNA3.1+ vector downstream of the CMV promoter. Flag (F)-tagged Magoh plasmid (p-F-Magoh), p-F-Y14 used for overexpression, pCR3.1-Flag-MLN51, and pCR3.1-Flag-NXF1, encoding nuclear RNA export factor 1 (NXF1), used for in vitro transcription, were created by cloning the respective human cDNAs in the pCR3.1 vector (Life Technologies) as described in ref. 2. Mutants of MLN51 were constructed by QuickChange mutagenesis using Phusion enzyme (Thermo Scientific), pcDNA3.1-MLN51-HF as a template, and the oligos TAA-GCA-TTT-GGA-TGA-TGA-TGA-AGA-caG-aAA-GAA-cCC-tGC-gTA-CAT-ACC-TCG-GAA-AGG-GCT-CTT-C/GAA-GAG-CCC-TTT-CCG-AGG-TAT-GTA-cGC-aGG-gTT-CTT-tCt-gTC-TTC-ATC-ATC-ATC-CAA-ATG-CTT-A (for the HF-tagged M523 siRNA-resistant plasmid, pCMV-MLN51r-HF) or GGA-AGG-ATG-AGG-GTC-GCT-GGG-AGg-cTG-ctA-AGT-TCC-GGG-AAG-ATG-AGC-AGG-CC/GGC-CTG-CTC-ATC-TTC-CCG-GAA-CTT-agC-Agc-CTC-CCA-GCG-ACC-CTC-ATC-CTT-CC [for pCMV-MLN51HD (MLN51 H220A/D221A mutant)-HF].

Protein Purification. Calmodulin-binding protein (CBP)-His and tandem-affinity purification (TAP)-His exon junction complex (EJC) proteins were expressed in *Escherichia coli* and purified as described in refs. 3 and 4. eIF4E protein was expressed in *E. coli* using the pGex-eIF4E plasmid. The protein was fixed on a GST column, and the elution was performed by cutting the GST tag using PreScission protease (GenScript). eIF4E was purified further using a MonoQ column (GE Healthcare). The eIF3 complex was purified from HeLa cells pellets essentially as described in refs. 5 and 6. 40S and 60S ribosomal subunits were purified from rabbit reticulocyte lysate (RRL) as previously described (7).

Cell Culture, Transfection, and siRNA Treatment. HEK293 cells (TRex Flp-In; Life Technologies) were cultivated in DMEM [(4.5 g/L), glucose (GIBCO)] supplemented with 10% (vol/vol) FCS (Life Technologies), and 1× penicillin/streptomycin (100× Pen/Strep mix, Life Technologies). HeLa cells were cultivated in the same medium but containing 1 g/L glucose. For EJC core protein overexpression, 1.5×10^5 HEK293 cells per well were seeded in 24-well plates. After 24 h, cells were transfected using JetPEI (Ozyme) according to the manufacturer's protocol with the following DNA mix: 10 ng of pcDNA3.1-TPIp-Firefly, 20 ng pcDNA3.1-TPIm-Firefly and/or 50 ng of pcDNA3.1-Renilla, and 0/0.4/0.8/1.2 μg of pCMV-MLN51-FH or pCMV-eIF4A3-FH or pCMV-F-Magoh/pCMV-F-Y14 mix, to a volume of 1.2 μg using pcDNA3.1+ vector. After 24 h of expression, luciferase assays and RNA quantification were done as described below.

For siRNA assays, 3.5×10^5 HEK293 cells per well were seeded in 12-well plates. Each well was transiently transfected 24 h later with 20 nM of siRNA (Eurogentec) and 1.5 μL RNAiMAX (Life Technologies) in 200 μL of OptiMEM (GIBCO) according to the manufacturer's protocol. Down-regulation experiments targeting GFP or MLN51 were performed using 5′-UGA-AUU-AGA-UGG-CGA-UGU-U-3′ (GFP), 5′-GAU-CGG-AAG-AAU-CCA-GCA-U-3′ (M523), or 5′-CAU-UCG-CUC-AGC-UCA-UAA-U-3′ (M72) (8). Twenty-four hours after siRNA transfection, cells were transfected using JetPEI (Ozyme) with the following DNA mix: 10 ng of pcDNA3.1-TPIp-Firefly or 20 ng of pcDNA3.1-TPIm-Firefly, 0/0.5/1.0/1.5 μg of pCMV-MLN51r-HF, completed to 2 μg with pcDNA3.1+ per well. After an additional 24 h, luciferase assays and RNA quantification were done as described below.

Luciferase Assays. To measure luciferase activity, cells were lysed in 1× Passive Lysis Buffer (Promega). Then 10 μL of lysate was used to detect luciferase activity. Light production was monitored using a Berthold Luminometer (Berthold Technologies) after the addition of 35 μL of the reagents provided (E1960 or E2810; Promega) with 1-s agitation, 1-s delay, and 10-s integration.

RNA Extraction and Analysis of the RNA Protection Assay. Total RNAs were extracted using Tri-Reagent (Sigma) according to the manufacturer's recommendations. For the RNA protection assay (RPA), radiolabeled probes were synthesized by T7 transcription (T7 RNA polymerase; Promega) with [α-32P]-CTP from templates generated by PCR: β-actin (gaa-ttc-gtt-atg-acc-atg-ggg-tca-gaa-gga-ttc-c/ cta-ata-cga-ctc-act-ata-ggg-gtt-atg-acc-atg-cag-aag-agg-tag-cgg-g), *Renilla* luciferase (gaa-ttc-gtt-atg-acc-atg-cca-tgt-tgc-cat-caa-aaa-tc/ cta-ata-cga-ctc-act-ata-ggg-gtt-atg-acc-atg-cat-ctt-ctt-gcg-aaa-aat-g), and Firefly luciferase (gaa-ttc-gtt-atg-acc-atg-cga-ggg-gga-tga-taa-a/ cta-ata-cga-ctc-act-ata-ggg-gtt-atg-acc-atg-cca-tcc-atc-ctt-gtc-aat-ca). Each probe was designed to span 200–300 nt of respective mRNA. RPAs were performed with 4 and 8 μg of total RNA plus 300 amol of probe as previously described (9). RNase digestions were performed with RNases A and T1 (Thermo Scientific). Gels were scanned with Typhoon FLA-9000, and quantifications were performed with ImageQuantTL (GE Healthcare). Firefly and *Renilla* luciferases mRNA levels were normalized to endogenous β-actin mRNA levels.

RRL. In vitro translation assays were performed with Flexi Rabbit Reticulocyte Lysates (micrococcal nuclease-treated RRL; Promega). For immunodepletion, 200 μL of RRL were incubated for 1 h at 4 °C rotating with (*i*) 50 μL Protein-A Dynabeads (Life Technologies) for mock RRL or (*ii*) 50 μL protein-A beads coupled to 14 μg anti-MLN51 for ΔMLN51 RRL; or (*iii*) 50 μL protein-A beads coupled to 14 μg anti-MLN51 and 50 μL protein-A beads coupled to 14 μg anti-eIF4A3 for ΔMLN51ΔeIF4A3 RRL. Protein depletion was monitored by analyzing 1 μL of each RRL by Western blot. Capped and poly(A)-tailed *Renilla* luciferase downstream of the globin 5′ UTR (glo-RLuc), F-NXF1, and F-MLN51 mRNAs were transcribed in vitro using the mMESSAGE mMACHINE kit from Ambion (Life Technologies) according to the manufacturer's protocol. Translation assays were performed in 10 μL reaction with 5 μL of RRL supplemented with 500 ng FLuc mRNA (uncapped mRNA provided by Promega), 15 ng glo-RLuc mRNA in the presence of 10 μM of amino acids minus Met or 10 μM of amino acids minus Leu, 0.1 mM KCl, and 0.5 mM $MgCl_2$. Reactions were incubated at 30 °C and quenched using 1 mM EDTA, and *Renilla* luciferase activity was measured as described above. The de novo synthesis of F-NXF1 and F-MLN51

was performed by incubating 500 ng of the respective in vitro capped and transcribed mRNAs in ΔMLN51 RRL. After 30 min at 30 °C, protein expression was assessed by Western blot analysis using anti-Flag antibody with 2 μL of reactions. The remaining 8 μL was complemented with 25 ng of glo-RLuc mRNA, 500 ng of competitor FLuc mRNA, and 5 μM of each of the amino acids mixes. After 30-min incubation the reaction was quenched with 1 mM EDTA, and *Renilla* luciferase activity was measured.

Immunoprecipitation. For each immunoprecipitation, a confluent 15-cm plate of HEK293 cells was washed in 1 mL PBS and lysed by incubation at 4 °C with 600 μL of RIPA lysis buffer [20 mM Tris·HCl (pH 7.5), 250 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Nonidet P-40 (Sigma), 1% sodium deoxycholate, 1% protease and phosphatase inhibitor (Pierce), and 6 U RQ1 DNase (Promega)]. Lysate was cleared by centrifugation at 16,000 × *g* for 10 min at 4 °C and was incubated for 5 min at 37 °C with or without 0.1 μg/μL of RNaseA (Fermentas). Then 10 μL of lysate was conserved as input, and the remainder was incubated for 2 h at 4 °C with 100 μL Protein-A Dynabeads (Life Technologies) crosslinked or not to antibodies (25 μg of purified anti-MLN51 or anti-eIF4A3). For crosslinking, beads were preblocked at 4 °C with 0.5 μg/μL BSA/0.1 μg/μL transfer RNA for 15 min, washed with IP150 buffer [10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 2.5 mM $MgCl_2$, 0.1% Nonidet P-40) and crosslinked using 25 mM dimethyl pimelimidate dihydrochloride (DMP; Sigma) in 0.2 M triethylamine (pH 8) for 30 min. To stop crosslinking, the buffer was replaced by 50 mM Tris·HCl (pH 7.5) for 15 min and washed twice with 0.1 M glycine (pH 3) to remove uncrosslinked antibodies. After incubation with radioimmuno-precipitation assay (RIPA) extract, beads were washed three times for 5 min with 1 mL IP150 buffer; then proteins were eluted by adding 10 μL of 1× loading dye [50 mM Tris·HCl (pH 6.8), 2% SDS, 10% (wt/vol) glycerol, 1.4 M β-mercaptoethanol, 0.05% bromophenol blue] and heating at 60 °C for 10 min. Input, supernatants, and precipitated proteins were resolved onto SDS/PAGE and blotted as described below.

Western Blot Analysis. Proteins were separated onto Tris-glycine SDS/PAGE or 4–12% 3-(N-morpholino)propanesulfonic acid (Mops) gels (Life Technologies) and were transferred onto 0.2-μm nitrocellulose membranes (Protan-BA83; GE Healthcare) using Bio-Rad Mini-Transblot systems (Bio-Rad). Membranes were blocked in PBS with 10% (wt/vol) milk and 0.1% Tween-20 (Euromedex). Polyclonal anti-eIF4A3 and anti-MLN51 (N-terminal) antibodies were produced and purified as described previously (10–12).

The following primary antibodies were used for detection: anti-Flag antibody (F7425; 1:1,000; Sigma); purified anti-eIF4A3 or anti-MLN51 (1:1,000); anti-RBM8A (Y14, NB100-55326; 1:1,000; Novus); anti-Magoh serum [1:1,000; a gift from E. Izaurralde (Max Planck Institute, Tübingen, Germany)]; anti-eIF2α (sc11386; 1:1,000; Santa Cruz Biotechnology); anti-eIF3a (NBP1-18891; 1:10000; Novus); anti-eIF3c (NB100-511; 1:15000; Novus); anti-eIF3d (NB10-93299; 1:4,000; Novus); anti-eIF4A1 (SC-14211; 1:1,000; Santa Cruz); monoclonal anti-eIF4E (610269; 1:1,000; BD); polyclonal anti-eIF4E (NB110-56952; 1:4,000; Novus); anti-eIF5 (611050; 1:4,000; BD); anti-rpS14 (ab66778; 1:4,000; Abcam); anti-rpL26 (ab59652; 1:4,000; Abcam); anti-PABP (NB120-6125; 1:5,000; Novus); anti-GAPDH (2118; 1:2,000; Cell Signaling); anti-tubulin (1:10,000; Covance Research Products); anti-HA (H6908; 1:1,000; Sigma); and anti-Rab5 [1:1,000; (10)]. After washing, membranes were incubated with stabilized goat anti-rabbit or goat anti–mouse-HRP secondary antibodies (1:8,000; Thermo Scientific) and visualized using SuperSignal West Femto (Thermo Scientific) with LAS4000mini (GE Healthcare). Signals were quantified with ImageQuantTL software (GE Healthcare) using the Rolling Ball background subtraction method.

Sucrose Gradient Centrifugation. HeLa cells were maintained in their respective medium supplemented with FCS at 37 °C. Cycloheximide (100 μg/mL) was added, and cells were incubated further for 30 min to stabilize polysomes. Cells were washed with cold PBS buffer containing 100 μg/mL cycloheximide nd then were collected and lysed in a hypotonic lysis buffer [200 mM Tris·HCl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 40 U/mL RNasin, 5 mM DTT, 1% Nonidet P-40, 1× complete EDTA-free (Roche)]. Lysates were loaded on top of 10–40% (wt/vol) sucrose density gradients [50 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl] and were sedimented by ultracentrifugation in a Beckmann SW41 at 36,000 rpm (250,000 × *g*) for 2 h at 4 °C. After centrifugation, gradients were fractionated from top to bottom, and the OD at 254 nm was recorded continuously using an ISCO fractionator (Teledyne ISCO). Each fraction was subjected to alcohol precipitation, and the subsequent pellet was suspended with SDS sample buffer. Proteins were separated onto 7.5–15% SDS/PAGE. Immunoblotting was performed as described above.

^{35}S-Met/Cys Metabolic Labeling. For metabolic labeling under siRNA conditions, 7.10^5 HEK293 cells per well were seeded in six-well plates containing 20 nM siRNA, 3 μL RNAiMAX, and 200 μL OptiMEM as described above and were incubated for 48 h. For overexpression experiments, 5.10^5 HEK293 cells were seeded in six-well plates, were transfected 24 h later with 3 μg of corresponding plasmid using JetPEI (Polyplus Transfection) according to the manufacturer's protocol, and were incubated for another 24 h. After siRNA or overexpression treatment, 100 μg/mL cycloheximide was added, and the cells were incubated further for 1 h. Then medium was replaced by Met/Cys-free DMEM [DMEM minus Met/Cys/Gln (Life Technologies] supplemented with 1× GlutaMAX (Life Technologies) and 100 μg/mL cycloheximide. After 20 min, the Met/Cys-free DMEM was replaced by radiolabeled DMEM [DMEM minus Met/Cys/Gln, 1× GlutaMAX, 10 μCi/mL EasyTag EXPRESS35S Met/Cys Protein Labeling Mix (Perkin-Elmer)]. After 1 h, medium was removed, and the cells were washed once with PBS1× and were lysed using 100 μL RIPA buffer. The protein concentration was determined using 1× Bradford reagent (Bio-Rad), and labeled cellular extracts were resolved onto SDS/PAGE. After gel drying, radioactivity in each sample was quantified using the Typhoon device and ImageQuantTL software (GE Healthware).

In Vitro Binding Assays. For binding assays, 10 μmol of each EJC-purified protein was incubated with 6 μmol of purified eIF3 in 30 μL 1× binding buffer (BB1) × 150 [20 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM $MgAc_2$, 2 mM imidazole, 2 mM $CaCl_2$, 0.1% Nonidet P-40, 10% (wt/vol) glycerol, 1 mM DTT]. After 2 h of interaction at 4 °C under rotation, 10 μL of calmodulin beads (Stratagene) and 190 μL of BB1 × 250 (250 mM NaCl) were added for 1.5 h. After three washes with 200 μL of BB1 × 200 (200 mM NaCl), proteins were eluted by incubation with 25 μL of elution buffer [10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM $MgAc_2$, 2 mM imidazole, 20 mM EGTA, 0.1% Nonidet P-40, 14% (wt/vol) glycerol, 10 mM β-mercaptoethanol] for 5 min at 30 °C, shaking at 1,400 rpm. Proteins bound to calmodulin beads were eluted with 10 μL of 1× loading dye and were separated onto SDS/PAGE gels before Coomassie staining or Western blotting. For interaction with EJC cores, EJC cores were preformed by mixing 200 μmol of eIF4A3, Magoh/Y14Δ50 (M/Y14Δ50), the MLN51 SELOR domain (MLN51.S) or the MLN51 N-terminal half (MLN51.Nt), and 0.5 μM of 20mer ssRNA with or without 2.5 μM ADPNP in 40 μL of BB1 × 150. After 30 min at 30 °C, 6 μL of Dynabeads (MyOne streptavidin T1 beads; Life Technologies) were added before rotation at 4 °C for 1 h. Beads were washed two times with 300 μL of BB1 × 500 and once with 300 μL of BB1 × 250. Half of

the beads were eluted using 15 μL of 1× loading dye and were resolved onto 13.5% SDS/PAGE gel before Coomassie staining. The remainder was resuspended in 30 μL of BB1 × 125 with 60 μmol of purified eIF3 and was incubated for 2 h at 4 °C. After three washes with 300 μL of BB1 × 200, proteins bound to streptavidin beads were eluted using 15 μL of 1× loading dye. The eluted proteins were separated onto 12% SDS/PAGE gel before Western blotting.

Far Western Blot. BSA (New England Biolabs) and HeLa-purified eIF3 were separated onto SDS Tris-glycine 13.5% PAGE or Mops 4–12% PAGE (Life Technologies) and were transferred onto nitrocellulose membrane. One eIF3 lane was Coomassie or silver stained according to the manufacturer's protocol (Life Technologies). Membranes then were placed at 4 °C under agitation and were incubated 10 min in 25 mL of denaturing buffer [basic buffer (BB): 20 mM Hepes (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40 with 6 M guanidine hydrochloride (Sigma)]. Then half of the denaturing buffer was exchanged for BB five times at 10-min intervals, followed by overnight incubation in 25 mL blocking buffer (1× BB, 5% milk). The next day, membranes were washed once with BB and were probed for 4 h with TAP-His, TAP-MLN51.Nt-His, TAP-MLN51 C-terminal half (TAP-MLN51.Ct)-His, or TAP-MLN51.S-His in interaction buffer [1× BB, 1% milk, 5% (wt/vol) glycerol]. Three 10-min washes were performed with washing buffer 1 (WB1) [1× PBS, 0.2% TritonX-100 (Euromedex)], two washes were performed with washing buffer 2 (1× WB1, 150 mM KCl), and one last wash was performed with WB1. TAP-tagged proteins were detected by Western blot using peroxidase anti-peroxidase (Sigma).

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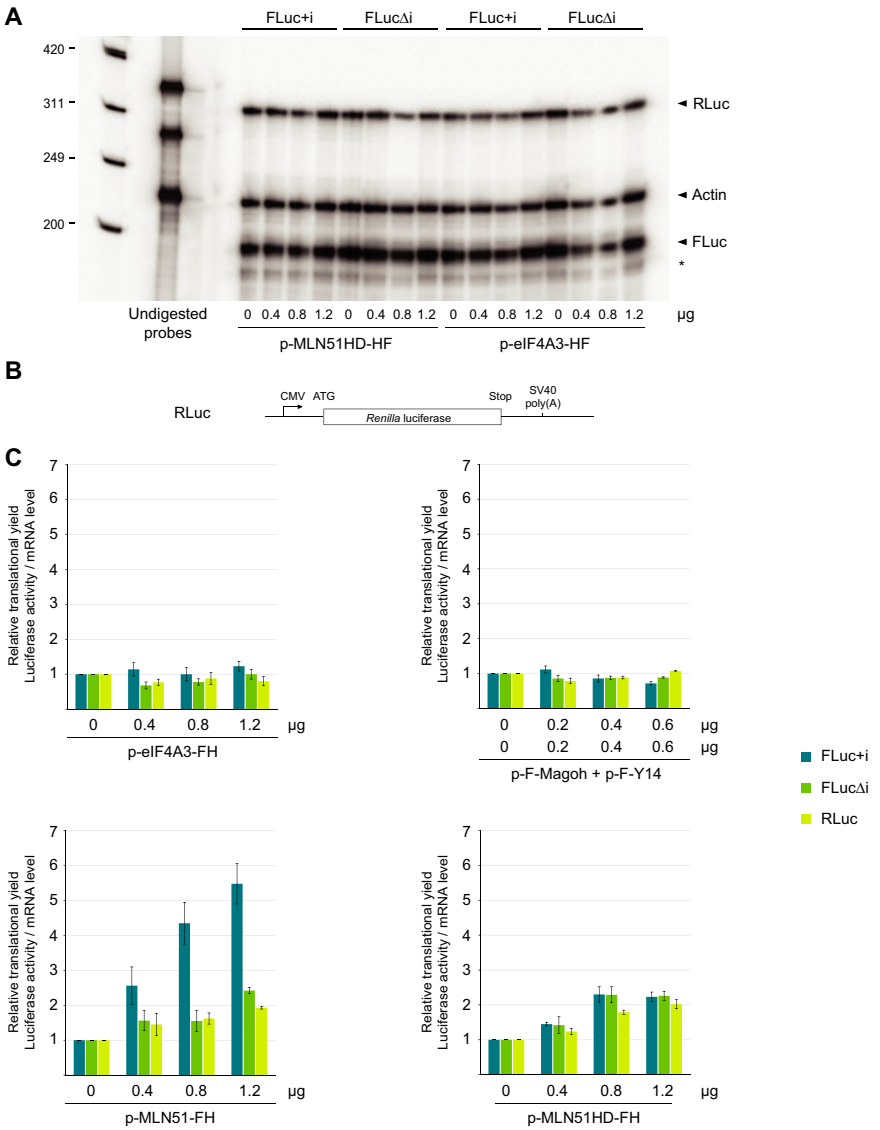

Fig. S1. (*A*) Quantification of FLuc, RLuc, and actin RNA levels by RPA in HEK293 cells expressing increasing amounts of the plasmids p-MLN51-HF and p-eIF4A3-HF as indicated. The molecular ladder is indicated on the left. FLuc-, RLuc-, and actin-digested probes are on the right. The asterisk marks a degradation of actin probe. (*B*) Schematic representation of the RLuc intronless construct. (*C*) Bar charts representing the translational yields of the intron-containing FLuc+i reporter (dark blue), the intronless FLucΔi reporter (light green), and intronless RLuc (orange) in cells transfected with the indicated amount of the plasmids expressing EJC core proteins. Translational efficiencies were normalized to the expression level of each reporter measured by RPA. Data represent mean values ± SD of three independent experiments.

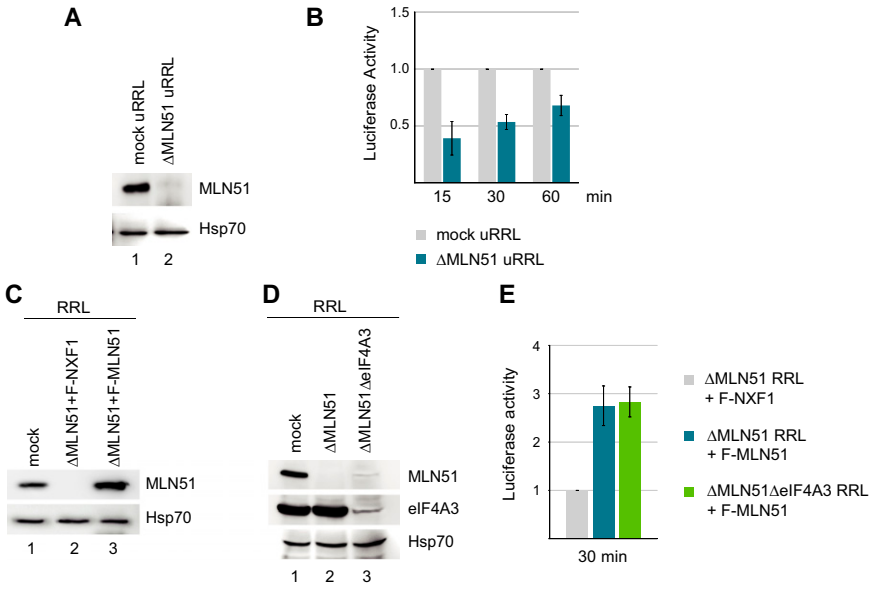

Fig. S2. MLN51 enhances translation efficiency in untreated RRL translation extracts. (*A*) Western blots with antibodies specific to the indicated protein in mock-depleted untreated RRL (uRRL, lane1) and in MLN51-depleted uRRL (lane 2). (*B*) Bar charts representing the luciferase activity of the glo-RLuc reporter in mock-depleted uRRL (gray bars) and ΔMLN51 uRRL (blue bars) after incubation for 15, 30, and 60 min. Data represent mean values ± SD measured from three independent experiments. (*C*) Western blots of MLN51 and heat shock protein 70 (Hsp 70) in mock-depleted (lane 1) and ΔMLN51 RRL under expression of F-NXF1 (lane 2) or F-MLN51 (lane 3). (*D*) Western blots of MLN51, eIF4A3, and Hsp70 in mock-depleted RRL (lane1), in MLN51-depleted RRL (ΔMLN51, lane 2), and in RRL depleted for both MLN51 and eIF4A3 (ΔMLN51ΔeIF4A3, lane 3). (*E*) Bar charts representing the luciferase activity of the glo-RLuc reporter in ΔMLN51 RRL expressing F-NXF1 (gray bars) or F-MLN51 (blue bars) and in ΔMLN51ΔeIF4A3 RRL expressing F-MLN51 (blue bars). Data represent mean values ± SD from three independent experiments.

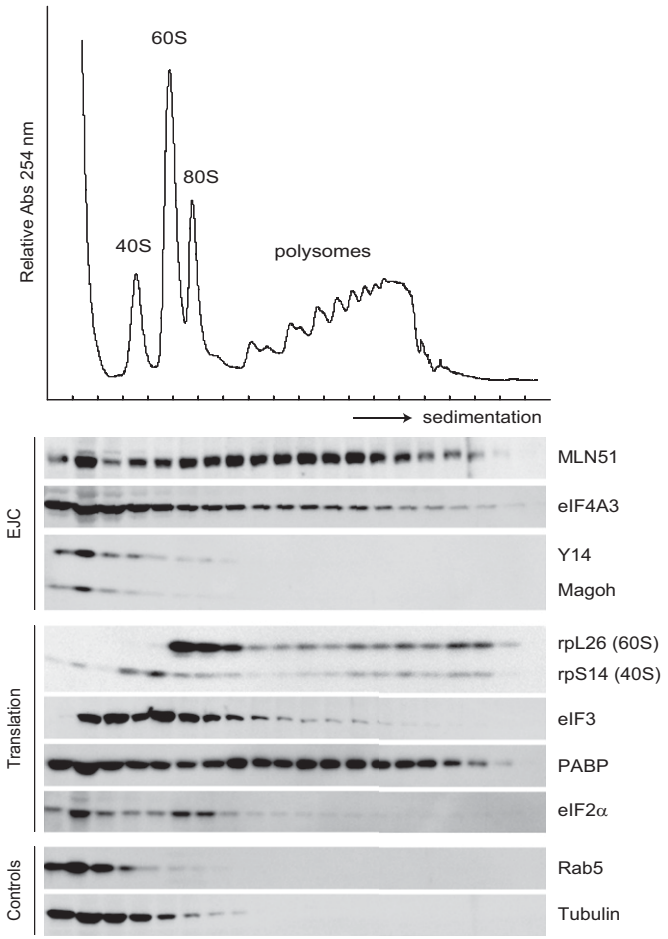

Fig. S3. Sucrose gradient fractionation of cytoplasmic HeLa cell extracts. (*Upper*) Relative absorbance at 254 nm. 40S, 60S, and 80S monosomes and polysome fractions are indicated. (*Lower*) Western blots with indicated antibodies targeting EJC, translation, or control proteins. PABP, poly(A)-binding protein; Rab5, ras-related small GTP binding protein 5.

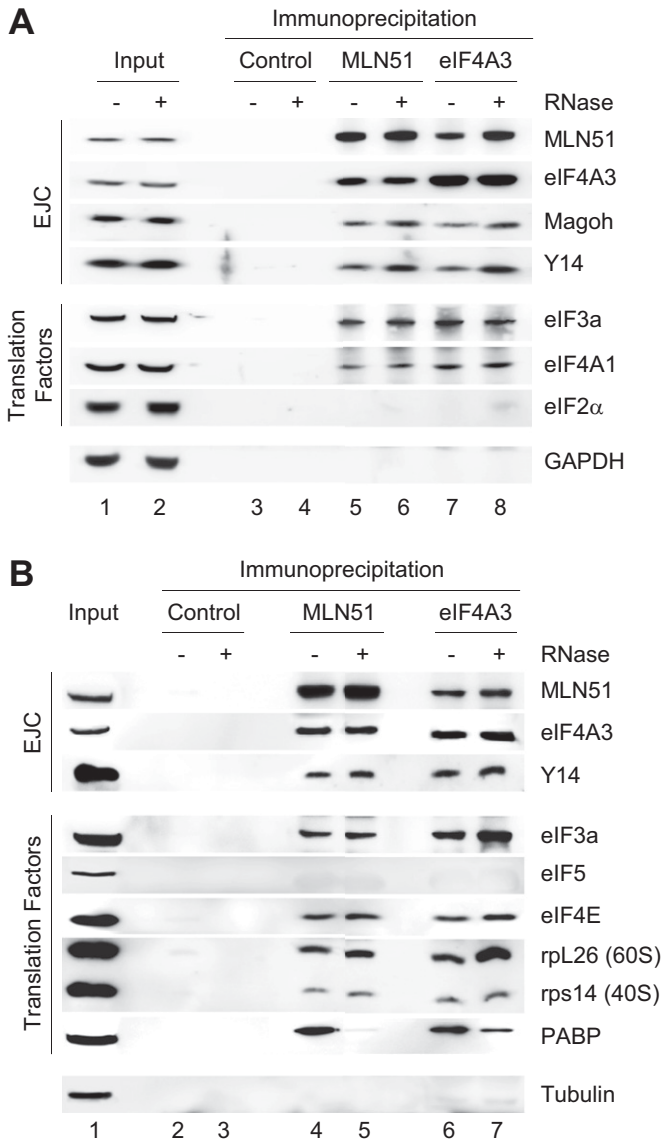

Fig. S4. MLN51 and eIF4A3 associate with EJC and translation factors in human cell extracts. (*A*) Protein extracts from HeLa cells treated by RNase or left untreated were subjected to immunoprecipitation without antibody (Control) or with the antibodies anti-MLN51 and anti-eIF4A3. Inputs (lane 1) and immunoprecipitation fractions (lanes 2–7) were resolved by SDS/PAGE and were immunoblotted with antibodies targeting EJC core proteins, translation factors, and control antibodies as indicated. (*B*) As in *A* with extracts from HEK293 cells.

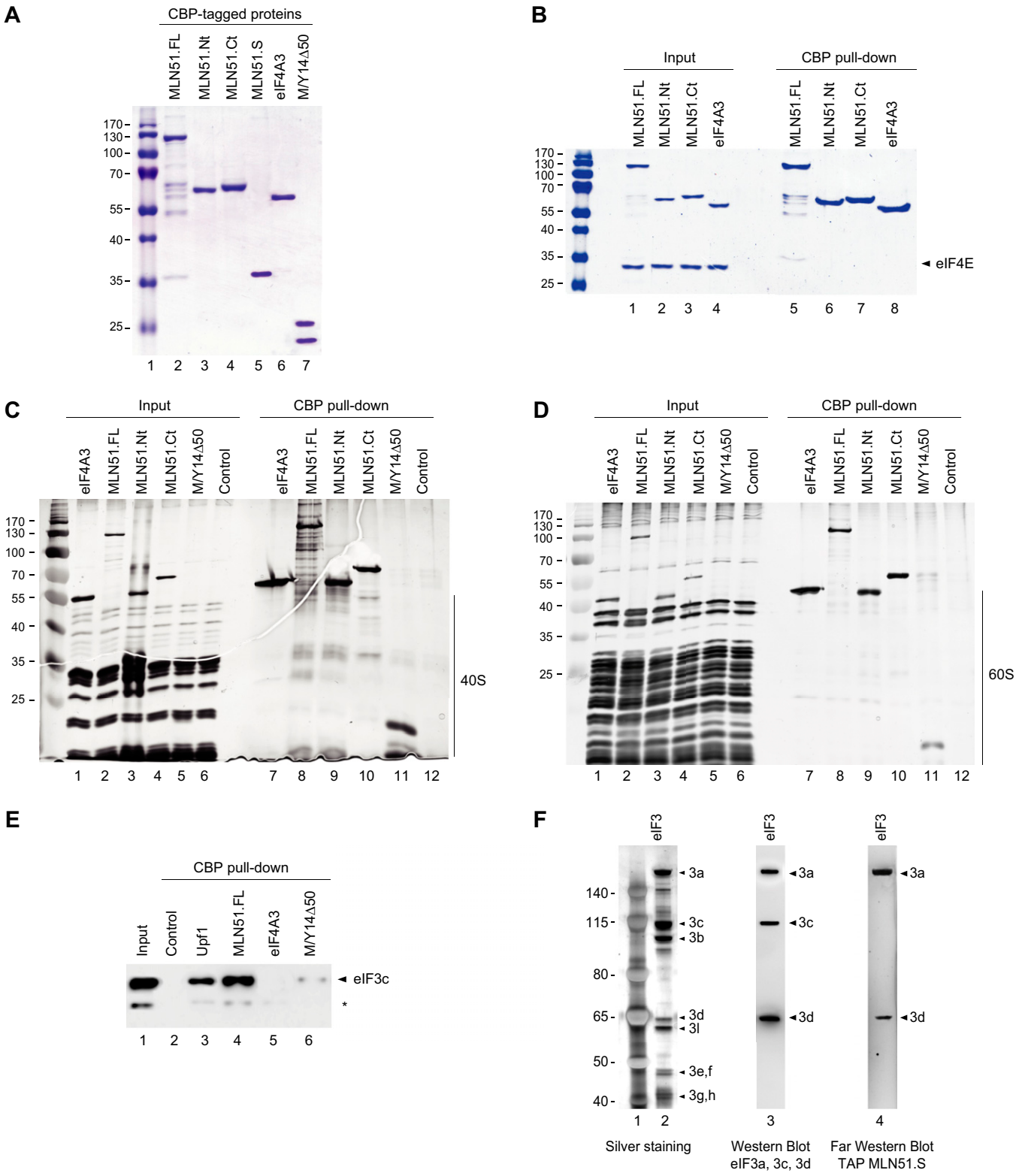

Fig. S5. Interaction CBP pull-down assays with purified EJC core proteins and eIF4E, 40S, or 60S ribosomal subunits. (*A*) Coomassie blue staining showing the different recombinant EJC core proteins purified from *E. coli*. Lane 1, protein molecular mass standards (kDa). (*B*) Interaction of eIF4E with CBP-tagged MLN51.FL, MLN51.Nt, MLN51.Ct, and eIF4A3. Protein mixtures before (10% of total, lanes 1–4) or after (lanes 5–8) precipitation were separated onto SDS/PAGE (13.5% acrylamide) before Coomassie staining. (*C*) Interaction of the 40S ribosomal subunit purified from RRL with the CBP-tagged proteins indicated on the top. Protein mixtures before (10% of total, lanes 1–4) or after (lanes 5–8) precipitation were separated onto SDS/PAGE (13.5% acrylamide) before silver staining. (*D*) As in *C* with the 60S ribosomal subunit purified from RRL. (*E*) CBP pull-down performed with CBP-tagged EJC proteins and HeLa purified eIF3. Western blotting using the anti-eIF3c antibody was used to monitor eIF3 coprecipitation in the absence of recombinant proteins (lane 2) or in the presence of the indicated proteins. The input lane (lane 1) represents 30% of the total. The asterisk marks a degraded fragment of eIF3c. (*F*) Far Western analyses. eIF3 complex resolved by 4–12% SDS/PAGE was analyzed by silver staining (lane 2), Western blotting with anti-eIF3a, anti-eIF3c, and anti-eIF3d antibodies (lane 3), and by Far Western blotting with TAP-MLN51.S as in *C* (lane 4).