SUPPLEMENTAL MATERIALS AND METHODS

Plasmids

Bacterial expression vectors for His_6-tagged eIF1, eIF1A, eIF4A, eIF4B, $eIF4G_{736-1115}$, eIF5, eRF1, eRF3aC that has a deletion of the first 138 amino acids (referred to as eRF3 in the text), Pelota, Hbs1 and *Escherichia coli* methionyl tRNA synthetase, as well as transcription vectors for $tRNA_i^{Met}$, $tRNA^{Leu}$-AAG, $tRNA^{Ser}$-AGA, $tRNA^{His}$-GUG and $tRNA^{Val}$-CAC have been described (Pestova et al., 1996, 1998, 2000; Lomakin et al., 2000, 2006; Pestova and Hellen 2001; Frolova et al., 1994, 1999; Pisarev et al., 2007a, 2010; Pisareva et al., 2011; Zinoviev et al., 2015). An expression vector for FLAG-tagged Pelota was prepared by inserting a FLAG-tag between the Pelota ORF and the C-terminal His_6-tag in the vector described in Pisareva et al., 2011 (GeneWiz, South Plainfield, NJ). Mammalian expression vectors pcDNA3.1+/C-(K)DYK for FLAG-tagged rat GTPBP2 and human Hbs1 were purchased from GenScript (Piscataway, NJ).

pDONR221-GTPBP1 and pCMV-SPORT6-GTPBP2 containing ORFs for GTPBP1 and GTPBP2, respectively, were obtained from the PlasmID Repository (DF/HCC DNA Resource Core, Harvard Medical School, Boston, MA). The full-length GTPBP1 (aa 1-669) and its two fragments (aa 152-669 and 152-586) were amplified with flanking BamHI and XhoI restriction sites and inserted into the pET28a vector, yielding vectors for expression of N-terminally His_6-tagged proteins. The full-length GTPBP2 (aa 1-602) and its two fragments (aa 89-602 and 164-602) were amplified with flanking NcoI and BamHI restriction sites and inserted into pET28a, yielding vectors for expression of C-terminally His_6-tagged proteins.

Human eEF1Bα (Genbank BC000211), eEF1Bγ (Genbank BC000384) and eEF1Bδ (Genbank BC009907) subunits of eEF1B were amplified from cDNA clones MGC:2349 (IMAGE:3353094), MGC:8423 (IMAGE:2821006) and 5267295 (IMAGE:2961609), respectively (American Type Culture Collection, Manassas, VA), and cloned into pET28a using BamHI/XhoI, HindIII/XhoI and BamHI/XhoI sites, respectively, yielding vectors for expression of N-terminally His_6-tagged proteins.

Transcription vectors for MF-STOP, MLHL-STOP, MSHL-STOP, MSSL-STOP, MLLSSF-STOP mRNAs were based on the previously described MVHL-STOP vector (Alkalaeva et al., 2006) that includes a 5'UTR derived from the native β-globin mRNA and a 3'UTR composed of nt 16-121 of β-globin ORF, and generated by inserting an appropriate DNA fragment flanked by a T7 promoter and HindIII restriction site into pUC57 (GeneWiz, South Plainfield, NJ). Transcription vectors for MF-1 and MF-2 mRNAs were based on the MF-STOP vector, but contained distinct 3'UTRs:

MF-1 3'UTR: 5'-UCUGUAAAGAAGUCUGCGGUCACUGCCCUGUGGGGAAGGUGAAUCUGGAA GAAGUUGGUGGUGAGGACCUGUCCUCUGCAAAUCCUGUUAAGAACAAUCCUAAGGAAGCU-3'

MF-2 3'UTR: 5'-UCUGUAAAUAAGUCUGCAGUCACUGAAAUCUGAAGAAAGAGAAUCUGAAA GAAGUUGGUAGUGAGGACCUGUAAUCUUCAAAUACUGUUAAGAACAAUAAUAAAAAAGCU-3'

Transcription vectors for human $tRNA^{Phe}$-GAA [C_{50}→G_{50}/G_{64}→C_{64}] (containing a double substitution that has no effect on activity; Nazarenko et al., 1992), murine $tRNA^{Arg}$-UCU and murine $tRNA^{Trp}$-CCA were made by inserting the appropriate DNA fragment flanked by a T7 promoter and a BstNI restriction site into pUC57, and human $tRNA^{Tyr}$-

GUA was made by inserting its DNA fragment flanked by a T7 promoter and a FokI restriction site into pUC57 (GeneWiz). All RNAs were transcribed using T7 RNA polymerase.

Purification of translation factors, ribosomal subunits and RNA exosome

Mammalian native 40S and 60S ribosomal subunits, eIF2, eIF3, eIF5B, eEF1H and eEF2 were purified from rabbit reticulocyte lysate (RRL) (Green Hectares, Oregon, WI) as described (Pestova and Hellen, 2003; Pisarev et al., 2007b). Recombinant His_6-tagged eIF1, eIF1A, eIF4A, eIF4B, $eIF4G_{736-1115}$, eIF5, eRF1, eRF3, Pelota, Hbs1 and *E. coli* methionyl tRNA synthetase were expressed in *E. coli* BL21 (DE3) and purified as described (Alkalaeva et al., 2006 and references therein; Pisarev et al., 2007b; Pisareva et al., 2011). Cell extracts from HeLa S3 and Krebs 2 cells were prepared as described (Pelletier and Sonenberg, 1988; Svitkin et al., 1985).

Native GTPBP1 was purified from RRL. Purification was monitored by Western blotting, using GTPBP1-specific antibodies (ab139088, Abcam). The 0-40% ammonium sulfate precipitation fraction of the 0.5 M KCl ribosomal salt wash that was prepared from ~10 L of RRL (Green Hectares) (Pisarev et al., 2007b) was dialyzed against buffer A (20mM Tris–HCl pH 7.5, 5% glycerol, 2mM DTT, 0.1mM EDTA) + 100 mM KCl and loaded on a DEAE (DE52) column equilibrated with buffer A + 100 mM KCl. The flow-through fraction was applied to a phosphocellulose (P11) column equilibrated with buffer A + 100 mM KCl. The column was washed with buffer A + 300 mM KCl, and GTPBP1 was eluted with buffer A + 400 mM KCl. The GTPBP1-containing fraction was dialyzed against buffer A + 100 mM KCl, diluted with 20 mM phosphate buffer (pH 7.5) supplemented with 5% glycerol and 1.5 mM β-mercaptoethanol, and subjected to FPLC on a hydroxyapatite column. Fractions were collected across a 20-500 mM phosphate buffer gradient, and GTPBP1 was eluted at ~180 mM phosphate buffer. GTPBP1 containing fractions were diluted with buffer B (20mM Hepes–KOH pH 7.5, 5% glycerol, 2mM DTT, 0.1mM EDTA) + 100 mM KCl and applied to a FPLC MonoS 5/50 GL column equilibrated with buffer B + 100 mM KCl. Fractions were collected across a 100-500 mM KCl gradient, and GTPBP1 was eluted at ~300 mM KCl.

Recombinant His_6-tagged GTPBP1, $GTPBP1_{152-669}$, $GTPBP1_{152-586}$, GTPBP2, $GTPBP2_{89-602}$ and $GTPBP2_{164-602}$ were expressed in 4-16 L of *E. coli* BL21 DE3 Star (Invitrogen). Protein production was induced by addition of 0.5 mM IPTG, after which cells were grown for 16 hours at 16ºC. All proteins were isolated by affinity chromatography on Ni-NTA agarose followed by FPLC on a MonoS 5/50 GL column.

Recombinant His_6-tagged eEF1Bα, eEF1Bγ and eEF1Bδ were expressed in 2 L of *E. coli* BL21 DE3 Star. Protein production was induced by addition of 1 mM IPTG, after which cells were incubated for 3 hours at 37ºC. Proteins were isolated by affinity chromatography on Ni-NTA agarose followed by FPLC on a MonoQ 5/50 GL column. Purified recombinant SMAD1 with a N-terminal GST-tag was purchased from Creative Biomart (Shirley, NY).

FLAG-tagged GTPBP2 and Hbs1 were expressed in HEK293T cells. Cells were grown in a standard, Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% FBS (Gibco), 1X Glutamax (Gibco) and 1x penicillin/streptomycin (Gibco) at 37°C with 5% CO_2 to 70%-90% confluency and transfected with an expression plasmid using Lipofectamine 3000 reagent (Invitrogen). After 72 hours, cells were harvested and lysed on ice in buffer C (50 mM Hepes pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 1x protease inhibitor cocktail, 2 mM DTT, 10%

glycerol, 1% Triton X-100). Cell debris was pelleted by centrifugation at 20,000 g for 10 min. The supernatants were loaded twice on a 100 μl anti-FLAG M2 affinity agarose gel (Sigma-Aldrich), washed with 6 ml buffer C, 6 ml buffer D (50 mM Hepes pH 7.4, 5 mM $MgCl_2$, 2 mM DTT, 10% glycerol) + 400 mM KCl, 6ml buffer D + 100 mM KCl and eluted by incubation with buffer D + 100 mM KCl supplemented with 0.2 mg/ml FLAG peptide for 30' at room temperature.

RNA exosome and DIS3 were purified by a single step FLAG affinity chromatography from EXOSC10-3xFLAG and DIS3-3xFLAG expressing HEK293 cells using "ExoI extraction solution" (20 mM Hepes-KOH, pH 7.4, 300 mM NaCl, 1% Triton X-100, 1x protease inhibitor cocktail, 5% glycerol), as described (Domanski et al, 2016).

Preparation and aminoacylation of tRNA

$tRNA_i^{Met}$, $tRNA^{Leu}$-AAG, $tRNA^{Ser}$-AGA, $tRNA^{Phe}$-GAA, $tRNA^{Arg}$-UCU, $tRNA^{His}$-GUG, $tRNA^{Trp}$-CCA, $tRNA^{Tyr}$-GUA and $tRNA^{Val}$-CAC were transcribed using T7 RNA polymerase. For filter binding experiments, $tRNA^{Phe}$-GAA was transcribed in the presence of [α-^{32}P]GTP. Yeast transcript $tRNA^{Cys}$-GCA was purchased from tRNA Probes (College Station, TX, USA). Native calf liver total tRNA was purchased from Promega. Yeast native $tRNA^{Phe}$ was purchased from Sigma or purified as described (Rodnina and Wintermeyer, 1992). Native yeast $tRNA^{Lys}$ and $tRNA^{Val}$ as well as the native HeLa total tRNA were purified, as described (Rodnina and Wintermeyer, 1992; Rezgui et al., 2013).

In vitro transcribed $tRNA_i^{Met}$ was aminoacylated using *E. coli* methionyl tRNA synthetase, as described (Pisarev et al., 2007b). Elongator tRNAs were aminoacylated with the matching amino acids using total native aminoacyl-tRNA synthetases or purified specific yeast tRNA synthetases, as described (Pisarev et al., 2007b; von der Haar 1979). For filter binding experiments, $tRNA^{Cys}$ was aminoacylated with [^{35}S]Cys. Leu-$tRNA^{Leu}$ and Phe-$tRNA^{Phe}$ were separated from non-aminoacylated tRNAs by HPLC on an RP18 column. Native total aa-tRNA, Lys-$tRNA^{Lys}$ and Val-$tRNA^{Val}$ were purified by binding to His_6-tagged EF-Tu, followed by affinity chromatography on Ni-NTA agarose, phenol-chloroform extraction and subsequent precipitation of the nucleic acids (Rezgui et al., 2013).

UV cross-linking

0.3-0.5 μM GTPBP1, $GTPBP1_{152-669}$, $GTPBP1_{152-586}$, native GTPBP1, GTPBP2, $GTPBP2_{89-602}$ or $GTPBP1_{162-602}$ were incubated for 5 minutes at 30ºC in 30 μl buffer E (20 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 100 mM KCl, 0.25 mM spermidine, 2 mM DTT) with 120 nM [α-^{32}P]GTP and combinations of 0.4 μM *in vitro* transcribed $tRNA^{Phe}$, $tRNA^{Leu}$, $tRNA^{Ser}$, $tRNA^{Arg}$, $tRNA^{Cys}$, $tRNA^{His}$, $tRNA^{Trp}$, $tRNA^{Tyr}$, or $tRNA^{Val}$, 0.4 μM native yeast $tRNA^{Phe}$, 0.7 μM native total calf liver tRNA, 0.4-0.7 μM *in vitro* transcribed Met-$tRNA_i^{Met}$, Leu-$tRNA^{Leu}$ or Ser-$tRNA^{Ser}$, 0.4 μM native yeast Phe-$tRNA^{Phe}$, Lys-$tRNA^{Lys}$, or Val-$tRNA^{Val}$, 0.7 μM native total calf liver aa-tRNA, 0.6 μM eRF1, 0.6 μM Pelota, 0.3 μM SMAD1, 0.5 μM eEF1Bα, 0.2 μM eEF1Bγ and 0.2 μM eEF1Bδ in the presence of 0.5 mg/ml casein. Assembled complexes were irradiated on ice for 10 min at 254 nm, treated with RNAses A, V1 and T1 for 15 min at 37°C, and analyzed by 4-12% SDS-PAGE followed by fluorescent SYPRO staining (Invitrogen) (to ensure equal loading) and autoradiography.

Fluorescence-based stopped-flow kinetic measurements

Fluorescence-based kinetic measurements were performed using a SX-20MV stopped-flow apparatus (Applied Photophysics) in which equal volumes of the reactants (60 µl each) were rapidly mixed and fluorescence changes were monitored with time. All reactions were performed in buffer E at 25ºC. The interaction between GTPBP1 and mant-GTP or mant-GDP was monitored by changes in FRET between the protein's tryptophan residues and the mant group. Tryptophan fluorescence was excited at 290 nm and FRET (peak at 440 nm) measured after passing through a KV408 filter (Schott). In the binding reactions, 50 nM GTPBP1, in the presence or absence of 0.3 µM yeast native Phe-tRNA^{Phe} or 0.3 µM yeast native tRNA^{Phe}, was mixed with 5 µM (or indicated concentrations) of mant-GTP/GDP, as indicated. In the dissociation reactions, 50 nM GTPBP1, in the presence or absence of 0.3 µM yeast native Phe-tRNA^{Phe}, 0.3 µM yeast native tRNA^{Phe}, 50 nM human native ∑aa-tRNA or 50 nM human native ∑tRNA, was preincubated with 5 µM mant-GTP or mant-GDP for 5 minutes at 25ºC and mixed with 0.5 mM GTP or GDP, respectively.

Time courses shown in the figures were derived from averaging 4-7 technical replicates. Data were evaluated by fitting the curves with a single or double exponential function containing the terms - apparent rate constant (k_{app}), amplitude (A) and final signal (F^{∞}) according to the equations $F=F^{\infty}+A\bullet\exp(-k_{app}\bullet t)$ or $F=F^{\infty}+A_1\bullet\exp(-k_{app1}\bullet t)+A_2\bullet\exp(-k_{app2}\bullet t)$. All calculations were performed using Prism (Graphpad Software).

Filter binding assay

0.3 nM [^{35}S]Cys-tRNA^{Cys} or [^{32}P]tRNA^{Phe} were incubated for 10 min at 37ºC in 50 µl buffer E with 0.5-2000 nM GTPBP1 in the presence/absence of 0.2 mM GTP or GDP. The resulting complexes were passed through a 0.45 µm nitrocellulose filter (Protran BA 85), which was subsequently washed with 5 ml buffer C, and the amount of retained [^{35}S]Cys-tRNA^{Cys} or [^{32}P]tRNA^{Phe} was determined by scintillation counting. The resulting curves were fitted to the Hill equation using GraphPad Prism software.

GTP hydrolysis assay (related to Figures 3, 5K, 6E-G and S5B)

0.25 µM native or recombinant full-length GTPBP1, GTPBP1$_{152-669}$, GTPBP1$_{152-586}$, GTPBP2$_{89-602}$, eRF3 or Hbs1 were incubated for 20 min at 37ºC in 10 µl buffer E with 40-80 nM [α-^{32}P]GTP in the presence/absence of different combinations of 0.1 µM 80S ribosomes, 10 nM purified 80S ECs (containing CUU, UCU or UUC codons in the A site), 0.5 µM tRNA^{Leu} or tRNA^{Phe}, 0.5 µM Leu-tRNA^{Leu} , Ser-tRNA^{Ser} or Phe-tRNA^{Phe}, 0.25 µM SMAD1, 0.25 µM Pelota and 0.25 µM eRF1. [α-^{32}P]GTP and [α-^{32}P]GDP in the reaction mixtures were separated using TLC on polyethyleneimine cellulose by spotting 1.5 µl aliquots onto the plates for chromatography done using 0.8 M LiCl/0.8 M acetic acid. The efficiency of GTP hydrolysis was quantified by cutting out areas corresponding to [α-^{32}P]GDP and [α-^{32}P]GTP and measuring their radioactivity by Cherenkov counting.

Assembly and analysis of ribosomal complexes by toe-printing

48S complexes were assembled by incubating 25 nM derivatives of β-globin mRNA with 60 nM 40S subunits, 350 nM eIF1, 350 nM eIF1A, 90 nM eIF2, 60 nM eIF3, 300 nM eIF4A, 60 nM eIF4B, 250 nM eIF4G$_{736-1115}$ and 100 nM Met-tRNA$_i^{Met}$ in buffer F (20mM Tris-HCl pH 7.5, 3.8 mM $MgCl_2$, 100 mM KCl, 0.25 mM spermidine, 2mM DTT) supplemented with 1 mM ATP, 0.3 mM GTP and 1 U/µl RiboLock RNase inhibitor (Thermo Scientific) for 10

minutes at 37ºC. To obtain 80S ICs, reaction mixtures were supplemented with 90 nM of 60S subunits, 200 nM eIF5 and 60 nM eIF5B, and incubation continued for an additional 10 min.

Elongation was carried out by mixing 80S IC reactions with various combinations of 60 nM eEF2, 150 nM eEF1H, 150 nM yeast eEF1A, 150 nM native or recombinant full-length GTPBP1, 150 nM $GTPBP1_{152-669}$, 150 nM $GTPBP1_{152-586}$, 150 nM GTPBP2, 150 nM $GTPBP2_{89-602}$, 150 nM $GTPBP2_{164-602}$, 150 nM SMAD1, 200 nM eEF1Bα, 200 nM eEF1Bγ, 200 nM eEF1Bδ, 1 μM native total calf liver aa-tRNA, 300 nM individual aa-tRNAs and 300 nM individual deacylated tRNAs (unless otherwise indicated), after which incubation continued at 37ºC for an additional 15 min (unless otherwise indicated).

80S ICs and ECs that were used in Figures 3A-D, 5K-N, 6E, 6K, 7C-H, S3A, S5B-D, S6B-C, S7C and S7E-G were prepared in 400 μl reaction mixtures and purified by centrifugation in a Beckman SW55 rotor for 1 hr 45 min at 4ºC and 50,000 rpm in 10%–30% linear sucrose density gradients (SDG) prepared in buffer E, and stored at -80ºC.

All resulting ribosomal complexes were analyzed by primer extension (Pisarev et al., 2007b) using AMV reverse transcriptase (Promega) and [^{32}P]-labeled primers complementary to nt 149-166 of MSHL-STOP, MSSL-STOP, and MLHL-STOP mRNAs or to nt 143-160 of MF-STOP mRNA. In time course experiments (Figure 4G), the elongation reactions were stopped by elevating the Mg^{2+} concentration to 20 mM before initiating primer extension. cDNA products were resolved in 6% polyacrylamide sequencing gels followed by autoradiography.

GTP hydrolysis and peptide bond formation (related to Figures 4H-J and S4)

The GTPBP1 ternary complex (TC) was assembled by incubating 2.3 μM GTPBP1, 3.3 μM Phe-$tRNA^{Phe}$ and 6.1 μM [α-^{32}P]GTP in 500 μl buffer E + 5% glycerol for 10 minutes at 37º, and purified by FPLC on a Superdex G75 column equilibrated with buffer E + 5% glycerol. The concentration of the TC was calculated on the basis of the [^{32}P] radioactivity present in the elution fractions.

To obtain 80S ICs used in figures 4H-J and S4A-D, 48S initiation complexes were assembled by incubating 200 nM MF mRNA with 150 nM 40S subunits, 650 nM eIF1, 650 nM eIF1A, 200 nM eIF2, 170 nM eIF3, 600 nM eIF4A, 150 nM eIF4B, 600 nM $eIF4G_{736-1115}$, 250 nM [^{35}S]Met-$tRNA_i^{Met}$ in 1.6 ml buffer F supplemented with 1 mM ATP, 0.3 mM GTP and 1 U/μl RiboLock RNase inhibitor for 15 minutes at 37ºC, after which reaction mixtures were supplemented with 250 nM of 60S subunits, 600 nM eIF5 and 170 nM eIF5B, and incubation continued for an additional 15 min. Assembled 80S ICs were purified by centrifugation in a Beckman SW55 rotor for 1 hr 45 min at 4ºC and 50,000 rpm in 10%–30% linear sucrose density gradients prepared in buffer E. The 80S peak fractions were combined, and the complex was pelleted by centrifugation in a Beckman TLS55 rotor at 55,000 rpm for 4 hours at 4ºC. The ribosomal pellet was resuspended in buffer E, and the concentration of 80S ICs was calculated on the basis of [^{35}S] incorporation.

100 nM of purified 80S ICs formed on MF mRNA in the presence of [^{35}S]Met-$tRNA_i^{Met}$ were incubated with 100 nM purified GTPBP1•[α-^{32}P]GTP•Phe-$tRNA^{Phe}$ TC in buffer E at 37ºC for different time periods. In control experiments 100 nM of the purified 80S ICs were mixed with different combinations of 1 μM GTPBP1, 1 μM eEF1H, 0.7 μM Phe-$tRNA^{Phe}$, 1 μM GTP, 1 μM GMPPNP in buffer E at 37ºC for different time periods. To analyze GTP hydrolysis, the reactions were stopped by addition of 0.1M EDTA, and 2 μl aliquots were spotted on

polyethyleneimine cellulose plates for chromatography done using 0.8 M LiCl/0.8 M acetic acid. For analysis of peptide bond formation, the MF peptides were released by incubation of the reaction mixtures with 0.5 M KOH for 30 min at 37ºC. Reaction mixtures were then neutralized by addition of 0.5 M acetic acid, and 2 µl aliquots were spotted on polyethyleneimine cellulose plates for chromatography in a solution of n-butanol:acetone:water:acetic acid at a 35:35:23:7 ratio. Formation of [α-^{32}P]GDP and [^{35}S]MF was quantified by Phosphorimager using ImageJ software.

In vitro translation

0.4 µg uncapped luciferase mRNA was incubated for 40 min at 37ºC in 20 µl Flexi Rabbit reticulocyte lysate System (Promega Corp.) supplemented with [^{35}S]Met in the presence/absence of indicated concentrations of GTPBP1, GTPBP1$_{152\text{-}586}$ or equivalent volumes of their respective buffers. Translation products were resolved by 4-12% SDS-PAGE and quantified by Phosphorimager.

Analysis of the interaction of GTPBP1 with eEF1B

0.5 µM GTPBP1 was incubated for 10 min at 37ºC in 300 µl buffer E with 0.25 µM eEF1Bα, 0.2 µM eEF1Bγ and 0.2 µM eEF1Bδ in the presence/absence of 1 mM GDP. The resulting complexes were loaded on a FPLC Superdex G200 column equilibrated with buffer E with/without 1 mM GDP. Fractions were collected and aliquots resolved using 4-12% SDS-PAGE followed by fluorescent SYPRO staining (Invitrogen).

Polysome analysis

HEK293T cells were grown in a standard, Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific), supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin at 37°C with 5% CO2 for 18-24 h to approximately 50%-70% confluency. For amino acid starvation, cells were further incubated with Krebs Ringer solution (Sigma-Aldrich) and 10% FBS for 6 hours, whereas non-starved cells continued to grow in DMEM. All cells were then treated with 100 μg/ml cycloheximide for 5 min followed by cell lysis on ice by passing through a 25 gauge syringe 10-15 times in buffer G (10 mM Hepes-KOH pH 7.9, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT) supplemented with 0.1% NP-40, 100 μg/ml cycloheximide and 100 U/ml RiboLock RNase inhibitor. Lysates' supernatants were loaded on 10–50% sucrose gradient in buffer G and centrifuged for 2.5 hours at 36,000 rpm, 4°C in a Beckman SW55 Ti rotor. Fractions were collected using the ISCO Programmable Density Gradient System with continuous monitoring at 254 nm using an ISCO UA-6 absorbance detector. Fractions were precipitated with 10% trichloroacetic acid overnight. The pellets were washed with acetone and 0.1 M Tris-HCl pH 7.9, air dried, resuspended in sample buffer, resolved using 4-12% SDS-PAGE followed by western blot analysis using specific antibodies for GTPBP1, GTPBP2, eEF1A and RPL11.

To assess protein expression in HEK293T cell lysates, proteins were resolved by 4-12% SDS-PAGE followed by western blotting using specific antibodies for GTPBP1, GTPBP2 and eEF1A (Abcam). Equal sample loading was confirmed by SimplyBlue staining.

Pull-down assay

Different combinations of 200nM FLAG-tagged GTPBP2, FLAG-tagged Hbs1, FLAG-His-tagged Pelota, His-tagged GTPBP2$_{89\text{-}602}$, His-tagged eRF1 and His-tagged Pelota were incubated in 100 μl buffer E supplemented with 200 μg/μl

casein and 0.2 mM GMPPNP or GTP at 37ºC for 15 minutes and applied to 30 μl of anti-FLAG M2 affinity agarose gel (Sigma-Aldrich) equilibrated with buffer E. The resin was washed with 3 ml buffer E supplemented with 200 μg/μl casein, and the bound proteins were eluted by incubation with buffer E supplemented with 200 μg/μl casein and 200 μg/μl 3xFLAG peptide for 20 minutes at room temperature. Proteins were resolved by 4-12% SDS-PAGE followed by western blotting with antibodies against FLAG-tag and His-tag.

Exosomal degradation of mRNA

MF-1 and MF-2 mRNAs were capped with [α-^{32}P]GTP using the T7 mScript Standard mRNA Production System (Cellscript, Madison, WI) according to the manufacturer's protocol. 80S ICs were assembled on [^{32}P]cap-labeled MF-1 and MF-2 mRNAs and isolated by SDG centrifugation as described above. 3 nM of purified 80S complexes or free [^{32}P]cap-labeled MF mRNAs were incubated with combinations of 15 nM Exo12$^{EXOSC10/SKIV2L2/C1D}$, 25 nM DIS3, 50 nM GTPBP1/GTPBP1$_{152-586}$/GTPBP2 and 100 nM tRNA^{Phe}/Phe-tRNA^{Phe}/Lys-tRNA^{Lys} in 20 μL buffer E supplemented with 250 nM cold MF mRNAs (as a competitor), 200 μg/μl casein and various combinations of 1 mM ATP, GTP, AMPPNP and GMPPNP at 37ºC for the indicated times. For the experiments shown in Figures S7C and S7G, the concentration of Exo 12 was reduced to 5 nM. The activity of DIS3 was confirmed using 3'[^{32}P]-phosphorylated MF-1 mRNA obtained by ligation with T4 RNA Ligase 1 and [^{32}P]pCp. After incubation, RNA was phenol-extracted, ethanol-precipitated and resolved on 6% polyacrylamide sequencing gels followed by autoradiography.

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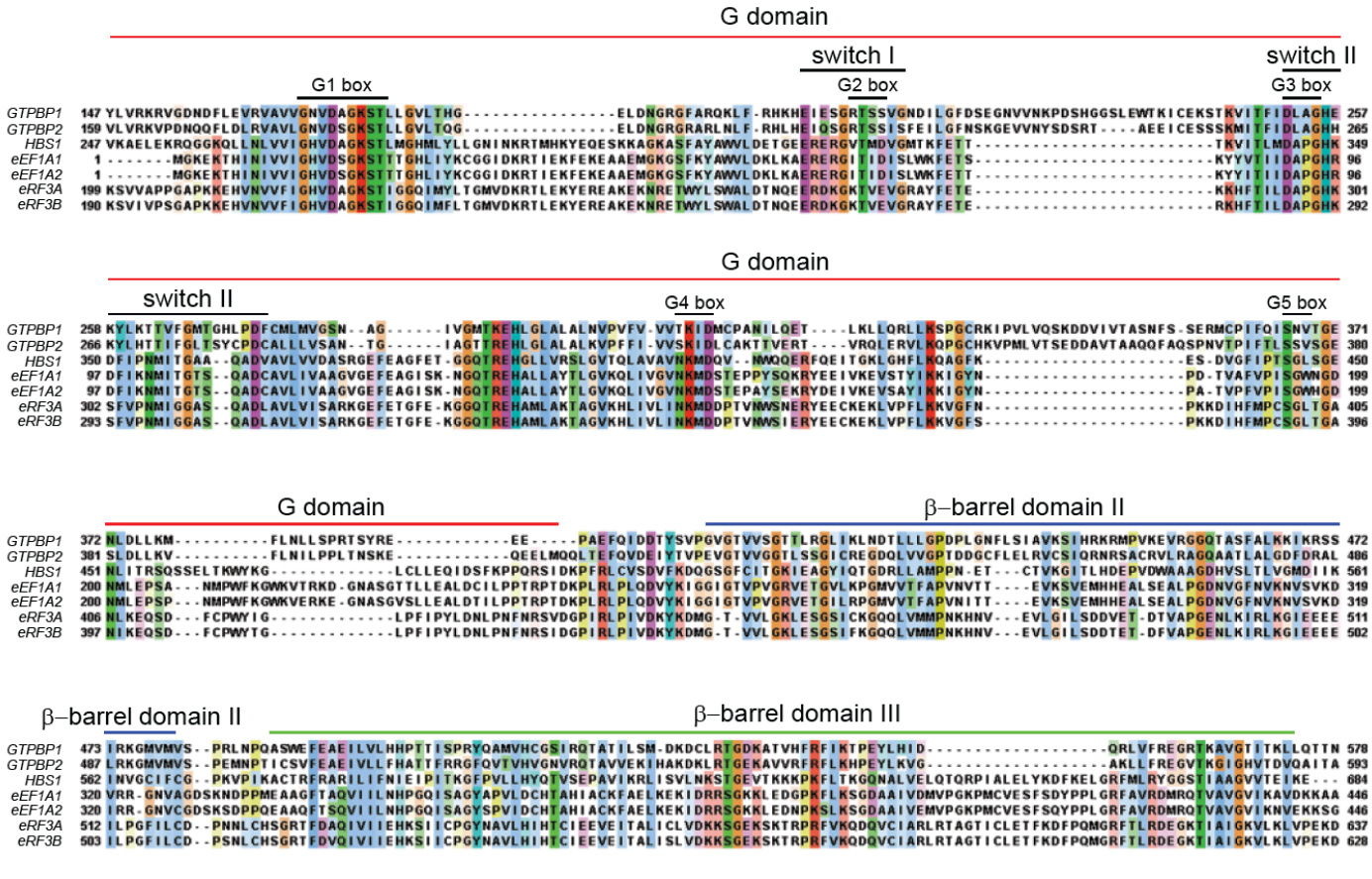
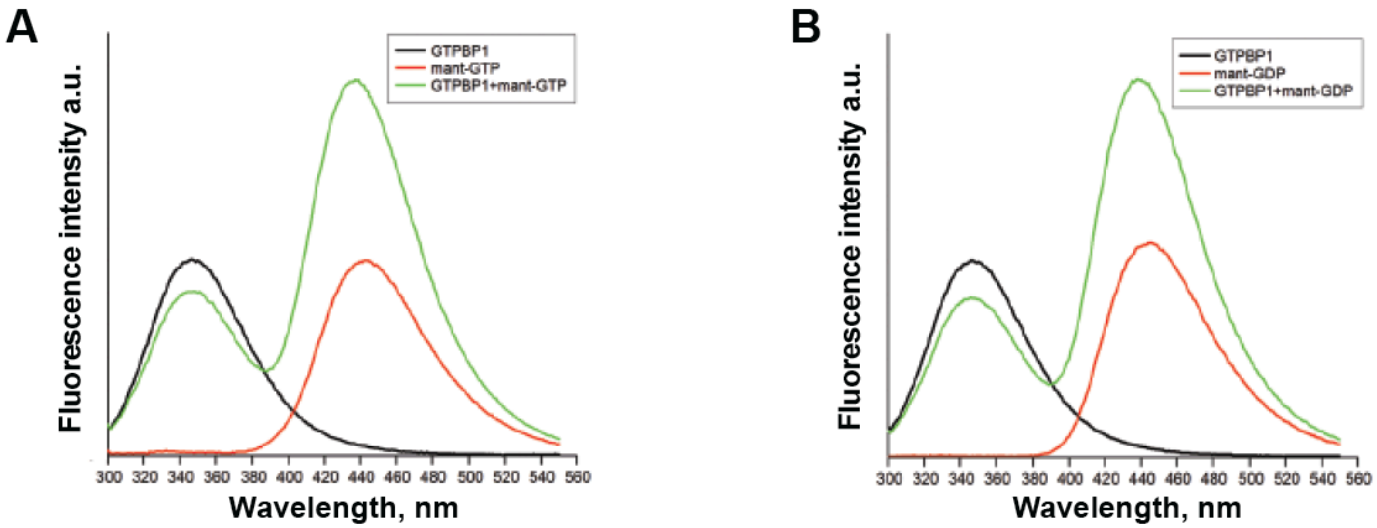
Zinoviev_Suplemental Fig. S1



Supplemental Figure S1. Multiple sequence alignment of the GTPase cores of *H. sapiens* GTPBP1, GTPBP2, Hbs1, eEF1A1, eEF1A2, eRF3A and eRF3B (related to Figure 1).

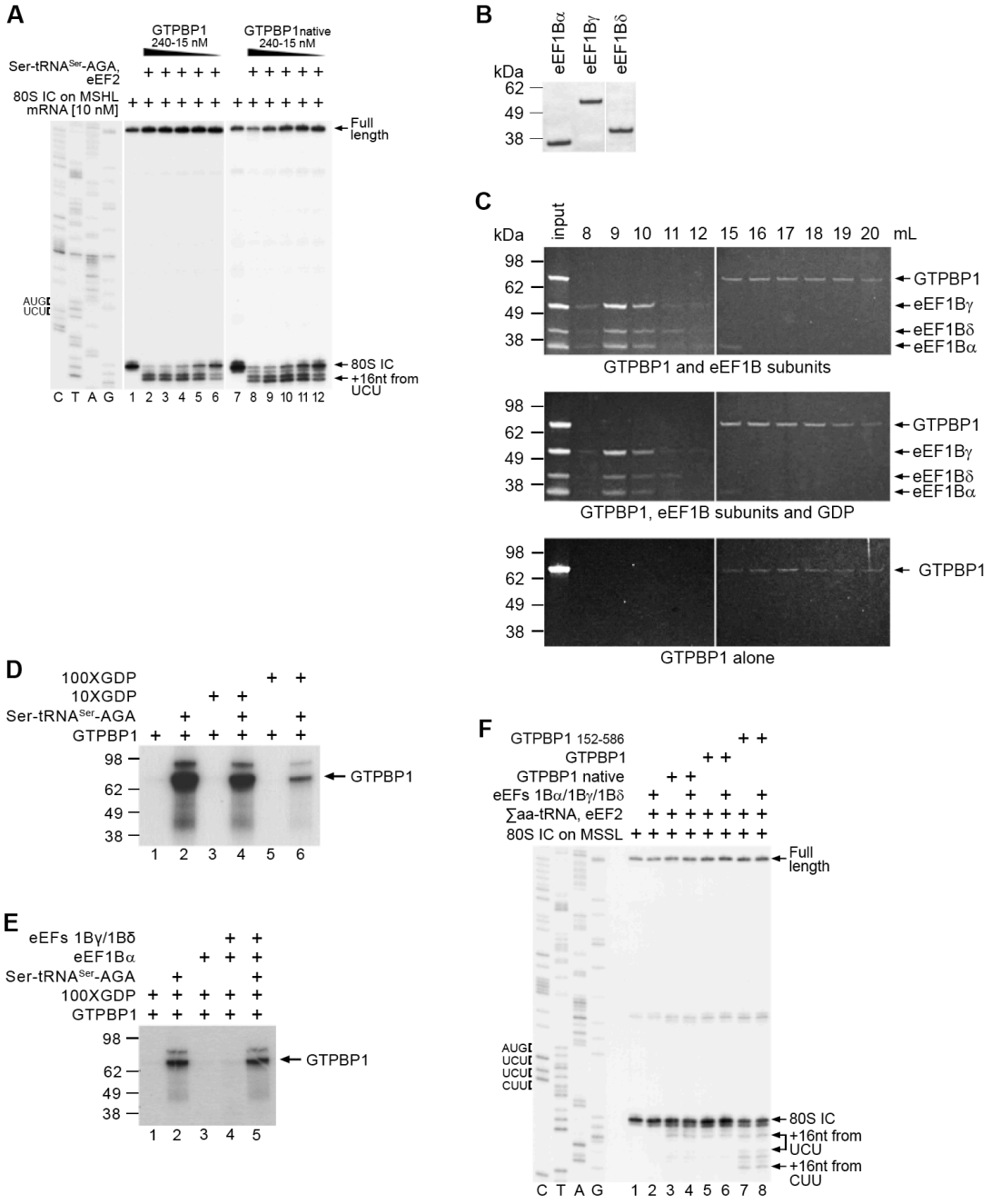
The alignment was made with ClustalW. NCBI accession numbers for *H. sapiens* proteins: eEF1A1 (NP_001393), eEF1A2 (NP_001949), GTPBP1 (NP_004277), GTPBP2 (NP_061969), eRF3A (NP_002085), eRF3B (NP_060564), Hbs1 (NP_006611). Domains, Switch I and Switch II elements and conserved G motifs (Leipe et al., 2002) are indicated.

Leipe DD, Wolf YI, Koonin EV, Aravind L. 2002. Classification and evolution of P-loop GTPases and related ATPases. *J Mol Biol* **317**: 41-72.



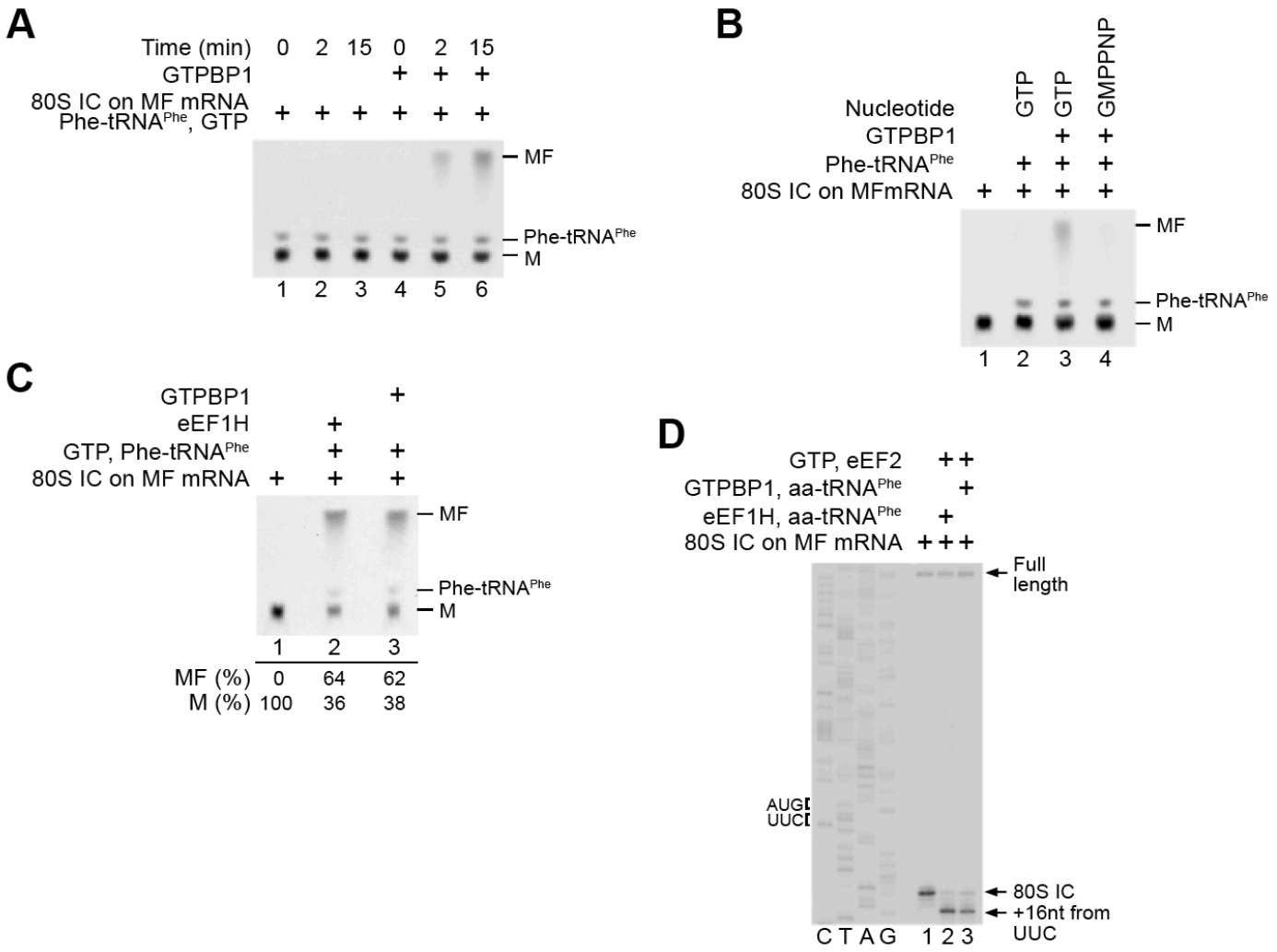
Supplemental Figure S2. Interaction of GTPBP1 with mant-guanine nucleotides (related to Figure 2).

(A) Emission spectra of 0.2 μM GTPBP1 (black), 3 μM mant-GTP (red), and their mixture (green) upon excitation at 290 nm. (B) Emission spectra of 0.2 μM GTPBP1 (black), 3 μM mant-GDP (red), and their mixture (green) upon excitation at 290 nm.



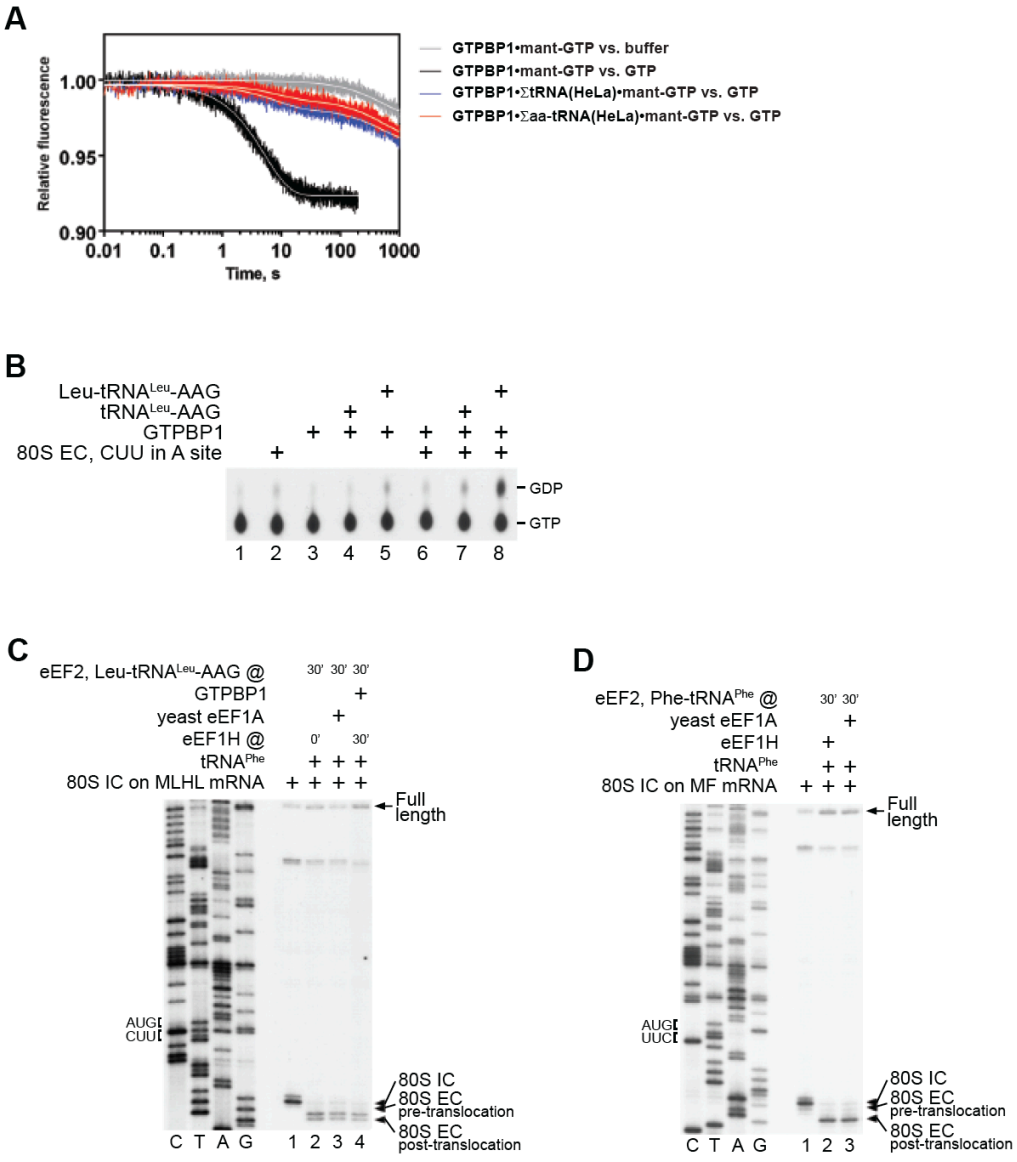
Supplemental Figure S3. Elongation activities of native and recombinant GTPBP1, and the influence of eEF1B on the activity of GTPBP1 (related to Figure 4).

(A) Titration of recombinant and native full-length GTPBP1 in one-cycle elongation on MSHL-STOP mRNA with cognate *in vitro* transcribed Ser-tRNA^{Ser}-AGA, assayed by toe-printing. (B) Purified recombinant eEF1Bα, eEF1Bγ and eEF1Bδ subunits, resolved by SDS-PAGE followed by SimplyBlue staining. (C) Elution from Superdex 75 column of GTPBP1 alone, or GTPBP1 incubated with eEF1B subunits in the presence/absence of GDP. Fluorescent SYPRO stained SDS-PAGE panels show the presence of GTPBP1 and eEF1B subunits in the elution peak fractions, as indicated. (D, E) UV cross-linking of GTPBP1 to [α-^{32}P]GTP in the presence of Ser-tRNA^{Ser}-AGA, excess GDP and eEF1B subunits, as indicated. Cross-linked proteins were separated by SDS-PAGE followed by autoradiography. (F) The activity of the different forms of GTPBP1 in three-cycle elongation on MSSL-STOP mRNA with native Σaa-tRNAs in the presence/absence of eEF1B subunits, assayed by toe-printing. (A, F) Positions of the 80S initiation complex (80S IC) and elongation complexes are indicated by arrows on the right. Lanes C/T/A/G depict corresponding DNA sequences.



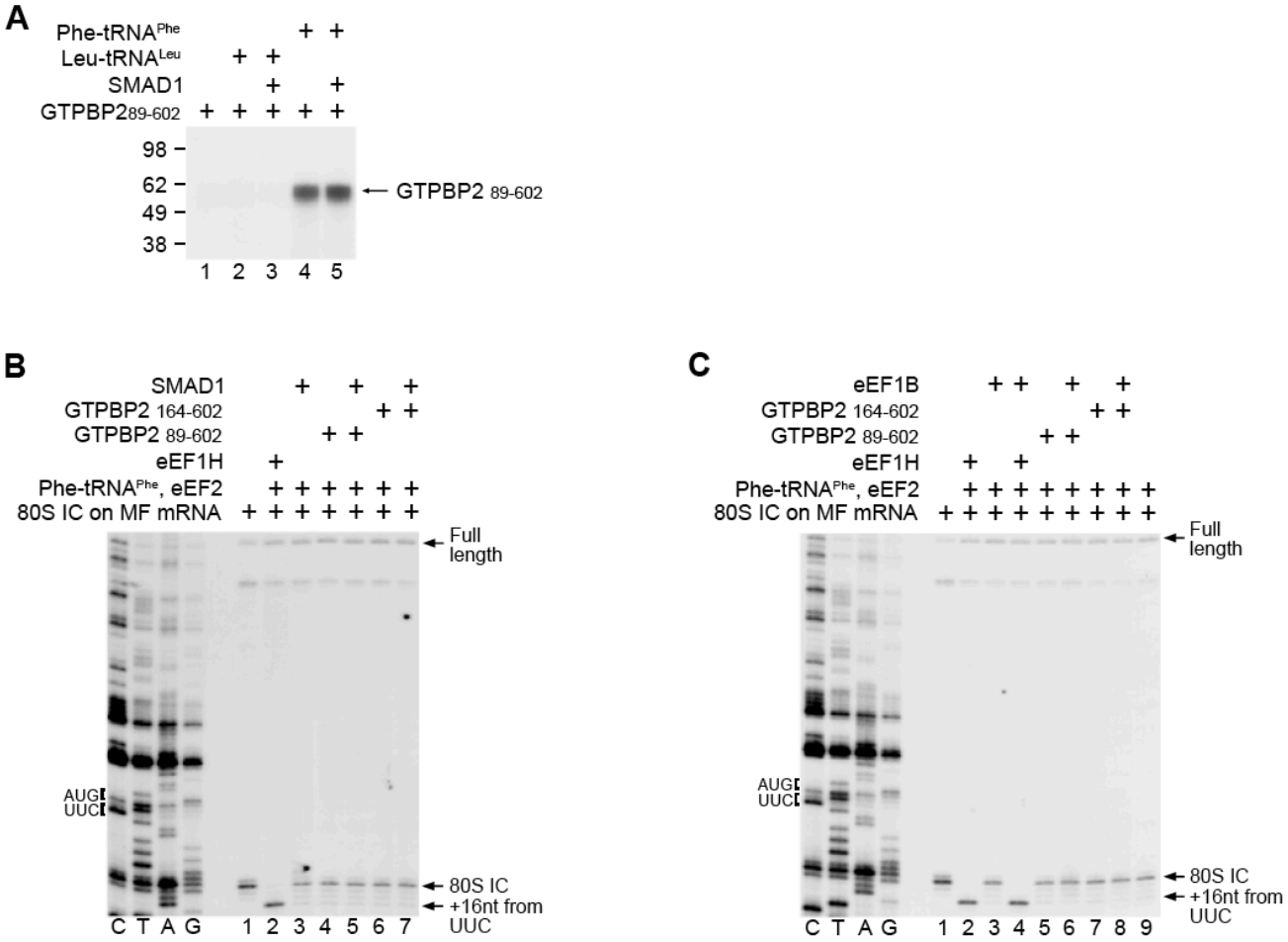
Supplemental Figure S4. GTPBP1-mediated elongation on 80S ICs assembled on MF-STOP mRNA (related to Figure 4).

(A) To confirm that dipeptide synthesis presented in Figure 4 of the main text did not occur by non-enzymatic loading of Phe-tRNA^{Phe} to the A site, purified 80S ICs assembled on MF mRNA with [^{35}S]Met-tRNA$_i^{Met}$, were incubated with excess [^{14}C]Phe-tRNA^{Phe} in the absence and in the presence of GTPBP1. Aliquots were removed at different time points, and formation of the MF dipeptide was analyzed by TLC. (B) To confirm that GTPBP1-mediated elongation required hydrolysis of GTP, purified 80S ICs assembled on MF mRNA with [^{35}S]Met-tRNA$_i^{Met}$ were incubated with GTPBP1 and [^{14}C]Phe-tRNA^{Phe} in the presence of GTP or GMPPNP, and formation of the MF dipeptide was assayed by TLC. (C) To determine the proportion of Met capable of peptide bond formation in the preparation of 80S ICs used in Figure 4 of the main text, 80S ICs assembled on the MF mRNA with [^{35}S]Met-tRNA$_i^{Met}$ were incubated with excess Phe-tRNA^{Phe}, GTP and eEF1H or GTPBP1 at 37ºC for 30 minutes, after which synthesis of the [^{35}S]MF peptide was analyzed by TLC with subsequent quantification by Phophorimager. In both cases, ~65% Met participated in peptide bond formation. (D) Analysis of the elongation competence of 80S ICs under identical conditions as (C) by toe-printing showed that nearly all complexes were able to participate in peptide bond formation. Comparison of (C) and (D) indicates that the 80S IC preparation contained Met-tRNA$_i^{Met}$-associated 80S ribosomes that were not programmed with the MF mRNA and therefore could not participate in peptide bond formation.



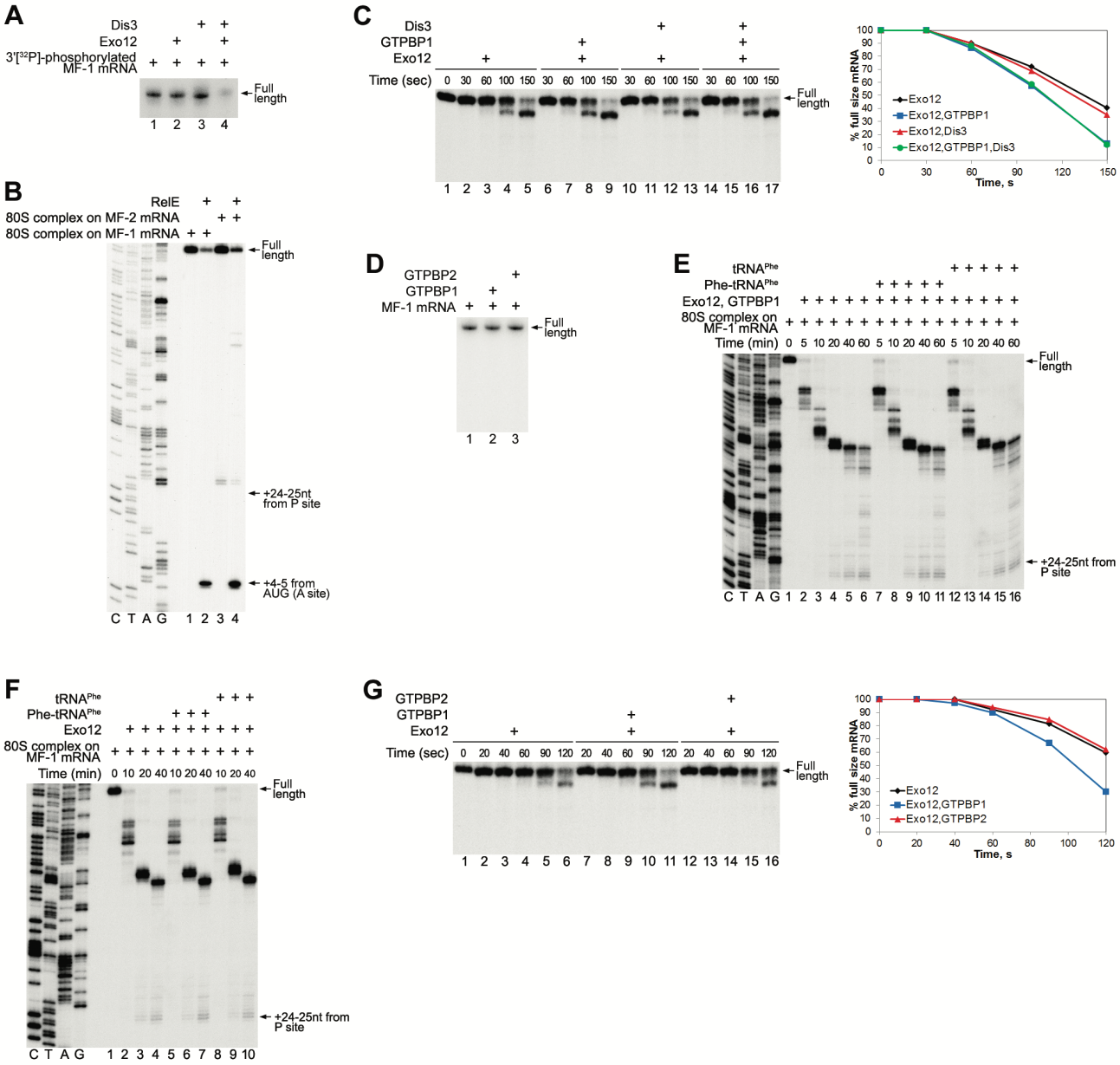
Supplemental Figure S5. Interaction of GTPBP1 with deacylated tRNA (related to Figure 5).

(A) Time courses of the dissociation of mant-GTP from GTPBP1 alone and in the presence of Σaa-tRNA(HeLa) or ΣtRNA(HeLa) upon chase with GTP (500 μM), monitored using the stopped-flow technique. (B) GTP hydrolysis by the full-length GTPBP1 in the presence/absence of different combinations of 80S ECs and cognate aminoacylated and deacylated $tRNA^{Leu}$-AAG, assayed by TLC and autoradiography. (C, D) Toe-printing analysis of the activity of eEF1H, yeast eEF1A and GTPBP1 in one-cycle elongation on MLHL-STOP (C) and MF-STOP (D) mRNAs with Leu-$tRNA^{Leu}$-AAG and native yeast Phe-$tRNA^{Phe}$ and $tRNA^{Phe}$, as indicated. Positions of the ORF codons are shown on the left. Positions of 80S ICs and pre-translocation and translocated elongation complexes are indicated on the right. Lanes C/T/A/G depict corresponding DNA sequences.



Supplemental Figure S6. The influence of SMAD1 and eEF1B on the activity of GTPBP2 (related to Figure 6).

(A) UV cross-linking of $GTPBP2_{89\text{-}602}$ to [α-^{32}P]GTP in the presence of different aa-tRNAs and SMAD1, as indicated. Cross-linked proteins were resolved by SDS-PAGE, followed by autoradiography. (B, C) Toe-printing analysis of the activity of $GTPBP2_{89\text{-}602}$ and $GTPBP2_{164\text{-}602}$ in single-cycle elongation on MF-STOP mRNA with cognate native yeast Phe-tRNA^{Phe} in the presence/absence of (B) SMAD1 or (C) eEF1B. Positions of the 80S initiation complexes (ICs) and elongation complexes are indicated by arrows on the right. Positions of the ORF codons are shown on the left. Lanes C/T/A/G depict corresponding DNA sequences.



Supplemental Figure S7. Controls for exosomal degradation experiments (related to Figure 7).
(A) To confirm the activity of purified DIS3, we assayed degradation of 3'[^{32}P]-phosphorylated MF-1 mRNA (obtained by ligation with [^{32}P]pCp) by Exo12$^{EXOSC10/SKIV2L2/C1D}$ and DIS3, individually or in combination. Consistent with the lack of activity of EXOSC10 on RNA substrates containing 3'-phosphate and the ability of DIS3 to degrade such RNAs (e.g. Domanski et al., 2016), degradation of 3'[^{32}P]-phosphorylated MF-1 mRNA was observed only in the presence of DIS3. (B) Assembly of 80S complexes on the initiation codons of [^{32}P]cap-labeled MF-1 and MF-2 mRNAs was confirmed by cleavage by RelE, which hydrolyzes mRNA in the A site (e.g. Pedersen et al., 2003). (C, G) RNA degradation intermediates obtained after incubation for the indicated time of 80S complexes assembled on the initiation codon of [^{32}P]cap-labeled MF-1 mRNA with Exo12 (5 nM) and different combinations of GTPBP1, Dis3 and GTPBP2 in the presence of ATP and GTP. The proportion of the remaining full-length mRNA was quantified by Phosphorimager. (D) The lack of nucleolytic activities in preparations of GTPBP1 and GTPBP2 was confirmed by investigation of their influence on the stability of [^{32}P]cap-labeled MF-1 mRNA. (E, F) RNA degradation intermediates obtained after incubation for the indicated time of 80S complexes assembled on the initiation codon of [^{32}P]cap-labeled MF-1 mRNA with Exo12 (15 nM) and different combinations of GTPBP1, Phe-tRNA^{Phe} and tRNA^{Phe} in the presence of ATP and GTP. tRNAs did not influence mRNA

degradation by Exo12 irrespective of the presence of GTPBP1. (A-G) In all cases, mRNA was phenol-extracted, ethanol-precipitated and analyzed by sequencing gel electrophoresis. (B, C, E-G) Lanes C/T/A/G depict DNA sequences employed for RNA size evaluation.

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Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K, Ehrenberg M. 2003. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* **112**: 131–140.