INVENTORY OF SUPPLEMENTAL INFORMATION

Supplemental data:

1. Supplemental Figure S1 showing the comparison of peptide release from pre-TCs containing either native or *in vitro* transcribed $tRNA^{Leu}$. This Figure is related to the first part of the Results section of the main text of the manuscript, and in particular, to Figure 1E.
2. Supplemental Figure S2 showing ribosomal recycling in RRL at different Mg^{2+} concentrations. It is related to the second part of the Results section.
3. Supplemental Table S1 showing identification of ABCE1 by LC/nanospray tandem mass-spectrometry of tryptic peptides. It is related to the second part of the Results section, and in particular, to Figure 2A.

Supplemental experimental procedures:

Contains the information concerning plasmid constructions and includes detailed protocols for all experimental procedures.

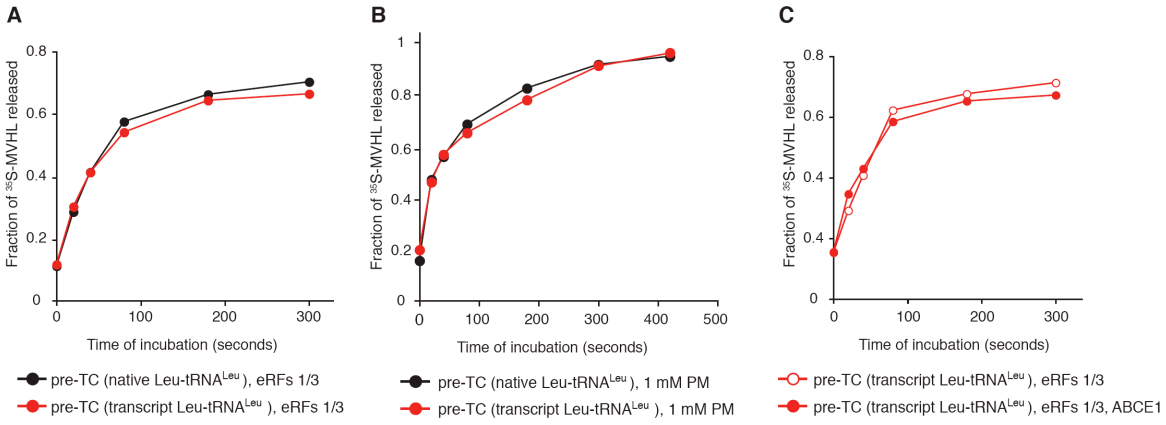
Supplemental references:

Contains references for Supplemental experimental procedures.

SUPPLEMENTAL DATA

Peptide release on pre-TCs containing in vitro transcribed tRNA^{Leu}

To compare peptide release from pre-TCs containing either native or *in vitro* transcribed tRNA^{Leu}, pre-TCs were assembled on MVHL-STOP mRNA (Fig. 1A of the main text) with either native or *in vitro* transcribed [^{35}S]Met-tRNA^{Met}, Val-tRNA^{Val}, His-tRNA^{His} and Leu-tRNA^{Leu} and purified by sucrose density gradient (SDG) centrifugation. Peptide release from pre-TCs containing *in vitro* transcribed tRNA^{Leu} occurred as efficiently as from pre-TCs with native tRNA^{Leu}, irrespective of whether it was induced by eRF1/eRF3 (Fig. S1A) or by puromycin (Fig. S1B). As expected, peptide release on pre-TCs assembled with *in vitro* transcribed tRNA^{Leu} was not influenced by ABCE1 (Fig. S1C).

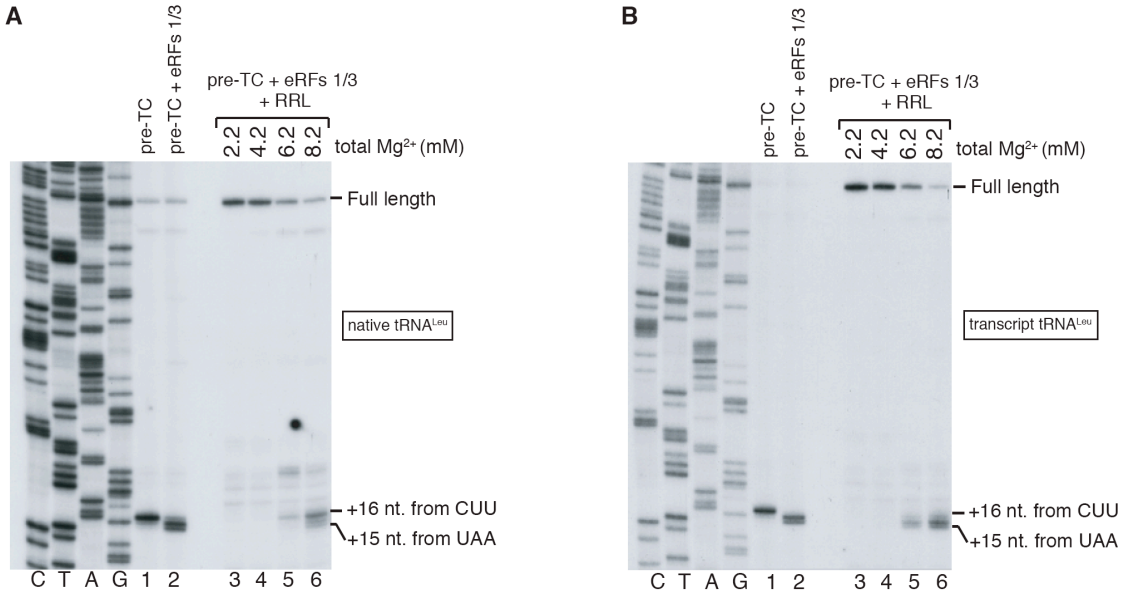


Supplemental Figure S1. Comparison of peptide release from pre-TCs containing either native or *in vitro* transcribed tRNA^{Leu}

Kinetics of [^{35}S]MVHL tetrapeptide release from pre-TC containing native (black circles) or *in vitro* transcribed (red circles) tRNA^{Leu} at 1 mM Mg^{2+} in the presence of (A) eRF1/eRF3, (B) 1 mM puromycin, (C) eRF1/eRF3 and ABCE1, as indicated.

Ribosomal recycling in RRL at different Mg^{2+} concentrations

To investigate the Mg^{2+}-dependence of ribosomal recycling in RRL, pre-TCs were assembled on MVHL-STOP mRNA (Fig. 1A of the main text) in an *in vitro* reconstituted translation system using purified 40S and 60S subunits, eIFs 2, 3, 1, 1A, 4A, 4B, 4F, 5 and 5B, elongation factors eEF1H and eEF2, and either native or *in vitro* transcribed Met-$tRNA^{Met}$, Val-$tRNA^{Val}$, His-$tRNA^{His}$ and Leu-$tRNA^{Leu}$, purified by SDG centrifugation and incubated with eRF1 and eRF3 to induce peptide release. The obtained post-TCs were then incubated in RRL at different Mg^{2+} concentrations, after which ribosomal association of MVHL mRNA was assayed by toe-printing. Incubation of post-TCs assembled with both native and *in vitro* transcribed $tRNA^{Leu}$ in RRL at 2.2 and at 4.2 mM total Mg^{2+} yielded almost exclusively full-length cDNA indicating near-complete recycling, and ~50% and ~5% of post-TCs were recycled at 6.2 and at 8.2 mM Mg^{2+}, respectively (Fig. S2).



Supplemental Figure S2. Mg^{2+}-dependence of ribosomal recycling in RRL of post-TCs containing native or *in vitro* transcribed $tRNA^{Leu}$

Toe-printing analysis of ribosomal complexes, which resulted from incubation in RRL at indicated Mg^{2+} concentrations of post-TCs, obtained by incubation with eRF1 and eRF3 of SDG-purified pre-TCs assembled on MVHL-STOP mRNA and containing (A) native or (B) *in vitro* transcribed $tRNA^{Leu}$. Lanes C, T, A, G depict cDNA sequences corresponding to MVHL-STOP mRNA. The positions of full-length cDNA and of toe-prints that correspond to ribosomal complexes are indicated.

Supplemental Table S1. Identification of ABCE1 by LC/nanospray tandem mass-spectrometry of tryptic peptides. Amino acid residues are numbered according to the sequence of *H. sapiens* ABCE1 (Genbank NP_001035809).

Deduced sequence	Amino acid residues
IAIVNHDK	8-15
LCIEVTPQSK	37-46
CPFGALSIVNLPSNLEK	65-81
YCANAFK	87-93
GSELQNYFTK	149-158
ILEDDLK	159-165
AIIKPQYVDQIPK	166-178
GTVGSILDR	182-190
TQAIVCQQLDLTHLK	196-210
NVEDLSGGELQR	213-224
FACAVVCIQK	225-234
ADIFMFDEPSSYLDVK	235-250
AAITIR	255-260
SLINPDR	261-267
EGINIFLDGYVPTENLR	307-323
FRDASLVFK	324-332
VAETANEEEVKK	333-343
MCMYK	345-349
TTFIR	386-390
LKPDEGGEVPVLNVSYKPQK	396-415
FILHAK	507-512
VIVFDGVPSK	532-540
NTVANSPQTLLAGMNK	542-557
RDPNNYRPR	568-576
INKLNSIK	577-584
LNSIKDVEQK	580-589
KSGNYFFLDD	590-599

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Expression vectors for His_6-tagged eIFs 1, 1A, 4A, 4B, 5, 3j, *wt* eRF1, eRF1(AGQ) mutant and eRF3aC lacking the N-terminal 138 a.a., which is referred to as eRF3 throughout the text, as well as transcription vectors for MVHL-STOP mRNA, $tRNA_i^{Met}$, $tRNA^{Val}$ (GUG) and $tRNA^{His}$ (CAC) have been described (Unbehaun et al., 2004; Alkalaeva et al., 2006 and references therein; Pisarev et al., 2007a). A transcription vector for $tRNA^{Leu}$ (CUU) was made by inserting DNA flanked by a T7 promoter and a *BstN*1 restriction site into pUC57 (GenScript Corp.). mRNA and tRNAs were transcribed using T7 RNA polymerase. ^{32}P-labeled MVHL-STOP mRNA ($1.5x10^6$ cpm/μg) was transcribed in the presence of [α^{32}P]ATP (222 Tbq/mmol).

Purification of factors and ribosomal subunits

Rabbit 40S and 60S subunits, eIFs 2, 3, 4F, 5B, and 6, eEF1H and eEF2, and recombinant His_6-tagged eIFs 1, 1A, 4A, 4B, 5, 3j, *wt* eRF1, eRF1(AGQ) and eRF3 were purified as described (Alkalaeva et al., 2006 and references therein; Si et al., 1997; Unbehaun et al., 2004). [^{32}P]eRF1 ($4x10^6$ cpm/pmol) and [^{32}P]60S subunits ($2x10^7$ cpm/pmol) were prepared using the catalytic subunit of cAMP-dependent protein kinase and casein kinase II, respectively (Pisarev et al., 2007a).

Aminoacylation of tRNA

Native [5'-^{32}P]$tRNA^{Leu}$ was prepared from total tRNA (Pisarev et al., 2007a). Rabbit aminoacyl-tRNA synthetases were purified, and native total rabbit tRNA (Novagen), native [5'-^{32}P]$tRNA^{Leu}$ and *in vitro* transcribed $tRNA^{Met}_i$, $tRNA^{Val}$, $tRNA^{His}$ and $tRNA^{Leu}$ were aminoacylated with Met, Val, His and Leu, as described (Pisarev et al., 2007a). For peptide release experiments, aminoacylation was done in the presence of [^{35}S]Met yielding [^{35}S]Met-$tRNA^{Met}$ with a specific activity of $6x10^5$ cpm/pmol.

Purification of native ABCE1

ABCE1 was purified from the 40-50% ammonium sulfate precipitation fraction of the 0.5 M KCl ribosomal salt wash that was prepared from 2.5 liters of RRL (Green Hectares, Oregon, WI) (Pisarev et al., 2007b). This fraction was dialyzed against buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 2 mM DTT, 0.1 mM EDTA) + 100 mM KCl and applied to a DEAE (D52) column equilibrated with buffer A + 100 mM KCl. ABCE1 was eluted in the flow-through fraction with buffer A + 100 mM KCl. This fraction was applied to a phosphocellulose (P11) column equilibrated with buffer A + 100 mM KCl. ABCE1 was eluted at 250-300 mM KCl. ABCE1-

containing fractions were dialyzed against buffer B (20 mM HEPES, pH 7.5, 20% glycerol, 2 mM DTT, 0.1 mM EDTA) + 50 mM KCl and applied to a FPLC MonoS HR 5/5 column. Fractions were collected across a 50-500 mM KCl gradient. ABCE1 eluted at ~120 mM KCl. ABCE1-containing fractions were diluted 3.5-fold with 20 mM phosphate buffer, pH 7.5 with 20% glycerol and applied to a hydroxyapatite column pre-equilibrated in the same phosphate buffer. Fractions were collected across a 20-500 mM phosphate buffer gradient. ABCE1 was eluted at ~140 mM phosphate buffer. ABCE1-containing fractions were concentrated and transferred into buffer A + 100 mM KCl on Microcon YM30. The yield of ABCE1 was 15-20 μg per 2.5 liters of RRL. The identity of ABCE1 was confirmed by LC-nanospray tandem mass spectrometry of peptides derived by in-gel tryptic digestion at the Rockefeller University Proteomics Resource Center.

Expression of recombinant ABCE1 in *E. coli* or in *S. cerevisiae* did not yield an active protein. We note that earlier attempts to purify overexpressed ABCE1 from *S. cerevisiae* likely also resulted in ABCE1's inactivation (Dong et al., 2004).

Assembly and purification of ribosomal complexes

Pre-TCs were assembled on MVHL-STOP mRNA and purified by sucrose density gradient centrifugation essentially as described (Alkalaeva et al., 2006). For subunit dissociation experiments, pre-TCs were assembled with [^{32}P]60S subunits and either total native tRNAs (Novagen) aminoacylated with Met, Val, His and Leu, or *in vitro* transcribed tRNA$^{Met}_{i}$, tRNA^{Val}, tRNA^{His} and tRNA^{Leu}. For mRNA release experiments, pre-TCs were assembled with [^{32}P]MVHL-STOP mRNA and total native tRNAs (Novagen) aminoacylated with Met, Val, His and Leu. For tRNA release experiments, pre-TCs were assembled with *in vitro* transcribed Met-tRNA$^{Met}_{i}$, Val-tRNA^{Val} and His-tRNA^{His} and native purified Leu-[5'-^{32}P]tRNA^{Leu}. For peptide release experiments, pre-TCs were assembled with either total native tRNAs (Novagen) aminoacylated with [^{35}S]Met, Val, His and Leu, or *in vitro* transcribed [^{35}S]Met-tRNA^{Met}, Val-tRNA^{Val}, His-tRNA^{His} and Leu-tRNA^{Leu}.

Dissociation of post-termination 80S ribosomes into subunits

Pre-TCs were assembled on MVHL-STOP mRNA with [^{32}P]60S subunits and either native or *in vitro* transcribed Leu-tRNA^{Leu} and purified by SDG centrifugation. For dissociation of post-TCs by eIFs (Figs. 1B, E), pre-TCs (0.5 pmol) were incubated with different combinations of 15 pmol *wt* eRF1, 15 pmol eRF3, 1 mM puromycin, 20 pmol eIF3, 30 pmol eIF1, 30 pmol eIF1A and 20 pmol eIF3j for 10 minutes at 37ºC in 120 μl buffer C (20 mM Tris, pH 7.5, 100 mM KCl, 0.25 mM spermidine, 2 mM DTT) supplemented with 1 mM ATP and 0.4 mM GTP and corresponding amounts of $MgCl_2$ to achieve the concentration of nucleotide-unbound (free) Mg^{2+} that is indicated

on each panel. For dissociation of post-TCs by ABCE1 (Figs. 2B-F, 4D, 6C-E), pre-TCs (0.5 pmol) were incubated with 2.5 pmol ABCE1 (all Figures), 20 pmol *wt* eRF1 and 20 pmol eRF3 (Figs. 2B-F, 4D, 6E), 80 pmol eRF1 (Figs. 4D, 6C, 6D), 1 mM puromycin (Fig. 4D), 2 pmol 60S subunits (Figs. 2B-E, 4D, 6 C-E), 10 pmol eIF6 (Figs. 2C) and 10 pmol eIF3, 30 pmol eIF1, 30 pmol eIF1A and 15 pmol eIF3j (Figs. 2C, F) for 10 minutes (except for the experiments shown in Fig. 2E, in which incubation for periods ranging from 30 seconds to 4 minutes, as indicated, was followed by quenching by elevation of Mg^{2+} concentration to 10 mM) at 37ºC in 80 μl buffer C supplemented with 0.2 mM ATP and 0.2 mM GTP (Figs. 2B-F, 4D), 0.4 mM ATP, GTP, CTP, UTP, ADP, AMPPNP or ATPγS (Figs. 6C, 6D), 15 μM GTP and 3 mM ATPγS (Fig. 6E) and corresponding amounts of $MgCl_2$ to achieve the concentration of nucleotide-unbound (free) Mg^{2+} indicated on each panel. The resulting ribosomal complexes were subjected to centrifugation through 10-30% sucrose density gradients prepared in buffer C + 2.5 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 110 minutes. Association of ribosomal subunits was measured by Cerenkov counting of an aliquot of each fraction.

tRNA release

SDG-purified pre-TCs (0.5 pmol) assembled on MVHL-STOP mRNA with native Leu-[5'-^{32}P]tRNA^{Leu} were incubated with different combinations of 30 pmol wt eRF1, 30 pmol eRF3, 3 pmol ABCE1, 10 pmol eIF6, 15 pmol eIF3, 30 pmol eIF1, 30 pmol eIF1A and 20 pmol 3j for 10 minutes at 37ºC in 120 μl buffer C supplemented with 0.2 mM ATP, 0.2 mM GTP and 2.9 mM $MgCl_2$, and subjected to centrifugation through 10-30% sucrose density gradients prepared in buffer C + 2.5 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. Ribosomal association of [5'-^{32}P]tRNA^{Leu} was measured by Cerenkov counting of an aliquot of each fraction.

mRNA release

SDG-purified pre-TCs (0.5 pmol) assembled on [^{32}P]MVHL-STOP mRNA with native Leu-tRNA^{Leu} were incubated with different combinations of 20 pmol *wt* eRF1, 20 pmol eRF3, 2 pmol ABCE1, 10 pmol eIF6, 10 pmol eIF3, 30 pmol eIF1, 30 pmol eIF1A and 15 pmol 3j for 10 minutes at 37ºC in 80 μl buffer C supplemented with 0.2 mM ATP, 0.2 mM GTP and 2.9 mM $MgCl_2$, and subjected to centrifugation through 10-30% sucrose density gradients prepared in buffer C + 2.5 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. Ribosomal association of mRNA was measured by Cerenkov counting of an aliquot of each fraction.

Toe-printing analysis of ribosomal complexes

SDG-purified pre-TCs (0.15 pmol) assembled on MVHL-STOP mRNAs and containing either native (Figs. 1C, 3E, 3F, 4E, 5F) or *in vitro* transcribed (1G, 6F) tRNA^{Leu} were incubated with

combinations of 5 pmol *wt* eRF1 and 5 pmol eRF3 (Figs. 1C, 1G, 3E-F, 5F, 6F), combinations of 10 pmol eRF1(AGQ), 10 or 50 pmol *wt* eRF1, 5 pmol eRF3 and 1mM puromycin (Fig. 4E), 0.5 pmol ABCE1 (3E, 3F, 4E, 6F), combinations of 7 pmol eIF3, 10 pmol eIF1, 10 pmol eIF1A and 7 pmol 3j (Figs. 3E, 4E, 6F) and 0.4 pmol SDG-purified 80S/eRF1/ABCE1 complexes (Fig. 5F) in 40 μl buffer C supplemented with 0.2 mM GTP and 0.2 mM ATP (Figs. 1C, 1G, 3E, 3F, 4E, 5F), 1 mM of ATP, GTP, UTP, CTP, ADP, GDP, GMPPNP or AMPPNP (Fig. 6F) and corresponding amounts of $MgCl_2$ to achieve the concentration of nucleotide-unbound (free) Mg^{2+} indicated on each panel. After incubation, the concentration of Mg^{2+} was elevated to 20 mM to prevent further recycling. Ribosomal complexes were analyzed by primer extension using AMV reverse transcriptase and a [^{32}P]primer complementary to nt 197-214 of β-globin mRNA, which was also done at 20 mM Mg^{2+} to avoid any possibility of recycling that might occur during primer extension.

Analysis of eRF1's association with post-TCs

For experiments shown in Figures 1D, 1F, 3G and 6G, SDG-purified pre-TCs (0.5 pmol) assembled on MVHL-STOP mRNA and containing native or *in vitro* transcribed $tRNA^{Leu}$ were incubated with 20 pmol [^{32}P]eRF1 (all Figures), 20 pmol eRF3 (Figs. 1D, 1F, 3G), 2 pmol ABCE1 (3G, 6G), 2 pmol 60S subunits (Fig. 3G) for 10 minutes at 37ºC in 120 μl buffer C supplemented with 1 mM GTP (Figs. 1D, 1F), 0.4 mM GTP and 1 mM ATP (Fig. 3G), 1 mM ADP or AMPPNP (Fig. 6G) and corresponding amounts of $MgCl_2$ to achieve the concentration of nucleotide-unbound (free) Mg^{2+} indicated on each panel. For experiments shown in Fig. 5E, individual 80S ribosomes (10 pmol) were incubated with 15 pmol [^{32}P]eRF1 in the absence/presence of 1.5 pmol ABCE1 and 30 pmol eRF3 for 10 minutes at 37ºC in 120 μl buffer C supplemented with 4 mM $MgCl_2$ and 1 mM ATP, ADP or AMPPNP, as indicated. After incubation, all reaction mixtures were subjected to centrifugation through 10-30% sucrose density gradients prepared in buffer C + 5 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. Ribosomal association of [^{32}P]eRF1 was measured by Cerenkov counting of an aliquot of each fraction.

Silencing of ABCE1 in HeLa cells

Adherently growing HeLa cells were cultured in DMEM (GIBCO) supplemented with 10 % fetal bovine serum. 1.7 x 10^6 HeLa cells were plated in a 10 cm dish and transfected 24 hour later with the indicated siRNA at a final concentration of 10 nM using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Silencer® Select validated ABCE1 siRNA (sense: 5' CCCACCUAAAAGAACGAAAtt 3'; antisense: 5' UUUCGUUCUUUUAGGUGGGtt 3') was purchased from Ambion Inc. The negative control GFP siRNA (sense: 5' GCAAGCUGACCCUGAAGUUCAU 3'; antisense: 5'

GAACUUCAGGGUCAGCUUGCCG 3') was purchased from Qiagen Inc. The following day, the cells were transferred to a 15 cm dish and harvested 48 hr later for cell extract preparation.

Preparation of HeLa cell extract

RNAi-silenced HeLa cells were harvested using trypsin/EDTA solution (GIBCO) according to the manufacturer's instructions and 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, one tablet complete EDTA free protease inhibitor cocktail, Roche) as described in Thoma et al. (2004). The cells were allowed to swell for 30 minutes on ice and were subsequently disrupted with approximately 20 strokes of a 1 ml syringe with a 27 G $^{3/4}$ needle. After lysis, the cell lysates were centrifuged at 13,000 g for 1 min at 4ºC. Supernatants were collected and stored at -80ºC.

Translation assay in HeLa cell extract

The translation assay for in vitro transcribed m7GpppG-capped, polyadenylated luciferase reporter mRNAs in the RNAi-silenced HeLa cell extract was performed as previously described in Thoma et al. (2004) in a final volume of 10 µl containing 4 µl of the cell lysate, 60 µM amino acids, 20 µM creatine phosphate, 40 ng/µl creatine phosphokinase, 16 mM HEPES buffer, pH 7.6, 0.8 mM ATP, 0.1 mM GTP, 0.05 mM spermidine, 40 mM potassium acetate, 2 mM magnesium acetate, and 2 fM of the mRNA transcript. The translation assay was carried out at 37ºC for 30 minutes. Luciferase activity was measured with luciferase substrate (Promega) using a 96-well plate luminometer (EG&G Berthold).

Analysis of ribosomal recycling in HeLa cell extracts

For toe-printing analysis of ribosomal recycling in HeLa cell extracts, 0.2 pmol pre-TCs, assembled on MVHL-STOP mRNAs with native aa-tRNAs and purified by SDG centrifugation, were first incubated with 2 pmol *wt* eRF1 and 2 pmol eRF3 in 7 µl buffer C supplemented with 0.2 GTP and 2.7 mM $MgCl_2$ for 10 minutes at 37ºC, and then with 0.2-2 µl of control or ABCE1-silenced HeLa cell extracts with/without addition of 0.5 pmol ABCE1 in a total volume of 40 µl buffer C supplemented with 0.2 mM ATP and 0.2 mM GTP and 2.9 mM $MgCl_2$ for 10 min at 37ºC. After incubation, the Mg^{2+} concentration was elevated to 20 mM to prevent further recycling. Ribosomal complexes were analyzed by primer extension using AMV reverse transcriptase and a [^{32}P]primer complementary to nt 197-214 of β-globin mRNA, which was also done at 20 mM Mg^{2+} to prevent any possibility that recycling might occur during primer extension.

Pull-down assay

For analysis of eRF1-ABCE1 interaction, 4 μl of anti-ABCE1 antibodies (ab32270, Abcam) were immobilized on 37.5 μl of Protein A Sepharose in the presence/absence of 0.6 μg of ABCE1 by incubation in 750 μl of buffer D (20 mM Tris, pH 7.5 and 100 mM NaCl) for 2 hours at 4ºC. Beads were washed 3 times with 1 ml buffer D and then incubated with 40 pmol eRF1 in 750 μl buffer C supplemented with 2.5 mM $MgCl_2$ and 0.1% Nonidet NP-40 for 3 hours at 4ºC. Beads were washed 3 times with 1 ml of the same buffer. Bound material was analyzed by western blotting using antibodies against eRF1 and ABCE1 (Abcam).

Analysis of ribosomal binding of ABCE1

For experiments shown in Figures 5A-B, ABCE1 (4 pmol) was incubated with 80S ribosomes (40 pmol), 40S subunits (40 pmol), 60S subunits (40 pmol) and 43S preinitiation complexes formed with eIFs 2/3/1/1A (40 pmol) in the presence/absence of 1 mM of various nucleotides, as indicated, for 10 minutes at 37ºC in 400 μl buffer C + 4 mM $MgCl_2$. For experiments shown in Figure 5C, ABCE1 (3 pmol) was incubated with SDG-purified pre-TCs (2.5 pmol) and different combinations of *wt* eRF1 (20 pmol), eRF1(AGQ) (20 pmol), eRF3 (20 pmol) and 1 mM puromycin in the presence of 1 mM of various nucleotides, as indicated, for 10 minutes at 37ºC in 400 μl buffer C + 4 mM $MgCl_2$. For experiments shown in Figure 5D, 80S ribosomes (40 pmol) were incubated with ABCE1 (10 pmol) and eRF1 (50 pmol) individually or in combination for 10 minutes at 37ºC in 400 μl buffer C + 4 mM $MgCl_2$. After incubation, reaction mixtures were subjected to centrifugation through 10-30% sucrose density gradients prepared in buffer C + 4 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. Fractions that corresponded to 80S ribosomal complexes were analyzed by western blotting using anti-ABCE1 and anti-eRF1 antibodies (Abcam).

NTPase assay

To determine the NTPase specificity of ABCE1 (Fig. 6A), 0.25 pmol ABCE1 was incubated in a 10 μl reaction mixture containing buffer C supplemented with 2.5 mM $MgCl_2$ and 0.33 μM [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]UTP, or [α-^{32}P]CTP in the presence/absence of 0.25 pmol 80S ribosomes at 37ºC for 30 minutes. 2 μl aliquots were spotted onto polyethyleneimine cellulose plates for chromatography done using 0.8 M LiCl/0.8 M acetic acid. The NTPase activity of ABCE1 was assayed by formation of [α-^{32}P]NDP. To investigate stimulation of the NTPase activity of ABCE1 by various ribosomal complexes (Figure 6B), 0.5 pmol ABCE1 were incubated with combinations of 0.5 pmol SDG-purified pre-TCs, 10 pmol eRF1 *wt*, 10 pmol eRF1(AGQ) mutant and 10 pmol eIF6 in 15 μl reaction mixtures containing buffer C supplemented with 0.33 μM [γ-

^{32}P]GTP and 2.5 mM $MgCl_2$ at 37ºC. 2 µl aliquots were removed after 2-30 minutes of incubation for PEI cellulose chromatography. The GTPase activity of ABCE1 was assayed by formation of [^{32}P]P_i.

Peptide release assay

SDG-purified pre-TCs (3 nM), assembled on MVHL-STOP mRNA with either native or *in vitro* transcribed [^{35}S]Met-tRNA^{Met}, Val-tRNA^{Val}, His-tRNA^{His} and Leu-tRNA^{Leu}, were incubated with 12.5 nM eRF1/eRF3, 1 mM puromycin and 12.5 nM ABCE1, as indicated, in 320 µl buffer C supplemented with 1.4 mM $MgCl_2$, 0.2 mM ATP and 0.2 mM GTP at 37ºC. Peptide release from 40 µl aliquots taken at different times was assayed by TCA precipitation (Zavialov et al., 2001).

Analysis of ribosomal recycling in RRL

For toe-printing analysis of ribosomal recycling in RRL, 0.2 pmol pre-TCs, assembled on MVHL-STOP mRNAs with either native or *in vitro* transcribed aa-tRNAs and purified by SDG centrifugation, were first incubated with 2 pmol *wt* eRF1 and 2 pmol eRF3 in 7 µl buffer C supplemented with 1mM GTP and 4 mM $MgCl_2$ for 10 minutes at 37ºC, and then with RRL (Promega) at 2.2-8.2 mM total $MgCl_2$ in a final volume of 40 µl for 10 min at 30ºC. After incubation, the concentration of Mg^{2+} was elevated to 20 mM to prevent further recycling. Ribosomal complexes were analyzed by primer extension using AMV reverse transcriptase and a [^{32}P]primer complementary to nt 197-214 of β-globin mRNA, which was also done at 20 mM Mg^{2+} to prevent the possibility that recycling might occur during primer extension.

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