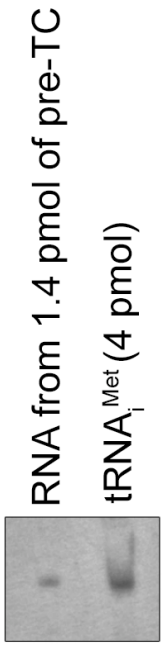
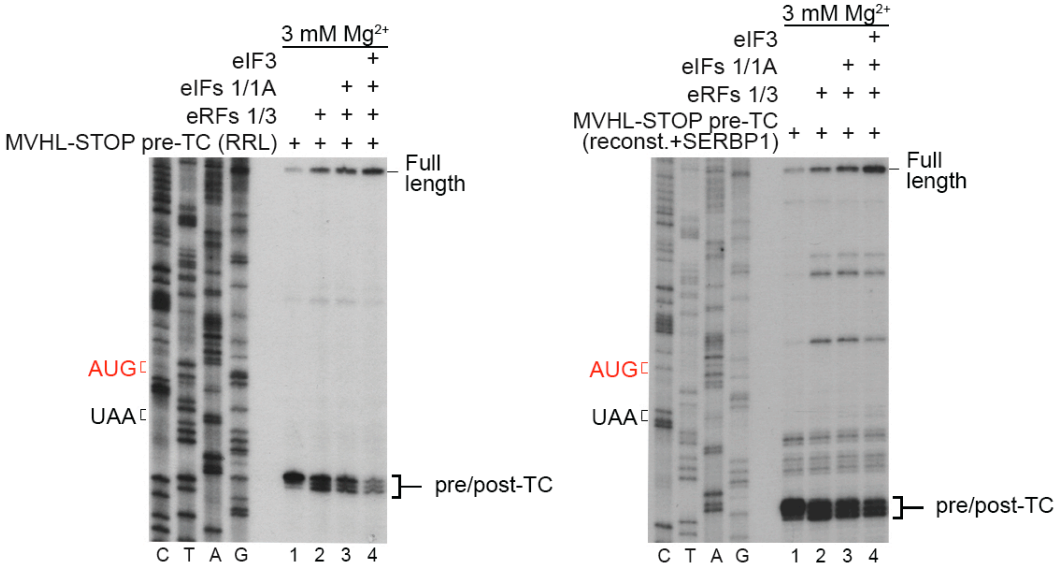
SUPPLEMENTAL DATA



Supplemental Figure S1. Presence of tRNA$_i^{Met}$ in preparations of RRL-derived pre-TCs assayed by Northern blotting, Related to Figure 3



Supplemental Figure S2. Recycling by eIFs of post-TCs assembled on MVHL-STOP mRNA, Related to Figure 4

Toe-printing analysis of the recycling activity of eIF1/eIF1A, individually and in combination with eIF3, on post-TCs assembled on MVHL-STOP mRNA in RRL (left panel) and in an *in vitro* reconstituted translation system in the presence of SERBP1 (right panel). Positions of pre/post-TCs on ORF1 (black brackets) and of full-length cDNA are shown on the right. Stop (black) and restart (red) codons are marked on the left. Free [Mg^{2+}] is indicated on each panel.

Supplemental Table S1.

Identification of SERBP1 by LC/nanospray tandem mass-spectrometry of tryptic peptides, Related to Figure 2

Deduced sequence	Amino acid Residues *
FDQLFDDESDPFEVLKAAENK	17-37
EAGGGGVGGPGAK	40-52
SAAQAAAQTNSNAAGKQLRK	53-72
KNPLPPNVGVVDKK	79-91
NPLPPNVGVVDKKEETQPPVALKK	80-103
RVGRRPDQQLQGEGK	108-122
RPDQQLQGEGKIIDR	112-126
RFEKPLEEK	137-145
GKREFDRHSGSDR	189-201
SSFSHYSGLKHEDKR	202-216
HEDKRGGSGSHNWGTVKDELTESPK	212-236
DRAKVEFNIR	299-308
KPNEGADGQWKK	309-320
SKSEEAHAEDSVMDHHFR	327-344
TDKSSASAPDVDDPEAFPALA	387-407

* Amino acid residues are numbered according to the sequence of *O. cuniculus* SERBP1 isoform 4 (NCBI Reference sequence XP_002715983).

Supplemental Table S2.

Identification of eEF2 by LC/nanospray tandem mass-spectrometry of tryptic peptides, Related to Figure 2

Deduced sequence	Amino acid Residues *
VNFTVDQIR	2-10
ANIRNMSVIAHVDHGK	17-32
AGIIASAR	43-50
FTDTRKDEQER	56-66
IKPVLMMNK	151-159
ALLELQLEPEELYQTFQR	163-180
FAAKGEGQLGPAER	236-249
AKKVEDMMK	250-258
KVEDMMKK	252-259
KLWGDRYFDPSNGKFSK	259-275 #
SATSPDGKKLPR	276-287
LIEKLDIKLDSEDKDK	315-330
LDIKLDSEDKDKEGKPLLK	319-337
WLPAGDALLQMITIHLPSPVTAQK	343-366
GPLMMYISK	392-400
FYAFGRVFSGLVSTGLKVR	410-428
IMGPNYTPGKKEDLYLKPIQR	429-449
TILMMGR	450-456
TGTITTFEHAHNMR	482-495
VAVEAKNPADLPK	507-519
SDPVVSYR	573-580
ARPFPDGLAEDIDKGEVSARQELK	606-629
YLAEKYEWDVAEARK	634-648
RGHVFEESQVAGTPMFVVK	760-778
AYLPVNESFGFTADLR	779-794
GLKEGIPALDNFLDKL	836-851

* Amino acid residues are numbered according to the sequence of *Cavia porcellus* eEF2 (NCBI Reference Sequence: XP_003461085.1), because neither GenBank nor Uniprot contain a sequence for eEF2 from *O. cuniculus*. Each peptide sequence matched the predicted *C. porcellus* sequence precisely, except for a single peptide (#), which has a two residue mismatch.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Expression vectors for His_6-tagged eIFs 1, 1A, 4A, 4B, $\Delta 4G_{736\text{-}1115}$ and 5, *wt* eRF1, $eRF1^{AGQ}$ mutant, eRF3aC that has deletion of the first 138 amino acids (referred to as eRF3 in the text), Ligatin, MCT1, and DENR, and transcription vectors for MVHL-STOP mRNA, $tRNA_i^{Met}$, $tRNA^{Leu}$ (CUU codon), and $tRNA^{His}$ (CAC codon) have been described (Pestova et al., 1996, 1998, 2000; Lomakin et al., 2000; Pestova and Hellen 2001; Frolova et al 1999; Seit-Nebi et al., 2001; Alkalaeva et al., 2006 and references therein; Pisarev et al., 2007a; Skabkin et al., 2010). The plasmid pET-15b PAI-RBP for expression of His_6-tagged SERBP1 (Heaton et al., 2001) was obtained from Addgene (Cambridge, MA). Transcription vectors for model *wt* and mutant RHDV mRNAs (HindIII), model *wt* and mutant NV mRNAs (SmaI), and $tRNA^{Ser}$ (UCU codon) (BstNI) were made by inserting appropriate DNA fragments flanked by a T7 promoter and the indicated restriction site into pUC57 (GenScript Corp.). All mRNAs and tRNAs were transcribed using T7 RNA polymerase.

Purification of factors and ribosomal subunits.

Native 40S and 60S ribosomal subunits, eIFs 2, 3 and 5B, eEF1H, eEF2, ABCE1 and ∑aminoacyl-tRNA synthetases were purified from rabbit reticulocyte lysate (RRL) (Green Hectares) (Pestova and Hellen, 2003; Pisarev et al., 2007b, 2010). Recombinant His_6-tagged eIFs 1, 1A, 4A, 4B, $\Delta 4G_{736\text{-}1115}$ and 5, eRF1 *wt*, $eRF1^{AGQ}$ mutant, eRF3, Ligatin, MCT1, and DENR were expressed in *E. coli* and purified as described (Seit-Nebi et al., 2001; Alkalaeva et al., 2006 and references therein; Lomakin et al., 2006; Pisarev et al., 2007b; Skabkin et al., 2010).

Aminoacylation of tRNA

Native total calf liver tRNA (Promega), and *in vitro* transcribed $tRNA^{Leu}$ and $tRNA_i^{Met}$ were aminoacylated using native aminoacyl-tRNA synthetases as described (Pisarev et al., 2007b).

Purification of SERBP1

Native SERBP1 was purified from the pellets of salt-washed ribosomes prepared from 600 ml of RRL (Pisarev et al., 2007b). Ribosomes were resuspended in buffer A (20 mM Tris-HCl pH 7.5, 50 mM KCl, 4 mM $MgCl_2$, 2 mM DTT) and incubated with 1 mM puromycin for 10 min on ice and then for 10 min at 37ºC, after which the concentration of KCl was elevated to 0.5 M. The obtained ribosomal suspension was the loaded onto 10 to 30% sucrose density gradients prepared in buffer B (20 mM Tris pH 7.5, 2 mM DTT, 4 mM $MgCl_2$, 0.5 M KCl) and centrifuged at 22,000 rpm in a Beckman SW28 rotor for 17 h at 4ºC. The upper gradient fractions containing SERBP1 were precipitated with 40% of ammonium sulfate, dialyzed against buffer C (20 mM HEPES pH 7.5, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 2 mM DTT) and then applied to a FPLC MonoS HR 5/5 column. Fractions were collected across a 100-500 mM KCl gradient. SERBP1 eluted at ~280 mM KCl.

Recombinant N-terminally His_6-tagged SERBP1 was expressed in 4 L of *E. coli* BL21 (DE3) after induction by 2 mM IPTG for 3 hours at 37ºC, and then purified by affinity chromatography on Ni-NTA-agarose followed by FPLC on a MonoS HR5/5 column.

Assembly of ribosomal complexes

Assembly of pre-TCs in an *in vitro* reconstituted system was done essentially as described (Alkalaeva et al., 2006). Briefly, 48S complexes were formed in 400 μl reaction mixtures (except Fig. 2G, where the reaction volume was 20 μl) by incubation of 25 nM of MVHL-stop mRNA with 100 nM 40S ribosomal subunits, 300 nM eIF1, 300 nM eIF1A, 200 nM eIF2, 150 nM eIF3, 400 nM eIF4A, 125 nM eIF4B, 300 nM $\Delta 4G_{736\text{-}1115}$ and 150 nM of *in vitro* transcribed Met-$tRNA_i^{Met}$ in buffer D (20 mM Tris-HCl pH 7.5, 100 mM KCl, 0.25 mM spermidine, 2 mM DTT) supplemented with 1 mM ATP, 0.3 mM GTP and 2.8 mM $MgCl_2$ for 10 min at 37ºC. When 48S complexes were formed in the presence of SERBP1, only 50 nM of 40S subunits were used, and they were preincubated with 150 nM SERBP1 for 10 min at 37ºC before addition to the reaction mixture. Assembled 48S complexes were then incubated with 200 nM 60S subunits, 300 nM eIF5 and 90 nM eIF5B for 10 min at 37ºC to allow formation of 80S initiation complexes. To form pre-TCs, 80S complexes were supplemented with 200 nM eEF1H, 500 nM eEF2 and appropriately aminoacylated native total tRNA (~250 nM each) and incubated for 10 min at 37ºC.

For assembly of pre-TCs by translation in RRL (Promega), 0.5 μM of RHDV, NV or MVHL-STOP mRNA were incubated with the lysate for 10 min at 37ºC in 200 μl reaction mixture supplemented with 1.25 μM $eRF1^{AGQ}$ and 1.25 μM eRF3 (that had been pre-incubated together for 10 min at 37ºC in the presence of 0.2 mM GTP) to arrest ribosomal complexes at the stop codon.

Pre-TCs of both types were purified by centrifugation through 10-30% sucrose density gradients prepared in buffer D supplemented with 2.5 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 105 min and stored at -80ºC.

Enzymatic footprinting

100 nM of individual RHDV mRNA or 22.5 nM of RHDV pre-TC were incubated with 0.01 U/μl or 0.025 U/μl of RNaseT1, respectively, in 20 μl of buffer D supplemented with 1 mM ATP, 0.3 mM GTP and 2.8 mM $MgCl_2$ for 10 min at 37ºC. Cleavage sites were identified by primer extension using AMV reverse transcriptase and ^{32}P-labeled primer complementary to nt 79-102 of the ORF2 of RHDV mRNA. cDNA products were resolved in 6% polyacrylamide sequencing gels followed by autoradiography.

Analysis of reinitiation by toe-printing

To investigate reinitiation (and recycling), 3.75 nM of SDG-purified pre-TCs were incubated for 10 min at 37ºC in 20 μl buffer D (supplemented with 0.3 mM GTP, 0.1 mM ATP and corresponding amounts of $MgCl_2$ to achieve the free [Mg^{2+}] indicated on each panel) with different combinations of the following factors and tRNAs: 50 nM eRF1, 100 nM eRF3, 9 nM Met-$tRNA_i^{Met}$, 9 nM Leu-$tRNA^{Leu}$, 40 nM $tRNA^{Ser}$,

40 nM $tRNA^{His}$, 50 nM eEF2, 100 nM eIF1, 100 nM eIF1A, 40 nM eIF2, 30 nM eIF3, 100 nM ABCE1, 50 nM Ligatin, 50 nM MCT1, and 50 nM DENR. To examine the elongation competence of 48S and 80S reinitiation complexes, reaction mixtures were supplemented with combinations of 50 nM eEF2 (where not added in a previous step), 50 nM eEF1H, 400 nM $eRF1^{AGQ}$ mutant, appropriately aminoacylated native total tRNA (6 nM each), 50 nM 60S subunits, 100 nM eIF5, and 90 nM eIF5B, and incubation was continued for another 10 min. Resulting complexes were analyzed by primer extension using AMV reverse transcriptase and ^{32}P-labeled primers complementary to nt 79-102 of the ORF2 of RHDV mRNA, nt 197-214 of β-globin mRNA, or nt 70-88 of the ORF2 of NV mRNA. cDNA products were resolved in 6% polyacrylamide sequencing gels followed by autoradiography.

Analysis of ribosomal association of SERBP1 and eEF2

To assay association of SERBP1 and eEF2 with 80S ribosomes and individual subunits, 290 nM SERBP1 and 290 nM eEF2 were incubated together or separately with 290 nM 40S subunits, 60S subunits or 80S ribosomes in 70 μL reaction mixtures containing buffer D supplemented with 1 mM ATP, 0.3 mM GTP and 2.8 mM $MgCl_2$ for 10 min at 37ºC. To assay association of SERBP1 and eEF2 with 43S complexes, 48S initiation complexes and pre-TCs, corresponding complexes were assembled in the presence of SERBP1 in 100 μL reaction mixtures as described above in the section "Assembly of ribosomal complexes". Resulting complexes were then separated by centrifugation through 10-30% sucrose density gradients prepared in buffer D supplemented with 2.5 mM $MgCl_2$ in a Beckman SW55 rotor at 53,000 rpm for 105 min. Ribosomal peak fractions were TCA precipitated and analyzed by 4-12% SDS-PAGE followed by SYPRO (Invitrogen) staining or western blotting using SERBP1 antibodies.

Northern blotting

RNA was isolated by phenol-extraction from 1.4 pmol of RRL-derived pre-TC assembled on NV mRNA, after which short (<100 nt) and long (>100nt) RNAs were separated by precipitation with 2.5M LiCl. Short RNAs and control $tRNA^{Met}$ were resolved on a denaturing 6% acrylamide gel, transferred to a nylon membrane, and immobilized by UV cross-linking at 254 nm for 1 min in a Stratalinker (Stratagene). The membrane was pre-hybridized by incubation for 2 h at 37ºC in 50% formamide, 5X SSC, 3X Denhardt's solution, 200 μg/mL herring testis carrier DNA, 0.1% SDS, after which $tRNA^{Met}$ was detected by incubation with a [^{32}P]-labeled primer (5'-TGGTAGCAGAGGATGG-3') in pre-hybridization solution for 16 h at 37ºC. After washing with 2X SSC/0.1% SDS for 10 min at 37ºC, the membrane was autoradiographed.

SUPPLEMENTAL REFERENCES

Alkalaeva, E.Z., Pisarev, A.V., Frolova, L.Y., Kisselev, L.L., and Pestova, T.V. (2006). In vitro reconstitution of eukaryotic translation reveals cooperativity between release factors eRF1 and eRF3. Cell *125*, 1125–1136.

Frolova, L.Y., Tsivkovskii, R.Y., Sivolobova, G.F., Oparina, N.Y., Serpinsky, O.I., Blinov, V.M., Tatkov, S.I, and Kisselev, L.L. (1999). Mutations in the highly conserved GGQ motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis. RNA *5*, 1014-1020.

Heaton, J.H., Dlakic, W.M., Dlakic, M., and Gelehrter, T.D. (2001). Identification and cDNA cloning of a novel RNA-binding protein that interacts with the cyclic nucleotide-responsive sequence in the Type-1 plasminogen activator inhibitor mRNA. J. Biol. Chem. *276*, 3341-3347.

Lomakin, I.B., Hellen, C.U., and Pestova, T.V. (2000). Physical association of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. Mol. Cell. Biol. *20*, 6019-6029.

Pestova, T.V., Hellen, C.U., and Shatsky, I.N. (1996). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol. Cell. Biol. *16*, 6859–6869.

Pestova, T.V., Borukhov, S.I., and Hellen, C.U. (1998). Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature *394*, 854-859.

Pestova, T.V., Lomakin, I.B., Lee, J.H., Choi, S.K., Dever, T.E. and Hellen, C.U. (2000). The joining of ribosomal subunits in eukaryotes requires eIF5B. Nature *403*, 332–335

Pestova, T.V., and Hellen, C.U. (2001). Preparation and activity of synthetic unmodified mammalian tRNAi(Met) in initiation of translation in vitro. RNA *7*, 1496–1505

Pestova, T.V., and Hellen, C.U. (2003). Translation elongation after assembly of ribosomes on the Cricket paralysis virus internal ribosomal entry site without initiation factors or initiator tRNA. Genes Dev. *17*, 181-186.

Pisarev, A.V., Hellen, C.U.T., and Pestova, T.V. (2007a). Recycling of eukaryotic posttermination ribosomal complexes. Cell *131*, 286-299.

Pisarev, A.V., Unbehaun, A., Hellen, C.U., and Pestova, T.V. (2007b). Assembly and analysis of eukaryotic translation initiation complexes. Methods Enzymol. *430*, 147-177.

Pisarev, A.V., Skabkin, M.A., Pisareva, V.P., Skabkina O.V., Rakotondrafara A.M., Hentze, M.W., Hellen, C.U.T., and Pestova, T.V. (2010). The role of ABCE1 in eukaryotic posttermination ribosomal recycling. Mol. Cell *37*, 196-210.

Seit-Nebi, A., Frolova, L., Justesen, J., and Kisselev, L. (2001). Class-1 translation termination factors: invariant GGQ minidomain is essential for release activity and ribosome binding but not for stop codon recognition. Nucleic Acids Res. *29*, 3982-3987.

Skabkin, M.A., Skabkina, O.V., Dhote, V., Komar, A., Hellen, C.U.T., and Pestova, T.V. (2010). Activities of Ligatin and MCT-1/DENR in eukaryotic translation initiation and ribosomal recycling. Genes Dev. *24*, 1787-1801.