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eIF3j facilitates loading of release factors into the ribosome

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ABSTRACT

eIF3j is one of the eukaryotic translation factors originally reported as the labile subunit of the eukaryotic translation initiation factor eIF3. The yeast homolog of this protein, Hcr1, has been implicated in stringent AUG recognition as well as in controlling translation termination and stop codon readthrough. Using a reconstituted mammalian *in vitro* translation system, we showed that the human protein eIF3j is also important for translation termination. We showed that eIF3j stimulates peptidyl-tRNA hydrolysis induced by a complex of eukaryotic release factors, eRF1-eRF3. Moreover, in combination with the initiation factor eIF3, which also stimulates peptide release, eIF3j activity in translation termination increases. We found that eIF3j interacts with the pre-termination ribosomal complex, and eRF3 destabilises this interaction. In the solution, these proteins bind to each other and to other participants of translation termination, eRF1 and PABP, in the presence of GTP. Using a toe-printing assay, we determined the stage at which eIF3j functions – binding of release factors to the A-site of the ribosome before GTP hydrolysis. Based on these data, we assumed that human eIF3j is involved in the regulation of translation termination by loading release factors into the ribosome.

INTRODUCTION

Eukaryotic translation termination requires two release factors: eRF1 and eRF3 (1,2). The termination process begins when a stop codon enters the ribosomal A-site. The complex of the release factors eRF1-eRF3 binds to the ribosomal A-site and eRF1 recognises the stop codon on mRNA, triggering eRF3-mediated GTP hydrolysis (1,3–7). Although it remains unclear whether eRF3 changes conformation or dissociates from the ribosome after GTP hydrolysis (8–10), eRF3 is known to induce a conformational rearrangement in eRF1 and promote accommodation of the GGQ motif of eRF1 in the peptidyl transferase centre (PTC) of the ribosome. In the PTC, eRF1 induces peptidyl tRNA hydrolysis and nascent polypeptide release. During the next stage of translation, called ribosome recycling, ribosomal complexes are disassembled into their components. Ribosome recycling begins with the binding of ATPase ABCE1 (Rli1 in yeast) to eRF1 to catalyse the dissociation of the 60S subunit from the 40S subunit, which is still bound to mRNA and deacylated tRNA (8,9,11,12). Additionally, it has been demonstrated that yeast Rli1 stimulates translation termination independently of ATP hydrolysis (8,13). In the next step, mediated by the eIF2D, MCT-1 and DENR proteins (Tma64, Tma20 and Tma22 in yeast), the mRNA and tRNA are released from the 40S subunit (14–16). While the functional activities of most of the participants in the final stages of translation are well studied, there are additional factors involved in this process. One of these factors is eIF3j.

eIF3j is a eukaryotic initiation factor originally reported as a loosely associated subunit of human eIF3 (17–19). Human eIF3j was shown to associate with 40S ribosomal subunits independently of the eIF3 holocomplex and critically improves the binding of the eIF3bgi subcomplex as well as that of the purified eIF3 (lacking eIF3j) to the 40S subunit *in vitro* (20). Likewise, 40S ribosomal subunit-binding of eIF3j was not affected in the individual knockdown of all 12 eIF3 subunits (21,22). It has been suggested that the yeast homolog of eIF3j, Hcr1, is an independent initiation factor that associates and closely cooperates with eIF3, without being an integral part of it (23). Moreover, Hcr1 is the only non-essential subunit in yeast (24).

eIF3j is involved in the initiation of translation. Directed hydroxyl radical probing and recent structural studies revealed that human and yeast eIF3j bind to the A-site of the ribosome and the mRNA entry channel of the 40S subunit, leading to the placement of eIF3j directly in the ribosomal decoding centre (25–27). However, the knockdown of eIF3j

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in HeLa and HEK293 cells had no effect on cell proliferation, only slightly reduced the initiation rates, and most importantly, showed no effect on eIF3 association with pre-initiation complexes *in vivo* (21). In yeast, it was shown that the deletion of *HCR1* leads to slow growth (Slg$^-$) phenotype and results in a severe leaky scanning phenotype indicating a defect in stringent AUG selection (28). Hcr1 was found to stimulate both the binding of eIF3 to the 40S subunit (28,29) and biogenesis of the 40S subunit (30). Hcr1 binds below the beak of the 40S subunit across the surfaces of eS30 and uS12 in the yeast 40S-eIF1-eIF1A-eIF3-eIF3j complex (31).

It was proposed that the major role of Hcr1 in yeast is the regulation of translation termination (23). *In vivo* experiments showed that the deletion of *HCR1* increases stop codon readthrough and results in the accumulation of eRF3 in fractions containing heavy polysomes that are enriched with termination complexes (23). However, other studies have suggested the involvement of eIF3j in ribosomal recycling. The role of Hcr1 in translation termination and recycling has also been investigated by ribosome profiling in yeast (32). It was shown that *hcr1Δ* cells have increased ribosome footprint occupancy in 3' untranslated regions (UTRs) and the level of translation reinitiation in 3′ UTRs, probably suppressing the function of the 60S recycling factor Rli1. Indeed, it was found that in *hcr1Δ* cells, the expression level of *RLI1* increased, which suppressed reinitiation induced by *HCR1* deletion. It was also demonstrated that Hcr1 is not involved in the recycling of the 40S subunit, as the overexpression of *HCR1* cannot compensate for the loss of the 40S subunit recycling factors Tma64 and Tma20 (32). Mammalian eIF3j is also involved in ribosomal recycling. It was demonstrated that the addition of the initiation factor eIF3 promotes splitting of the 60S subunit from the 40S subunit, and the initiation factors eIF3j, eIF1, and eIF1A enhance its activity *in vitro*. eIF1 mediates the release of tRNA from the P site, and eIF3j facilitates subsequent dissociation of mRNA from the 40S subunit (12,33). It was also demonstrated that eIF3j interacts with eIF1A and reduces the affinity of the 40S subunit for mRNA (25). Recently, it was shown that in the presence of the purified splitting factors Dom34, Hbs1, ABCE1, and eIF6, the reaction of splitting of the 80S ribosome is stimulated by eIF3j (26). Moreover, the N-terminal region of eIF3j in the 43S complex extends toward the GTPase binding region of the 40S subunit and ATP-binding domain of ABCE1 (26,27). This interaction probably prevents ATP hydrolysis and ABCE1 dissociation from the 40S subunit of the ribosome.

Thus, eIF3j is probably able to function independently in eukaryotic translation, and there is evidence supporting its involvement in initiation, termination, and ribosome recycling. However, there are no reported studies that clearly elucidate the mechanisms of eIF3j activity in translation termination and identify the molecules with which eIF3j interacts. To decipher the role of eIF3j in translation termination, we performed a functional study on human eIF3j using a reconstituted mammalian translation system (1). We showed that human eIF3j stimulates translation termination and determines the stage at which it functions. Experiments on the binding of eIF3j with the release factors and ribosomal complexes confirmed its direct involvement in translation termination. We concluded that eIF3j ensures the binding of the eRF1-eRF3-GTP complex to the A-site of the ribosome.

MATERIALS AND METHODS

Cloning and purification of eIF3j and its mutant forms

Human eIF3j cDNA (corresponding to isoform 1 mRNA) was cloned into the pET-28b(+) vector (Novagen) between the NdeI/XhoI sites. Based on the construct pET-28b-eIF3j, mutant W52A with single amino acid substitution was obtained using the QuikChange Site-Directed Mutagenesis Kit (Agilent, CA, USA), isoforms 2 and 3 of eIF3j and shortened variants Δ1–15 and Δ243–258 were obtained using the Gibson Assembly assay (NEB, MA, USA). The resulting His-tagged proteins were expressed in *Escherichia coli* BL21 cells after induction with 1 mM IPTG at 25°C overnight, followed by purification using a His-Select Nickel Affinity Gel (Sigma-Aldrich, MO, USA) and elution with 300 mM imidazole in buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 2 mM DTT and 10% glycerol. After dialysis, the eluted protein was applied to a HiTrapQ column (GE Healthcare, IL, USA). The N-terminal His-tag was cleaved with thrombin using a Thrombin Cleavage Capture Kit (Novagen).

In vitro pull-down assay using purified proteins

To study protein–protein interactions, in vitro pull-down assays were performed using purified proteins as described in (34) with modifications. All experiments were performed with 5 μM His-containing protein (eRF3a-His or eRF1-His) and 1 μM of the tested proteins (eRF1, eIF3j and PABP) and 0.2 mM nucleoside triphosphate (GTP, GDP, or GDPCP) in a final reaction volume of 20 μl in buffer P (20 mM Tris–HCl pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 10 mM imidazole pH 7.5, 1 mM DTT). The protein mixtures were incubated for 1 h on ice; subsequently, 20 μl of His-Select® Nickel Affinity Gel (Sigma-Aldrich, MO, USA) was added to the mixture and incubated for 1 h on ice. The reaction mixtures were washed six times with 200 μl of buffer P, and the proteins were eluted using buffer P supplemented with 200 mM imidazole. SDS loading dye (7 μl) was mixed with 20 μl of the eluted complexes. Then, 10 μl of the resulting samples and the samples of input proteins (deluted 20 times) were loaded onto a 10% or 12.5% SDS–PAGE gel. Proteins were detected by western blot analysis using the following antibodies: anti-eIF3j (Abcam, 71416), anti-eRF3 (Cell Signalling Technology, 14980S), anti-eRF1 (Abcam, 153731) and anti-PABP (Abcam, 21060). The intensity of the bands was measured using Image Lab program (BioRad, version 6.0.1). All experiments were performed in three replicates. Histogram data are presented as mean relative intensity ± standard error of the mean. A two-tailed *t*-test was used to compare the mean values between the two groups. The difference was considered significant when the *P*-value was less than 0.05.

Pre-termination complex on MVHL mRNA (preTC(MVHL)) assembly

The 40S and 60S ribosomal subunits as well as eukaryotic translation factors eIF2, eIF3, eEF1H, and eEF2, were purified from a rabbit reticulocyte lysate, as previously described (1). The human translation factors eIF1, eIF1A, eIF4A, eIF4B, ΔeIF4G, ΔeIF5B, eIF5, eRF1 and eRF3c were produced as recombinant proteins in *E. coli* strain BL21 with subsequent protein purification on Ni-NTA agarose and ion-exchange chromatography columns (1). Human eRF3a was expressed in insect cells Sf21 from the pFastBac-Htb-GSPT1 vector (35) and purified by affinity chromatography using a HisTrap HP column (GE Healthcare, IL, USA) followed by anion-exchange chromatography using a MonoQ column (GE Healthcare, IL, USA) (35).

MVHL-UAA mRNA was transcribed using T7 RNA polymerase from the pET28-MVHL-UAA plasmid. The pET28-MVHL-UAA plasmid contains a T7 promoter, four CAA repeats, the β-globin 5' UTR, an open reading frame (encoding the peptide MVHL), followed by the stop codon UAA and a 3' UTR comprising the rest of the natural β-globin coding sequence. To perform run-off transcription the mRNA plasmid was linearized using XhoI.

Either [^{35}S]-labelled or unlabelled eukaryotic preTCs on MVHL-UAA mRNA were assembled and purified as previously described (36). Briefly, initiation complexes were assembled in a 500-μl solution containing 37 pmol MVHL-UAA mRNA, 200 pmol Met-tRNA^{iMet} or [^{35}S]-labelled Met-tRNA^{iMet}, 90 pmol 40S and 60S ribosomal subunits, 200 pmol eIF2, 90 pmol eIF3, and 125 pmol of eIF4A, ΔeIF4G, eIF4B, eIF1, eIF1A, eIF5, ΔeIF5B supplemented with buffer A (25 mM Tris–HCl (pH 7.5), 50 mM KOAc, 2.5 mM $MgCl_2$, 2 mM DTT, 0.3 U/μl RNase inhibitor, 1 mM ATP, 0.25 mM spermidine, and 0.2 mM GTP). The reaction mixture was maintained at 37°C for 15 min to allow the ribosomal-mRNA complex formation. Peptide elongation was performed by adding 200 pmol total tRNA (acylated with all or individual amino acids), 200 pmol eEF1H, and 50 pmol eEF2 to the initiation complex, followed by incubation for another 15 min at 37°C. The ribosomal complexes were centrifuged in a Beckman SW55 rotor for 95 min at 4°C and 50 000 rpm in a linear sucrose density gradient (10–30%, w/w) prepared in buffer A containing 5 mM $MgCl_2$. After centrifugation, 13 lower gradient fractions of 150 μl were collected, and fractions corresponding to preTC complexes were detected by a toe-printing assay and by the presence of [^{35}S]-Met. Additionally, 8 μl of each fraction was analysed by western blotting using antibodies against eIF3j (Abcam, 71416), eIF3a (Abcam 86146), rpL9 (Abcam 182556) and rpS15 (Abcam 157193). The preTC fractions were combined and diluted 3-fold with buffer A containing 1.25 mM $MgCl_2$ (to a final concentration of 2.5 mM Mg^{2+}) and used in the peptide-release assay, for conformation-rearrangement analysis (toe-printing) or preTC binding assay.

Peptide-release assay

Radiolabelled preTCs (0.5 pmol), assembled on MVHL mRNA, were preincubated in 150 μl of the reaction mixture with 100 pmol eIF3j or GST at 37°C for 3 min, followed by the addition of 2.5 pmol eRF1 and incubation at 37°C for 15 min. Otherwise 150 μl aliquots containing 0.5 pmol preTCs were incubated with 50 pmol eIF3j or GST at 37°C for 3 min, followed by addition of 1.25 pmol eRF1 and 1.25 pmol eRF3a with 0.2 mM GTP and 0.2 mM $MgCl_2$ and incubation at 37°C for 3 min. In experiments on the influence of the initiation factor eIF3 on eIF3j in peptide-release assay, we preincubated 30 μl of the reaction mixture with 0.25 pmol eIF3 or 0.25 pmol eIF3 and 10 pmol eIF3j at 37°C for 3 min, followed by addition of 0.25 pmol eRF1 and 0.25 pmol eRF3a with 0.2 mM GTP and 0.2 mM $MgCl_2$ and incubation at 37°C for 3 min. Ribosomes and tRNA were pelleted with ice-cold 5% trichloroacetic acid (TCA) and centrifuged at 14 000 g at 4°C for 15 min. The amount of released [^{35}S]-MVHL tetrapeptide was determined by scintillation counting supernatants using an Intertechnique SL-30 liquid scintillation spectrometer (36).

Toe-printing assay

For conformational rearrangement (or toe-printing) analysis 10 μl aliquots containing 0.03 pmol preTCs were incubated at 37°C for 15 min with 0.25 pmol of eRF1 or 0.25 pmol eRF1(AGQ) mutant and 7 pmol of eIF3j or GST. Otherwise, 10 μl aliquots containing 0.03 pmol preTCs were incubated at 37°C for 15 min with 0.045 pmol of eRF1 and 0.015 eRF3a with 0.2 mM GTP or 0.045 pmol of eRF1 and 0.015 eRF3a with 0.2 mM GDPCP or with 0.045 pmol of eRF1(AGQ) mutant and 0.015 eRF3a with 0.2 mM GTP and 7 pmol of eIF3j or GST. Nucleotides were supplemented with equimolar amounts of $MgCl_2$.

The samples were analysed using a primer extension protocol. Toe-printing analysis was performed with AMV reverse transcriptase and a 5'-FAM-labelled primer complementary to the 3' UTR sequence. cDNAs were separated by electrophoresis using standard GeneScan® conditions on an ABI Prism® Genetic Analyser 3100 (Applera).

Conformational rearrangements corresponding to the TC or postTC formation were detected as a +2 nt shift of toe-print peaks, as previously described (35–38). Ribosomal shift efficiency was calculated using the formula TC/(TC + preTC).

Termi-Luc peptide release assay

A peptide release with nanoluciferase (NLuc) was performed as previously described (39) with modifications. The assay allows the measurement of NLuc release from TCs assembled on NLuc mRNA in the RRL. NLuc folds into the catalytically active form only after its release from the ribosome, which leads to luminescence in the presence of the substrate. NanoLuc mRNA was transcribed *in vitro* (T7 RiboMAX Express Large Scale RNA Production System, Promega, WI, USA) from a template containing β-globin 5' UTR, NLuc CDS, 3' UTR derived from pNL1.1[Nluc] vector (Promega, WI, USA), and 50 nt poly(A) tail. RRL lysate (1 ml; Green Hectares, USA) was preincubated in a mixture containing 1.5 u/μl Micrococcal nuclease (Thermo Fisher Scientific, MA, USA) and 1 mM $CaCl_2$ at 30°C for 10 min, followed by the addition of EGTA to a final concentra-

tion of 2 mM. A reaction mixture containing 70% nuclease-treated RRL was supplemented with 20 mM HEPES–KOH (pH 7.5), 80 mM KOAc, 0.5 mM $Mg(OAc)_2$, 0.36 mM ATP, 0.2 GTP, 0.05 mM each of 20 amino acids (Promega, WI, USA), 0.5 mM spermidine, 5 ng/μl total rabbit tRNAs, 10 mM creatine phosphate, 0.003 u/μl creatine kinase (Sigma-Aldrich, MO, USA), 2 mM DTT, and 0.2 U/μl Ribolock (Thermo Fisher Scientific, MA, USA) in a volume of 1 ml. The mixture was preincubated with 1 μM eRF1(AGQ) at 30°C for 10 min, followed by the addition of NLuc mRNA to a final concentration of 8 μg/ml, resulting in the formation of TCs. Next, the concentration of KOAc was adjusted to 300 mM and the mixture was layered on a 10–35% linear sucrose gradient in a buffer containing 50 mM HEPES–KOH, pH 7.5, 7.5 mM $Mg(OAc)_2$, 300 mM KOAc and 2 mM DTT. The gradients were centrifuged in a SW-41 Ti (Beckman Coulter) rotor at 18 000 rpm for 14 h. Fractions enriched with preTCs were collected. PreTC was aliquoted, flash-frozen in liquid nitrogen, and stored at − 80°C. Peptide release was performed in a solution containing 8 nM preTC, 50 mM HEPES–KOH, pH 7.5, 0.25 mM spermidine, 2 mM DTT, 0.2 mM GTP, and 1% NLuc substrate (Nano-Glo, Promega, WI, USA) in the presence of 8 nM eRF1, 8–20 nM eRF3a/c, 8 nM eIF3 and 160 nM eIF3j or its mutants/isoforms or GST as a negative control. Luminescence was measured for 1 h at 30°C using a Tecan Infinite 200Pro (Tecan, Männedorf, Switzerland). Peptide release kinetic curves were generated, and the standard deviation was calculated.

GTPase assay

Ribosomal 40S and 60S subunits (1 pmol each) were incubated at 37°C for 10 min. Then, GTPase buffer (10 mM Tris–HCl pH 7.5, 35 mM NH_4Cl, 18 mM $MgCl_2$, and 0.5 mM GTP), 1 pmol of eRF1, 3 pmol of eRF3a and 0, 5, 10 or 20 pmol of eIF3j were added. The final reaction volume was 10 μl. After incubation for 15 min at 37°C, the amount of released phosphate was estimated using the Malachite Green Phosphate Assay (Sigma-Aldrich, MO, USA), according to the manufacturer's protocol.

PreTC binding assay

A sample of 1.5 pmol of purified preTC(MVHL), 1.35 pmol of purified preTC(NL), or 1.5 pmol of 80S ribosomes was incubated with 15 pmol eRF3a, 15 pmol eIF3j, and 15 pmol eRF1 in the buffer containing 0.2 mM GTP/GDPCP/GDP supplemented with 0.2 mM $MgCl_2$ at 37°C for 10 min. The reaction volume was 500 μl. Subsequently, TCs were incubated with 1% formaldehyde at 4°C for 1 h (40). Glycine was added up to 0.1 M to stop the cross-linking reaction. The preTCs were centrifuged in a 10–30% (w/w) linear SDG, as described above. The whole gradient was fractionated into 14 equal fractions (360 μl), followed by precipitation in 10% TCA. The protein pellets were dried and analysed by western blotting using antibodies against eIF3j (Abcam, 71416), eRF3 (Cell Signalling Technology, 14980S), eRF1 (Abcam, 153731) and rpL9 (Abcam 182556).

RESULTS

eIF3j enhances the peptidyl-tRNA hydrolysis induced by release factors eRF1 and eRF3

To study the activity of human eIF3j in translation termination, we assembled a pre-termination complex on the model MVHL-UAA mRNA, the preTC(MVHL), in the mammalian reconstituted translation system by sequentially carrying out the translation initiation and elongation stages. The preTC(MVHL) was purified by SDG centrifugation. The addition of human recombinant eIF3j and release factors to this complex allowed us to study the effect of eIF3j on translation termination *in vitro*. The efficiency of peptidyl–tRNA hydrolysis was determined by quantifying the radioactive MVHL peptide released from the ribosomal complexes into solution. We found that the tetrapeptide release efficiency was not significantly changed after the addition of eIF3j to the reaction mixture in the presence of eRF1 (Figure 1A). In contrast, when both release factors eRF1 and eRF3a were present, eIF3j slightly stimulated peptide release (Figure 1B).

To study the effect of eIF3j on peptide release by another approach, we obtained the preTC on the nanoluciferase mRNA, the preTC(NL), using the Termi-luc method (39). The preTC(NL) contains long polypeptide–nanoluciferase (19.1 kDa, 171 amino acids). Nluc release from the ribosome during translation termination causes luminescence of the substrate. We revealed that in the presence of eRF1 alone, the efficiency of polypeptide release was slightly stimulated by eIF3j (Figure 1A), but in the presence of both eRF1 and eRF3a, eIF3j significantly increased polypeptide release (Figure 1B). eIF3j alone did not induce peptide release. To show that the effect of eIF3j on peptide release is not associated with the stabilisation of release factors or the nonspecific prevention of their sorption in test tubes due to low eRFs concentrations, we used glutathione S-transferase (GST) as a negative control (Figure 1A, B). The addition of the same amount of GST as eIF3j did not affect the activity of the release factors. Thus, the effect of eIF3j on peptide release is specific and reflects its functional activity in cells. We also tested the activity of lower concentrations of eIF3j in peptide release (Supplementary Figure S1A) and demonstrated that eIF3j can function under such conditions, confirming its specificity for peptide release.

To study the participation of eRF3 in multiple rounds of peptide release in the presence of eIF3j, we determined the activity of eIF3j at higher concentrations of eRF3a (Figure 1C). We demonstrated that excess of eRF3a reduced the positive effect of eIF3j on translation termination. Therefore, eIF3j increases the activity of eRF3 or its working concentration.

Furthermore, we compared eIF3j activity in the presence of different eRF3 variants, full-size eRF3a, and an N-terminally truncated form eRF3c that lacked the first 138 amino acids (Supplementary Figure S1B). We revealed that the presence of the N-terminal domain of eRF3a is important for the stimulation of translation termination by eIF3j.

Since eIF3j strongly activates peptidyl-tRNA hydrolysis in the presence of eRF3, we suggest that eIF3j can affect the GTPase activity of eRF3. eRF3 is known to exhibit

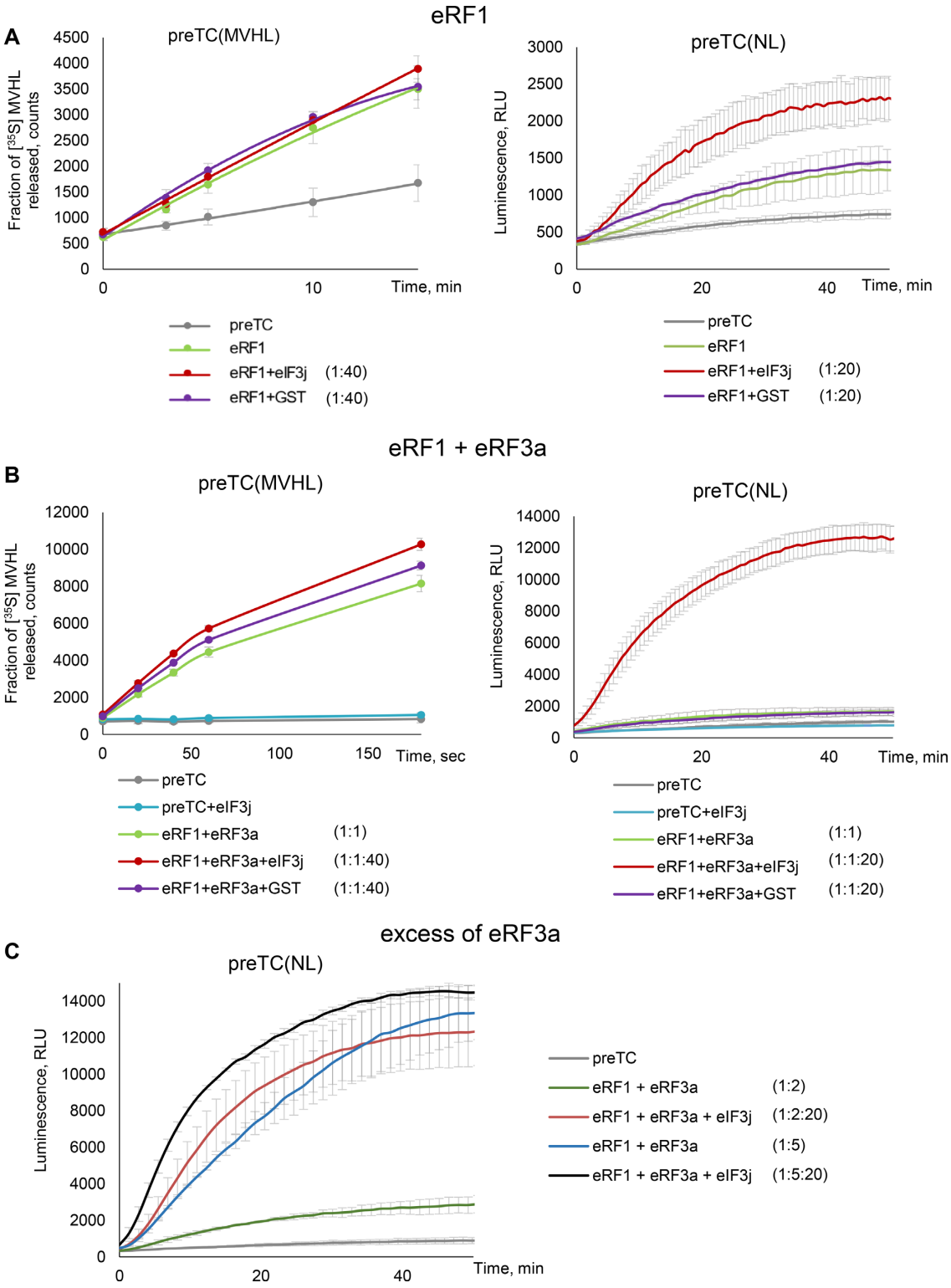

Figure 1. eIF3j increases the efficiency of peptidyl–tRNA hydrolysis induced by release factors eRF1 and eRF3. (**A**) Hydrolysis of peptidyl-tRNA on the preTC(MVHL) and preTC(NL) induced by the addition of eRF1 in the presence/absence of eIF3j ($n = 3$). (**B**) Hydrolysis of peptidyl-tRNA on the preTC(MVHL) and preTC(NL) induced by the addition of eRF1 and eRF3a-GTP in the presence/absence of eIF3j ($n = 3$). (**C**) Hydrolysis of peptidyl–tRNA on the preTC(NL) induced by the addition of eRF1 and 2-fold or 5-fold excess of eRF3a-GTP in the presence/absence of eIF3j ($n = 3$). The error bars represent the standard deviation; RLU, relative luminescence units.

GTPase activity in the presence of the 80S ribosome and eRF1. The addition of eIF3j to the GTPase reaction did not affect the ability of human eRF3a to hydrolyse GTP (Supplementary Figure S1C). Thus, we have shown that eIF3j stimulates peptidyl-tRNA hydrolysis induced by release factors and does not affect the GTPase activity of eRF3, which is consistent with the data obtained for the yeast homolog (23).

Both eIF3j and eIF3 stimulate peptide release in a cooperative way

Since eIF3j works differently on different preTCs, we compared their composition by western blotting. We did not find eIF3j in the fractions corresponding to both preTCs (Figure 2A). We hypothesised that during SDG centrifugation, the labile subunit eIF3j dissociated from the ribosome, since proteins in the complexes were not immobilised with formaldehyde before centrifugation. However, we revealed that eIF3 remains bound to the formed on the short coding sequence preTC(MVHL) even after purification of the complex by ultracentrifugation in an SDG (Figure 2A). Therefore, the preTC(MVHL) is free of eIF3j but contains the eIF3 holocomplex. The presence of eIF3 in the preTC(MVHL) is consistent with its role in termination and stop codon readthrough (41). We also determined the amount of eIF3 in the preTC(NL) (Figure 2A). We detected only a small amount of eIF3 bound to the preTC formed on the long coding sequence. Therefore, we obtained preTCs containing different amounts of eIF3.

We then determined the effect of eIF3 on the activity of eIF3j in both preTCs. The addition of eIF3 to the preTC(NL) significantly promoted peptide release from this complex and slightly increased the activity of eIF3j. In contrast, the addition of extra eIF3 to the preTC(MVHL) slightly stimulated peptide release and did not significantly influence the activity of eIF3j, because the preTC composition did not change (Figure 2B). Therefore, we revealed that both eIF3j and eIF3 stimulate peptide release. Moreover, when combined, these proteins slightly enhance the individual activity of each other during translation termination. This fact explains the weak effect of eIF3j on peptide release on the preTC(MVHL) and strong stimulation on the preTC(NL) (Figures 1B, 2B). Comparing these data with the activities of eIF3j on different complexes (Figure 1A, B), we concluded that the presence of the eIF3 holocomplex in the preTC masks the activity of eIF3j in translation termination and that eIF3j can function in translation termination in the absence of eIF3.

eIF3j binds to eRF3a and the eRF1-eRF3a complex in the presence of GTP

Since the activity of eIF3j in translation termination is directly related to the presence of eRF3, we tested their ability to interact with each other in the solution. For this, we performed a pull-down assay using His-tagged eRF3a bound to Ni-NTA agarose (Figure 3A). We revealed that in the presence of GTP, eIF3j binds to eRF3a alone as well as when eRF3a is in the complex with eRF1 (Figure 3A). We also observed that in the presence of a non-hydrolysable analogue of GTP, GDPCP, eIF3j interacts only with the complex of release factors eRF3a-eRF1 and does not bind to eRF3a alone (Figure 3A). Probably GDPCP locks eRF3 in a specific conformation that prevents its binding to eIF3j and eRF1 overcomes it. In the presence of GDP, no complex of release factors with eIF3j was detected. The analysis of the amount of eRF1 in such complexes showed that eIF3j did not affect the interaction of eRF1 and eRF3a with each other (Figure 3A).

Similar experiments were performed to study the interaction between eRF1 and eIF3j in the solution. Using His-tagged eRF1 bound to Ni-NTA agarose in the pull-down assay, we showed that eIF3j was unable to bind to eRF1 (Figure 3B). Considering this, we speculated that in the triple complex, eIF3j interacts with eRF1 through eRF3.

Therefore, pull-down analysis indicated that eIF3j could interact with eRF3a in the solution in a nucleotide-dependent manner (Figure 3C), which is consistent with previously obtained results and published data. In yeast, *in vivo* pull-down experiments demonstrated that Hcr1 was co-purified with selected eIF3 subunits, Rli1, eRF3 (about 9%), and eRF1 (about 2%). However, when *in vitro* GST pull-down assays for Hcr1 were performed, the authors could not see any binding with release factors, possibly because the GST-tag prevented binding (23).

Binding of eIF3j and release factors to the termination complexes

Previous studies involving SDG centrifugation and immunoblotting of formaldehyde cross-linked whole-cell extracts reported the detection of Hcr1, along with the release factors Sup35 (eRF3) and Sup45 (eRF1) in the polysome fraction (23).

Since the binding of eIF3j to the eIF3 holocomplex is not required for the activity of eIF3j in translation termination (Figure 1B), we proposed that the initiation factor eIF3, when associated with the preTC, can attract eIF3j to the complex. This binding is likely to be labile since it was not observed after purification of the preTCs with SDG (Figure 2A). To check the ability of eIF3j to bind purified preTCs, formaldehyde was added in the binding reaction of eIF3j with the preTC, by analogy with the study performed on yeast (23), and then the complexes and free proteins were separated by SDG centrifugation. eIF3j was detected in the gradient fractions by western blotting. The preTC(MVHL) was detected in fractions 7–13 and the preTC(NL) in fractions 10–12 by the presence of 60S ribosomal subunits. As expected, recombinant eIF3j was detected in the same fractions (Figure 4). It should be noted that eIF3j binds directly to the ribosome in the pretermination ribosomal complex because the preTC(NL) contains very small amounts of eIF3 to bind eIF3j (Figure 2A). To be sure that after the second round of SDG performed during the binding experiments with the preTC, eIF3 did not dissociate from the preTC(MVHL), we determined eIF3 in the fractions by western blot analysis after repeated SDG (Supplementary Figure S2A). Indeed, we observed a large amount of eIF3 in the preTC(MVHL) and very few traces of eIF3 in the preTC(NL). Thus, we confirmed that eIF3 remained

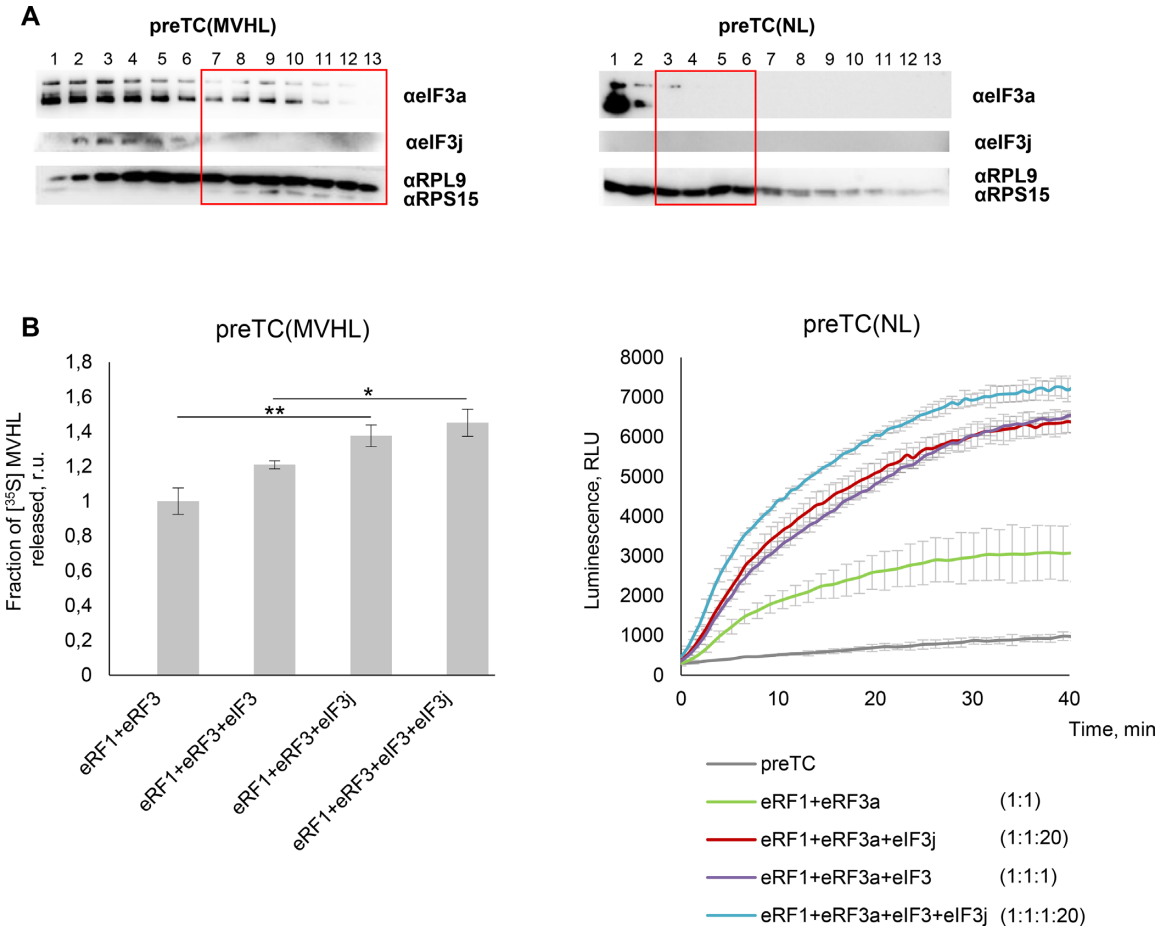

Figure 2. eIF3j does not require binding to eIF3 during translation termination**.** (**A**) Western blot analysis of the preTC gradient after SDG. Antibodies raised against eIF3j, eIF3a, rpL9 and rpS15 were used for detection. The fractions of the SDG are indicated above the western blots; fraction 1 corresponds to the top of the gradient and 13 to the bottom of the gradient. Boxes indicate the fractions that contain ribosomal complexes according to the toe-printing. (**B**) Hydrolysis of peptidyl-tRNA on the preTC(MVHL) and preTC(NL) induced by the addition of eRF1 and eRF3a in the presence of eIF3 and eIF3j ($n = 3$). For preTC(MVHL), the background was subtracted, and each mean value was normalized to the value of eRF1 activity. The error bars represent the standard deviation, ** represents a significant difference from the respective control $P < 0.01$, * represents $P < 0.05$, *t*-test. r.u., relative units; RLU, relative luminescence units.

bound to the preTC(MVHL) during the purification of the binding mixture. Therefore, we can confidently assert that eIF3j directly binds to the ribosomes in the preTC(NL) and eRF3a indirectly interacts with eIF3 via eIF3j in the preTC(MVHL). To exclude the aggregation of eIF3j, we tested the distribution of this protein in SDG in the absence of the ribosomal complexes. eIF3j alone can be detected only in the top fractions of the gradient (fractions 1-3) (Supplementary Figure S2B).

Since we demonstrated the interaction between eIF3j and eRF3a (Figure 3A), we determined the pattern of eIF3j binding to the ribosomes in the presence of eRF3a-GTP and formaldehyde. A small amount of eRF3a was detected in the preTC(MVHL), and we did not observe any eRF3a in the preTC(NL) (Figure 4). However, when the preTCs were incubated simultaneously with eIF3j and eRF3a-GTP, in the case of the preTC(MVHL) complex, we detected very good binding of eIF3j which promotes binding of eRF3a to the preTC (Figure 4A). In the case of the preTC(NL), only a small fraction of eRF3a and no eIF3j were associated with the preTC (Figure 4B).

In addition, we performed control experiments with empty 80S ribosomes (Supplementary Figure S2C). We observed binding of eIF3j and lack of binding of eRF3a-GTP with the 80S ribosomes and 40S ribosomal subunits. Moreover, the presence of eIF3j did not promote binding of eRF3a to the 80S ribosome. Consequently, joint binding of eRF3a and eIF3j to the preTC(MVHL) is specific to the preTC.

To study the binding of both release factors to the preTC in the presence of eIF3j, we performed similar experiments with the binding of eIF3j and eRF1-eRF3a complex to the preTC(MVHL) in the presence of GTP, GDPCP, and GDP (Supplementary Figure S2D). We found that all three proteins bind to the preTC, regardless of the nucleotide type. It is likely that eIF3j and release factors could remain bound to the ribosome, interacting with the A-site or eIF3, depending on whether a ternary protein complex is formed in the presence of GTP or a double protein complex is formed in the presence of GDP (Figure 3C).

Thus, we found that eRF3a-GTP destabilises the binding of eIF3j to the preTC in the absence of eIF3 and remains

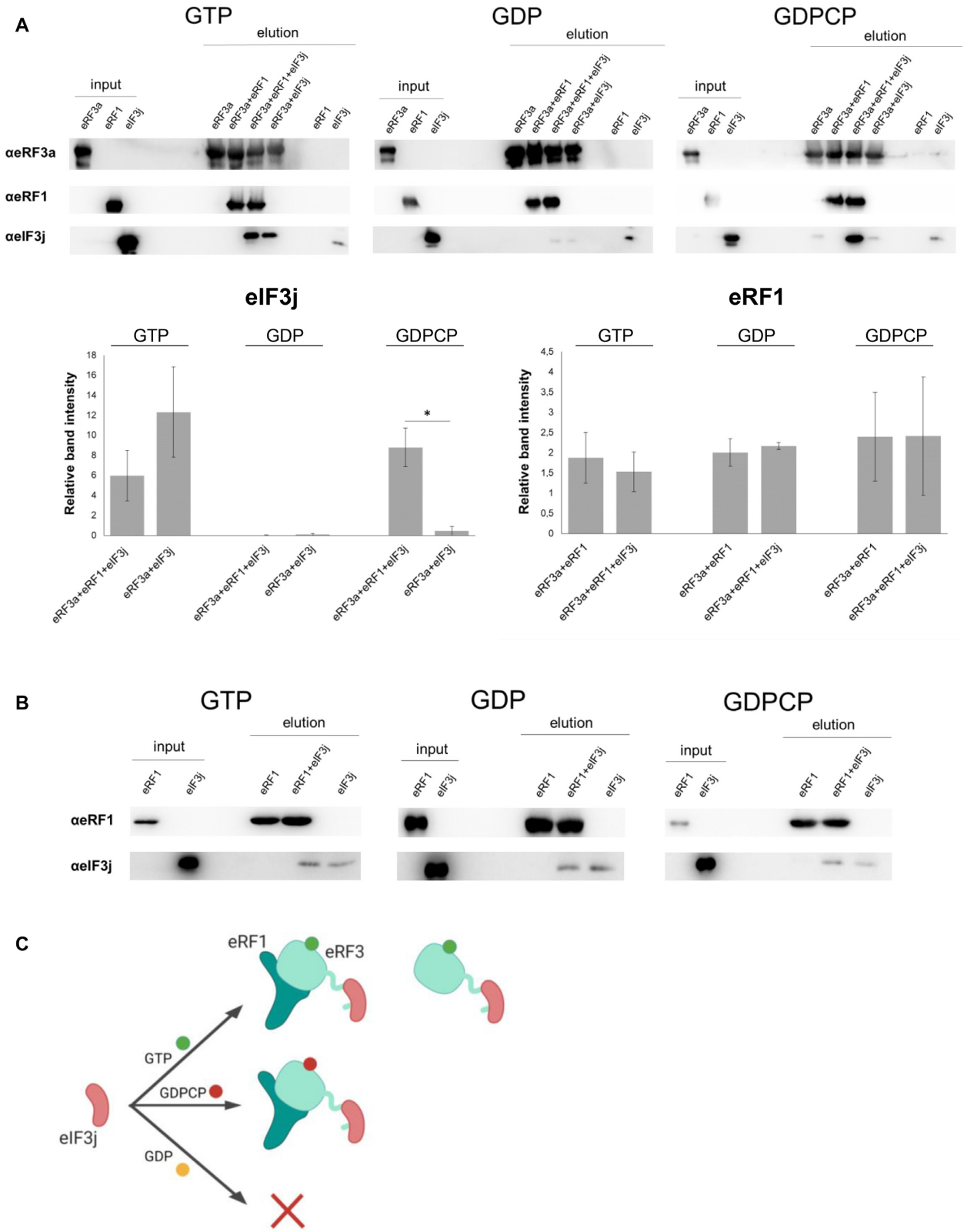

Figure 3. eIF3j interacts with eRF3a-GTP in the pull-down assay. (**A**) eIF3j binding with His-eRF3a and His-eRF3a + eRF1 in the presence of GTP, GDPCP and GDP. (**B**) eIF3j binding with His-eRF1 in the presence of GTP, GDPCP and GDP. Protein samples before loading onto the resin (input) or after elution (elution) were analysed by western-blot. For detection, antibodies raised against eRF1, eRF3 and eIF3j were used. All experiments were carried out in three replicates. The intensity of each band was normalized to the intensity of the control band. Histogram data are presented as mean relative intensity ± standard error of the mean. The difference was considered significant when P value (two-tailed t-test) was less than 0.05 (*). (**C**) Schematic representation of interactions of eIF3j with release factors as revealed by pull-down assay.

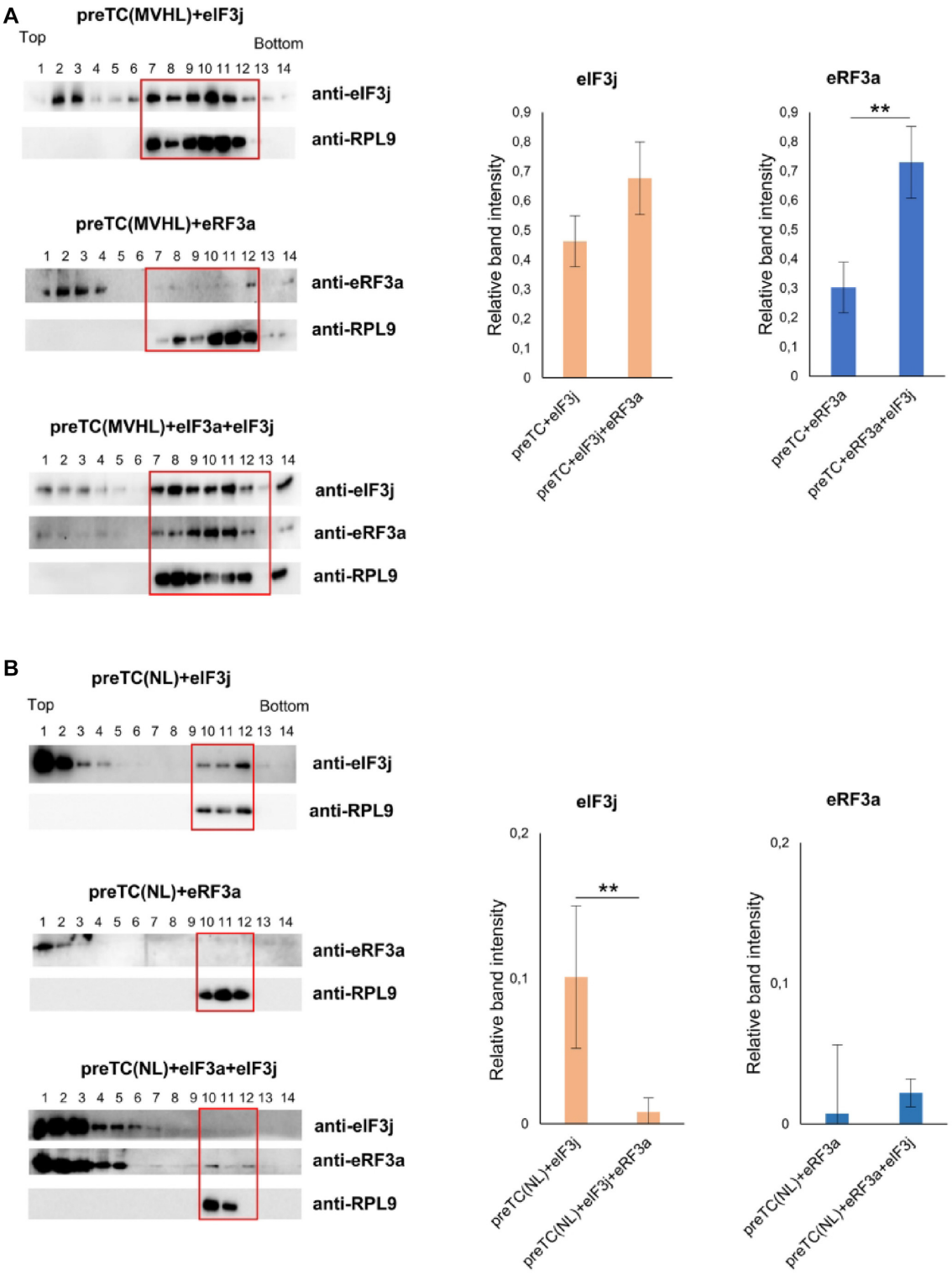

Figure 4. Binding of eIF3j and eRF3a to the preTCs. Western blot analyses of binding of eIF3j and eRF3a in the presence of GTP to purified (**A**) preTC(MVHL) and (**B**) preTC(NL), separated via SDG centrifugation. Antibodies raised against eIF3j, eRF3a and ribosomal protein L9 were used for detection. The fractions of the SDG are indicated; fraction 1 corresponds to the top of the gradient and 14 to the bottom of the gradient. Boxes indicate the fractions that contain ribosomal complexes. All experiments were carried out in three replicates. The intensity of each band was normalized to the intensity of the corresponding rpL9 band. Histogram data are presented as mean relative intensity ± standard error of the mean. The difference was considered significant when *P* value (two-tailed *t*-test) was less than 0.01 (**).

bound to the preTC in the presence of eIF3, probably as part of the complex eIF3–eIF3j–eRF3a–GTP. Joint dissociation of eRF3a and eIF3j from the preTC in the presence of GTP is consistent with the data obtained for *HCR1*, the deletion of which causes accumulation of Sup35 (eRF3) in the polysomes (23).

eIF3j affects termination complexes formation

To determine the stage of the translation termination in which eIF3j works, we used a fluorescence toe-printing assay that allows the determination of the position of the ribosomal complex on mRNA (37,38).

It should be noted that translation termination occurs in several consecutive steps (Figure 5A). First, the binding of release factors to the ribosome and recognition of the stop codon by eRF1 occurs, resulting in the formation of termination complex 1 (TC1). Next, GTP is hydrolysed by eRF3; however, it is not completely clear what happens next, eRF3-GDP either dissociates spontaneously from the ribosome or changes its conformation so that the M domain of eRF1 is accommodated in the PTC, forming termination complex 2 (TC2). As soon as the GGQ motif in the M domain of eRF1 enters the PTC, peptidyl-tRNA is hydrolysed and the newly synthesized peptide is released from the ribosome. Post-termination complex 1 (postTC1) is formed, in which eRF1 is still associated with the ribosome. As a result of eRF1 dissociation from the ribosome after the termination of translation, post-termination complex 2 (postTC2) is formed. The addition of various modified translation termination components stabilises certain termination complexes. To freeze TC1, we used the non-hydrolysable GTP analogue (GDPCP). To stabilise TC2, we used a mutant form of eRF1, eRF1(AGQ), which can bind to the stop codon and accommodate the M domain in the PTC after GTP hydrolysis but cannot induce peptidyl-tRNA hydrolysis. Using of eRF1 + eRF3 + GTP allowed us to obtain a mixture of postTC1 and postTC2.

During stop codon recognition by eRF1, the ribosome protects additional nucleotides on mRNA, which is detectable in the toe-printing assay as a one- or two-nucleotide shift of the ribosomal complex (1,3,42). As a result, depending on the components added to the reaction, either TC1/2 or postTC1 could be detected at the same mRNA position. The postTC2 in the toe-printing assay could be detected at the same position as the preTC because after the dissociation of eRF1 from the ribosome, the mRNA conformation returned to its original state (Figure 5A).

For the toe-printing assay, preTC(MVHL) was used. The raw data for fluorescent toe-printing are shown in Supplementary Figure S3. Toe-printing revealed that the addition of eIF3j to the translation termination reaction with eRF1 or eRF1(AGQ) alone did not have a significant effect on TC and postTC formation (Figure 5B, Supplementary Figure S3B), which is consistent with the results obtained in the peptidyl-tRNA hydrolysis assay (Figure 1A). The effect of eIF3j on TC formation in the presence of both release factors was determined at a 3-fold lower eRF3a concentration relative to eRF1. This allowed us to activate termination in the deficiency of eRF3a and to investigate the stimulatory activity of eIF3j. We observed that eIF3j significantly stimulated the formation of TC1 in the presence of GDPCP (Figure 5C, Supplementary Figure S3C) and slightly stimulated the formation of TC2 in the presence of eRF1(AGQ) (Figure 5C, Supplementary Figure S3C). These experiments indicate that the stimulation of translation termination by eIF3j occurs before or independently of GTP hydrolysis by eRF3a. Our results showed that eIF3j exerted no stimulatory effect on postTC1 formation in the presence of eRF1, eRF3a, and GTP (Figure 5C, Supplementary Figure S3C). This can be explained by the dissociation of eRF1 from the ribosome after peptidyl-tRNA hydrolysis and the transition of postTC1 to postTC2. PostTC2 cannot be distinguished from the preTC by the toe-printing assay. GST was used as a negative control for the action of eIF3j in termination complex formation, similar to the peptide release experiments. PreTC(MVHL) contains significant amounts of eIF3 (Figure 2A), and to distinguish between the effects of eIF3j and eIF3 on TC formation, we used the preTC(MVHL)-HS. The preTC(MVHL)-HS was obtained using SDG containing potassium acetate in high concentration. Western blot analysis of this complex showed the absence of eIF3 and eIF3j in the samples (Supplementary Figure S4A). The toe-printing assay on the preTC(MVHL)-HS in the presence of GDPCP demonstrated similar stimulatory activity of eIF3j on TC1 formation as for the preTC (MVHL) (Supplementary Figure S4B, C). Therefore, we assumed that eIF3j promotes binding of the eRF1–eRF3–GTP complex to the preTC before GTP hydrolysis.

Activities of the mutant forms of human eIF3j in translation termination

We investigated the translation termination activity of human eIF3j isoforms resulting from alternative splicing and eIF3j mutants (Figure 6A). The main isoform of eIF3j in human cells is isoform 1 (iso 1), which consists of 258 amino acids. The sequence of isoform 2 (iso 2) differs from iso1 in the replacement of aa 137-191 by a single Val. Compared with iso1, isoform 3 (iso 3) lacks aa 50–98. Additionally, we obtained the C-terminally truncated form of eIF3j-Δ (243–258). The C-terminus was identified as a target of cleavage by caspase 3 between residues 242 and 243, resulting in a protein lacking 16 amino acid residues (20). This form of eIF3j binds poorly to the 40S subunit, which in turn worsens the binding of the holocomplex eIF3 to the 40S subunit. We also obtained an N-terminal truncated form of eIF3j-Δ(1–15) to reveal the role of terminal amino acid residues in translation termination. In addition, we tested the activity of the W52A mutant of eIF3j, a form of eIF3j with a mutation that greatly reduces its ability to bind to the eIF3b subunit (28).

In peptidyl-tRNA hydrolysis experiments using the preTC(NL), it was found that both isoforms of eIF3j and the N-terminal deletion variant of eIF3j-Δ(1–15) were completely inactive (Figure 6B). The C-terminal deleted variant Δ(243–258) demonstrated ∼30% of eIF3j activity and W52A demonstrated approximately 15% of eIF3j activity. Thus, all tested mutant variants of eIF3j are important for its activity in translation termination, although the residues responsible for binding with eIF3 (W52A) and the

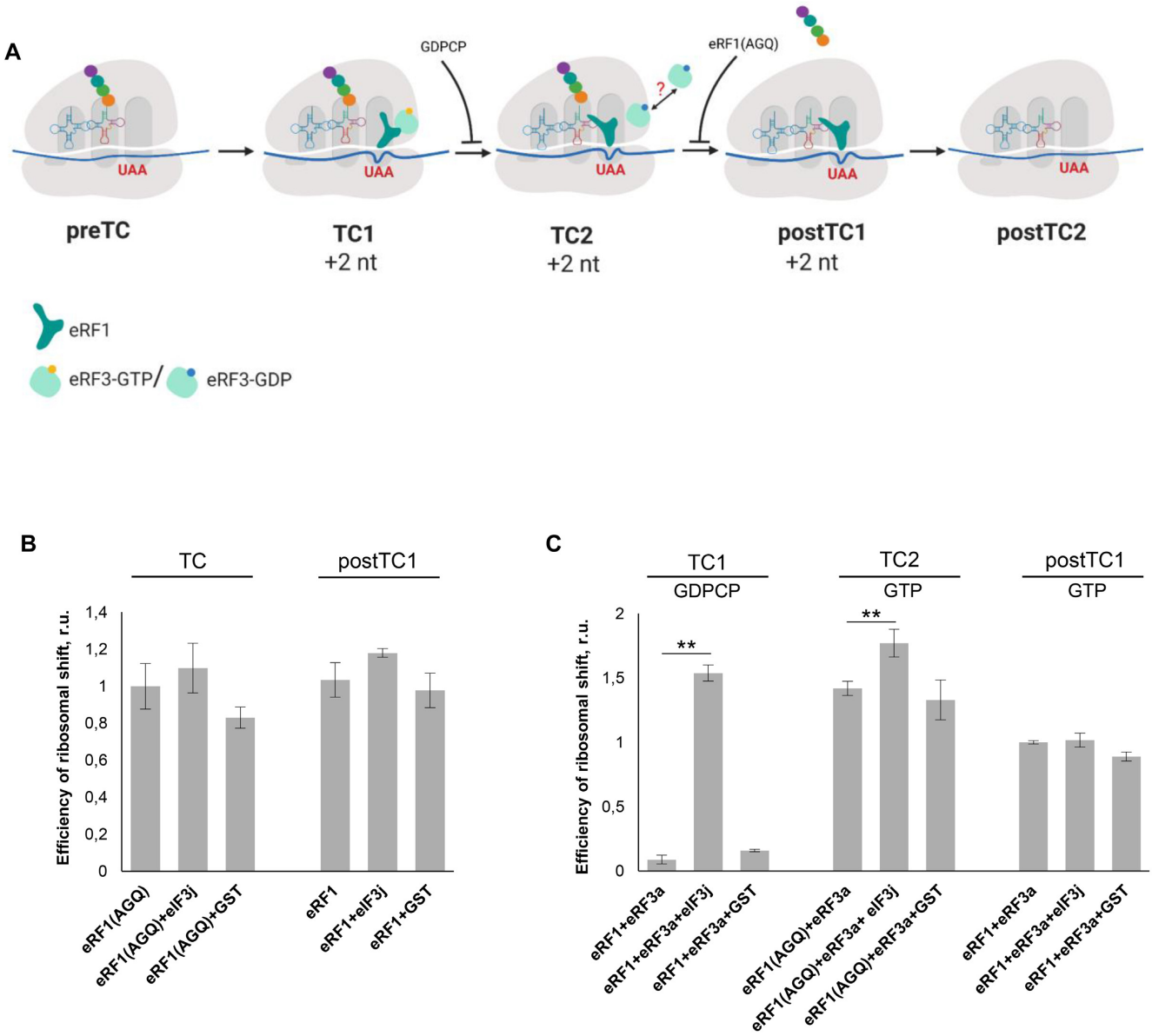

Figure 5. Effect of eIF3j on the termination and post-termination complexes formation. (**A**) Outline of the termination process related to the toe-printing assay (see Results). (**B**) Effect of eIF3j on TC and postTC formation in the presence of eRF1(AGQ) or eRF1 ($n = 3$). The background was subtracted and each mean value was normalized to the value of eRF1 activity. (**C**) Effect of eIF3j on TC1, TC2 and postTC formation in the presence of eRF1 + eRF3a + GDPCP, eRF1(AGQ) + eRF3a + GTP, or eRF1 + eRF3a + GTP, respectively ($n = 3$). The background was subtracted and each mean value was normalized to the value of eRF1 + eRF3a + GTP activity. The error bars represent the standard deviation, ** represents a significant difference from the respective control $P < 0.01$, *t*-test. *n*, number of repeats; r.u., relative units.

mRNA entry channel of the 40S subunit (Δ(243–258)) have a smaller contribution to the termination activity of eIF3j than others. It should be noted that residues that are more important are located in the structured core region of eIF3j; however, the deletion of the unstructured N-terminal region completely inactivated its activity in translation termination (Figure 6C). This indicates the direct involvement of this region in the termination process.

eIF3j can function in termination in the presence of PABP

Since the activity of eIF3j was significantly increased in the presence of the N domain of eRF3a (Supplementary Figure S1B), we assumed that eRF3a interacts with eIF3j in this domain. It is known that eRF3a binds to poly(A)-binding protein (PABP) via the N domain. Therefore, it became necessary to determine whether eIF3j and PABP are antagonists in translation termination. We performed peptide release experiments using the preTC(NL) and determined that simultaneously added eIF3j and PABP worked independently and did not suppress each other (Supplementary Figure S5A). We detected even a slight co-stimulation of these proteins during peptide release. Moreover, pull-down experiments showed that eRF1, eRF3a, eIF3j and PABP form a quadruple complex in the presence of GTP (Supplementary Figure S5B). Thus, eIF3j and PABP probably

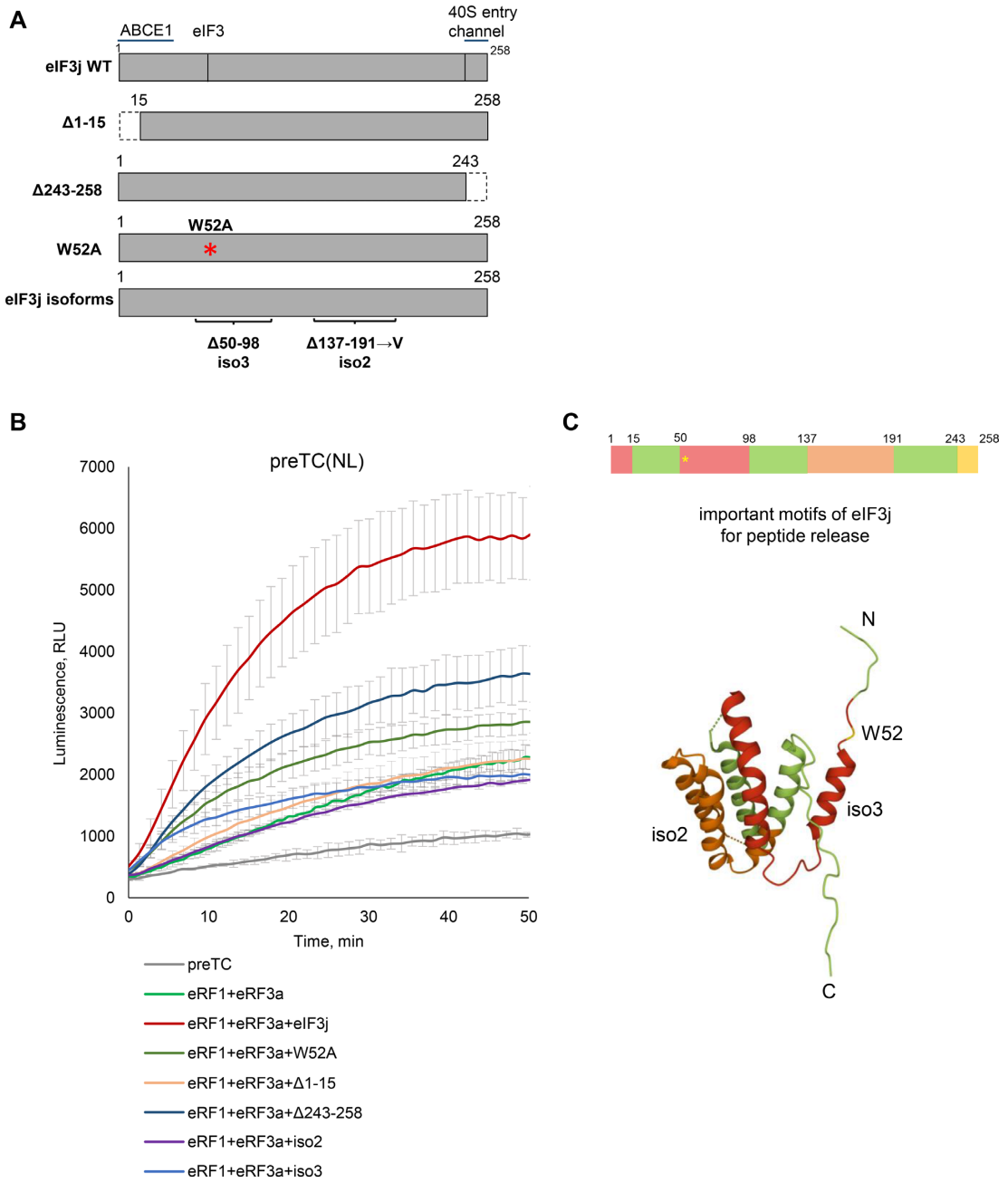

Figure 6. eIF3j isoforms and mutants activity in peptide release. (**A**) Schematic representation of the eIF3j mutants used in this study. (**B**) Peptide release on the preTC(NL) induced by the addition of eRF1 and eRF3a in the presence of eIF3j, eIF3j isoforms, or eIF3j mutants ($n = 3$). The error bars represent the standard deviation; RLU, Relative Luminescence Units. (**C**) Motifs of eIF3j important for peptide release represented on the structure of eIF3j ((27); PDB 6YBW chain z, lacking 43 residues at the N-terminus, 23 residues at the C-terminus, and residues (97–112) and (136–152)).

interact with different regions of the N domain of eRF3a and can be involved in translation termination at the same stage.

DISCUSSION

In this study, we revealed the role in translation termination of the human translation factor eIF3j, originally reported as the labile subunit of eIF3. We showed that eIF3j increases the efficiency of peptidyl-tRNA hydrolysis in the presence of release factors eRF1 and eRF3 (Figure 1), and that the N-terminal domain of eRF3a is important for this reaction (Supplementary Figure S1B). Weak peptide release stimulation in the presence of eRF1 alone is possibly caused by the effect of eIF3j on the A-site of the ribosome, since it cannot interact with eRF1 in the solution (Figure 3B), but binds to the preTC (Figure 4). Thus, we found that human eIF3j, like its yeast homolog Hcr1, is involved in translation termination. In yeast, the deletion of the non-essential *HCR1* encoding eIF3j causes a significant increase in stop codon readthrough and accumulation of eRF3 in the fractions containing heavy polysomes that are enriched with ter-

mination complexes, without affecting the levels of eRF1 and eRF3 in the cells (23).

Furthermore, using a pull-down assay, we showed that human eIF3j can bind to eRF3a alone or in combination with eRF1 only in the presence of GTP (Figure 3). Previously, *in vivo* co-immunoprecipitation experiments using formaldehyde pre-treated cells showed that its yeast homolog, Hcr1, was associated with eRF3 (about 9%) and eRF1 (about 2%), but it was not clear whether they bind as a single complex, or whether these proteins can interact individually (23). The authors also attempted to perform *in vitro* pull-down of GST-tagged Hcr1 with radioactively labelled eRF1 and eRF3 but could not detect binding of the yeast proteins; therefore, they suggested that the binding of eIF3j and eRF3 occurs only in the postTC (23). However, our results indicate that human eIF3j binds to eRF3 in the solution, and this binding does not interfere with the interaction between eRF1 and eRF3. Since the N-terminal domain of eRF3a significantly stimulates eIF3j activity during translation termination (Supplementary Figure S1B), we suggest that this is the region of eRF3a that is involved in the interaction of eRF3a with eIF3j.

The influence of eIF3j on translation termination was found not only in peptidyl–tRNA hydrolysis, but also in the toe-printing assay of termination and post-termination complexes (Figure 5). Using this method, we accurately determined the step at which eIF3j functions - that is, binding to the A-site of the ribosome before GTP hydrolysis by eRF3. We also demonstrated that the function of human eIF3j in translation termination does not require its interaction with eIF3, since eIF3j significantly stimulates peptide release in the preTC(NL), lacking eIF3 (Figure 2). This conclusion is supported by the fact that the W52A mutation of eIF3j, which weakens the interaction of eIF3j with eIF3, does not completely reduce protein activity (Figure 6B). This is also consistent with previously published data for the yeast homologue of this protein, which indicated that mutations disrupting the interaction of Hcr1 with eIF3 did not affect the stop codon readthrough (23), and with the results of ribosomal profiling of yeast cells (32). In agreement with this, eIF3j is loosely associated with the other eIF3 subunits, and when the eIF3 complex is purified, eIF3j is often missing from the pool of 12 co-purifying subunits (17–19). Hcr1 was also shown to interact independently with both sides of the 40S subunit mRNA entry channel (28).

On the other hand, we have also demonstrated that eukaryotic initiation factor 3, irrespective of eIF3j, significantly stimulates peptide release (Figure 2B) and that the *j* subunit together with this protein enhance individual activities of each other during translation termination (Figure 2B). Thus, eIF3j activity in translation termination does not require the presence of eIF3, but can be regulated by this protein.

To study the activity of eIF3j in translation termination, we used two types of preTCs assembled on the different model mRNAs, coding short tetrapeptide MVHL, preTC(MVHL), and long polypeptide nanoluciferase, preTC(NL). These complexes simulate various termination processes occurring on mRNA during the translation of long coding sequences and short uORFs. We determined the composition of these complexes and showed that preTC(MVHL) contains a large amount of initiation factor eIF3 (Figure 2A), which does not dissociate after initiation during the synthesis of a short peptide and remains bound to the ribosome. At the same time, preTC(NL) contains only traces of eIF3 (Figure 2A). The presence of eIF3 significantly affects the activity of eIF3j in the translation termination - the preTC(NL) is strongly activated by eIF3j during peptide release, in contrast to the preTC(MVHL) (Figure 2B). Moreover, the presence of eIF3 affected the binding of the eIF3j-eRF3a-GTP complex with the preTC (Figure 4). eIF3j binds to both types of preTCs in the absence of release factors. While eRF3a does not bind to preTCs or poorly binds to them, the eIF3j–eRF3a–GTP complex binds only with the preTC(MVHL), containing eIF3, and does not interact with the preTC(NL). We proposed that in the presence of eIF3, eIF3j attracts eRF3a into the preTC, probably via binding with the initiation factor 3 (Figure 4). The joint dissociation of the eRF3 and eIF3j from the ribosome is determined by the low ability of eRF3a to bind to the ribosome in the absence of eRF1 (Figure 4, Supplementary Figure S2), which causes dissociation of the eIF3j–eRF3–GTP complex. The interaction between eRF3-GTP and eIF3j probably prevents eRF3 binding to the A-site of the ribosome in the absence of eRF1, to ensure accurate translation termination. The binding of the eIF3j–eRF3a–GTP complex to eIF3 on the 40S subunit can additionally position release factors near stop codons of uORFs to enhance the efficiency of translation termination. uORFs can be located at a great distance from the poly(A) tail of mRNA associated with PABP if the closed-loop structure is not formed. Thus, eIF3, together with eIF3j, can serve as an additional depot for release factors inducing peptide release during the translation termination of uORFs.

As we showed earlier, PABP is necessary for loading eRF3a into the ribosome during translation termination (35). Our data showed that PABP did not suppress eIF3j activity during peptide release (Supplementary Figure S5A). Consequently, these proteins can function in translation termination simultaneously, facilitating the loading of eRF3a to the ribosome.

Summarising the obtained results, we concluded that eIF3j is regulating protein, promoting the loading of the eRF1-eRF3-GTP complex to the ribosome. We propose the following model for eIF3j in translation termination on the long CDS (Figure 7A): (I) eIF3j binds to the A-site of the preTC before the release factors, bound with PABP on poly(A) tail; (II) then eIF3j attracts the eRF1-eRF3-GTP-PABP complex to the A-site of the ribosome; (III) after stop codon recognition by eRF1 and GTP hydrolysis by eRF3, which promotes the accommodation of the M domain of eRF1 in the peptidyl-transferase centre and peptide release, eIF3j dissociates from the eRF1-eRF3-GDP complex. The attraction of eRFs and PABP to the preTC by eIF3j enhances translation termination. On the third step, eIF3j probably remains bound near the A-site of the ribosome to be involved in the next stage of ribosomal recycling. An alternative model for eIF3j activity in translation termination, occurring at the uORFs, involves the participation of eIF3 and the absence of PABP due to a significant distance to the poly(A) tail (Figure 7B): (I) eIF3j binds to the A-site of the preTC before the release factors and probably

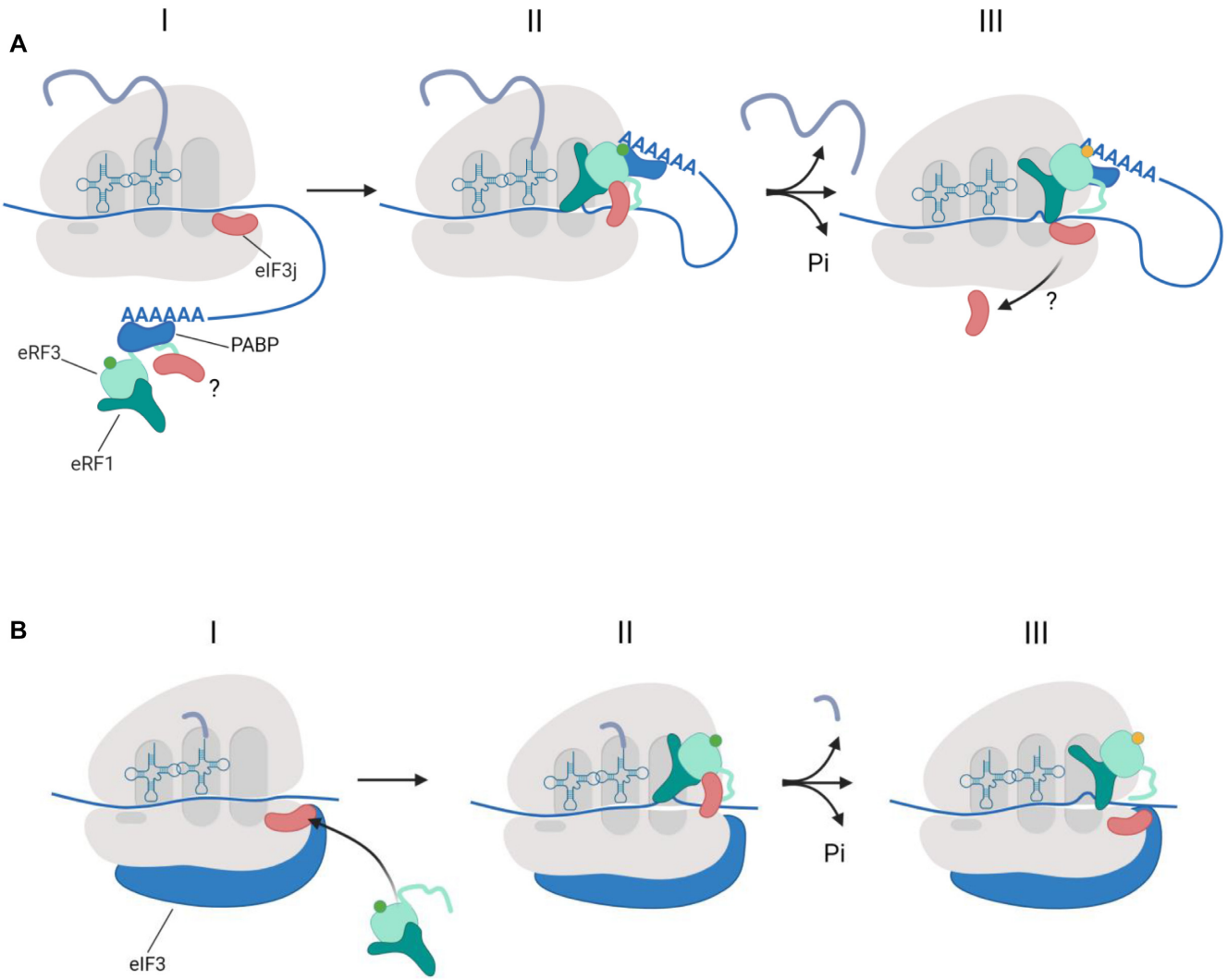

Figure 7. Model for eIF3j-stimulated translation termination. (**A**) Translation termination on the long CDS. (I) eIF3j binds to the preTC before release factors; (II) the eRF1-eRF3a-GTP-PABP complex binds to eIF3j to the A-site of the preTC; (III) after the stop codon recognition, GTP hydrolysis, and peptide release, eIF3j dissociates from eRF3-GDP. **(B)** Translation termination on the uORFs. (I) eIF3j binds to the preTC and possibly to eIF3; (II) the eRF1-eRF3a-GTP complex binds to eIF3j to the A-site of the preTC; (III) after the stop codon recognition, GTP hydrolysis, and peptide release, eIF3j dissociates from eRF3-GDP and remains associated with eIF3 on the 40S subunit.

with eIF3, bound with the 40S subunit; (II) then eIF3j attracts the eRF1–eRF3–GTP complex to the A-site of the ribosome; (III) after stop codon recognition, GTP hydrolysis, and peptide release, eIF3j dissociates from the eRF1–eRF3–GDP complex, but remains bound to eIF3 near the A-site of the ribosome. Two proteins, eIF3j and eIF3, bind with each other and together enhance translation termination in the absence of PABP.

The function of eIF3j as an eRF3 recycling factor was proposed earlier in the study of the yeast homolog Hcr1 (23). The authors suggested that after recognition of the stop codon and GTP hydrolysis, Hcr1 stimulates the release of eRF3-GDP, but from the postTC, allowing Rli1 to bind eRF1 since eRF3 and Rli1 bind at the same postTC site. The obtained results do not confirm the assumption that eIF3j alone acts as a recycling factor for eRF3. eIF3j does not induce dissociation of eRF3-GDP from the terminating ribosome after GTP hydrolysis, since eIF3j cannot bind to eRF1-eRF3a or eRF3a in the presence of GDP (Figure 3A, C). Experiments on the binding of the release factors to the preTC in the presence of different nucleotides clearly demonstrate the stability of the eRF1-eRF3a-GDP complex in the TC, regardless of the presence of eIF3j (Supplementary Figure S2D). However, how to explain the accumulation of eRF3 in the fractions containing heavy polysomes that are enriched with the termination complexes after deletion of *HCR1* in yeast (23)? One possible explanation is that eIF3j may be involved in the recycling of eRF3 as a partner of another protein, for example ABCE1.

Overexpression of *RLI1* restored the normal distribution of eRF3 during polysome profiling in cells with a deletion of *HCR1*. In addition, an increase in *RLI1* expression eliminated *hcr1-Δ* cell growth defects (23). Thus, Rli1 can compensate for the lack of Hcr1 during translation termination. Recently, ribosomal profiling of yeast cells with *hcr1-Δ* was published, in which *RLI1* expression increased in the ab-

sence of *HCR1*, that is, the yeast cells respond to the absence of Hcr1, and the effects of its absence can be offset by an increase in the amount of Rli1 in the cells (32). It should also be noted that in the recently obtained structures of the 43S and 48S initiation complexes, the interaction of eIF3j and ABCE1 on the 40S subunit was found, which suppressed ATP hydrolysis and the release of ABCE1 from the ribosome (26,27). At the same time, there are data on the stimulation by eIF3j of 80S ribosome splitting performed by ABCE1, Dom34, Hbs1 and eIF6, after which eIF3j and ABCE1 remain associated with the 40S subunit (26). These data indicate an active participation of eIF3j in the recruitment and retention of ABCE1 on the ribosome. ABCE1 binds to the same regions as eRF3 on the ribosome (GTPase binding region) and eRF1 (5), and this can facilitate the dissociation of eRF3 from the termination complex. This hypothesis was also confirmed by the peptide release stimulating activity of ABCE1 (8). Thus, the deletion of the eIF3j gene from the cells, presumably attracting ABCE1 to the termination complex, would inevitably lead to a decrease in the ABCE1 binding to the complex of release factors. This, in turn, suppresses the dissociation of eRF3-GDP from the ribosome and its accumulation in polysome fractions, and also causes a significant suppression of ribosome recycling. Increasing the concentration of Rli1 in the cell shifts the equilibrium toward the effective competition of Rli1 with eRF3-GDP for binding to eRF1 and the ribosome.

DATA AVAILABILITY

The data that support the findings of this study are contained within the article and the supporting information. All source data generated for this study are available from the corresponding author (Dr Elena Alkalaeva; alkalaeva@eimb.ru) upon reasonable request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. 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.
2. Zhouravleva,G., Frolova,L., Le Goff,X., Le Guellec,R., Inge-Vechtomov,S., Kisselev,L. and Philippe,M. (1995) Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *EMBO J.*, **14**, 4065–4072.
3. Brown,A., Shao,S., Murray,J., Hegde,R.S. and Ramakrishnan,V. (2015) Structural basis for stop codon recognition in eukaryotes. *Nature*, **524**, 493–496.
4. des Georges,A., Hashem,Y., Unbehaun,A., Grassucci,R.A., Taylor,D., Hellen,C.U.T., Pestova,T.V. and Frank,J. (2014) Structure of the mammalian ribosomal pre-termination complex associated with eRF1•eRF3•GDPNP. *Nucleic Acids Res.*, **42**, 3409–3418.
5. Preis,A., Heuer,A., Barrio-Garcia,C., Hauser,A., Eyler,D.E., Berninghausen,O., Green,R., Becker,T. and Beckmann,R. (2014) Cryoelectron microscopic structures of eukaryotic translation termination complexes containing eRF1-eRF3 or eRF1-ABCE1. *Cell Rep.*, **8**, 59–65.
6. Song,H., Mugnier,P., Das,A.K., Webb,H.M., Evans,D.R., Tuite,M.F., Hemmings,B.A. and Barford,D. (2000) The crystal structure of human eukaryotic release factor eRF1–mechanism of stop codon recognition and peptidyl-tRNA hydrolysis. *Cell*, **100**, 311–321.
7. Dubovaia,V.I., Kolosov,P.M., Alkalaeva,E.Z., Frolova,L.I. and Kiselev,L.L. (2006) Influence of individual domains of the translation termination factor eRF1 on induction of the GTPase activity of the translation termination factor eRF3. *Mol. Biol. (Mosk).*, **40**, 310–316.
8. Shoemaker,C.J. and Green,R. (2011) Kinetic analysis reveals the ordered coupling of translation termination and ribosome recycling in yeast. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, E1392–E1398.
9. Becker,T., Franckenberg,S., Wickles,S., Shoemaker,C.J., Anger,A.M., Armache,J.-P., Sieber,H., Ungewickell,C., Berninghausen,O., Daberkow,I. *et al.* (2012) Structural basis of highly conserved ribosome recycling in eukaryotes and archaea. *Nature*, **482**, 501–506.
10. Hellen,C.U.T. (2018) Translation Termination and Ribosome Recycling in Eukaryotes. *Cold Spring Harb. Perspect. Biol.*, **10**, a032656.
11. Young,D.J., Guydosh,N.R., Zhang,F., Hinnebusch,A.G. and Green,R. (2015) Rli1/ABCE1 recycles terminating ribosomes and controls translation reinitiation in 3'UTRs in vivo. *Cell*, **162**, 872–884.
12. 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.
13. Khoshnevis,S., Gross,T., Rotte,C., Baierlein,C., Ficner,R. and Krebber,H. (2010) The iron-sulphur protein RNase L inhibitor functions in translation termination. *EMBO Rep.*, **11**, 214–219.
14. Skabkin,M.A., Skabkina,O.V., Dhote,V., Komar,A.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.
15. Young,D.J., Makeeva,D.S., Zhang,F., Anisimova,A.S., Stolboushkina,E.A., Ghobakhlou,F., Shatsky,I.N., Dmitriev,S.E., Hinnebusch,A.G. and Guydosh,N.R. (2018) Tma64/eIF2D, Tma20/MCT-1, and Tma22/DENR recycle post-termination 40S subunits in vivo. *Mol. Cell*, **71**, 761–774.
16. Lomakin,I.B., Stolboushkina,E.A., Vaidya,A.T., Zhao,C., Garber,M.B., Dmitriev,S.E. and Steitz,T.A. (2017) Crystal structure of the human ribosome in complex with DENR-MCT-1. *Cell Rep.*, **20**, 521–528.
17. Hashem,Y., des Georges,A., Dhote,V., Langlois,R., Liao,H.Y., Grassucci,R.A., Hellen,C.U.T., Pestova,T.V. and Frank,J. (2013)

Structure of the mammalian ribosomal 43S preinitiation complex bound to the scanning factor DHX29. *Cell*, **153**, 1108–1119.
18. Querol-Audi,J., Sun,C., Vogan,J.M., Smith,M.D., Gu,Y., Cate,J.H.D. and Nogales,E. (2013) Architecture of human translation initiation factor 3. *Structure*, **21**, 920–928.
19. Zhou,M., Sandercock,A.M., Fraser,C.S., Ridlova,G., Stephens,E., Schenauer,M.R., Yokoi-Fong,T., Barsky,D., Leary,J.A., Hershey,J.W. *et al.* (2008) Mass spectrometry reveals modularity and a complete subunit interaction map of the eukaryotic translation factor eIF3. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 18139–18144.
20. Fraser,C.S., Lee,J.Y., Mayeur,G.L., Bushell,M., Doudna,J.A. and Hershey,J.W.B. (2004) The j-subunit of human translation initiation factor eIF3 is required for the stable binding of eIF3 and its subcomplexes to 40 S ribosomal subunits in vitro. *J. Biol. Chem.*, **279**, 8946–8956.
21. Wagner,S., Herrmannová,A., Malík,R., Peclinovská,L. and Valášek,L.S. (2014) Functional and biochemical characterization of human eukaryotic translation initiation factor 3 in living cells. *Mol. Cell. Biol.*, **34**, 3041–3052.
22. Herrmannová,A., Prilepskaja,T., Wagner,S., Šikrová,D., Zeman,J., Poncová,K. and Valášek,L.S. (2020) Adapted formaldehyde gradient cross-linking protocol implicates human eIF3d and eIF3c, k and l subunits in the 43S and 48S pre-initiation complex assembly, respectively. *Nucleic Acids Res.*, **48**, 1969–1984.
23. Beznosková,P., Cuchalová,L., Wagner,S., Shoemaker,C.J., Gunišová,S., von der Haar,T. and Valášek,L.S. (2013) Translation initiation factors eIF3 and HCR1 control translation termination and stop codon read-through in yeast cells. *PLos Genet.*, **9**, e1003962.
24. Valášek,L., Hašek,J., Trachsel,H., Imre,E.M. and Ruis,H. (1999) The *Saccharomyces cerevisiae* HCR1 gene encoding a homologue of the p35 subunit of human translation initiation factor 3 (eIF3) is a high copy suppressor of a temperature-sensitive mutation in the Rpg1p subunit of yeast eIF3. *J. Biol. Chem.*, **274**, 27567–27572.
25. Fraser,C.S., Berry,K.E., Hershey,J.W.B. and Doudna,J.A. (2007) eIF3j is located in the decoding center of the human 40S ribosomal subunit. *Mol. Cell*, **26**, 811–819.
26. Kratzat,H., Mackens-Kiani,T., Ameismeier,M., Potocnjak,M., Cheng,J., Dacheux,E., Namane,A., Berninghausen,O., Herzog,F., Fromont-Racine,M. *et al.* (2021) A structural inventory of native ribosomal ABCE1-43S pre-initiation complexes. *EMBO J.*, **40**, e105179.
27. Brito Querido,J., Sokabe,M., Kraatz,S., Gordiyenko,Y., Skehel,J.M., Fraser,C.S. and Ramakrishnan,V. (2020) Structure of a human 48 S translational initiation complex. *Science*, **369**, 1220–1227.
28. ElAntak,L., Wagner,S., Herrmannová,A., Karásková,M., Rutkai,E., Lukavsky,P.J. and Valášek,L. (2010) The indispensable N-terminal half of eIF3j/HCR1 cooperates with its structurally conserved binding partner eIF3b/PRT1-RRM and with eIF1A in stringent AUG selection. *J. Mol. Biol.*, **396**, 1097–1116.
29. Nielsen,K.H., Valásek,L., Sykes,C., Jivotovskaya,A. and Hinnebusch,A.G. (2006) Interaction of the RNP1 motif in PRT1 with HCR1 promotes 40S binding of eukaryotic initiation factor 3 in yeast. *Mol. Cell. Biol.*, **26**, 2984–2998.
30. Valášek,L., Hašek,J., Nielsen,K.H. and Hinnebusch,A.G. (2001) Dual function of eIF3j/Hcr1p in processing 20 S pre-rRNA and translation initiation. *J. Biol. Chem.*, **276**, 43351–43360.
31. Aylett,C.H.S., Boehringer,D., Erzberger,J.P., Schaefer,T. and Ban,N. (2015) Structure of a yeast 40S-eIF1-eIF1A-eIF3-eIF3j initiation complex. *Nat. Struct. Mol. Biol.*, **22**, 269–271.
32. Young,D.J. and Guydosh,N.R. (2019) Hcr1/eIF3j is a 60S ribosomal subunit recycling accessory factor in bivo. *Cell Rep.*, **28**, 39–50.
33. Pisarev,A.V., Hellen,C.U.T. and Pestova,T.V. (2007) Recycling of eukaryotic posttermination ribosomal complexes. *Cell*, **131**, 286–299.
34. Neu-Yilik,G., Raimondeau,E., Eliseev,B., Yeramala,L., Amthor,B., Deniaud,A., Huard,K., Kerschgens,K., Hentze,M.W., Schaffitzel,C. *et al.* (2017) Dual function of UPF3B in early and late translation termination. *EMBO J.*, **36**, 2968–2986.
35. Ivanov,A., Mikhailova,T., Eliseev,B., Yeramala,L., Sokolova,E., Susorov,D., Shuvalov,A., Schaffitzel,C. and Alkalaeva,E. (2016) PABP enhances release factor recruitment and stop codon recognition during translation termination. *Nucleic Acids Res.*, **44**, 7766–7776.
36. Mikhailova,T., Shuvalova,E., Ivanov,A., Susorov,D., Shuvalov,A., Kolosov,P.M. and Alkalaeva,E. (2017) RNA helicase DDX19 stabilizes ribosomal elongation and termination complexes. *Nucleic Acids Res.*, **45**, 1307–1318.
37. Shirokikh,N.E., Alkalaeva,E.Z., Vassilenko,K.S., Afonina,Z.A., Alekhina,O.M., Kisselev,L.L. and Spirin,A.S. (2010) Quantitative analysis of ribosome-mRNA complexes at different translation stages. *Nucleic Acids Res.*, **38**, e15.
38. Egorova,T., Sokolova,E., Shuvalova,E., Matrosova,V., Shuvalov,A. and Alkalaeva,E. (2019) Fluorescent toeprinting to study the dynamics of ribosomal complexes. *Methods*, **162–163**, 54–59.
39. Susorov,D., Egri,S. and Korostelev,A.A. (2020) Termi-Luc: a versatile assay to monitor full-protein release from ribosomes. *RNA*, **26**, 2044–2050.
40. Valášek,L., Szamecz,B., Hinnebusch,A.G. and Nielsen,K.H. (2007) In vivo stabilization of preinitiation complexes by formaldehyde cross-linking. In: *Methods in Enzymology*. pp. 163–183.
41. Beznosková,P., Wagner,S., Jansen,M.E., von der Haar,T. and Valášek,L.S. (2015) Translation initiation factor eIF3 promotes programmed stop codon readthrough. *Nucleic Acids Res.*, **43**, 5099–5111.
42. Matheisl,S., Berninghausen,O., Becker,T. and Beckmann,R. (2015) Structure of a human translation termination complex. *Nucleic Acids Res.*, **43**, 8615–8626.