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Misdecoding of rare CGA codon by translation termination factors, eRF1/eRF3, suggests novel class of ribosome rescue pathway in *S. cerevisiae*

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The CGA arginine codon is a rare codon in *Saccharomyces cerevisiae*. Thus, full-length mature protein synthesis from reporter genes with internal CGA codon repeats are markedly reduced, and the reporters, instead, produce short-sized polypeptides via an unknown mechanism. Considering the product size and similar properties between CGA sense and UGA stop codons, we hypothesized that eukaryote polypeptide-chain release factor complex eRF1/eRF3 catalyses polypeptide release at CGA repeats. Herein, we performed a series of analyses and report that the CGA codon can be, to a certain extent, decoded as a stop codon in yeast. This also raises an intriguing possibility that translation termination factors eRF1/eRF3 rescue ribosomes stalled at CGA codons, releasing premature polypeptides, and competing with canonical $tRNA^{ICG}$ to the CGA codon. Our results suggest an alternative ribosomal rescue pathway in eukaryotes. The present results suggest that misdecoding of low efficient codons may play a novel role in global translation regulation in *S. cerevisiae*.

The CGA codon is one of six arginine-encoding codons, as shown in the standard genetic code. In *Saccharomyces cerevisiae*, CGA is a rare codon decoded by arginyl-$tRNA^{ICG}$, which is biosynthesized by deamination of the wobble position adenine of $tRNA^{ACG}$ [1]. Arginine codon repeats, which are composed solely or partly of CGA rare codons, have been used for reporter gene constructs, hereafter referred to as 'CGA reporters', to assess the effects of ribosome stalling on mRNAs or translation inhibition [2–7]. However, few studies have focused on the decoding aspect [2,4]. Recent ribosome profiling analyses in *S. cerevisiae* revealed that the use of translation inhibitors, including cycloheximide, to halt protein synthesis resulted in a differential profile at the CGA codon [8], suggesting that CGA decoding in *S. cerevisiae* could have certain distinctive physiological underpinnings.

Quality control systems for mRNA and nascent polypeptides have been reported in eukaryotic protein synthesis [9–11]. Aberrant mRNAs with premature stop codons are degraded via the nonsense mediated mRNA decay (NMD) pathway, with widely conserved UPF factors, and translation termination factors eRF1/eRF3 [12,13]. Aberrant mRNAs stalling ribosomes via formation of stem-loop secondary structures are targeted for degradation via the no-go decay pathway, which

Abbreviations

eRF3c, eRF3 C-terminal region; his3t, HIS3 3′UTR and terminator; luc2, luciferase2; NMD, nonsense-mediated mRNA decay; Rluc, *Renilla* luciferase; RQC, ribosome-associated quality control; Sc-eRF1, *Saccharomyces cerevisiae* eRF1.

includes translation termination factor homologues, Pelota (Dom34 in *S. cerevisiae*) and Hbs1, on the ribosome [14,15]. Aberrant mRNAs lacking a stop codon are also targeted for degradation via the nonstop decay pathway, wherein in addition to Pelota and HBS1, specific mRNA decay factors, Ski7, the ski complex (ski2/ski3/ski8) and the exosome complex function in mRNA decay [16–18]. Ribosomal arrest caused by polybasic residues (such as poly-lysine) or others were reported to cause mRNA cleavage; moreover, nascent polypeptides are degraded via ribosome-associated quality control (RQC) mechanisms [19]. Intriguingly, in the previous analyses, CGA reporters accumulate premature polypeptides (or arrest products) approximately corresponding to the size of the peptide up to the CGA position [2,4,5,20]. Upon knockout of Zuo1, Asc1 affects frame maintenance, which contributes to premature polypeptide synthesis [19,21]. Among those studies on RQC defective conditions including ΔLtn1 or ΔRqcs, CGA reporters yielded premature products with additional mRNA-independent alanine-threonine tails (CAT-tail) at the C-terminal of nascent-peptides [6,22]. In addition, recent reports on RQC revealed that responses to ribosome arrest or stalling are not homogeneous, depending on the species (*S. cerevisiae* and mammalian cell line) [20,23,24]. In any eukaryotic processes, ribosomes are supposed to be rescued without polypeptide-chain release from P-site peptidyl-tRNA [10,25]. However, in bacteria, three types of ribosome rescue systems are known to function upon ribosomal stalling. tmRNA/SmpB, ArfA/RF2 and ArfB function in three pathways. Interestingly, ArfB has homologous domains including the GGQ motif to class-I peptide-chain release factors, RF1 and RF2; thus, all three pathways rely on RF or RF-like peptidyl-tRNA hydrolysis activity to release polypeptides from stalled ribosomes [26–28].

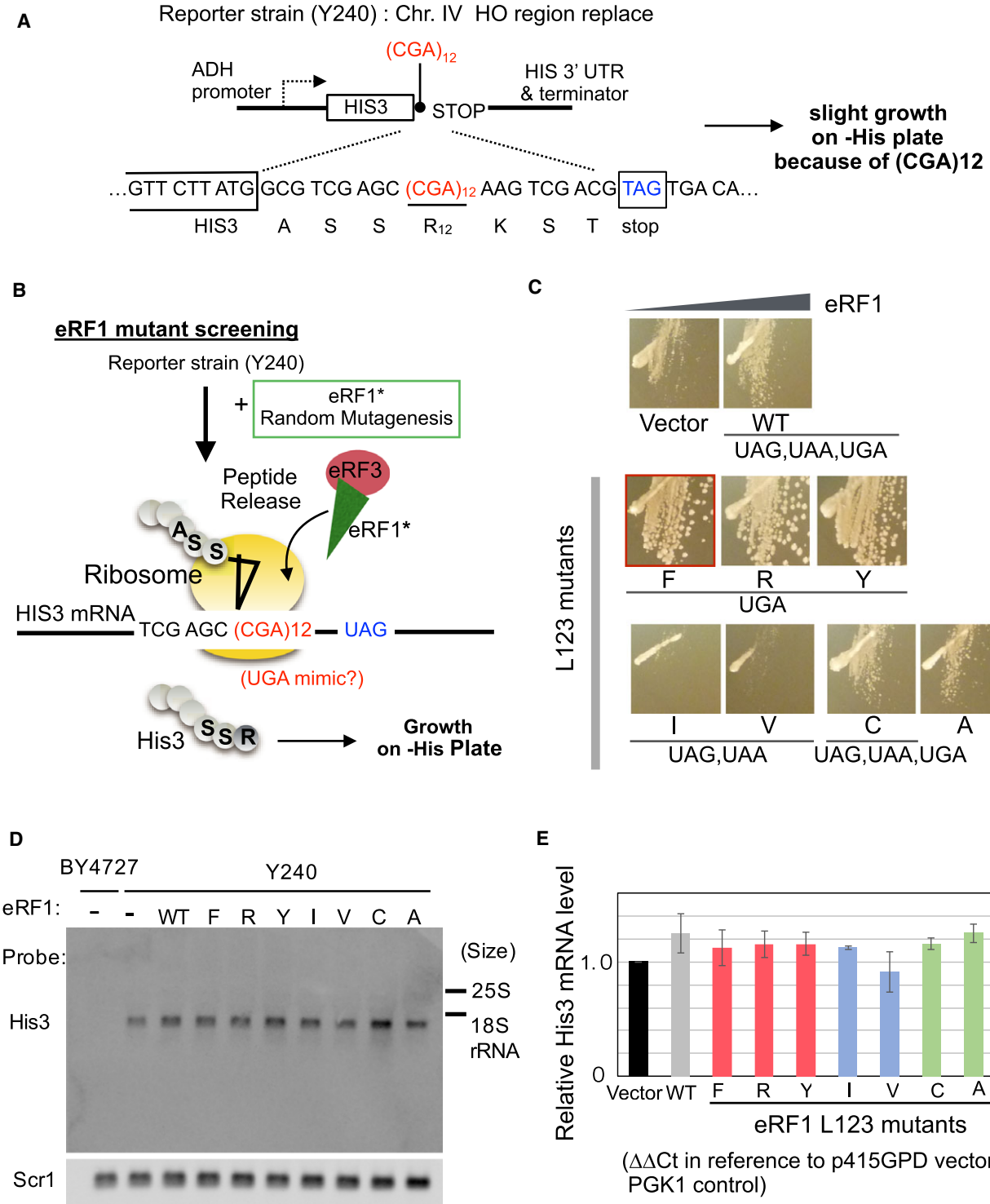
eRF1 (Sup45 in *S. cerevisiae*) is a eukaryotic class I polypeptide-chain release factor that directly deciphers UAA, UAG and UGA stop codons in the ribosomal A-site and catalyses the polypeptide chain releasing reaction. Similar to the EF1A/tRNA complex in translation elongation, eRF1 functions in complex with eRF3 (Sup35 in *S. cerevisiae*) and is referred to as tRNA mimicry [29–32]. Cooperative interaction between eRF1 and eRF3 is crucial for precise stop codon recognition and translation termination in eukaryotes [32–37]. GTPase activity of eRF3 is both eRF1- and ribosome-dependent [33], and their precise interactions on the ribosome have been reported in recent structural analyses [15,31,38–40]. eRF3 is composed of an N-terminal regulatory region and a C-terminal GTPase catalytic region (eRF3c), which is essential for translation termination. The eRF3 N-terminal region interacts with various factors [41,42], and contains unique motif sequences that can induce a [PSI+] prion-like phenotypes [43]. Unlike eukaryotes, in prokaryotes, class I release factors, RF1 and RF2, decipher UAG/UAA and UGA/UAA codons respectively [32,44]. To understand the omnipotency of eRF1, its codon recognition mechanism has been extensively studied [45–50]. Well-conserved motifs, known as TASNICS and Y-C-F, are involved in codon recognition at the tip of the codon recognition domain. An eRF1 residue, L123, inside the Y^{122}-C^{124}-F^{128} motif (*S. cerevisiae* numbering) plays a distinctive role in stop codon discrimination by directly interacting with the ribosome [35,50,51].

Herein, we hypothesized that the CGA codon can be recognized as a stop codon to a certain extent by eRF1/eRF3 translation termination complex in *S. cerevisiae*, resulting premature polypeptide release and rescue for stalling ribosome on mRNA. We conducted a selection of eRF1 mutants with enhanced termination at CGA codons, and further showed that the wild-type eRF1/eRF3 complex can cause peptide release at the CGA codon *in vivo*. The putative physiological underpinnings of this phenomenon are also discussed herein.

Results

Isolation of an eRF1 mutant enhancing the viability of the conditional lethal CGA ribosomal stall strain

To assess the effect of eRF1 on CGA codon repeats based on growth, we generated a reporter strain. Consecutive 12 CGA codon repeats were cloned immediately upstream to the stop codon of *HIS3*, and this reporter was introduced into the *S. cerevisiae* genome, exchanging the HO region to produce the CGA ribosomal stall conditional lethal strain, Y240 (Fig. 1A,B), thus leaving the endogenous wild-type eRF1 and eRF3 chromosomal genes intact. This strain exhibited slight growth on histidine-lacking (–His) media because the consecutive CGA repeats downregulated reporter HIS3 protein (Fig. 1C, 'Vector'). Therefore, growth on histidine-lacking media can be considered to assess the expression of active HIS3 polypeptides, those with CGA codon repeat-mediated prematurely terminated translation and full-length HIS3-R_{12} products. To examine the hypothesis that peptide release factor, eRF1, decodes the CGA codon as a stop codon, eRF1 mutants were screened for the enhanced phenotype. *S. cerevisiae* eRF1 (hereafter 'Sc-eRF1') gene cloned in



expression vector, p415GPD (LEU2 selection marker), was randomly mutated via hydroxylamine treatment, introduced into the reporter strain, and viable colonies on SC -His-Leu plates were selected. From approximately 1×10^4 eRF1 clones, one most prominently fast-growing clone was specified, and sequencing revealed that the eRF1 mutation was a leucine to phenylalanine substitution (G^{369} to C) at position 123

Fig. 1. eRF1 mutants that confer tolerance to CGA repeats. (A) A schematic representation of the CGA ribosomal stall conditional lethal stain (Y240). (B) Screening strategy of eRF1 mutants based on growth. Randomly mutated eRF1 expression plasmids (pMW1111) were transformed into the reporter strain and screened. (C) The effects of eRF1 overexpression on the growth of the CGA ribosomal stall conditional lethal strains. Growth of the Y240 strain transformed with vector (p415GPD) and wild-type (WT) eRF1 on -His plates is shown in the first row. Growth of the strains transformed with eRF1 L123 mutants cloned in p415GPD (pMW1112s) is shown along with their stop codon preference. The screened L123F mutant is indicated with a red frame. (D) The His3 mRNA level of the transformants in (C) evaluated via northern blot analysis. (E) Relative His3 mRNA levels of transformants in (C) examined via quantitative PCR. The values shown are calculated as $2^{-\Delta\Delta Ct}$ relative to the vector transformant. The experiments are performed twice in triplicate ($n = 3$) and the error bars represent ±SD.

(Fig. 1C L123 mutants, F). We decided to focus on the mutant, since eRF1 mutations at this position were extensively analysed [35,51].

Previously, eRF1 position 123 reportedly discriminated the second base of stop codons in an eRF3-dependent manner. In the previous study by Saito *et al.* [35], the eRF1 L123 mutations showed either 'UAA and UAG dual-biased', 'UGA biased' or omnipotent 'UAA, UAG and UGA' specificity similar to wild-type eRF1, via relative read-through efficiency studied in conditional eRF1ts mutant strains. To examine correlations between CGA assay strain growths and stop codon specificities of eRF1 L123 mutants, a set of eRF1 L123 variants (wild-type, F, R, Y, I, V, C and A) was introduced into the reporter strain, and growth was monitored on –His plates (Fig. 1C). The UGA-biased eRF1 mutant transformants, L123F, L123R and L123Y showed marked growth, indicating stable HIS3 expression. eRF1 mutant transformants, L123A and L123C, exhibited growth similar to that of wild-type eRF1. The UAA- and UAG-biased eRF1 mutant transformants, L123I and L123V, showed markedly lesser growth than the vector only control and wild-type eRF1 (Fig. 1C). Most growth tendencies shown by the L123 mutants were consistent with eRF1-mediated relative termination activity at the UGA stop codon. Notably, the HIS3-CGA12 reporter gene synthesized sufficient levels of functional HIS3 polypeptide, which is supposed to be released from the stalled ribosome upon expression of mutant eRF1.

To avoid possibility of marked mRNA decay by cellular surveillance, His3 mRNA levels of each transformant were examined via northern blot analysis and quantitative PCR analysis. No significant differences in mRNA levels in eRF1 mutants were observed in our reporter strain (Fig. 1D,E), indicating that the growth of the transformants depends on the efficiency of cellular protein synthesis. These results suggest that eRF1 can potentially recognize CGA codons as stop codons, resulting in the release of premature products.

CGA termination activity of mutant eRF1 assessed via the dual-luciferase assay

To further investigate the CGA decoding efficiency of eRF1, quantitative assay strains were generated. Assay strains with dual-luciferase reporter gene constructs, with 12 or 6 CGA codon repeats between *Renilla* luciferase (Rluc) and luciferase2 (luc2) are hereafter referred to as CGA12-luc reporter and CGA6-luc reporter respectively. The ratio of luc2/Rluc to that of blank assay strain (Fig. 2) provided an index of the relative levels of the two different sized products, named 'CGA read-through index'. The smaller index putatively shows efficient peptide release at CGA codon repeats. Overexpression of WT eRF1 does not change the CGA read-through index. Using the CGA12-luc reporter, L123F, L123R and L123Y decreased the CGA read-through index, suggesting enhanced peptide release at CGA codon repeats (Fig. 2A). In contrast, the CGA read-through index was increased by the L123I and L123V mutants. L123A and L123C did not appear to affect CGA read-through. Together, these results indicate that the CGA read-through profile of eRF1 mutants is consistent with the reported 'UGA-specific' read-through profile of eRF1 [21]. Although endogenous eRF1 alleles between previous and the present assay systems are different, the CGA read-through activity is greatly correlated with the UGA read-through activities of the eRF1 variants. This strongly suggests that eRF1 might produce short-sized products from CGA codon repeats by terminating within the CGA codon repeats. Similar CGA read-through index profiles were also observed for eRF1 mutants in the CGA6-luc reporter strain (Fig. 2B). However, read-through index was far higher for the CGA6-luc reporter than for the CGA12-luc reporter. These results indicate that in CGA-luc reporter assays, CGA read-through index ratios are probably the sum of probable peptide release at the CGA codon and reductions in full-length protein by CGA ribosomal stalling, not a simple reflection of eRF1-mediated termination at CGA codon repeats.

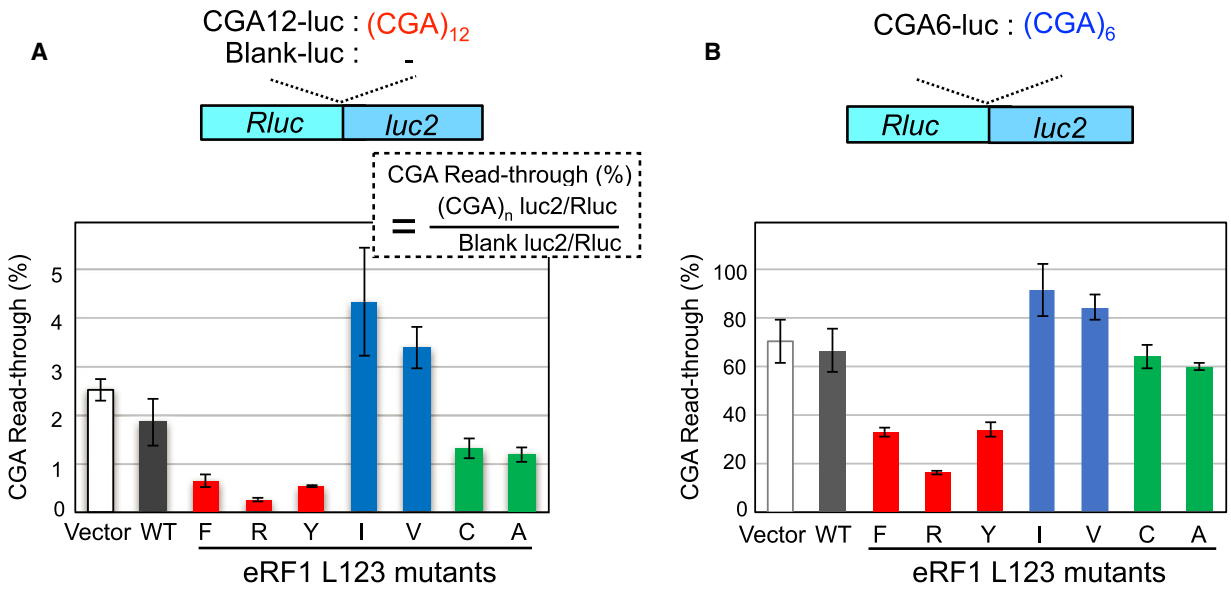


Fig. 2. CGA termination activity of eRF1s assessed via a dual-luciferase assay. CGA codon repeats were placed between Rluc and luc2 genes to construct reporters and replaced the HO region of the genome. (A) CGA12-luc and (B) CGA6-luc reporter strains transformed with either vector, wild-type (WT) eRF1 (pMW1111), or L123 mutant genes (encoding F, R, Y, I, V, C and A) cloned into p415GPD (pMW1112s) were analysed using dual-luciferase assays. Rluc and luc2 activity were measured, and CGA read-through (%) was calculated as the luc2/Rluc ratio compared to that of corresponding Blank-luc control. The experiments are performed in triplicate ($n = 3$) and the error bars represent ±SD.

Wild-type and mutant eRF1 affects the cellular levels of premature termination products from CGA reporters

To directly monitor the cellular levels of premature and full-length products from CGA6-luc and CGA12-luc reporters in cells (Fig. 3A), western blot analyses were conducted with the anti-*Renilla* luciferase (anti-Rluc, αRluc) antibody (Fig. 3B). Since the Rluc ORF is before the CGA codon repeats in the reporter construct, the anti-Rluc antibody should react with full-length and short-sized (premature) products. As size controls, Rluc-stop (UAA; corresponding to the size of Rluc) and Rluc-R $(AGA)_{12}$-stop (corresponding to the size of Rluc-R_{12}) constructs were included (Fig. 3A). The blot was probed with anti-PGK1 antibody as a loading control (Fig. 3B, lower). In both CGA6-luc and CGA12-luc reporter strains, Rluc-size premature products gradually increased in the order of the vector, wild-type (WT), L123F and L123R (Fig. 3B). Even upon only vector addition in CGA repeat strains, a small amount of short-sized Rluc bands were observed (It is used as basis for the bands ratio). It could be derived from degradation of larger bands, however, even a wild-type effect is evident by quantification. As all strains used have endogenous eRF1 and eRF3, those probably contribute to the production of short-sized Rluc bands at the CGA repeats. These results confirmed that the short-sized products are Rluc released by eRF1. There was no difference in the sizes of premature products produced from CGA6-luc and CGA12-luc reporter strains. From the comparison with Rluc and Rluc-R_{12} size markers, surprisingly, only one, or a few, CGA codon(s) seem to be required to cause peptide release in *S. cerevisiae*, although many CGA codons are required to investigate this effect in reporter systems. Although similar size bands were observed in blank strain transformants, they are independent of the CGA presence and insensitive to eRF1 overproduction. Full-length product levels were not prominently reduced upon an increase in the short-sized products in the CGA6-luc reporter strain (Fig. 3B CGA6-luc), although the amount of the full-length product is much lesser than those of the blank strain (Fig. 3B Blank). Full-length products were only faintly observed in the CGA12-luc reporter strain probably owing to a pleiotropic effect by accumulated translation inefficiency through a much longer CGA repeat.

Northern blot analysis revealed that the mRNA levels detected by Rluc probe in each sample are comparable (Fig. 3C), indicating that CGA6-luc and CGA12-luc reporter mRNAs are not subjected to significant intracellular mRNA decay.

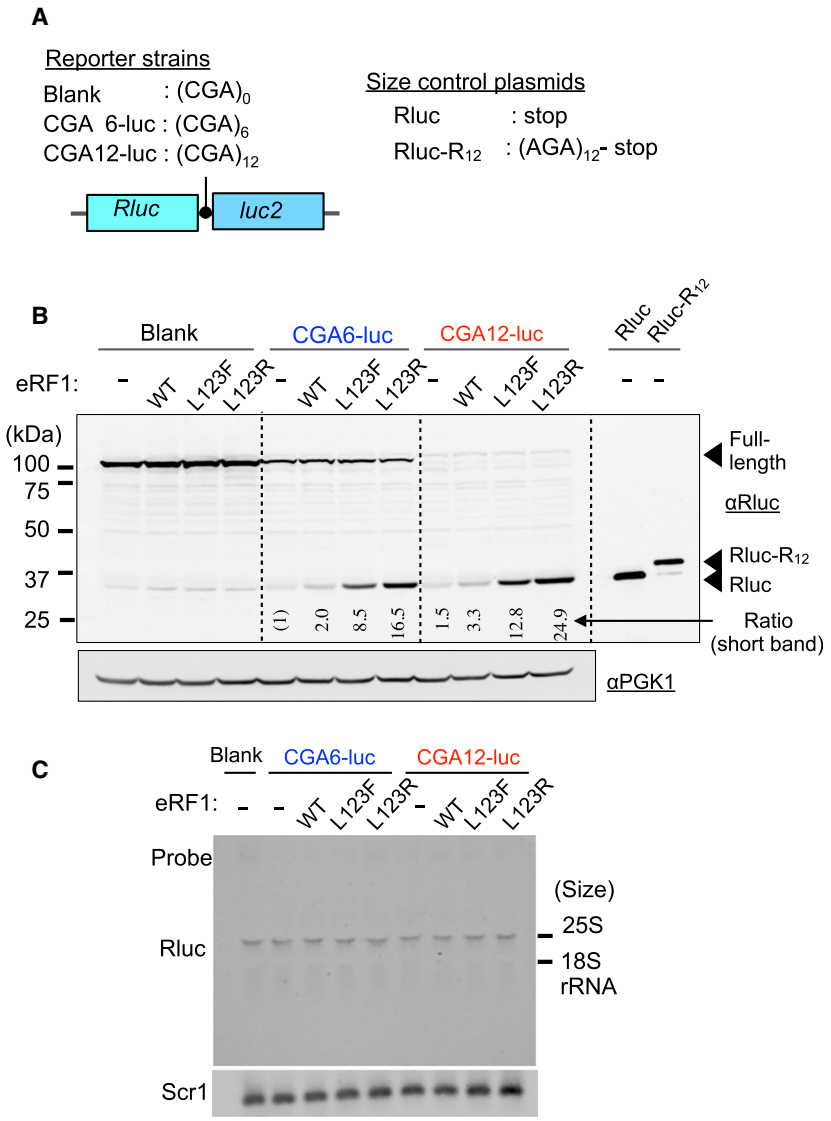


Fig. 3. Effect of eRF1 on CGA repeat reporter products. (A) A schematic representation of CGA6-luc and CGA12-luc reporter strains, and Rluc only and Rluc-R12 size control plasmid reporters. (B) Profiling of luciferase reporter expression via western blot analysis, using anti-Rluc antibody. Samples were prepared from the indicated strain transformant cells with eRF1s encoded on p415GPD vector (pMW1111, pMW1112 L123F and pMW1112 L123R). Size controls; BY4727 strain transformed with Rluc or Rluc-R12 genes cloned in p416GPD vector (pMW1252 and pMW1253). Short-sized band ratios relative to CGA6-luc transformed with vector (all are corrected with PGK1 control) are shown under the bands. (C) Northern blot analysis of the samples prepared from the identical transformant cells with (B), detected using a Rluc probe. The lower panel shows Scr1 RNA as a control.

eRF1 competes with tRNA^{Arg} at CGA codon *in vivo*

To examine the competition between eRF1 and tRNAs in the CGA6-luc reporter strain, tRNA^{ACG} and tRNA^{UCG} expressing plasmids were constructed. Since CGA arginine codon is deciphered by tRNA^{ICG} from tRNA^{ACG} via post-transcriptional modification in *S. cerevisiae*, tRNA^{ACG} gene encoding tRNA^{ICG} precursor that is believed to be less efficient for decoding, and mutant tRNA^{UCG}, which is mutated from tRNA^{ACG} to perfectly match to decipher CGA without cellular modification, were cloned into pRS416 (single copy plasmid). Either of vector (p415GPD), WT, L123F and L123R eRF1 in p415GPD was co-introduced with either of indicated tRNA-encoding plasmids into the reporter strain and products were detected via western blot analysis (Fig. 4A). Consequently, reduction in the short-sized premature products (Rluc*) and reciprocal increase in the full-length products were observed with tRNA^{UCG} in all of vector, WT, L123F and L123R eRF1 expressing strains. The effect was not evident with tRNA^{ACG}. The result seems ambiguous from the point of competition with eRF1 and tRNA^{ICG}, that should exist a lot *in vivo*, in this experiment. However, instead, it shows rather strong effect of eRF1 at CGA.

Homogeneous eRF3 cooperatively enhances eRF1-mediated CGA decoding

To compare the manner of recognition of the CGA codon by the eRF1/eRF3 translation termination complex with the canonical translation termination at

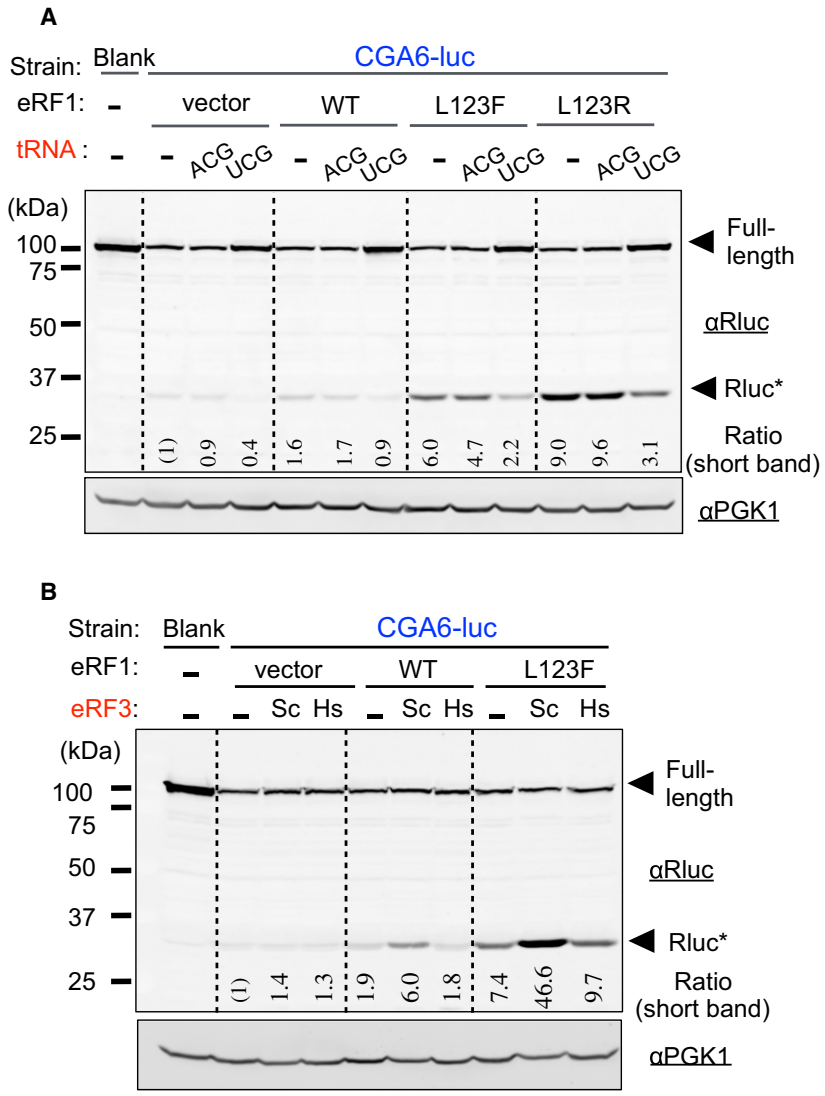


Fig. 4. tRNA mimicry by eRF1/eRF3 at CGA. (A) Analysis of the competition between the eRF1s (vector, WT, L123F and L123R) and $tRNA^{ACG}$ (tR(ACG)J) or $tRNA^{UCG}$ (tR(ACG)J mutant with indicated exchange). Rluc products from the CGA6-luc strain transformants, expressing eRF1 (WT, L123F, L123R or vector control p415GPD) and $tRNA^{ACG}$ or $tRNA^{UCG}$ from pRS416 vector (pMW1096 or pMW1097) were assessed by western blot analysis. PGK1 was detected using anti-PGK1 antibody as the loading control. Short-sized band ratios relative to CGA6-luc transformed with vector (all are corrected with PGK1 control) are shown under the bands. (B) Assessment of the combined effects on Rluc-size product production via overexpression of eRF1 and eRF3s of different species via western blot analysis. eRF1 (p415GPD vector, WT, or L123F; pMW1111 and pMW1112 L123F), and eRF3 (-: p416GPD vector, Sc: *S. cerevisiae* eRF3c, and Hs: human eRF3a (GSPT1c); pMW659 and pMW1259) were co-overexpressed from plasmids in the CGA6-luc reporter strain and analysed. PGK1 was detected as loading control. Short-sized band ratios relative to CGA6-luc transformed with vector (all are corrected with PGK1 control) are shown under the bands. Rluc*: Short-sized Rluc products that might have a few arginine translated from CGA repeats.

stop codons, both eRF1 and eRF3 (*S. cerevisiae* eRF3: Sc-eRF3c (Sc), or Human eRF3: GSPT1c (Hs)) were overexpressed in the CGA6-luc reporter strain and assessed via western blot analysis (Fig. 4B). Co-overproduction of homogeneous yeast eRF1 and eRF3 enhanced the production of short-sized premature polypeptides (Fig. 4B, WT). This effect was more prominent upon expression of the L123F eRF1 mutant. On the contrary, heterogeneous expression of human eRF3 (GSPT1c) with yeast eRF1 hardly enhanced the production of short-sized premature polypeptides, despite GSPT1c's replaceability the endogenous activity of yeast eRF3. The present results show that the homogeneous combination of yeast eRF1/eRF3 recognizes the CGA codon in a manner similar to that of stop codons during translation termination.

A single CGA codon at the end of the gene is detectably decoded as the stop codon

The present results indicate that only a few CGA codons are sufficient for the eRF1/eRF3 complex to cause peptide release within the repeats in a competitive manner with 'inefficient' decoding of CGA by the tRNA/EF1A complex. Therefore, we investigated the effect of a single CGA codon under a condition favourable for the eRF1/eRF3 complex, that is at a position for normal stop codons within the mRNA (Fig. 5). The Rluc-TGA-his3t (HIS3 3′UTR and terminator) plasmid reporter was constructed, and the TGA stop codon of the reporter was exchanged for CGA, and AGA, GGA and AGG control codons (Fig. 5A). The BY4727 wild-type strain was transformed with reporter plasmids and Rluc expression was examined

via western blot analysis. Using the Rluc-CGA-his3t reporter, we observed slight but significant synthesis of the Rluc product, whereas no Rluc product was observed in transformants expressing reporters with AGA, GGA and AGG codons (Fig. 5B left). Overexpression of eRF1 L123R mutant with the Rluc-CGA reporter revealed enhanced Rluc products (Fig. 5B right). These data provide strong evidence that the endogenous eRF1/eRF3 translation termination complex can recognize CGA to release polypeptides. Although signal enhancement by WT eRF1 overproduction is not observed in the system, it probably reflects unknown requirements for peptide release at single CGA, showing clear difference in efficiency with proper translation termination in the context.

Discussion

In this study, several artificial reporter systems were introduced to confirm that the eRF1/eRF3 complex can cause premature peptide release at CGA sense codons in *S. cerevisiae*. A specific sense codon, CGA, is decoded as a stop codon by the translation termination complex, eRF1/eRF3. The biological underpinnings of its latent nature are yet unclear. However, recent ribosome profiling data exhibited potential slow decoding of CGA codons on various mRNAs, suggesting their regulatory significance together with its low codon usage [8,52]. To decode the CGA codon as a stop codon, the eRF1/eRF3 complex would compete well with $tRNA^{ICG}$/EF1A complex. $tRNA^{ICG}$ is synthesized via deamination of $tRNA^{ACG}$, and decodes CGA via I-A wobble base pairing [1]. Thus, inefficient $tRNA^{ICG}$ biosynthesis, or inefficient decoding of CGA by $tRNA^{ICG}$ may allow for misdecoding of CGA by eRF1/eRF3 as a stop codon, thus serving as a type of regulatory mechanism. Interestingly, the CGA codon is decoded by mostly either $tRNA^{ICG}$ or $tRNA^{UCG}$ depending on the species, $tRNA^{ICG}$ in *S. cerevisiae*, $tRNA^{UCG}$ in humans. The regulatory aspect of $tRNA^{ICG}$ biosynthesis is not fully elucidated; however, biosynthesis might be coupled with global decoding profiles of protein synthesis. *S. cerevisiae* and other organisms using $tRNA^{ICG}$ to decode CGA may use similar regulatory systems. Although eRF1 reads three stop codons cooperatively with eRF3, the requirement for eRF3 differed on the basis of specific stop codons, UAA/UAG versus UGA [53]. This may be related to

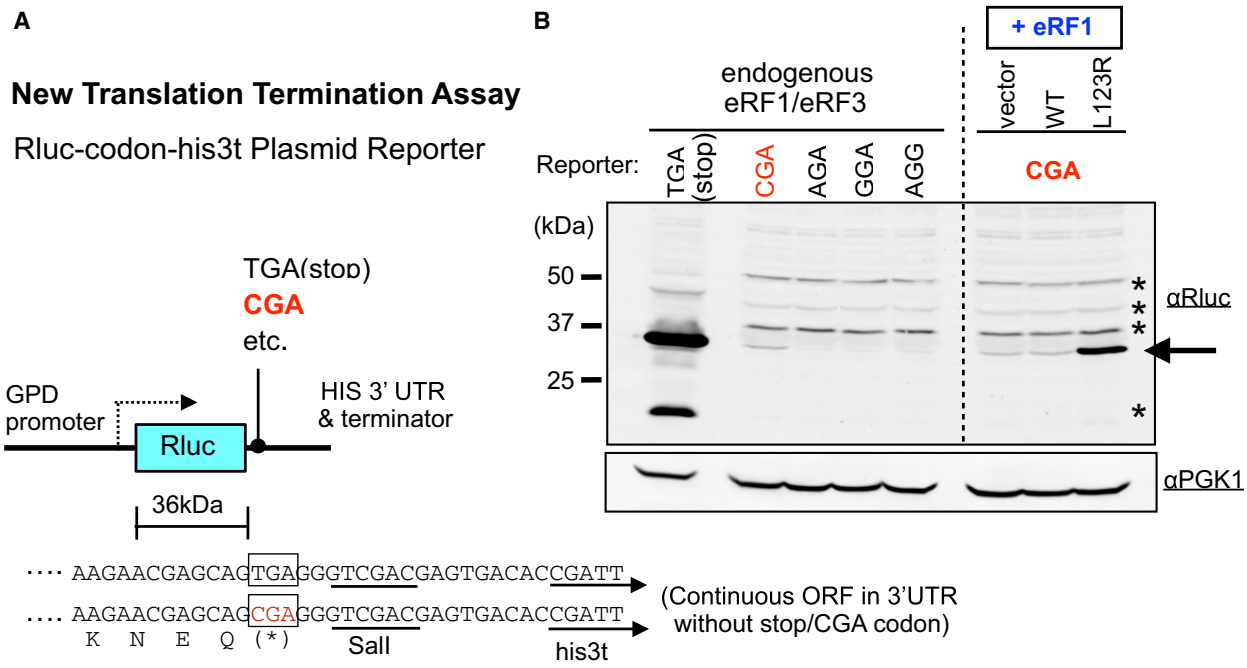


Fig. 5. A single CGA codon is enough for eRF1/eRF3 complex to be decoded. (A) Introduction of translation termination assay at mRNA terminal region. Schematic representation of the Rluc-codon-his3t plasmid reporters. Stop, CGA and other codons were cloned immediately downstream, of the Rluc gene at the stop codon site, to be in-frame with the HIS3 terminator lacking stop or CGA codons (pMW1321s). (B) Left; BY4727 (WT) strains transformed with each of Rluc-codon-his3t expression plasmids (pMW1321s) were examined for Rluc expression with anti-Rluc antibody by western blot analysis. Right; The effect of eRF1 expression (vector, wild-type or L123R; pMW1111 and pMW1112 L123R) on the Rluc expression profile of the Rluc-CGA-his3t plasmid containing strain. *unknown bands.

the capacity to read CGA codons. As eRF3 has N-terminal regulatory region that interacts with plural factors [41,54], direct and indirect interactions with the factors or mRNA may affect, as it were, 'CGA recoding', in addition to $tRNA^{ICG}$ availability [55,56]. Recent reports evidenced the dual use of stop codons as 'stop' and 'sense' codons in some organisms, depending on their location [57–60]. CGA codon deciphered via translation termination factors, the eRF1/eRF3 protein complex, may be an evolutionary deviation demonstrating a flexible eukaryotic translation termination system.

From the viewpoint of quality control, CGA repeats could provide a distinctive mode of ribosome stalling among other ribosome stall reporters, as shown via mRNA quantitation analyses performed herein (Figs 1D,E and 3C) and previously [2,5,20]. On performing western blot analyses in this study, the CGA-luc reporters yielded two distinctive protein products, that is short-sized and full-length ones. The amount of the full-length product contrasts the number of CGA, as is previously reported with reporter constructs [2,20] and seemingly unaffected by eRF activities. However, intriguingly, the amount of the short-sized product is seemingly unaffected by the number of CGA, while being susceptible to the putative CGA misdecoding activity of wild-type eRF1 and to that of L123 mutated eRF1 in a cooperative manner with eRF3. We believe that short-sized product seems to be released from the stalled ribosomes by decoding of early CGA within consecutive CGAs, whereas full-length product synthesis is inhibited depending on the number of CGAs. The precise mechanism underlying the reduction of the full-length product at the CGA repeats is yet unknown; however, we hypothesize that inefficient decoding by $tRNA^{ICG}$ is involved and it also enables easy visualization of rather inefficient short-sized band production in the system. Our hypothesis is not inconsistent with the latest ribosome profiling analysis of the stalled ribosomes on poly-Arg codons containing CGAs [20].

Recent advancements in cryo-EM revealed structural aspects of stop codon decoding by eRF1 in the ribosome [38,39,61–63]. In the pre-GTP hydrolysis mode [38] and in the accommodation mode [61], conformation of domain1 of eRF1 and A-site stop codon appears similar (Fig. 6), suggesting that domain1 conformation is not largely affected during this process. Based on those latest structures, the trinucleotides region of the stop codon, surprisingly, form U-turns, and the structure seems to be primarily fixed by interactions between $NIKS^{58\text{-}61}$ motif (Hs (Human numbering):$NIKS^{61\text{-}64}$) and 1st base U, 3rd nucleotide backbone, between A1825 (A2256 in *S. cerevisiae*, A1493 in *Escherichia coli*) of 18S rRNA h44 and 2nd base. The NIKS motif interacts with 1st base of the codon, and part of Y^{122}-C^{124}-F^{128} (Hs:Y^{125}-C^{127}-F^{131})motif and E^{52} (Hs:E^{55}) with 2nd and 3rd bases [61]. These interactions coincide with those reported in previous genetic studies [35,47,64,65]. However, in our previous study, we speculated functional counterpart regions between eRF1 and tRNA, by structural superimposition of EF-Tu/tRNA complex and archeal translation termination aEF1A/aRF1 complex based on tRNA mimicry [34]. In this prediction, L123 residue (R126 in *A. pernix*) corresponds to the backbone moieties around the 38th and 39th nucleotides of tRNA in close proximity of the 36th base of tRNA complementary to the 1st base of A-site codon, possibly affecting decoding fidelity. The protruding side chain moiety of L123 are proximal to and potentially interact with both 25S rRNA (A3731 of helix69, Hs numbering) and 18S rRNA (A1825 that makes stack with 2nd base of stop codon), whereas the side chains of adjacent residues Y^{122} and C^{124} is directed to the opposite direction to form stop codon binding pocket in the ribosomal complex structures. E^{52} of eRF1 occupies rather specific position that reported to interact the 2nd and the 3rd bases depending on the stop codons, that is it may contribute to stop codon discrimination directly [61]. The L123 of eRF1 was speculated to play its role through the 2nd base discrimination by Y^{122}-C^{124} and E^{52}.

The C4 carbonyl of uracil at the 1st base of stop codon interacts with K60 (Hs:K^{63}) within the NIKS motif. For the putative CGA misdecoding in our hypothesis, an amino group of cytosine at the same position would deter its interaction with K60, without largely loosing other interactions. Although K60 comprises important conserved motifs, in previous reports, single K60 mutant eRF1s did not completely lose their activity towards stop codons, suggesting potential recognition of CGA by eRF1[49,64]. L123 of eRF1 may be functionally sensitive for the distance between the 1st base and ribosomal interaction subunits, making bulkier mutations (F, R, Y) functionally easier in both stop and CGA codons. Interestingly, in human eRF1, K60 (Hs: K63), which is proximal to the 1st base of the stop codon, within TASNIKS motif was reported to be modified C4 lysyl hydroxylation by JMJD4. However, the modification enzyme was not found in *S. cerevisiae;* thus, the 1st base decoding might be differently regulated among species. Sense codon misdecoding capacities of eRF1 should be studied in detail in future studies. The physiological

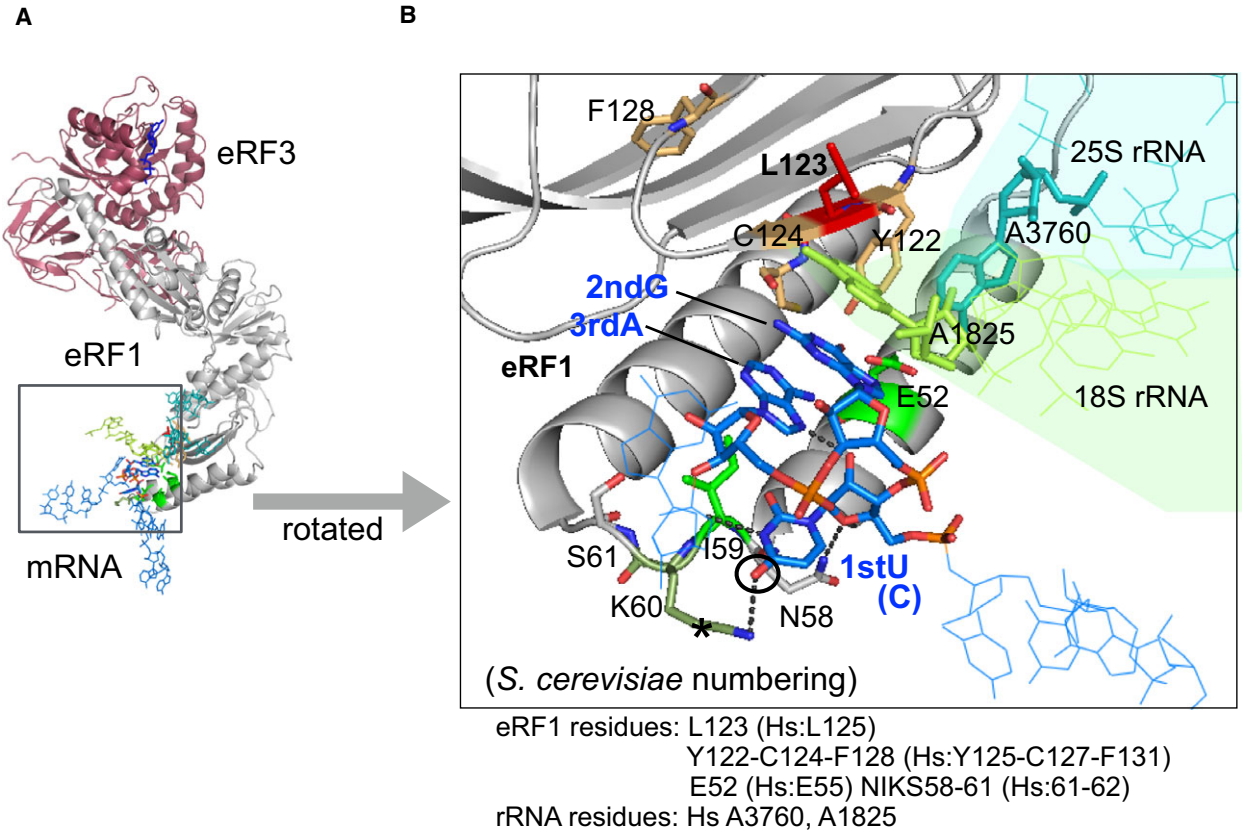


Fig. 6. Structural model of CGA decoded by eRF1. (A) eRF1•eRF3•GMPPCP-mRNA complex structure derived from the cryo-EM structure, PDB 5LZT (mammalian ribosome, human eRF1, eRF3; from Shao *et al.*, [38]). (B) eRF1 and the UGA codon interaction site is shown in a part of the magnified structure of (A). The UGA stop codon is shown in blue and indicated. C4-carbonyl of 1st Uracil base that distinguished from cytosine is indicated by black open circle. eRF1 residues are indicated as follows, L123 in red, Y122-C124-F128 in pale orange, E52 and I59 in green, K60 in forest-green. A1825 of 18S rRNA and A3760 of 25S rRNA (rRNA residues are in human numbering in accordance with the PDB 5LZT) close to Y122-L123-C124 are also indicated.

underpinnings and their regulatory mechanism of putative CGA decoding by translation termination complex in the cell are yet unknown.

Intriguingly, if eRF1/eRF3 complex can rescue stalled ribosomes, producing premature products within inefficiently translated mRNA contexts such like CGA repeats, it is tempting to assume that it could provide an analogous situation as NMD, wherein eRF1/eRF3 releases the polypeptide at the premature termination codon. Recently, NMD was reported to be involved in translational fidelity [13] and physiological regulation [66], but not only for unexpected decay of aberrant mRNA. Therefore, mis-decoding by eRF1/eRF3, as in CGA codon repeats, is strongly suggested to have global regulatory underpinnings, which are yet unknown. It is interesting to note that codon usage and the corresponding tRNA repertoire in organisms may be a part of sophisticated translational regulatory systems.

Materials and methods

Strains and plasmids

The strains and the plasmids used in this study are listed in Table 1 and 2. DNA fragments for pMW1252 and pMW1253 were amplified via PCR from pMW1210 with the vector promoter primer and P830 (5′-GGGTCGACT TAGCTCGAGGCTAGCGAGCTCAGGTACC-3′), P831 (5′-GGGTCGACTTATCTTCTTCTTCTTCTTCTTCTTCT TCTTCTTCTTCTGCTCGAGGCTAGC-3′) and cloned into p416GPD vector at EcoRI and SalI restriction sites. DNA fragments for pMW1321-TGA, pMW1321-CGA, pMW1321-AGA, pMW1321-GGA and pMW1321-AGG were amplified via PCR from pMW1210 with vector promoter primer and P891 (5′-CCCGTCGACCCTCACTGCTCGTTCTTCAG CACGC-3′), P892 (5′-CCCGTCGACCCTCGCTGCTCGT TCTTCAGCACGC-3′), P893 (5′-CCCGTCGACCCTCTCT GCTCGTTCTTCAGCACGC-3′), P894 (5′-CCCGTCGA CCCTCCCTGCTCGTTCTTCAGCACGC-3′) and P895

Table 1. List of the yeast strains used in the study.

Strain name	Genotype	Source
HIS3-CGA-stop growth reporter (Y240)	MAT alpha, his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-kanMX-ADH-HIS3 -R(CGA)12-STOP-HIS3t	This work
Blank-luc control reporter (Y261)	MAT alpha, his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-TEFp-Rluc-blank-luc2-CYCt-hphMX-HO	Derivative of Saito *et al.* [5]
CGA12-luc reporter (Y262)	MAT alpha, his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-TEFp-Rluc-CGA12-luc2-CYCt-hphMX-HO	Derivative of Saito *et al.* [5]
CGA6-luc reporter (Y294)	MAT alpha, his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-TEFp-Rluc-CGA6-luc2-CYCt-hphMX-HO	This work
BY4727	MAT alpha, his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0	Brachmann *et al.* [68]

(5′-CCCGTCGACCCCCTCTGCTCGTTCTTCAGCAC GC-3′) and cloned into p416GPD vector at EcoRI and SalI restriction sites.

Mutant isolation and growth assay

The eRF1 gene cloned into p415GPD (pMW1111) was randomly mutated via hydroxylamine treatment. Five micrograms of pMW1111 was added to hydroxylamine solution (1 M hydroxylamine, 0.4 μM EDTA, pH 6.0) and incubated at 37 °C for 20 h. Plasmids were ethanol precipitated and washed with 70% ethanol, twice. The treated pMW1111 plasmids were introduced into the HIS3-CGA_{12}-stop growth reporter strain, Y240, via transformation. Y240 transformants were then incubated on SC-His-Leu agar plates at 30 °C to select for growing colonies (pMW1106). For growth assay, Y240 strain was transformed with vector or eRF1 variants (pMW1111 and pMW1112s; pMW1112 L123F is equivalent to the screened pMW1106) and incubated on SC-Leu agar plates at 30 °C. Obtained transformants were seeded onto SC-His-Leu agar plates and incubated at 30 °C to examine growth.

CGA read-through assay

The dual-luciferase translational read-through assay was conducted in accordance with a previously reported method [35]. Y262, Y294 and Y261 (blank) strains were transformed with p415GPD vector or eRF1 variants cloned in p415GPD. The transformants were cultured at 30 °C, cells were harvested and suspended in lysis buffer, and cell lysates were prepared by vigorously shaking with glass beads (Sigma-Aldrich, St. Louis, MO, USA), using Fastprep24 (MP biomedicals, Santa Ana, CA, USA). The dual-luciferase assay was conducted with Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The percentage of CGA

Table 2. List of the plasmids used in the study.

Plasmid name	Construct	Source
p415GPD	P415GPD vector	Mumberg *et al.* [69]
pMW1111	p415GPD-BamHI-SceRF1-SalI	Vector exchange of SceRF1 (Wada *et al.* [34])
pMW1106	Screened mutant (eRF1 L123F)	
pMW1112s	P415GPD-BamHI-SceRF1 L123 mutant-SalI	Vector exchange of SceRF1 L123 mutants (Saito *et al.* [35])
pMW1210	p414TEF-EcoRI-Rluc-Arg(CGA)12-Luc2-SalI	Saito *et al.* [5]
pMW1252 (size control)	p416GPD-EcoRI-Rluc-stop-SalI	This study
pMW1253 (size control)	p416GPD-EcoRI-Rluc-Arg(AGA)12-SalI	This study
pMW1096	pRS416-BamHI-tR(ACG)J (−549 + 301)-XhoI	Laboratory stock
pMW1097	pRS416-BamHI-tR(UCG)J (−549 + 301)-XhoI	Laboratory stock
p416GPD	P416GPD vector	Mumberg *et al.* [69]
pMW659	p416GPD-BamHI-SceRF3c(nt760-2058)-XhoI/SalI	Wada *et al.* [34]
pMW1259	p416GPD-EcoRI-GSPT1c (aa188-637 of human GSPT1 P15170-3)-SalI	Laboratory stock
pMW1321-TGA	p416-GPD-EcoRI-Rluc-TGA-SalI-his3t	This study
pMW1321-CGA	p416-GPD-EcoRI-Rluc-CGA-SalI-his3t	This study
pMW1321-AGA	p416-GPD-EcoRI-Rluc-AGA-SalI-his3t	This study
pMW1321-GGA	p416-GPD-EcoRI-Rluc-GGA-SalI-his3t	This study
pMW1321-AGG	p416-GPD-EcoRI-Rluc-AGG-SalI-his3t	This study
pMW1208	p416TEFkan-EcoRI-Rluc-(CGA)12-luc2-SalI	Laboratory stock

read-through was determined from the ratio of luc2/Rluc compared to that of corresponding blank control.

Western blot analysis

The transformant cells were cultured in appropriate media till the mid-log phase (OD_{600} of approximately 0.75). The cells were harvested, treated with 10% TCA for 30 min on ice and harvested. Thereafter, the samples were suspended in sample buffer (Laemmli, Sigma-Aldrich), and the neutral pH was adjusted with 4 N NaOH and the mixture was vigorously shaken with glass beads (Sigma-Aldrich), using Fastprep24 (MP biomedicals). The protein in the lysates were separated via SDS/PAGE (10% resolving gel) and transferred to Hybond-ECL (GE Healthcare, Little Chalfont, UK) via the semidry method. The filters were treated with either anti-Rluc Antibody, clone 5B11.2 (anti-Rluc; Merck Millipore, Billerica, MA, USA) or PGK1 monoclonal antibody (anti-PGK; 22C5D8; ThermoFisher), and Anti-Mouse IgG, HRP-linked Whole Ab Sheep (GE Healthcare). Signals were detected with ImmunoStar LD (Wako Laboratory Chemicals, Osaka, Japan) and LAS-3000 mini (Fujifilm, Tokyo, Japan). Quantification of bands was conducted by Multi Gauge software (Fujifilm). To detect anti-Rluc antibody, the Can Get Signal Immunoreaction enhancer (Toyobo, Osaka, Japan) was used to enhance the reaction.

Northern blot analysis

The transformant cells were cultured in the appropriate media to the mid-log phase. The cells were harvested and samples were prepared as described previously [67], via hot phenol treatment and ethanol precipitation, and dissolved in H_2O. In 20 μg of each RNA sample, MOPS buffer with formaldehyde and formamide was added, and incubated at 55 °C for 10 min. RNA samples were separated via electrophoresis on a 1.2% agarose gel with 6.7% formaldehyde, then transferred to Hybond-N+ (GE Healthcare) and fixed via UV irradiation. RNAs were detected using the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare) in accordance with the manufacturer's protocol. The His3 probe was amplified via PCR from p413GPD, using primers P768/P769 (5′-ACGACCATCACACCACTGAA-3′/5′-GCGAGGTGGCTTCTCTTATG-3′). The Rluc probe was amplified via PCR from pMW1208 with primers P828/P829 (5′- CGAGCACCAAGACAAGATCA -3′/5′- CATTTCAT CTGGAGCGTCCT -3′), and the SCR1 probe was amplified from pMW1326 with primers P384/P385 (5′-AGGCTGTA ATGGCTTTCTGGTGGGATGGGA-3′/5′-GATATGTGCT ATCCCGGCCGCCTCCATCAC-3′). The probes were labelled and used for hybridization to the filter. The filter was washed with the primary wash buffer (0.5 × SSC, 0.4% SDS and 6 M urea) and the secondary wash buffer (2 × SSC), followed by detection with the detection solution and measured using LAS-3000 mini (Fujifilm).

Quantitative PCR

RNA samples obtained from transformant cells, for northern blot analysis, were reverse transcribed by ReverTra Ace (Toyobo) with poly d(T) primers. Quantitative PCR was conducted with His3 primers (P852/P853 5′-CACTGAA GACTGCGGGATTG-3′/5′-TGGAAAGTGCCTCATCCA AA-3′) and Pgk1 primers (P947/P948 5′-GGCCAAG GGTGTCGAAGTCG-3′/5′-CAACCCTTGCCAGCCAGC TG-3′) as a control in QuantStudio (ThermoFisher, Fremont, CA, USA). Relative mRNA expression levels were normalized to those of vector controls via the $2^{-\Delta\Delta Ct}$ method to quantify show the relative His3 levels to the vector transformed control.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

KI and MW conceived and designed the study. MW performed the experiments. KI and MW wrote the article.

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