Research Article

The CGA Codon Decoding through Arg-tRNA^ICG Supply Governed by Tad2/Tad3 in Saccharomyces cerevisiae

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The CGA codon is a rare codon in *Saccharomyces cerevisiae* and is known to be inefficiently decoded by wobble pairing with Arg-tRNA^{ICG}. Experimental consecutive CGA codons cause ribosome stalling to result in a reduction of the encoding protein product. In this study, an additional supply of Arg-tRNA(ACG) genes that produce decoding Arg-tRNA^{ICG} recovered the product level from the CGA12-luc reporter, revealing that the product reduction is essentially due to inefficient decoding and deficiency in the tRNA supply. The mature tRNA^{ICG} and the precursor tRNA^{ACG} ratios examined for cellular tRNA fraction determined that the tRNA^{ICG} ratio is maintained at less than 30%, and is responsive to the anticodon first adenosine deamination enzyme, Tad2/Tad3, expression level.

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Introduction

The decoding of codons by tRNAs in the ribosome is not simply determined by three Watson-Crick type base pairings, instead, it is affected by the repertoire of tRNAs of the organism, modifications, structures, and ribosomal decoding environment. An important aspect of the decoding exists on the tRNA side. Not all tRNAs with anticodons corresponding to 61 sense codons are encoded in the genomes of all known organisms [1] (GtRNAdb: http://gtrnadb.ucsc.edu, SGD: https://www.yeastgenome.org). There are wobble pairings between a codon's third base and first base

of anticodon (thirty-fourth base of tRNA) that allow non-perfect match base pairing [2], resolving the lack of perfect match tRNA genes. Firstly, in eukaryotes, most of the wobble position adenines of tRNAs are edited to hypoxanthine (inosine as nucleoside) [3][4][5]. Secondly, wobble position uracil pairs with guanine as observed in other RNA secondary structures. In addition, some of the anticodon bases are modified for finetuning without changing main base structures [6][7][8][9] (Modomics: https://iimcb.genesilico.pl/modomics/). While a lot of detailed tRNA modification analyses were reported, the inventory of codon-tRNA relationship analyses would further proceed our understanding of decoding. We propose the concept of "anticodonome" which is a set of total anticodons of tRNAs that function in the ribosome for the decoding of whole genetic codes in an organism.

In *Saccharomyces cerevisiae*, the CGA codon is a rare codon, known to be inefficiently decoded[10][11][12]. The tRNA entity responsible for the decoding of CGA in *S. cerevisiae* is reported to be arginyl-tRNA^{ICG} which is produced from arginyl-tRNA^{ACG} by Tad2/Tad3 adenine deamination complex (ADAT2/ADAT3 in higher eukaryotes)[13][14]. In *Escherichia coli*, TadA homodimer functions the reaction to produce arginyl-tRNA^{ICG} and the arginyl-tRNA^{ICG} is the only inosine-using tRNA[15][16]. The tRNA^{ICG} is uniquely used in some yeasts and bacteria, including *S. cerevisiae*, for the CGA codon decoding, and the base pair is specially referred to A:I wobble (codon third base "A" to anticodon first base "I" in the pairing)[17][18]. We abbreviate arginyl-tRNA^{ICG} and arginyl-tRNA^{ACG} to tRNA^{ICG} and tRNA^{ACG} respectively, for simplification. Most eukaryotes use seven to eight inosine-containing tRNAs for decoding except for A:I wobble. Thus, those organisms should have different "anticodonome" for the decoding. The decoding with A:I wobble pairing is reported inefficient[10]. The CGA codon repeats are reported to cause ribosome stall leading to products reduction[11][19][20]. In relation to inefficient decoding of the CGA codon, we previously reported that the translation termination factor complex, eRF1/eRF3, could cause termination at the CGA codon (repeats) in the mis-decoding level[12].

Here we confirmed CGA codon decoding is accomplished by anticodon-ICG-tRNA, through post-transcriptional adenosine deamination, despite inefficient pairing. The *in vivo* tRNA^{ICG} supply has been subjected to the enzyme Tad2/Tad3 expression level, which brought various tRNA$^{ICG/ACG}$ ratios.

Materials and Methods

Yeast strains

Strain name	Genotype	Source
BY4727	*MATα his3Δ200 leu2Δ200 lys2Δ200 met15Δ0 trp1Δ63 ura3Δ0*	Brachmann et al. (1998)[21]
Y352	*MATα Δnkp2 his3Δ200 leu2Δ200 lys2Δ200 ura3Δ0 tad3-1 (Δ1081T)*	YKOα Δnkp2 Open Biosystems[22] (22)
Blank-luc reporter (Y261)	*MATα his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-TEFp-Rluc-blank- luc2-CYCt-hphMX-HO*	Derivative of Saito et al.(2015)[20]
CGA6-luc reporter (Y294)	*MATαhis3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-TEFp-Rluc-CGA6- luc2-CYCt-hphMX-HO*	Wada et al. (2019)[12]
CGA12-luc reporter (Y262)	*MATα his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 HO-TEFp-Rluc-CGA12-luc2-CYCt-hphMX-HO*	Derivative of Saito et al.(2015)[20]
FLAG-Tad3 (Y355)	*MATα his3Δ200 leu2Δ200 lys2Δ200 met15Δ0 trp1Δ63 ura3Δ0 FLAG-Tad3*	Present study

Plasmids

Plasmid name	Construct	Source
pRS426		
pMW1347	pRS426-BamHI-(-110)-tR(ACG)D-(+271)-SalI	Present study
pMW1385	pRS426-BamHI-(-101)-tR(ACG)E-(+271)-SalI	Present study
pMW1386	pRS426-BamHI-(-101)-tR(ACG)K-(+274)-SalI	Present study
pMW1387	pRS426-BamHI-(-104)-tR(ACG)L-(+258)-SalI	Present study
pMW1388	pRS426-BamHI-(-101)-tR(ACG)O-(+274)-SalI	Present study
pMW1348	pRS426-BamHI-(-110)-tR(ACG)J-(+274)-SalI	Present study
pMW1586	pRS426-BamHI-(-100)-tR(CCG)-(+276)-SalI	Present study
pMW1588	pRS426-BamHI-(-110)-tR(UCG)D-(+271)-SalI	Present study
pMW1590	pRS426-BamHI-(-110)-tR(UCG)J-(+274)-SalI	Present study
p416GPD		
pMW1334	p416CYC-BamHI-Tad3-ShoI/SalI	Present study
pMW1255	p416GPD-EcoRI-Tad3-XhoI	Present study
pMW1254	p416GPD-BamHI-Tad2-SalI	Present study
pMW1420	p416CYC-EcoRI-FLAG-Tad3-XhoI/SalI	Present study
pMW1421	FLAG-Tad3 in p416ADH	Present study
pMW1378	p416 -HindIII-Ptad3-EcoRI-FLAG-Tad3-XhoI	Present study

Western blot analysis

Samples were cultured, collected, and treated with a 10% TCA solution. After centrifugation, the samples were suspended with a sample buffer for SDS-PAGE, pH was neutralized and then, the cells were disrupted with glass beads by Fastprep24 (MP Biomedicals). The supernatants from each sample were applied to SDS-PAGE, transferred to a nitrocellulose filter, and reacted with either of the antibodies. For Luc-CGA reporters, the anti-Renilla Luciferase antibody (Millipore, clone 5B11.2) was

used as the primary antibody and HRP conjugated anti-mouse antibody (Cytiva) was used as the secondary antibody. For FLAG-Tad3 detection, an anti-FLAG antibody (Sigma-Aldrich, F3165) as first antibody, and HRP conjugated anti-mouse antibody was used secondary antibody. For PGK1 detection, the anti-PGK1 antibody (Molecular Probes 459250) was used as the primary antibody instead. The reaction was detected using an Immunostar-LD (Fujifilm Wako Pure Chemicals Corporation, Osaka, Japan) by Las 3000mini (Fujifilm).

Complementation analysis

The yeast strains, BY4727 and Y352, were transformed with the plasmids, and transformants were examined for growth at 37˚C on SC-Ura plates.

Inosine nucleotide and tRNA analysis by RT-PCR sequencing

For confirmation of inosine nucleotide by RT-PCR sequencing, inosine containing oligo RNA named mini-tRNA^{ICG} was ordered (Fasmac). The RNA was reverse transcribed by a ReverTra Ace kit (Toyobo) with a P1169 primer. The reverse transcribed sample was applied to PCR with primers P1168 and P1169. The PCR product was tread with $CHCl_3$, blunted with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase and cloned into pUC119, and sequenced by Applied biosystems 3500 (Thermo Fisher Scientific). P1168: AATGGTCACGGCGTCTG, P1169: CCTGGAATCTTCTGGTT

For tRNAs analysis, RNAs were purified from the cells by employing the hot phenol method, and small RNAs were purified with RNA clean and concentrator (Zymoresearch). The RNAs were reverse transcribed and treated basically the same as described above, with primer P1048 for RT-PCR and primers P1043 and P1048 for PCR. P1043: TCGTGGCCCAATGGTCA, P1048: CTTCCCCGCCAGGACT

Results

tR(ACG) gene oversupply upregulates CGA repeat containing reporter product level

The strains with dual-luciferase reporter gene constructs with 0, 6 and 12 CGA codon repeats reduced full-length Rluc-luc2 products level in line with the increase of CGA codon number, as previously reported (Fig. 1A)[12]. In contrast, Rluc-size bands that were thought to be terminated by eRF1/eRF3 increased slightly, although this was not comparable to a full-length product level decrease. Interestingly, in *S. cerevisiae,* the anticodon edited Arg-tRNA^{ICG} decipher CGA codon, similarly to

bacteria, instead of Arg-tRNA^{UCG} in most eukaryotes. In *S. cerevisiae*, six tR (ACG) genes, D, E, K, L, O and J (composed of two groups of sequences, D-O and J), whose anticodon adenines were thought to be deaminated to inosine, were encoded in the genome (Fig. 1B). All the tRNA genes were cloned with their context sequences respectively (approximately 100 bp upstream and 250 bp downstream), and expressed to examine their effects to the product level of consecutive 12 CGA containing dual-luciferase reporter (CGA12-luc) product level (Fig. 1C). Overproduction of all the six tRNA genes all promoted full-length CGA12-luc product levels in different extents. The expression of artificial perfect match tRNA genes for the CGA codon, tR (UCG)D and tR (UCG)J, produced more abundant full-length products. In contrast, control tR (CCG) overproduction which deciphers the CGG codon for Arg did not affect the full-length product level, compared to the vector. The CGA codon was confirmed not to be read by tRNA^{CCG}. These results verified that the tR(ACG) genes produced tRNAs for the CGA codon, most likely tRNA^{ICG}, and were less effective than artificial tRNA^{UCG} that perfectly match with CGA in sequence. The results strongly indicate that the fundamental reason for reduced product level from reporters with consecutive CGA codons can be attributed to the decoding process by the tRNAs and their supply. Consecutive CGA codons might cause a local shortage of tRNA^{ICG}. In addition, the difference between tRNA^{ACG} and tRNA^{UCG} overproduction to the product level indicates that tRNA^{ACG} modification to tRNA^{ICG} would retain important physiological meaning through the codon decoding. (Fig. 1A). Similar results were reported by Lezring et al. with a reporter assay system[10], thus together, the inefficiency of CGA codon decoding has been thought to be established in *S. cerevisiae*.

Reconfirmation of tRNA^{ICG}-inosine read by sequencing

The CGA codon is deciphered by Arg-tRNA^{ICG} in *S. cerevisiae* (Fig. 2A). Fig. 2A is a part of the table we named "decoding table" of *S. cerevisiae*, which simply summarizes codon-anticodon base pairings in the ribosome, is based on the information of tRNA genes and prior studies. The third column in the table shows the total possible anticodons, a part of the "anticodonome" of *S. cerevisiae*. Importantly, the decoding table has not been established yet. The codon-anticodon relationship is complicated in the CGU/C/A codon box shown by the red box (Fig. 2A). From the tRNA^{ICG} perspective, decoding codons and what could be decoded by the tRNA in the ribosome remains ambiguous. The A:I pairing at wobble position is rather unusual even among inosine-containing tRNA using organisms[10]. In sequencing, inosine (hypoxanthine as base) was reported to be read as guanine (G)[13], because of the

fundamental limitation of the general sequencing method. It is equipped to distinguish four bases in genes. When inosine-containing RNA was reverse-transcribed to cDNA, inosine was read as G[23][24]. This has been reconfirmed using synthetic oligoRNA (mini-tRNA^{ICG}) which contains part of the tRNA^{ICG} (Fig. 2B). The oligoRNA was reverse transcribed with primer B and PCR amplified with primers A and B. The sequencing showed guanine at the site of inosine (Fig. 2B), confirming the previous reports. It should be emphasized that the discrepancy between the genome sequence and the cDNA sequence could be attributed to RNA modification or editing. The principle allows us to obtain the ratio of unmodified tRNAs in cellular RNA preparations.

Functional cooperation of Tad2/Tad3

tRNA^{ICG} is biosynthesized from tRNA^{ACG} through adenosine deamination by Tad2/Tad3 complex in *S. cerevisiae* (ADAT2/ADAT3 complex in higher eukaryotes) (Fig. 3A). To examine functional cooperation between Tad2 and Tad3 *in vivo*, a tad3 defective strain was prepared (Fig. 3B). The Tad3 gene in the genome was connected to the Nkp2 gene at a tail-to-tail manner without any additional terminator sequence (SGD database). We identified that the Δnkp2 strain of the YSC1054 YKO strain collection possessed two bases deletion close to the terminal of the Tad3 gene and the strain showed temperature sensitivity (ts) at 37°C. Thus, the tad3 temperature-sensitive strain (Y352) was used to examine tad3 functionality. Tad3 encoding plasmids from low expression CYC1 and high expression GPD promoters both supported the growth of the *tad3* ts strain at the restrictive temperature (Fig.3C). In addition, Tad2 encoding plasmids from GPD promoter also supported the growth of the strain (Fig. 3C). Nkp2 encoding plasmids could not support the growth of the strain at 37°C, ensuring that the defect was due to tad3 mutation. The result strongly suggests the co-functionality of Tad2 and Tad3, likely making a complex, *in vivo*. However, the growth observed by the Tad2 or Tad3 effect is not necessarily due only to Arg-tRNA^{ICG} but also to the other inosine-containing tRNAs.

The Tad3 expression level from the native promoter was examined by constructing a N-terminally FLAG-tagged Tad3 strain because the Tad3 expression from the CYC1 promoter complemented the growth of the tad3 ts strain. For comparison, FLAG-Tad3 expression plasmids with Ptad3, native Tad3 promoter, CYC1 and ADH promoters were constructed. When their expression level was compared via FLAG-tag detection, Tad3 from the native promoter was expressed less than from the low expression CYC1 promoter, indicating that a small level of Tad3 was expressed in general culture (Fig. 3D). The FLAG-Tad3 cloned in plasmid under a Ptad3 promoter, expressed similar amounts of FLAG-Tad3,

supporting the low-level expression of Tad3 (Fig. 3D). The Tad3 expression level was unexpectedly low considering seven kinds of tRNAs that use inosine were modified by Tad2/Tad3 adenosine deaminase.

Ratio of tRNA^{ACG} and tRNA^{ICG} in S. cerevisiae

Inosine in tRNA was confirmed to be read as guanine (Fig. 2B), therefore, the anticodon ACG and edited ICG should be distinguished by sequencing. To examine the adenosine deamination level of tRNA^{ACG} to tRNA^{ICG}, tRNA-containing small-size RNAs were prepared from cultures and converted to cDNA, amplified with common primers for tRNA$^{ACG/ICG}$, and then cloned for sequencing (Fig. 4A). As a known difference between tRNA^{ACG} and tRNA^{ICG} does not exist, other than at the deamination site, the sequences were not expected to be biased. In the wild-type strain, three trials of a total of 82 clones revealed inosine ratios of 27%, 7%, and 21% (Fig. 4B, and 4C). In the tad3 defective ts strain, no ICG anticodon tRNA was found in a total of 25 clones (Fig. 4B and 4C). Tad3 and Tad2 overproduction from p416GPD plasmids resulted in 47% and 39% inosine ratios in 19 and 41 examined clones, respectively (Fig. 4B and 4C). The results clearly demonstrated that the tRNA ICG/ACG ratio responded to Tad3 and Tad2 expression levels. Intriguingly, ICG/ACG ratio was lower than expected, correlating with low-level expression of Tad3 (Fig. 3D). This may reflect the modification status of tRNAs that undergo high numbers of maturation steps after transcription, such as aminoacylation, high levels of modification other than at the anticodon site, or low amounts of the Tad2/Tad3 complex may exist close to limitation in translation.

Discussion

A large number of ribosome stalling studies used consecutive CGA or CGN containing reporters to examine their effects. In this present study, the decoding aspect of CGA codon by the tRNA^{ICG} in translation was aimed to further understanding. Our previous study indicated that even a single CGA could be deciphered by translation termination machinery, eRF1 and eRF3, at low level depending on a condition[12]. Although we have still used consecutive CGA12 containing dual-luciferase reporter as a good indicator, simple overproduction of tRNA^{ACG} genes revealed apparent recovery of full-length product level, though not to blank level (Fig. 1C). This strongly suggests that product level reduction caused by consecutive CGA codons is primarily a cumulative result of inefficient decoding and deficiency in tRNA^{ICG} supply. It does not exclude secondary effects caused by inefficient decoding of

CGA codons, as the product reduction is quite impressive. However, as no more than two consecutive CGA codon repeats exist in the *S. cerevisiae* genome, it would not be programmed in a general translation process.

A lot of information regarding tRNA modifications had been revealed. However, wobble inosine usage in tRNA anticodons still remains a mystery since the wobble hypothesis by Francis Crick. In eukaryotes, seven or eight inosine-containing tRNAs at anticodon first position are used for decoding[25][3]. Among the seven *S. cerevisiae* tRNAs, Arg-tRNA^{ICG} is unique as it decodes rare CGA codon by A:I pairing, while the other inosines pair with cytosine (C) and/or uracil (U). Thus, it was thought to have inefficient nature, and has been revealed inefficient. In this study, tRNA^{ICG} ratios to the total tRNA$^{(ICG+ACG)}$ examined were low, at less than 30% in the wild-type strain, and increased by Tad3 and Tad2 overproduction. In tad3 defective strain, no tRNA^{ICG} was observed. This raises the question of the necessity of tRNA^{ICG} for the expression of CGA codon-containing genes in general culture, requiring further investigation. Even in Tad3 or Tad2 overproduction, the tRNA^{ICG} ratio did not exceed 50%. The meaning of the ratio could be 1) a maximum ratio of ICG within the tRNA maturation steps, 2) existence of Arg-tRNA^{ACG} as pooled precursors[26], or 3) genuine working tRNA ratio (i.e. not only tRNA^{ICG} but tRNA^{ACG} is involved in decoding, likely to read CGU and/or CGC codons). The deamination of adenosine to inosine of Arg-tRNA^{ACG} may be slow to limit tRNA^{ICG} availability. In accordance with this, expression levels of Tad3 were quite low considering a large amount of tRNA's anticodon first adenosine deamination. Thus, the amount of each tRNAs requiring adenosine deamination would also affect translational efficiency[27]. In some bacteria, mycoplasmas, and mollicutes, a similar tRNA^{ICG} study was reported in relation to the retention of bacterial ortholog of Tad2/Tad3, TadA enzyme genes, to give quite a different situation with eukaryotes[28].

In humans, the Tad3 ortholog ADAT3 mutant (V144M) was identified to be a cause of intellectual disability[29][30]. The mutant site amino acid V144 is conserved among eukaryotes (Supplementary Fig. S1) and was reported to reduce at least some of position 34 inosine-containing tRNAs (i-tRNAs hereafter). Thus, it is readily conceivable that Tad2/Tad3 complex would affect all of the i-tRNAs in each species. That is an intriguing point of i-tRNA repertories between humans and *S. cerevisiae*. While in *S. cerevisiae*, the CGA codon is deciphered by i-tRNA, arg-tRNA^{ICG}, but in humans, the CGA codon is read by non-i-tRNA, arg-tRNA^{UCG}. Hence, humans should be free from apparent inefficient decoding

of CGA codon by $tRNA^{ICG}$ observed in this study in *S. cerevisiae*. In spite that the Tad2/Tad3 (ADAT2/ADAT3) complex owes fundamental function for tRNA biogenesis for gene translation, and Tad2 and Tad3 have interesting regional length variation (Supplementary Fig. S1 and S2) that might contribute to their tRNA substrate selection and variation of "anticodonome". The Tad2 (ADAT2) of the complex was reported to have oxidative deamination enzyme activity having homology to bacterial TadA in enzyme activity site, and presumable severe effect of tad2 mutation might be a reason for no tad2 mutations are reported in clinical cases until now.

This study confirmed the CGA codon—$tRNA^{ICG}$ correspondence in *S. cerevisiae.* Left to determine in the part of the table (Fig. 2A red box) is whether $tRNA^{ACG}$, observed in ICG/ACG ratio, decipher codon(s), in other words, whether the $tRNA^{ACG}$ is included in the "anticodonome" of *S. cerevisiae*. If $tRNA^{ACG}$ is included, then, are each CGU, CGC, and CGA codon decoded by either/or both $tRNA^{ICG}$ and $tRNA^{ACG}$? To establish the "anticodonome", codon-anticodon base pairings in the ribosome, rather than free tRNAs, should be determined.

The inefficiency of CGA codon decoding has been strongly suggested to contribute to the mis-decoding level termination by the eRF1/eRF3 complex in prior and present studies[12]. If the mis-decoding is programmed for any cellular process or response, the anticodon portion of the translation termination factor, "peptide anticodon"[31][32], could be included in the "Anticodonome". The mis-decoding by eRF1/eRF3 in *S. cerevisiae* could rescue the stalled ribosome with consecutive CGA codons, in addition to the ribosome quality control reported. Since repertoires of tRNAs are unique to species/organisms, each species/organism should possess its own "anticodonome" and the decoding table. *In vivo* reflected studies are necessary to clarify the "anticodonome"s, as it should be translational machinery dependent and so many modifications of tRNAs would affect decoding.

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Figures

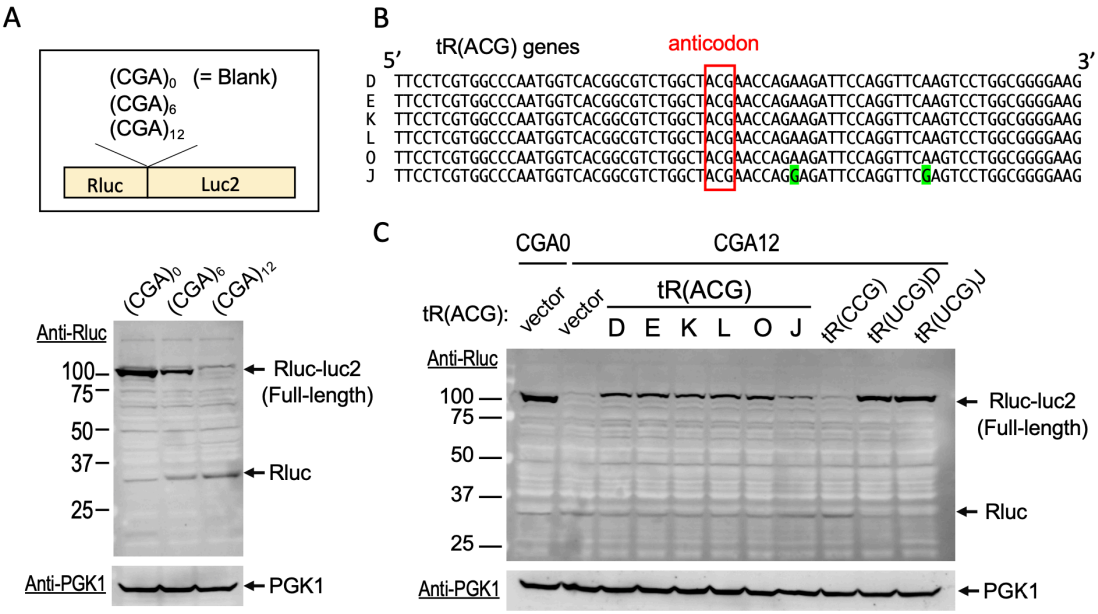

Figure 1. *Effects of tRNA overproduction to the consecutive CGA decoding. (A) The strains, Y261, Y294, Y262, with dual-luciferase reporters, CGA0-luc, CGA6-luc, CGA12-luc, respectively, were cultured and their product levels were analyzed by western blot analysis with anti-Rluc antibody. The detection by the anti-PGK1 antibody was used as a control. (B) Alignment of tR(ACG) genes of S. cerevisiae. Anticodon encoding residues are indicated by a red box. Sequence variants are indicated in green. (C) The overexpression effect of each tR(ACG) gene, D, E, K, L, O, and J to CGA12-luc reporter products were examined by western blot analysis with anti-Rluc antibody. Controls were the CGA0-luc strain, and CGA12-luc strain transformed with pRS425 vector, plasmids with tR(CCG)gene, and perfect-match artificial tR(UCG)D and tR(UCG)J genes. The detection by the anti-PGK1 antibody was used as a control.*

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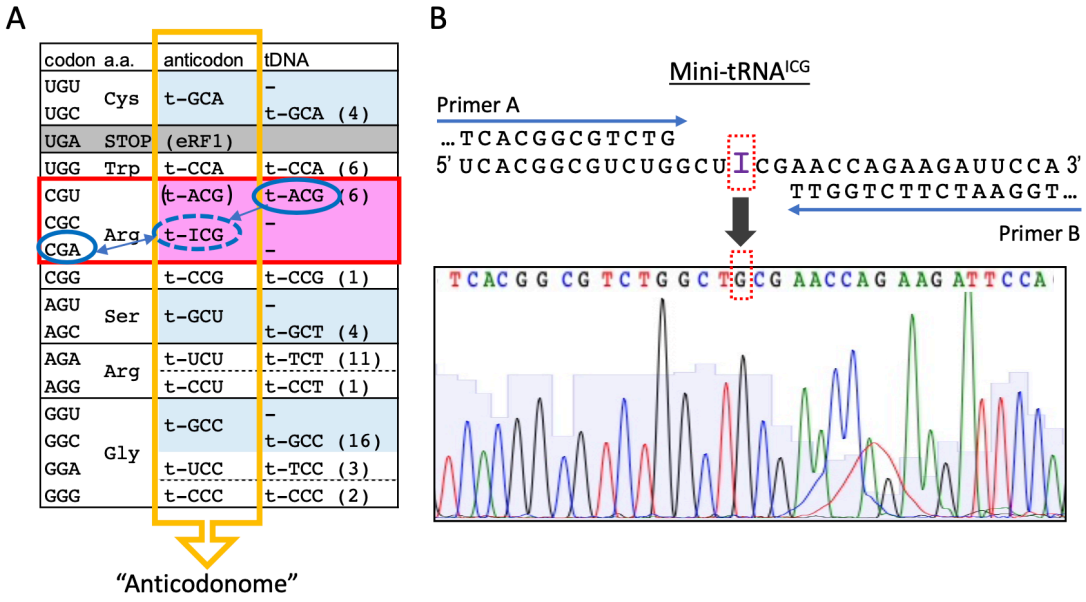

Figure 2. *A part of the "anticodonome" and tRNA^{ICG} analysis by sequencing. (A) A part of the table that explains codon-anticodon pairings in S. cerevisiae. The first column shows the codon, and the second column shows corresponding amino acids (a.a.). The fourth column is anticodon portion sequences of tRNA genes (tDNA) that recognize the codons in the first column. The third column is the tRNA anticodons derived from the fourth column tDNA anticodons, and filled up vacancy with reported knowledge, and is not completely fixed yet. The compiled anticodons from the third columns corresponding to all codons should be called "anticodonome". The hyphens in the fourth column indicate that no corresponding anticodon encoding tRNA genes existed in the genome. When a perfect match anticodon that pairs with the first column codon were not identified, reported wobbles were considered, including inosine. The numbers in the parentheses in the tDNAs indicate the number of genes in the genome. The red box shows the CGU/C/A codon box reported to be read by tRNA^{ICG}. (B) Sequence of Mini-tRNA^{ICG}, (commercially supplied oligo-RNA) that was reverse transcribed with primer A, and PCR amplified with primers A and B.*

doi.org/10.32388/HEN7AF

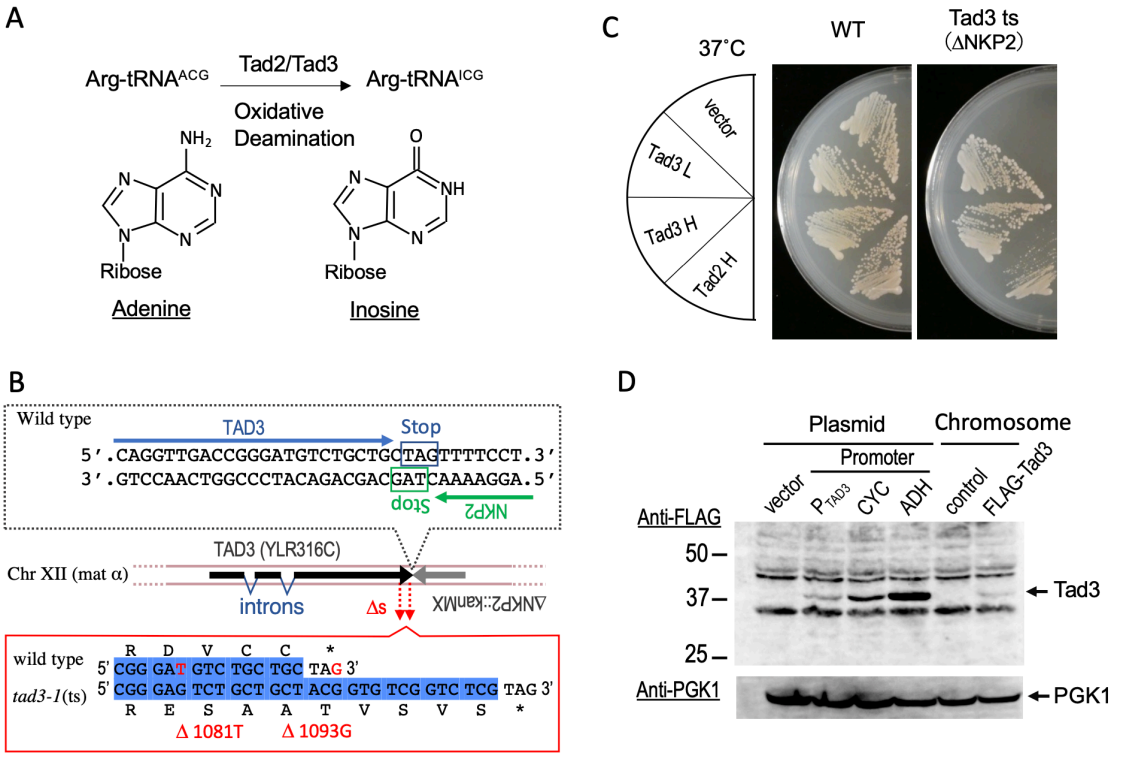

Figure 3. *Tad2 and Tad3 functionality and expression in vivo. (A) Adenosine deamination reaction by Tad2/Tad3. (B) The Tad3 locus of S. cerevisiae chromosome XII. The Tad3 gene retains two introns and is connected to the Nkp2 gene in a tail-to-tail manner with no spacer. In the tad3 allele of the Δnkp2 strain of yeast knockout collection, two nucleotide deletions, Δ1081T and Δ1093G have been found. The tad3 allele showed temperature sensitivity and was named tad3-1. (C) Complementation analysis of tad3 ts strain. The wild type, BY4727 strain, and the tad3 temperature-sensitive (ts) strain transformants with p416GPD vector, Tad3L(Tad3 in p416CYC), Tad3H(Tad3 in p416GPD) and Tad2H(Tad2 in p416GPD) were examined for growth at a restrictive temperature (37°C). (D) The Tad3 expression level was examined by introducing FLAG-tag in the Tad3 genome locus of the wild type (BY4727) strain (chromosome). The strain control was BY4727. The Tad3 plasmids with different promoters, Ptad3, CYC1, and ADH were introduced into the wild-type strain and used as expression controls. The strains and transformants were cultured, and Tad3 expression was detected by western blot analysis with the anti-FLAG antibody and anti-PGK1 antibody as a control.*

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A

Arg-tRNA I(G)CG / ACG

5′ TTCCTCGTGGCCCAATGGTCACGGCGTCTGGCTXCGAACCAGAAGATTCCAGGTTCAAGTCCTGGCGGGGAAG 3′

P1043 P1048 RT

X = A or I(G)

B

Strains	Trials	Number of Samples		Nubmer of clones		Ratio
		Each	Total	ACG	I(G)CG	ICG (%)
WT	1	14	82	11	3	27
	2	30		28	2	7
	3	38		30	8	21
tad3 ts	1	11	25	11	0	0
	2	14		14	0	0
+Tad3	1	19	19	10	9	47
+Tad2	1	41	41	25	16	39

C

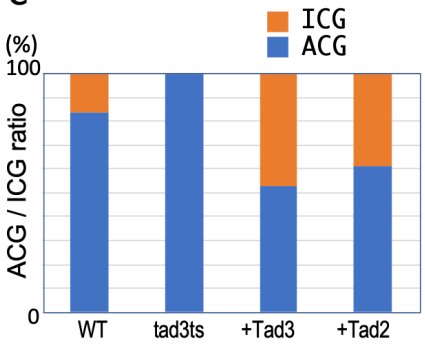

Figure 4. *tRNA-I(G)CG/ACG ratio in S. cerevisiae strains. (A) A strategy to examine $tRNA^{ICG/ACG}$ ratio. (B) $tRNA^{ICG}/tRNA^{ACG}$ ratio of wild type (wt), tad3 ts strains, and Tad3 or Tad2 overexpression transformants were examined. The numbers in the trials column indicate the number of different RNA preparations from cell cultures. The columns of "Number of samples" indicate clones applied to sequencing, and "Number of clones" shows the number of clones with "ACG" and "ICG=(G)CG" anticodons, respectively. The ratio indicates the I(G)CG ratios in sequenced clones. (C) Graphic summary of ICG and ACG clone ratios of the strains. Orange shows the ICG ratio, and blue shows the ACG ratio. The ratios of WT and tad3ts are averages of the trials in (B).*

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