Supporting Information

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SI Materials and Methods

Luciferase Assays. Luciferase assays were performed on clarified lysates, as previously described (1). Briefly, cell pellets were resuspended in buffer W [100 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA; supplemented with 0.1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture], and enzyme activity was measured after the addition of 400 μL of luciferase assay buffer [25 mM Hepes (pH 7.8), 15 mM potassium phosphate (pH 7.8), 15 mM magnesium sulfate, 4 mM EGTA, 2 mM ATP, and 1 mM DTT]. Assays were performed in a GloMax 20/20 luminometer by injecting 100 μL of 200 mM D-luciferin (Analytical Luminescence Labs). Luciferase activity was normalized to total protein concentration of the lysates measured with the Bradford assay (BioRad).

RNA Analyses. Cells expressing either HA-*LUC*-SF or HA-$LUC_{(PTC20)}$-SF were grown in synthetic complete (SC) media lacking Leu. Total RNA isolation and Northern blot analyses were performed as described previously (1). Random-primed DNA probes made from the 1.6-Kb NcoI-XhoI luciferase fragment were used to detect *LUC* mRNA. Randomly primed DNA probes made from the 0.6-Kb EcoRI-HindIII *CYH2* fragment or the 0.5-Kb EcoRI *SCR1* fragment were used to detect the *CYH2* pre-mRNA, *CYH2* mRNA, and *SCR1* RNA as controls for NMD and normalization, respectively. Transcript-specific signals were determined with a FUJI BAS-2500 analyzer (Fuji Electric).

Western Analyses. Cells representing 0.4 OD_{600} units were harvested and resuspended in 200 μL of sample buffer as described previously (2). Samples were resolved by 8% SDS/PAGE, transferred to Immobilon-P (Millipore) membranes, and incubated with anti-HA antibody (Sigma) or anti-FLAG antibody (Sigma).

Protein Purification and Analyses. Cells expressing HA-*LUC*-SF or HA-LUC_{PTC20}-SF were grown in SC medium lacking Leu. Cells were collected by rapid filtration and resuspended in buffer W [100 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA; supplemented with 0.1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture (Roche)]. Cells were lysed using a cryoMill (Retsch) at 10 Hz for 20 min with continuous liquid nitrogen cooling. Cell lysates were clarified by centrifugation at 12,000 rpm (Sorvall SS34) for 10 min at 4 °C, and supernatants were filtered with 0.2 μM filters. Luciferase was purified using Strep-Tactin resin (IBA) according to the manufacturer's protocol. Briefly, clarified lysates were bound to Strep-Tactin Superflow columns (IBA) and washed three times with buffer W. Elutions were performed with buffer E (buffer W supplemented with 2.5 mM desthiobiotin, 0.1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture) by collecting multiple fractions. The elution profile of luciferase protein was monitored by analyzing fractions for luciferase activity, as well as Western blot analyses using anti-FLAG antibody (Sigma). Elution fractions from Strep-Tactin purification were concentrated using Amicon Ultra-15 30K NMWL filters (EMD Millipore), followed by analysis on 8% SDS/PAGE to resolve the readthrough product. Gels were silver-stained (Proteosilver; Sigma) and processed for MS analysis. Three independent experiments were performed for each condition (one codon under one readthrough-inducing condition).

Sample Preparation for MS. Destained gel bands were cut into 1 × 1-mm pieces and placed in 1.5-mL Eppendorf tubes with 1 mL of water for 30 min. The water was removed, and 200 μL of 250 mM ammonium bicarbonate was added. For reduction, 30 μL of a 45 mM solution of 1,4 dithiothreitol was added and the samples were incubated at 50 °C for 30 min. The samples were cooled to room temperature, and then, for alkylation, 30 μL of a 100 mM iodoacetamide solution was added and allowed to react for 30 min. The gel slices were washed twice with 1-mL aliquots of distilled, deionized water. The water was removed, 1 mL of a 1:1 mixture of 50 mM ammonium bicarbonate and acetonitrile was placed in each tube, and samples were incubated at room temperature for 1 h. The solution was then removed, and 200 μL of acetonitrile was added to each tube, at which point the gels slices turned opaque white. The acetonitrile was removed, and gel slices were further dried in a Speed Vac (Savant Instruments). Gel slices were rehydrated in 100 μL of 4 ng/μL endoproteinase Lys-C (Roche Diagnostics) in 0.01% ProteaseMAX Surfactant (Promega) and 50 mM ammonium bicarbonate. Additional bicarbonate buffer was added to ensure complete submersion of the gel slices. Samples were incubated at 37 °C for 18 h. The supernatant of each sample was then removed and placed in individual 0.5-mL Eppendorf tubes. Gel slices were further extracted with 200 μL of 80:20 (acetonitrile/1% formic acid). The extracts were combined with the supernatants of each sample. The samples were then completely dried down in a Speed Vac.

Liquid Chromatography/Tandem MS Analysis. Endoproteinase Lys-C peptide digests were reconstituted in 20 μL of 5% acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid and separated on a NanoAcquity (Waters) UPLC (ultraperformance liquid chromatography). In brief, a 4-μL injection was loaded in 5% acetonitrile containing 0.1% formic acid at 4 $\mu L \cdot min^{-1}$ for 4 min onto a 100-μm i.d. fused-silica precolumn packed with 2 cm of 5-μm (200 Å) Magic C18AQ (Bruker–Michrom) and eluted using a gradient at 300 $nL \cdot min^{-1}$ onto a 75-μm i.d. analytical column packed with 25 cm of 3-μm (100 Å) Magic C18AQ particles to a gravity-pulled tip. The solvents were 0.1% formic acid (solvent A) and acetonitrile in 0.1% formic acid (solvent B). A linear gradient was developed from 5% (vol/vol) solvent A to 35% (vol/vol) solvent B in 35 min. Ions were introduced by positive electrospray ionization via liquid junction into a Q Exactive hybrid mass spectrometer (Thermo). Mass spectra were acquired over *m/z* 300–1,750 at a resolution of 70,000 (*m/z* 200), and data-dependent acquisition selected the top 12 most abundant precursor ions for tandem MS by high-energy collisional dissociation fragmentation using an isolation width of 1.2 Da, collision energy of 27, and resolution of 35,000. The run conditions follow the "sensitive" settings recommended by Kelstrup et al. (3) for optimizing the Q Exactive mass spectrometer for low-abundance proteins.

Data Analysis. Raw data files were processed with Proteome Discoverer (version 1.4; Thermo) or Mascot Distiller (version 2.4; Matrix Science, Inc.) before database searching with Mascot Server (version 2.4; Matrix Sciences, Ltd.) against the UniProt index of *Saccharomyces cerevisiae* containing construct sequences of all 20 potential codon 20 mutations of the *LUC* gene. Search parameters included endoproteinase Lys-C specificity with two missed cleavages and the variable modifications of oxidized Met, pyroglutamic acid for N-terminal Gln, N-terminal acetylation of the protein, and a fixed modification for carbamidomethyl Cys. The mass tolerances were 10 ppm for the precursor and 0.05 Da for the fragments. Precursor intensity data of individual peptides

were extracted using Mascot Distiller or Proteome Discoverer before loading into the Scaffold viewer (Proteome Software, Inc.). To calculate the relative abundance of each mutant in the sample, the corresponding precursor intensity of each endo Lys-C peptide containing codon 20 was added to produce a total peptide abundance that was then used to calculate the percentage of contribution of each specific mutant. All charge states observed were taken into account in calculating relative abundances, and the assumption was made that single mutations within a larger peptide did not significantly alter the peptide's ionization potential.

MALDI-TOF/TOF Analysis. Samples digested as described in liquid chromatography-tandem MS (MS/MS) analysis were further purified via a micro–zip-tip cleanup. Briefly, 10-μL volumes were acidified with 1.5 μL of 5% (vol/vol) TFA. Samples were loaded on a μC18 zip-tip (Millipore Corp.) after pre-equilibration in 0.1% TFA. After washing with 2 × 10-μL aliquots of 0.1% TFA, samples were deposited directly onto the MALDI sample target in 1 μL of acetonitrile in 0.1% TFA, followed by the addition of 0.5 μL of matrix solution, which consisted of 5 mg/mL alpha-cyano-4-hydroxycinnamic acid in acetonitrile in 0.1% TFA. Samples were allowed to air-dry before insertion into the mass spectrometer. Analyses were performed on a Shimadzu Biotech Axima TOF^2 (Shimadzu Instruments) MALDI-TOF mass spectrometer. Peptides were analyzed in positive ion reflectron mode. The instrument was externally calibrated using a local spot to the sample of interest with angiotensin II (1,046.54 Da), P14R (1,533.86 Da), and ACTH (18–39) (adrenocorticotropic hormone residues 18–39) (2,465.20 Da). Collisionally induced dissociation (CID) analysis was performed on the same instrument using a dual-timed ion gate for high-resolution precursor selection with a laser power about 20% higher than for MS acquisition. CID fragments were separated in a curved field reflectron, which allowed for a seamless full mass range acquisition of the MS/MS spectrum. All spectra were processed with Mascot Distiller before database searching. Database searches were performed in-house with Mascot Server. For MS searches, the peptide mass fingerprint program was used with a peptide mass tolerance of 100 ppm. For MS/MS searching (CID spectra), the MS/MS ion search program was used with a precursor tolerance of 100 ppm and a fragment tolerance of 1.5 Da.

1. Ghosh S, Ganesan R, Amrani N, Jacobson A (2010) Translational competence of ribosomes released from a premature termination codon is modulated by NMD factors. *RNA* 16(9):1832–1847.
2. Fourati Z, et al. (2014) A highly conserved region essential for NMD in the Upf2 N-terminal domain. *J Mol Biol* 426(22):3689–3702.
3. Kelstrup CD, Young C, Lavallee R, Nielsen ML, Olsen JV (2012) Optimized fast and sensitive acquisition methods for shotgun proteomics on a quadrupole orbitrap mass spectrometer. *J Proteome Res* 11(6):3487–3497.

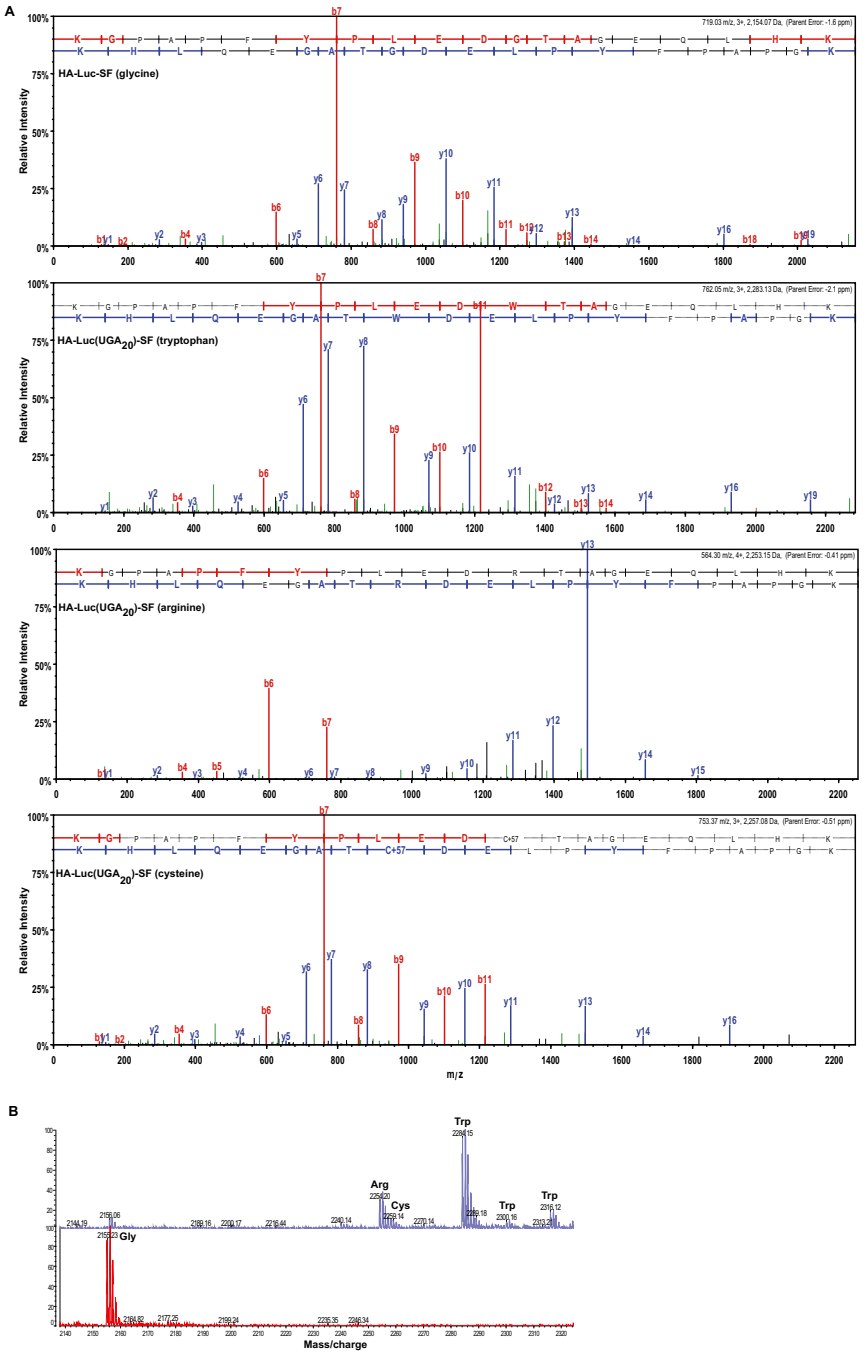

Fig. S1. (*A*) Representative MS/MS spectra generated via high-energy collisional dissociation (HCD) of the endo-LysC peptides showing WT luciferase containing Gly and luciferase readthrough products containing Trp, Arg, and Cys. Fragment ions are designated as either "y," where charge retention is on the C termini, or "b," where charge retention is on the N termini. The mass difference between subsequent fragment ions of the same type is equal to the mass of the amino acid in the sequence. The mutated amino acid is clearly defined by the corresponding mass difference between the y8 and y9 fragment ions in each spectrum. The corresponding precursor mass and charge state with mass error in parts per million are shown in the upper right corner of each panel. (*B*) MALDI-TOF MS spectra showing the relative abundances of the singly charged mutant endo-LysC peptides containing Trp, Arg, and Cys (*Upper*) in place of Gly (*Lower*) in HA-Luc-UGA$_{20}$-SF readthrough products.

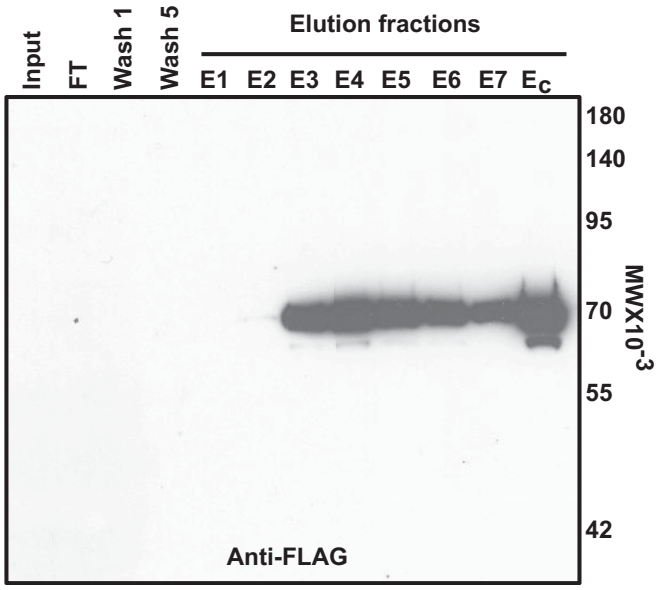

Fig. S2. Strep-Tactin purification of HA-Luc$_{(PTC20)}$-SF products. Western blot analysis was performed using anti-FLAG antibody of the full-length readthrough products purified from *upf1Δ* [*PSI-*] cells expressing HA-*LUC*$_{(UGA20)}$-SF reporter. E_c, pooled (fractions E3–E7) and concentrated elution fractions.

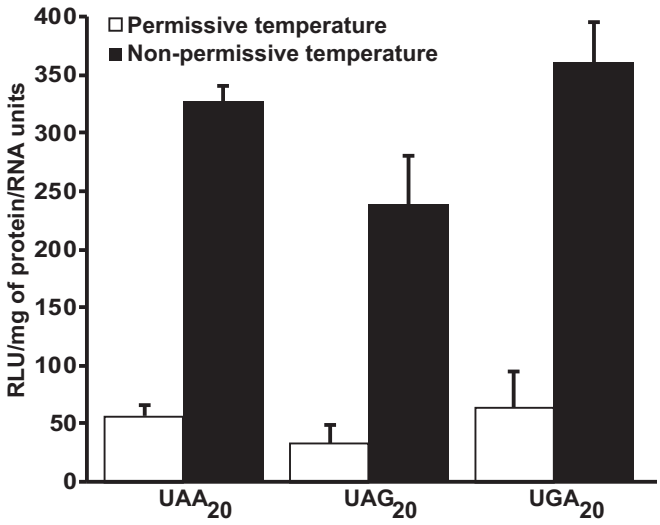

Fig. S3. *sup45-2* [*PSI*$^-$] cells show increased readthrough after temperature shift. Luciferase activity representing the extent of readthrough from *sup45-2* [*PSI*$^-$] cells expressing HA-*LUC*$_{(PTC20)}$-SF reporters before (gray bar) and after (black bar) temperature shift to 37 °C for 30 min is shown. Luciferase activity is expressed as relative luciferase units (RLU) per microgram of protein/RNA units (n = 3; error bars represent SD from the mean).

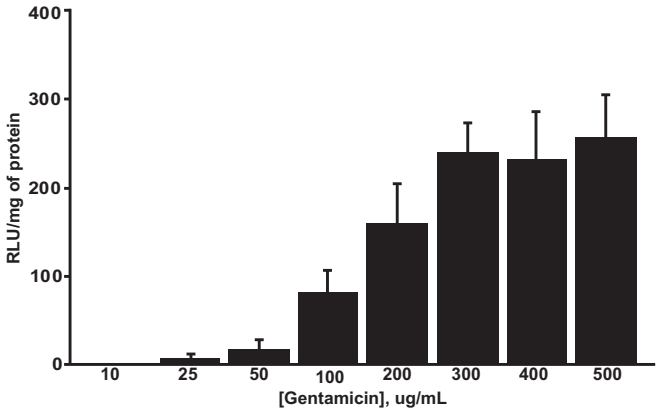

Fig. S4. Readthrough efficiency after gentamicin treatment. Luciferase activity is expressed as RLU per microgram of protein after treatment with varying concentrations of gentamicin (n = 3; error bars represent SD from the mean).

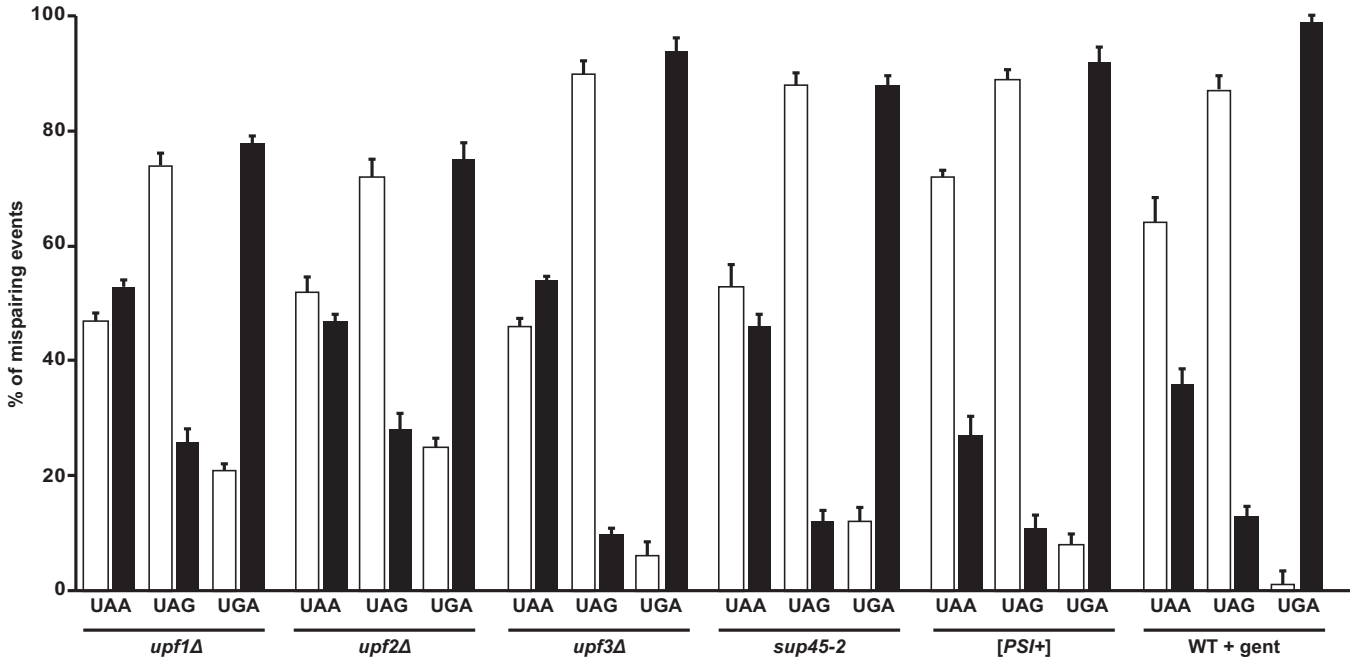

Fig. S5. Readthrough from position 1 and position 3 mispairing. Comparison of position 1 (white bar) or position 3 (black bar) mispairing for each termination codon leading to amino acid insertion at PTCs under multiple conditions.

Table S1. Oligonucleotides used in this study

Oligonucleotide name	Nucleotide sequence (5′–3′)
TPI1 promoter-PstI (For)	AACTGCAGGAGTTATAATAATCCTACGTTAGTGTGAGCGGG
TPI1 promoter-SalI (Rev)	ACGCGTCGACTTTTAGTTTATGTATGTGTTTTTTGTAGTTATAGATTTAAGC
3HA-SalI (For)	GCGCGTCGACAAAATGGGATATCCATATG
3HA-NcoI (Rev)	GCGCCCATGGCGTAATCTGGCACGTC
Luc-NcoI (For)	CCAGCCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCC
Luc-XhoI (Rev)	CTCCGCTCGAGCACGGCGATCTTGCCGCCCTTCTTGGCC
SF-XhoI (For)	GCGCCTCGAGGGCTGGTCCCACCCCCA
SF-XbaI (Rev)	GCGCTCTAGATTATTTGTCATCATC
TPI1 3′UTR-XbaI (For)	GCTCTAGATTAATATAATTATATAAAAATATTATCTTCTTTTCTTTATATCTAGTG
TPI1 3′UTR-EcoRI (Rev)	CGGAATTCTGAGTAACCCATATAGAGATCGTACACATTTTACAAGG
UAA20 (For)	CACTCGAAGACTAAACCGCCGGCG
UAA20 (Rev)	CGCCGGCGGTTTAGTCTTCGAGTG
UAG20 (For)	CACTCGAAGACTAGACCGCCGGCG
UAG20 (Rev)	CGCCGGCGGTCTAGTCTTCGAGTG
UGA20 (For)	CACTCGAAGACTGAACCGCCGGCG
UGA20 (Rev)	CGCCGGCGGTTCAGTCTTCGAGTG

For, forward; Rev, reverse.

Table S2. Possible mispairings at PTCs

UAA				UAG				UGA			
Position of mispairing in codon	Potential decoding tRNA sequence (5′–3′)	AA inserted	BP created	Position of mispairing in codon	Potential decoding tRNA sequence (5′–3′)	AA inserted	BP created	Position of mispairing in codon	Potential decoding tRNA sequence (5′–3′)	AA inserted	BP created
UAA (position 1)	UUU	Lys	U-U	UAG (position 1)	CUU	Lys	U-U	UGA (position 1)	UCU	Arg	U-U
	UUG	Gln	U-G		CUG	Gln	U-G		UCG	Arg†	U-G
	UUC	Glu	U-C		CUC	Glu	U-C		UCC	Gly	U-C
	UUA*		U-A		CUA*		U-A		UCA*		U-A
UAA (position 2)	UUA*		A-U	UAG (position 2)	CUA*		A-U	UGA (position 2)	UUA*		G-U
	UGA	Ser	A-G		CGA	Ser	A-G		UGA	Ser	G-G
	UCA*		A-C		CCA	Trp	A-C		UCA*		G-C
	UAA	Leu	A-A		CAA	Leu	A-A		UAA	Leu	G-A
UAA (position 3)	UUA*		A-U	UAG (position 3)	UUA*		G-U	UGA (position 3)	UCA*		A-U
	GUA	Tyr	A-G		GUA	Tyr	G-G		GCA	Cys	A-G
	CUA*		A-C		CUA*		G-C		CCA	Trp	A-C
	AUA	Tyr†	A-A		AUA	Tyr†	G-A		ACA	Cys†	A-A

Underlining denotes codon positions that are mispaired.
*tRNA unknown in available database.
†tRNA detectable in some organisms but absent in yeast.