Supplementary Information for:

Natural human tRNA^{Ala} anticodon variants mistranslate the genetic code

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SUPPLEMENTARY METHODS

In-solution digest samples were prepared for LC-MS/MS analysis at Bioinformatics Solutions Inc. (Waterloo, Ontario, Canada). Briefly, samples were reduced with 10 mM dithiothreitol (Sigma-Aldrich, Missouri, USA), alkylated with 20 mM iodoacetamide (Sigma-Aldrich, Missouri, USA) and precipitated in acetone at -80 °C. After removing the acetone, the samples were then digested overnight with MS grade trypsin (Promega, Wisconsin, USA). Digested peptides were desalted with in-house made C18 spin columns. The desalted samples were dried down and stored at -20 °C until analysis

Samples were resuspended in 0.1% formic acid prior to MS analysis. For each run, the resuspended sample was separated by nanoflow liquid chromatography using an Ultimate 3000 chromatography system (ThermoFisher, Massachusetts, USA), then injected into the Thermo Orbitrap Fusion Lumos (ThermoFisher, Massachusetts, USA). Liquid chromatography was performed using a constant flow of 0.25 µL/min and a 15 cm reversed-phased column with a 75 µm inner diameter filled with Reprosil C18 (PepSep, Bruker, Germany). Mobile phase A was 0.1% formic acid and Mobile phase B was 99.9% acetonitrile, 0.1% formic acid. The separation was carried out over 120 minutes as follows: linearly 4% B to 35% B over 110 minutes with an increase to 95% B over 0.1 minute and held constant for 4.9 minutes to clean the column. Then the B percentage was set back to 4% in the final 5 minutes.

MS/MS data acquired on Thermo Orbitrap Fusion Lumos for each sample were carried out in data-dependent acquisition mode with a cycle time of three seconds. In the first round, MS1 scan data were obtained at 120,000 resolution (at 400 m/z) with a mass range of 400–1,600 m/z. The automatic gain control (AGC) was set to standard, with an auto maximum ion injection time. The radio frequency (RF) lens was set to 30%. The charge state filter was set to 2-8 and the dynamic exclusion was set to 15 seconds. Isolation for MS2 scans was performed in the quadrupole, with an isolation window of 0.7 Da. MS2 scan data were acquired at a resolution of 15,000 m/z in the orbitrap, with a standard AGC target and an auto ion injection time. The scan range of MS2 was also set to auto. Higher energy collisional dissociation (HCD; fixed normalized collision energy: 30%) was used for generating MS2 spectra, with the number of microscans set to 1.

MS Raw Files were processed using PEAKS 11 (v11.5, Bioinformatics Solutions Inc., Ontario, Canada). The data was searched against a custom database containing the mCherry sequence in

conjunction with the human Uniprot reviewed database. Precursor ion mass error tolerance was set to 10 ppm and fragment ion mass error tolerance was set to 0.02 Da. Semi-specific cleavage with chymotrypsin was selected with a maximum of 2 missed cleavages. A fixed modification of carbamidomethylation (+57.02 Da) on cysteine residues were specified. Variable modifications of deamidation (+0.98 Da) on asparagine and glutamine, as well as oxidation (15.99 Da) on methionine, were specified. The false discovery rate threshold was set to 1% for the database search. Ala misincorporation was identified as a -28.03 Da mass shift from Val, a -30.01 Da mass shift from Thr, or a -58.01 Da mass shift from Glu as indicated. Only confident mutations were reported: at least three major fragment ions (b- or y-ions) must be found consecutively at the mutation site with a minimum relative ion intensity of 2% in each spectrum to ensure confidence in the identity of the mutation. MS Raw files and Search files were deposited into the PRIDE Repository (see Data Availability).

SUPPLEMENTARY TABLES

Table S1. List of oligonucleotides used to construct $tRNA^{Ala}$ variants

tRNA gene	Primer Name	Sequence (5′to 3′)	Used for:
$tRNA^{Ala}_{CGC}$ 1-1	Ala-CGC-1-1-500U-F	AGTTCTTGGTTTCACCTGC	Genomic PCR of ~500 bp up-and downstream of tRNA sequence
	Ala-CGC-1-1-500D-R	CGTGCTGAGCTGAGATTCA	
	Ala-CGC-1-1-300U-F	CAGACT**ACATGT**TTAGTTTCAGCAGTCCGC	PCR to add PciI restriction site to ends of sequence for plasmid ligation
	Ala-CGC-1-1-300D-R	CAGACT**ACATGT**GCCCTAAAGGCAAACCATATA	
	Ala-CGC-1-1-G35T-F	TAGAGCGCATGCTTCTCATGTATGAGG	SOE PCR to make mutant tRNA variant
	Ala-CGC-8-1-G35T-R	ATCGAACCCGGGACCTCATACATGAGAAGC	
$tRNA^{Ala}_{TGC}$ 4-1	Ala-TGC-4-1-500U-F	AACTCTCCTTGGAGGGG	Genomic PCR of ~500 bp up-and downstream of tRNA sequence
	Ala-TGC-4-1-500D-R	GTCTCCAGGACTGGACTATAA	
	Ala-TGC-4-1-300U-F	CAGACT**ACATGT**TATGTCCATACCATTCTCTCGC	PCR to add PciI restriction site to ends of sequence for plasmid ligation
	Ala-TGC-4-1-300D-R	CAGACT**ACATGT**CTGGGAGTATTCGTTTCAAA	
	Ala-TGC-4-1-G35A-F	TCAGTGGTAGAGCGCATGCTTTACACG	SOE PCR to make mutant tRNA variant
	Ala-TGC-4-1-G35A-R	TCATACGTGTAAAGCATGCGCTCTACC	
$tRNA^{Ala}_{AGC}$ 8-1	Ala-AGC-8-1-500U-F	TATCAGCAGCATCTTATCGC	Genomic PCR of ~500 bp up-and downstream of tRNA sequence
	Ala-AGC-8-1-500D-R	CTCTGCCAGGAAACAAGA	
	Ala-AGC-8-1-300U-F	CAGACT**ACATGT**AGAATCTTGCACATGGATGC	PCR to add PciI restriction site to ends of sequence for plasmid ligation
	Ala-AGC-8-1-300D-R	CAGACT**ACATGT**GGGAGGCGGGAAAGATAAA	
	Ala-AGC-8-1-C36T-F	ATGGTAGAGCGCTCGCTTAGTATGCG	SOE PCR to make mutant tRNA variant
	Ala-AGC-8-1-C36T-R	TACCTCTCGCATACTAAGCGAGCGCTC	

SUPPLEMENTARY FIGURES

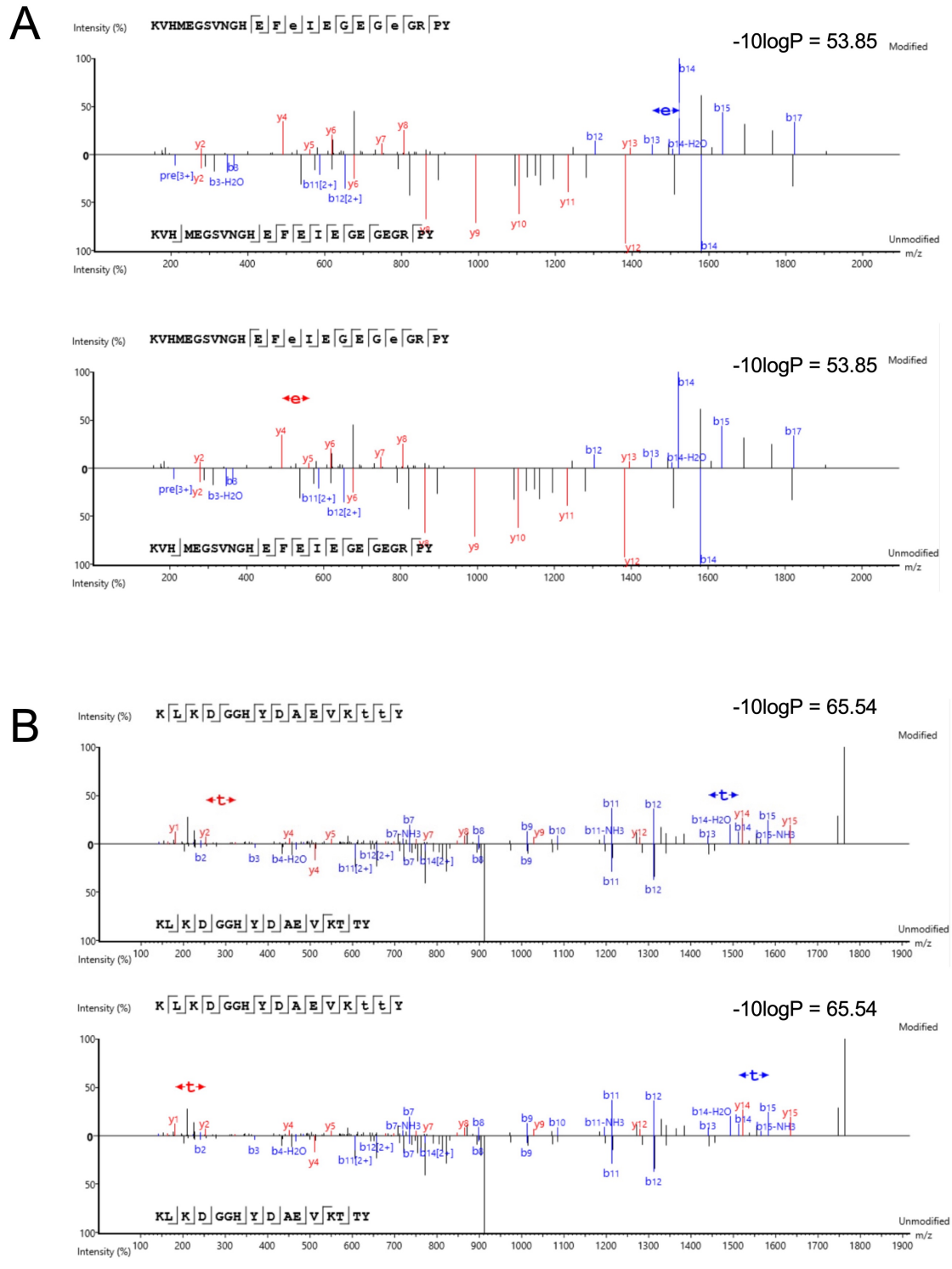

Figure S1. Tandem mass spectra of dual mis-incorporation of Ala in individual mCherry peptides isolated from HEK293T cells. Cells expressing the (A) $tRNA^{Ala}_{CGC}$ G35T mutant had double misincorporation at Glu residues 33 and 39, and (B) $tRNA^{Ala}_{AGC}$ C36T mutant had double misincorporation at Thr residues 184 and 185.

Figure S2. Mistranslation events in mCherry detected by tandem mass spectrometry from HEK293T cells expressing wildtype tRNA$^{Ala}_{CGC}$. Letters within black boxes indicate sites of mistranslation. No alanine misincorporation events were detected.

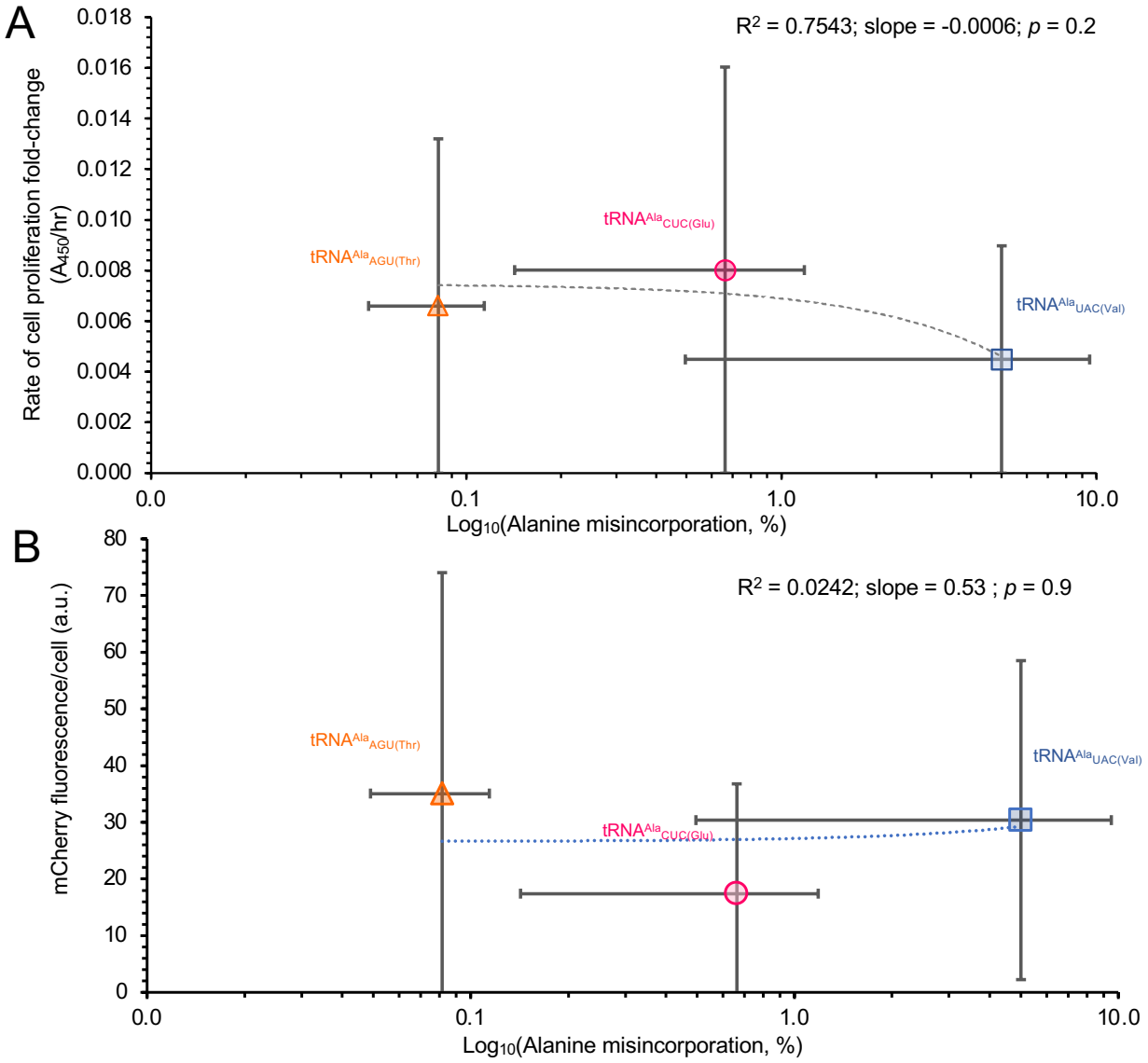

Figure S3. Comparing cell growth and protein production to mistranslation frequency in cells expressing tRNA^{Ala} anticodon variants. (A) Cell proliferation (y-axis) data (Fig. 5) are plotted as a function of the Log_{10} of the mis-incorporation frequency (x-axis) data from Fig. 7. (B) The average mCherry fluorescence per cell (Fig. 3) was also plotted as a function of the Log_{10} of the mis-incorporation frequency. The (A) grey dashed and (B) blue dotted lines represent the lines of best fit between the two indicated variables, which show no significant correlation. Error bars show ±1 standard deviation about the mean of at least n = 3 biological replicates.

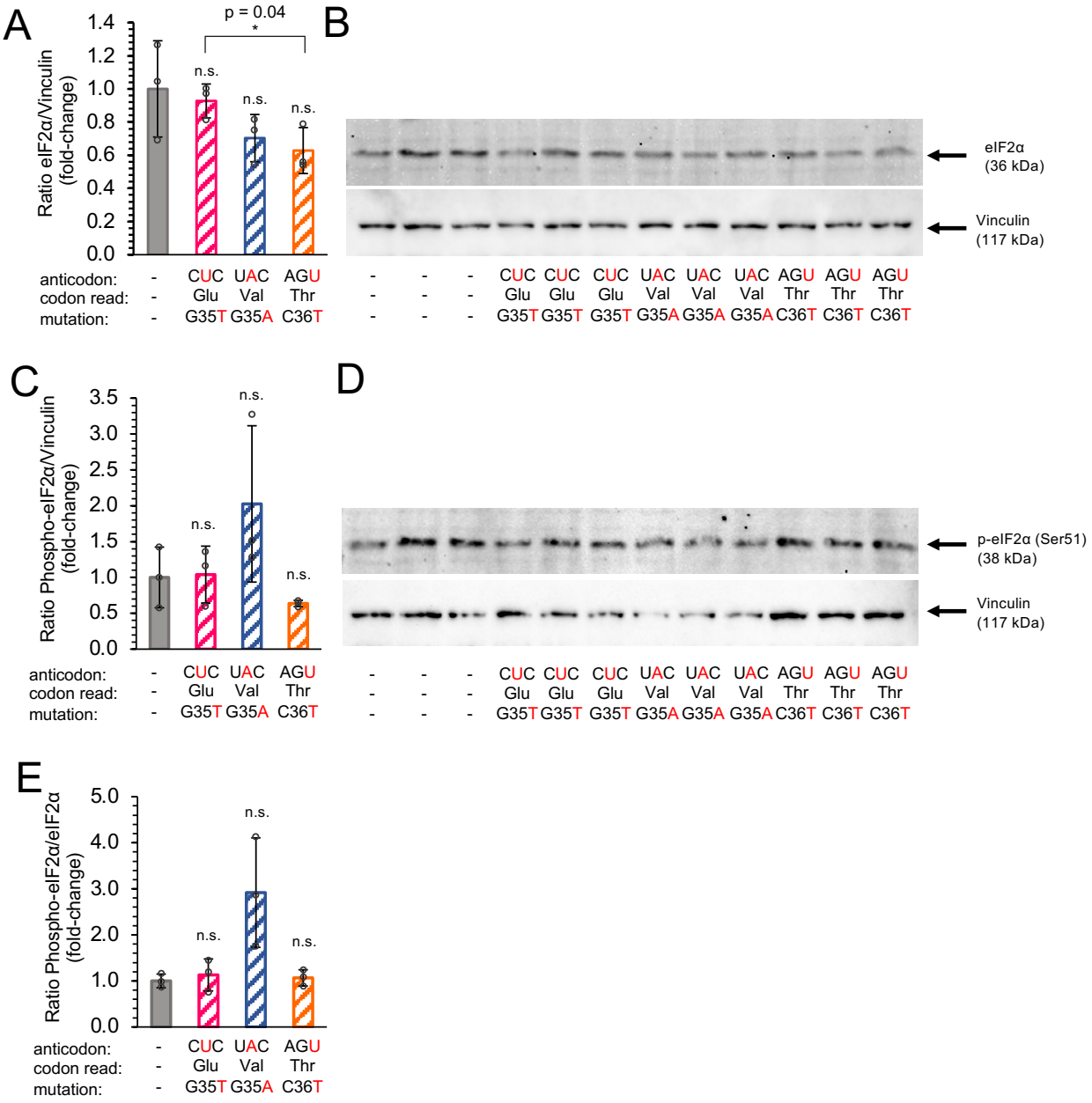

Figure S4. Probing the integrated stress response. In cells transfected with a plasmid lacking an additional tRNA (-) and in cells transfected with each of the indicated $tRNA^{Ala}$ anticodon variants, we used western blotting to measure the levels of (A,B) eIF2α and (C,D) phosphorylated eIF2α (p-eIF2α) relative to a vinculin loading control. (E) The relative phosphorylation level of eIF2α (p-eIF2α/eIF2α) was also plotted. Independent sample t-tests were (n.s. = not significant, * $p < 0.05$) based on at least n = 3 biological replicates and error bars show ±1 standard deviation of the mean.