SUPPLEMENTAL INFORMATION

EXPERIMENTAL PROCEDURES

Construction of yeast strains and plasmids

List of all strains used throughout this study can be found in Table S1.

List of all plasmids and PCR primers used throughout this study can be found in Tables S2 and S3, respectively.

Plasmid PBB123 was created using fusion PCR with pTH335 serving as a template and following combinations of primers: (i) PB105/PB108 and (ii) PB106/PB107. Primers PB105 and PB106 were then used in the third reaction with a 1:1 ratio of PCR products from the first and second reactions as templates. The resulting PCR product was digested with *Xho*I and *Bam*HI and inserted into *Xho*I/*Bam*HI cut pTH335.

Plasmid PBB125 was created using fusion PCR with PBB97 serving as a template and following combinations of primers: (i) PB94/PB110 and (ii) PB95/PB109. Primers PB94 and PB95 were then used in the third reaction with a 1:1 ratio of PCR products from the first and second reactions as templates. The resulting PCR product was digested with *Xho*I and *Bam*HI and inserted into *Xho*I/*Bam*HI cut pTH335.

PBB135 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB115 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB136 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB116 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB137 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB117 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB138 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB118 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB139 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB119 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB140 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB120 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB141 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB121 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB142 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB122 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB143 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB123 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

PBB144 was created by inserting the *Sal*I-*Not*I digested PCR product obtained with primers PB124 and PBRFNotI using pTH477 as template into *Sal*I-*Not*I digested pTH477.

Table S1. Yeast strains used in this study.

Strain	Genotype	Source or reference
74D-694[a]	*MAT*a *ade1-14 trp1-289 his3-Δ200 leu2-3,112 ura3-52*	(1)
L2327[a]	*MAT*a *ade1-14 trp1-289 his3-Δ200 leu2-3,112 ura3-52 sup45-M48I*	(2)
L2521[a]	*MAT*a *ade1-14 trp1-289 his3-Δ200 leu2-3,112 ura3-52 sup45-Y410S*	(2)
PBH140[a]	*MAT*a *ade1-14 trp1-289 his3-Δ200 leu2-3,112 ura3-52 tif35Δ* (YCp22-g/TIF35-screen)	(3)
W303-1a	*MATa ade2 can1-100 his3-11 his3-15 leu2-3 leu2-112 trp1-1 ura3-1*	(4)
H2879	*MATa leu2-3 leu2-112 ura3-52 PRT1*	(5)

[a] indicate isogenic strain background

Table S2. Plasmids used in this study.

Plasmid	Description	Source of reference
YCp22-g/TIF35-screen	single copy wt *TIF35-His* in *TRP1* plasmid from YCplac22	(6)
YCp22-g/TIF35-KLF	single copy *tif35KLF-His* in*TRP1* plasmid from YCplac22	(6)
pTH460	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is CAA-CCGUUC; for read-through measurements) in *URA3* plasmid from YEplac195	(7)
pTH477	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-CCGUUC; for read-through measurements) in *URA3* plasmid from YEplac195	(7)
YEp-R/T-UGAC-L	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-C; for read-through measurements) in *LEU2* plasmid from YEplac181	(8)
YEp-R/T-CAAC-L	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is replaced with CAA-C [coding triplet]; for control read-through measurements) in *LEU2* plasmid from YEplac181	(8)
PBB75	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-A; for read-through measurements) in *LEU2* plasmid from YEplac181	(3)
PBB76	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-G; for read-through measurements) in *LEU2* plasmid from YEplac181	(3)
PBB77	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-U; for read-through measurements) in *LEU2* plasmid from YEplac181	(3)
pTH461	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAA-C; for read-through measurements) in *URA3* plasmid from YEplac195	(7)
pTH469	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAG-C; for read-through	(7)

	measurements) in *URA3* plasmid from YEplac195	
pDB689	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAA-A; for read-through measurements) in *URA3* plasmid from YEplac195	D. Bedwell
pDB725	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAA-G; for read-through measurements) in *URA3* plasmid from YEplac195	D. Bedwell
pDB727	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAA-U; for read-through measurements) in *URA3* plasmid from YEplac195	D. Bedwell
pDB730	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAG-A; for read-through measurements) in *URA3* plasmid from YEplac195	D. Bedwell
pDB731	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAG-G; for read-through measurements) in *URA3* plasmid from YEplac195	D. Bedwell
pDB718	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UAG-U; for read-through measurements) in *URA3* plasmid from YEplac195	D. Bedwell
YEplac195	high copy cloning vector, *URA3*	(9)
pTH335	high copy *URA3* vector (pRS426) containing genomic DNA surrounding the tW(CCA)G1 gene	(3)
PBB97	high copy *tC(GCA)P1* in *URA3* plasmid from pRS426	(3)
PBB99	high copy *tR(UCU)E* in *URA3* plasmid from pRS426	(3)
PBB100	high copy *tG(UCC)O* in *URA3* plasmid from pRS426	(3)
PBB123	high copy *URA3* vector (pRS426) containing genomic DNA surrounding the tW*(CCA)G1 gene	this study
PBB125	high copy *tC*(GCA)P1* in *URA3* plasmid from pRS426	this study
PBB135	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-ACCGAT; for read-through	this study

	measurements) in *URA3* plasmid from YEplac195	
PBB136	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-ATTTAT; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB137	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-AATTTT; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB138	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-CGGTTA; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB139	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is UGA-TTTCAT; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB140	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is GCTTGC-TAA-ACCGAT; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB141	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is ATGTGT-TAA-ATTTAT; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB142	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is TACCCG-TAG-AATTTT; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB143	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is TTTCCG-TAA-CGGTTA; for read-through measurements) in *URA3* plasmid from YEplac195	this study
PBB144	high copy PGK-Renilla-Firefly R/T cassette (stop codon of Renilla is GAACGC-TGA-TTTCAT; for read-through measurements) in *URA3* plasmid from YEplac195	this study

Table S3. Primers used in this study.

Primer name	Primer sequence (5´to 3´)
PBRFNotI	CTCGAAGCGGCCGCTCTAGAATTACAC
PB94	AATAACTCGAGTTGCGTGGATAAGTGTTATTATTCTATTGCC
PB95	AATAAGGATCCAAAGCCGTACAGGCGAACGTATATAATTAAAATTC
PB105	CCCCCCTCGAGATTTTTTACATTTGTTCTATCAG
PB106	CTAGTGGATCCTATAAAAAGAACATATTCATAC
PB107	CAATGGTAGAGCTTTCGATTCCAATTAAATCTTGG
PB108	CCAAGATTTAATTGGAATCGAAAGCTCTACCATTG
PB109	GTGGTAGCGCAGCAGACTGCAAATCTGTTGGTCCTTAG
PB110	CTAAGGACCAACAGATTTGCAGTCTGCTGCGCTACCAC
PB115	CAAATGTCGACGTGCGATTGAACCGATCCGTTCGGATCCTTCAACTTCCCTGAG
PB116	CAAATGTCGACGTGCGATTGAATTTATCCGTTCGGATCCTTCAACTTCCCTGAG
PB117	CAAATGTCGACGTGCGATTGAAATTTTCCGTTCGGATCCTTCAACTTCCCTGAG
PB118	CAAATGTCGACGTGCGATTGACGGTTACCGTTCGGATCCTTCAACTTCCCTGAG
PB119	CAAATGTCGACGTGCGATTGATTTCATCCGTTCGGATCCTTCAACTTCCCTGAG
PB120	CAAATGTCGACGTGCGATGCTTGCTAAACCGATGGATCCTTCAACTTCCCTGAGCTCG
PB121	CAAATGTCGACGTGCGATATGTGTTAAATTTATGGATCCTTCAACTTCCCTGAGCTCG
PB122	CAAATGTCGACGTGCGATTACCCGTAGAATTTTGGATCCTTCAACTTCCCTGAGCTCG
PB123	CAAATGTCGACGTGCGATTTTCCGTAACGGTTAGGATCCTTCAACTTCCCTGAGCTCG
PB124	CAAATGTCGACGTGCGATGAACGCTGATTTCATGGATCCTTCAACTTCCCTGAGCTCG
tRNA-C	AGCTCGCACTCAGGATCGAAC
tRNA-G	TGAGCGGTACGAGAATCGAACC
tRNA-R	CACTCACGATGGGGGTCGAA
tRNA-W	TGAACCTGCAACCCTTCGATTTGGAGTCGAAAGCTCTACC
5.8S rRNA	GCTGCGTTCTTCATCGATGCGAGAACCAA

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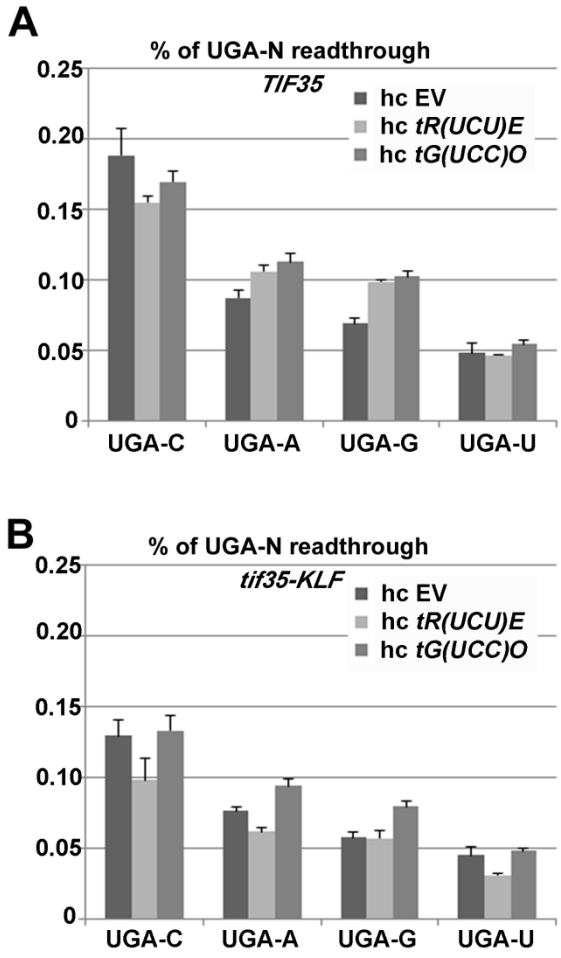
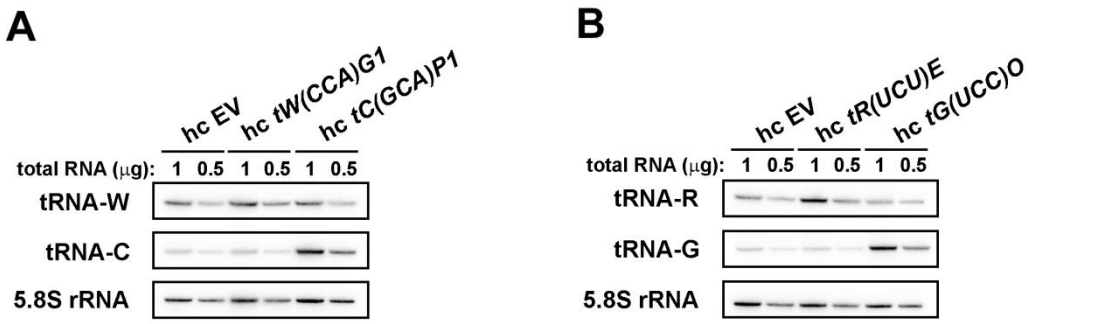


Figure S1. Increased gene dosage of arginine or glycine tRNAs affect readthrough neither in wt (A) nor in mutant *tif35-KLF* (B) cells at any UGA-N tetranucleotide. (**A**) The PBH140 derivative bearing the *TIF35* wt allele was transformed with either empty vector (EV), high copy (hc) tR(UCU)E or hc tG(UCC)O and the resulting transformants were grown and processed for stop codon readthrough measurements as described in Figure 1. (**B**) The PBH140 derivative the bearing *tif35-KLF* mutant allele was transformed with either empty vector (EV), hc tR(UCU)E or hc tG(UCC)O and the resulting transformants were grown and processed for stop codon readthrough measurements as described in Figure 1.

Beznoskova_SuplFig2



C

	fold increase normalized to 5.8S rRNA
tRNA-W	1.45 ± 0.025
tRNA-C	2.78 ± 0.319
tRNA-R	1.88 ± 0.360
tRNA-G	7.00 ± 0.787

Figure S2. Increased gene dosage of the selected nc-tRNAs increases their cellular levels *in vivo*. (**A**) Total RNAs were extracted from the PBH140 strain bearing a plasmid indicated at the top of each panel and 1 μg or 0.5 μg aliquots were loaded onto the Criterion Precast gels and subjected to Northern blotting with ^{32}P-labelled probes shown to the left. (**B**) Quantification of signals shown in panels A and B. Northern blots were quantified using the NIH ImageJ program and the signals were first normalized to the control 5.8S rRNA. The resulting values obtained with cells bearing an empty plasmid „hc EV“ were then set to 1.00 and those obtained with cells bearing a gene for a given nc-tRNA were expressed relative to the „hc EV“. Standard deviations from three individual experiments are given.

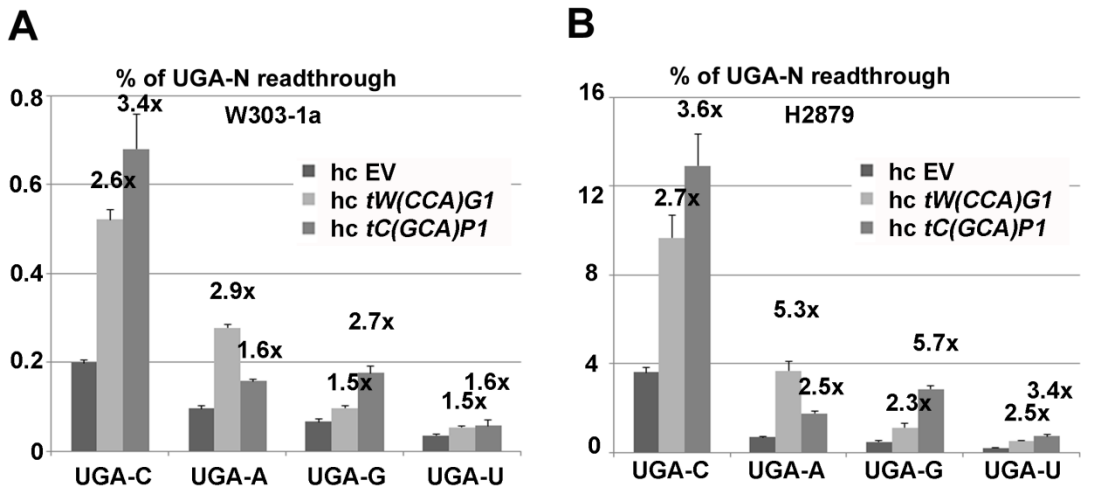


Figure S3. Preferential decoding of UGA-N by tryptophan and cysteine nc-tRNAs in two other genetically unrelated yeast strain backgrounds. Yeast strains W303-1a (**A**) and H2879 (**B**) were transformed with either empty vector (EV), high copy (hc) *tW(CCA)G1* or hc *tC(GCA)P1* and the resulting transformants were grown and processed for stop codon readthrough measurements as described in Figure 1.

% of readthrough (*TIF35*)

	n.t.	paromomycin	
UGA-C	0.217 ± 0.0003	1.168 ± 0.2141	5.4x
UGA-A	0.088 ± 0.0064	0.329 ± 0.0602	3.7x
UGA-G	0.066 ± 0.0029	0.260 ± 0.0102	4.4x
UGA-U	0.032 ± 0.0020	0.182 ± 0 .0429	5.7x

Figure S4. The effect of paromomycin on readthrough at all four UGA tetranucleotides in wild type cells. The PBH140 derivative bearing *TIF35* wt was transformed with empty vector and the resulting transformants were grown in SD without or with 200μg/ml paromomycin for six hours and processed for stop codon readthrough measurements as described in Figure 1; n.t. – non-treated.

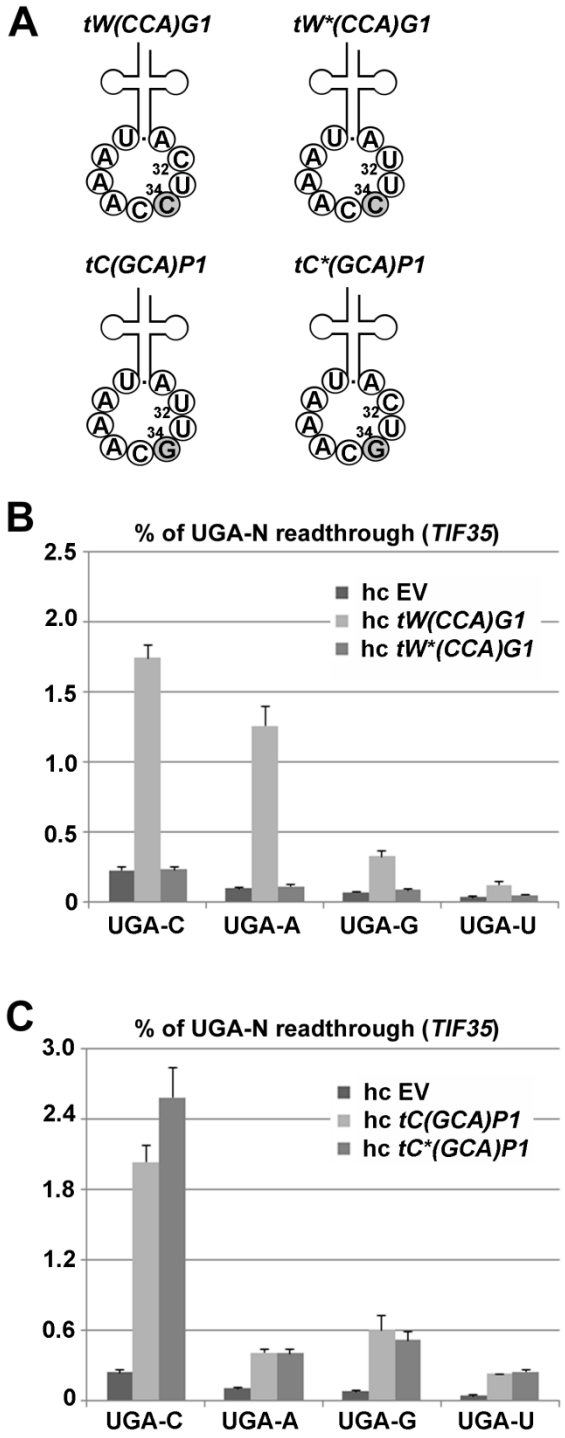
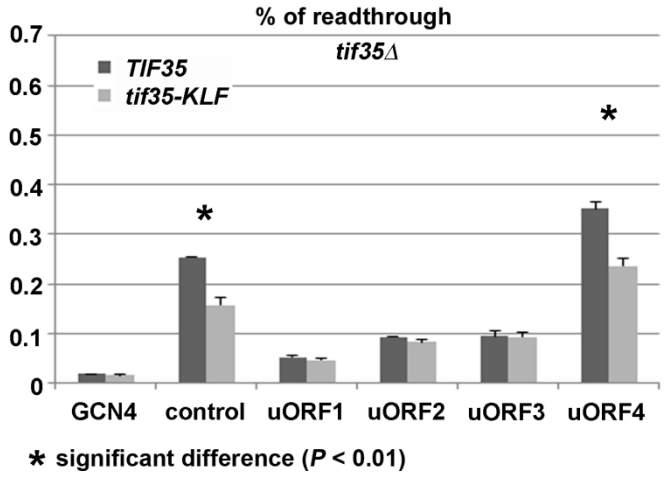


Figure S5. The effect of N_{32} on the nc-tRNA preference for UGA-A or UGA-G tetranucleotides (**A**) Schematics of wt and mutant Trp and Cys nc-tRNAs. Only the nucleotides of the anticodon loop are shown with N_{32} indicated. The only differing base between tryptophan and cysteine tRNA anticodons is highlighted in grey. (**B**) The impact of the C32U substitution in the tryptophan nc-tRNA on its ability to promote readthrough in wt cells. The PBH140 derivative bearing *TIF35* wt was transformed with either empty vector (EV), hc tW(CCA)G1 or mutant hc tW*(CCA)G1 and the resulting transformants were grown in SD and processed for stop codon

readthrough measurements as described in Figure 1. (**C**) The impact of U32C substitution in the cysteine nc-tRNA on its ability to promote readthrough in wt cells. The PBH140 derivative bearing *TIF35* wt was transformed with either empty vector (EV), hc tC(GCA)P1 or mutant hc tC*(GCA)P1 and the resulting transformants were grown in SD and processed for stop codon readthrough measurements as described in Figure 1.

Beznoskova_SuplFig6



* significant difference ($P < 0.01$)

GCN4	GAACGC **UGA** UUUCAU
control	UGCGAU **UGA** CCGUUC
uORF1	GCUUGC **UAA** ACCGAU
uORF2	AUGUGU **UAA** AUUUAU
uORF3	UACCCG **UAG** AAUUUU
uORF4	UUUCCG **UAA** CGGUUA

Figure S6. The genuine 6 nt-long sequences flanking stop codons of uORF1 – 3 from both sides are not subject to eIF3-dependent readthrough. The PBH140 derivatives bearing *TIF35* wt and *tif35-KLF* mutant alleles were grown in SD and processed for stop codon readthrough measurements using standard dual luciferase readthrough reporter constructs pTH460; pTH477; PBB140; PBB141; PBB142; PBB143 and PBB144 as described in Materials and Methods. Changes in the measured readthrough values between *TIF35* and *tif35-KLF* cells were analyzed by the student´s *t*-test (mean±SE; n=6) and shown to be statistically significant only for those cases marked with the asterisk ($P < 0.01$).