

Supplementary Material

Supplementary Figure Legends

Fig. S1. *S. pombe* *pus1Δ* strains are temperature sensitive and lack Ψ in bulk tRNA. (A) *S.*

***pombe pus1Δ* strains are temperature sensitive.** Independent strains of WT and *pus1Δ* cells were grown overnight in YES media at 30°C, and cells were diluted to an OD₆₀₀ of 0.5, serially diluted 10-fold, and spotted for growth analysis at indicated temperatures on EMMC-His and YES media for 4 days (YES) or 8 days (EMMC-His). **(B) Bulk tRNA purified from *S. pombe pus1Δ* strains has reduced Ψ and increased uridine, relative to WT strains.** Strains were grown in triplicate in YES media at 30°C, and bulk canonical-sized tRNA was purified and digested to nucleosides, which were analyzed by HPLC. Levels of nucleosides in WT and *pus1Δ* strains are shown in blue and green, respectively. Standard deviations and statistical significance are indicated.

Fig. S2. *S. pombe pus1Δ* strains are temperature sensitive due to reduced levels of tRNA^{Ile(UAU)}.

(A) *S. pombe pus1Δ* strains have reduced growth at 38.5 °C. *S. pombe* WT and *pus1Δ* strains were grown in biological triplicate in YES media at 30°C and shifted to 38.5°C. Growth was monitored by measuring the OD₆₀₀ every hour. Standard deviations are indicated. **(B) *S. pombe* WT and *pus1Δ* cells are viable after incubation at 38.5°C for 6 hours.** After incubation at 30.0°C or at 38.5°C for 6 hours, cells of independent *S. pombe* WT and *pus1Δ* strains were diluted to OD₆₀₀ of 0.25, serially diluted 10-fold, and spotted for growth analysis at indicated temperatures on YES media for 4 days. **(C) Northern analysis of *S. pombe* WT and *pus1Δ* strains during growth at 30°C, and after shift to 38.5°C.** The Northern analysis in Fig. 2A was continued to show levels of different tRNAs in *S. pombe* WT and *pus1Δ* strains in YES media at 30°C and after shift to 38.5°C. Note that the four hybridizations in Fig. 2A are the same as the first four rows of this extended set of hybridizations with different tRNA probes.

Fig. S3. Quantification of tRNA levels in *S. pombe* WT and *pus1Δ* strains at 30°C and 38.5°C.

The quantitative analysis shown in Fig. 2B was continued to show levels of different tRNAs in *S. pombe* WT **(A)** and *pus1Δ* **(B)** strains in YES media at 30°C and after shift to 38.5°C. The bar chart shows relative levels of tRNA species, in progressively lighter shades of blue and green for levels of tRNAs in WT and *pus1Δ* cells respectively after shift to 38.5°C. Standard deviations are shown.

Fig. S4. Overexpression of tRNA^{Ile(UAU)} in *S. pombe pus1Δ* strains restores tRNA^{Ile(UAU)} levels.

(A) Northern analysis of levels of tRNA^{Ile(UAU)} and control tRNAs in *S. pombe pus1Δ* strains overexpressing tRNA^{Ile(UAU)}. Independent [*tl(UAU) leu2⁺*] or [*leu2⁺*] transformants of *S. pombe* WT and *pus1Δ* strains were grown in EMMC-Leu media at 30°C and shifted to 38°C for 6 hours, and then bulk RNA was analyzed by Northern blotting with probes as shown. **(B) Quantification of tRNA levels in *S. pombe* WT and *pus1Δ* strains overexpressing tRNA^{Ile(UAU)}.**

Fig. S5. Mutations in genes of the rapid tRNA decay pathway lead to restoration of growth in *S.*

***pombe pus1Δ* strains. (A) A reconstructed *pus1Δ dhp1-S737P* strain suppresses the temperature sensitive phenotype of *S. pombe pus1Δ* strains.** A *pus1Δ dhp1-S737P* strain and a WT *dhp1-S737P* strain were constructed as described, and then assessed for growth on media as indicated. **(B) Overexpression of *TOL1* complements the suppression phenotype of spontaneous suppressor *pus1Δ tol1-3*.** Independent [*TOL1⁺ leu2⁺*] or [*leu2⁺*] transformants of *S. pombe* WT, *pus1Δ*, and *pus1Δ tol1-3* strains were grown in EMMC-Leu media at 30°C, and cells were analyzed for growth at indicated temperatures on EMMC-Leu and YES media for 3 days.

Fig. S6. Spontaneous *pus1Δ* suppressors carrying mutations in *dhp1* or *tol1* restore tRNA^{Ile(UAU)}

levels in *S. pombe pus1Δ* strains at 30°C and 38.5°C. (A) Northern blot analysis of the experiment in Fig. 3B, showing partial restoration of tRNA^{Ile(UAU)} levels in *pus1Δ dhp1* and *pus1Δ tol1* strains. (B) Quantification of tRNA^{Cys(GCA)} levels. The quantitative analysis shown in

Fig. 3C was continued to show levels of tRNA^{Cys(GCA)} in *S. pombe* WT and *pus1Δ* strains before and after temperature shift.

Fig. S7. Deletion of *cid14/trf4* of the nuclear surveillance pathway leads to minimal restoration of growth in *S. pombe pus1Δ* strains. Biologically independent *pus1Δ*, *pus1Δ cid14Δ*, and *cid14Δ* strains were cultivated in YES media at 30°C, dilute, and analyzed for growth at indicated temperatures on EMMC-His and YES media for 4 days.

Fig. S8. Several suppressors of the *S. pombe pus1Δ* growth defect have mutations predicted to affect ribosome levels. *S. pombe* WT, *pus1Δ*, and *pus1Δ* suppressor strains with mutations as indicated were grown ON in YES media at 30°C, and then diluted, and spotted as indicated on YES media, YES + 5-FU, EMMC-His, and EMMC-His + 3-AT.

Fig. S9. tRNA^{Ile(UAU)} purified from *S. pombe* WT and *pus1Δ* strains contains Ψ₅₅. Purified tRNA was treated with CMCT and analyzed by primer extension, as described in Materials and Methods, with primer OFS046 (complementary to tRNA^{Ile(UAU)} nt 75-57). The primer extension stop corresponding to Ψ₅₅ is prominent in tRNA^{Ile(UAU)} purified from WT and from *pus1Δ* strains, demonstrating that this modification is not introduced by Pus1.

Figure S10. Quantification of levels of tRNA^{Ile(UAU)}, tRNA^{Ala(AGC)}, unspliced pre-tRNA^{Ile(UAU)}, and unspliced pre-tRNA^{Arg(CCU)} in *S. pombe* WT and *pus1Δ* strains at 30°C and after shift to 38.5°C. Note that the data at 38.5°C is plotted in Fig. 6B and Fig. 6C.

Figure S11. A schematic of the structure of WT tRNA^{Ile(UAU)}, and three variants predicted to have structure compatible with splicing. The schematics indicate the entire predicted pre-tRNA^{Ile(UAU)} structure, as well as that of the designed variants, with the intron sequence one residue after the UAU anticodon (boxed in red). Structures were predicted using the RNAstructure program

(<https://rna.urmc.rochester.edu/RNAstructureWeb/>). Arrows indicate the 5' and 3' splice site, and numbers at the bottom indicate the predicted folding free energy (kcal/mol) of the pre-tRNA. The colors of nucleotides are as in Fig. 7A, corresponding to the probability of each base pair.

Figure S12. A *pus1Δ tl(UAU)i-var2* strain has increased levels of tRNA^{Ile(UAU)} and undergoes little obvious decay of tRNA^{Ile(UAU)}. Quantification of levels of tRNA^{Ile(UAU)}, tRNA^{Ala(AGC)}, and unspliced pre-tRNA^{Ile(UAU)} in *S. pombe* WT and *pus1Δ* strains at 30°C and after temperature shift to 38.5°C. Note that the data at 38.5°C is plotted in Fig. 8B.

Table S1. *S. pombe* strains used in this study

Strain name	Parent strain	Genotype	Source
YFS001		Wild type	(De Zoysa and Phizicky 2020)
YFS044	YFS001	<i>ura4⁺::dhp1⁺</i>	This study
YFS045	YFS001	<i>ura4⁺::dhp1-S737P</i>	This study
YFS009	YFS001	<i>cid14Δ::hygR</i>	(Tasak and Phizicky 2022)
YFS170	YFS001	<i>ura4⁺::SIV</i>	This study
YFS171	YFS001	<i>ura4⁺::tl(UAU)-SIV</i>	This study
YFS172	YFS001	<i>ura4⁺::tl(UAU)-U27C-SIV</i>	This study
YFS173	YFS001	<i>ura4⁺::tl(UAU)-iΔ-SIV</i>	This study
YFS178	YFS171	<i>tl(UAU)Δ::hygR ura4⁺::tl(UAU)-SIV</i>	This study
YFS179	YFS172	<i>tl(UAU)Δ::hygR ura4⁺::tl(UAU)-U27C-SIV</i>	This study
YFS180	YFS173	<i>tl(UAU)Δ::hygR ura4⁺::tl(UAU)-iΔ-SIV</i>	This study
YFS005	YFS001	<i>pus1Δ::hygR</i>	This study
YFS027-2	YFS005	<i>pus1Δ::kanR</i>	This study
YFS027-3/4	YFS001	<i>pus1Δ::kanR</i>	This study
YFS048	YFS005	<i>pus1Δ::hygR ura4⁺::dhp1⁺</i>	This study
YFS049	YFS005	<i>pus1Δ::hygR ura4⁺::dhp1-S737P</i>	This study
YFS050	YFS009	<i>pus1Δ::kanR cid14Δ::hygR</i>	This study
YFS174	YFS027-2	<i>pus1Δ::kanR ura4⁺::SIV</i>	This study
YFS175	YFS027-2	<i>pus1Δ::kanR ura4⁺::tl(UAU)-SIV</i>	This study
YFS176	YFS027-2	<i>pus1Δ::kanR ura4⁺::tl(UAU)-U27C-SIV</i>	This study
YFS177	YFS027-2	<i>pus1Δ::kanR ura4⁺::tl(UAU)-iΔ-SIV</i>	This study
YFS181	YFS175	<i>pus1Δ::kanR tl(UAU)Δ::hygR ura4⁺::tl(UAU)-SIV</i>	This study
YFS182	YFS176	<i>pus1Δ::kanR tl(UAU)Δ::hygR ura4⁺::tl(UAU)-U27C-SIV</i>	This study
YFS183	YFS177	<i>pus1Δ::kanR tl(UAU)Δ::hygR ura4⁺::tl(UAU)-iΔ-SIV</i>	This study
YFS006-1G	YFS005-1A	<i>pus1Δ::hygR rpl1101-N7fs</i>	This study
YFS006-1H	YFS005-1A	<i>pus1Δ::hygR rpl502-R23-R24del</i>	This study
YFS007-1I	YFS005-2A	<i>pus1Δ::hygR tol1-W285L (tol1-4)</i>	This study
YFS007-2I	YFS005-2A	<i>pus1Δ::hygR dhp1-S737P (dhp1-13)</i>	This study
YFS007-4K	YFS005-2A	<i>pus1Δ::hygR rps1901-P31del</i>	This study
YFS007-4P	YFS005-2A	<i>pus1Δ::hygR tol1-C153Y (tol1-3)</i>	This study
YFS008-1H	YFS005-3A	<i>pus1Δ::hygR dbp7-R388X</i>	This study
YFS008-2H	YFS005-3A	<i>pus1Δ::hygR dbp7-L193X</i>	This study
YFS008-4L	YFS005-3A	<i>pus1Δ::hygR rpl1001-F119fs</i>	This study
YSN003-1G	YFS005-1A	<i>pus1Δ::hygR dhp1-dup(Y368-G373) (dhp1-16)</i>	This study
YSN004-2F	YFS005-2A	<i>pus1Δ::hygR dhp1-W623L (dhp1-14)</i>	This study
YSN005-2G	YFS005-3A	<i>pus1Δ::hygR dhp1-A107T (dhp1-15)</i>	This study

Table S2. Plasmids used in this study

Plasmid	Parent	Description	Source
pREP3x		<i>leu2⁺</i> P _{nmt1}	(Forsburg 1993)
EFS10	pREP3x	<i>leu2⁺</i> <i>S. pombe</i> tI(UAU)	This study
EEM64		<i>ura4⁺</i> single integrating vector (SIV)	(Vjestica et al. 2020)
EEM79-1	EEM64	<i>ura4⁺</i> (SIV) <i>S. pombe</i> tI(UAU)-WT	This study
EEM80-1	EEM64	<i>ura4⁺</i> (SIV) <i>S. pombe</i> tI(UAU)-U27C	This study
EEM81-1	EEM64	<i>ura4⁺</i> (SIV) <i>S. pombe</i> tI(UAU)-iΔ	This study

Table S3. Oligomers used for northern analysis of *S. pombe* strains

Name	Target tRNA	Probe 5'-3'	Sequence
AH480	tL(AAG)	57-38	CACGaGCTTTCGCACTAGTG
AH481	tL(UAA)	57-38	CGCGTTTTACAACAGCAGGG
OFS003	tI(UAU)	74-60	GTGCTCCCGGCGAGG
OFS008	tR(ACG)	57-38	CGAACCTGGAATCTCTTGAT
OFS009	tI(AAU)	75-59	GGTGGTCACAGCCGGGT
OFS059	tMe(CAU)	75-59	TGGTGCTCCTGGTGAGGC
OFS060	tT(AGU)	57-38	TGAACACTGGGCCTCTTGCTT
OFS061	tW(CCA)	76-59	TGGTGACCCCTAAGTGAC
OFS086	tK(CUU)	76-59	TGGCTCCCAAGGCGAGAC
OFS087	tK(UUU)	76-59	TGGCTCCCACTGCGAGAC
OFS105	tI(UAU)	44-i11	CCACGTaacacacctcctaag
OFS118	tR(CCU)	i6-i24	ctccccaacaacgatcagg
LH735	tL(UAA)	73-54	TGCGGCCAGAGAGGTTTCGAA
OMT530	tF(GAA)	58-37	TCGAACCGATGACCAACAGATC
OMT602	tI(CAU)	57-38	CGATCCTGGGACCTACGGGT
OMT623	tA(AGC)	57-39	CGAATCTGGGACCTCTTCC
OMT624	tT(AGU)	19-1	ACCACTGAGCTACAAGAGC
OMT625	tG(GCC)	76-59	TGGTGCTTTGGCCGGGAA
OMT626	tY(GUA)	57-39	CGAACTAGCGACCAACCGG
OMT627	tP(AGG)	76-59	TGGGGGCTGTTGTGGGAA
ONV16	tL(UAG)	69-51	GGGACTCGAACCCGCGAG
TDZ128	tY(GUA)	73-52	TCTCCTGAGCCAGAATCGAACT
TDZ184	tC(GCA)	22-1	CTAACCACTGAGCTATGACCCC

Table S4. Oligomers used for tRNA purifications

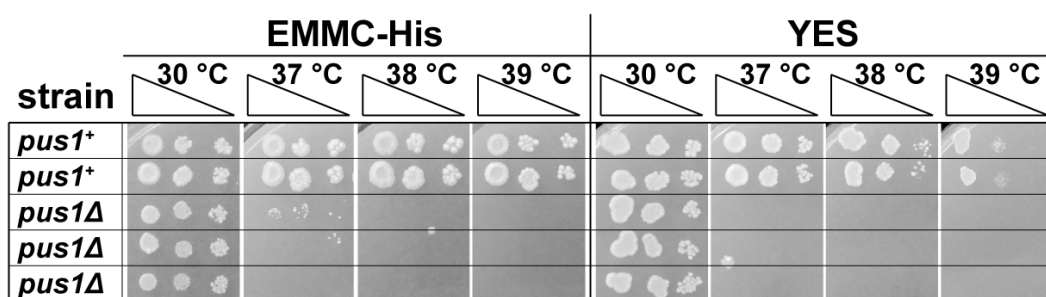
Name	Target tRNA	Probe 5'-3'	Sequence
OFS012	<i>S. pombe</i> tI(UAU)	76-60	biotin-TGGTGCTCCCGGCGAGG
OFS080	<i>S. pombe</i> tK(CUU)	76-59	biotin-TGGCTCCCAAGGCGAGAC

Table S5. Oligomers used for CMCT assays

Name	Target tRNA	Probe 5'- 3'	Sequence
OFS046	<i>S. pombe</i> tI(UAU)	75-57	GGTGCTCCCGGCGAGGATC
OFS049	<i>S. pombe</i> tI(UAU)	57-39	CGAACTCGCGATTTCCACG

Figure S1

A



B

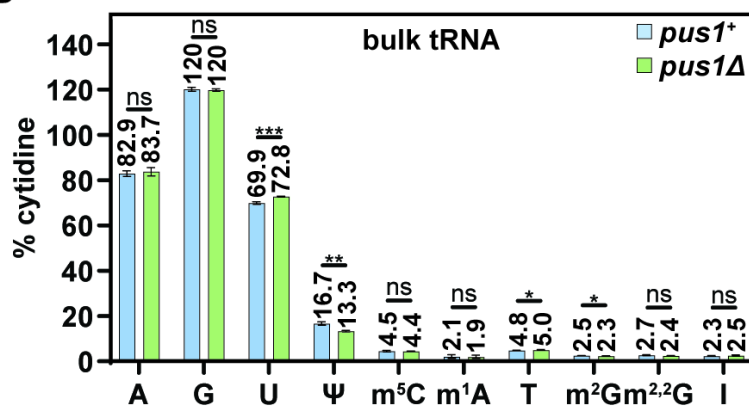
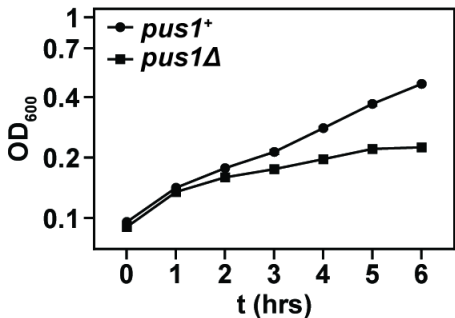
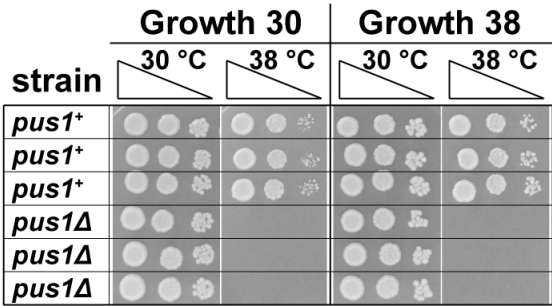


Figure S2

A



B



C

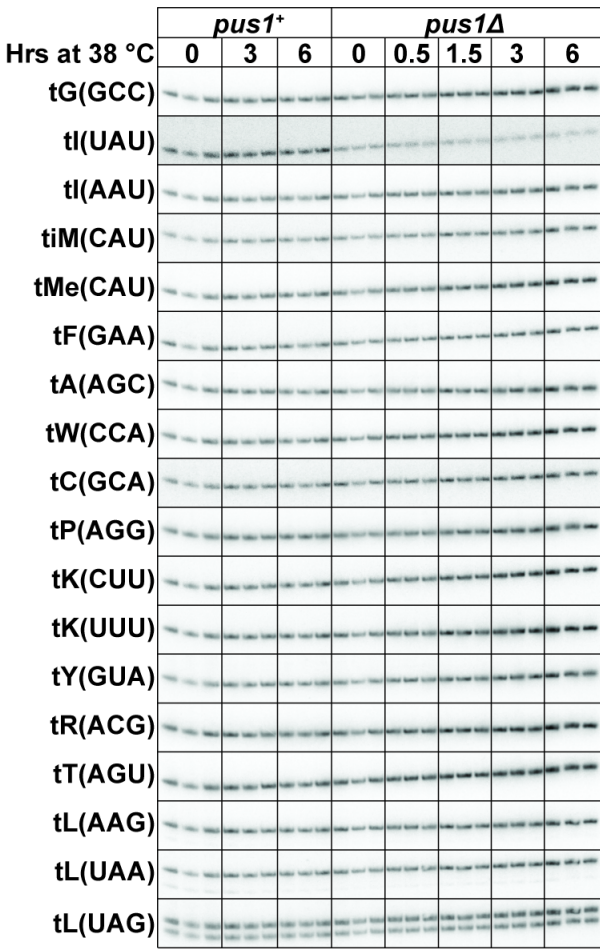
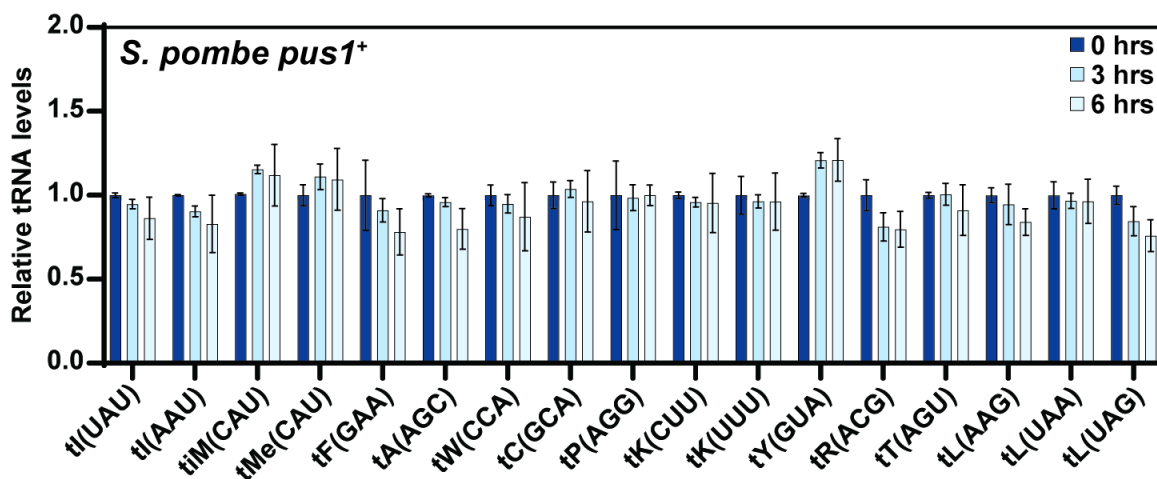


Figure S3

A



B

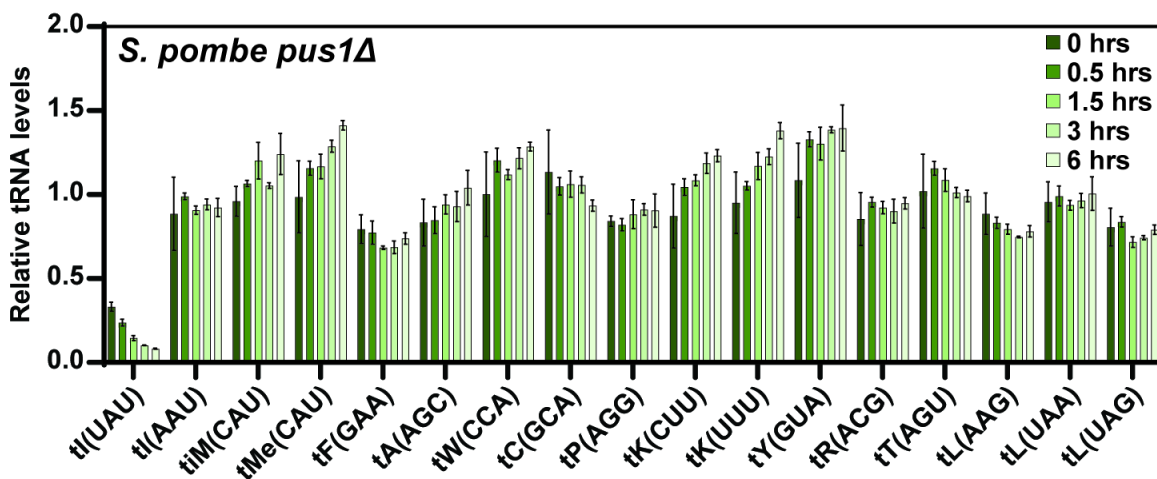
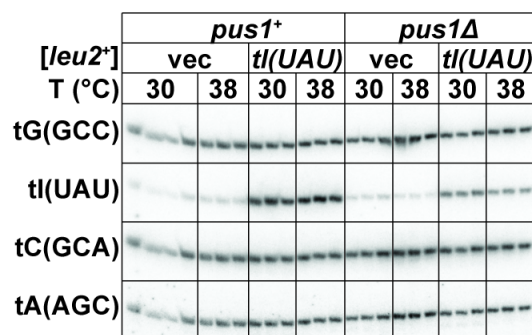


Figure S4

A



B

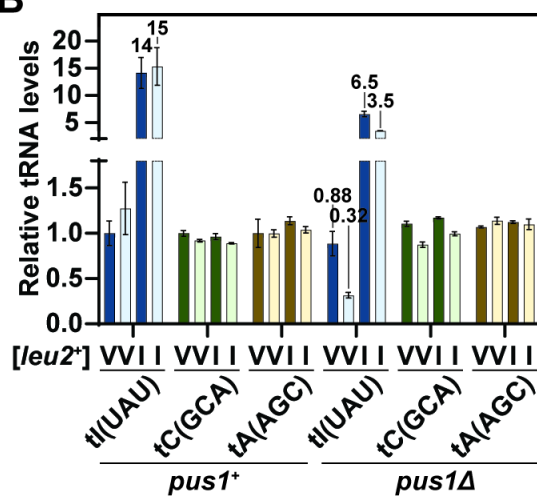


Figure S5

A

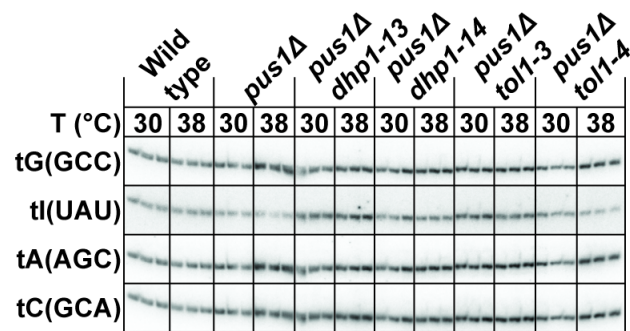
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	33 °C	37 °C	38 °C	39 °C	33 °C	37 °C	38 °C	39 °C
<i>pus1⁺ dhp1⁺</i>								
<i>pus1⁺ dhp1⁺</i>								
<i>pus1⁺ dhp1-S737P</i>								
<i>pus1⁺ dhp1-S737P</i>								
<i>pus1Δ dhp1⁺</i>								
<i>pus1Δ dhp1⁺</i>								
<i>pus1Δ dhp1-S737P</i>								
<i>pus1Δ dhp1-S737P</i>								

B

strain	[<i>leu2⁺</i>]	EMMC-Leu			YES		
		33 °C	37 °C	39 °C	33 °C	37 °C	39 °C
<i>pus1⁺</i>	vector						
<i>pus1⁺</i>	vector						
<i>pus1⁺</i>	<i>TOL1</i>						
<i>pus1⁺</i>	<i>TOL1</i>						
<i>pus1Δ</i>	vector						
<i>pus1Δ</i>	vector						
<i>pus1Δ</i>	<i>TOL1</i>						
<i>pus1Δ</i>	<i>TOL1</i>						
<i>pus1Δ tol1-3</i>	vector						
<i>pus1Δ tol1-3</i>	vector						
<i>pus1Δ tol1-3</i>	<i>TOL1</i>						
<i>pus1Δ tol1-3</i>	<i>TOL1</i>						

Figure S6

A



B

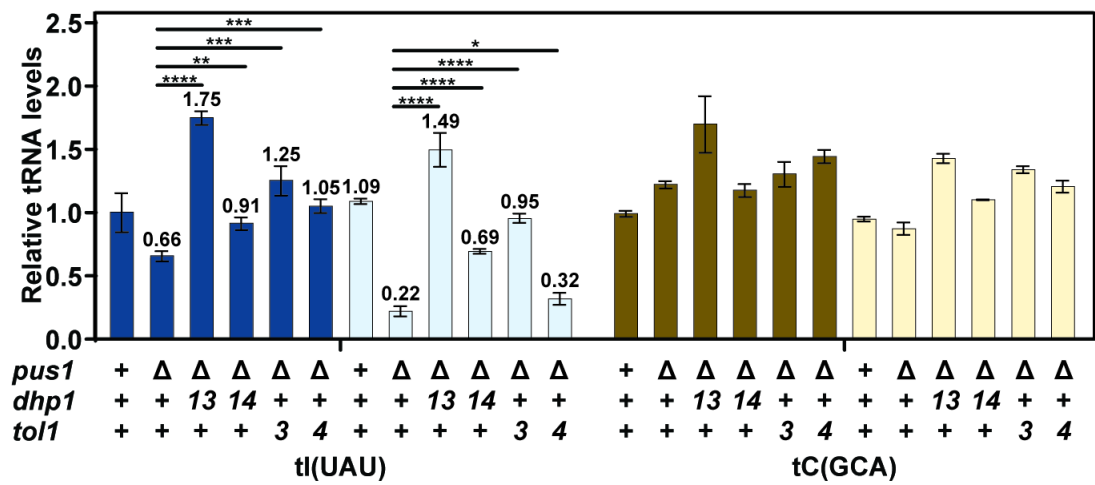


Figure S7

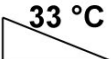
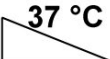
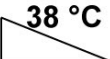
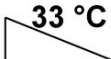
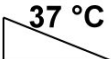
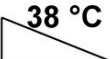
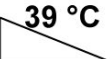
strain	EMMC-His				YES			
	33 °C	37 °C	38 °C	39 °C	33 °C	37 °C	38 °C	39 °C
								
<i>pus1⁺ cid14⁺</i>								
<i>pus1⁺ cid14⁺</i>								
<i>pus1Δ cid14⁺</i>								
<i>pus1Δ cid14⁺</i>								
<i>pus1⁺ cid14Δ</i>								
<i>pus1⁺ cid14Δ</i>								
<i>pus1Δ cid14Δ</i>								
<i>pus1Δ cid14Δ</i>								

Figure S8

	EMMC-His		EMMC -His + 3-AT	YES			YES + 5-FU
	37 °C	38 °C	37 °C	33 °C	37 °C	38 °C	37 °C
<i>pus1</i> ⁺							
<i>pus1</i> Δ							
<i>pus1</i> Δ <i>tol1-3</i>							
<i>pus1</i> Δ <i>dhp1-13</i>							
<i>pus1</i> Δ <i>dhp1-14</i>							
<i>pus1</i> Δ <i>dhp1-15</i>							
<i>pus1</i> Δ <i>rpl1101-N7fs</i>							
<i>pus1</i> Δ <i>rpl502-R23,24Δ</i>							
<i>pus1</i> Δ <i>rpl1001-F119fs</i>							
<i>pus1</i> Δ <i>rps1901-P31Δ</i>							
<i>pus1</i> Δ <i>dbp7-L193*</i>							
<i>pus1</i> Δ <i>dbp7-R388*</i>							

Figure S9

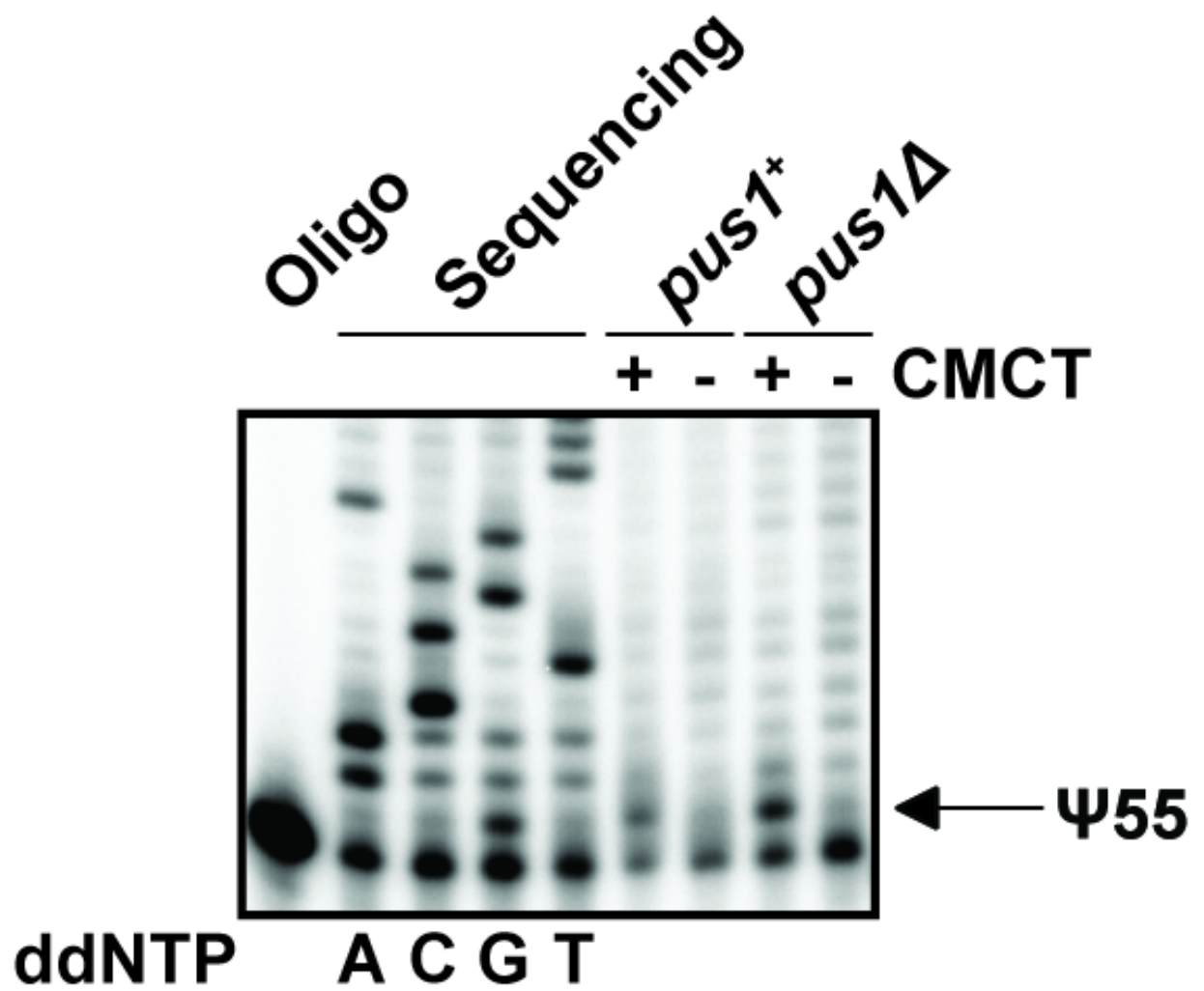
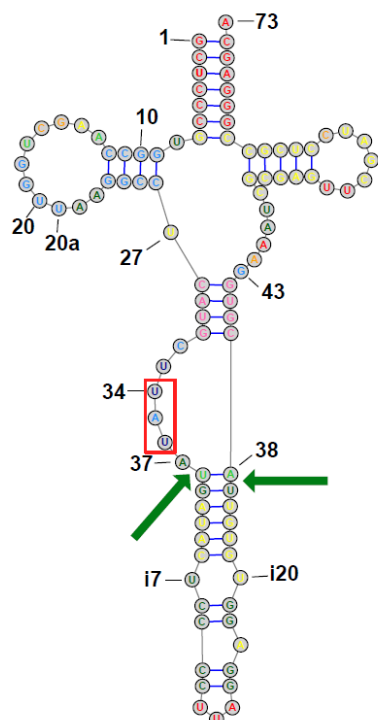


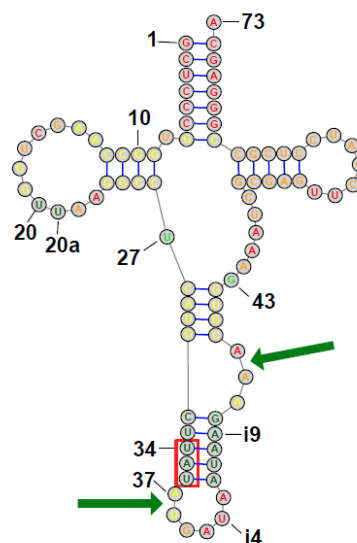
Figure S10

Strain	tl(UAU) variant	Temp.	tl(UAU)	STD	tA(AGC)	STD	pre-tl(UAU)	STD	pre-tr(CCU)	STD
WT	tl(UAU)-WT	30°C	1.00	0.14	1.00	0.16	1.00	0.03	1.00	0.08
WT	tl(UAU)-U27C	30°C	0.56	0.07	0.96	0.10	0.48	0.09	0.90	0.05
WT	tl(UAU)-iΔ	30°C	0.85	0.04	1.00	0.04	0.05	0.04	0.69	0.04
<i>pus1Δ</i>	tl(UAU)-WT	30°C	0.63	0.03	1.06	0.08	2.42	0.28	0.64	0.05
<i>pus1Δ</i>	tl(UAU)-U27C	30°C	0.44	0.04	0.85	0.06	0.66	0.02	0.85	0.20
<i>pus1Δ</i>	tl(UAU)-iΔ	30°C	0.73	0.09	0.84	0.06	0.00	0.06	0.76	0.18
WT	tl(UAU)-WT	38.5°C	1.01	0.10	0.80	0.06	0.85	0.14	0.35	0.02
WT	tl(UAU)-U27C	38.5°C	0.87	0.06	1.14	0.17	0.74	0.07	0.42	0.07
WT	tl(UAU)-iΔ	38.5°C	1.30	0.22	1.10	0.14	-0.04	0.05	0.56	0.10
<i>pus1Δ</i>	tl(UAU)-WT	38.5°C	0.27	0.03	1.14	0.11	4.29	0.54	0.69	0.10
<i>pus1Δ</i>	tl(UAU)-U27C	38.5°C	0.60	0.11	0.89	0.11	0.64	0.03	0.55	0.05
<i>pus1Δ</i>	tl(UAU)-iΔ	38.5°C	0.99	0.27	1.12	0.17	-0.05	0.03	0.64	0.19

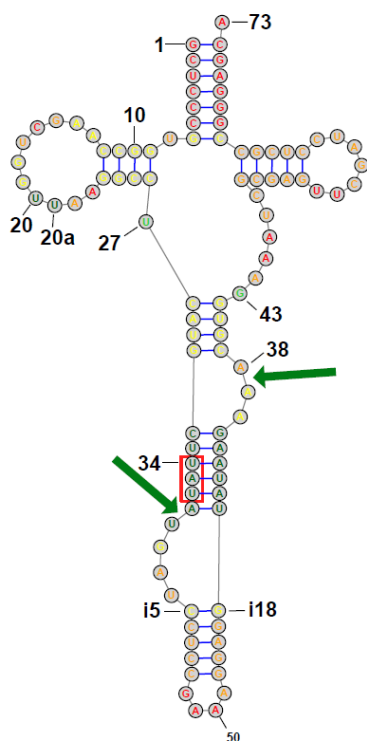
Figure S11



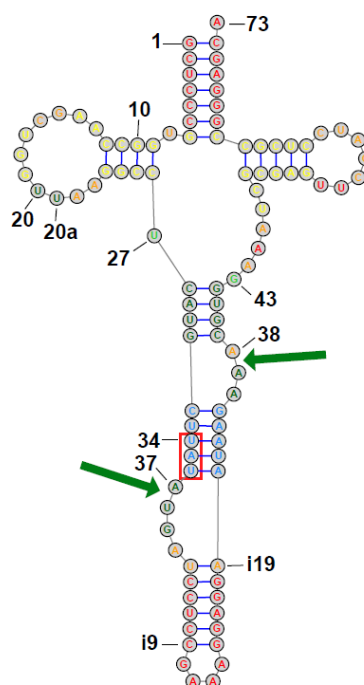
WT (-36.7 kcal/mol)



i-var1 (-31.2 kcal/mol)



i-var2 (-41.1 kcal/mol)



i-var3 (-41.7 kcal/mol)

Figure S12

Strain	tl(UAU) variant	Temp.	tl(UAU)	STD	tA(AGC)	STD	pre-tl(UAU)	STD
WT	tl(UAU)-WT	30°C	1.00	0.05	1.00	0.05	1.00	0.03
WT	tl(UAU)i-var2	30°C	0.85	0.08	0.96	0.01	0.00	0.03
<i>pus1Δ</i>	tl(UAU)-WT	30°C	0.69	0.02	1.01	0.08	1.47	0.16
<i>pus1Δ</i>	tl(UAU)i-var2	30°C	0.88	0.01	1.03	0.06	-0.06	0.04
WT	tl(UAU)-WT	38.5°C	1.08	0.05	0.97	0.07	1.00	0.03
WT	tl(UAU)i-var2	38.5°C	0.76	0.08	0.89	0.08	0.00	0.09
<i>pus1Δ</i>	tl(UAU)-WT	38.5°C	0.21	0.01	1.07	0.03	2.66	0.23
<i>pus1Δ</i>	tl(UAU)i-var2	38.5°C	0.46	0.01	0.98	0.05	0.03	0.07
<i>pus1Δ</i>	tl(UAU)-U27C	38.5°C	0.48	0.03	0.92	0.04	0.51	0.14
<i>pus1Δ</i>	tl(UAU)-iΔ	38.5°C	0.69	0.02	0.92	0.08	-0.12	0.06