Supplemental Material

Mammalian nuclear TRUB1, mitochondrial TRUB2 and cytoplasmic PUS10 produce conserved pseudouridine 55 in different sets of tRNA

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METHODS

PUS1, PUS2 and tRNA Ψ31 synthase activities for use as controls for the tRNA Ψ55 synthase activities in the extracts of TRUB1, TRUB2 and PUS10 knockdown strains

Although TRUB1 and TRUB2 are located in the nucleus and mitochondria, respectively (Rintala-Dempsey and Kothe 2017), both can produce Ψ27 in tRNAs. To determine this activity, we treated [α-^{32}P]UTP-labeled tRNA^{Phe} with the extracts and quantitated the amount of labeled Ψp after RNase T2 digestion of the product, separation by 2D-TLC and phosphorimaging. In these digests, a labeled modified or unmodified U would be derived from the first of the two consecutive U's present in the tRNA. Human tRNA^{Phe} contains UU sequences at three positions: 16-17, 27-28 and 54-55 (Supplemental Fig. S1). Dihydrouridine (D), Ψ and T are present at positions 16, 27 and 54 respectively, in the native human tRNA^{Phe} (Juhling et al. 2009). Dp sometimes merges with and is indistinguishable from Up in the TLC separations. Generally, Tp is not produced due to the lack of methyl-donor SAM in the extracts. Previously, by treating the U27A mutant of tRNA^{Phe} with total cell extracts, we confirmed that the Ψ from [α-^{32}P]UTP-labeled tRNA^{Phe} is derived from U27 (Deogharia et al. 2019). No Ψ was produced in that case. In the experiments here, we observe labeled Ψp only with the nuclear and mitochondrial extracts, which would be the products of Ψ27-synthase activities of PUS1 and PUS2, respectively (Supplemental Fig. S2A). Although, human cytoplasmic PUS10 can convert U54 to a Ψ in

certain tRNAs, this is not the case here, because it does not produce Ψ54 in $tRNA^{Phe}$ (Deogharia et al. 2019). This is again confirmed here by the lack of Ψ after treatment with cytoplasmic extract (Supplemental Fig. S2A, panel at the bottom). The Ψ27 synthase activities of both PUS1 and PUS2 remain nearly the same in the nuclear and mitochondrial extracts of the knockdown strains when compared with their activities with the extracts of the wild type (Supplemental Fig. S2A). Therefore, Ψ55 synthase activities in the nuclear and mitochondrial extracts of the knockdown strains are measured relative to their Ψ27 synthase activities as shown in Fig. 2C.

So far, $tRNA^{Met}$ is the only known cytoplasmic tRNA of eukaryotes that contains Ψ31 (Juhling et al. 2009). Pus6 is reported to produce Ψ31 in the cytoplasmic and mitochondrial tRNAs of yeast and it is not present in the nucleus (Ansmant et al. 2001). PUSD1 is suggested to do the same modification in human tRNAs (Spenkuch et al. 2014). Therefore, we used tRNA Ψ31 synthase activity as a control for cytoplasmic extracts of the knockdown strains. Since U31 of $tRNA^{Met}$ is followed by a C32, we used [α-^{32}P]CTP-labeled $tRNA^{Met}$ to determine the Ψ31 synthase activity of the extracts. However, this tRNA contains UC at four positions: 27-28, 31-32, 39-40 and 55-56 (Supplemental Fig. S1). Therefore, we used two mutant versions of this tRNA. In one case, three U's, i.e., at positions 27, 39 and 55 were changed to C, C and A, respectively (referred as Mut-$tRNA^{Met}$ in Supplemental Fig. S2B), thus leaving UC only at position 31-32, and in the other case, U31 was also changed to a C (referred as U31C mut). PCR products of two oligonucleotides that overlapped at their 3' ends, were used to prepare transcripts of these two tRNAs. Treatment of the three U's mutant $tRNA^{Met}$ (Mut-$tRNA^{Met}$) showed labeled Ψp only with the cytoplasmic extracts, but this was absent when the four U's mutant tRNA was used (Supplemental Fig. S2B). This suggested that the Ψp observed here is derived from the position 31. Mitochondrial and nuclear extracts did not show any Ψp (bottom two panels in

Supplemental Fig. S2B). Apparently our $tRNA^{Met}$ transcript is not a substrate for the human mitochondrial Ψ31 synthase activity. Sequence of human mitochondrial $tRNA^{Met}$ is very different from the cytoplasmic $tRNA^{Met}$ and mammalian mitochondrial $tRNA^{Met}$ genes contain G not T at position 31 (Juhling et al. 2009). Ψ31 synthase activity remains nearly the same in the cytoplasmic extracts of the knockdown strains when compared with their activities in the wild type (Supplemental Fig. S2B). Therefore, Ψ55 synthase activities in the cytoplasmic extracts of the knockdown strains are measured relative to their Ψ31 synthase activity as shown in Fig. 2C.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Sequences of tRNA transcripts used in this study. The original A-U pair at position 1-72 of the $tRNA^{iMet}$ was changed to G-C for the ease of transcription. Relevant Ψ modifications observed in native mammalian tRNAs are shown in parentheses. Mutations of residues in $tRNA^{Ala}$, $tRNA^{iMet}$, $tRNA^{AU\text{-}Ala}$ and $tRNA^{Met}$ used in this work are indicated.

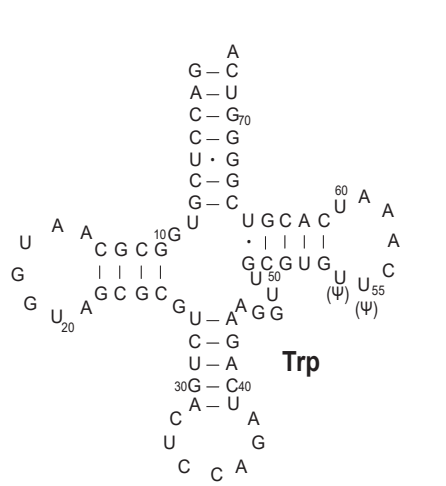
Supplemental Figure S2. PUS1, PUS2 and tRNA Ψ31 synthase activities of the extracts. (A) [α-^{32}P]UTP-labeled transcripts of $tRNA^{Phe}$ were incubated with nuclear (NE) and mitochondrial (ME) extracts of WT and TRUB1, TRUB2, PUS10 knockdown strains of PC3 cells as well as with extracts where all the three proteins were depleted (All 3-KD). They were analyzed as in Fig. 1. Ψ in all cases is derived from positions 27 of the tRNAs. Unrelated luciferase knockdown (siLuc) is a control showing there is no affect in Ψ synthesis due to the transfection itself. Cytoplasmic extract (CE, bottom panel) showed no Ψ27 synthesis. (B) [α-^{32}P]CTP-labeled transcripts of Mut-$tRNA^{Met}$ (U27C, U39C and U55A mutations-containing $tRNA^{Met}$) were incubated with cytoplasmic (CE) extracts of WT and TRUB1, TRUB2, PUS10 and luciferase

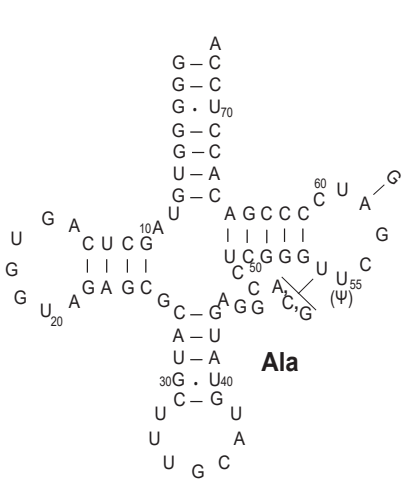
knockdown strains of PC3 cells as well as with extracts when all the three proteins were depleted (All 3-KD). They were analyzed as in (*A*). Absence of Ψ in U31C mutant of this tRNA (WT+U31C) indicates that Ψ in all cases is derived from positions 31 of these tRNAs. Nuclear and mitochondrial extracts (bottom two panel) showed no Ψ31 synthesis.

Supplemental Figure S3. Immunoblots of sub-cellular fractions. (A) Purity of nuclear (NE) and total cytoplasmic (TCE) fractions of HEK293T cells transiently transfected with His-tagged human PUS10 clone. Blots were probed with antibodies against lamin A (ab26300, Abcam) and tubulin (ab6046, Abcam), which were used as nuclear and cytoplasmic markers, respectively. (B) Recombinant proteins isolated from nuclear (nh-PUS10) and total cytoplasmic (ch-PUS10) fractions shown in (*A*) are indeed His-tagged PUS10 as determined by anti-PUS10 (HPA049582, Sigma) and anti-His (2365, Cell Signal Technology) antibodies. (C) Purity of nuclear (NE), cytoplasmic (CE) and mitochondrial (ME) fractions of PC3 cells were determined by anti-lamin A, anti-tubulin and anti-porin (ab15895, Abcam) antibodies as nuclear, cytoplasmic and mitochondrial markers, respectively. (D) Purity of nuclear (NE) and total cytoplasmic (TCE) fractions of mouse liver cells were determined by using anti-lamin A and anti-tubulin antibodies as nuclear and cytoplasmic markers, respectively.

Supplemental Figure S4. Determination of protein knock down. (A) Immunoblot analyses of cell lysates of PC3 cells after performing knockdowns (KD) for TRUB1, PUS10 and TRUB2 showed a decreased amount of these proteins in the cells. Lysates of normal PC3 (WT) and siLuc transfected PC3 cells were used as controls. Blots were probed with antibodies against TRUB1 (PA5-58163, Invitrogen), PUS10 (HPA049582, Sigma), TRUB2 (sc-514573, Santa Cruz

Biotechnology) and β-actin (ab8227, Abcam). β-actin was used as the loading control. The blots were normalized with respect to β-actin and a fold change was calculated with respect to the WT signal. The fold changes are indicated below each lane in the blot. (B) qPCR was used to compare the amount of PUS10, TRUB1 and TRUB2 mRNA in PC3 cells after performing knockdowns (KD) for the proteins. WT and siLuc transfected PC3 cells were again used as a control. Results are normalized with respect to HPRT mRNA. Values are the mean ± S.E. (n=3).


Trp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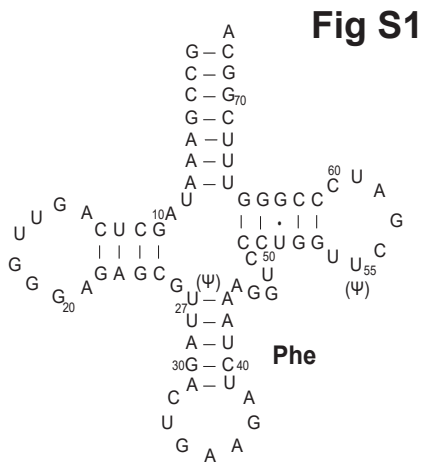
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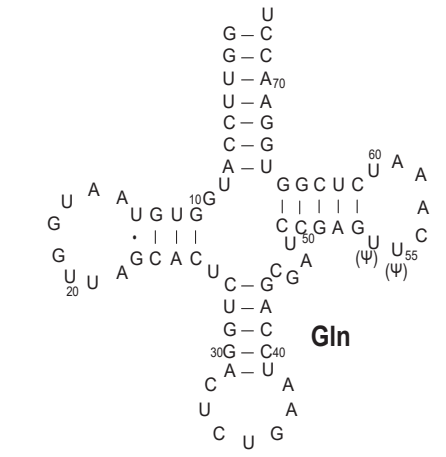
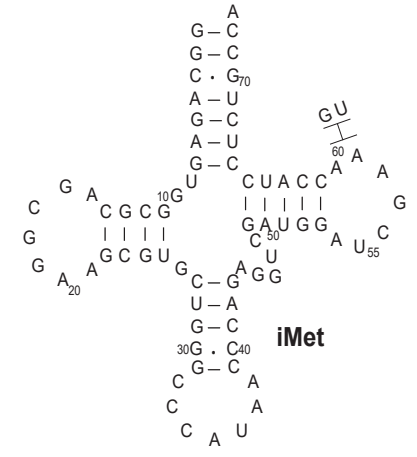
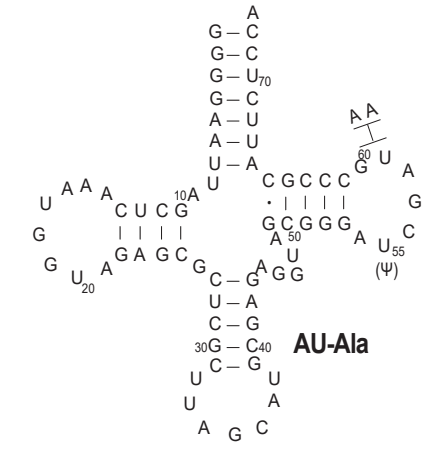

Gln


iMet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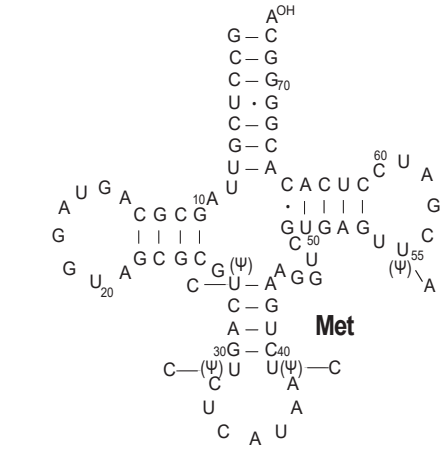

Met

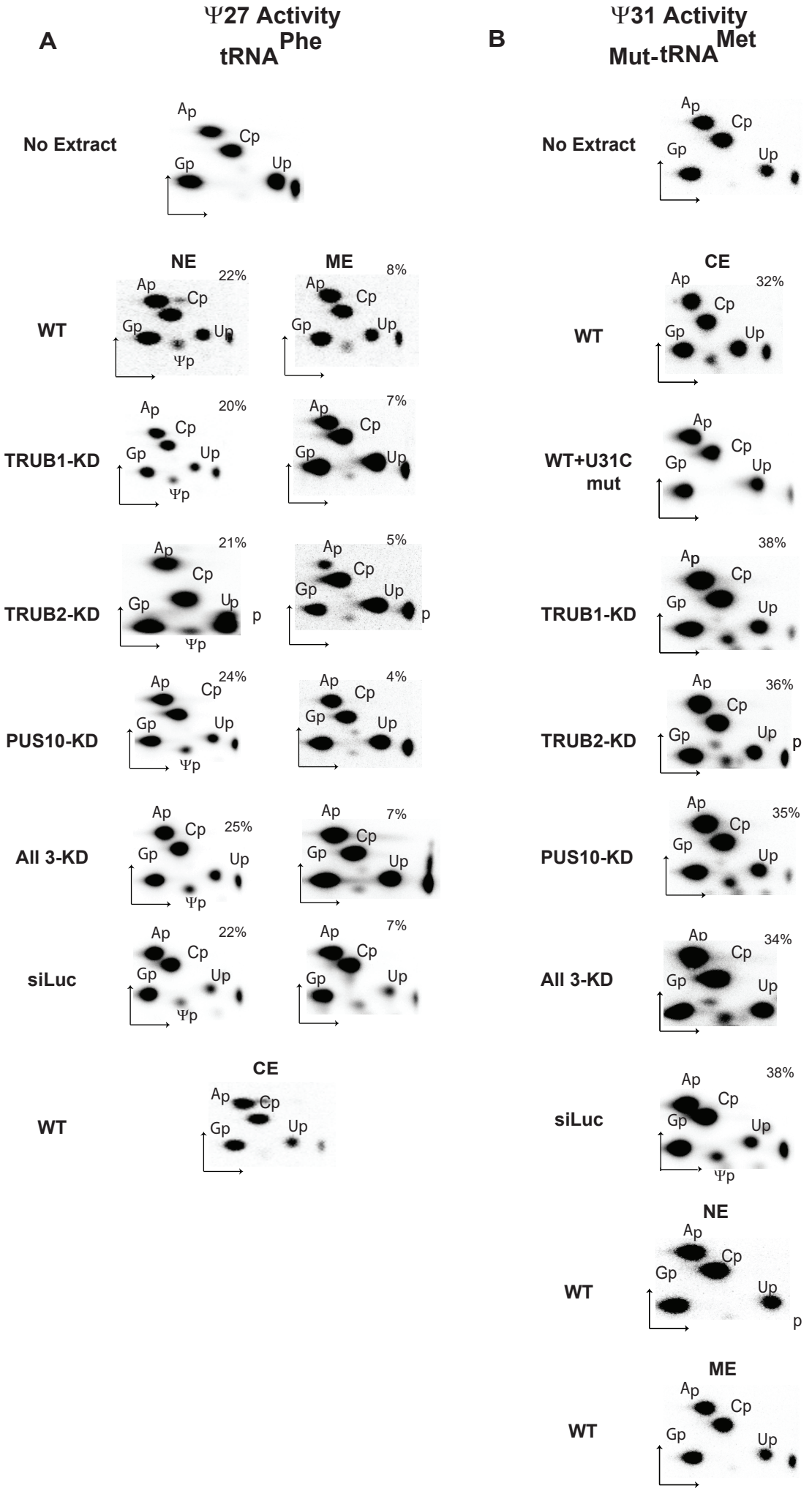

A
Ψ27 Activity
tRNA Phe
No Extract
NE
ME
WT
22%
8%
TRUB1-KD
20%
7%
TRUB2-KD
21%
5%
PUS10-KD
24%
4%
All 3-KD
25%
7%
siLuc
22%
7%
CE
WT
B
Ψ31 Activity
Mut-tRNA Met
No Extract
CE
WT
32%
WT+U31C mut
TRUB1-KD
38%
TRUB2-KD
36%
PUS10-KD
35%
All 3-KD
34%
siLuc
38%
NE
WT
ME
WT
Ap
Cp
Gp
Up
Ψp
p

Figure S3

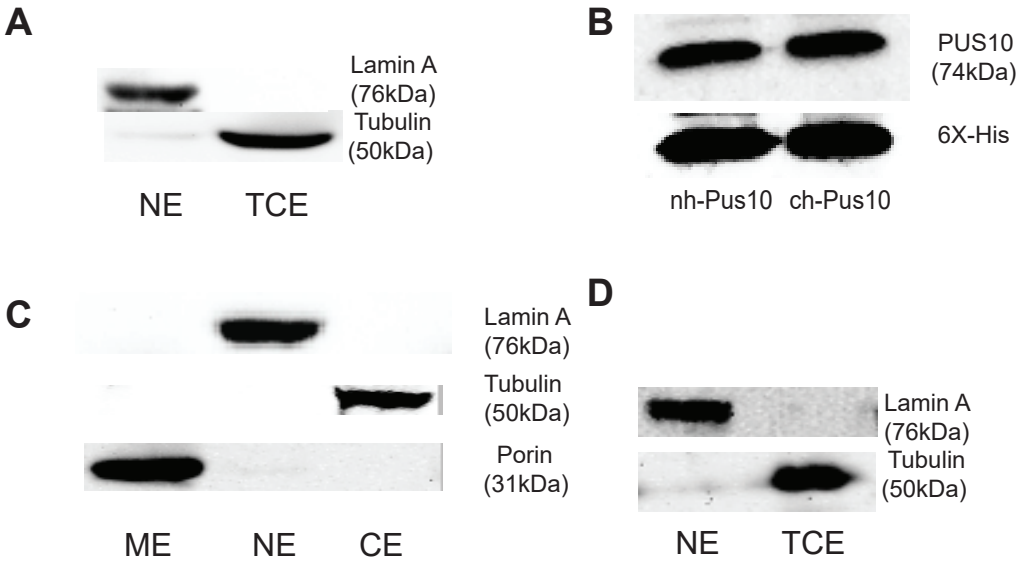
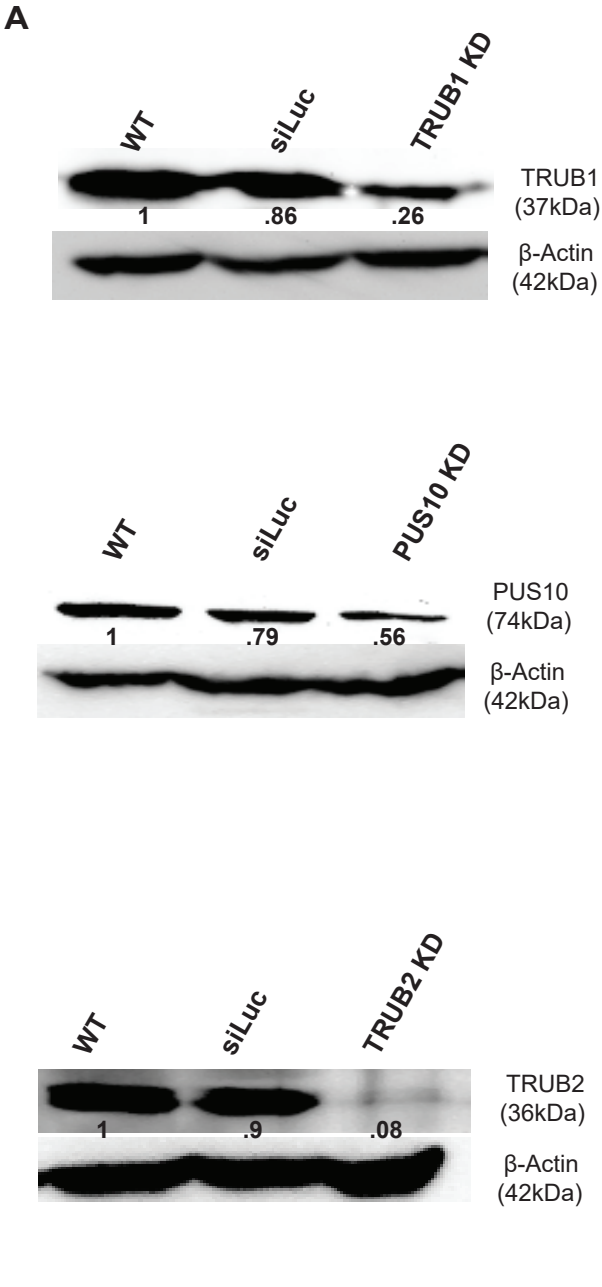
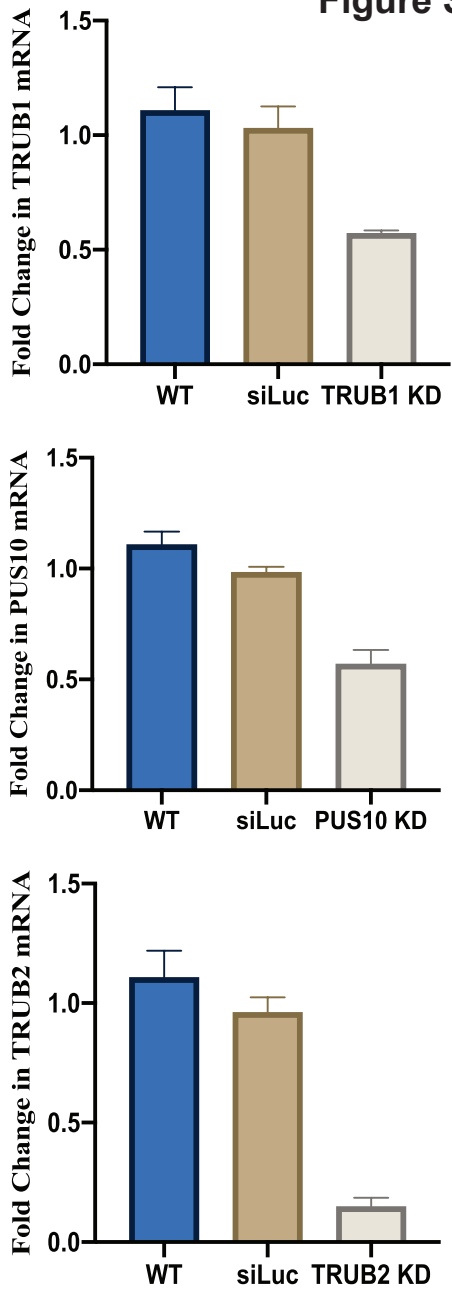

A
Lamin A
(76kDa)
Tubulin
(50kDa)
NE
TCE
B
PUS10
(74kDa)
6X-His
nh-Pus10
ch-Pus10
C
Lamin A
(76kDa)
Tubulin
(50kDa)
Porin
(31kDa)
ME
NE
CE
D
Lamin A
(76kDa)
Tubulin
(50kDa)
NE
TCE

Figure S4


A
WT
siLuc
TRUB1 KD
TRUB1
(37kDa)
1
.86
.26
β-Actin
(42kDa)
WT
siLuc
PUS10 KD
PUS10
(74kDa)
1
.79
.56
β-Actin
(42kDa)
WT
siLuc
TRUB2 KD
TRUB2
(36kDa)
1
.9
.08
β-Actin
(42kDa)


B
Fold Change in TRUB1 mRNA
1.5
1.0
0.5
0.0
WT
siLuc
TRUB1 KD
Fold Change in PUS10 mRNA
1.5
1.0
0.5
0.0
WT
siLuc
PUS10 KD
Fold Change in TRUB2 mRNA
1.5
1.0
0.5
0.0
WT
siLuc
TRUB2 KD

Supplemental Table 1: p values

NE	Fig 2C			
	Trp		Ala	
	p value	Summary	p value	Summary
WT vs siLuc	>0.9999	ns	0.8126	ns
WT vs TRUB1 KD	0.0049	**	0.0286	*
WT vs TRUB2 KD	0.4062	ns	0.9999	ns
WT vs PUS10 KD	0.3853	ns	0.9221	ns
WT vs B1+B2+P10	<0.0001	****	0.0022	**
CE	Trp		Ala	
	p value	Summary	p value	Summary
WT vs siLuc	0.7423	ns	0.9654	ns
WT vs TRUB1 KD	0.9898	ns	0.9996	ns
WT vs TRUB2 KD	0.9998	ns	0.2509	ns
WT vs PUS10 KD	<0.0001	****	0.0474	*
WT vs B1+B2+P10	<.0001	****	0.0002	***
ME	Trp		Ala	
	p value	Summary	p value	Summary
WT vs siLuc	>0.9999	ns	0.9813	ns
WT vs TRUB1 KD	>0.9999	ns	0.9924	ns
WT vs TRUB2 KD	0.0001	***	0.0002	***
WT vs PUS10 KD	0.984	ns	>0.9999	ns
WT vs B1+B2+P10	0.0002	***	0.001	**

+TRUB1 vs h-PUS10+TRUB1	Fig 6A	
	p value	Summary
Trp	0.002	**
Ala	0.818	ns
iMet	0.02	*
AU-Ala	>0.9999	ns

	Fig 6C			
	Trp		Ala	
	p value	Summary	p value	Summary
NE vs ch-PUS10+NE	0.009	**	0.013	*
NE vs nh-PUS10+NE	0.0056	**	>0.9999	ns
CE vs ch-PUS10+CE	0.1689	ns	0.0409	*
CE vs nh-PUS10+CE	0.027	*	0.9999	ns

*p<0.05,**p<0.01,***p<0.001 and ****p<0.0001, ns = not significant.

Supplemental Table S2: List of oligonucleotides used in this work and their functions

Oligonucleotide	Sequence	Purpose
Oligonucleotides used for primer extensions		
MUTRP3R	TGG TGA CCC CGA CGT	CMCT-primer extension of Trp tRNA
MUGLN3R	TGG AGG TCC CAC CGA	CMCT-primer extension of Gln tRNA
MUALA3R	TGG AGG TGT CGG GGA	CMCT-primer extension of Ala tRNA
MUPHE3R	TGG TGC CGA AAC CCG	CMCT-primer extension of Phe tRNA
MUiMET3R	TGG TAG CAG AGG ATG	CMCT-primer extension of iMet tRNA
MUAU-ALA3R	TGG TGG AGA ATG CGG	CMCT-primer extension of AU-Ala tRNA
Oligonucleotides used to generate templates for in vitro tRNA transcription		
T7HUTRP5F	TAA TAC GAC TCA CTA TAG GTT CCA TGG TGT AAT GGT TAG C	To generate templates to transcribe Trp tRNA
HUTRP-3R	TGA CCC CGA CGT GAT TTG AAC	
T7HUALA5F	TAA TAC GAC TCA CTA TAG GGG GTG TAG CTC AGT GG	To generate templates to transcribe Ala tRNA

HUALA-3R	TGG AGG TGT CGG GGA TCG AAC CCG AGG	
HUALA-3R2	TGG AGG TGT CGG GGA CCG AAC CCG AGG	To generate A58G mutation in Ala tRNA
HUALA-3R3	TGG AGG TGT CGG GGA TCG AGC CCG AGG	To generate U54C mutation in Ala tRNA
HUALA-3R4	TGG AGG TGT CGG GGA TCG ACC CCG AGG	To generate U54G mutation in Ala tRNA
HUALA-3R5	TGG AGG TGT CGG GGA TCG ATC CCG AGG	To generate U54A mutation in Ala tRNA
T7HU-iMET5F	TAA TAC GAC TCA CTA TAG GCA GAG TGG CGC AGC GGA AGC GTG CTG GGC CC	To generate templates to transcribe iMet tRNA
HU-iMET-3R	TGG CAG AGG ATG GTT TCG ATC CAT CGA CCT CTG GGT TAT GGG CCC AGC ACG CTT CC	
HUiMET-3R2	TGG CAG AGG ATG GCA TCG ATC CAT CGA CCT CTG GGT TAT GGG CCC AGC ACG CTT CC	To generate AA59-60UG mutation in iMet tRNA
T7HUAU-ALA5F	TAA TAC GAC TCA CTA TAG GGG AAT TAG CTC AAA TGG TAG AGC GCT CGC TTA G	To generate templates to transcribe AU-Ala tRNA
HUAU-ALA-3R	TGG AGA ATG CGG GCA TCG ATC CCG CTA CCT CTC GCA TGC TAA GCG AGC GCT CTA CC	
HUAU-ALA-3R2	TGG AGA ATG CGG GTT TCG ATC CCG CTA CCT CTC GCA TGC TAA GCG AGC GCT CTA CC	To generate UG59-60AA mutation in AU-Ala tRNA
T7HUPHE5F	TAA TAC GAC TCA CTA TAG CCG AAA TAG CTC AGT TGG G	To generate templates to transcribe Phe tRNA

HUPHE-3R	TGC CGA AAC CCG GGA TCG AAC	
T7HUMUT-MET-5F	TAA TAC GAC TCA CTA TAG CCT CGT TAG CGC AGT AGG TAG CGC GCC AGT CTC	To generate templates to transcribe elongator Met tRNA with U27C,U55A and U39C change
HUMUT-MET-3R	TGC CCC GTG TGA GGA TCG TAC TCA CGA CCT TCA GGT TAT GAG ACT GGC GCG CTA CC	
HUMUT-MET-3R2	TGC CCC GTG TGA GGA TCG TAC TCA CGA CCT TCA GGT TAT GAG GCT GGC GCG CTA CC	To generate U27C,U55A, U39C and U31C mutation in elongator Met tRNA
T7HUGLN5F	TAA TAC GAC TCA CTA TAG GTT CCA TGG TGT AAT GGT TAG C	To generate templates to transcribe Gln tRNA
HUGLN-3R	AGG TTC CAC CGA GAT TTG AAC TCG	

Oligonucleotides used to generate and check knockdowns

siLuc_ss	GCA CAU AUC GAG GUG AAC ATT	Sense(ss) and Antisense(as) siRNA sequences used for transient knock-down of Luciferase gene which was used as a control
siLuc_as	UGU UCA CCU CGA UAU GUG	
siTRUB1_ss	GCA GAA GAC AGC UCC UUU ATT	Sense(ss) and Antisense(as) siRNA sequences used for transient knock-down of TRUB1 gene
siTRUB1_as	UAA AGG AGC UGU CUU CUG CTT	
siTRUB2_ss	CCC AGG AGU UUA AGG UUG UTT	Sense(ss) and Antisense(as) siRNA sequences used for transient knock-down of TRUB2 gene

siTRUB2_as	ACA ACC UUA AAC UCC UGG GTT	
HUPUS10-F	CTC ACC AAA GGC TGT ATG C	Primers used to perform qPCR to estimate the amount of PUS10 mRNA
HUPUS10-R	CCA AGG AGT TTG TGG TAG ATT CC	
HUTRUB1-F	ACA CAA GAA GAT ATT GAA GGC A	Primers used to perform qPCR to estimate the amount of TRUB1 mRNA
HUTRUB1-R	CCT CTC TTC ATC AAA GTC GAA A	
HUTRUB2-F	GCT GAT AAC TGG CAT CCG ATG C	Primers used to perform qPCR to estimate the amount of TRUB2 mRNA
HUTRUB2-R	GCA GTG GTC TTT AGT TCC AGG C	
HUHPRT-F	TGACACTGGCAAAACAA GCA	Primers used to perform qPCR to estimate the amount of HPRT mRNA
HUHPRT-R	GGTCCTTTTCACCAGCAA GCT	
Oligonucleotides used to clone genes		
HUTRUB1-F2	CCT GCC ATG GCC GCT TCT GAG GCG GCG	Forward primer to clone TruB1 gene in *Nco*I (CCATGG) site of pET28a

HUTRUB1-R2	CTG CTC GAG GCT GCC GCG CGG CAC CAG ACA CGT CTT AAT TAC ATC ATC	Reverse primer to clone TruB1 gene, has an *Xho*I (CTCGAG) site and sequence complementary to codons of thrombin cut site (GCT GCC GCG CGG CAC CAG)
HUTRUB2-F2	CGC TCT AGA AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CCA TGG GGT CTG CTG GCT TGT	Forward primer to clone TruB2 gene in *Xba*I (TCTAGA) site of pET28a
HUTRUB2-R2	CTG CTC GAG GCT GCC GCG CGG CAC CAG CTG CCC CGC ACC CCT C	Reverse primer to clone TruB2 gene, has an *Xho*I (CTCGAG) site and sequence complementary to codons of thrombin cut site (GCT GCC GCG CGG CAC CAG).