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Supplemental Information

A tRNA-Derived Small RNA Regulates Ribosomal Protein S28 Protein Levels after Translation Initiation in Humans and Mice

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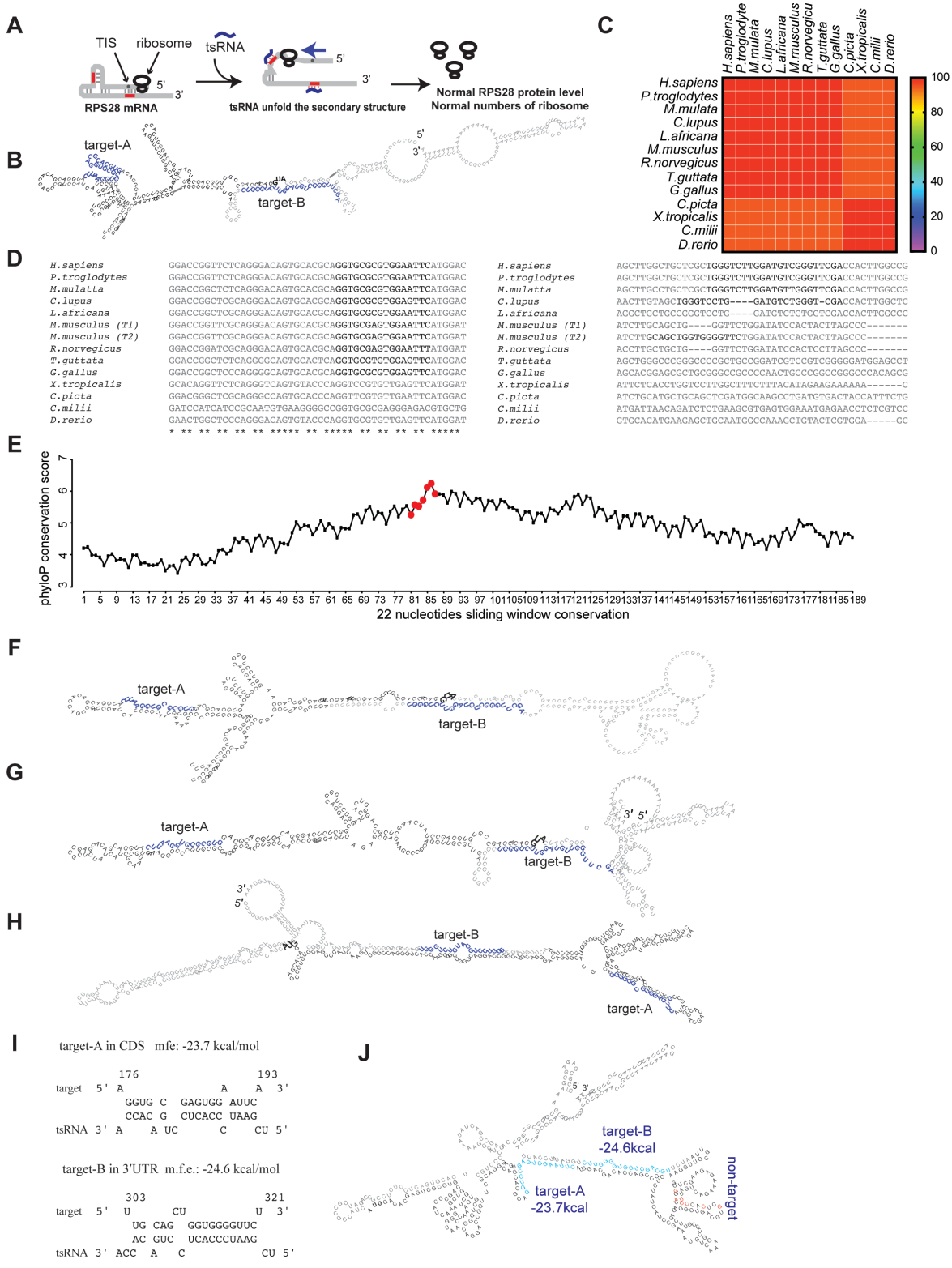
A
TIS
ribosome
tsRNA
5'
3'
RPS28 mRNA
tsRNA unfold the secondary structure
Normal RPS28 protein level
Normal numbers of ribosome
B
target-A
target-B
C
H.sapiens
P.troglodytes
M.mulata
C.lupus
L.africana
M.musculus
R.norvegicus
T.guttata
G.gallus
C.picta
X.tropicalis
C.milii
D.rerio
100
80
60
40
20
0
D
H.sapiens GGACCGGTTCTCAGGGACAGTGCACGCAGGTGCGCGTGGAATTCATGGAC
P.troglodytes GGACCGGTTCTCAGGGACAGTGCACGCAGGTGCGCGTGGAATTCATGGAC
M.mulatta GGACCGGCTCTCAGGGACAGTGCACGCAGGTGCGCGTGGAATTCATGGAC
C.lupus GGACCGGCTCGCAGGGACAGTGCACGCAGGTGCGCGTGGAGTTCATGGAC
L.africana GGACCGGCTCGCAGGGACAGTGCACGCAGGTGCGCGTGGAATTCATGGAC
M.musculus (T1) GGACCGGTTCGCAGGGACAGTGCACGCAGGTGCGAGTGGAATTCATGGAT
M.musculus (T2) GGACCGGTTCGCAGGGACAGTGCACGCAGGTGCGAGTGGAATTCATGGAT
R.norvegicus GGACCGGATCGCAGGGACAGTGCACGCAGGTGCGAGTGGAATTTATGGAT
T.guttata GGACCGGCTCTCAGGGGCAGTGCACTCAGGTGCGTGTGGAGTTCATGGAC
G.gallus GGACCGGCTCCCAGGGGCAGTGCACGCAGGTGCGCGTGGAGTTCATGGAC
X.tropicalis GCACAGGTTCTCAGGGTCAGTGTACCCAGGTCCGTGTTGAGTTCATGGAT
C.picta GGACGGGCTCGCAGGGCCAGTGCACCCAGGTTCGTGTTGAATTCATGGAC
C.milii GATCCATCATCCGCAATGTGAAGGGGCCGGTGCGCGAGGGAGACGTGCTG
D.rerio GAACTGGCTCCCAGGGACAGTGTACCCAGGTGCGTGTTGAGTTCATGGAT
H.sapiens AGCTTGGCTGCTCGCTGGGTCTTGGATGTCGGGTTCGACCACTTGGCCG
P.troglodytes AGCTTGGCTGCTCGCTGGGTCTTGGATGTCGGGTTCGACCACTTGGCCG
M.mulatta AGCTTGCCTGCTCGCTGGGTCTTGGATGTTGGGTTCGACCACTTGGCCG
C.lupus AACTTGTAGCTGGGTCCTG----GATGTCTGGGT-CGACCACTTGGCTC
L.africana AGGCTGCTGCCGGGTCCTG----GATGTCTGTGGTCGACCACTTGGCCC
M.musculus (T1) ATCTTGCAGCTG----GGTTCTGGATATCCACTACTTAGCCC-------
M.musculus (T2) ATCTTGCAGCTGGTGGGGTTCTGGATATCCACTACTTAGCCC-------
R.norvegicus ACCTTGCTGCTG----GGTTCTGGATATCCACTCCTTAGCCC-------
T.guttata AGCTGGGCCCGGGCCCCGCTGCCGGATCGTCCGTCGGGGGATGGAGCCT
G.gallus AGCACGGAGCGCTGCGGGGCCCGCCCCAACTGCCCGGCCGGGCCCACAGCG
X.tropicalis ATTCTCACCTGGTCCTTGGCTTTCTTTACATAGAAGAAAAAA------C
C.picta ATCTGCATGCTGCAGCTCGATGGCAAGCCTGATGTGACTACCATTTCTG
C.milii ATGATTAACAGATCTCTGAAGCGTGAGTGGAAATGAGAACCTCTCGTCC
D.rerio GTGCACATGAAGAGCTGCAATGGCCAAAGCTGTACTCGTGGA-----GC
E
phyloP conservation score
22 nucleotides sliding window conservation
F
target-A
target-B
G
target-A
target-B
3' 5'
H
3'
5'
target-B
target-A
I
target-A in CDS mfe: -23.7 kcal/mol
176 193
target 5' A A A 3'
GGUG C GAGUGG AUUC
CCAC G CUCACC UAAG
tsRNA 3' A A UC C CU 5'
target-B in 3'UTR m.f.e.: -24.6 kcal/mol
303 321
target 5' U CU U 3'
UG CAG GGUGGGGUUC
AC GUC UCACCCUAAG
tsRNA 3' ACC A C CU 5'
J
5' 3'
target-B
-24.6kcal
target-A
-23.7kcal
non-target

Figure S1. Related to Figure 1 – LeuCAG3′tsRNA and its target site in CDS of *RPS28* mRNA is conserved between vertebrate species. (A) Proposed model of LeuCAG3′tsRNA-mediated translation regulation in human cells (Kim et al., 2017). The LeuCAG3′tsRNA is processed from the mature Leu-CAG tRNA by an unknown process. It binds to two target sites (red colored region) in human *RPS28* mRNA and unwinds the secondary structure of the target sites during translation, maintaining a specific concentration of RPS28 protein and 40S ribosomes. **(B)** The detailed human *RPS28* mRNA (NM_001031.4) secondary structure is depicted. Grey, untranslated region (UTR); Black, coding sequences (CDS); Blue, the potential binding sites of the LeuCAG3′tsRNA. Translation initiation site (TIS) is indicated as black-bold characters. **(C)** The percent identity across 13 vertebrate species is depicted. Each percent identity is 95 or 100 demonstrating that LeuCAG3´tsRNA sequence is either identical or different by 1 nt between diverse vertebrate species. **(D)** The sequence alignments of target-A (left) and target-B sites (right) in *RPS28* mRNA for LeuCAG3′tsRNA across different species. The targets of LeuCAG3′tsRNA in *RPS28* mRNA of each species were predicted using RNAhybrid. *M. musculus* (T1) and (T2) are mouse *Rps28* transcript 1 and transcript 2, respectively. Black-colored nucleotides are the predicted target sites in each species. *, conserved site. **(E)** The average phyloP conservation score (y-axis) of each 22-nucleotide-long sliding window (x-axis) through the *RPS28* CDS across 100 vertebrate species. The red-colored consecutive seven, 22-nucleotide windows span the entire LeuCAG3′tsRNA target site. The average conservation score of a group of these seven windows is 5.75, which is the second highest score among those of all grouped consecutive seven, 22-nucleotide windows that span the whole *RPS28* CDS. (F, G, H) Schematic of the RPS28 mRNA secondary structure predicted by RNAfold. *P.troglodytes* RPS28 (XM_003953343.3) **(F)**. *M.mulatta* RPS28 (NM_001193436.1) **(G)**. *C.lupus* RPS28 (XM_542128.5) **(H)**. Grey, untranslated region (UTR); Black, coding sequences (CDS); Blue, the potential binding sites of the LeuCAG3´tsRNA. Translation initiation site (TIS) is indicated as black-bold characters. **(I)** The thermodynamic potential of LeuCAG3′tsRNA binding sites in the mouse *Rps28* mRNA: the target-A (nt 176 to 193) in the CDS and the target-B (nt 303 to 321) in the 3′ UTR. The binding site is predicted by RNAhybrid. Indicated numbers on each diagram represents the 5′ end and 3′ end position of the target site of the mouse *Rps*28 mRNA, respectively. **(J)** Schematic of the mouse *Rps28* mRNA (NM_001355384.1) secondary structure predicted by RNAfold. Blue, the possible binding sites of the LeuCAG3′tsRNA. Red, the altered nucleotides of the non-target RPS28 mutant.

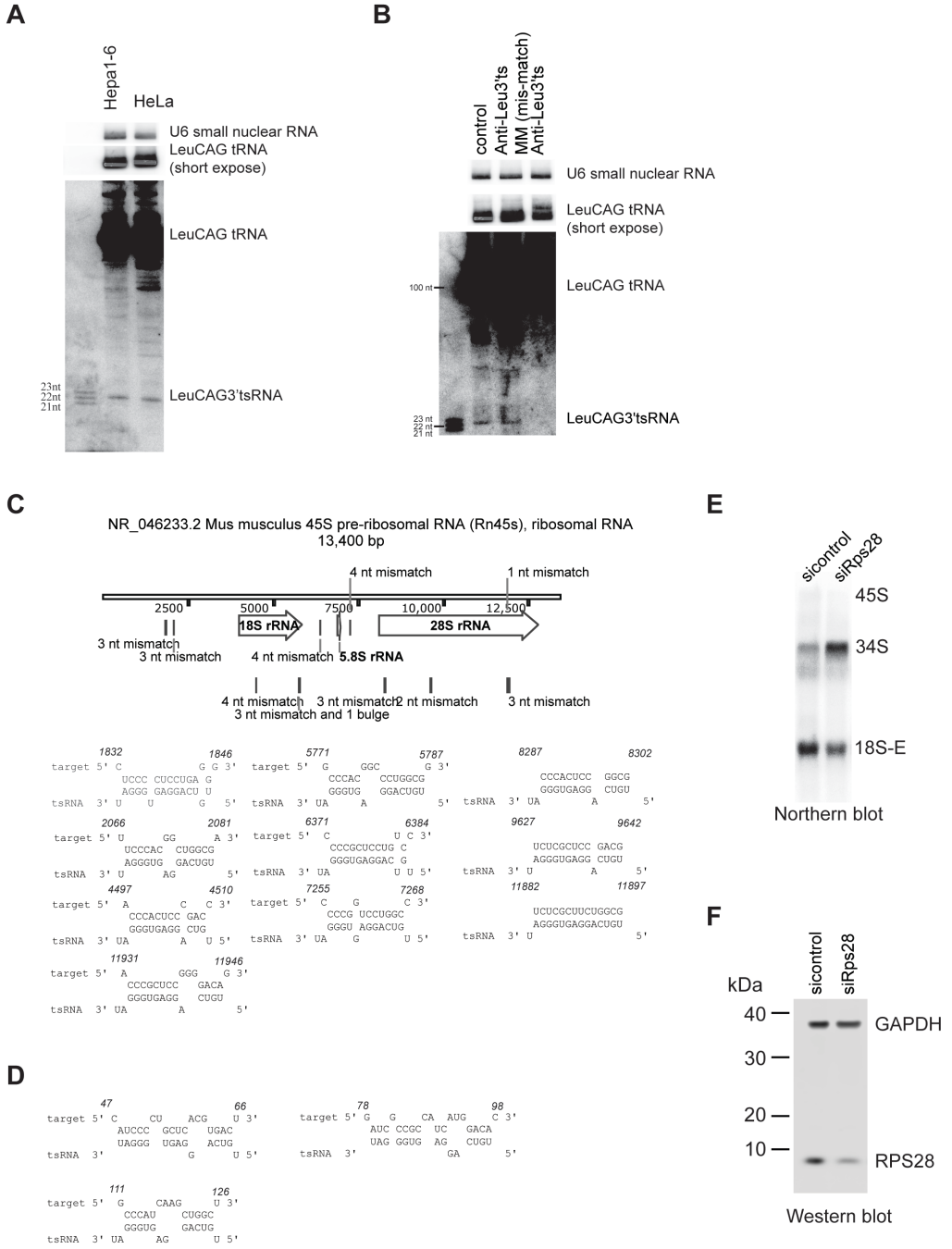
A
Hepa1-6
HeLa
U6 small nuclear RNA
LeuCAG tRNA
(short expose)
LeuCAG tRNA
23nt
22nt
21nt
LeuCAG3'tsRNA
B
control
Anti-Leu3'ts
MM (mis-match)
Anti-Leu3'ts
U6 small nuclear RNA
LeuCAG tRNA
(short expose)
100 nt
LeuCAG tRNA
23 nt
22 nt
21 nt
LeuCAG3'tsRNA
C
NR_046233.2 Mus musculus 45S pre-ribosomal RNA (Rn45s), ribosomal RNA
13,400 bp
4 nt mismatch
1 nt mismatch
2500
5000
7500
10,000
12,500
18S rRNA
28S rRNA
3 nt mismatch
3 nt mismatch
4 nt mismatch
5.8S rRNA
4 nt mismatch
3 nt mismatch and 1 bulge
3 nt mismatch
2 nt mismatch
3 nt mismatch
target 5' C G G 3'
UCCC CUCCUGA G
AGGG GAGGACU U
tsRNA 3' U U G 5'
1832
1846
target 5' G GGC G 3'
CCCAC CCUGGCG
GGGUG GGACUGU
tsRNA 3' UA A 5'
5771
5787
target 5' U GG A 3'
UCCCAC CUGGCG
AGGGUG GACUGU
tsRNA 3' U AG 5'
2066
2081
target 5' C U C 3'
CCCGCUCCUG C
GGGUGAGGAC G
tsRNA 3' UA U U 5'
6371
6384
target 5' A C C 3'
CCCACUCC GAC
GGGUGAGG CUG
tsRNA 3' UA A U 5'
4497
4510
target 5' C G C 3'
CCCG UCCUGGC
GGGU AGGACUG
tsRNA 3' UA G U 5'
7255
7268
target 5' A GGG G 3'
CCCGCUCC GACA
GGGUGAGG CUGU
tsRNA 3' UA A 5'
11931
11946
target 5' A C 3'
CCCACUCC GGCG
GGGUGAGG CUGU
tsRNA 3' UA A 5'
8287
8302
target 5' G C 3'
UCUCGCUCC GACG
AGGGUGAGG CUGU
tsRNA 3' U A 5'
9627
9642
target 5' U C 3'
UCUCGCUUCUGGCG
AGGGUGAGGACUGU
tsRNA 3' U 5'
11882
11897
D
target 5' C CU ACG U 3'
AUCCC GCUC UGAC
UAGGG UGAG ACUG
tsRNA 3' G U 5'
47
66
target 5' G G CA AUG C 3'
AUC CCGC UC GACA
UAG GGUG AG CUGU
tsRNA 3' GA 5'
78
98
target 5' G CAAG U 3'
CCCAU CUGGC
GGGUG GACUG
tsRNA 3' UA AG U 5'
111
126
E
sicontrol
siRps28
45S
34S
18S-E
Northern blot
F
kDa
sicontrol
siRps28
40
30
20
10
GAPDH
RPS28
Western blot

Figure S2. Related to Figure 2 – Antisense oligonucleotides targeting the LeuCAG3′tsRNA specifically inhibits the LeuCAG3′tsRNA and mouse RPS28 is required for ribosomal RNA processing in a mouse hepatoma cell line. (A) Total RNA was extracted from both cells and subjected to northern hybridization. The markers are synthesized oligonucleotides containing LeuCAG3′tsRNA sequences. U6 small nuclear RNA and LeuCAG tRNA are loading controls (n=2 independent experiments). **(B)** Northern analysis of the LeuCAG3′tsRNA and mature Leu-CAG tRNA post-transfection. The tsRNA (22 nt) and mature tRNA (around 100 nt) were detected with the same probe. To quantify and compare the relative amount of the mature tRNA, the blot was exposed for a shorter time after detecting the tsRNA (n=2 independent experiments). U6 snRNA and mature LeuCAG tRNA are the loading controls. **(C)** Schematic picture showing putative binding sites and binding structure of the anti-Leu3′ts ASO on the 45S primary transcript (45S pre-rRNA). To identify the tsRNA binding sites in the 45S pre-rRNA, we used the RNAhybrid program and 22-nt sequence from the 3′ end of LeuCAG-tRNA. The resulting two putative binding sites were positioned in the 5′ETS, two sites in 18S rRNA, one site in ITS1, one site in ITS2, and four sites in 28S rRNA, all of which have more than two mis-matches or a bulge except for the one binding site in 28S rRNA. The putative anti-Leu3′ts oligonucleotide binding site is indicated as a bar. **(D)** The potential interaction of Anti-LeuCAG3′tsRNA oligonucleotide and mouse *Rps28* mRNA: All of the potential interactions have more than two mis-matches. All binding sites were predicted by RNAhybrid. Indicated numbers on each diagram represents the 5′ end and 3′ end position of the target site on mouse 45S pre-rRNA or *Rps*28 mRNA, respectively. **(E, F)** Knockdown of the *Rps28* mRNA accumulated 34S pre-rRNA in Hepa 1-6 cells. Northern hybridization was performed with total RNA from Hepa 1-6 cells 24 h post-transfection (n=2 independent experiments). The ITS1 probe detects the 45S primary transcript and intermediate forms of the mature 18S rRNA including 34S, 29S, and 18S-E pre-rRNAs. The 47S and 29S pre-rRNAs are normally short-lived in cells due to their rapid processing. Mouse ribosomal RNA processing is depicted in Figure 2B **(E)**. The RPS28 protein level was measured by western blot 24 h post-transfection (n=2 independent experiments) **(F)**.

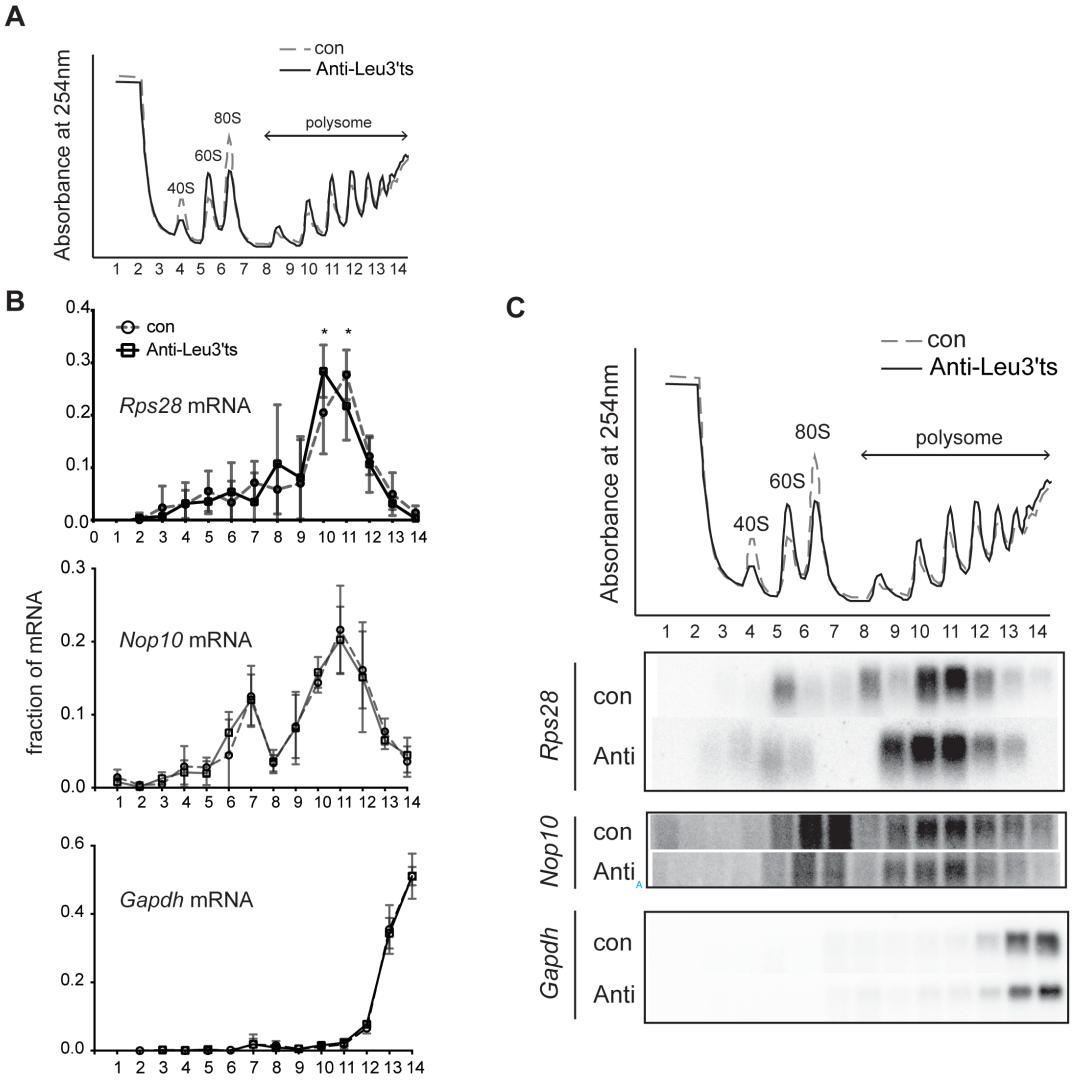

Figure S3. Related to Figure 3 – Inhibition of LeuCAG3′tsRNA reduced the number of ribosomes associated with *Rps28* mRNA, while *Nop10* and *Gapdh* mRNA were unaffected. Polysome analysis was performed using sucrose gradient fractionation 24 h post-transfection and subsequently northern hybridization was performed with the extracted RNA from each fraction (for *RPS28*, n=5 independent experiments; for *Nop10* and *GAPDH*, n=3 independent experiments). **(A)** Polysome profiles indicates the position of the 40S and 60S ribosomal free subunit, 80S monosome, and polysomes for each fraction. **(B)** Each fraction value for the specific mRNA was normalized to the sum of the mRNA level across all gradient fractions. **(C)** A represented northern image. X-axis, fraction number. con, control; Anti, Anti-LeuCAG3´tsRNA. The mean is indicated. Error bar, s.d. *, P<0.05 by two-tailed t-test.

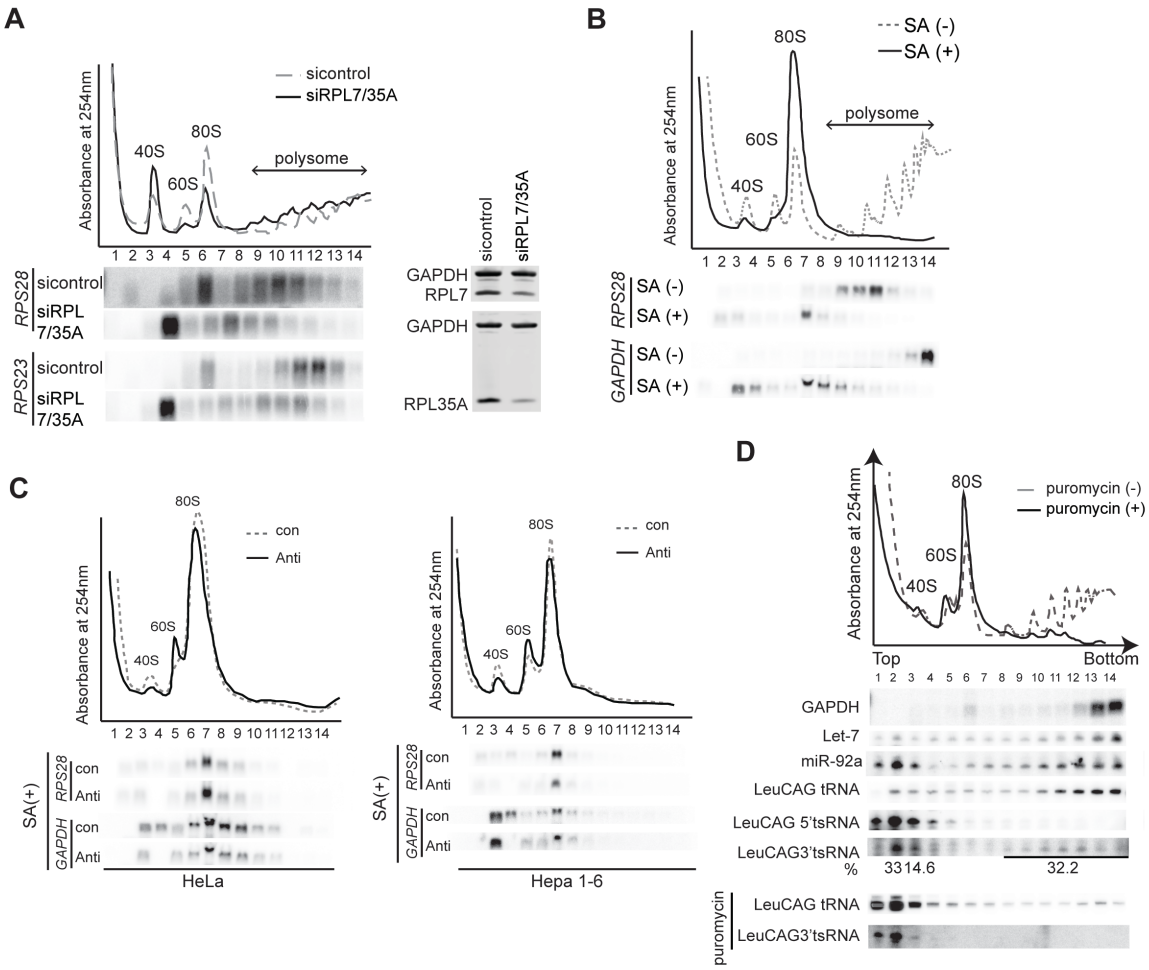

Figure S4. Related to Figure 4 – LeuCAG3´tsRNA regulates RPS28 post-translational initiation in both HeLa and Hepa 1-6 cells. The polysome profile (top) and northern hybridization (bottom) in each panel were analyzed 24 h post-transfection. The polysome profile indicates the position of ribosomal free subunit, monosome, and polysomes for each designated fraction and shows that the treatment with sodium arsenite (solid line) inhibits the formation of polysomes. **(A)** In HeLa cells, depletion of RPL7 and RPL35A, components of the 60S ribosome stalls RPS28 and RPS23 mRNA migration to fractions near the 40S subunit (n=2 independent experiments). Western blot showing depleted RPL7 and RPL35A protein levels by siRNA knockdown in the right panel. GAPDH is a loading control. **(B)** Sodium arsenite treatment stalls *RPS28* and *GAPDH* mRNA at the 80S monosome in HeLa cells (n=2 independent experiments). **(C)** Sodium arsenite treatment stalls RPS28 and GAPDH mRNA at the 80S monosome regardless of the inhibition of LeuCAG3´tsRNA in HeLa (left) and Hepa1-6 (right) cells (n=2 independent experiments). SA (+), treatment of sodium arsenite; SA (-), no treatment of sodium arsenite; con, control; Anti, Anti-LeuCAG3´tsRNA. **(D)** LeuCAG3´tsRNA co-migrates with polysomal fractions. Cytoplasmic lysates from HeLa cells were treated with cycloheximide or puromycin and separated by ultracentrifugation in 10−50% sucrose gradients. Total RNAs were extracted from each fraction, separated on a denaturing 15% acrylamide gel, and subjected to northern hybridization (n=2 independent experiments). Upper graph is the ribosomal profile detected at 254nm UV showing sucrose gradient fractionation discriminates the 40S and 60S ribosomal free subunits, 80S monosome, and polysomes. The percentage below the LeuCAG3´tsRNA northern result indicates the relative LeuCAG3´tsRNA abundance in fractions normalized to the sum of the LeuCAG3´tsRNA level across all gradient fractions.

sample	Transcript 1 (short form)	Transcript 2 (long form)	others	GEO accession number
liver_control_rep1		44	3	GSM1694991
liver_control_rep2		89		GSM1694992
liver_U6-sh-hAAT_25nt_rep1		52	2	GSM1694993
liver_U6-sh-hAAT_25nt_rep2		58		GSM1694994
liver_H1-sh-hAAT_25nt_rep1	1	43		GSM1694995
liver_H1-sh-hAAT_25nt_rep2		43		GSM1694996

Table S1. Related to Figure 1 – Relative expression of mouse *Rps28* transcript variants in whole liver. The number in each transcript column indicates the number of reads that span the splice junction (overlapping both exons).

NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL		
1.000	1.000	0.000	0.381	0.033	0.120	0.296	0.556	0.315	0.381	0.330
0.365	0.058	0.108	0.333	0.377	0.061	0.061	0.118	0.029	0.073	0.141
0.040	0.151	0.344	0.000	0.186	0.199	0.063	0.042	0.233	0.118	0.110
0.056	0.022	0.096	0.087	0.197	0.059	0.048	0.022	0.030	0.008	0.012
0.010	0.007	0.049	0.004	0.000	0.000	0.000	0.058	0.014	0.000	0.057
0.076	0.052	0.042	0.017	0.034	0.039	0.004	0.134	0.112	0.068	0.122
0.306	0.381	0.483	0.104	0.000	0.035	0.201	0.192	0.199	0.259	0.131
0.058	0.042	0.032	0.023	0.059	0.051	0.046	0.084	0.128	0.423	0.162
0.004	0.003	0.169	0.148	0.371	0.069	0.129	0.130	0.192	0.151	0.057
0.045	0.283	0.152	0.133	0.231	0.292	0.232	0.070	0.035	0.002	0.035
0.152	0.327	0.213	0.420	0.122	0.245	0.127	0.106	0.171	0.124	0.063
0.020	0.029	0.009	0.120	0.118	0.175	0.038	0.000	0.128	0.103	0.075
0.089	0.090	0.019	0.330	0.233	0.119	0.114	0.006	0.031	0.071	0.091
0.000	0.025	0.050	0.019	0.000	0.134	0.305	0.762	0.120	0.301	0.384
0.247	0.040	0.230	0.423	0.695	1.000	0.430	0.404	0.980	0.269	0.497
0.711	0.580	0.240	0.262	0.132	0.066	0.000	0.000	0.103	0.504	0.452
0.124	0.028	0.181	0.097	0.206	0.020	0.018	0.012	0.017	0.007	0.028
0.000	0.024	0.300	0.164	0.022	0.458	0.000	0.000	0.000	0.000	0.000
0.000	0.156	0.523	0.281	0.151	0.151	0.222	0.365	0.678	0.267	0.152
0.409	0.622	1.000	0.786	0.478	0.630	0.393	0.910	0.794	0.569	0.462
0.350	0.142	0.128	0.651	0.646	0.249	0.399	0.402	0.418	0.585	0.224
0.296	0.724	0.739	0.845	1.000	1.000	0.393	0.468	0.471	0.368	0.052
0.000	0.020	0.019	0.000	0.031	0.113	0.001	0.013	0.004	0.001	0.040
0.021	0.040	0.015	0.011	0.013	0.023	0.052	0.098	0.246	0.870	0.171
0.000	0.000	0.039	0.003	0.040	0.071	0.056	0.086	0.123	0.113	0.023
0.000	0.000	0.000	0.022	0.004	0.017	0.028	0.084	0.027	0.022	0.013
0.015	0.009	0.000	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL
NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL	NULL

Table S2. Related to Figure 1 – icSHAPE scores for the full-length mouse *Rps28* transcript 2 (ENSMUST00000173844.7). Each number represents the scores for each nucleotide.

Human *GAPDH* mRNA: 5′-agatcatcagcaatgcctcct-3′ and 5′-tggtcatgagtccttccacg-3′	IDT	N/A
Human *RPS28* mRNA: 5′-gccgccatggacaccagccgtgtgc-3′ and 5′-tcagcgcaacctccgggcttc-3′	IDT	N/A
Human *RPS23* mRNA: 5′-gccgccatgggcaagtgtcgtggac-3′ and 5′-ttatgatcttggtctttccttc-3′	IDT	N/A
Mouse *Gapdh* mRNA: 5′-acccctcattgacctcaacta-3′ and 5′-cttctccatggtggtgaagac -3′	IDT	N/A
Mouse *Rps28*: 5′-ctcgcgagagcgaaagtgag-3′ and 5′-taatataaatgctttatttaacagttgcag-3′	IDT	N/A

Table S3. Related to STAR Methods – PCR primers used as northern probes for mRNAs.

Mouse *Rps28* target-A mutant: 5′-gagcggctggtgtcatccataaactcgaccctgacttgcgtgcactgtccctgcga-3′ and 5′-tcgcagggacagtgcacgcaagtcagggtcgagtttatggatgacaccagccgctc-3′	IDT	N/A
Mouse Rps28 target-B mutant: 5′-agagaagctcgaaggttgcgttaatcttggatatccactac-3′ and 5′-gtagtggatatccaagattaacgcaaccttcgagcttctct-3′	IDT	N/A
Mouse Rps28 non-target mutant: 5′-ttcgagcttctctttctgactgcagcaatgtcaggacgtcaccctctcgaacggggcc-3′ and 5′-ggccccgttcgagagggtgacgtcctgacattgctgcagtcagaaagagaagctcgaa-3′	IDT	N/A

Table S4. Related to STAR Methods – Oligonucleotides used for site-directed mutagenesis.