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Supplemental Information

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SUPPLEMENTAL INFORMATION

VELCRO-IP RNA-seq reveals ribosome expansion segment function in translation genome-wide

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This document includes:

Figures S1 to S6
Tables S1 to S3

Other supplementary material for this manuscript includes the following:

Table S4: mRNA regions enriched by VELCRO-IP RNA-seq
Table S5: GO term analysis for all genes enriched with hES9S

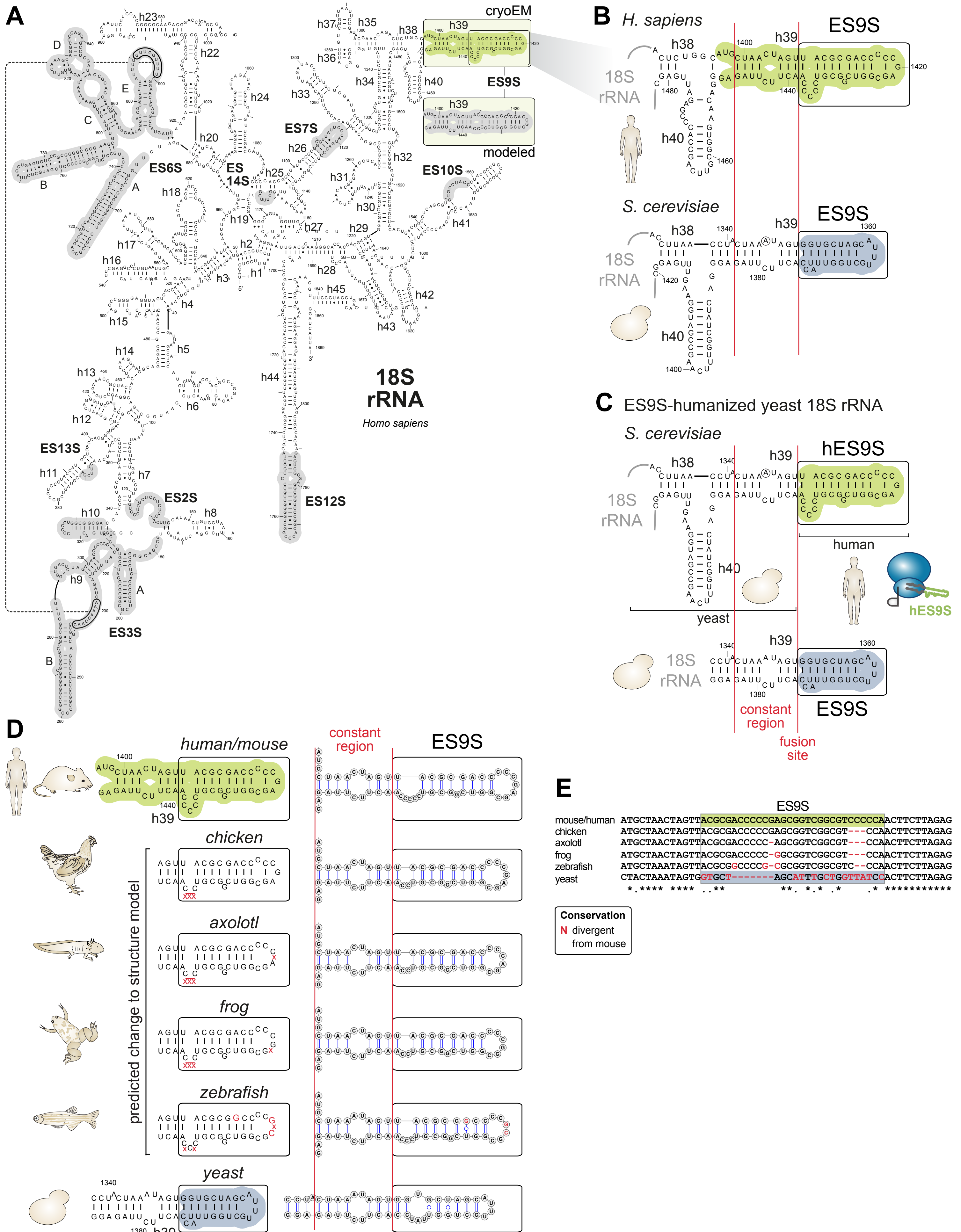
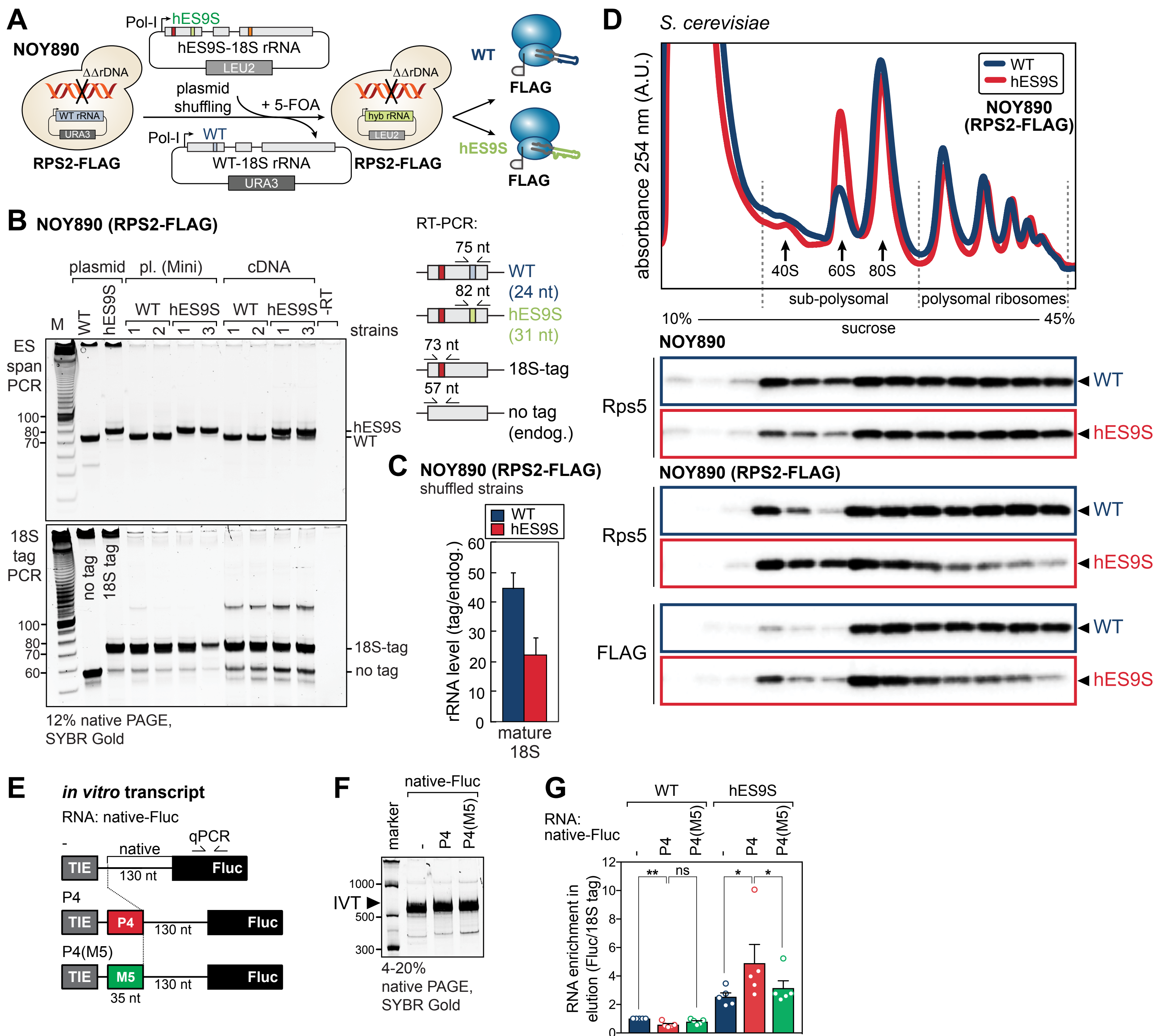

A
18S rRNA
Homo sapiens
cryoEM
modeled
ES9S
ES6S
ES 14S
ES7S
ES10S
ES13S
ES2S
ES3S
ES12S
h1
h2
h3
h4
h5
h6
h7
h8
h9
h10
h11
h12
h13
h14
h15
h16
h17
h18
h19
h20
h22
h23
h24
h25
h26
h27
h28
h29
h30
h31
h32
h33
h34
h35
h36
h37
h38
h39
h40
h41
h42
h43
h44
h45
B
H. sapiens
ES9S
18S rRNA
S. cerevisiae
ES9S
C
ES9S-humanized yeast 18S rRNA
S. cerevisiae
hES9S
human
yeast
hES9S
constant region
fusion site
ES9S
D
human/mouse
chicken
axolotl
frog
zebrafish
yeast
predicted change to structure model
constant region
ES9S
E
ES9S
mouse/human ATGCTAACTAGTTACGCGACCCCCGAGCGGTCGGCGTCCCCCAACTTCTTAGAG
chicken ATGCTAACTAGTTACGCGACCCCCGAGCGGTCGGCGT---CCAACTTCTTAGAG
axolotl ATGCTAACTAGTTACGCGACCCCC-AGCGGTCGGCGT---CCAACTTCTTAGAG
frog ATGCTAACTAGTTACGCGACCCCC-GGCGGTCGGCGT---CCAACTTCTTAGAG
zebrafish ATGCTAAATAGTTACGCGGCCCCG-GCGGTCGGCGTC--CCAACTTCTTAGAG
yeast CTACTAAATAGTGGTGCT-------AGCATTTGCTGGTTATCCACTTCTTAGAG
Conservation
N divergent from mouse

Figure S1. Confirmation of interspecies sequence variation of ES9S rRNA region. Related to Figure 1.

(**A**) Secondary structure model of the human (*H. sapiens*) 18S rRNA adapted from (Anger et al., 2013). rRNA expansion segment regions are highlighted in grey. Nucleotide positions, helices and ESs are numbered. The boxed region shows the ES9S structure based on either our cryo-EM data (green; (Leppek et al., 2020)) or based on a previous model (grey; (Anger et al., 2013)).
(**B**) Secondary structure models of the human and baker's yeast (*S. cerevisiae*) 18S rRNA region containing ES9S, highlighted in green and blue, respectively. The structure of the distal human ES9S (boxed region in A and B) was revised based on cryo-EM data (Leppek et al., 2020).
(**C**) Secondary structure model of the engineered yeast 18S rRNA after exchange of the yeast ES9S with the human one (hES9S, green). Constant region (h39) and ES9S-fusion site selected for engineering chimeric 18S rRNA are indicated in red.
(**D**) Predicted structural changes in the ES9S region of 18S rRNA across different species. Sequence changes and their predicted effects on the ES9S structure are indicated in red. Human/mouse ES9S (identical sequence) is the reference for the comparison. The variable sequences across the species are obtained by RT-PCR from total RNA extracts of the different species (E11.5, stage E11.5 FVB mouse embryo; chicken, *Gallus gallus*; axolotl, *Ambystoma mexicanum*; frog, X. l., *Xenopus laevis*; zebrafish, *Danio rerio*; yeast, S. c., *Saccharomyces cerevisiae*) using primers specific for the 18S rRNA region containing ES9S in the center (see **Figure 1A-C**, partially reproduced from **Figure 1A**). Secondary structures of ES9S of different species were modeled using Vienna RNAfold (http://rna.tbi.univie.ac.at) and visualized using VARNA (http://varna.lri.fr) with default settings.
(**E**) Multiple sequence alignment of RT-PCR-confirmed ES9S sequences from the different species used for the structure models in (D). Partially reproduced from **Figure 1C**.



A
NOY890
Pol-I
hES9S
hES9S-18S rRNA
LEU2
ΔΔrDNA
WT rRNA
URA3
plasmid shuffling
+ 5-FOA
Pol-I
WT
WT-18S rRNA
URA3
RPS2-FLAG
hyb rRNA
LEU2
RPS2-FLAG
WT
FLAG
hES9S
FLAG
B NOY890 (RPS2-FLAG)
plasmid
pl. (Mini)
cDNA
M
WT
hES9S
WT
hES9S
WT
hES9S
-RT
strains
ES span PCR
18S tag PCR
no tag
18S tag
hES9S
WT
18S-tag
no tag
12% native PAGE, SYBR Gold
RT-PCR:
75 nt
WT (24 nt)
82 nt
hES9S (31 nt)
73 nt
18S-tag
57 nt
no tag (endog.)
C NOY890 (RPS2-FLAG)
shuffled strains
WT
hES9S
rRNA level (tag/endog.)
mature 18S
D S. cerevisiae
absorbance 254 nm (A.U.)
WT
hES9S
NOY890 (RPS2-FLAG)
40S
60S
80S
sub-polysomal
polysomal ribosomes
10%
sucrose
45%
NOY890
Rps5
WT
hES9S
NOY890 (RPS2-FLAG)
Rps5
WT
hES9S
FLAG
WT
hES9S
E in vitro transcript
RNA: native-Fluc
native
130 nt
qPCR
TIE
Fluc
P4
130 nt
P4(M5)
M5
35 nt
F
native-Fluc
marker
P4
P4(M5)
IVT
4-20% native PAGE, SYBR Gold
G
WT
hES9S
RNA: native-Fluc
RNA enrichment in elution (Fluc/18S tag)
**
ns
*
*

Figure S2. Plasmid shuffling and yeast strain characterization; and VELCRO-IP qRT-PCR serves as a proof-of-principle to identify novel hES9S-interacting 5' UTRs. Related to Figure 1, 2, 3.

(**A**) A yeast strain containing the plasmid-encoded chimeric 18S rRNA is generated by plasmid shuffling. Schematic of the plasmid shuffling approach to generate yeast strains (NOY890, RPS2-FLAG) that contain a homozygous knock-out of the rDNA locus (NOY890), resulting in rRNA transcription exclusively from the plasmids. All rDNA plasmids contain unique 18S and 25S rRNA sequence tags. 5-FOA-based selection of transformed yeast cells allows for isolation of clones that retain a transformed *LEU2*-plasmid (pNOY373) and lost the original *URA3*-plasmid (pNOY373). Successful plasmid exchange from *URA3* (WT) to *LEU2* (tagged WT or hES9S)-plasmids in isolates is achieved by growth on SD-*LEU2*, and SD+5-FOA but not on SD-*LEU/URA*.

(**B**) RT-PCR analysis using ES9S-specific primers that span ES9S allow analysis of expression of WT or hES9S 18S rRNA since there is a 7 nt difference in the length of the PCR products between WT and hES9S (ES span PCR). Similarly, the presence of the 18S tag can be distinguished from WT rRNA (18S tag PCR). Total RNA for cDNA synthesis or plasmid DNA was extracted from clones and used for RT-PCR. Plasmid-derived PCR products serve as controls. PCR products were resolved by 12% native PAGE and stained with SYBR Gold. Two independent isolates of tagged-WT and tagged-hES9S strains (NOY890/RPS2-FLAG background) used in this study are presented. RT-PCR specific for the 18S rRNA tag confirms the presence of the tag in transformed plasmid-derived mature 18S rRNA. A 10 bp DNA ladder (Invitrogen) was loaded as reference.

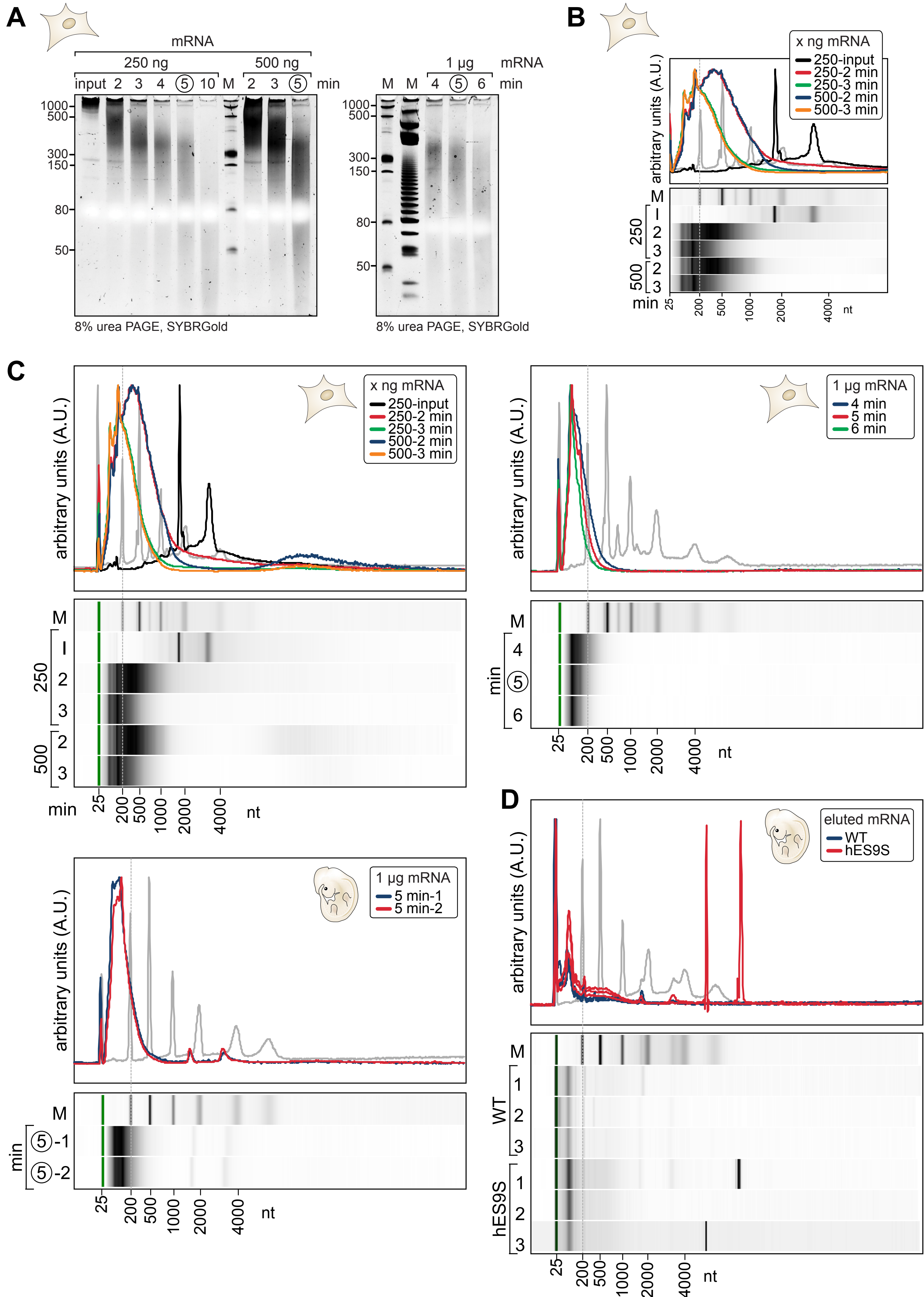
(**C**) Yeast strain characterization after plasmid shuffling and isolation of clones. qRT-PCR analysis with specific primers for rRNA tags and endogenous rRNAs is used to quantify tag/endogenous rRNA levels (i.e. the substitution rates of WT with tagged-WT or tagged-hES9S ribosomes present in isolated strains). For NOY890/RPS2-FLAG strains, the qRT-PCR analysis determined that only one endogenous plasmid-derived WT ribosomes still remained per every 44 tagged WT or every 22 tagged hES9S ribosomes.

(**D**) Sucrose gradient fractionation analysis of yeast lysates derived from WT and hES9S-stains in the background of NOY890 and NOY890/RPS2-FLAG, containing scarless C-terminal Rps2-FLAG (Jan et al., 2014), on 10-45% sucrose gradients (n = 3). Compared to WT rRNA-containing cells, humanized ribosome-containing cells show a slight growth defect. Polysome traces demonstrate proper ribosomal assembly. Incorporation of the FLAG tag into polysomes demonstrates its non-perturbative nature.

(**E**) Schematic of *in vitro* transcripts used for the proof-of-principle experiment of the VELCRO-IP qRT-PCR. Reproduced from **Figure 3B**.

(**F**) For qualitative analysis of the integrity of *in vitro* transcripts, RNAs were subjected to 4-20% polyacrylamide/TBE/native PAGE and visualized by SYBR Gold staining.

(**G**) Analysis of total RNA in the 3xFLAG peptide elution by qRT-PCR using same volumes of RNA per sample for the RT. Normalization of Ct values for Fluc to the 18S rRNA tag internally controls for ribosome-IP efficiency per sample. The native/WT sample was used to normalize for fold enrichment of RNA binding (set to 1). The same data as in **Figure 3D** is plotted differently. Average RNA fold enrichment, SEM, n = 5; ns, not significant.



A
mRNA
250 ng
500 ng
input 2 3 4 5 10 M 2 3 5 min
1000
500
300
150
80
50
8% urea PAGE, SYBRGold
1 µg mRNA
M M 4 5 6 min
8% urea PAGE, SYBRGold
B
x ng mRNA
250-input
250-2 min
250-3 min
500-2 min
500-3 min
arbitrary units (A.U.)
M
I
250
500
min
25 200 500 1000 2000 4000 nt
C
1 µg mRNA
4 min
5 min
6 min
5 min-1
5 min-2
D
eluted mRNA
WT
hES9S

Figure S3. Controlled mRNA fragmentation. Related to Figure 3.

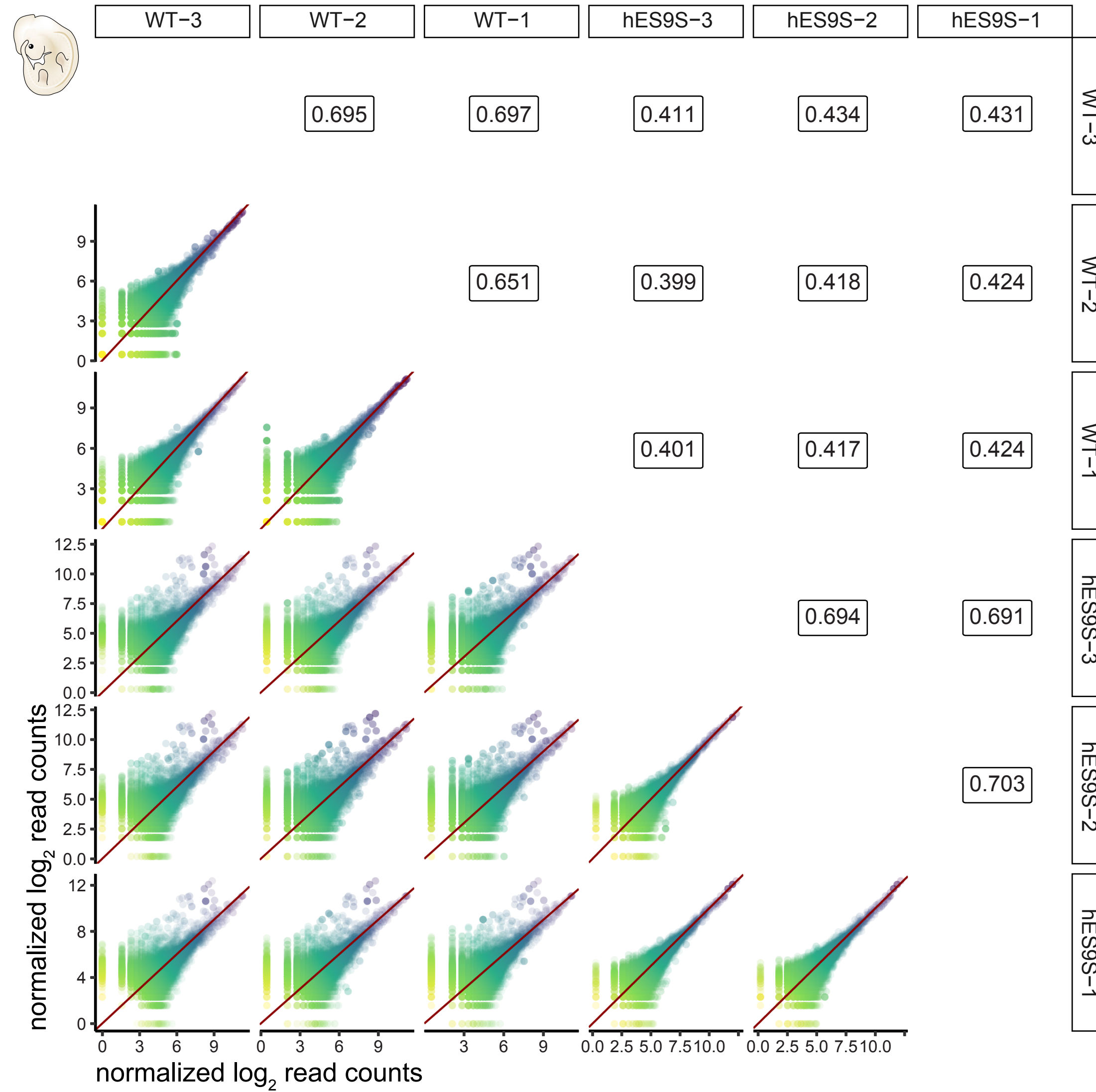
(**A**) Fragmented mouse mRNA from C3H/10T1/2 cells in different amounts (250 ng, 500 ng, and 1 μg) and timepoints of fragmentation, analyzed by 8% denaturing urea PAGE and visualized by SYBR Gold. The left-most lane shows the 250 ng mRNA input without fragmentation for reference. Ladders: Low Range ssRNA Ladder (NEB); 20 bp Bayou DNA Ladder (Bayou Biolabs).

(**B**) Fragmented mouse mRNA from C3H/10T1/2 cells in different amounts (250 and 500 ng) and timepoints of fragmentation (2 and 3 min) and the 250 ng mRNA input, analyzed on a mRNA Pico Chip (Agilent) on a Bioanalyzer (Agilent). Zoomed-in view of the Bioanalyzer quantification (top) and virtual gel images (bottom) is shown. Grey line plots the marker (lane M in virtual gel images) for reference. See also (**C**).

(**C**) Optimization of mouse mRNA fragmentation from C3H/10T1/2 cells and stage E11.5 mouse embryos. Full views of the Bioanalyzer (Agilent) analyses shown in **Figures S3B, 3F and 3G.** Grey lines plot the markers (lane M in virtual gel images) for reference.

(**D**) Full view of the Bioanalyzer (Agilent) quantification and virtual gel images in **Figure 4B** is shown for the eluted and yeast rRNA-depleted mouse embryo RNA from three independent replicates of WT and hES9S VELCRO-IP experiments. Grey lines plot the markers (lane M in virtual gel images) for reference.

A VELCRO-IP: reproducibility



B VELCRO-IP: enriched mRNA fragments

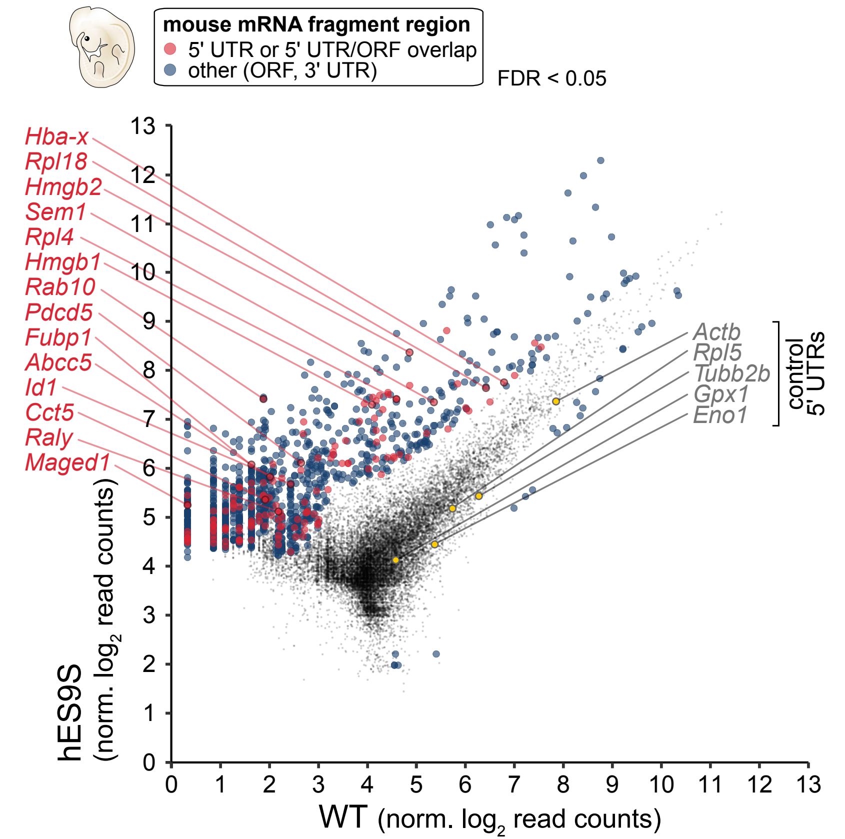
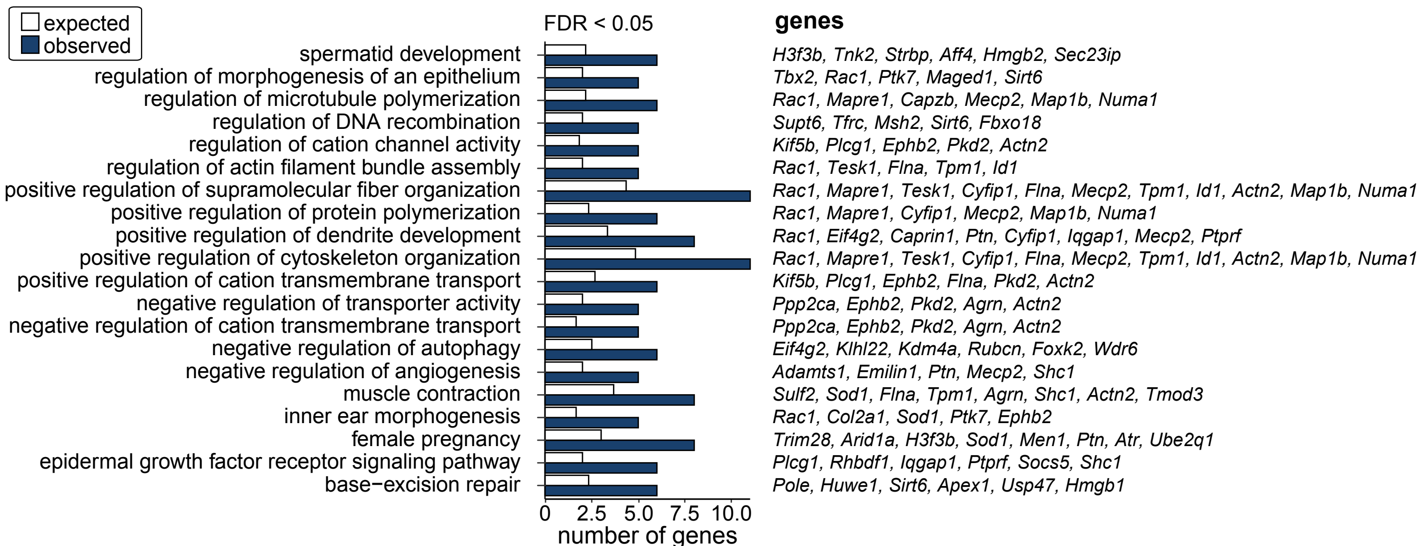
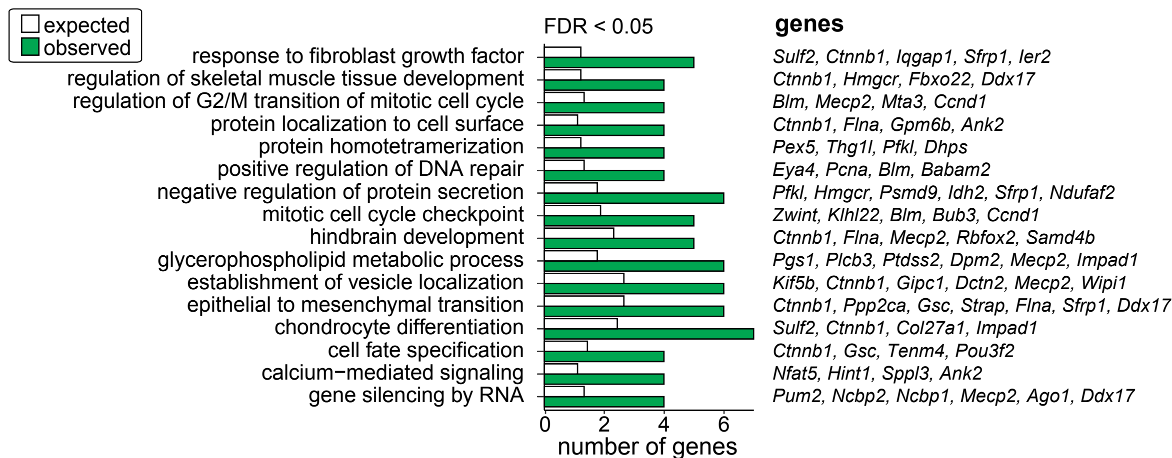


Figure S4. Reproducibility of VELCRO-IP RNA-seq and identification of hES9S-interacting 5' UTRs. Related to Figure 4.

(**A**) A matrix comparing every possible pair of individual VELCRO-IP RNA-seq samples (three replicate samples per condition, hES9S and WT). Lower triangle: scatter plots of normalized log read counts, colored by expression level. Upper triangle: Pearson correlation coefficient.

(**B**) RNA-seq results of independent replicates (n = 3) for each WT and hES9S samples. Normalized log read counts are presented for WT and hES9S-enriched mRNA fragments. Fragments less than FDR < 0.05 are colored according to the region in the mRNA. Fragments mapping to 5' UTR and overlapping 5' UTR/ORF (red) are highlighted compared to other regions (ORF and 3' UTR, blue). We label mouse genes for which we identified enriched fragments in the 5' UTR and/or 5' region of the ORF and for whose 5' UTRs we performed validation experiments. Five control 5' UTRs are highlighted in yellow that are equally bound to both WT and hES9S 40S subunits and served as negative controls. Corresponds to **Figure 4E**. See also **Table S4**.


A GO term (biological process) for enriched ORF
expected
observed
FDR < 0.05
genes
spermatid development
regulation of morphogenesis of an epithelium
regulation of microtubule polymerization
regulation of DNA recombination
regulation of cation channel activity
regulation of actin filament bundle assembly
positive regulation of supramolecular fiber organization
positive regulation of protein polymerization
positive regulation of dendrite development
positive regulation of cytoskeleton organization
positive regulation of cation transmembrane transport
negative regulation of transporter activity
negative regulation of cation transmembrane transport
negative regulation of autophagy
negative regulation of angiogenesis
muscle contraction
inner ear morphogenesis
female pregnancy
epidermal growth factor receptor signaling pathway
base−excision repair
H3f3b, Tnk2, Strbp, Aff4, Hmgb2, Sec23ip
Tbx2, Rac1, Ptk7, Maged1, Sirt6
Rac1, Mapre1, Capzb, Mecp2, Map1b, Numa1
Supt6, Tfrc, Msh2, Sirt6, Fbxo18
Kif5b, Plcg1, Ephb2, Pkd2, Actn2
Rac1, Tesk1, Flna, Tpm1, Id1
Rac1, Mapre1, Tesk1, Cyfip1, Flna, Mecp2, Tpm1, Id1, Actn2, Map1b, Numa1
Rac1, Mapre1, Cyfip1, Mecp2, Map1b, Numa1
Rac1, Eif4g2, Caprin1, Ptn, Cyfip1, Iqgap1, Mecp2, Ptprf
Rac1, Mapre1, Tesk1, Cyfip1, Flna, Mecp2, Tpm1, Id1, Actn2, Map1b, Numa1
Kif5b, Plcg1, Ephb2, Flna, Pkd2, Actn2
Ppp2ca, Ephb2, Pkd2, Agrn, Actn2
Ppp2ca, Ephb2, Pkd2, Agrn, Actn2
Eif4g2, Klhl22, Kdm4a, Rubcn, Foxk2, Wdr6
Adamts1, Emilin1, Ptn, Mecp2, Shc1
Sulf2, Sod1, Flna, Tpm1, Agrn, Shc1, Actn2, Tmod3
Rac1, Col2a1, Sod1, Ptk7, Ephb2
Trim28, Arid1a, H3f3b, Sod1, Men1, Ptn, Atr, Ube2q1
Plcg1, Rhbdf1, Iqgap1, Ptprf, Socs5, Shc1
Pole, Huwe1, Sirt6, Apex1, Usp47, Hmgb1
0 2.5 5.0 7.5 10.0
number of genes


B GO term (biological process) for enriched 3' UTRs
expected
observed
FDR < 0.05
genes
response to fibroblast growth factor
regulation of skeletal muscle tissue development
regulation of G2/M transition of mitotic cell cycle
protein localization to cell surface
protein homotetramerization
positive regulation of DNA repair
negative regulation of protein secretion
mitotic cell cycle checkpoint
hindbrain development
glycerophospholipid metabolic process
establishment of vesicle localization
epithelial to mesenchymal transition
chondrocyte differentiation
cell fate specification
calcium−mediated signaling
gene silencing by RNA
Sulf2, Ctnnb1, Iqgap1, Sfrp1, Ier2
Ctnnb1, Hmgcr, Fbxo22, Ddx17
Blm, Mecp2, Mta3, Ccnd1
Ctnnb1, Flna, Gpm6b, Ank2
Pex5, Thg1l, Pfkl, Dhps
Eya4, Pcna, Blm, Babam2
Pfkl, Hmgcr, Psmd9, Idh2, Sfrp1, Ndufaf2
Zwint, Klhl22, Blm, Bub3, Ccnd1
Ctnnb1, Flna, Mecp2, Rbfox2, Samd4b
Pgs1, Plcb3, Ptdss2, Dpm2, Mecp2, Impad1
Kif5b, Ctnnb1, Gipc1, Dctn2, Mecp2, Wipi1
Ctnnb1, Ppp2ca, Gsc, Strap, Flna, Sfrp1, Ddx17
Sulf2, Ctnnb1, Col27a1, Impad1
Ctnnb1, Gsc, Tenm4, Pou3f2
Nfat5, Hint1, Sppl3, Ank2
Pum2, Ncbp2, Ncbp1, Mecp2, Ago1, Ddx17
0 2 4 6
number of genes

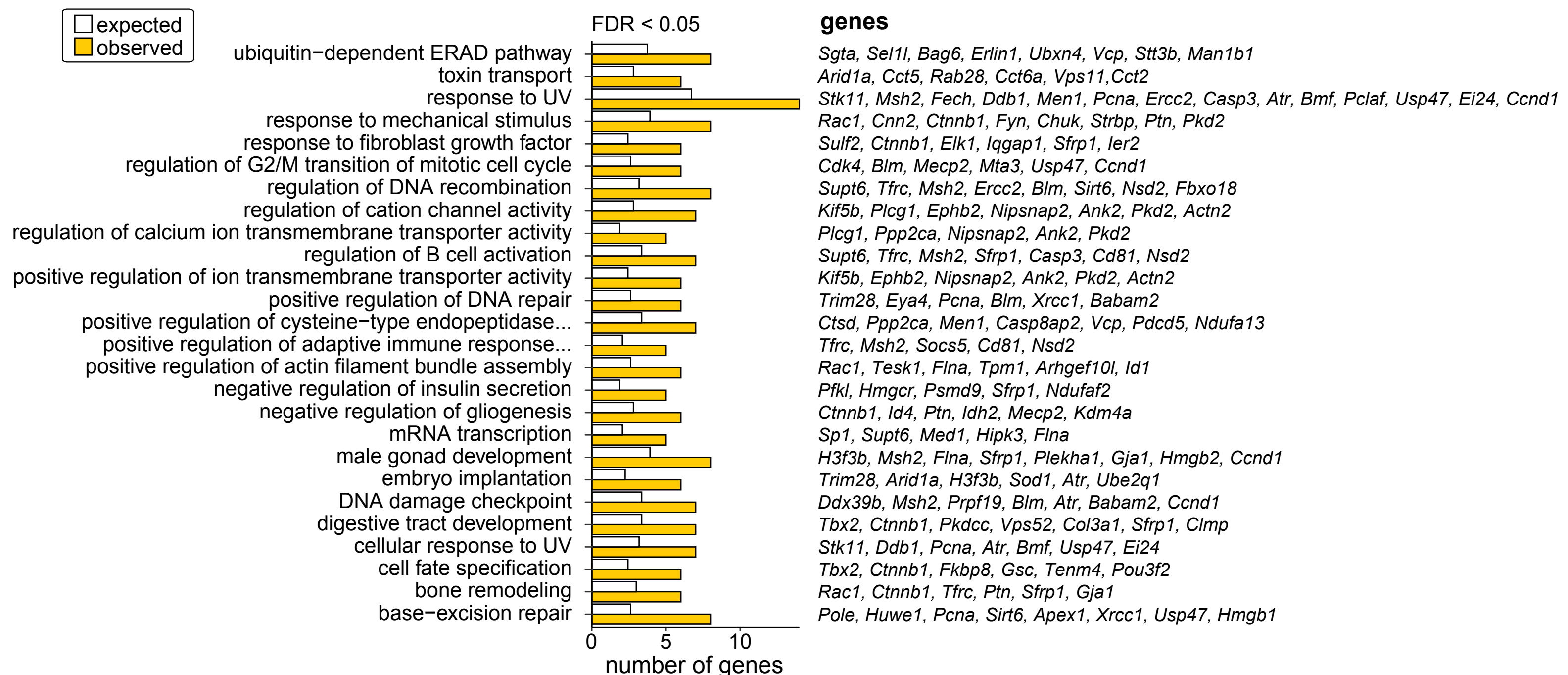

C GO term (biological process) for enriched mRNA (all regions)
expected
observed
FDR < 0.05
genes
ubiquitin−dependent ERAD pathway
toxin transport
response to UV
response to mechanical stimulus
response to fibroblast growth factor
regulation of G2/M transition of mitotic cell cycle
regulation of DNA recombination
regulation of cation channel activity
regulation of calcium ion transmembrane transporter activity
regulation of B cell activation
positive regulation of ion transmembrane transporter activity
positive regulation of DNA repair
positive regulation of cysteine−type endopeptidase...
positive regulation of adaptive immune response...
positive regulation of actin filament bundle assembly
negative regulation of insulin secretion
negative regulation of gliogenesis
mRNA transcription
male gonad development
embryo implantation
DNA damage checkpoint
digestive tract development
cellular response to UV
cell fate specification
bone remodeling
base−excision repair
Sgta, Sel1l, Bag6, Erlin1, Ubxn4, Vcp, Stt3b, Man1b1
Arid1a, Cct5, Rab28, Cct6a, Vps11,Cct2
Stk11, Msh2, Fech, Ddb1, Men1, Pcna, Ercc2, Casp3, Atr, Bmf, Pclaf, Usp47, Ei24, Ccnd1
Rac1, Cnn2, Ctnnb1, Fyn, Chuk, Strbp, Ptn, Pkd2
Sulf2, Ctnnb1, Elk1, Iqgap1, Sfrp1, Ier2
Cdk4, Blm, Mecp2, Mta3, Usp47, Ccnd1
Supt6, Tfrc, Msh2, Ercc2, Blm, Sirt6, Nsd2, Fbxo18
Kif5b, Plcg1, Ephb2, Nipsnap2, Ank2, Pkd2, Actn2
Plcg1, Ppp2ca, Nipsnap2, Ank2, Pkd2
Supt6, Tfrc, Msh2, Sfrp1, Casp3, Cd81, Nsd2
Kif5b, Ephb2, Nipsnap2, Ank2, Pkd2, Actn2
Trim28, Eya4, Pcna, Blm, Xrcc1, Babam2
Ctsd, Ppp2ca, Men1, Casp8ap2, Vcp, Pdcd5, Ndufa13
Tfrc, Msh2, Socs5, Cd81, Nsd2
Rac1, Tesk1, Flna, Tpm1, Arhgef10l, Id1
Pfkl, Hmgcr, Psmd9, Sfrp1, Ndufaf2
Ctnnb1, Id4, Ptn, Idh2, Mecp2, Kdm4a
Sp1, Supt6, Med1, Hipk3, Flna
H3f3b, Msh2, Flna, Sfrp1, Plekha1, Gja1, Hmgb2, Ccnd1
Trim28, Arid1a, H3f3b, Sod1, Atr, Ube2q1
Ddx39b, Msh2, Prpf19, Blm, Atr, Babam2, Ccnd1
Tbx2, Ctnnb1, Pkdcc, Vps52, Col3a1, Sfrp1, Clmp
Stk11, Ddb1, Pcna, Atr, Bmf, Usp47, Ei24
Tbx2, Ctnnb1, Fkbp8, Gsc, Tenm4, Pou3f2
Rac1, Ctnnb1, Tfrc, Ptn, Sfrp1, Gja1
Pole, Huwe1, Pcna, Sirt6, Apex1, Xrcc1, Usp47, Hmgb1
0 5 10
number of genes

Figure S5. GO-terms of hES9S-interacting mRNA regions. Related to Figure 4.

(**A**) GO term analysis as in **Figure 4H** for biological process of ORF regions (FDR < 0.05, n = 3) enriched by hES9S. Displayed are the expected and observed frequency of genes for the significant terms (FDR < 0.05, expressed mRNA regions were used as the background; see methods for details of the thresholds used). Also see **Table S5**.
(**B**) GO term analysis as in (A) for biological process of 3' UTR regions (FDR < 0.05, n = 3) enriched by hES9S.
(**C**) GO term analysis as in (A) for biological process of the full mRNA (any region) (FDR < 0.05, n = 3) enriched by hES9S.

A

target mRNAs - VELCRO-IP: fragment binding profile

scaled fragment to mRNA length

246 nt

Rpl18

hES9S

WT

input

normalized coverage

* 5' UTR (66 nt)

Hmgb2

hES9S

WT

input

normalized coverage

* 5' UTR (123 nt)

Pdcd5

hES9S

WT

input

normalized coverage

* 5' UTR (150 nt)

Fubp1

hES9S

WT

input

normalized coverage

* 5' UTR (74 nt)

Id1

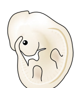
hES9S

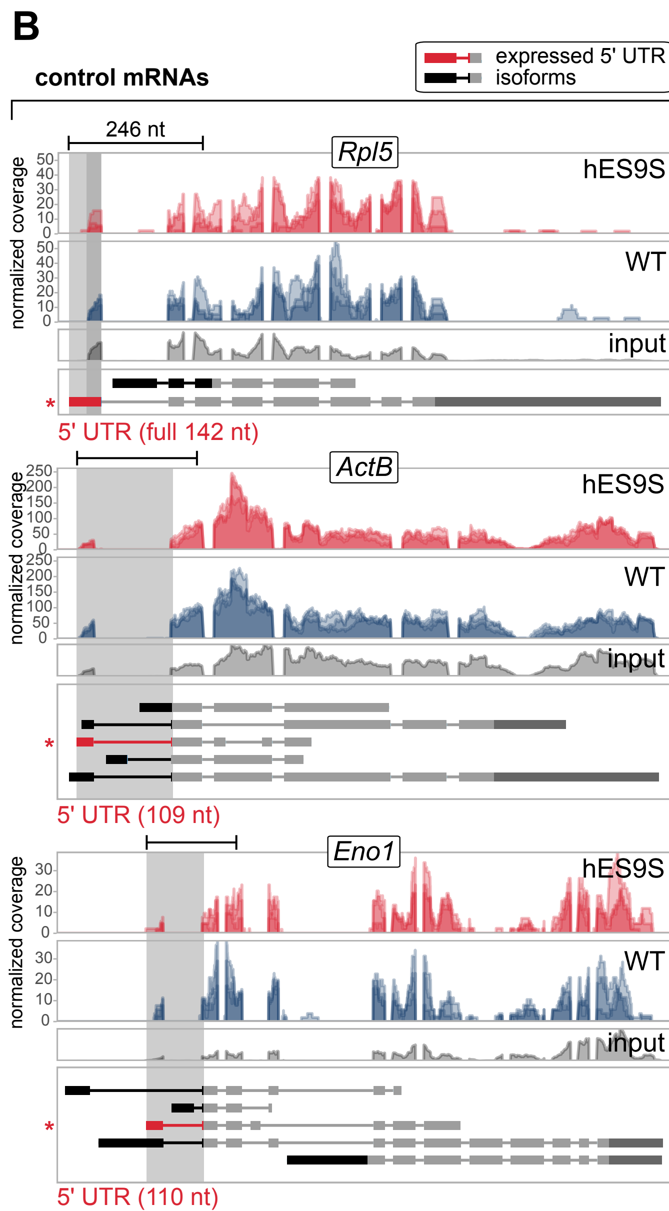
WT

input

normalized coverage

* 5' UTR (90 nt)





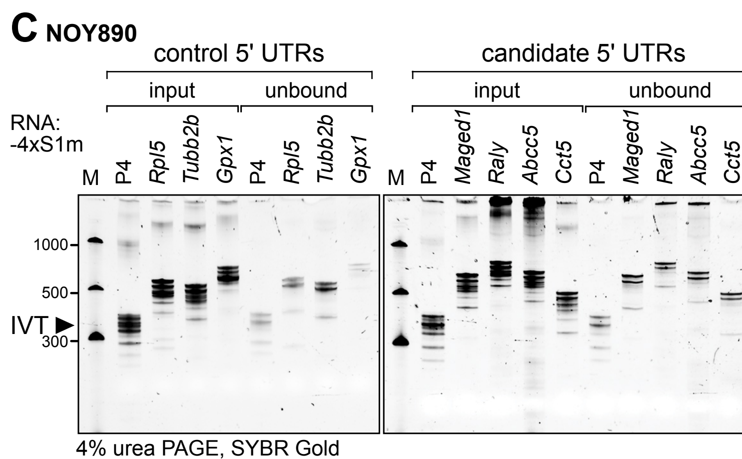


Figure S6. VELCRO-IP mRNA binding pattern and validation of hES9S-interacting 5' UTRs. Related to Figure 5, 6.

(**A**) mRNA binding profile as coverage plots for candidate hES9S-target genes whose 5' UTR-overlapping windows are significantly enriched in the hES9S over WT samples (FDR < 0.05, n = 3). The other five out of the total tested 14 genes not shown in **Figure 5C, D** are shown here. Normalized per base coverage of individual biological replicate libraries for WT (blue) and hES9S (red) samples is plotted (above). All mRNA isoforms annotated in ENSEMBL are displayed below. Exon lengths are to scale while intron lengths are pseudo-scaled. The read coverage of the input mRNA fragments (grey) are also plotted for reference. 5' UTR regions for the most likely expressed mRNA isoform in embryos is highlighted in red and the corresponding regions in the tracks is shaded in yellow. The 5' UTR region picked for further experimental validation corresponds to the asterisk-marked isoform. The mRNA fragment length for each gene is scaled according to the mRNA length for the individual genes presented. See also **Figure 5C**.

(**B**) The same analysis as in (A) was performed for the other three of total five control 5' UTRs where no enrichment of hES9S interaction over WT was found. 5' UTR regions for the most likely expressed mRNA isoform in embryos is highlighted in red and the corresponding regions in the tracks are shaded in gray. Corresponds to **Figure 5D**.

(**C**) A 4xS1m pulldown experiment with the focus on the comparison of full-length control and candidate hES9S-interacting 5' UTRs for their ability to bind to tagged-WT and tagged-humanized 40S subunits was performed. *In vitro* transcribed RNAs fused to 4xS1m aptamers were coupled to SA-sepharose beads for 4xS1m pulldown using WT and hES9S ribosome expressing yeast strains to generate cellular extracts as input. Coupled beads were incubated with cell extracts, washed and eluted using RNase A to release RNA-bound proteins. Input and unbound samples were taken before and after incubation of RNAs with beads. To monitor coupling efficiency, 10% of the input and unbound RNA fraction of each sample was resolved by 4% denaturing polyacrylamide/TBE/urea PAGE and visualized by SYBR Gold. Representative of n = 3 is shown. Low Range ssRNA Ladder (NEB) was loaded for reference. Corresponds to **Figure 6B**.

SUPPLEMENTAL TABLES

Table S1: Plasmids used in this study. Related to STAR Methods.

All plasmids used for *in vitro* transcription and mammalian transient transfection or yeast transformation are listed in the table.

Table S1. List of plasmids		
Plasmid	**Notes**	**Reference**
***In vitro* transcription constructs**		
pSP73	SP6 promoter, kindly provided by G. Stoecklin	Promega
pSP73-4xS1m	p2880, kindly provided by G. Stoecklin	(Leppek and Stoecklin, 2014)
pSP73-4xS1m(MCS)		(Leppek et al., 2020)
pSP73-a9(P4)-4xS1m(MCS)		(Leppek et al., 2020)
pSP73-Rpl5-4xS1m(MCS)		This study
pSP73-Tubb2b-4xS1m(MCS)		This study
pSP73-Gpx1-4xS1m(MCS)		This study
pSP73-Maged1-4xS1m(MCS)		This study
pSP73-Raly-4xS1m(MCS)		This study
pSP73-Abcc5-4xS1m(MCS)		This study
pSP73-Cct5-4xS1m(MCS)		This study
Mammalian cells		
Expression constructs		
pRF	SV40 promoter, kindly provided by D. Ruggero	
pRF-HCV IRES	kindly provided by D. Ruggero	
pRF-EMCV IRES	kindly provided by D. Ruggero	
pRF-a9-IRES FL		(Xue et al., 2015)
pRF-a9-P4-native		(Leppek et al., 2020)
pGL3-FLB-stop-TIE-native		(Leppek et al., 2020)
pGL3-FLB-stop-TIE-P4-native		(Leppek et al., 2020)
pGL3-FLB-stop-TIE-P4(M5)-native		(Leppek et al., 2020)
pRF-Abcc5	full-length 5' UTR, 199 nt	This study
pRF-Raly	full-length 5' UTR, 289 nt	This study
pRF-Cct5	full-length 5' UTR, 99 nt	This study
pRF-Maged1	184 nt most 3' of full-length 5' UTR, 184 nt	This study
pRF-Rpl18	full-length 5' UTR, 66 nt	This study
pRF-Hmgb2	full-length 5' UTR, 123 nt	This study
pRF-Pdcd5	full-length 5' UTR, 150 nt	This study
pRF-Fubp1	full-length 5' UTR, 74 nt	This study
pRF-Id1	full-length 5' UTR, 90 nt	This study
pRF-Hba-x	full-length 5' UTR, 264 nt	This study
pRF-Rab10	200 nt most 3' of full-length 5' UTR, 200 nt	This study
pRF-Sem1	full-length 5' UTR, 104 nt	This study

pRF-Hmgb1	full-length 5' UTR, 155 nt	This study
pRF-Rpl4	full-length 5' UTR, 56 nt	This study
pRF-Rpl5	full-length 5' UTR, 142 nt	This study
pRF-ActB	full-length 5' UTR, 109 nt	This study
pRF-Tubb2b	full-length 5' UTR, 121 nt	This study
pRF-Eno1	full-length 5' UTR, 110 nt	This study
pRF-Gpx1	full-length 5' UTR, 238 nt	This study
Yeast		
rDNA constructs		
pNOY373-18S25Stag	*LEU2, 2μ, Pol1-rDNA- tagged rRNA*	(Leppek et al., 2020)
pNOY373-18S25Stag-hES9S	*LEU2, 2μ, Pol1-rDNA- tagged rRNA-hES9S*	(Leppek et al., 2020)

Table S2: Yeast strains used in this study. Related to STAR Methods.

All yeast strains used and/or generated for this study are listed in the table.

Table S2. List of yeast strains

Strain	Genotype and Notes	Reference
KAY488 (NOY890)	*MATA ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 rdnaΔΔ::HIS3 carrying pRDN-hyg::URA3*	(Nemoto et al., 2010)
NOY890 WT rRNA	*MATA ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 rdnaΔΔ::HIS3 carrying pNOY373-WT rRNA::LEU2*	(Leppek et al., 2020)
NOY890 tagged-hES9S	*MATA ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 rdnaΔΔ::HIS3 carrying tagged pNOY373-rRNA-hES9S::LEU2*	(Leppek et al., 2020)
RPS2-FLAG	*MATA ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 rdnaΔΔ::HIS3 RPS2-FLAG::kanMX6 carrying pRDN-hyg::URA3*	This study
RPS2-FLAG WT rRNA	*MATA ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 rdnaΔΔ::HIS3 RPS2-FLAG::kanMX6 carrying pNOY373-WT rRNA-hES9S::LEU2*	This study
RPS2-FLAG tagged-hES9S	*MATA ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 rdnaΔΔ::HIS3 RPS2-FLAG::kanMX6 carrying pNOY373-tagged rRNA-hES9S::LEU2*	This study

Table S3: DNA Oligonucleotides used in this study. Related to STAR Methods.

All DNA oligonucleotides used for cloning, RT-PCR, and qRT-PCR are listed in the table. F, forward primer; R, reverse primer.

Table S3. DNA oligonucleotides

Name	Sequence	Description
	qPCR primer	
KL050	TGGAGAATAACTTCTTCGTGGA	Rluc qPCR F
KL051	TTGGACGACGAACTTCACC	Rluc qPCR R
KL052	AAGAGATACGCCCTGGTTC	Fluc qPCR F
KL053	TTGTATTCAGCCCATATCGTTTC	Fluc qPCR R
KL318	TGCAAACTCCTTGGTCACAC	y-UsnRNA1(SNR19) qPCR F

KL319	CAAACTTCTCCAGGCAGAAG	y-UsnRNA1(SNR19) qPCR R
KL320	CCATCATGAAGTGTGATGTC	y-*actin1* qPCR F
KL321	GACCTTCATGGAAGATGGAG	y-*actin1* qPCR R
	qPCR primer for rRNA detection	
KL300	CTAGGCGAACAATGTTCTTAAAG	pre-mature 25S rRNA F
KL301	GACCTCAAATCAGGTAGGAGTACCC	mature 25S rRNA F
KL302	CACCGAAGGTACACTCGAGAGCTTC	tagged 25S rRNA R
KL303	CACCGAAGGTACCAGATTTC	endogenous 25S rRNA R
KL304	GCTTGTTGCTTCTTCTTTTAAGATAG	pre-mature 18S rRNA F
KL305	TACAGTGAAACTGCGAATGGC	mature 18S rRNA F
KL306	ATCTCTTCCAAAGGGTCGAG	endogenous 18S rRNA R
KL307	CGAGGATTCAGGCTTTGG	tagged 18S R
	PCR primer for rRNA strain characterization and ES9S sequencing	
KL314	GAACGAGACCTTAACCTACTAAATAGT	ES9S-span RT-PCR F
KL315	AAACCGATAGTCCCTCTAAGAAGT	ES9S-span RT-PCR R
KL316	GCTAATACATGCTTAAAATCTCGA	18Stag-span RT-PCR F
KL317	TTTTTATCTAATAAATACATCTCTTCCAA	18Stag-span RT-PCR R
KL473	TCGATTCCGTGGGTGGTGG	18S rRNA-seq primer F
KL474	TAGCGCGCGTGCAGC	18S rRNA-seq primer R
	In vitro transcription DNA template primer	
KL414	GCCGATTTAGGTGACACTATA**G**AAGAGctctggttgctctgtggg	IVT SP6-TIE primer F
KL415	CGGCATAAAGAATTGAAGAGAGTTTTCAC	IVT Fluc primer R
	5' UTR-specific PCR primer	
KL433	gagcaagggtgatctggccgGAATTCCTTTTCCTGTGGGAGCAGCC	T-*Rpl4* Gibson F
KL435	gagcaagggtgatctggccgGAATTCAAGAGGCTGGGGATTGCGTTA	T-*Hmgb2* Gibson F
KL437	gagcaagggtgatctggccgGAATTCAACACCCCTCCTAAGGCC	T-*Hba-x* Gibson F
KL438	gagcaagggtgatctggccgGAATTCTCGTCTCTATGGTTGCGCC	T-*Sem1* Gibson F
KL441	gagcaagggtgatctggccgGAATTCTCTCATTGTACAACCTTTCTTCAACTTCTTGT	T-*Id1* Gibson F
KL444	gagcaagggtgatctggccgGAATTCGGATTCTGCGTCCTCTCGC	T-*Cct5* Gibson F
KL445	gagcaagggtgatctggccgGAATTCTTCCTTTCTTAGCAGTTAACCGAGAGC	T-*Fubp1* Gibson F
KL447	gagcaagggtgatctggccgGAATTCGATGCCTGAGCATCACTCGC	T-*Pdcd5* Gibson F
KL449	ATGTTTTTGGCGTCTTCCATGACGGGGAGAGGAGAAGG	T-*Rpl4* Gibson R
KL451	ATGTTTTTGGCGTCTTCCATGACGACGGCGCGG	T-*Hmgb2* Gibson R
KL452	ATGTTTTTGGCGTCTTCCATTGGGAGGAGCGGCTC	T-*Rab10* Gibson R
KL453	ATGTTTTTGGCGTCTTCCATGGTGGTGGTGGTGGTGA	T-*Hba-x* Gibson R
KL454	ATGTTTTTGGCGTCTTCCATCGCGCCGCGCC	T-*Sem1* Gibson R
KL457	ATGTTTTTGGCGTCTTCCATGATCCTGAGAACAGGCGGAG	T-*Id1* Gibson R
KL460	ATGTTTTTGGCGTCTTCCATGGTGGACGAACTAGAACGAGC	T-*Cct5* Gibson R
KL461	ATGTTTTTGGCGTCTTCCATACCCACGCTACAGCACAC	T-*Fubp1* Gibson R
KL463	ATGTTTTTGGCGTCTTCCATGGCGCGGCTGTCC	T-*Pdcd5* Gibson R
KL466	CTCGAATCACTAGTCAGCTGGAATTC	pRF-EcoRI F Gibson
KL469	ATGTTTTTGGCGTCTTCCAT	Fluc-R Gibson
KL472	CTCGAATCACTAGTCAGCTGGAATTCGCACGGGCGCCG	EcoRI-*Rab10*(200nt) Gib. F
KL529	CTCGAATCACTAGTCAGCTGGAATTCAATGTTACAGAGCGGAGAGAGTGAG	*Hmgb1* Gib F
KL530	CTCGAATCACTAGTCAGCTGGAATTCGCTCTTTCCCCGCCCA	*Rpl18* Gib F
KL532	CTCGAATCACTAGTCAGCTGGAATTCGTCAGTGCGGCGGG	*Raly* Gib F
KL533	CTCGAATCACTAGTCAGCTGGAATTCGATTCCCTTCGGTCTTGCG	*Abcc5* Gib F
KL534	CTCGAATCACTAGTCAGCTGGAATTCGGCGGAGAGGCGG	*Maged1* Gib F
KL536	ATGTTTTTGGCGTCTTCCATGTTTAGTTGATTTTCCTCCGCGAGG	*Hmgb1* Gib R
KL537	ATGTTTTTGGCGTCTTCCATGATGGCGCCTCCTGCT	*Rpl18* Gib R

KL539	ATGTTTTTGGCGTCTTCCATGGTGTTCACCAGTACCAAGAATGAG	*Raly* Gib R
KL540	ATGTTTTTGGCGTCTTCCATCTTCACCACACAGAGGACCA	*Abcc5* Gib R
KL541	ATGTTTTTGGCGTCTTCCATAGCTCTCGTCTCCCTGG	*Maged1* Gib R
KL554	CTCGAATCACTAGTCAGCTGGAATTCCAGCCACTCTTTCTCACGTCG	*Rpl5* Gib F
KL555	CTCGAATCACTAGTCAGCTGGAATTCAGTTAAAAGGAGGTGCAGGGCC	*Gpx1* Gib F
KL556	CTCGAATCACTAGTCAGCTGGAATTCCTCAGCCCGTAGCCCG	*Tubb2b* Gib F
KL557	CTCGAATCACTAGTCAGCTGGAATTCAGTGCTGCTCCGGTACAGG	*Eno1* Gib F
KL558	ATGTTTTTGGCGTCTTCCATCCTGCGGAATAGAGACCCG	*Rpl5* Gib R
KL559	ATGTTTTTGGCGTCTTCCATCTCGGTGTAGTCCCGGATC	*Gpx1* Gib R
KL560	ATGTTTTTGGCGTCTTCCATGGTGCCTGGTTAGCTTCTTGC	*Tubb2b* Gib R
KL561	ATGTTTTTGGCGTCTTCCATGGCGAATTTCTGGCAGTAGGATC	*Eno1* Gib R
KL562	CTCGAATCACTAGTCAGCTGGAATTCGCTCTTTCCCCGCCCACTCCGGCGCGGTTCCGTC	*Rpl18*-full Gib F
KL563	GTTTTTGGCGTCTTCCATGATGGCGCCTCCTGCTCGGCCAGGTCCGGAAAGACGGAACCG	*Rpl18*-full Gib R
KL565	CTCGAATCACTAGTCAGCTGGAATTCTATAAAACCCGGCGGCGC	*ActB* Gib F
KL566	ATGTTTTTGGCGTCTTCCATGGCGAACTGGTGGCG	*ActB* Gib R
	Hybrid ES9S sequences	
24 nt	CCTACTAAATAGT**GGTGCTAGCATTTGCTGGTTATCC**ACTTCTTAGAGG	Yeast WT ES9S
31 nt	CCTACTAAATAGT**TACGCGACCCCCGAGCGGTCGGCGTCCCCCA**ACTTCTTAGAGG	hES9S
16 nt	AAAGCCTGAATCCTCG	18S rRNA sequence tag
24 nt	GGTACTGAAGCTCTCGAGTGTACC	25S rRNA sequence tag