SUPPLEMENTARY MATERIALS AND METHODS

Biofluid samples

CSF was obtained from a patient with progressive supranuclear palsy at the Movement Disorder Center of the University Of California San Diego School Of Medicine, following approved procedures and protocols. The sample was centrifugated at 2,000 x g for 10 min at room temperature before freezing at -80 ºC and de-identified before use.

Blood and urine samples were collected at the Pasteur Institute of Montevideo and de-identified before use. Blood samples were obtained by venipuncture from a healthy donor in serum blood collection tubes and centrifuged at 2,500 rpm for 15 min to separate serum, which was stored at -20 ºC until use.

20 mL of a human urine sample was retrieved within 1 h of collection from a healthy donor in a sterile container. The sample was transferred to a 10 mL Falcon tube and centrifuged at 300 x g and 4 °C for 10 minutes, followed by centrifugation at 2000 x g and 4 °C for 20 minutes. The supernatant was then stored at -20 °C until use.

Cell culture

U2-OS cells were cultured in DMEM (Gibco) with 4.5 g/L D-glucose and 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) (Gibco), without antibiotics. For assays performed under serum-free conditions, cells were washed and incubated in MEGM (Lonza), without the inclusion of bovine pituitary extracts. When indicated, 2 µL (80 U) recombinant murine RNase inhibitor (RI, New England Biolabs) were added to fresh media.

Generation of stress-induced tRNA halves and intracellular RNA purification

For the generation of stress-induced tRNA halves (tiRNAs), 500 µM of freshly made sodium arsenite (Sigma-Aldrich) were added to U2-OS cells grown to 90% confluence in DMEM + 10% FBS. After 2 h at 37°C, the medium was removed and cells were washed with warm 1X PBS prior to RNA extraction using a Total RNA Miniprep Kit (NEB). Manufacturer's instructions were followed, except for two modifications: i) cell lysis was performed by adding 2 mL of lysis buffer directly to the cell monolayer, followed by a short (5 min) incubation at room temperature; and ii) twice the indicated volume of ethanol was added to the RNA sample after elution from the RNA-binding column.

Purification of intracellular ribosomes

Ribosomes were purified from U2-OS cells following published methods with modifications (1,2). Briefly, U2-OS cells were cultured in T75 flasks with DMEM supplemented with 10% FBS and no antibiotics. When cells reached 95-100% confluency, CCM was discarded and cells were washed three times with DPBS (without Ca^{2+} or Mg^{2+}) to remove any trace of RNAse activity. Cells were detached with a scraper, suspended in DPBS, and pelleted by centrifugation (10min, 500g, 4°C). Pelleted cells were gently resuspended in 2ml of mild lysis buffer (20 mM Tris-Cl, pH=7.4; 300mM NaCl; 6mM $MgCl_2$; EDTA-free protease inhibitor cocktail (Roche); 200 U murine RNase inhibitor; and 0.5% v/v IGEPAL CA-630 (Sigma-Aldrich)) and kept on ice for 25 min to release their cytoplasmic content. Unlysed cells, nuclei, and mitochondria were removed by sequential centrifugation at 750 x g and 12,500 x g for 10 min at 4°C. The remaining supernatant was layered on top of a 1 M sucrose cushion prepared in purification buffer (50 mM Tris-Cl, pH = 7.4; 150 mM KCl, 5 mM $MgCl_2$, 200 U murine RNase Inhibitor) and centrifuged for 3 h at 250.000 x g and 4 °C using a Sorvall Discovery M120 SE with a S100-AT6 rotor. After removing the supernatant, a translucent pellet containing purified ribosomes was clearly visible. The pellet was washed two times with 300 µl of ice-cold ultrapure RNase-free water, resuspended in ice-cold working buffer (50mM Tris-Cl, pH = 7.4; 25 mM KCl; 5 mM $MgCl_2$) and stored at -80 °C until use. Ribosome quality was assessed by RNA polyacrylamide gel electrophoresis under denaturing conditions.

RNA purification by solid phase extraction (SPE)

SPE was carried out using Monarch RNA Cleanup Kits (10 µg binding capacity columns, NEB) except for the purification of stress-induced tRNA-derived fragments from U2-OS cells, where the Total RNA Miniprep Kit (NEB) was used. Purification was carried out following manufacturer's instructions, but in several experiments, the volume of ethanol added to the binding buffer was doubled to avoid small RNA loss.

RNA decay assays and in vitro RNA digestion

To study the effect of proteins on rRNA stability, 25 µg total RNA (purified from U2-OS cells by TRIzol extraction) or 3 µg of rRNA present in purified ribosomes from U2-OS cells, were incubated in 600 µL RPMI containing 10% FBS, with or without the addition of 200 U of RI. 100 µL aliquots were taken at predetermined times, and total RNAs contained in these aliquots were extracted after mixing with 900 µL of TRIzol (ThermoScientific).

For determination of RNA half-lives in biofluids, 1 µg of heated and refolded (1 min at 90 °C followed by 30 min at room temp.) U2-OS total RNA was added to 50 µL undiluted human CSF, undiluted human urine, undiluted human serum, diluted human serum or FBS (both 10% in 1X PBS). Additionally, 1 µg of heated and refolded total RNA was incubated at 37 ºC for variable periods with recombinant human RNase 5 at 55 µg/mL (r-RNase 5; R&D Systems) or recombinant human RNase 1 at 0.5 µg/mL (r-RNase 1; Bon Opus

Bio). Reactions were stopped by the addition of SPE binding buffer and RNA cleanup. In all cases, samples were analyzed by northern blot.

For in vitro generation of nicked tRNAs and/or tDRs, 1 µg heated and refolded U2-OS total RNA was mixed with 40 µL of r-RNase 1 (Bon Opus Bio; diluted in PBS at 0.0625 µg/mL) for 15, 30 or 60 minutes at 37 °C. For RNA digestion in human CSF, 1 µg heated and refolded U2-OS total RNA was added to 10 µL of CSF, and incubated for 1 or 60 min at 37 °C.

Kinetic analysis of RNA decay

The half-lives of the RNA fragments in different environments were obtained by fitting a single exponential function or a model for an intermediate in consecutive first-order reactions to the intensities of the northern blot bands *vs* time (3). If the decay of the original full-length RNA band could not be observed within the first incubation minute, a half-life of < 6 s was assigned.

A single exponential model (Intensity = Amp*exp(-k*t) + Y, where Amp is the amplitude, t is time, k is the first-order rate constant and Y is an offset value) was fitted to the decay of the initial full-length RNA (when possible) or to the first degradation product (when the decay of the parent full-length RNA was immediate). Transient intermediates that build up and later fall were considered to follow an irreversible model A → B → C. A function (Intensity = (k1*Amp)/(k2-k1)*(exp(-k1*t)-exp(-k2*t)) + Y, where k1 is the first-order rate constant for the decay of the parent fragment A and k2 is the constant for the decay of fragment B, was fitted to the band intensities to obtain k2. Intensity = 0 at t = ∞ was included as a constrain.

Half-lives were calculated as $t_{1/2}$ = ln (2)/k. Errors were calculated based on the adjustment error while plotting and fitting together all available independent replicates of the experiment. OriginPro 8.6 was used to analyze the data.

Northern Blotting

For denaturing northern blots, 5 µL of RNA was mixed with 5 µL of loading buffer containing 95% formamide, 1 mM EDTA, 0.02% SDS, 0.02% bromophenol blue and 0.01% xylene cyanol, heated to 65 ºC for 5 min and run in 10 x 10 cm 10% polyacrylamide gels containing 7M urea in 1X Tris-borate EDTA (TBE, pH 8.4). For non-denaturing northern blots, the RNA samples were mixed with 1 µL of 6X native loading buffer and run in gels containing 1X Tris-borate (pH 8.3) and 10 mM $MgCl_2$ (TB + Mg^{2+} gels). Gels were run for 80 minutes in 0.5X TBE or 0.5X TB + Mg^{2+} running buffer at room temperature, stained with 1X SYBR gold (Invitrogen) and then transferred to positively charged nylon membranes (Roche) using a semi-dry Trans-Blot Turbo Transfer System (Bio-Rad) in 0.5X TBE at constant I = 0.3 A for 30 minutes.

Membranes were UV cross-linked and hybridized for 16 h at 42 °C with digoxigenin-labeled DNA probes in DIG Easy Hyb solution (Roche). After hybridization, membranes were washed for 5 minutes at room temperature with low stringency wash buffer (twice, 2X SSC / 0.1% SDS), 5 minutes at 42 °C with high stringency wash buffer (1X SSC / 0.1% SDS), blocked for 30 minutes at room temperature with 1X blocking solution (Roche) and probed for 30 minutes with an alkaline phosphatase-labeled anti-digoxigenin antibody (Roche). Membranes were washed twice with 1X TBS-T for 5 min and then incubated in detection buffer (Roche). Signals were then visualized with CDP-Star, ready-to-use (Roche) and detected using an Amersham ImageQuant 800 imager (GE Healthcare/Cytiva).

Probes (5' to 3'):

rRNA 28S 5': CACGTCTGATCTGAGGTCGC
rRNA 18S 5': ATGCTACTGGCAGGATCAAC
rRNA 5.8S: CGCACGAGCCGAGTGATCCAC
rRNA 5S 5': GGTGGTATGGCCGTAGAC
7SL: CACTACAGCCCAGAACTCCTGGACT
$tRNA^{Gly}_{GCC}$ 5': CTACCACTGAACCACCCATGC
$tRNA^{Lys}_{UUU}$ 5': CTGATGCTCTACCGACTGAGCTATCCGGGC
$tRNA^{Asp}_{GUC}$ 5': TCACCACTATACTAACGAGGA
$tRNA^{Glu}_{GUC}$ 5': TAACCACTAGACCACCAG
$tRNA^{Gly}_{GCC}$ 3': GCCGGGAATCGAACCCGGGCCTCCCGCG
$tRNA^{Gly}_{GCC}$/$^{Asp}_{GUC}$ ACL: TCCCGCGTGGCAGGCGAGAA
$tRNA^{Lys}_{UUU}$ ACL: CCTCAGATTAAAAGTCTGATG

All oligonucleotides were obtained from Integrated DNA Technologies (IDT, USA), and labeled with DIG Oligonucleotide Tailing Kit, 2nd generation (Roche), following manufactuer's instructions.

Stem-loop RT-qPCR for tDRs.

Specific cDNA of $tRNA^{Gly}_{GCC}$ 5' halves and miR-21-5p was obtained with SuperScript II (ThermoScientific) based on the stem-loop RT-qPCR method, as previously described (Tosar et al. 2018). Purified RNAs were heated at 90 °C for 1 minute and immediately placed on ice before reverse transcription. qPCR was performed using a QuantStudio 3 Real Time PCR System (ThermoScientific) with FastStart Universal SYBR Green Master (Rox; Roche). 2^{-Cq} values were obtained and normalized against the fraction containing the highest signal.

Primers (5' to 3')

Stem-loop RT primer (“X” denotes assay-specific 3’ overhangs):
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACXXXXXX
$tRNA^{Gly}_{GCC}$ (5' half, 35 nt, 3' overhang): GGCAGG
$tRNA^{Gly}_{GCC}$ (5' half, 30 nt, 3' overhang): GCGAGA
miR-21-5p (3' overhang): GTCAAC

$tRNA^{Gly}_{GCC}$ (5' half, F-primer): CCGCATTGGTTCAGTGGT
miR-21-5p (F-primer): gccccgTAGCTTATCAGACTGATGT
Universal reverse primer: GTGCAGGGTCCGAGGT

Lower case letters indicate added bases to increase melting temperature.

All primers were obtained from Integrated DNA Technologies (IDT, USA).

RT-PCR for full-length tRNAs.

2 µL of 10 ng/µL U2-OS total RNA (input) or RNase 1-treated RNAs (starting from an equivalent amount of input RNA) were mixed with 1 µL of 2 µM gene-specific RT primer and 1 µL of 10 mM (each) dNTP mix in a reaction volume of 11 µL, incubated for 5 min at 65 °C, and chilled on ice for at least 1 min. Annealed RNAs were mixed with 4 µL 5X SuperScript IV (SSIV, Thermo) buffer, 1 µL 0.1M DTT, 20 U RI and 20 U SSIV RT. The reaction was incubated for 10 min at 65 °C and then for 10 min at 80 °C. The cDNA was diluted 1/2 prior to qPCR or 1/10 for endpoint PCR.

Gene-specific RT primer for $tRNA^{Gly}_{GCC}$1-4: GCGTCTCACTTATGCACAGCGAACT**TGCATGGGCCGGG**
$tRNA^{Gly}_{GCC}$-1 (F-primer): GCATGGGTGGTTCAGTGGTA
Universal tRNA reverse primer: GTCTCACTTATGCACAGCGAA

RNA ligation assays

For ligation assays involving T4 RNA ligases, 5 µL of in vitro-digested RNA (or RNA purified from CCM) were incubated for 1 h at 37 °C in a 10 µL reaction containing 20 U of RI, 1 mM ATP, 1X T4 PNK reaction buffer, and desired enzyme combinations. Enzymatic cocktails included: 10 U T4 RNA ligase 1 (Rnl1, NEB) or T4 RNA ligase 2 (Rnl2, NEB) and/or 10 U of T4 PNK (wild-type or 3' phosphatase minus, NEB). As a control, RNA was heat-denatured before enzymatic treatment (1 min at 90 °C and immediately placed on ice).

For RtcB ligation, reaction mixtures (10 µL; 1 h at 37 °C) contained 5 µL of in vitro-digested RNA (with or without previous heat denaturation), 20 U of RI, 1X RtcB ligase buffer, 1 mM GTP, 1 mM Mn^{2+} and 1 µM RtcB ligase from *E. coli* (NEB). The reactions were stopped by addition of 2X RNA Loading Dye and analyzed by northern blot.

RNase R treatment

RNA from CCM treated with T4 PNK + T4 Rnl1 was purified by SPE. Reaction mixtures containing 10 µL of ligated RNAs with or without heat denaturation, 1X RNase R Reaction buffer and 20 U of RNase R (Lucigen), in a 20 µL final volume, were incubated for 1 h at 37 °C. Reactions were stopped by the addition of 100 µL of the RNA Binding buffer included in SPE kits.

Analysis of nonvesicular RNAs by density gradient separation

U2-OS cells were incubated with fresh complete medium (DMEM + 10% FBS, both Gibco) in a T150 culture flask until 90% confluency. Fresh medium was added containing or not 500 µM sodium arsenite (Sigma-Aldrich) for 2 hours. Cells were then washed with 1x DPBS (Gibco) and incubated in complete medium for 6 additional hours. The CCM was centrifuged at 300 x g and then at 2,000 x g at 4 ºC (20 min) and concentrated to 1 mL by ultrafiltration, using Vivaspin centrifugal filters (Sartorius) with a cut-off of 10 kDa. The CCM was then loaded on the bottom of a 12 – 36% iodixanol gradient prepared as described in Tosar et al. 2020 (4) and centrifuged overnight at 150,000 x g and 4 ºC (SW 40 Ti rotor, Beckman Coulter) using an Optima XPN ultracentrifuge (Beckman Coulter). 12 fractions of 1 mL were collected from the top of the tube. Fractions >7 are EV-depleted (Tosar et al. 2020; Jeppesen et al. 2019). RNA from a volume equivalent to 50% of each fraction was extracted by Trizol LS (ThermoScientific), following manufacturer's instructions, and analyzed by northern blot.

Fractionation of cell-conditioned media by SEC (IZON columns).

U2-OS cell-conditioned medium (CCM) obtained in MEGM + 80 U RI (1x T75 flask at 80% confluency, conditioning time = 24 h) was harvested, centrifuged at 2,000 x g, and concentrated to 500 µL by ultrafiltration, using Vivaspin centrifugal filters (Sartorius) with a cut-off of 10 kDa. The concentrated CCM was then fractionated using a 70 nm qEVoriginal column (IZON) and an IZON automatic fraction collector, using PBS as the mobile phase. All procedures were done at 4ºC. The columns were pre-chilled by running 1 column volume of chilled PBS before injecting the samples. Thirteen fractions of 1 mL were collected after the void volume had passed through the column. Fractions #1 and #2 contained most EVs and were analyzed in parallel for other projects. EV-depleted fractions #5 to #8 were isopropanol-precipitated, pooled, and the RNA was purified from these fractions by SPE.

Identification of nonvesicular nicked tRNAs in biofluids

A 200 µL healthy donor human serum sample was thawed and centrifuged at 2,000 x g and 4 °C for 10 min and diluted in 12 mL of PBS. EV depletion was performed by ultracentrifugation at 256,000 x g and 4 °C for 1 h in an Optima XPN ultracentrifuge (Beckman Coulter) with a SW 40 Ti rotor. The supernatant was then concentrated by ultrafiltration to 200 µL, using 10 kDa MWCO Amicon Ultra-15 Centrifugal filters (Merck). Then, 440 µL of the RNA Binding Buffer included in RNA Cleanup kits (NEB) was added to the concentrated supernatant and treated with 20 µL Proteinase K (QIAgen) for 30 minutes at 37 °C. Nucleic acids were then purified by SPE and eluted in 50 µL nuclease free H_2O (Invitrogen). An identical procedure was followed for CSF (input: 400 µL). Samples were analyzed by size-exclusion chromatography using an FPLC system.

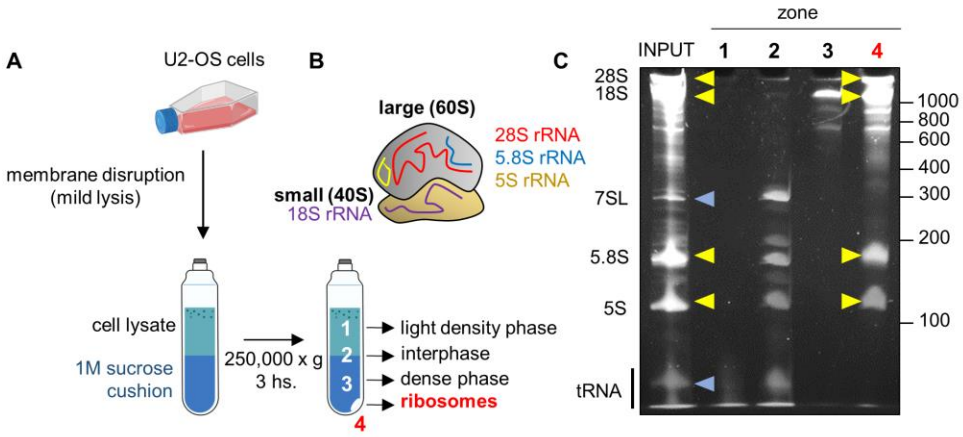
Size exclusion chromatography (FPLC)

Nonvesicular samples from human serum and CSF, or total RNA from U2-OS cells stressed with sodium arsenite (with or without heat denaturation and refolding) were diluted in 1X PBS (500 µL) and centrifuged at 10.000 x g and 4 °C for 10 min prior to injection into a Superdex 75 10/300 column (GE). Size-exclusion chromatography (SEC) was performed at 0.5 mL/min in 0.2 µm-filtered 1X PBS with an Äkta Pure FPLC system and 0.2 mL fractions were collected while monitoring the absorbance at 260 and 280 nm. Nucleic acids from selected fractions were ethanol-precipitated (700 µL absolute anhydrous ethanol; 100 µL 3 M NaAc, pH = 5.2; 0.5 µL Glycogen Blue) overnight at -20ºC and centrifuged at 12.000 x g and 4 ºC for 15 minutes. The pellet was washed with 500 µL 75% ethanol, centrifuged at 12.000 x g and 4 ºC for 15 minutes and resuspended in 10 µL of nuclease-free water (Invitrogen) before northern blot or stem-loop RT-qPCR.

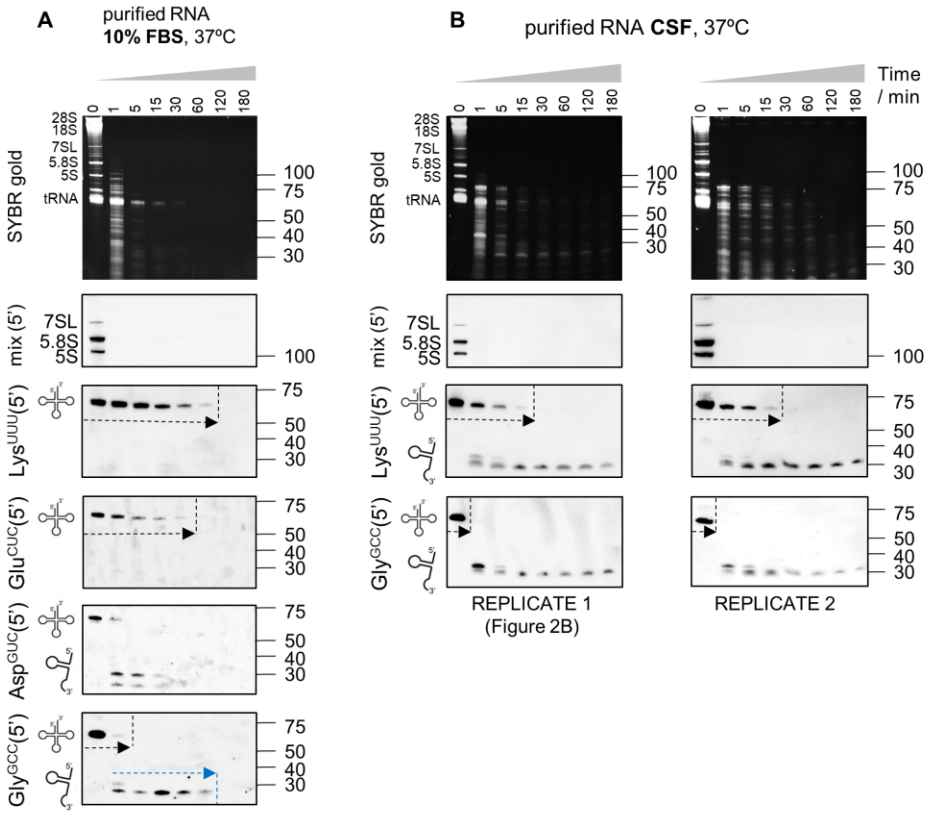
References

1. Khatter, H. *et al.* Purification, characterization and crystallization of the human 80S ribosome. *Nucleic Acids Res* **42**, e49–e49 (2014).
2. Belin, S. *et al.* Purification of Ribosomes from Human Cell Lines. *Curr Protoc Cell Biology* **49**, 3.40.1-3.40.11 (2010).
3. Espenson, J. Chemical Kinetics and Reaction Mechanisms 2nd Edition, (1994)
4. Tosar, J.P. *et al.* Fragmentation of extracellular ribosomes and tRNAs shapes the extracellular RNAome. *Nucleic Acids Res* **48**, 12874-12888 (2020).

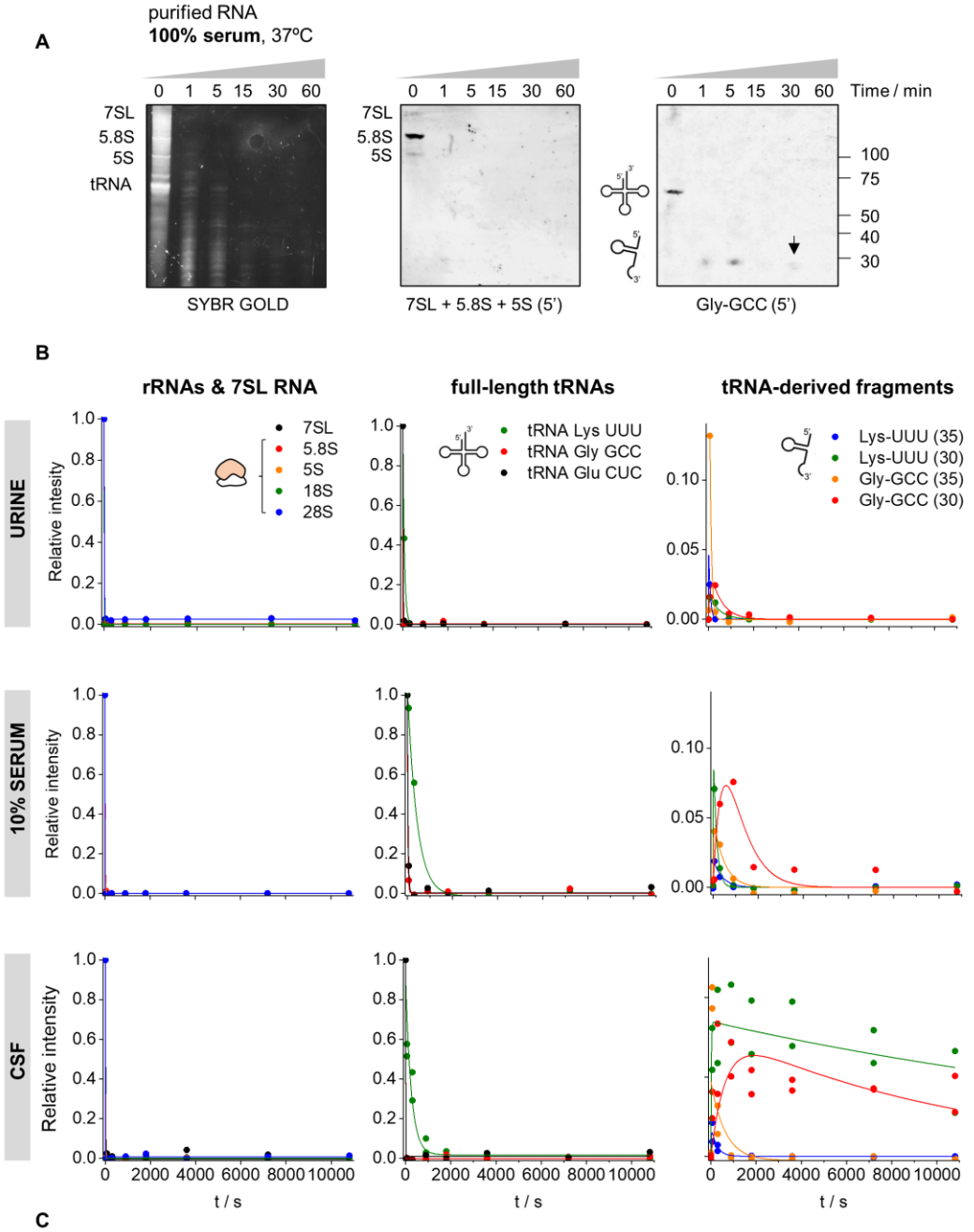
SUPPLEMENTARY FIGURES S1 – S8



Supplementary Figure 1: A) Purification of intracellular ribosomes. B) Schematic representation of an eukaryotic 80S ribosome with the different rRNA species associated with each ribosomal subunit. C) 7M Urea PAGE analysis of purified ribosomes (lane 4) vs. RNAs from the different fractions collected by ultracentrifugation (A). rRNA bands are shown in yellow.



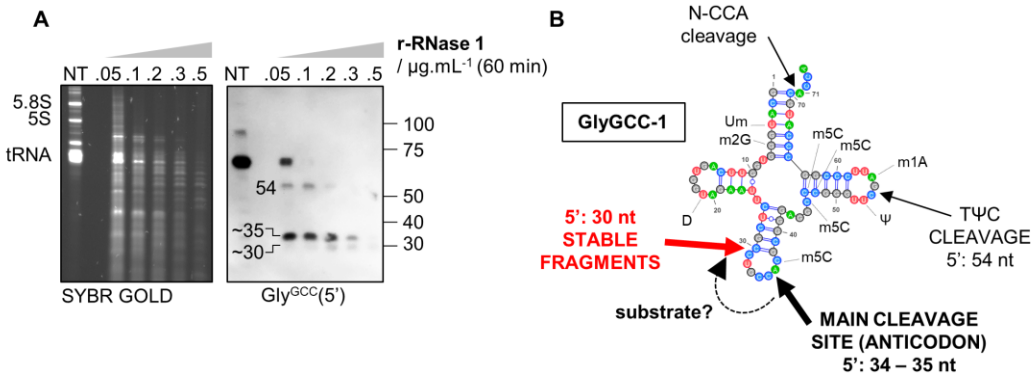
Supplementary Figure 2: A) Northern blots from Figure 2A, with the addition of data obtained with probes directed to the 5' end of $tRNA^{Glu}_{CUC}$ and $tRNA^{Asp}_{GUC}$. B) Reproducibility of results shown in Figure 2B. A different aliquot of CSF was thawed and RNA decay assays were performed using the same protocol, obtaining highly consistent results.



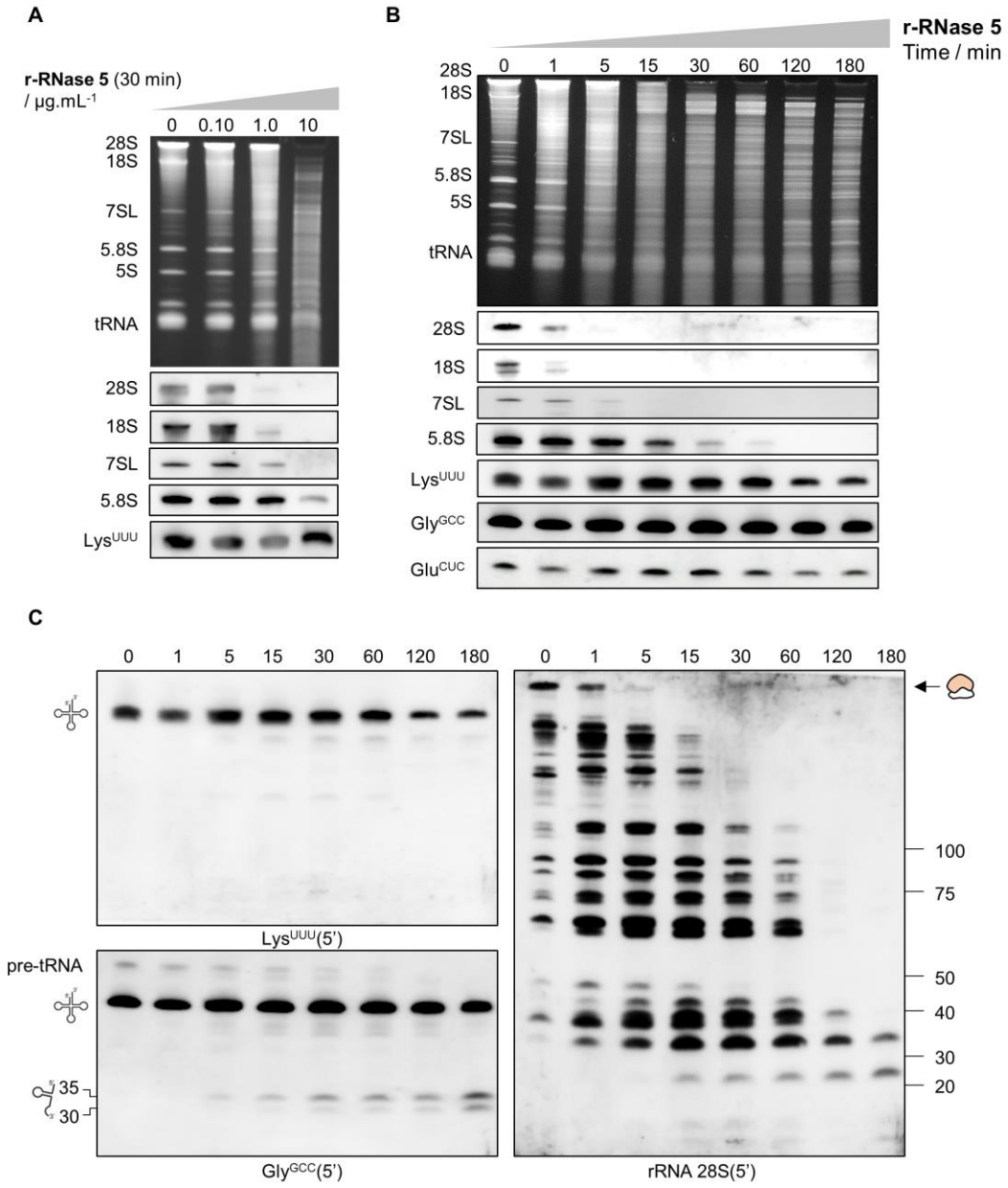
C

BIOFLUID	rRNA				tRNA			5' tRNA halves			
	5.8s	5s	18s	28s	LysUUU	GlyGCC	GluCUC	Lys (35)	Lys (30)	Gly (35)	Gly (30)
urine	< 6	< 6	< 6	< 6	49.5	< 6	< 6	< 6	319	52	341
10% serum	< 6	< 6	< 6	< 6	289	15	21	< 6	103	332	481
CSF	< 6	< 6	< 6	< 6	174	< 6	< 6	12.8	17953	439	7529

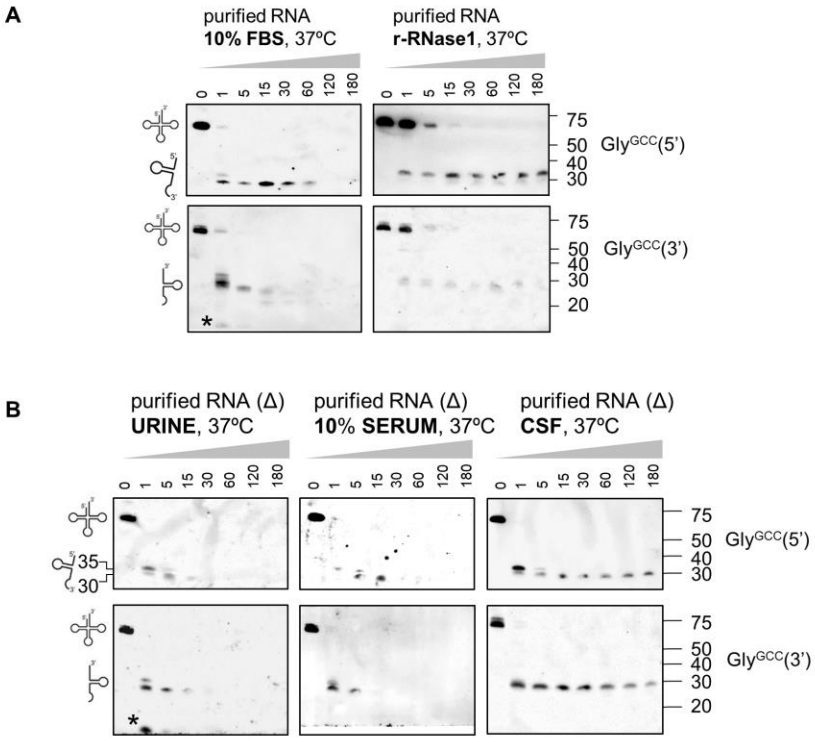
Supplementary Figure 3: A) RNA decay in undiluted human serum. Time = 0 corresponds to RNAs added to serum and immediately placed on ice. B) Kinetic modeling of RNA decay in human biofluids was done according to the procedure described in supplementary methods. Single exponential decay or transient intermediate models were fitted to band intensities obtained from Figure 2 and Figure S2 to determine half-lives for different RNA species incubated in different biofluids. C) Half-lives (in seconds) obtained from kinetic modeling (B), as explained in supplementary methods.



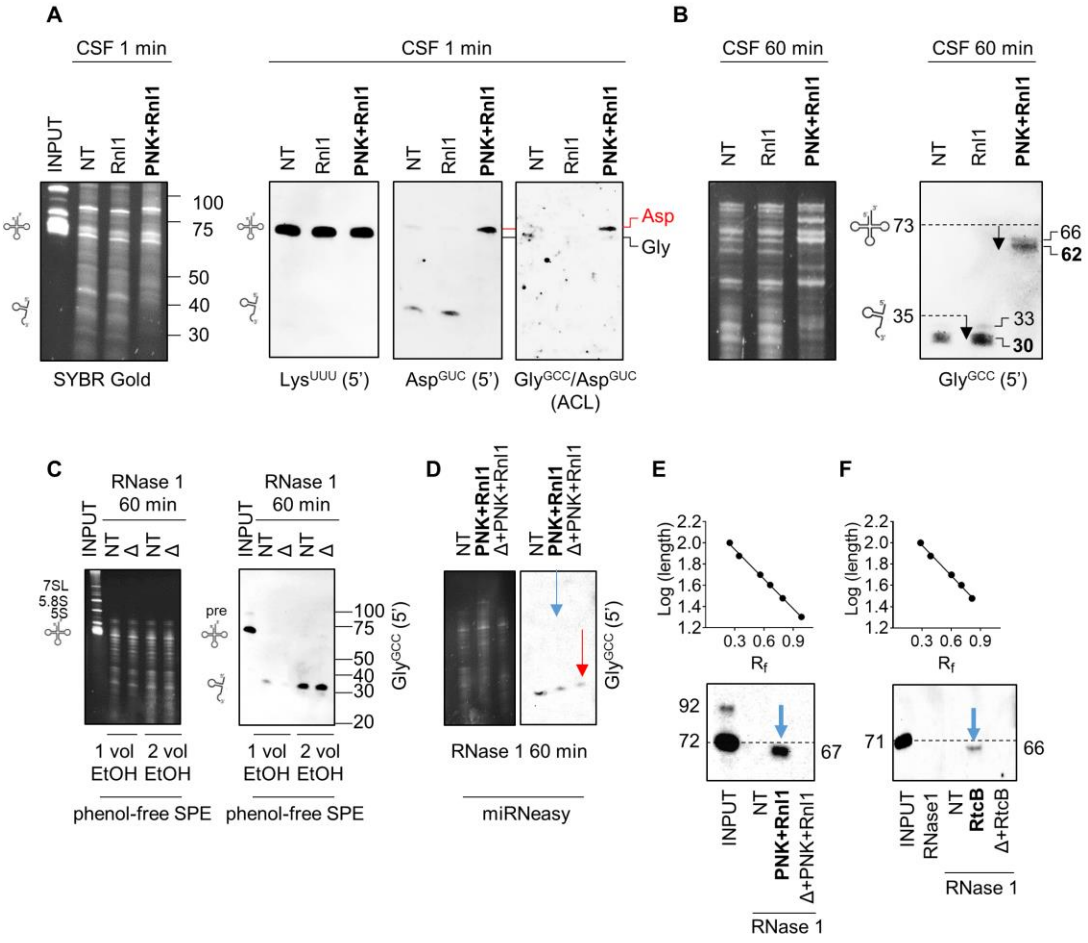
Supplementary Figure 4: A) Northern blot using a probe complementary to the 5' end of $tRNA^{Gly}_{GCC}$ after exposure of TRIzol-purified total U2-OS RNA (NT) to different concentrations of recombinant human RNase 1 at 37ºC for 60 min. B) Cloverleaf diagram of human $tRNA^{Gly}_{GCC}$-1 showing modified bases as described in the modomics database (http://genesilico.pl/modomics/) and predicted cleavage sites based on data presented in this study. It is not clear from our results whether cleavage sites at position 30 and 34 – 35 are independent, or whether cleavage at position 34 – 35 is a requisite for efficient cleavage at position 30.



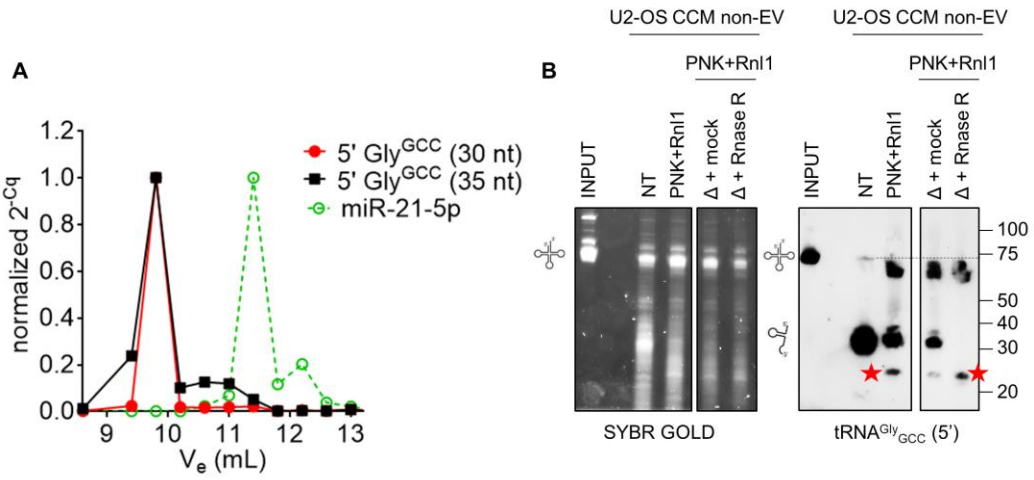
Supplementary Figure 5: A) Northern blot analysis of different full-length transcripts after incubating total U2-OS RNA with increasing concentrations of recombinant human RNase 5, for 30 min at 37ºC. B) Same as (A), but the concentration of RNase 5 was fixed and the analysis was performed by varying the incubation time. C) Expanded blots from (B), to show the generation and decay of tRNA-derived fragments (left) and 28S rRNA-derived fragments (right).



Supplementary Figure 6: Northern blots using probes complementary to the 5' and 3' halves of $tRNA^{Gly}_{GCC}$, corresponding to the assays shown in Figure 2, A (A) and Figure 2, B (B).



Supplementary Figure 7: A-B) incubation of purified RNA in CSF for 1 min (A) or 1 h (B) at 37ºC, followed by enzymatic repair of nicked tRNAs. The northern blot band for $tRNA^{Lys}_{UUU}$ serves as a loading control, because $tRNA^{Lys}_{UUU}$ is not significantly degraded by short incubations in CSF. The repair of $tRNA^{Asp}_{GUC}$ (A, red) is analyzed by using either a 5' (center) or an anticodon-targeting probe (right). NT: fragmented RNA not treated with the enzymatic repair cocktail. C) purification of RNAse1-treated RNA (with or without heat denaturation, Δ) by SPE, following manufacturer's instructions (1 vol EtOH) or duplicating the volume of ethanol added to the binding buffer (2 vol EtOH). D) Enzymatic repair (PNK + Rnl1) of RNase-1 treated RNA purified with the miRNeasy kit. E) Determination of the size of the repaired products in Figure 4, B, based on the R_f method. Migration of the small RNA ladder was used to construct the calibration curve. F) Same as (D), but for Figure 4, D.



Supplementary Figure 8: A) Data from Figure 5, C, but overlapping the signal obtained when using primers specific for $tRNA^{Gly}_{GCC}$ 5' fragments of exactly 35 nt (black). B) A replicate of the assay shown in Figure 6, B, but the ligated RNA was now heated after PNK + Rnl1 treatment (Δ), cooled down to room temperature, and incubated with RNase R to degrade single-stranded linear and unstructured RNAs. Red stars: short, RNase R-resistant ligation products, presumably corresponding to circularized single-stranded 5' $tRNA^{Gly}_{GCC}$ halves.