Supplementary Materials

Poly(A)-specific ribonuclease regulates processing of small subunit rRNA in human cells

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Running Title: PARN and ribosome biogenesis

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EXPERIMENTAL PROCEDURES

Antibodies

The antibody sources and dilution ratios used in this study were as follows: rabbit polyclonal anti-PARN (Abcam, 27778; 1:2,000 for immunoblot analysis [IB], 1:250 for immunofluorescence staining [IF]), goat polyclonal anti-Bystin (Santa Cruz Biotechnology, 1:500 for IB), mouse monoclonal anti-Bystin (A-10) (Santa Cruz Biotechnology, 1:1,000 for IB), rabbit polyclonal anti-RIO2 (Bethyl Laboratories, 1:2,000 for IB), mouse monoclonal anti-FLAG (M2) (Sigma, 1:2,000 for IB), mouse monoclonal anti-Myc (4A6) (Upstate, 1:2,000 for IB), rabbit polyclonal anti-NOB1 (GeneTex, 1:2,000 for IB), mouse monoclonal anti-RPS3 (82-K) (Santa Cruz Biotechnology, 1:1,000 for IB), mouse monoclonal anti-beta-Actin Antibody (AC-15) (Santa Cruz Biotechnology, 1:2,000 for IB), mouse monoclonal anti-CKIe (4D7) (Santa Cruz Biotechnology, 1:1,000 for IB), mouse monoclonal anti-CKId (E-10) (Santa Cruz Biotechnology, 1:1,000 for IB), rabbit polyclonal anti-TSR1 (Abcam, 1:500 for IB), mouse polyclonal anti-UTP14A (Abnova, H00010813-B01P; 1:1,000 for IB), rabbit polyclonal anti-PNO1 (D-14) (Santa Cruz Biotechnology, 1:1,000 for IB), rabbit polyclonal anti-RRP12 (E-14) (Santa Cruz Biotechnology, 1:1,000 for IB), rabbit polyclonal anti-NOB1 (GeneTex, 1:2,000 for IB), rabbit polyclonal anti-EXOSC10 (Sigma, 1:1,000 for IB), rabbit polyclonal anti-NOC4L (G-22) (Santa Cruz Biotechnology, 1:1,000 for IB), rabbit polyclonal anti-FBL (H-140) (Santa Cruz Biotechnology, 1:1,000 for IB), mouse monoclonal anti-FBL (Cytoskeleton, 1:2,000 for IF), mouse monoclonal anti-UBF (F-9) (Santa Cruz Biotechnology,1:50 for IF), rabbit polyclonal anti-B23 (C-19) (Santa Cruz Biotechnology, 1:1,000 for IB), mouse monoclonal, anti-B23 (Zymed, 1:400 for IF) and mouse monoclonal anti-GAPDH (6C5) (Ambion, 1:10,000 for IB). For immunofluorescence staining, the following secondary antibodies were used: FITC-conjugated anti-rabbit IgG (American Qualex, 1:500), Cy3-conjugated anti-mouse IgG (Sigma, 1:500) and Cy3-conjugated anti-rabbit IgG (Sigma, 1:500). For immunoblotting following secondary antibodies were used: HRP-linked anti-mouse IgG (Cell Signaling Technology, 1:10,000), HRP-linked anti-rabbit IgG (Cell Signaling Technology, 1:10,000), Anti-Goat IgG-Peroxidase antibody (Sigma, 1:10,000), alkaline phosphatase conjugated anti-mouse IgG (Cell Signaling Technology, 1:10,000) and alkaline phosphatase conjugated anti-rabbit IgG (Cell Signaling Technology, 1:10,000).

Construction of Epitope-tagged Expression Plasmids—For pcDNA3.1(+)-HF construction, 5’-GAAGAAGATATCGGGTTCTGGTGCCTACCCATATGACGTCCCGGACTACGCCGGATCTG GAGATTACAAGGATGACGACGATAAGTAACTCGAGTTG-3’ and 5’-CAACTCGAGTTACTTATCGTCGTCATCCTTGTAATCTCCAGATCCG GCGTAGTCCGGGACGTCATATGGGTAGGCACCAGAACCCGATATCTTCTTC-3’ oligonucleotides were annealed, and was ligated into the *EcoR*V/*Xho*I sites of pcDNA3.1(+) vector. For the construction of pcDNA3.1(+)-HEF vector to express N-terminal HEF tagged protein, 5’-GAAGAAGCTAGCCACCATGGGATACCCATATGACGTCCCGGACTACGCCGGTTCTGGT GAGAATTTGTACTTCCAGGGTTCTGGTGCCGATTACAAGGATGACGACGATAAGGGTTCTG GTGCCAAGCTTGAAGAA-3’ complementary oligonucleotide was synthesized with four oligonucleotides, 5’-GAAGAAGCTAGCCACCATGGGATACCCATATGACGTCCCGGACTACGCC-3’, 5’-GAACCCTGGAAGTACAAATTCTCACCAGAACCGGCGTAGTCCGGGACGT-3’,

5’-GAATTTGTACTTCCAGGGTTCTGGTGCCGATTACAAGGATGACGACGAT-3’ and 5’-TTCTTCAAGCTTGGCACCAGAACCCTTATCGTCGTCATCCTTGTAATCG-3’ by PCR based extension. The synthesized oligonucleotide was ligated into *Nhe*I/*Hind*III sites of pcDNA3.1(+) vector. For the construction of pcDNA3.1(+)-HEF vector to express C-terminal HEF tag fusion protein, four oligonucleotide, 5’-GAAGATATCGGGTTCTGGTGCCGATTACAAGGATGACGACGATAAGG-3’, 5’-TGGAAGTACAAATTCTCGGCACCAGAACCCTTATCGTCGTCATCCTT-3’, 5’-CCGAGAATTTGTACTTCCAGGGTTCTGGTGCCTACCCATATGACG-3’ and 5’-CAACTCGAGTTAGGCGTAGTCCGGGACGTCATATGGGTAGGCA-3’, were used to synthesize 5’-GAAGATATCGGGTTCTGGTGCCGATTACAAGGATGACGACGATAAGG GTTCTGGTGCCGAGAATTTGTACTTCCAGGGTTCTGGTGCCTACCCATATGACGTCCCGGA CTACGCCTAACTCGAGTTG-3’ complementary oligonucleotide. The oligonucleotide was ligated into *Eco*RV/*Xho*I sites of pcDNA3.1(+) vector. For pcDNA5-FRT/TO-Myc construction, we ligated the annealed 5’-GAAGATATCGGAACAAAAACTCATCTCAGAAGAGGATCTGCTCGAGTGACATATGCTCG AGCATCATCACCATCACCATGTCTAGAGAA-3’ and 5’-TTCTCTAGACATGGTGATGGTGATGATGCTCGAGCATATGTCACTCGAGCAGATCCTCTT CTGAGATGAGTTTTTGTTCCGATATCTTC-3’ oligonucleotides first into the *EcoR*V/*Xba*I sites of pcDNA3.1(+) vector (pcDNA3.1(+)-Myc vector), cut out the DNA fragment encoding Myc-tag sequence with *Eco*RV/*Apa*I from the pcDNA3.1(+)-Myc vector, and ligated the fragment into *Eco*RV/*Apa*I sites of pcDNA5-FRT/TO. All constructs were verified by DNA sequencing.

Construction of Epitope-tagged Expression Plasmids—RT-PCR was performed with the Super Script II kit (Invitrogen) according to the manufacturer’s instructions using total mRNA as template that was prepared from 293EBNA cells (Invitrogen). LTV1 (NM_032860) cDNA, Bystin (NM_004053) cDNA, PARN (NM_002582) cDNA, were amplified by PCR using primer sets 5’-GAAGAAAAGCTTCACCATGGGACCTCACAGGAAGAAAAAGCC-3’ and 5’-CAAGAATTCCTAGCTTTAGACCCTCAACATTC-3’ for LTV1, 5’-GAAGAAAAGCTTCACCATGGGACCCAAATTCAAGGCGGCCCGT-3’ and 5’-CAAGAATTCCCTCCACGGTGATGGGAACATCT-3’ for Bystin, and 5’-GAAGAAAGATCTCACCATGGAGATAATCAGGAGCAATTTTAAGA-3’ and 5’-CAACTCGAGCCCGGGCCCCATGTGTCAGGAACTTCAAAGA-3’ for PARN. The PCR products were cloned into the *Hind*III/*Eco*RI sites of pcDNA3.1(+) for LTV1 and Bystin or into the *Bam*HI/*Xho*I sites for PARN cDNA. All cloned cDNAs were verified by DNA sequencing. The cloned cDNAs were sub-cloned into pcDNA3.1(+)-HF or pcDNA3.1(+)-HEF vector to fuse DNA fragment coding epitope tag sequence. DNA fragment coding tagged protein sequence was cut out and ligated into pcDNA5-FRT/TO vector. N-terminally tagged proteins were denoted as HEF-[protein name], and C-terminally tagged proteins were denoted as [protein name]-HF or HEF, respectively.

For tag fusion, Cloned LTV1 was cut out with *Hind*III/*Eco*RV, then ligated into the sites of *Hind*III/*Eco*RV of pcDNA3.1(+)-HF or pcDNA3.1(+)-HEF vector. The tag fused protein coding sequence was cut out with *Hind*III/*Xho*I and then ligated into the sites of *Hind*III/*Xho*I of pcDNA5-FRT/TO to make pcDNA5-FRT/TO-LTV1-HF or LTV1-HEF.

To make pcDNA5-FRT/TO-Bystin-HEF, firstly LTV1 coding sequence was removed from pcDNA5-FRT/TO-LTV1-HEF with *Hind*III/*Eco*RV, leaving tag coding sequence and then this vector was ligated with Bystin coding sequence, cut out from pcDNA3.1(+)-Bystin with *Hind*III/*Eco*RV.

To make pcDNA-FRT/TO-HEF-PARN, pcDNA3.1(+)-PARN was amplified by PCR with primer sets 5'-GAAGAAAGATCTATGGAGATAATCAGGAGCAATTTTAAGA-3' and 5'-CAACTCGAGTTACCATGTGTCAGGAACTTCAAAGA -3' for adjust frame and insert termination codon. Amplified sequence was ligated into pcDNA3.1(+)-HEF vector for N-terminally HEF tag fusion. Then pcDNA5-FRT/TO vector and pcDNA3.1(+)-HEF-PARN were cut with *Hind*III and *Nhe*I respectively, and klenow fill-in reaction was performed to convert protruding end to blunt end, and then cut with *Xho*I, and then HEF-PARN fragment and vector was ligated to make pcDNA-FRT/TO-PARN.

To make pcDNA5-FRT/TO-PARN-Myc, PARN coding sequence was cut out from pcDNA3.1(+)-PARN with *Kpn*I/*Sma*I, then the fragment was ligated into *Kpn*I/*Eco*RV sites of pcDNA5-FRT/TO-Myc vector. All constructs were verified by DNA sequencing.

FLAG based Immunoprecipitation— 48 hour after 1 μg/ml doxycycline addition, doxycycline inducible epitope tagged protein expression cells were harvested from three 90-mm dishes, once washed with PBS and lysed by vigorous vortexing for 30 s in 1 ml lysis buffer (50 mM Tris-HCl pH 8.0, containing 150 mM NaCl, 0.5% IGEPAL-CA630 containing 1mM PMSF), and incubated on ice for 30 min. The soluble fraction was obtained by centrifugation at 20,000 × *g* for 30 min at 4°C. Total cell lysate was incubated with 10-15 μl anti-FLAG M2 agarose beads for 3 hour at 4°C. After washing the agarose beads five times with 1 ml lysis buffer and once with 50 mM Tris-HCl pH 8.0, containing 150 mM NaCl, the complexes bound to the agarose beads were eluted with 500 μg/ml FLAG peptide. The eluted complexes were submitted to further experiment.

Cell Fractionation immunoprecipitation —Sub-confluent T-Rex-293 cells, inducible LTV1-HEF or Bystin-HEF expression (Flp-In T-Rex-293) cells, were collected from 140-mm dish, lysed by vortexing for 10 sec with 500 μl buffer A (16.7 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF) containing 0.1% Triton X-100, incubated for 5 min on ice, and centrifuged at 1,200 × *g* for 5 min. The supernatant was collected and pellet was once again suspended in 500 μl buffer A, then vortexing for 10 sec and centrifuged at 1,200 × *g* for 5 min. The supernatant was collected and merged with precedent supernatant and treated as the cytoplasmic fraction. NaCl concentration of cytoplasmic fraction was adjusted by 5M NaCl to become 150 mM. On the other hand, pellet was lysed with 1 ml Buffer B (16.7 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF) containing 0.1% Triton X-100, incubated for 20 min on ice. Cytoplasmic fraction and suspension of pellet was centrifuged at 20,000 × *g* for 30 min at 4°C and obtained supernatant was treated as cytoplasmic and nuclear fraction, respectively. Each fraction was subsequently submitted to FLAG based immunoprecipitation assay described above.

Protein and RNA separation from ribonucleoprotein complex—Eluate from two-step

immunoprecipitation (HF) or one-step immunoprecipitation (FLAG) was added with equal volume of 2x RNA extraction solution and phenol/chloroform (pH 8.0) and vigorously vortex mixed. Then aqueous phase and organic phase was separated with centrifugation at 20,000 x g for 30 min at 4°C. Aqueous phase containing RNA was collected to another tube and equal volume of 2-propanol was added. Four volume of 2-propanol was added to organic phase containing protein to become monophasic. RNA and protein were precipitated with centrifugation at 20,000 x g for 30 min at 4°C, respectively. Then the precipitates were rinsed with 75% ethanol, and air-dried. RNA and protein were submitted to further analysis.

Immunoblotting of proteins—Precipitated protein were dissolved in sample buffer then separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were blocked with 5% non-fat dried skim milk in PBS at 4°C at least for 1 hour, and incubated with the appropriate primary antibody overnight at 4°C. The membranes were washed three times with TBST (TBS containing 0.1% (w/v) Tween 20) for 10 min, incubated with secondary antibody conjugated with alkaline phosphatase for 1 hour at room temperature, washed three times with TBST for 10 min, and washed once with TBS for 5 min. Staining was performed in NBT (nitro-blue tetrazolium chloride) / BCIP (5-bromo-4-chloro-3'-Indolylphosphatase *p*-toluidine salt) solution, prepared by a (1:50) dilution of NBT/BCIP stock solution with alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$).

Northern blotting—Precipitated RNA from ribonucleoprotein complex or total RNA from whole cells were analyzed by non-RI based northern blotting method. Total RNA was isolated from whole cells using the RNAgent total RNA isolation system (Promega) according to the manufacturer's instructions. Without any mention, 2.5 μg of total RNA was loaded per lane. The RNA was separated on a 0.8% agarose/formaldehyde gel and electrophoresed in MOPS running buffer at 4 V/cm for 2.5 hour for analysis of large rRNAs, and a 9% polyacrylamide/7.5 M urea gel in 0.5x TBE running buffer at 12.5 V/cm for 2.5 hour for small RNAs. Large RNA separated by agarose was capillary transferred in 20x SSC buffer and small RNA separated by PAGE was electrotransferred using semi-dry blotting apparatus with 0.5x TBE buffer to Hybond-N+ membrane (GE Healthcare). Blotted membrane was dried and UV-cross linked then blocked with pre-hybridization buffer (containing 5X SSC, 20 mM NaH2PO4 pH 7.5, 7% SDS, 2X Denhardt's Solution and 40 μg/ml sheared salmon sperm DNA) at least for 1 hour. After blocking, oligonucleotide end labeled with biotin was added to each blot, and then hybridized overnight at 50°C. Then, the membrane was washed twice with Non-Stringent Wash Solution (3X SSC, 25 mM NaH2PO4 pH 7.5, 5% SDS) for 15 min, and washed twice with Stringent Wash Solution (1X SSC, 1% SDS) for 15 min. Membrane was submit to subsequent chemiluminescent based method with chemiluminescent nucleic acid detection module (Thermo Fisher scientific), and detected with LAS4000 luminescent image analyzer (FUJI FILM).

Biotin end labeling with 16-dUTP—Following oligonucleotides were end labeled with biotin using Biotin 3' End DNA Labeling kit (Pierce); 18S probe (5'-GGCGACTACCATCGAAAGTTGATAG-3'), ITS1 probe (5'-AACGCGCTAGGTACCTGGACGG-3'), 5.8S probe (5'-AGACAGGCGTAGCCCCGGGAGGAA-3'), 18S probe

(5'-GGCGACTACCATCGAAAGTTGATAG-3'), 28S probe (5'-TTCGGAGGGAACCAGCTACTAGAT-3'), 5'ITS1 probe (5'-CCTCGCCCTCCGGGCTCCGTTAATTGATC-3') (1), U3 probe (5'-ATCATCAATGGCTGACGGCAGTTG-3'), 7SK probe (5'-AGGCAGACTGCCACATGCAG-3'), U6 probe (5'-ACGAATTTGCGTGTCATCCTTGCG-3'), 5S probe (5'-TTCCGAGATCAGACGAGATCGG-3'), 18S-3' probe (1837-1856 of 18S rRNA: 5'-AGGTTCACCTACGGAAACCT-3').

Fluorescent in situ hybridization (FISH)—FISH was performed as described (1). In brief, HeLa cells were seeded on collagen coated culture slide, and fixed with 4% formaldehyde in PBS, washed twice with PBS and permeabilized for 16 hour at 4°C in 70% ethanol. After washing the cells two times with 2x SSC, 10% formamide Rehydrate, hybridization was performed for 3 hour at 37°C in hybridization solution consisting of 10% formamide, 2x SSC, 0.5 mg/ml yeast tRNA, 10% dextran sulfate, 50 mg/ml BSA and 10 mM ribonucleoside vanadyl complexes containing 0.5 ng/ml of 3'-Cy3 labeled 5'ITS1 (5'-CCTCGCCCTCCGGGCTCCGTTAATGATC-3') oligonucleotide. The slides were then washed twice with 2x SSC, 10% formamide and once with PBS. If required, following immunofluorescent staining was performed and then mounted with Vectashield. The resulting slides were observed with an Axiovert 200 M microscope (Carl Zeiss, Germany).

Immunofluorescence Staining—Cells were washed with PBS followed by fixation with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing twice with PBST (0.05% w/v Tween 20), the cells were permeabilized with PBS containing 0.1% (w/v) Triton X-100 for 5 min at room temperature and washed once with PBST. The cells were then blocked with 3% (w/v) non-fat dried milk in PBS for 1 h, and incubated with the appropriate primary antibody for 1 hour at room temperature. The cells were washed three times in PBST for 10 min and then incubated with fluorochrome-conjugated secondary antibody for 1 hour at room temperature. The cells were washed three times in PBST for 10 min and then mounted with Vectashield. The resulting cells were examined with an Axiovert 200 M microscope (Carl Zeiss, Germany).

Proliferation Assay—CFDA (carboxyfluorescein diacetate, succinimidyl ester) is a cell permeable fluorochrome, emits its fluorescent after cleavage of intracellular esterase. CFDA also reacts with intracellular amines (e.g., proteins), and is retained in the cell unless cells are proliferated. After the cell division, this fluorochrome is diluted, so that highly proliferative cells show weaker staining than quiescent ones. At 24 hours after stealth siRNA transfection, 293 EBNA were labeled with CFDA according to manufacture's instruction. The cells were cultivated further for 48 hours, and submitted to immunofluorescence staining as described above.

Salt sensitivity of LTV1 associated pre-40S complex— The complexes bound to the anti FLAG conjugated agarose beads were incubated buffer containing 50 mM Tris-HCl pH 8.0 and indicated concentration of NaCl incubated for 30 min on ice. Then the beads were washed twice with lysis buffer and once with 50 mM Tris-HCl pH 8.0, 150 mM NaCl and eluted with buffer containing the FLAG peptide as described above.

Ribonuclease Treatment of the Immunoprecipitated Protein Complexes prepared by two-step purification—Pre-40S complex precipitated with LTV1 or Bystin in FLAG eluate were incubated with 50 mM Tris-HCl pH 8.0, 150 mM NaCl containing 10 μg/ml RNase A for 10 min at 37°C, incubated for 30 min on ice, then submitted to following HA-Pull-down described above.

Glycerol Density Gradient Ultracentrifugation of protein complex—LTV1-associated pre-40S complex pulled down with anti-FLAG beads was layered on 10-25% (v/v) Glycerol density gradient buffered with 50 mM Tris-HCl pH8.0, 150 mM NaCl, and centrifuged with Beckman MLS50 rotor at 45,000 rpm (ave. 162,500 × *g*) for 4 hour at 4°C, and then separated into 10 fractions (500 μl each). Eluate was measured at 540 nm with ProTeam LCTM System210 (ISCO, Inc Lincoln NE, USA).

Polysome analysis with sucrose density gradient—72 hour from transfection, cells were harvested by 5 min after the addition of 50 μg /ml cycloheximide to the medium. Equal numbers of cells were pelleted by centrifugation at 1,200 x g for 5 min at 4°C. Cells were lysed in 20 mM Tris-HCl (pH 7.2), 130 mM KCl, 10 mM $MgCl_2$, 2.5 mM DTT, 0.5% IGEPAL-CA630, 0.5% sodium deoxycholate, 10 μg /ml Cycloheximide, 0.2 mg/ml heparin for 15 min at 4°C. The cell suspensions were centrifuged at 8,000 x g for 10 min, and the supernatants were layered on 10 to 45% (wt/wt) sucrose density gradients in 10 mM Tris-HCl (pH 7.2), 60 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml heparin. The gradients were centrifuged at 45,000 rpm for 99 min at 4°C with Beckman MLS50 rotor and fractionated into 10 fractions. The absorbance at 254 nm was measured continuously with ProTeam LCTM System210 (ISCO, Inc Lincoln NE, USA).

Supplementary Figure Legends

Supplementary Figure S1. Assignment of LTV1- or Bystin-associated pre-rRNAs
A) LTV1-associated RNAs were separated by agarose gel electrophoresis and stained with ethidium bromide. Lane 1, total RNA (stained bands corresponding to 28S or 18S rRNAs are indicated to the left); lanes 2 and 3, control and LTV1-HF–associated RNAs isolated during the first step of purification, respectively; lanes 4 and 5, control and LTV1-HF–associated RNAs isolated during the second step of purification. **B)** Structure of the primary rRNA transcript (47S), outline of pre-rRNA processing in mammalian cells modified from Yoshikawa et al. (1), and oligonucleotide probes used for hybridization analysis. The main processing sites 1 and 3 denote the mature ends of 18S rRNA, site 4' denotes the 3' end of 5.8S, and sites 3' and 02 are for 28S (2). The external and internal transcribed spacers (ETS and ITS, respectively) contain additional cleavage sites designated 01, A0, E, C, 2, and 4a. Pre-rRNAs (45S, 41S, 30S, 36S, 21S, 18S-E, 32S, and 12S) produced by cleavage at those sites are indicated. **C)** LTV1-associated RNAs prepared by two-step purification were detected by northern blotting with probes for 18S, 5'ITS1, and 28S as indicated below the corresponding blots. For each probe, lane 1 corresponds to total RNA, lane 2 to the control, and lane 3 to LTV1-HF–associated RNAs. **D)** Schematic diagram of the RNase H targeting method for detecting the 3'-end sequence of pre- or mature 18S rRNA. DNA-RNA hybrids were formed by annealing pre- and mature 18S rRNAs with a DNA oligonucleotide corresponding to bases 1800–1823 of the mature 18S rRNA sequence, with subsequent digestion with RNase H. The resulting RNA fragments are detected by northern blotting with probe 18S-3' (corresponding to 1837–1856) or 5'ITS1 (corresponding to 1863–1891). **E)** RNA fragments prepared from LTV1- or Bystin-associated pre-40S particles were detected by SYBR Gold staining or by northern blotting with probe 18S-3' or 5'ITS1 after denaturing urea-PAGE**.** In each stained gel, lanes 1–4 (lanes 2–5 for SYBR Gold staining) represent reactions that lacked a targeting probe, and lanes 5–8 (6–9 for SYBR Gold staining) represent RNA fragments produced by targeted RNase H digestion. Marker oligonucleotides and sizes are indicated to the left of the SYBR Gold–stained gel. Arrows indicate oligonucleotides containing approximately 50, 60, and 90 nucleotides generated from 18S', 18S'E1, and 18S'E'2, respectively, by targeted RNase H digestion.

Supplementary Figure S2. Nuclear localization of PARN and 18S-E
A) LTV1- or Bystin-associated pre-40S particles were isolated from cytoplasmic extract (Cy) or nuclear extract (Nu) prepared from T-Rex 293 cells or Flp-In T-Rex 293 cells expressing LTV1-HEF or Bystin-HEF inducibly with doxycycline. RNAs are separated by agarose gel electrophoresis and detected by northern blotting with the probes indicated. Sizes of the corresponding pre-rRNAs are indicated to the left. **B)** HEF-PARN was immunoprecipitated (IP) with anti-FLAG–conjugated beads from HEF-PARN–expressing cells that were transfected (TF) with (+) or without (–) LTV1-Myc vector. Proteins were detected by immunoblotting with the antibodies against proteins indicated to the left. **C)** FISH analysis of RNase-treated HeLa cells. After RNase treatment, RNA species were visualized with the 5'ITS1 probe (red). The cells were also subjected to immunocytochemical analysis with anti-PARN (green). 5'ITS1 image on the right, and anti-PARN image on the left. Both images were acquired at the same time point. Scale

bar, 10 μm.

Supplementary Figure S3. Effect of PARN knockdown on 293EBNA cell proliferation and processing of pre-rRNAs

A) siRNA-treated cells were stained with anti-PARN and secondary antibody conjugated with Cy3 (red) or with carboxyfluorescein diacetate ester (CFDA SE, green). DAPI staining was overlaid with the Merge image (Merge + DAPI). Scale bar, 10 μm. **B)** After treatment with control scRNA (sc) or siRNA (si), RNAs in 293EBNA cells were detected by northern blotting with probes for 18S, 5'ITS1, 28S, and ITS1. Northern blotting was also done using probes for the RNAs U3, 7SK, U6, and 5S after separation by denaturing urea-PAGE. PARN and GAPDH were detected by immunoblotting (IB). rRNAs were separated with 0.8% agarose gel electrophoresis.

Supplementary Figure S4. Function of PARN in Bystin-associated pre-40S particles

A) RNAs extracted from HeLa cells transfected (TF) with control scRNA (sc) or siRNA (si) for PARN knockdown were co-transfected for 48 h with empty vector (Vec), expression vector containing an siRNA-resistant sequence encoding PARN-WT-Myc, or expression vector containing an siRNA-resistant sequence encoding PARN-D28A-Myc. RNAs were detected by northern blotting with the probes indicated under each blot. Proteins were detected by immunoblotting (IB) with the antibodies indicated. **B)** The plasmid encoding PARN-WT-Myc was mixed with pcDNA3.1 empty vector (proportion: 0:1, 1:4, 1:2, or 1:0) and transfected into Flp-In T-Rex 293 cells expressing Bystin-HEF. The protein components of the isolated Bystin-HEF–associated pre-40S particles were detected by immunoblotting with the antibodies indicated, and the RNA components were detected by northern blotting with probes for 18S ITS1, and 5'ITS1. **C)** Bystin-HEF–associated pre-40S particles were immunoprecipitated (IP) with anti-FLAG–conjugated beads from cytoplasmic (Cy) or nuclear (Nu) extract prepared from Flp-In T-Rex 293 cells expressing Bystin-HEF that were transfected with PARN-WT-Myc or PARN-D28A-Myc expression vector. The protein components of the isolated pre-40S particles were detected by immunoblotting with the antibodies indicated to the left.

(1) Yoshikawa H., Ishikawa H., Izumikawa K., Miura Y., Hayano T., Isobe T., Simpson R.J. and Takahashi N., Human nucleolar protein Nop52 (RRP1/NNP-1) is involved in site 2 cleavage in internal transcribed spacer 1 of pre-rRNAs at early stages of ribosome biogenesis. Nuc. Acids Res. 2015, 43(11):5524-5536

(2) Sato, S., Ishikawa, H., Yoshikawa, H., Izumikawa, K., Simpson, R.J. and Takahashi, N., Collaborator of alternative reading frame protein (CARF) regulates early processing of pre-ribosomal RNA by retaining XRN2 (5'-3' exoribonuclease) in the nucleoplasm. Nucleic Acids Research, 43(21):10397-10410, 2015

Supplementary Table 1 The LTV1-associated proteins identified by mass-based analysis

Proteins involved or expected to be involved in ribosome biogenesis are indicated. Proteins are grouped based on SwissProt classification. Proteins were identified by LC-MS/MS (Q-Tof2). Proteins detected in mock are not listed in this table. Uniprot KB accession numbers, entry names, protein names as well as gene symbols are shown. For proteins having yeast orthologs, the gene names are indicated (obtained by Blink analysis of the NCBI database).

Uniprot KB accession	Uniprot KB Entry Name	protein name	Gene name	Yeast Homolog
40S subunit trans-acting factor (Yeast homolog)				
O95453	PARN_HUMAN	Poly(A)-specific ribonuclease PARN	PARN	
Q2NL82	TSR1_HUMAN	Pre-rRNA-processing protein TSR1 homolog	TSR1	TSR1
Q96GA3	LTV1_HUMAN	Protein LTV1 homolog	LTV1	LTV1
Q13895	BYST_HUMAN	Bystin	BYSL	ENP1
Q5JTH9	RRP12_HUMAN	RRP12-like protein	RRP12	RRP12
Q9ULX3	NOB1_HUMAN	RNA-binding protein NOB1	NOB1	NOB1
Q9NRX1	PNO1_HUMAN	RNA-binding protein PNO, DIM2	PNO1	PNO1
Q9BVS4	RIOK2_HUMAN	Serine/threonine-protein kinase RIO2	RIOK2	RIOK2
Q9NSI2	CU070_HUMAN	Uncharacterized protein C21orf70	C21orf70	
rRNA processing				
P19338	NUCL_HUMAN	Nucleolin	NCL	
Q9BVJ6	UT14A_HUMAN	U3 small nucleolar RNA-associated protein 14 homolog A	UTP14A	
Q9H6R4	NOL6_HUMAN	Nucleolar protein 6	NOL6	
Q9NR30	DDX21_HUMAN	Nucleolar RNA helicase 2	DDX21	
RNA Helicase				
Q08211	DHX9_HUMAN	ATP-dependent RNA helicase A	DHX9	
P17844	DDX5_HUMAN	Probable ATP-dependent RNA helicase DDX5	DDX5	
Q9UJV9	DDX41_HUMAN	Probable ATP-dependent RNA helicase DDX41	DDX41	
Q6P158	DHX57_HUMAN	Putative ATP-dependent RNA helicase DHX57	DHX57	

Supplementary Table 2 Identification of proteins associated with the LTV1 by LC-MS/MS analysis with Q-Tof2

Summary of the LTV1-associated proteins identified by LC-MS/MS analysis with Q-Tof2. a. Positions of amino- (Start) and carboxyl- (End) termini of the identified peptides within the amino acid sequence are shown. b. The significance threshold (p = 0.05) and probability-based MOWSE scores obtained from a database search by MASCOT are shown. Scores that exceed their threshold are statistically confident with more than 95% certainty. c. The charge state and observed precursor mass are indicated.

Uniprot KB accession	Uniprot KB Entry Name	protein name	Gene Symbol	Start	Sequence	End	Charge State	Mascot Score	Observed Precursor Mass
40S subunit trans-acting factor (Yeast homolog)									
Q2NL82	TSR1_HUMAN	Pre-rRNA-processing protein TSR1 homolog	TSR1	241	QQHLAFR	247	2+	41.35	450.24
				280	GQTLNVNR	287	2+	41.81	451.25
				66	EAVLAEKR	73	2+	59.48	458.28
				2	AAHRPGPLK	10	2+	33.45	473.79
				601	DPGNTEPVK	609	2+	58.68	478.75
				697	VVLSGHPFK	705	2+	54.71	492.31
				591	MSVLNMVVR	599	2+	56.29	540.79
				518	ENLPQDYAR	526	2+	49.9	553.34
				761	SQDTVLMNLYK	771	2+	53.91	664.38
				625	ASPLFSQHTAADK	637	3+	67.9	458.29
				527	IFQFQNFTNTR	537	2+	70.51	708.47
				761	SQDTVLMNLYKR	772	2+	48.54	742.47
				94	ISLPEAMQLLQDR	106	2+	74.28	757.5
				288	LLHIVGYGDFQMK	300	3+	42.51	507.67
				94	ISLPEAMQLLQDR	106	2+	60.11	765.45
				721	EDVLWFKPVELR	732	2+	49.08	766.02
				301	QIDAPGDPFPLNPR	314	2+	44.46	768.99
				288	LLHIVGYGDFQMK	300	2+	36.62	769
				527	IFQFQNFTNTRK	538	2+	38.96	772.45
				79	DGPPHQVLVVPLHSR	93	3+	57.72	551
				220	LLLLDTQQEAGMLLR	234	2+	108.75	857.6
				575	QGTPLIAFSLLPHEQK	590	2+	62.01	881.6
				777	WTYDPYVPEPVPWLK	791	2+	42.04	945.6
				511	TSPWDPKENLPQDYAR	526	3+	41.63	639.71

				672	SNGMHSLIATGHLMSVDPDR	691	3+	36.9	724.09
				280	GQTLNVNRLLHIVGYGDFQMK	300	3+	34.81	801.86
				251	AYLFAHAVDFVPSEENNLVGTLK	273	3+	63.66	845.49
Q96GA3	LTV1_HUMAN	Protein LTV1 homolog	LTV1	431	KQAIKEER	438	2+	40.96	501.32
				382	TGIPLNVLPK	391	2+	40.4	526.38
				15	AVSFHLVHR	23	2+	57.16	533.35
				14	KAVSFHLVHR	23	2+	76.51	597.4
				402	IQMINGSDLPK	412	2+	51.22	616.33
				253	NEQLTLHDER	262	2+	75.74	627.86
				27	DPLAADESAPQR	38	2+	88.42	635.41
				294	LQEVLNDYYK	303	2+	83.04	642.88
				97	EETLVIPSTGIK	108	2+	43.99	643.92
				170	ATGEEEGMDIQK	181	2+	62.72	662.34
				252	RNEQLTLHDER	262	3+	44.89	470.94
				240	FTEYSMTSSVMR	251	2+	75.95	727.87
				294	LQEVLNDYYKEK	305	3+	32.26	514.64
				225	AIADHLFWSEETK	237	2+	77.15	773.95
				24	SQRDPLAADESAPQR	38	3+	65.24	547.65
				253	NEQLTLHDERFEK	265	3+	30.66	553.65
				93	REEKEETLVIPSTGIK	108	3+	59.21	610.44
				74	EPSGPSELIPSSTFSAHNR	92	3+	55.76	671.71
Q13895	BYST_HUMAN	Bystin	BYSL	190	EVLSK	194	1+	33.44	575.45
				420	ELQSAVPR	427	2+	62.08	450.32
				399	EALLELLR	406	2+	65.49	478.87
				146	TLADIIMEK	154	2+	63.59	517.33
				419	RELQSAVPR	427	2+	30.34	528.38
				258	VRDDVAEYK	266	2+	39.82	547.86
				249	FYNLVLLPR	257	2+	50.65	567.88
				304	EAIIVGSIITK	314	2+	58.84	572.4
				389	YKADLATDQK	398	2+	51.16	576.89
				145	RTLADIIMEK	154	2+	53.05	595.43
				258	VRDDVAEYKR	267	2+	30.17	625.92
				268	LNFHLYMALK	277	2+	59.33	633.44
				41	GTGEAEEEYVGPR	53	2+	52.75	697.37

				407	LQPHPQLSPEIR	418	3+	50.68	472.34
				267	RLNFHLYMALK	277	3+	34.36	474.64
				16	HAPLADQILAGNAVR	30	2+	45.13	773.55
				329	IAEMEYSGANSIFLR	343	2+	65.7	859.05
Q5JTH9	RRP12_HUMAN	RRP12-like protein	RRP12	266	GSEFMFEK	273	2+	34.93	495.79
				1129	VLATQPGPGR	1138	2+	50.53	498.34
				740	VLDPASSDFTR	750	2+	50.37	604.38
				600	AMDLAQAGSTVESK	613	2+	53.85	704.39
Q9NRX1	PNO1_HUMAN	RNA-binding protein PNO	PNO1	192	FTIENVTR	199	2+	37.12	490.35
				12	AEEGFTQVTR	21	2+	51.66	569.34
				47	MDTEEARPAK	56	2+	59.42	582.32
				31	QAEQLSAAGEGGDAGR	46	2+	47.93	758.89
				95	IFTPIVEHLGLQIR	108	3+	60.21	546.04
Q9BVS4	RIOK2_HUMAN	Serine/threonine-protein kinase RIO2	RIOK2	75	LTNAGYDYLALK	86	2+	67.03	671.41
				172	KFPVPKPIDYNR	183	3+	42.79	491.99
				277	FSYESELFPTFK	288	2+	36.36	747.94
				29	NHEIVPGSLIASIASLK	45	3+	67.55	583.78
Q9ULX3	NOB1_HUMAN	RNA-binding protein NOB1	NOB1	398	RLNPNASR	405	2+	33.84	464.32
				46	LAVLPYELR	54	2+	30.56	537.41
				32	EVVTEIRDK	40	2+	33.68	544.83
				334	YAINPHLTEDQR	345	3+	31.84	486.27
				2	APVEHVVADAGAFLR	16	2+	68.48	776.54
rRNA processing									
P19338	NUCL_HUMAN	Nucleolin	NCL	325	TGISDVFAK	333	2+	79.21	469.29
				478	NSTWSGESK	486	2+	47.82	498.24
				334	NDLAVVDVR	342	2+	54.98	500.84
				640	VTLDWAKPK	648	2+	46.76	529.33
				428	SKGIAYIEFK	437	2+	62.36	578.4
				458	SISLYYTGEK	467	2+	39.95	580.87
				600	IVTDRETGSSK	610	2+	44.1	596.83
				578	GLSEDTTEETLK	589	2+	50.71	661.9
Q9BVJ6	UT14A_HUMAN	U3 small nucleolar RNA-associated protein 14	UTP14A	321	QAMQEQLSK	329	2+	30.94	539.78
				232	ALQSYYEAK	240	2+	19.38	536.79
				478	ASSEGTIPQVQR	489	2+	21.12	636.87

				153	QAEQLVFPLEK	163	2+	23.75	651.38
Q9H6R4	NOL6_HUMAN	Nucleolar protein 6	NOL6	942	AQLPVMVIVTPQDR	955	2+	35.35	792.03
				808	EVQSPEGMISLR	819	2+	12.07	681.43
				281	GQSPAGDGSPEPPTPR	296	2+	21.44	775.4
				942	AQLPVMVIVTPQDR	955	2+	14.74	783.99
RNA Helicase									
Q08211	DHX9_HUMAN	ATP-dependent RNA helicase A	DHX9	810	LGGIGQFLAK	819	2+	33.82	502.36
				930	MGGEEAEIR	938	2+	57.21	504.26
				622	LSMSQLNEK	630	2+	39.48	533.28
				529	DVVQAYPEVR	538	2+	43.5	588.32
				1105	AAMEALVVEVTK	1116	2+	33.55	638.87
				669	HLEMNPHFGSHR	680	3+	45.29	493.28
				249	QLYHLGVVEAYSGLTK	264	3+	49.88	593.4
				1137	QISRPSAAGINLMIGSTR	1154	3+	36.23	630.03
P17844	DDX5_HUMAN	Probable ATP-dependent RNA helicase DDX5	DDX5	471	LLQLVEDR	478	2+	34.62	493.37
				392	APILIATDVASR	403	2+	67.71	613.96
				438	TGTAYTFFTPNNIK	451	2+	42.78	788.03
Q9UJV9	DDX41_HUMAN	Probable ATP-dependent RNA helicase DDX4	DDX41	116	ILESVAEGR	124	2+	40.87	487.34
				456	GVEAVAIHGGK	466	2+	58.62	519.35
Q6P158	DHX57_HUMAN	Putative ATP-dependent RNA helicase DHX57	DHX57	604	ISAISVAER	612	2+	42.21	473.33
				533	QFQSILQER	541	2+	29.85	574.88
Q9NR30	DDX21_HUMAN	Nucleolar RNA helicase 2	DDX21	237	TFSFAIPLIEK	247	2+	31.36	633.47
				33	KEKPK	37	1+	11.73	629.46
				205	GVTFLFPIQAK	215	2+	25.86	610.91

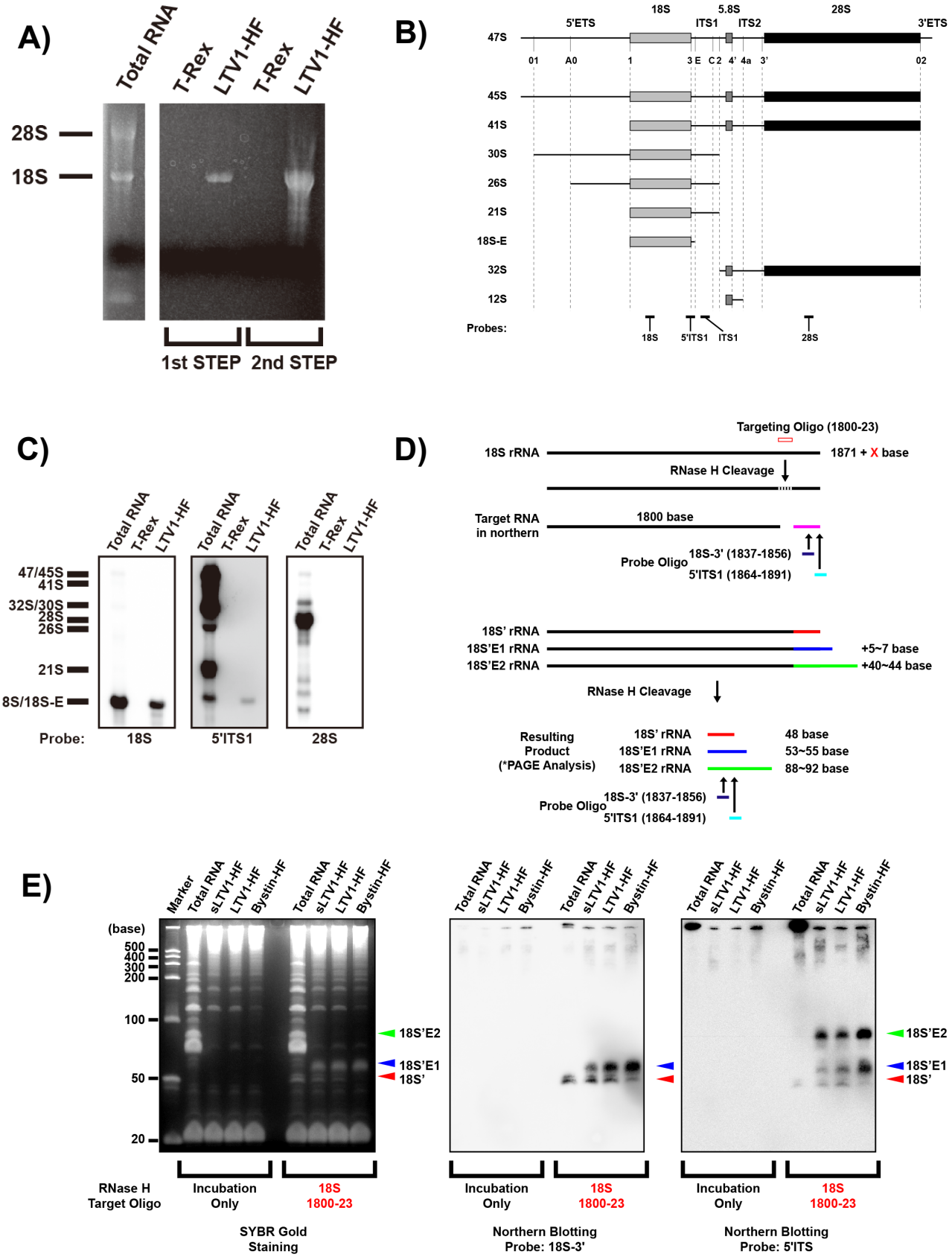
Supplementary Table 3. Summary of the PARN-associated proteins identified by MALDI-MS/MS analysis with TOF5800

Uniprot KB accession	Uniprot KB entry name	Protein name	Gene symbol	Start[a]	Sequence	End[a]	Charge[c] state	Mascot[b] score	Observed[c] precursor mass
PARN IP1									
Q00839	HNRPU_HUMAN	Heterogeneous nuclear ribonucleoprotein U	HNRNPU	576	NFILDQTNVSAAAQR	590	1+	62.15	1646.79
				256	GYFEYIEENKYSR	268	1+	41.09	1696.73
				187	SSGPTSLFAVTVAPPGAR	204	1+	80.98	1713.86
Q9BVJ6	UT14A_HUMAN	U3 small nucleolar RNA-associated protein 14 homolog A	UTP14A	263	KALKEFEQLR	272	1+	16.06	1260.67
				115	TVELPLNKEEIER	127	1+	46.34	1568.8
				721	VVTKPGHIINPIKAEDVGYR	740	1+	25.03	2205.17
PARN IP2									
Q2NL82	TSR1_HUMAN	Pre-rRNA-processing protein TSR1 homolog	TSR1	715	YMFFNR	720	1+	32.43	876.37
				715	YMFFNR	720	1+	10.47	892.35
				241	QQHLAFR	247	1+	19.8	898.45
				518	ENLPQDYAR	526	1+	57.8	1104.49
				527	IFQFQNFTNTR	537	1+	90.3	1414.69
				610	AKEELIFHCGFR	621	1+	25.68	1448.69
				94	ISLPEAMQLLQDR	106	1+	79.23	1512.78
				301	QIDAPGDPFPLNPR	314	1+	46.99	1535.75
				480	LEEMFPDEVDTPR	492	1+	78.22	1576.67
				79	DGPPHQVLVVPLHSR	93	1+	130.21	1649.88
				220	LLLLDTQQEAGMLLR	234	1+	20.84	1712.92
				511	TSPWDPKENLPQDYAR	526	1+	28.7	1915.88
PARN IP3									
O95453	PARN_HUMAN	Poly(A)-specific ribonuclease PARN	PARN	129	NGIPYLNQEEER	140	1+	45.53	1460.66
				87	SFNFYVFPKPFNR	99	1+	32.08	1661.8
				257	HAKEQEELNDAVGFSR	272	1+	14.18	1828.83
Q9BVI4	NOC4L_HUMAN	Nucleolar complex protein 4 homolog	NOC4L	17	RLEAVLASR	25	1+	15.45	1013.56
				137	SKWEGNYLFPR	147	1+	17.71	1395.66
				430	ALESSLWELQALQR	443	1+	45.5	1642.83
PARN IP4									
Q13895	BYST_HUMAN	Bystin	BYSL	420	ELQSAVPR	427	1+	54.08	898.44

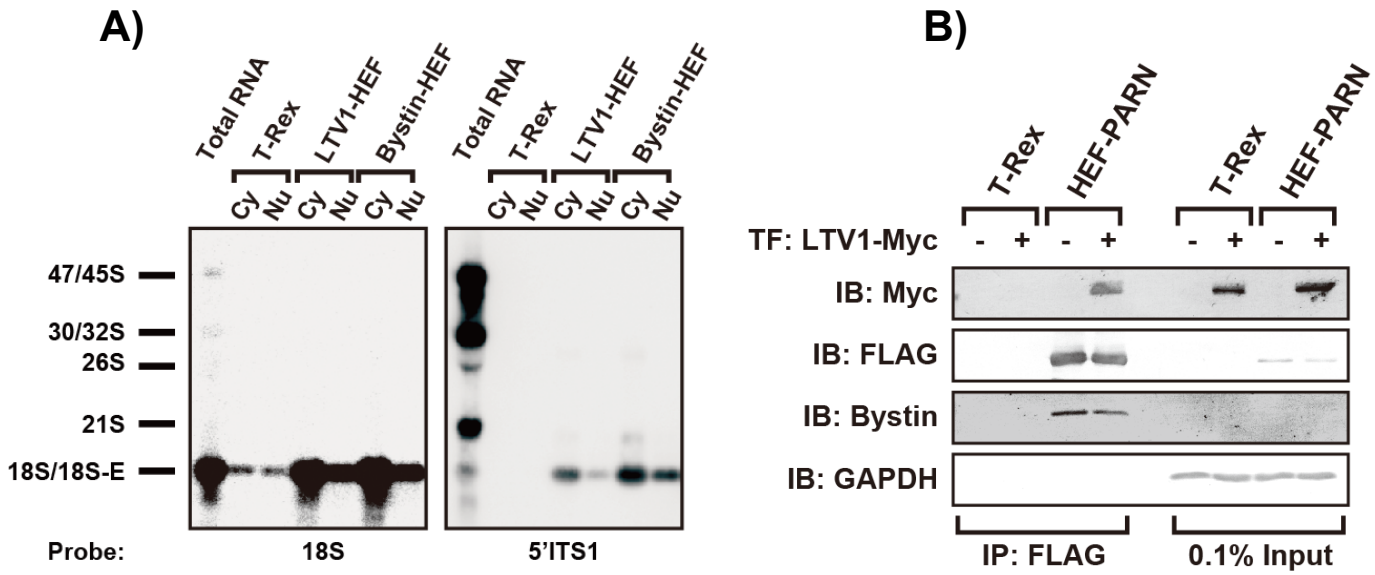
				399	EALLELLR	406	1+	19.84	955.52
				249	FYNLVLLPR	257	1+	64.36	1133.62
				41	GTGEAEEEYVGPR	53	1+	82.81	1392.57
				407	LQPHPQLSPEIR	418	1+	82.81	1413.73
				16	HAPLADQILAGNAVR	30	1+	125.64	1544.8
				407	LQPHPQLSPEIRR	419	1+	41.19	1569.82
				39	GRGTGEAEEEYVGPR	53	1+	40.49	1605.68
				391	ADLATDQKEALLELLR	406	1+	30.23	1797.93
				389	YKADLATDQKEALLELLR	406	1+	38.12	2089.08
				64	QQQEELEAEHGTGDKPAAPR	83	1+	70.4	2189.97
PARN IP6									
P61247	RS3A_HUMAN	40S ribosomal protein S3a	RPS3A	35	APAMFNIR	42	1+	32.67	918.44
				153	TSYAQHQQVR	162	1+	30.15	1216.57
				66	VFEVSLADLQNDEVAFR	82	1+	20.9	1950.93
				66	VFEVSLADLQNDEVAFRK	83	1+	38.86	2079.02
PARN IP8									
Q9Y3B7	RM11_HUMAN	39S ribosomal protein L11, mitochondrial	MRPL11	26	AGLAMPGPPLGPVLGQR	42	1+	69.45	1645.89
				123	IKAQDEAFALQDVPLSSVVR	142	1+	18.7	2185.19

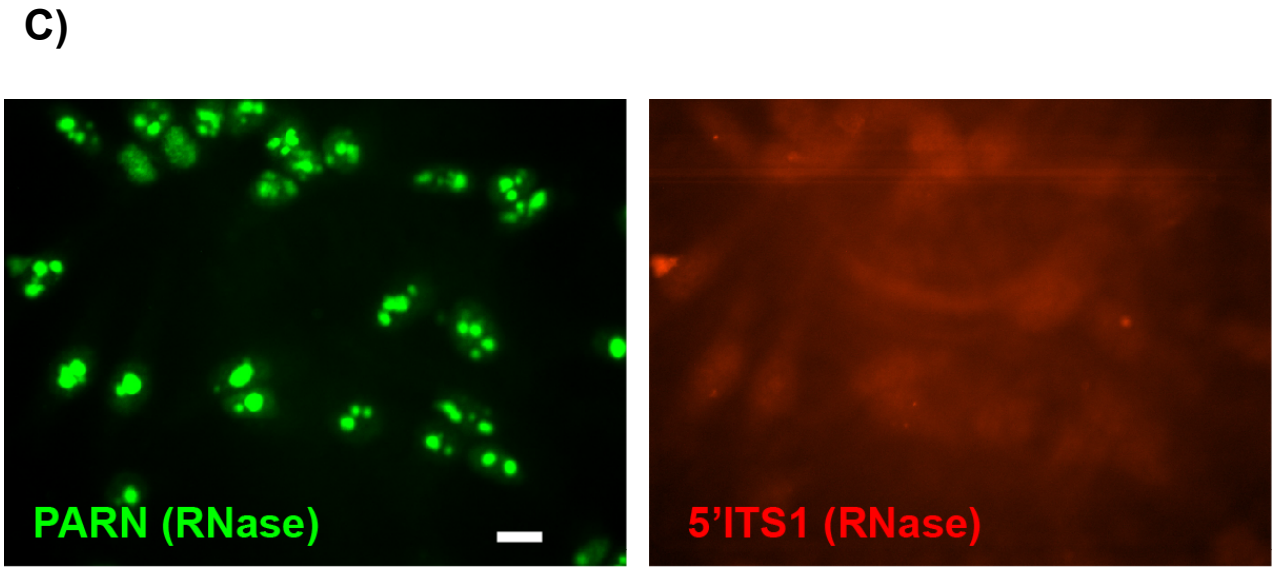
a. The positions are indicated for the amino- (Start) and carboxyl- (End) termini of the identified peptides. b. The significance threshold (P = 0.05) and probability-based MOWSE scores are shown, which were obtained from a database search by MASCOT (23-25). Scores that exceed their threshold are statistically confident with >95% certainty. c. The charge state and observed precursor mass are indicated.

Supplementary Fig. S1

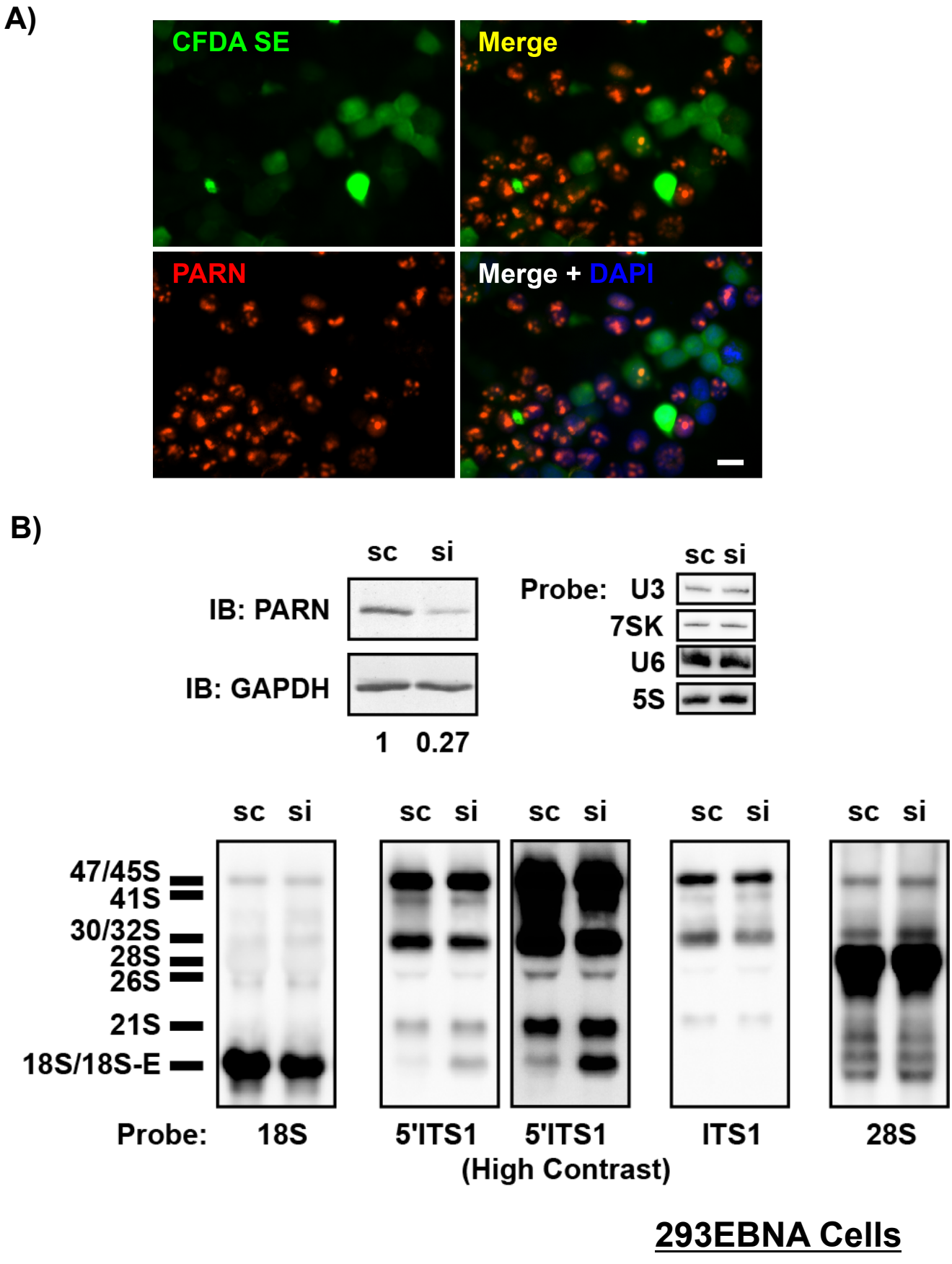


Supplementary Fig. S2





Supplementary Fig. S3



Supplementary Fig. S4