

## **SUPPLEMENTARY MATERIAL**

### **Distinct steps of nuclear maturation of human pre-60S complexes require the activity of GTPases including GNL3L**

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#### **SUPPLEMENTARY TABLES – see associated .xlsx sheet.**

**Supplementary Table S1.** Plasmids used in this study.

**Supplementary Table S2.** Oligonucleotides used in this study.

**Supplementary Table S3.** Antibodies used in this study.

**Supplementary Table S4.** siRNAs used in this study.

**Supplementary Table S5.** Proteins identified by MS in GTPBP4-His-FLAG or GNL2-His-FLAG IPs.

**Supplementary Table S6.** Proteins identified by MS in GNL3-His-FLAG or GNL3L-His-FLAG IPs in the presence of GTP.

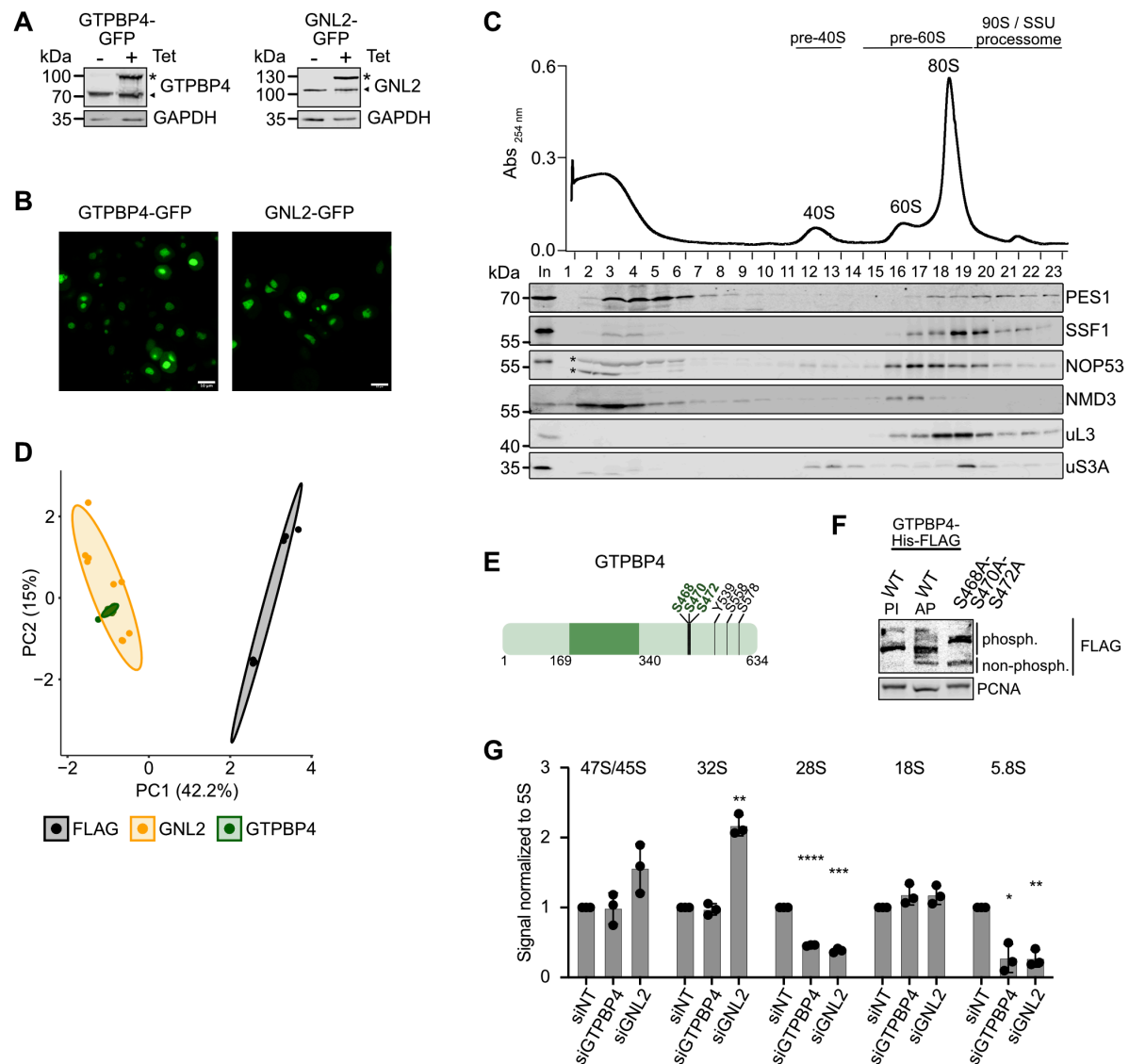
**Supplementary Table S7.** Proteins identified by MS in GNL3L-His-FLAG IPs in the absence of GTP.

**Supplementary Table S8.** RNA contact sites mapped on GNL3L after UV crosslinking and MS analysis.

**Supplementary Table S9.** Genomic coordinates, log2 fold-changes and p-values of peaks identified in GNL3L CRAC analysis.

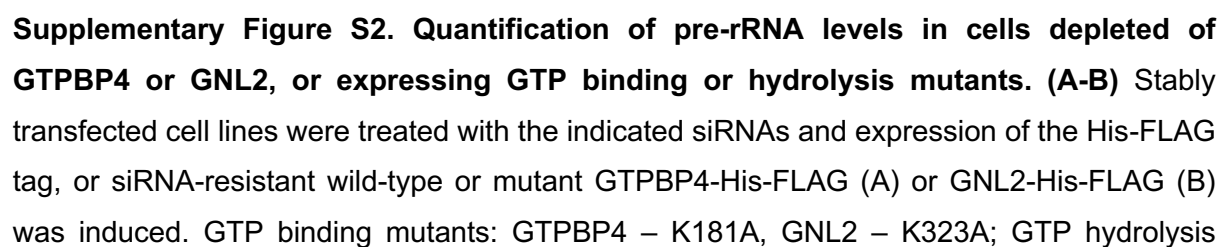
**Supplementary Table S10.** Proteins identified by MS in GNL3L WT, K265A and G306A-His-FLAG IPs.

## SUPPLEMENTARY FIGURES AND LEGENDS

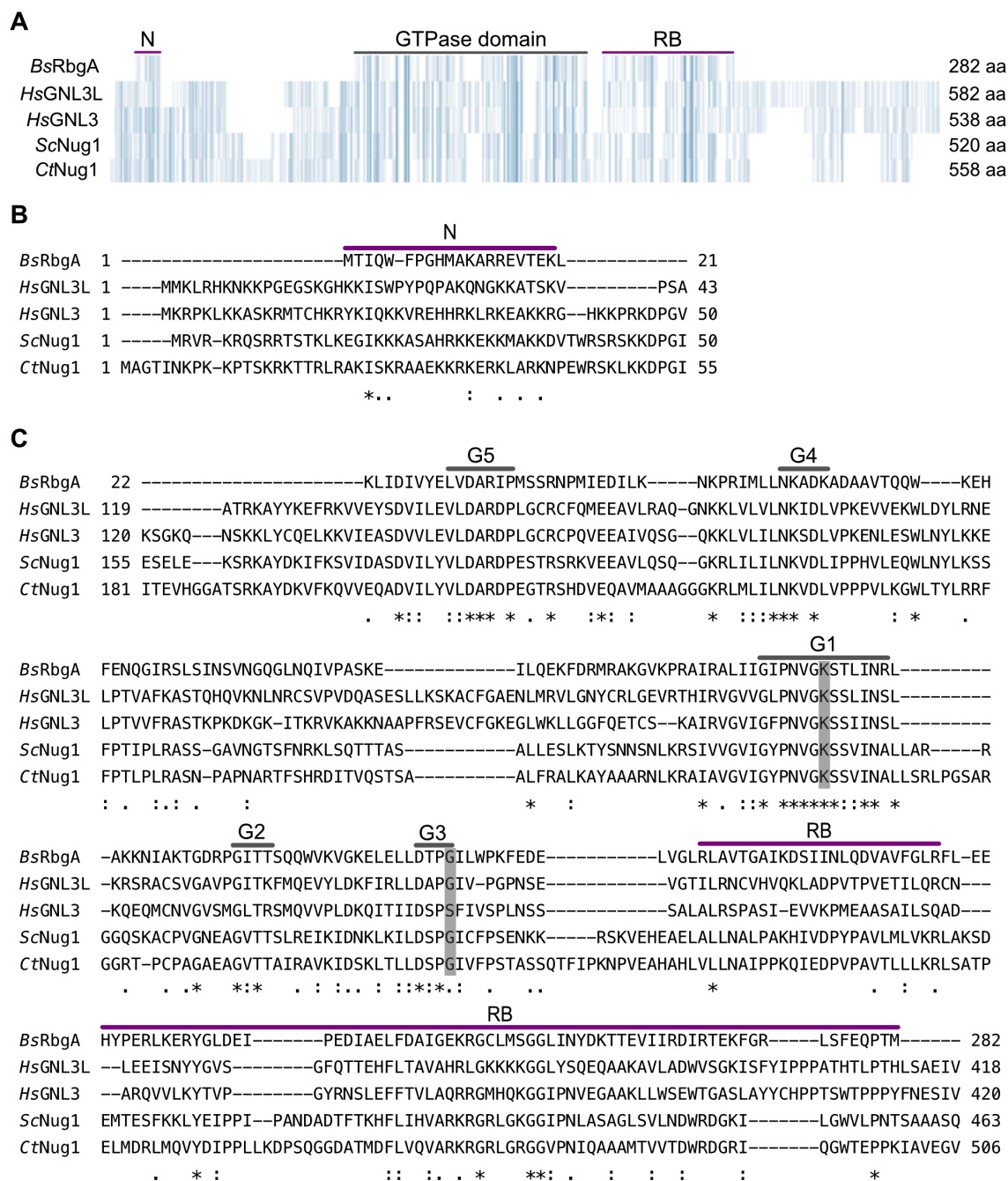


**Supplementary Figure S1. Analyses of GTPBP4 or GNL2 interactors and requirement for nascent rRNA production.** (A) Western blot analysis of stably transfected HEK293 cells expressing GTPBP4-GFP or GNL2-GFP from a tetracycline (Tet)-inducible promoter. Arrow heads and asterisks indicate endogenous and GFP-tagged proteins, respectively. (B) Live cell imaging on GTPBP4-GFP and GNL2-GFP. Scale bar = 10  $\mu$ m. (C) HEK293 whole cell extracts were separated by sucrose density gradient centrifugation. Peaks corresponding to ribosomes (80S) and ribosomal subunits (40S and 60S) are marked on an absorbance (Abs) profile at 254 nm (top). Proteins present in the cell extract (In) and each fraction were western blotted using the indicated antibodies. Asterisk indicates a suspected unspecific interaction of the anti-NOP53 polyclonal antibody with proteins present in fractions containing free proteins and low molecular weight complexes. PES1 and SSF1 are recruited to early nucleolar pre-60S particles, whereas NOP53 and NMD3 associate with mid- and late nucleoplasmic pre-60S

particles, respectively (1, 2). **(D)** Extracts from cells expressing GTPBP4-His-FLAG, GNL2-His-FLAG or the His-FLAG tag were used for anti-FLAG IP experiments and eluates were analyzed by MS. Principal component (PC) analysis of proteins identified in the individual replicates (three biological and three technical) for each sample is shown. **(E)** Scheme of the domain architecture of GTPBP4 (Q9BZE4). The GTPase domain is indicated in dark green and the positions of phosphorylations identified in >10 high-throughput screens (PhosphositePlus; (3)) are indicated. Numbers indicate amino acids at domain boundaries. **(F)** Phosphatase inhibitor (PI)- and alkaline phosphatase (AP)-treated extracts from cells expressing GTPBP4-His-FLAG (WT) or GTPBP4-S468A-S470A-S472A-His-FLAG were separated on a Phos-tag gel, and analyzed by western blotting using anti-FLAG and anti-PCNA antibodies. The migration positions of phosphorylated (phosph.) and non-phosphorylated (non-phosph.) forms of GTPBP4-His-FLAG are indicated. Three experiments were performed and representative data are shown. **(G)** HEK293 cells transfected with non-target siRNAs (siNT) or those targeting GTPBP4 or GNL2 were subjected to pulse-chase metabolic labeling and extracted RNAs were separated by denaturing agarose (47S/45S, 32S, 28S, 18S) or acrylamide (5.8S, 5S) gel electrophoresis. After transfer to a membrane, abundant labeled RNA species were detected using a phosphorimager (Figure 2B). Signals were quantified and normalized according to the 5S rRNA (see also heatmap in Figure 2C). Data are presented as mean  $\pm$  standard deviation of three independent experiments, scaled to siNT values. Individual data points are shown and error bars represent standard deviation. Statistical analysis was performed using one sample t-test in comparison to siNT (\* $p < 0.05$ , \*\* $p < 0.01$ ).

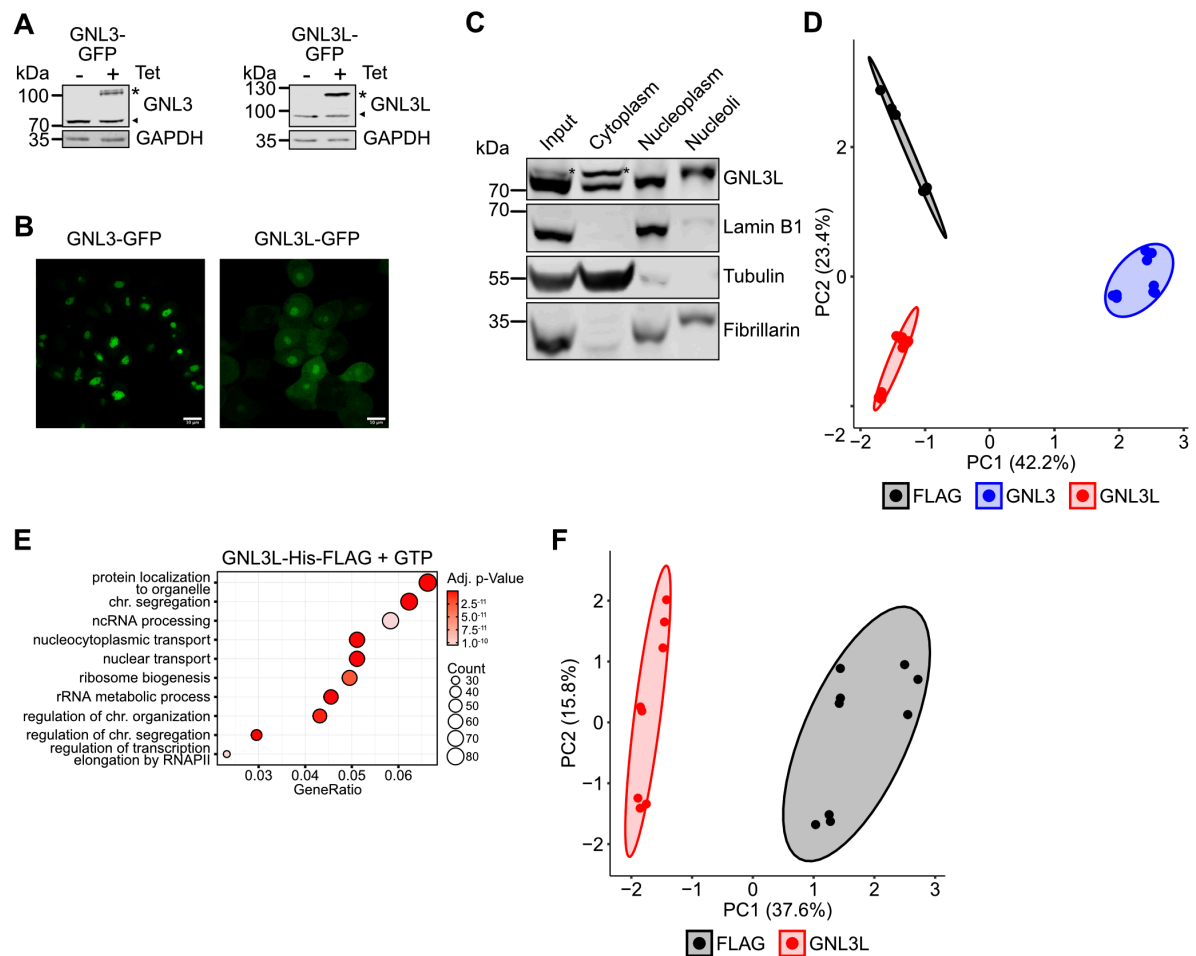


mutants: GTPBP4 – G224A, GNL2 – G364A. Total RNAs were separated by denaturing gel electrophoresis analyzed by northern blotting using probes indicated in Figure 2D. Pre-rRNA intermediates were quantified and normalized according to the mitochondrial 16S rRNA or the U6 snRNA (see also Figure 2G, H). Data are presented as mean  $\pm$  standard deviation of three independent experiments, scaled to siNT values. Individual data points are shown and error bars represent standard deviation. Statistical analysis was performed using one sample t-test in comparison to siNT (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).



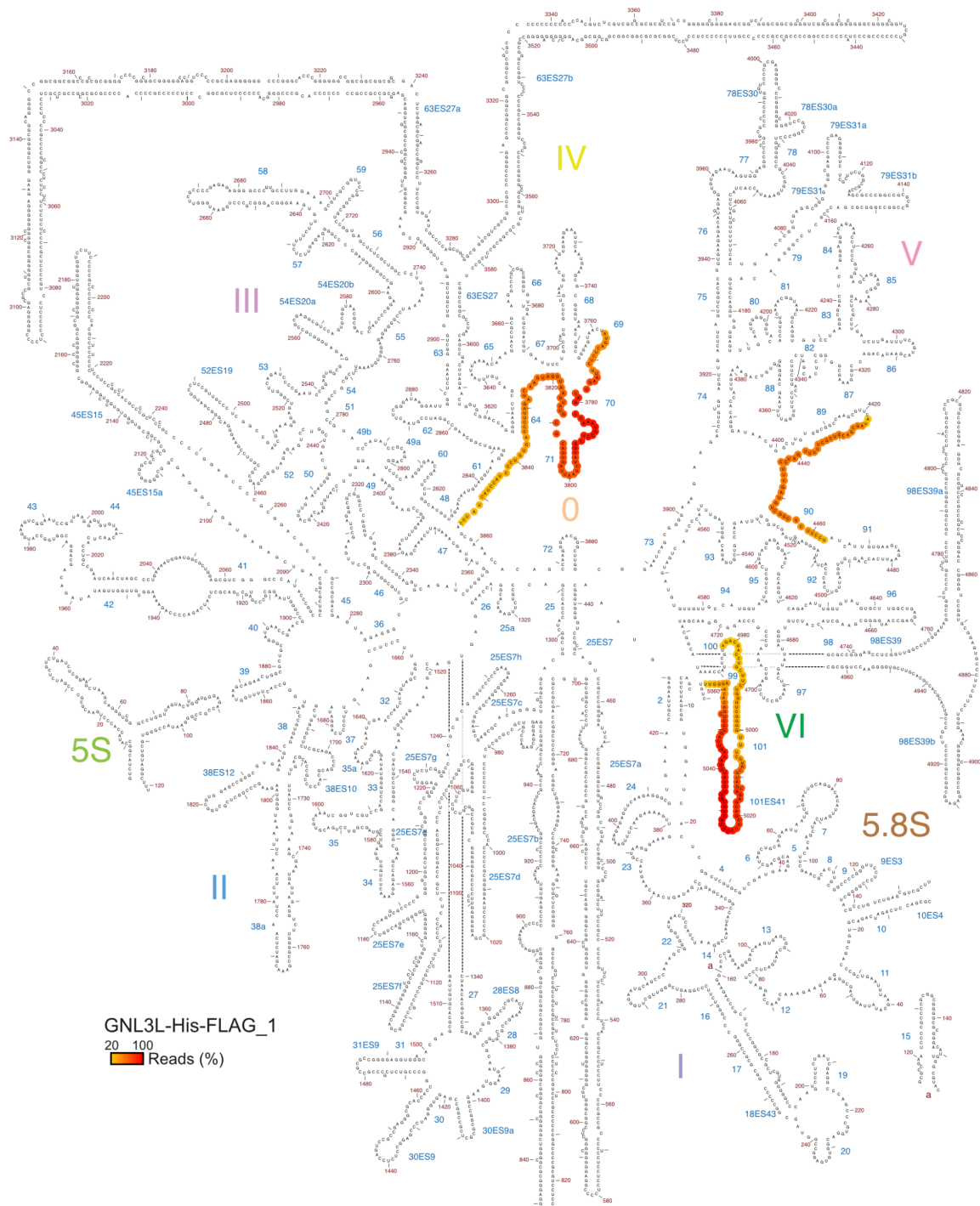
**Supplementary Figure S3. Multiple sequence alignment of GNL3 and GNL3L with yeast and bacterial homologues. (A)** Multiple sequence alignment of amino acid sequences of Nug1-like proteins from *Bacillus subtilis* (*BsRbgA* - NP\_389487.1), humans (*HsGNL3L* - NP\_001171748.1; *HsGNL3* - NP\_055181.3), *Saccharomyces cerevisiae* (*ScNug1*; NP\_010921.1) and *Chaetomium thermophilum* (*CtNug1*; XP\_006696310.1) using MUSCLE. Polar amino acids are highlighted in blue. Number of amino acids (aa) in each protein are indicated on the right. Regions of conserved amino acids at the N-terminus (N) and the *BsRbgA* ribosome-binding (RB) site are indicated by purple bars. GTPase domain is indicated by a grey bar. **(B)** Detailed view of amino acid sequences surrounding the N region. **(C)** Detailed view of the circularly permuted GTPase domain and RB region. Conserved amino acids in G1 and G3

motifs required for GTP-binding and hydrolysis, respectively are highlighted in grey. Symbols below the amino acids indicate the degree of conservation.

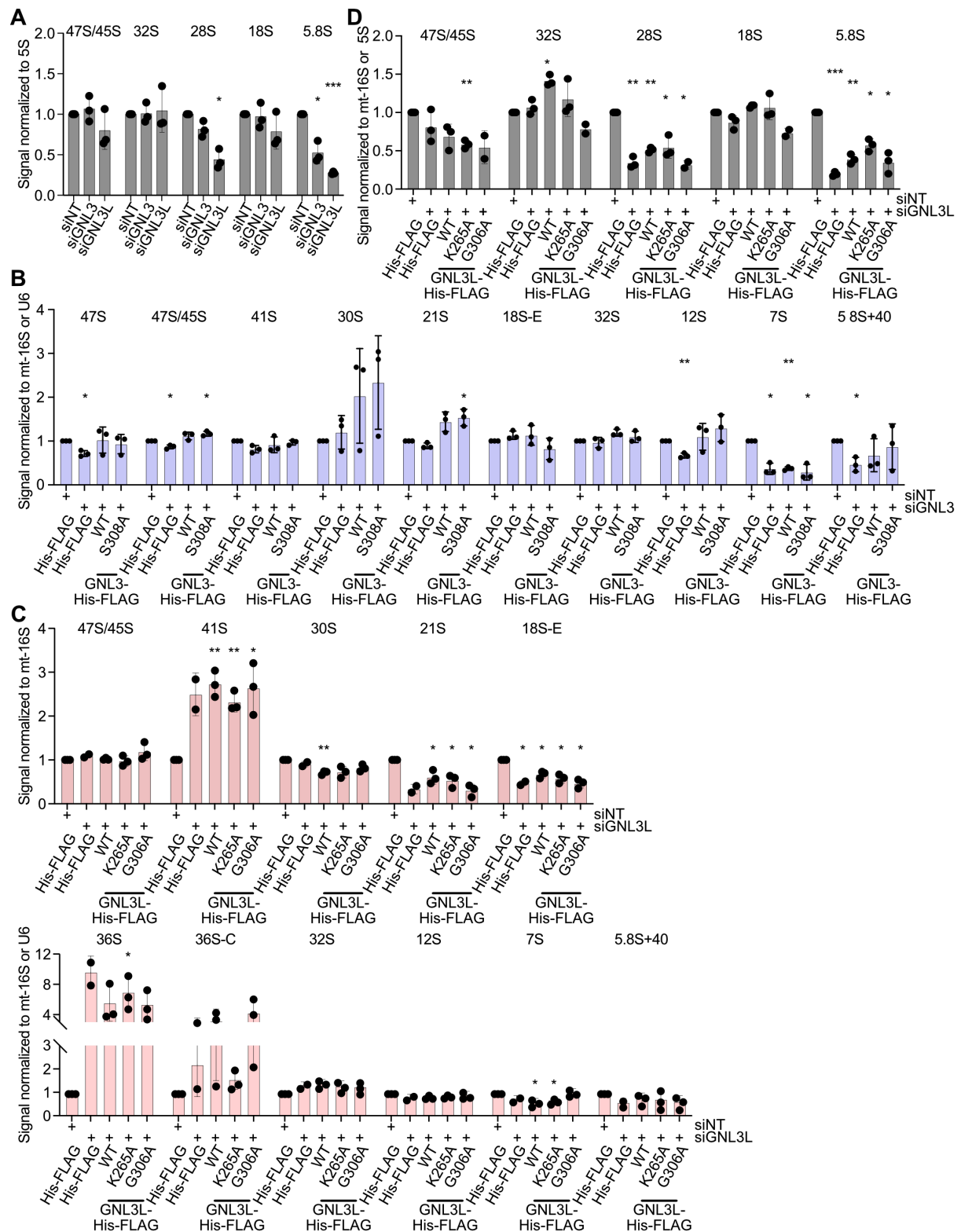


**Supplementary Figure S4. Analyses of GNL3L localization, and GNL3 and GNL3L interactomes.** **(A)** Western blot analysis of stably transfected HEK293 cells expressing GNL3-GFP or GNL3L-GFP from a tetracycline (Tet)-inducible promoter. Arrow heads and asterisks indicate endogenous and GFP-tagged proteins, respectively. **(B)** Live cell imaging on GNL3-GFP and GNL3L-GFP. Scale bar = 10  $\mu$ m. **(C)** HEK293 cells were fractionated into cytoplasm, nucleoplasm and nucleoli. Proteins in the whole extract (Input; 0.5%), and cytosolic (1%), nucleoplasmic (1%), and nucleolar (5%) fractions were analyzed by western blotting with antibodies against the indicated proteins. Tubulin, Lamin B1, and Fibrillarin are markers of the cytosolic, nucleoplasmic and nucleolar compartments, respectively. Asterisks indicate unspecific interactions of the anti-GNL3L polyclonal antibody with a protein present in the cytoplasmic fraction. **(D)** Extracts from cells expressing GNL3-His-FLAG, GNL3L-His-FLAG or the His-FLAG tag were used for anti-FLAG immunoprecipitation experiments in the presence of exogenous GTP, and eluates were analyzed by MS. Principal component (PC) analysis of proteins identified in the individual replicates of each of the indicated samples is shown. **(E)** GO analysis of proteins significantly ( $\log_2$  fold-change (FC) > 0.58 and a p-value < 0.05) enriched with GNL3L-His-FLAG compared to the His-FLAG control in the presence of GTP. **(F)** Extracts from cells expressing GNL3L-His-FLAG or the His-FLAG tag were used for

anti-FLAG immunoprecipitation experiments in the absence of exogenous GTP, and eluates were analyzed by mass spectrometry. PC analysis of proteins identified in the individual replicates of each of the indicated samples is shown. For panels C-E, averaged data from three biological and three technical replicates was used.

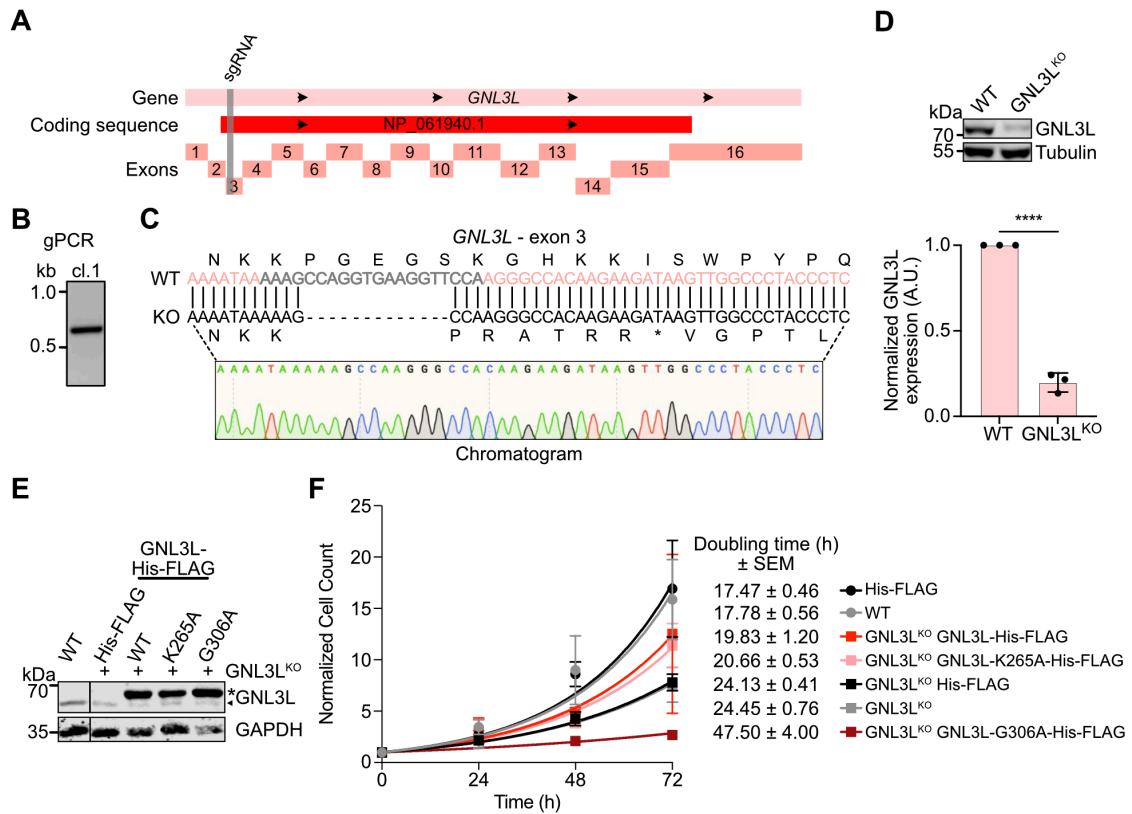


**Supplementary Figure S5. Mapping of GNL3L crosslinking sites on the secondary structure of the 60S rRNAs.** The binding sites of GNL3L-His-FLAG are mapped on a secondary structure model of the rRNAs of the large ribosomal subunit (28S, 5.8S and 5S) (4) in the indicated color scale with the highest peak shown in red. Two CRAC experiments were performed and data from replicate 1 are shown.



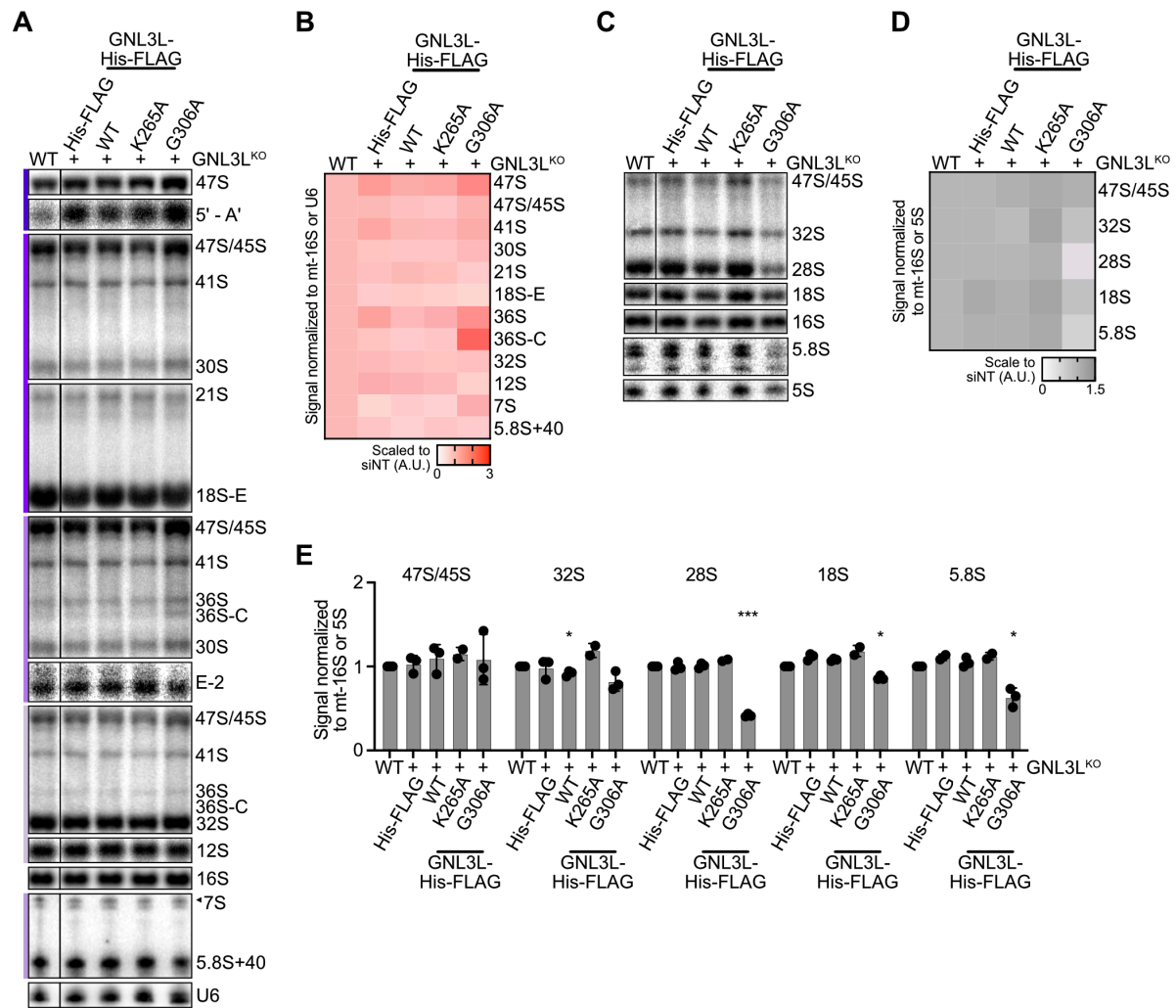
**Supplementary Figure S6. Quantification of pre-rRNA processing defects in cells lacking GNL3 or GNL3L, or expressing GTP binding or hydrolysis mutants. (A)** HEK293 cells transfected with non-target siRNAs (siNT) or those targeting GNL3 or GNL3L were pulse-chase metabolically labeled and extracted RNAs were separated by denaturing agarose (47S/45S, 32S, 28S, 18S) or acrylamide (5.8S, 5S) gel electrophoresis. After transfer to a

membrane, abundant labeled RNA species were detected using a phosphorimager. Signals were quantified and normalized according to the 5S rRNA (see also Figure 5B, C). **(B, C)** Stably transfected cell lines were treated with the indicated siRNAs (+) and expression of the His-FLAG tag, or siRNA-resistant wild-type or mutant GNL3/GNL3L-His-FLAG was induced. GTP binding mutant: GNL3L – K265A; GTP hydrolysis mutants: GNL3 – S308A, GNL3L – G306A. Total RNAs were separated by denaturing gel electrophoresis analyzed by northern blotting using probes indicated in Figure 2D. Pre-rRNA intermediates were quantified and normalized according to the mitochondrial (mt-) 16S rRNA or the U6 snRNA (see also heatmaps in Figure 5G). Data are presented as mean  $\pm$  standard deviation of three independent experiments, scaled to siNT values. Individual data points are shown and error bars represent standard deviation. Statistical analysis was performed using one sample t-test in comparison to siNT (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). **(D)** Cells treated as in (B, C) were subjected to pulse-chase metabolic labeling and extracted RNAs were separated by denaturing agarose (47S/45S, 32S, 28S, 18S) or acrylamide (5.8S, 5S) gel electrophoresis. After transfer to a membrane, abundant labeled RNA species were detected using a phosphorimager. Signals were quantified and normalized according to the mt-16S rRNA or the 5S rRNA (see also Figure 5H, I). Data are presented as mean  $\pm$  standard deviation of three independent experiments, scaled to siNT values. Individual data points are shown and error bars represent standard deviation. Statistical analysis was performed using one sample t-test in comparison to siNT (\* $p < 0.05$ , \*\*\* $p < 0.001$ ).



**Supplementary Figure S7. Generation and characterization of a *GNL3L* knockout cell line.** **(A)** Schematic representation of the gene, exons and protein coding sequence (NP\_061940.1) of *GNL3L*. The target region of the small guide RNA (sgRNA) in exon 3 of *GNL3L* gene is indicated. **(B)** After transfection of HEK293 cells with the CRISPR-Cas9-sgRNA plasmid, transfected cells were selected with puromycin and genomic DNA from isolated clones (cl.1) was used to PCR amplify the region targeted by the sgRNA. **(C)** Sanger sequencing of the PCR product from cl.1 shows 13 nucleotide deletion at the sgRNA target site, which leads to a ribosome frame-shift and introduction of a premature stop codon (\*) in the knockout (KO) cells compared to the parental (WT) cells. Chromatogram indicates the homozygosity of the *GNL3L*<sup>KO</sup> cell line. **(D)** Western blot analysis of WT and *GNL3L*<sup>KO</sup> cells using antibodies against endogenous *GNL3L* and Tubulin. For quantification of protein-expression levels, *GNL3L* expression values were normalized relative to Tubulin, scaled to WT values and are presented as mean ± standard deviation of three independent experiments. Statistical analysis was performed with two-tailed unpaired t-test and significant values are indicated as \*\*\*\*p<0.0001. **(E)** Proteins from stably transfected WT or *GNL3L*<sup>KO</sup> cell lines expressing of the His-FLAG tag, or wild-type or mutant *GNL3L*-His-FLAG were analyzed by western blotting using the indicated antibodies. **(F)** The growth of wild-type HEK293 Flp-In (WT) cells, *GNL3L*<sup>KO</sup> cells and *GNL3L*<sup>KO</sup> cells expressing His-FLAG tagged,

wild-type, K265A or G306A GNL3L was monitored and the growth rates determined (see also Figure 6F).



**Supplementary Figure S8. Analysis of pre-rRNA processing in GNL3L<sup>KO</sup> cells expressing WT GNL3L or GTP binding or hydrolysis mutants. (A-B)** Wild-type (WT) or GNL3L knockout (GNL3L<sup>KO</sup>) cells were induced to express the His-FLAG tag, or wild-type or mutant GNL3L-His-FLAG as indicated. GTP binding mutant: K265A; GTP hydrolysis mutant: G306A. Total RNAs were separated by denaturing gel electrophoresis analyzed by northern blotting using probes indicated in Figure 2D to detect the pre-rRNA species indicated on the right (A). Signals were quantified, normalized according to the mitochondrial (mt-) 16S rRNA or the U6 snRNA and are shown as a heatmap (B). **(C-E)** Cells as in (A) were subjected to pulse-chase metabolic labeling and extracted RNAs were separated by denaturing agarose (47S/45S, 32S, 28S, 18S) or acrylamide (5.8S, 5S) gel electrophoresis. After transfer to a membrane, abundant labeled RNA species were detected using a phosphorimager (C). Signals were quantified, normalized according to the mt-16S rRNA or the 5S rRNA and are shown as a heatmap (D) or bar chart (E). Data are presented as mean  $\pm$  standard deviation of three independent experiments, scaled to siNT values. Individual data points are shown

and error bars represent standard deviation. Statistical analysis was performed using one sample t-test in comparison to siNT (\* $p < 0.05$ , \*\*\* $p < 0.001$ ).

## SUPPLEMENTARY METHODS

### *Live-cell imaging*

Stably transfected HEK293 Flp-In T-REx cell lines for the inducible expression of C-terminally GFP tagged proteins were grown on Nunc Lab-Tek Chambered Coverglass and treated with 50 ng/mL (GNL2, GNL3L) or 1 µg/mL (GTPBP4, GNL3) tetracycline for 24 h before imaging. Cells were imaged using a LEICA STELLARIS 5 confocal microscope using a 63x oil-immersion objective. Acquired images were analyzed and processed using ImageJ (v2.14.0) software.

### *Phos-tag gels*

Protein samples for Phos-tag gels were prepared as described in the Materials and Methods section, with the addition of 1x EDTA-free cOmplete Mini Protease Inhibitor Cocktail (Roche) and either phosphatase inhibitor (PhosSTOP, Roche) or alkaline phosphatase (10 U of FastAP Thermosensitive Alkaline Phosphatase per 0.2 mg/mL phosphoprotein, Thermo Fisher Scientific) to the RIPA buffer. Samples were incubated for 45 min at 4 °C (phosphatase inhibitor-treated) or 37 °C (alkaline phosphatase-treated), but otherwise protein extraction followed the method as described. Protein pellets were, after quantification, resuspended in 1x NuPAGE LDS sample buffer (Invitrogen) containing 50 mM dithiothreitol (DTT). 20 µg of protein were separated by electrophoresis in 8% Bis-Tris gels containing 50 µM Phos-tag solution (Fujifilm Wako Pure Chemical Corporation) and 100 µM ZnCl<sub>2</sub>. Tris-MOPS (50 mM Tris, 50 mM MOPS, 0.1% SDS) running buffer supplemented with NaHSO<sub>3</sub> and 200 mM Tris/HCl pH 9 anode buffer were used. Gel electrophoresis was carried out at constant 40 mA per gel under maximum 90 V until the loading dye front was out of the gel. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories) by wet-transfer, with the transfer buffer supplemented with 1 mM DTT. Consequent steps of western blotting were carried out as described using antibodies listed in **Supplementary Table S3**.

### *Protein sequence alignment*

The similarities between protein sequences of GNL3L and GNL3 with fungal and bacterial homologues were determined by multiple sequence alignment using MUSCLE (v3.8). Amino acid sequences from *BsRbgA* (NP\_389487.1), *HsGNL3L* (NP\_001171748.1), *HsGNL3* (NP\_055181.3), *ScNug1* (NP\_010921.1), *CtNug1* (XP\_006696310.1) were aligned using MUSCLE's default settings. The Percentage Identity Matrix calculated by Clustal 2.1 indicates the degree of similarity between sequences. Clustal 2.1 is part of MUSCLE available at EMBL-EBI (<https://www.ebi.ac.uk/>).

### **Cellular fractionation**

HEK293 Flp-In T-REx cells ( $1.25 \times 10^7$  cells/mL) were gently resuspended for 5 min in transport buffer (20 mM HEPES-NaOH pH 7.3, 110 mM KAc, 2 mM EGTA-NaOH pH 7.3, 2 mM MgAc, 5 mM NaAc, 1x protease inhibitor) containing 100 µg/mL of digitonin and 100 µg/mL of wheat germ agglutinin (WGA) pre-warmed at 25 °C. Cell lysate was centrifuged at 1,000 x g for 1 min at room temperature and the supernatant was collected as cytoplasm fraction. Pellet was washed with transfer buffer containing WGA and centrifuged to collect the supernatant, which was added to the cytoplasm fraction. To isolate the nucleoplasm, pellet was resuspended in 3 mL of S1 buffer (250 mM sucrose, 10 mM MgCl<sub>2</sub>) and layered over 3 mL of S2 buffer (350 mM sucrose, 0.5 mM MgCl<sub>2</sub>), followed by centrifugation at 1,430 x g for 10 min at room temperature. Pellet was resuspended in 3 mL of S2 buffer and sonicated on ice six times for 10 s at 20% amplitude (pulses 0.3 s ON/0.7 s OFF) with 20 s intervals. Lysed sample containing intact nucleoli was layered on top of 3 mL S3 buffer (880 mM sucrose, 0.5 mM MgCl<sub>2</sub>) and centrifuged at 3,000 x g for 10 min at 4°C. The supernatant was collected as nucleoplasm fraction. The pellet was washed in S2 buffer (500 µL) with centrifugation at 3,000 xg for 5 min at 4°C and resuspended in 400 µL of high salt buffer (20 mM HEPES pH 8.0, 500 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.8% Triton X-100, 0.4% CHAPS, 1x protease inhibitor) with 80 U RNase-free DNase I and incubated on ice for 30 min. Afterwards, solution was sonicated on ice three times for 15 s at 20% amplitude (pulses 0.3 s ON/0.7 s OFF) with 20 s intervals and centrifuged at 20,000 x g for 15 min at 4°C. To obtain the nucleoli fraction, supernatant was diluted 1:4 in buffer without salt (20 mM HEPES pH 8.0, 2 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 13.3% glycerol, 1x protease inhibitor). Proteins in subcellular fractions were precipitated with 12.5% TCA (v/v), separated by SDS-PAGE and analysed by western blotting using antibodies listed in **Supplementary Table S3**.

## SUPPLEMENTARY REFERENCES

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