Perlman syndrome nuclease DIS3L2 controls cytoplasmic non-coding RNAs and provides surveillance pathway for maturing snRNAs

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Supplementary Data

Supplementary Materials and Methods

Generation of cellular models to study DIS3L2 and DIS3L substrates.

The multistep cloning procedure described below was used to generate vectors for co-expression of recoded C-terminal FLAG-tagged DIS3L2 [wild type (WT) variant or its catalytic mutant counterpart (mut)] and sh-miRNAs directed against endogenous DIS3L2 mRNA (Supplementary Figure S12).

We used the BLOCK-iT™ RNAi Designer tool from Invitrogen (with "*miR RNAi*" option) (http://rnaidesigner.invitrogen.com/rnaiexpress) to search for 3 miRNA sequences that should specifically and efficiently target endogenous DIS3L2 mRNA. We chose candidate sequences starting at nt positions 1473, 1583 and 1647 of the DIS3L2 ORF. Based on the general principles of BLOCK-iT™ Pol II miR RNAi Expression Vector Kits from Invitrogen (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/rnai/Vector-based-RNAi/Pol-II-miR-RNAi-Vectors.html), we then designed a synthetic DNA fragment that encompassed a combination of the miRNA sequences listed above. This fragment encoded three tandemly-positioned shRNAs that corresponded to the pre-designed miRNAs-so-called sh-miRs- where sense and antisense miRNA sequences were separated by a loop element that could form a hairpin (Supplementary Figure S13a). Each of the sh-miR sequences was flanked at both termini with motifs to ensure correct miRNA processing from the artificial pre-miRNA precursor that followed a natural miRNA biogenesis pathway in human cells. In addition, polyadenylation signals derived from the gene encoding the herpes simplex virus thymidylate kinase (HSV-TK-PA) were placed at the 3'-end of this synthetic cassette to allow for correct termination of transcription in human cells. The cassette contained EcoRI/SalI and ClaI/HindIII restriction site combinations at the 5'- and 3'-ends, respectively, which were used in subsequent cloning steps. The cassette was synthesized by the BlueHeronBio company (http://blueheronbio.com) and inserted between the EcoRI and

HindIII sites of the pUCAmpMinusMCS vector. Next, a eGFP sequence that allowed monitoring of the expression of the artificial pre-miRNA was amplified by PCR using an eGFPFor-eGFPRev primer pair (see Supplementary Table S2 for sequences) and pEGFP-N1 plasmid (Clontech) as a template and inserted into the EcoRI and SalI sites of the provided "[pUCAmpMinusMCS] tri-miR-L2" plasmid, to yield the "[pUCAmpMinusMCS'] eGFP-tri-miR-L2" transitory construct (Supplementary Figure S12c). Additionally, a site recognized by the XmaJI restriction endonuclease was introduced into the eGFPFor oligonucleotide upstream of the 5'-end of the eGFP ORF, which was used at a later cloning stage.

To use the BI-16 vector (1), we designed a cloning strategy to replace the existing hRLUC ORF with a DIS3L2 version that is insensitive to the action of microRNAs. Because in later stages of the cloning procedure the ApaI enzyme was used, we mutagenized the ApaI site present in the DIS3L2 ORF using mutD3L2F and mutD3L2R primers. Successful mutagenesis resulted in the temporary plasmids "p-ApaI-L2_WT" and "p-ApaI-L2_mut" that were confirmed by ApaI digestion and sequencing with DIS3L2_2, DIS3L2_3, DIS3L2_4, and DIS3L2_5 primers. After mutagenesis, inserts encompassing open reading frames coding for WT and mut variants of DIS3L2 were amplified with the DIS3L2F and DIS3L2R primer pair using "p-ApaI-L2_WT" and "p-ApaI-L2_mut" constructs as respective templates (the forward primer also introduced a MluI restriction site and a Kozak sequence at the 5' -end; the reverse primer carried a sequence encoding a FLAG-tag, STOP codon and restriction site (ApaI) at the 3' end). Next, the inserts were cloned into the MluI and ApaI sites of the BI-16 vector to replace the hRLUC ORF in the MH1 *E. coli* strain (Supplementary Figure S12a). Through these procedures the "[BI-16'] L2-FLAG_WT" and "[BI-16'] L2-FLAG_mut" transitory vectors were constructed. The next phase of construct generation required modification of exogenous DIS3L2 ORF sequences to render it insensitive to miRNA action. To this end, we had the recoded fragment of DIS3L2 ORF synthesized (see Supplementary

Figure S14a for nucleotide sequence alignment of original and recoded DIS3L2 ORF fragment). Recoding allowed the introduction of synonymous mutations into all possible codons (at those positions where the degenerative nature of the genetic code could be used and codon usage frequency taken into account) within the fragment containing sites recognized by miRNA, so that the sequence would diverge as much as possible from the original. We recoded a 600 nt-long fragment (nt 1261-1860 of the open reading frame) around the sh-miR target sites. This fragment covered all three sites that could be targeted by miRNAs yet was located outside the region where the catalytic mutation (D391N; change GAT to AAT at position 1171 of the ORF) is introduced. Our goal was to replace the endogenous DIS3L2 sequence with a recoded sequence using an overlap PCR method based on that described by Bryksin and Matsumura (2). To this end, we ordered a recoded fragment surrounded by 75 nt flanking regions that were fully complementary to the initial sequence and terminated at both ends with sites recognized by the SchI restriction enzyme, which cleaves DNA at some distance from its site and yields blunt ends following cleavage. The ordered fragment was provided by BlueHeronBio as an insert that could be cloned into pUCAmpMinusMCS and then excised from the provided plasmid (propagated in the MH1 strain) with SchI. The resulting fragment was used as a “megaprimer” in overlap PCR to produce WT and mut recoded versions of DIS3L2 (250x molar excess of primer to plasmid DNA template “[BI-16’] L2-FLAG_WT” and “[BI-16’’] L2-FLAG_mut”, respectively) (Supplementary Figure S12b). For this PCR, we used the Phusion DNA polymerase and 23 cycles of amplification (30 s denaturation at 98°C, 90 s annealing at 55°C and 15 min 30 s elongation at 72°C, followed by 20 min of final elongation at 72°C). Next, we digested the overlap PCR products with DpnI and transformed the MH1 strain to yield “[BI-16’’] L2rec-FLAG_WT” and “[BI-16’’] L2rec-FLAG_mut” plasmids. The presence of the recoded fragment was checked by NdeI digestion and sequencing.

In the ultimate cloning stage we transferred a DNA fragment containing a co-cistron of the eGFP coding sequence and pre-miRNA/HSV-TK-pA from the "[pUCAmpMinusMCS'] eGFP-tri-miR-L2" construct to the BI-16 vector derivatives from the previous step ("[BI-16'') L2rec-FLAG_WT" and "[BI-16'') L2rec-FLAG_mut") by replacing the FLUC ORF present in the latter construct. All plasmids were propagated in an *E. coli* dam-/dcm- strain prior to the standard cloning procedure that targeted the XmaJI and ClaI restriction sites (Supplementary Figure S12d), followed by transformation of the ligation products into *E. coli* MH1. These procedures generated the final constructs **pAL_01** ([BI-16''') L2rec-FLAG_WT; eGFP-tri-miR-L2/HSV-TK-pA) and **pAL_02** ([BI-16''') L2rec-FLAG_mut; eGFP-tri-miR-L2/HSV-TK-pA). Both DIS3L2 and eGFP-tri-miR-L2 inserts were sequenced.

We also used model cell lines for DIS3, EXOSC10 and DIS3L exosome catalytic subunits that were analogous to those described for DIS3L2. Some lines were previously established in our laboratory [DIS3 and EXOSC10 (3)], while the DIS3L lines were created for this study. Their generation is described below.

The cloning procedure to generate vectors that co-express recoded C-terminal FLAG-tagged DIS3L [wild type (WT) variant or its catalytic mutant counterpart (mut)] and sh-miRNAs directed against endogenous DIS3L mRNA was identical to that for DIS3L2, with the following exceptions:

1. Candidate sequences constituting miRNA targets started at nt positions 2414, 2621 and 2746 of the DIS3L ORF.
2. The BI-16 vector hRLUC ORF was replaced with miRNA-insensitive DIS3L. Because in the later stages of the cloning procedure the ClaI enzyme was used, we first removed the existing ClaI site in the DIS3L ORF by mutagenesis with the mutDISLF and mutDISLR primers. Successful mutagenesis resulted in the temporary plasmids "p-ClaI-

L_WT" and "p-ClaI-L_mut" and was confirmed by ClaI digestion and sequencing (primers: E11fw, E13fw, E15rv, E5fw, E5rw, E8fw). After mutagenesis, inserts encompassing ORFs coding for WT and mut variants of DIS3L were amplified with the DIS3LF and DIS3LR primer pair using "p-ClaI-L_WT" and "p-ClaI-L_mut" constructs as respective templates. Inserts were cloned into the BI-16 vector as described for the DIS3L2 protein.

3. To generate miRNA-insensitive DIS3L, we ordered the synthesis of a recoded fragment of DIS3L ORF (see Supplementary Figure S14b for nucleotide sequence alignment of the original and recoded DIS3L ORF fragment). We recoded a 621 nt fragment (nucleotides 2275-2895 of the ORF) around the target sites of sh-miRs that covered all three sites to be targeted by miRNAs and was located outside the region where a catalytic mutation (D486N; change GAT to AAT at position 1456 of the ORF) was introduced. After overlap PCR (conducted as for DIS3L2) the presence of recoded fragment was checked by digestion with PstI restriction enzyme and sequencing.
4. Final cloning states generated the final constructs: **pAL_03** ([BI-16'''']Lrec-FLAG_WT; eGFP-tri-miR-L2/HSV-TK-pA) and **pAL_04** ([BI-16'''']Lrec-FLAG_mut; eGFP-tri-miR-L2/HSV-TK-pA). Both DIS3L and eGFP-tri-miR-L inserts were sequenced.

This cellular model proved to be functional, because endogenous DIS3L transcript expression was reduced by 80% and we observed production of all relevant system components (data not shown).

Preparation of pcDNA5/FRT/TO plasmids for TUT4 and TUT7 overexpression

Parental pcDNA5/FRT/TO plasmids (ThermoFisher Scientific) were digested with FD BamHI and FD EcoRV (ThermoFisher Scientific). A cassette with 2 TEV protease cleavage site sequences was incorporated through two rounds of PCR using primer pairs p0_for1+p0_rev1, p0_for2+p0_rev2 (Supplementary Table S2) to amplify the entire plasmid

sequence and add overhangs. The product of PCR2 was ligated using the SLIC approach (4) to generate plasmid p0. The final sequence of the incorporated cassette was: (BamHI)GAAAACCTGTACTTCCAAGGA**ACCGGT**GGTCACCTGTACAATCGATCCT GCAGGA***GCTAGC***GAGAATTTGTATTTTCAGGGT(EcoRV).

This cassette contained the TEV sites (underlined) and AgeI and NheI restriction sites that are marked in bold and bold+italics, respectively.

To generate plasmids for N' tagging with eGFP, p0 was digested with AflII and BamHI (both within the parental pcDNA5/FRT/TO sequence) and the eGFP sequence was amplified by PCR using the pEGFP-C1 plasmid as a template and the primer pair p2_for+p2_rev comprising extensions for SLIC. The resulting DNAs were joined by SLIC to create the p2 plasmid.

The CDS of TUT4 (NCBI accession number NM_001009881) or TUT7 (NM_001185059) were amplified by standard PCR using the primer pairs ZCCHC11_F_FRTT+, ZCCHC11_R_FRTT_stop and ZCCHC6_F_FRTT+ZCCHC6_R_FRTT_stop. The PCR products were cloned into an AgeI- and NheI-cleaved p2 plasmid prepared as described above using the SLIC approach to generate pZW_GZ11 (TUT4) and pZW_GZ6 (TUT7).

Plasmids pZW_GZ11cm and pZW_GZ6cm for overexpression of catalytically-inactive TUT mutants TUT4 D1011A and TUT7 D1060A were generated by a standard site-directed mutagenesis protocol (5) using pZW_GZ11 and pZW_GZ6, respectively, as templates.

Immunofluorescence and microscopy analysis

Stable inducible human Flp-In T-REx 293 cell lines were plated at a density of 3×10^4 cells per well in a Nunc Lab-Tek II Chamber Slide™ System (Thermo Scientific) [wells were pre-

coated with Poly-D-Lysine (Sigma-Aldrich)]. Cells were cultured for 24 hours in medium with or without the doxycycline inducer.

After 24 hours, the medium was aspirated and the cells were washed (each washing step in the immunofluorescence protocol consisted of three short washes with PBS) and fixed with PBS solution containing 3.7% formaldehyde and 5% (w/v) sucrose for 25 min. The cells were then washed and permeabilized with PBS solution supplemented with 0.5% Triton X-100 and 10% FBS for 15 min at room temperature. Specimens were washed again and PBS with 10% FBS was used for blocking (30 min). The cells were incubated for one hour at room temperature with the primary antibody - rabbit polyclonal anti-FLAG (Sigma-Aldrich, F7425, 1:400 in 10% FBS in PBS), washed, and incubated for one hour in the dark with Alexa Fluor 555-conjugated goat anti-rabbit IgG (Molecular Probes, 1:800 in 10% FBS in PBS) as the secondary antibody. After incubation with the secondary antibody the cells were washed again, and the nuclei were stained with DAPI (Invitrogen, 2.5 μg/ml in PBS) for 7 min, and the washing was repeated. All procedures were performed at room temperature. Specimens were mounted with ProLong Gold Antifade Mountant (Molecular Probes). Images were collected with a FluoView1000 confocal microscope (Olympus) using a 60x/1.40 oil immersion objective and processed with FluoView software.

Flow cytometry analysis of eGFP expression

Stable inducible human Flp-In T-REx 293 cell lines were plated at a density of 3×10^5 cells per well in a 6-well plate, and cultured for 24 hours in medium with or without doxycycline. The cells were then detached from the plate by scraping in cell culture medium, centrifuged at 500 g for 3 min, washed with PBS, centrifuged again and resuspended in 0.5 ml PBS. eGFP signals were measured using a FACScalibur instrument (BD Biosciences) and the data were analysed using Cyflogic software (CyFlo Ltd.).

Western blotting

Stable inducible human Flp-In T-REx 293 cell lines were plated at a density of 1×10^6 cells per well in 6-well plates and cultured for 24 hours in medium without inducer or with doxycycline, with the exception of experiment with inducer titration when also tetracycline was used. The cells were detached from the plate by scraping in cell culture medium, centrifuged at 500 g for 3 min, washed with phosphate buffered saline (PBS), centrifuged again and resuspended in (100µl/cells from a single 6-well plate well) lysis buffer (RIPA) and sonicated (Supplementary Figure S8b) or in buffer containing 0.5X PBS, 0.1% NP40, protease inhibitors and 2u/µl viscolase (A&A Biotechnology) and incubated at 37°C for 20 min to digest chromatin (Supplementary Figure S1d,e and Supplementary Figure S5a). Whole cell extracts were resolved in 10% SDS-PAGE gels and transferred to a Protran nitrocellulose membrane (Whatman) by electrotransfer using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Suppl. Fig S8b) or wet transfer (Suppl. Figs. S1b,c and S5a). Membranes were stained with Ponceau S Red (Sigma-Aldrich; 0.1% in 3% acetic acid), destained with water and blocked by incubating twice with 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 60 min and then incubated overnight at 10°C with primary antibodies. The following antibodies were used:

1) mouse monoclonal α-FLAG (Sigma Aldrich, M2 F1804, 1:300),

2) rabbit polyclonal anti-FTL (Sigma Aldrich, F8556, 1:2000),

3) rabbit polyclonal α-TUT4 (ProteinTech, 18980-1-AP, 1:2500),

4) rabbit polyclonal α-TUT7 (Sigma-Aldrich, HPA020620, 1:2500),

5) rabbit polyclonal α-GAPDH (Novus Biologicals, NB300-327, 1:6000),

6) rabbit polyclonal α-DIS3L2 (1:1500, the same as (6)).

On the next day, the membranes were washed four times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies for 3 hours (Calbiochem, 401393 or 401215, 1:3000 for 1-2, 1:20000 for 3-4, 1:40000 for 5 and 1:10000 for 6).

Blots were washed four times for 10 min with $TBST_{20}$ and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocol. Digital images were obtained with a CCD camera (Alpha Innotech) or blots were exposed to CL-Exposure films (ThermoFisher Scientific) and developed in an AGFA Curix CP-1000 apparatus.

Small RNA-Seq (detailed protocol)

The first steps of the small RNA-Seq protocol were identical to those of the standard RNA-Seq protocol (DNase treatment and ribodepletion of 10 µg total RNA). We then treated ribodepleted DNA-free RNA molecules with TAP (Epicentre, 5 U enzyme, 40 U RiboLock RNase Inhibitor) at 37°C for 2 h. After phenol:chloroform extraction and ethanol precipitation, pre-adenylated linker RA3 (sequence of Illumina; synthesis by IDT) was ligated to the RNA for 1 hour at 25°C (an initial denaturation at 70°C was conducted for 2 min without enzyme).

Reagent		Volume [µl]
Precipitated RNA		10.7
T4 RNA Ligation buffer (NEB)	10 x	2
RA3	30 µM	1
PEG 8000 (NEB)	50%	4.8
RiboLock RNase Inhibitor	40 U/µl	0.5
T4 RNA Ligase 2, Truncated (NEB)	200 U/µl	1
	TOTAL	20

The RTP primer (Illumina) hybridization (30 pmol in 1.5 µl per sample) involved incubation of the RNA according to following scheme: 5 min at 75°C, 30 min at 37°C and 15 min at 25°C.

The RA5 linker (Illumina) was then ligated for 1 hour at 25°C

Reagent		Volume [µl]
RNA from previous steps		21.5
H_2O		3.8
T4 RNA Ligation buffer (NEB)	10 x	1
RA5	20 µM	1.5
ATP	25 mM	1.2
T4 RNA Ligase 1 (NEB)	10 U/µl	1
	TOTAL	30

Subsequently, one third of the reaction mixture was used for reverse transcription carried out with SuperScript III Reverse Transcriptase for 1 hour at 42°C (following an initial 2 min incubation at 42°C without enzyme).

Reagent		Volume [µl]
RNA from previous steps		10
H_2O		1.5
First Strand Buffer	5 x	4
dNTP	10 mM	1
RiboLock RNase Inhibitor	40 U/µl	0.5
DTT	0.1 M	2
SuperScript III Reverse Transcriptase	200 U/µl	1
	TOTAL	20

Finally, libraries were amplified in a PCR reaction with the RP1 and RPI_X primers (where X represents index number) (sequences by Illumina, DNA synthesis by Sigma-Aldrich):

Reagent		Volume [µl]
DNA from previous steps		20
H_2O		8.5
RP1	20 µM	1
RPI_X	20 µM	1
dNTP	10 mM	1
HF Buffer	5 x	8
Phusion High-Fidelity DNA Polymerase (Thermo Scientific)	2 U/µl	0.5
	TOTAL	40

We used the following PCR program:

98°C for 30 seconds	
98°C for 10 seconds	10 cycles
60°C for 30 seconds	
72°C for 30 seconds	
72°C for 5 minutes	

Following PCR, libraries (volume 40 µl) were purified with 60 µl Magnetic Beads (Agencourt AMPure XP; Beckman Coulter) according to the manufacturer's instructions, eluted in 12 µl RSB Buffer (Illumina) and separated in a 6% native polyacrylamide gel. Bands of ~140 to ~320 bp were excised, released from the acrylamide by a crush-and-soak method and incubated overnight with elution buffer (0.5 M ammonium acetate, 0.001 M EDTA and 0.1% SDS). Libraries were then precipitated with isopropanol and 2 µl glycogen (20 mg/ml) in a final volume of 20 µl and again purified with 40 µl Magnetic Beads. The quality of the prepared libraries was checked using a Bioanalyzer and High Sensitivity DNA kit (Agilent Technologies).

Flow cytometry analysis of cell cycle

Stable inducible human HEK293 cell lines were cultured in 100 mm dishes and treated with doxycycline (100 ng/ml) for 3 days. To analyse cell cycle, staining with propidium iodide (PI) was performed using a previously described procedure (7) with slight modifications. These modifications included: 1) changed centrifugation parameters to 4 min at 500 g; 2) changed RNase A concentration to 100 µg/ml; 3) changed staining time and temperature to 45 min at 37°C. Cells were read on an Attune NxT acoustic focusing cytometer (ThermoFisher) and fractions were calculated using dedicated software.

Primer Extension Analysis

Primer Extension Analysis was performed as previously described (8). Sequencing ladders for regions under analysis were prepared from plasmids with a cloned genomic DNA fragment encompassing the *vtRNA1-1* and *Y4* genes (see Supplementary Table S2 for oligonucleotides used for sequencing and primer extension).

Cell fractionation

Stable inducible human HEK293 cell lines were cultured in 100 mm dishes and treated with doxycycline (100 ng/ml) for 3 days. After induction, cells were fractionated to obtain RNAs from different cellular compartments. The previously described procedure (9) was slightly modified as described below.

Cells were centrifuged at 500 g at 4°C for 5 min, resuspended in 380 µl ice-cold HLB supplemented with 100 U RiboLock RNase Inhibitor, incubated for 8 min on ice, vortexed briefly and centrifuged again at 500 g at 4°C for 3 min. The resulting supernatant contained cytoplasmic RNAs and the pellet corresponded to the nuclear fraction.

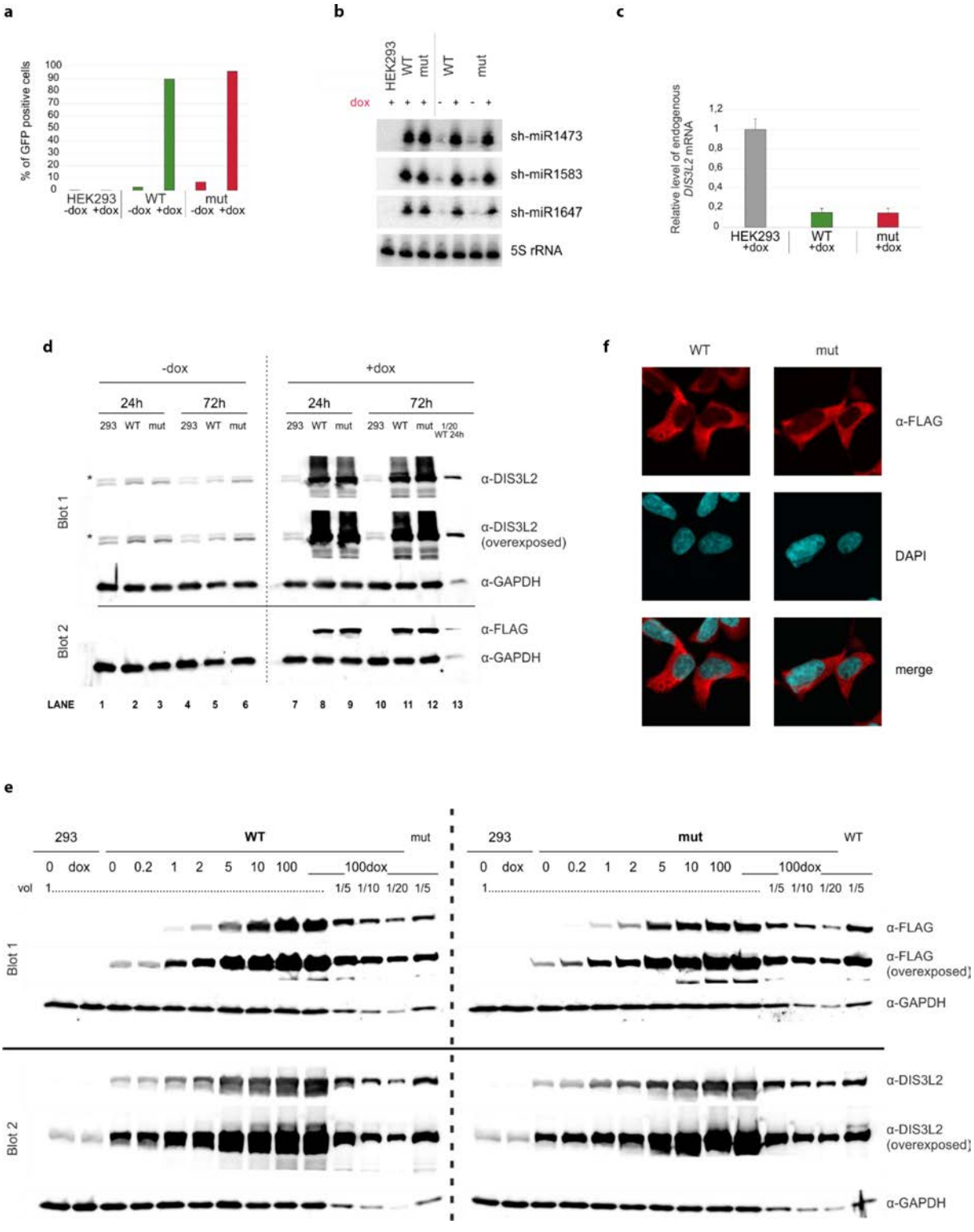
The supernatant was transferred to a new tube, 1 ml RPS was added and samples were stored at -20°C for at least 1 hour. Samples were then vortexed for 30 s and centrifuged at 18,000 g at 4°C for 15 min. The pellet was washed with ice-cold, 70% ethanol and centrifuged again at 18,000 g at 4°C for 5 min and partially air-dried before 1 ml TRI Reagent was added. In the meantime, the nuclear fraction was washed gently 3x with 1 ml ice-cold HLB and centrifuged at 500 g at 4°C for 2 min. Afterwards, 1 ml TRI Reagent was added. Before standard RNA isolation, 10 µl 0.5 M EDTA was added to the samples in TRI Reagent and the pellets were heated to 65°C with vortexing until they dissolved.

4sU labelling

Stable inducible human HEK Flp-In T-REx cell lines producing WT/mut versions of DIS3L2 were cultured in 100 mm dishes and treated with doxycycline (100 ng/ml) for 3 days. Then, a previously described procedure (10) was carried out with minor modification as described below.

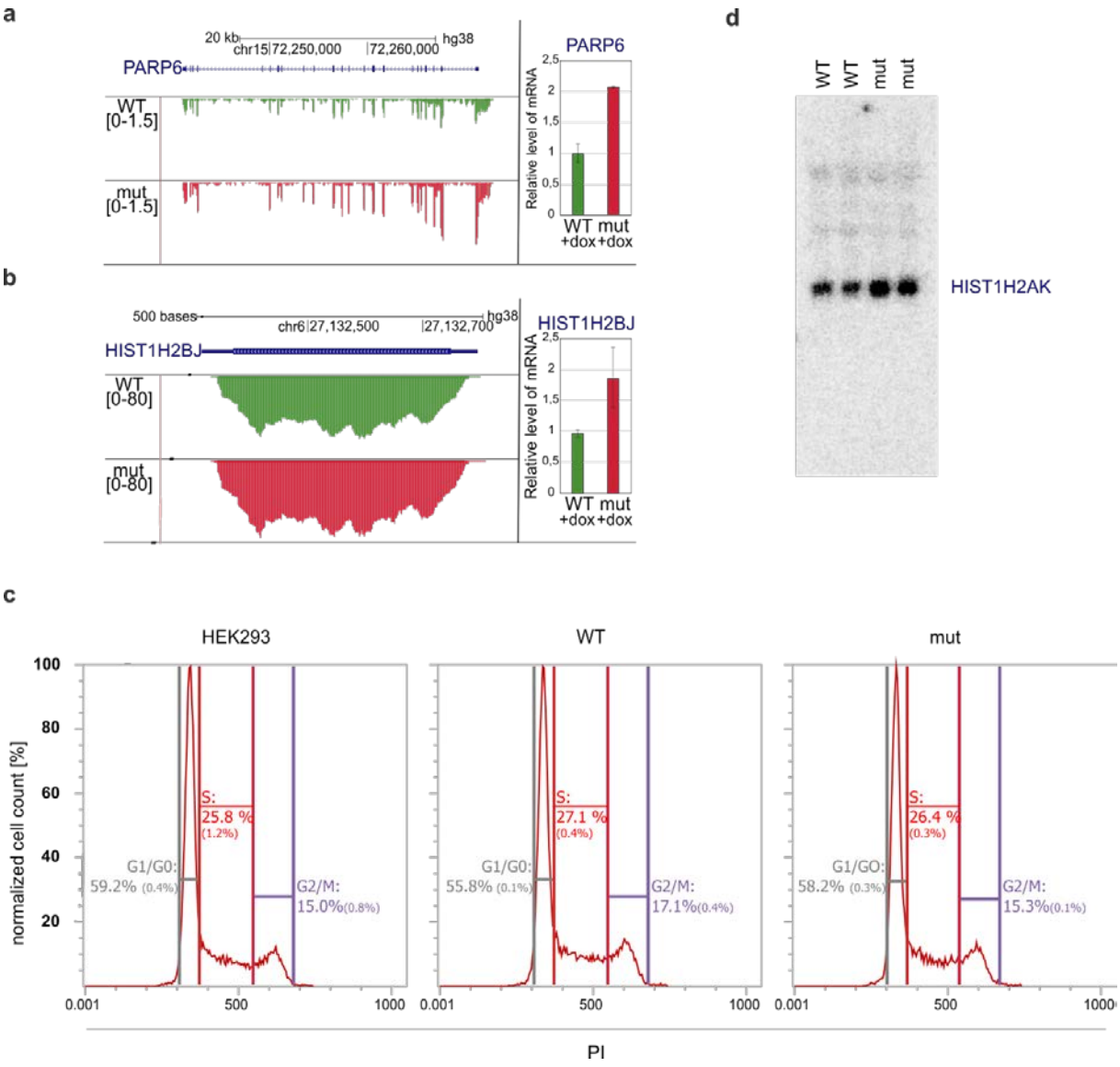
On the last day of induction, 4sU (Sigma) was added to the cell culture medium to a final concentration of 250 μM. Cells were incubated with 4sU for the indicated time periods, up to 120 min. The labelling reaction was terminated by the addition of TRI Reagent. RNA was then isolated as described previously, including non-treated controls. RNA (100 μg) was biotinylated by the addition of 100 μl 10x biotinylation buffer (100 mM Tris–HCl, pH 7.4, 10 mM EDTA), 200 μL EZ-Link HPDP-Biotin (Pierce) and 600 μL RNase-free water. The biotinylation reaction was continued for 90 min with rotation at room temperature. The RNA was then purified by phenol-chloroform extraction, precipitated and separated into previously existing and newly synthesized fractions by incubation with streptavidin-coupled Dynabeads MyOne T1 (Invitrogen) according to the manufacturer's instructions. One μl streptavidin-coupled Dynabeads was used per 1 μg biotinylated RNA with 100 mM Tris–HCl, pH 7.4, 10 mM EDTA, 1 M NaCl, 0.1% Tween20 as the wash buffer. The unbound fraction represented preexisting RNAs and the newly synthesized RNAs were eluted from the beads with 100 mM DTT for 3 min at room temperature. RNA was then recovered by precipitation with 2-propanol and subjected to northern blot analysis (1/2 of obtained volume).

Supplementary Figures

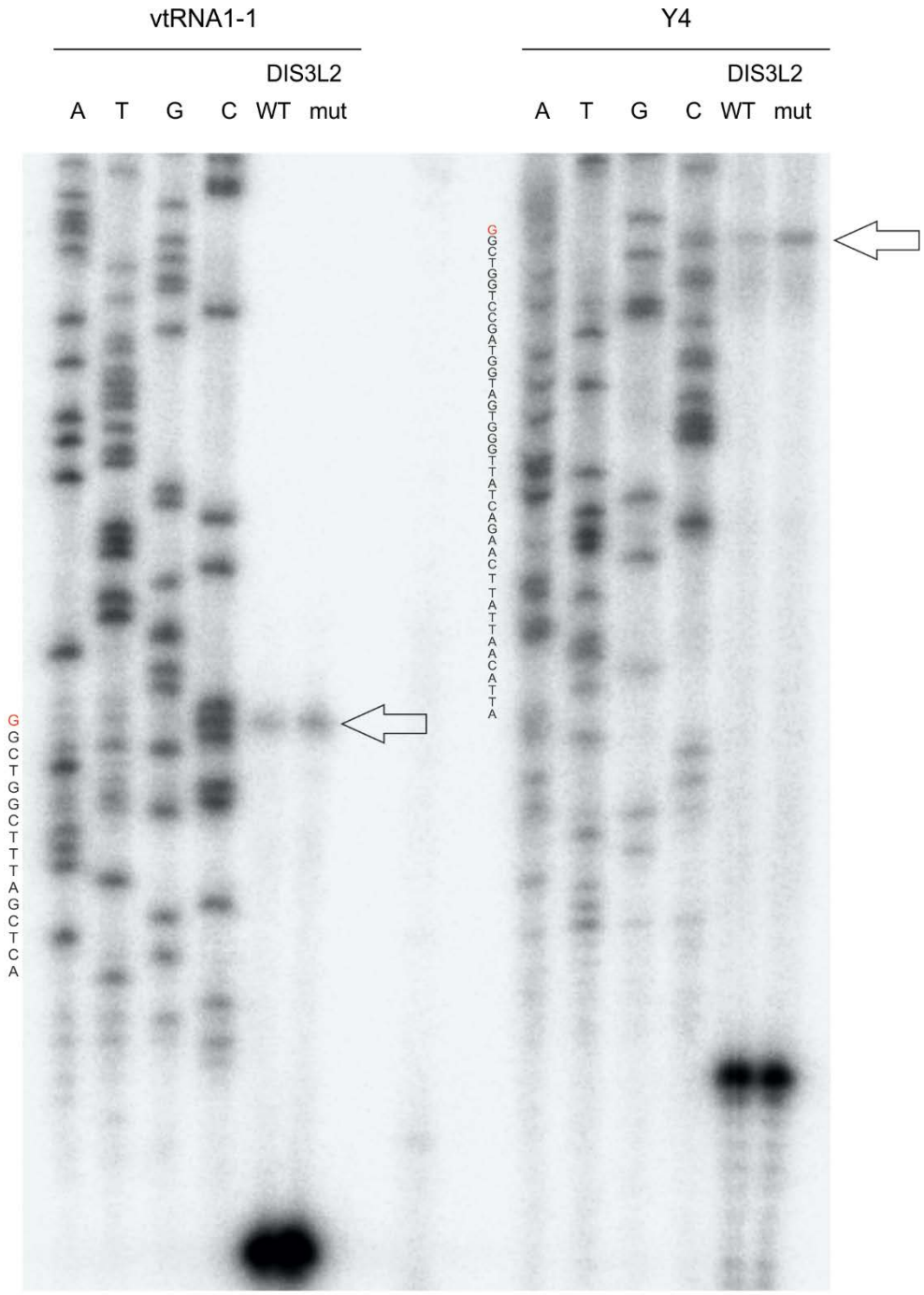


Supplementary Figure S1. Verification of model cell lines co-expressing sh-miRNAs for knockdown of endogenous DIS3L2 and producing exogenous copies of wild-type or mutant miRNA-insensitive DIS3L2 **(a)** Flow cytometry analysis of eGFP in parental HEK293 cells

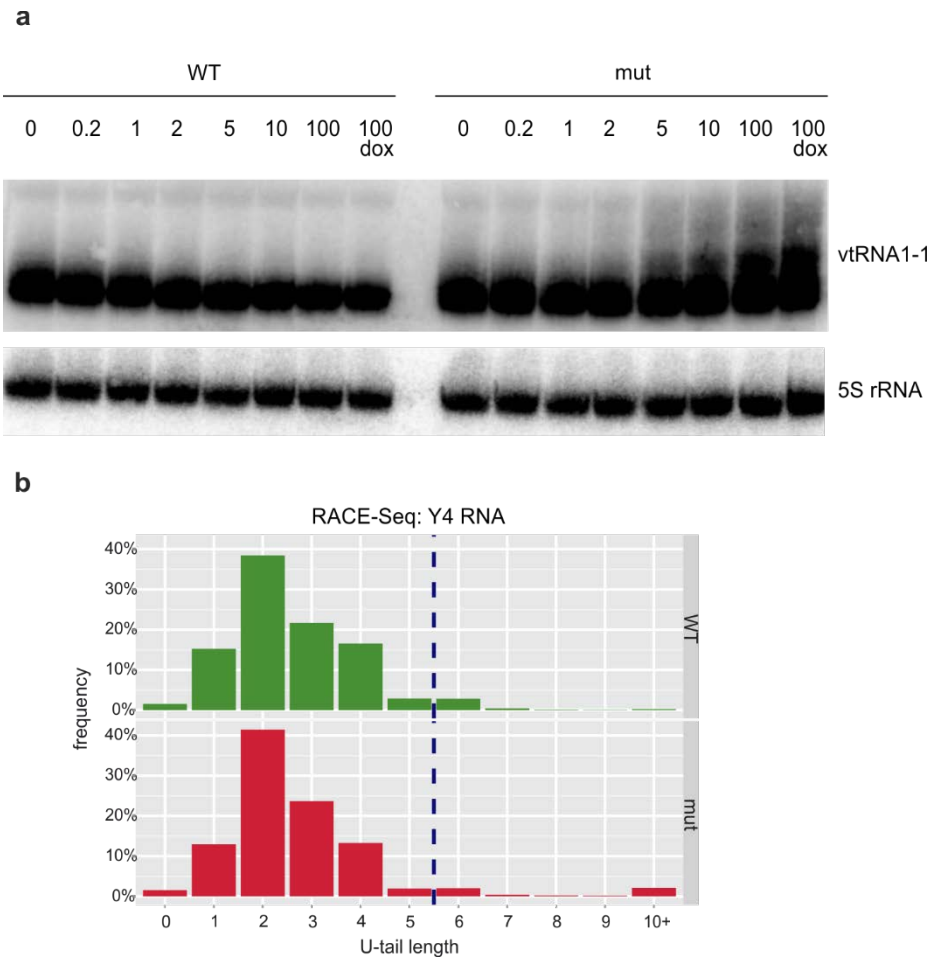
and the model cells grown without ("-dox") or with doxycycline induction ("+dox"). eGFP was used as a tag of the sh-miRNA cassette. Without induction only a minor fraction (≤7%) of the cells expressed eGFP (sh-miRNA) at a low level (not shown). After induction of the cells with doxycycline ≥90% model cells expressed high levels of eGFP, which demonstrates high homogeneity and tightness of our experimental system. **(b)** Northern blot confirmed the inducible expression of three sh-miRNAs targeting endogenous DIS3L2. 5S rRNA was used as an internal control. **(c)** Quantitative PCR (qPCR) was employed to verify silencing efficiency of endogenous DIS3L2 using primers complementary to the region of the endogenous transcript that corresponds to the recoded part of the exogenous version. Analysis showed that, relative to parental HEK293 cells, around 80% silencing was achieved upon sh-miRNA induction in both wild-type and mutant model cell lines. **(d)** Western blotting with α-FLAG-antibody confirmed production of exogenous protein variants following induction (for 24h or 72h). Incubation with α-DIS3L2-antibody allows for comparison of endogenous and exogenous DIS3L2 protein levels between parental HEK293 cells and the established cell lines. α-GAPDH-antibody staining was used as a loading control. Asterisks denote unspecific bands. In lane 13, 1/20 volume of sample from lane 8 was loaded. **(e)** Western blotting with α-FLAG-antibody and α-DIS3L2-antibody after titration of inducer for 72h allows for observation of exogenous protein expression also at lower inducer's concentrations. α-GAPDH-antibody staining was used as a loading control. **(f)** Immunofluorescence analysis with α-FLAG antibody verified that the recoded exogenous DIS3L2-FLAG variants localized to the cytoplasm in model cell lines.



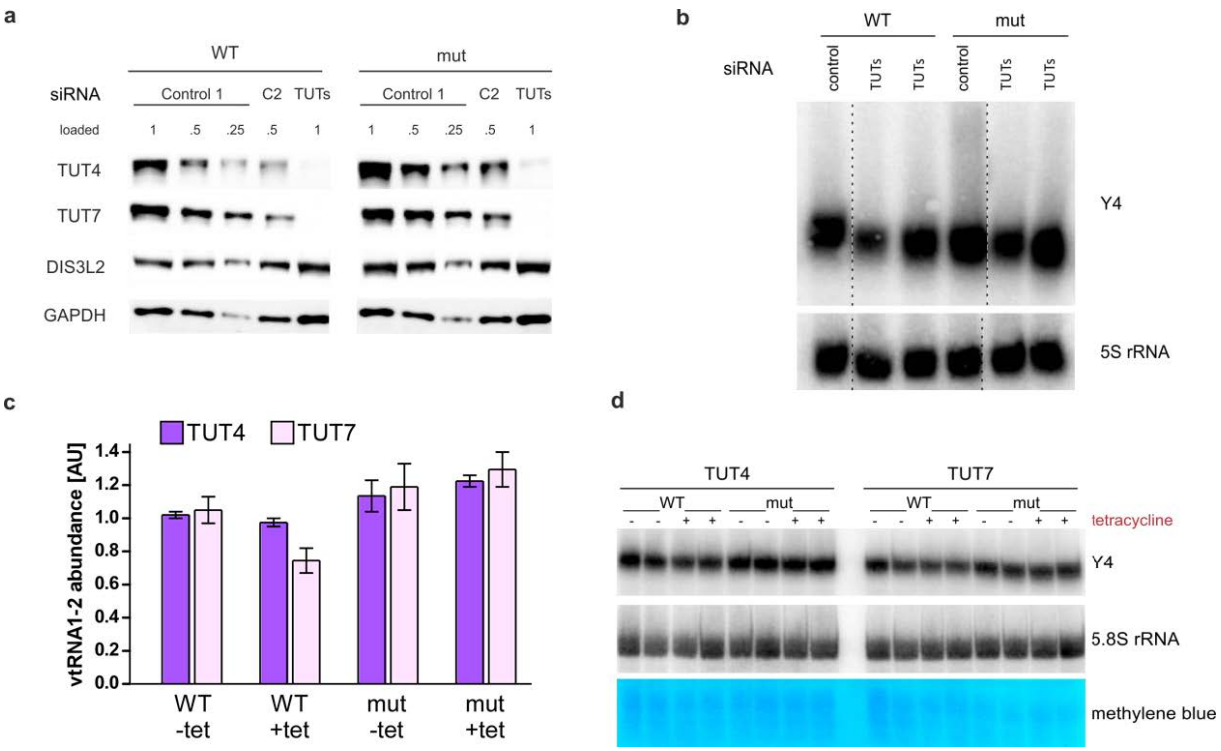
Supplementary Figure S2. mRNAs are DIS3L2 substrates. **(a,b)** Screenshot from Genome Browser showing genomic regions encoding PARP6 mRNA (**a**) or HIST1H2BJ mRNA (**b**), with reads from deep sequencing for WT and mut DIS3L2. Number denotes the normalized expression measured by RNA-Seq (*left*); qPCR validation of accumulation of PARP6 mRNA (**a**) and HIST1H2BJ mRNA (**b**) in mut DIS3L2 (bars illustrate the standard deviation for three biological replicates) (*right*); **(c)** Flow cytometry analysis of propidium iodide stained cells (HEK293, WT and mut) indicating no significant changes in cell cycle distribution. Fractions are indicated. Numbers in parentheses represent standard deviation. **(d)** Validation of HIST1H2AK mRNA accumulation in mut DIS3L2 by northern-blot – uncropped blot image from **Figure 1d**.



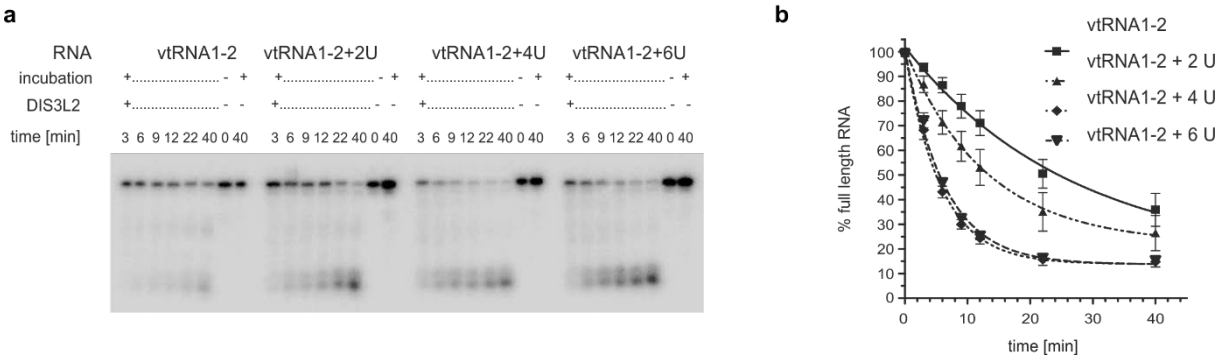
Supplementary Figure S3. Primer extension analysis of the transcription start site for vtRNA1-1 and Y4 in WT and mut DIS3L2 cells. Following total RNA isolation, primer extension was performed using 5' terminally labelled oligonucleotides (listed in Supplementary Table S2). The same oligonucleotides were used to generate sequencing ladders with DNA templates encompassing cloned genomic DNA fragments with vtRNA1-1 and Y4 genes. Both primer extension products and sequencing ladders were subjected to 6% denaturing PAGE and analysed by phosphorimaging. Reference sequences are shown next to the sequencing results.



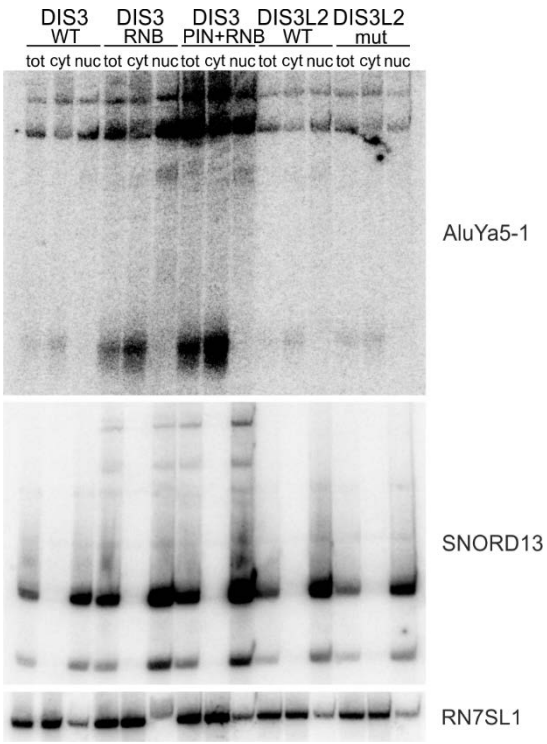
Supplementary Figure S4. Vault and Y RNAs are DIS3L2 substrates. **(a)** Validation of vtRNA1-1 accumulation at lower inducer's concentrations, as assessed by northern blot. 5S rRNA was used as an internal control. **(b)** RACE-Seq results showing that Y4 transcripts bearing 3' non-templated nucleotide additions accumulated in cells expressing catalytically inactive DIS3L2. Since Y4 is a polymerase III transcript, it has five uridines encoded in the genome (left side of dashed line). The distribution of reads with the selected U-tail length is shown (bar represent frequency of reads with selected U-tail length normalized to total read counts).



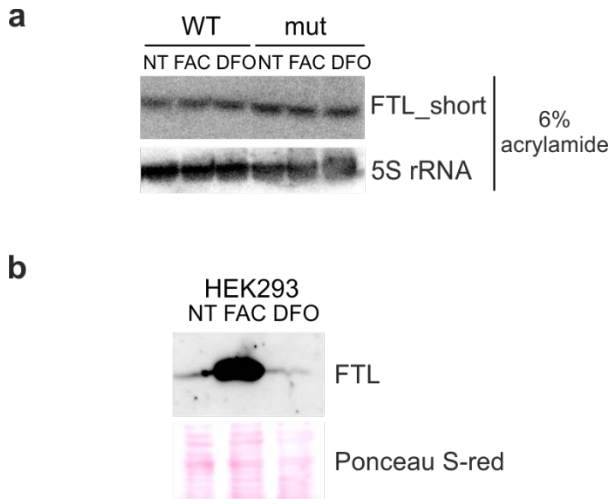
Supplementary Figure S5. Influence of endogenous TUTs on *in vivo* Y4 RNA uridylation. **(a)** Western blot analysis of TUT4 and TUT7 protein levels in model cell lines transfected with two sets of control siRNAs (Control 1 and C2), or a pool of siRNAs against TUT4 and TUT7 (TUTs). Specific antibodies were used to detect TUT4, TUT7, DIS3L2 and GAPDH as described in the Methods section. **(b)** Northern blot analysis of Y4 in WT and mut DIS3L2 cell lines after siRNA-mediated repression of TUTase expression (lanes marked "TUTs"). 5S rRNA was used as an internal control. Dashed lines indicate excised lanes (third biological replicate). **(c)** Quantification (mean and SEM of two replicates) of northern blot data from **Figure 3g** conducted with MultiGauge software (Fuji) and GraphPadPrism. **(d)** Northern blot analysis of Y4 RNA in HEK293 parental cells after transfection with plasmids for tetracycline-inducible overexpression of wild type or catalytically inactive TUT4 or TUT7. "+" indicates induction with tetracycline, and "-": lack of induction. 5.8S rRNA was used as an internal control. Additionally methylene blue staining of the membrane with RNAs of ~70-100 nt in lengths is shown.



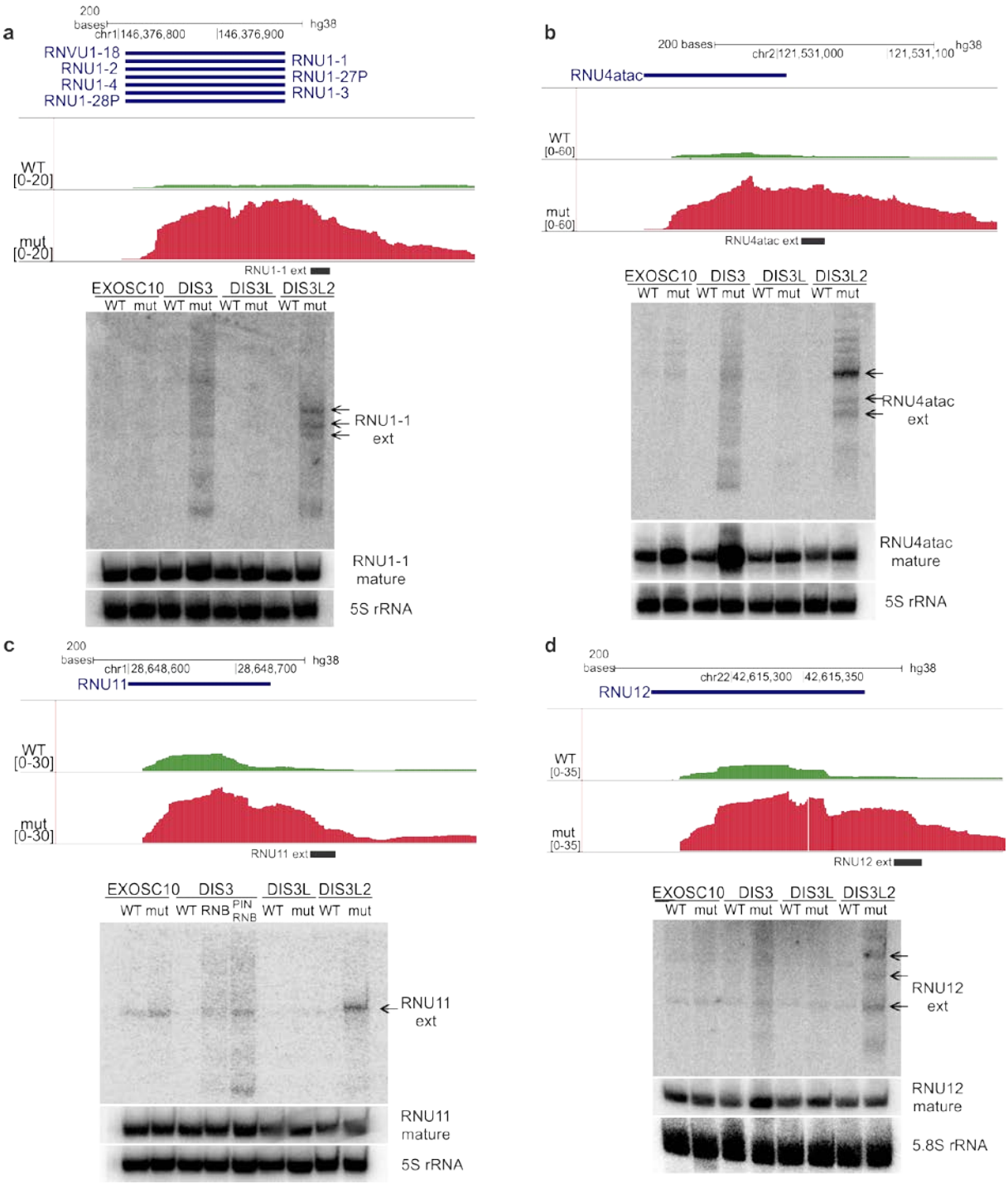
Supplementary Figure S6. Non-templated 3' uridines enhance DIS3L2-mediated degradation kinetics of vtRNA1-2. **(a)** *In vitro* transcribed and PAGE purified vtRNA1-2 and its variants comprising 2, 4 and 6 non-templated Us at the 3'-ends were subjected to DIS3L2 degradation time-course assays. Aliquots were withdrawn at the indicated time points and the reactions rapidly stopped and visualized as in **Figure 4a (b)** The percentages of full-length RNAs remaining in the reactions at the indicated time points in 5 replicate experiments are plotted. The data points were fit using Graph Pad Prism software and single exponential decay equations.



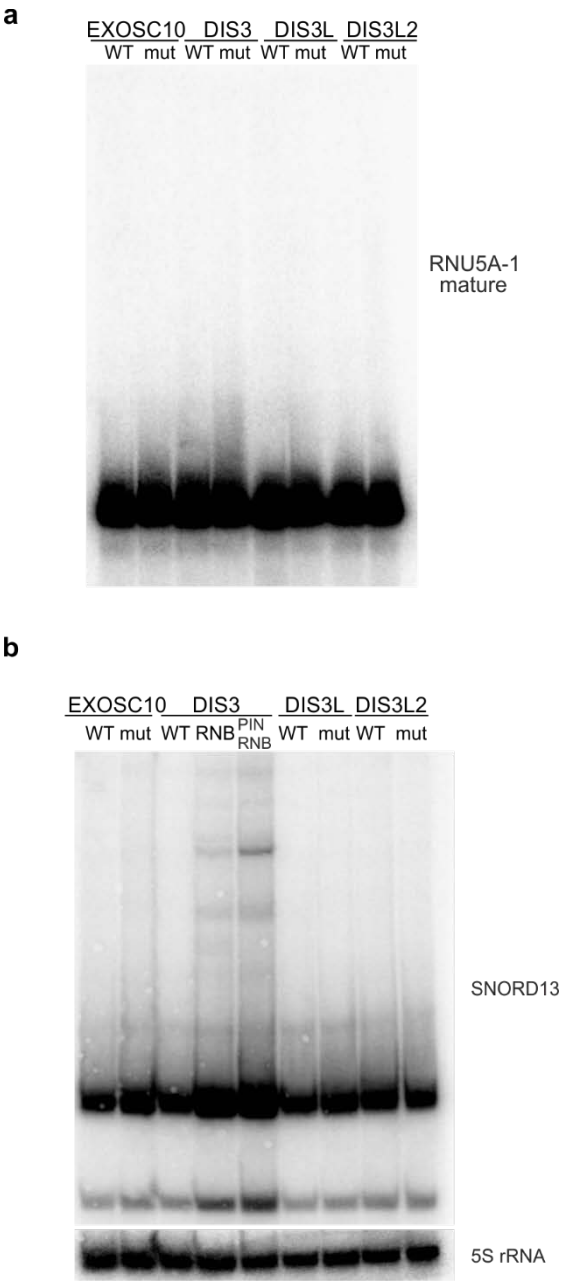
Supplementary Figure S7. Localization of AluYa5 transcripts in different cellular compartments. Cell fractionation and northern blot were used for analysis. Lanes are marked 'tot' for total RNA, 'nuc' for nuclear RNA and 'cyt' for cytoplasmic RNA. RNB indicates the DIS3 mutant of exonucleolytic activity, while PIN+RNB denotes double endo- and exonucleolytic mutations in DIS3. Cell fractionation was monitored by assessing the presence of transcripts prevailing in a given cellular compartment – i.e., SNORD13 (nucleus) and RN7SL1 (cytoplasm).



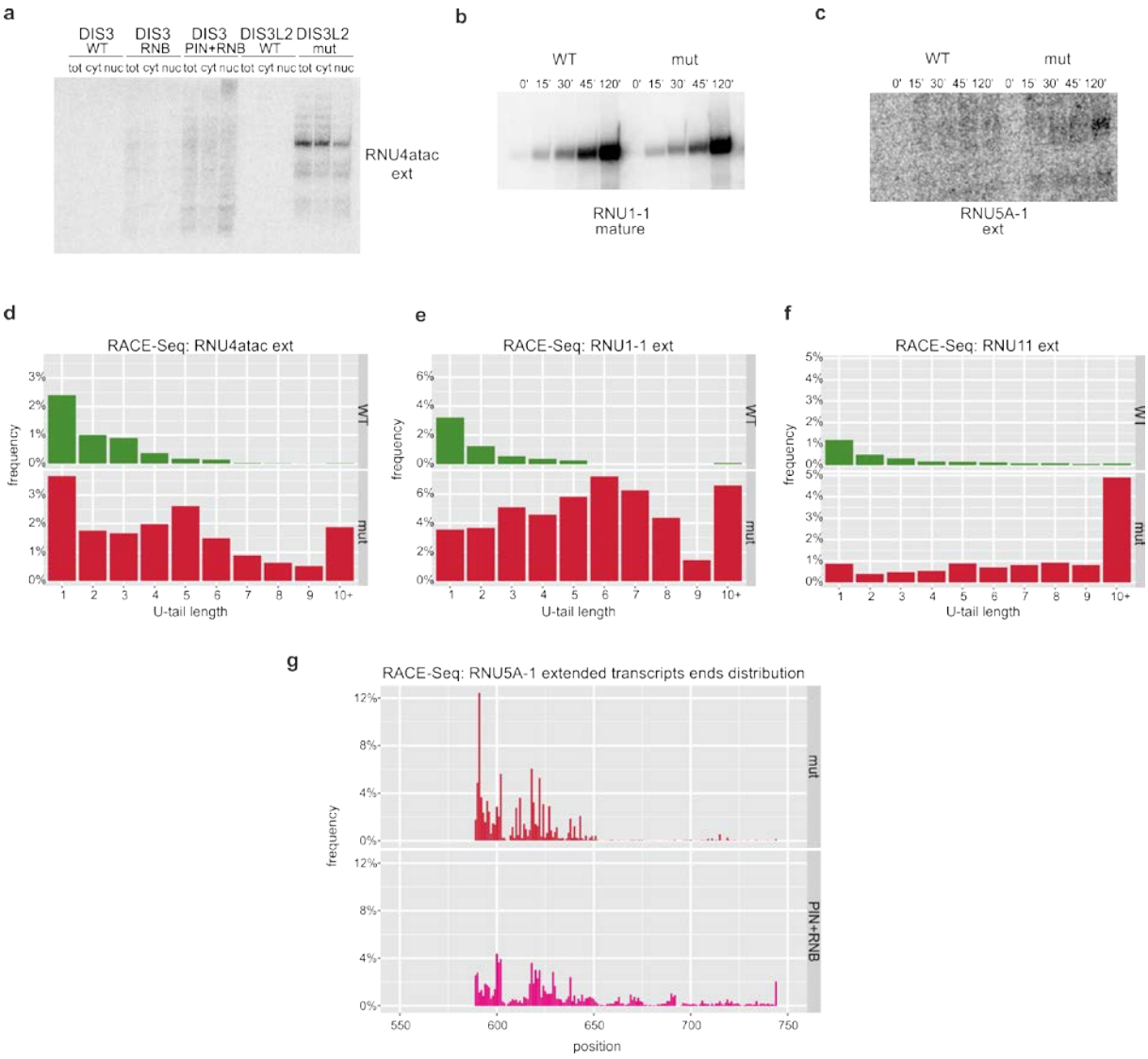
Supplementary Figure S8. FTL_short possibly does not function in iron metabolism. **(a)** After induction with doxycycline, WT and mut cells were treated for 24 hours with substances that alter environmental iron levels: FAC (ferric ammonium citrate) and DFO (deferoxamine). The amounts of FTL_short transcript were then analysed by northern blot. 5S rRNA was used as an internal control. **(b)** Validation of FAC and DFO activity by western blot analysis of FTL protein levels in HEK293 cells following FAC and DFO treatment. Specific antibodies were used for detection as described in the Methods section. Ponceau S-Red staining was used as a loading control.



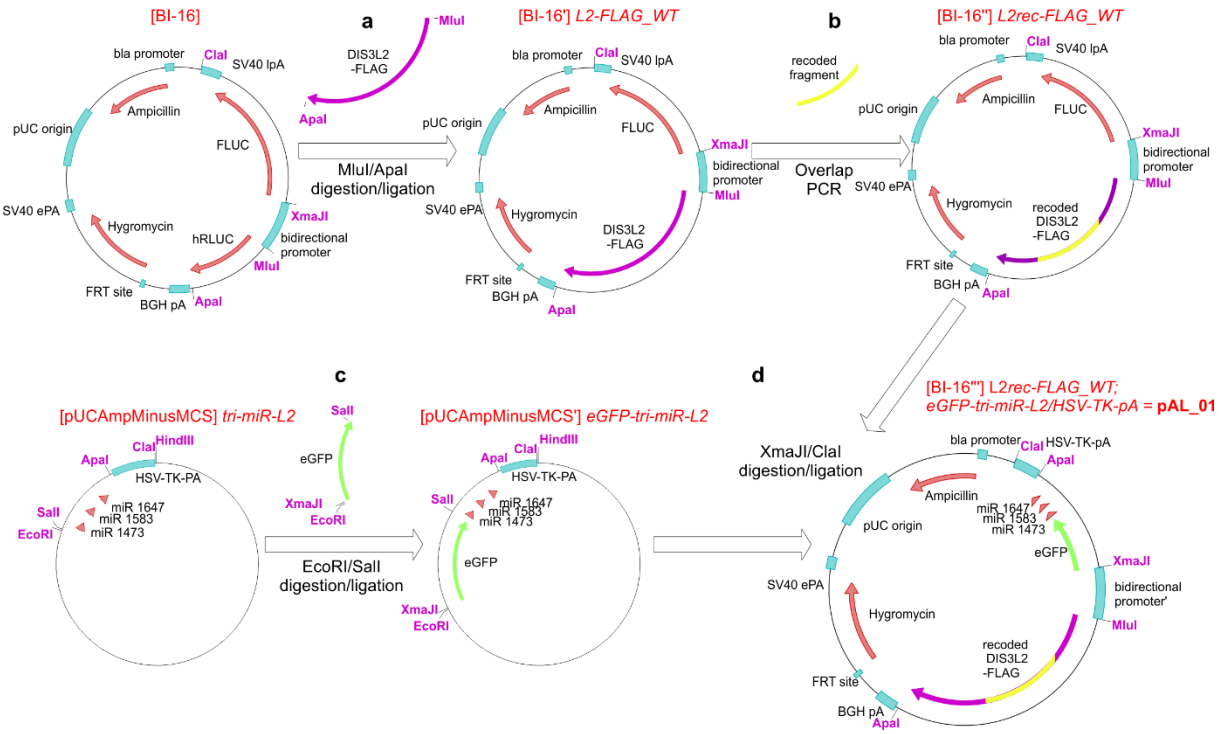
Supplementary Figure S9. Extended snRNAs ("ext") accumulate upon DIS3L2 mutation. **(a-d;** *top***)** Screenshots from Genome Browser showing the genomic region encoding U1 (RNU1-1) snRNA – **a**, U4atac (RNU4atac) – **b**, U11 (RNU11) – **c** and U12 (RNU12) – **d**, with deep sequencing reads for WT and mut DIS3L2. Number denotes the normalized expression measured by RNA-Seq. (**a-d**; *bottom*) Northern blot validation of the presence of extended snRNAs (U1-1 snRNA – **a**, U4atac – **b**, U11 – **c** and U12 – **d**) in model cell lines producing wild-type or mutated EXOSC10, DIS3, DIS3L or DIS3L2. Mature snRNA levels were also analysed. 5S and 5.8S rRNA were used as internal controls. RNB indicates the DIS3 mutant of exonucleolytic activity, while PIN+RNB denotes double endo- and exonucleolytic mutations in DIS3.



Supplementary Figure S10. Mature snRNAs and snoRNAs are not DIS3L2 substrates **(a)** Northern blot verification of mature snRNA level in model cell lines producing wild-type or mutated EXOSC10, DIS3, DIS3L or DIS3L2 – uncropped and overexposed blot image from **Figure 7b. (b)** SNORD13 transcript analysis. Expression of mature SNORD13 RNA and its precursors in the model cell lines with wild type or mutated EXOSC10, DIS3, DIS3L or DIS3L2 was monitored by northern blot. 5S rRNA was used as an internal control.



Supplementary Figure S11. DIS3L2 surveillance of maturing snRNAs. **(a)** Extended U4atac snRNAs ("ext") accumulating upon DIS3 and DIS3L2 mutations are present in the cytoplasm and nucleus. Localization of extended snRNAs in different cellular compartments was assessed by cell fractionation and northern blot analysis (tot – total RNA, nuc – nuclear RNA, cyt – cytoplasmic RNA). RNB indicates DIS3 mutant of exonucleolytic activity, while PIN+RNB denotes double endo- and exonucleolytic mutations in DIS3. **(b,c)** 4-thiouridine (4sU) labelling was used to study the kinetics of mature snRNA production. RNA was collected after labelling and the reaction was terminated at the indicated time points. RNA labelled with 4sU was retrieved by biotinylation and separation on streptavidin beads. **(b)** Presence of mutated DIS3L2 does not influence kinetics of mature U1 snRNA production 15 min to 120 min after the 4sU chase. **(c)** Extended U5 snRNA accumulation after the 4sU chase. **(d-f)** Extended U4atac **(d)**, U1-1 **(e)** and U11 **(f)** transcripts bearing 3' non-templated nucleotide additions accumulate in the cells expressing catalytically inactive DIS3L2 as shown by RACE-Seq. Distribution of reads with selected U-tail length is shown (bar represent frequency of reads with selected U-tail length normalized to total read counts). **(g)** Distribution of U5 extended snRNA transcripts ends in cells expressing mut DIS3L2 and double mutant PIN+RNB DIS3. Mature U5 snRNA transcript ends at position 570 nt.



Supplementary Figure S12. Schematic representation of the cloning procedure used to produce model cells in which endogenous *DIS3L2* gene expression is silenced by three sh-miRs and exogenous DIS3L2 is synthesized in either wild type (on the scheme) or mutant versions as described in the Supplementary Materials and Methods. **(a)** FLAG-tagged DIS3L2 was cloned into the BI-16 vector at the ApaI and MluI sites. **(b)** In an overlap PCR reaction, the DIS3L2 fragment was recoded to introduce sh-miR resistance. **(c)** A parallel synthetic construct with three designed sh-miRs was used as a backbone to insert an eGFP coding sequence (utilizing EcoRI and SalI sites), which was later engaged to monitor sh-miR expression. **(d)** Sequences encoding sh-miRs together with eGFP were cloned into the vector derived in **(b)** using XmaJI/ClaI sites to produce the final construct.

(a)

GAATTCATATAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGCTGTATGCTGTGTGCATG
CTCGTAGCTAAGTGTTTTGGCCACTGACTGACACTTAGCTGAGCATGCACACAGGACACAAG
GCCTGTTACTAGCACTCACATGGAACAAATGGCCCAGATCCTGGAGGCTTGCTGAAGGCTGT
ATGCTGTTCCGTGGAGATTCAAGACGGGTTTTGGCCACTGACTGACCCGTCTTGTCTCCACG
GAACAGGACACAAGGCCTGTTACTAGCACTCACATGGAACAAATGGCCCAGATCCTGGAGGC
TTGCTGAAGGCTGTATGCTGAGCTTTAGCTGATCCAAACGAGTTTTGGCCACTGACTGACTC
GTTTGGCAGCTAAAGCTCAGGACACAAGGCCTGTTACTAGCACTCACATGGAACAAATGGCC
CAGATCTGGCCGCACTCGAGATATCTAGTGATCTAGAGGGCCCGCGGTTCGCTGATGGGGGA
GGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAA
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GCTGGCACTCTGTCGATACCCCACCGTGACCCCATTGGGGCCAATACGCCCGCGTTTCTTCC
TTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGGGC
GGCAGGCCCTGCCATAGCATCGATCGCAAGCTT

(b)

GAATTCATATAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGCTGTATGCTGTTAACAAG
CGGTGTACTACAAGTTTTGGCCACTGACTGACTTGTAGTACCGCTTGTTAACAGGACACAAG
GCCTGTTACTAGCACTCACATGGAACAAATGGCCCAGATCCTGGAGGCTTGCTGAAGGCTGT
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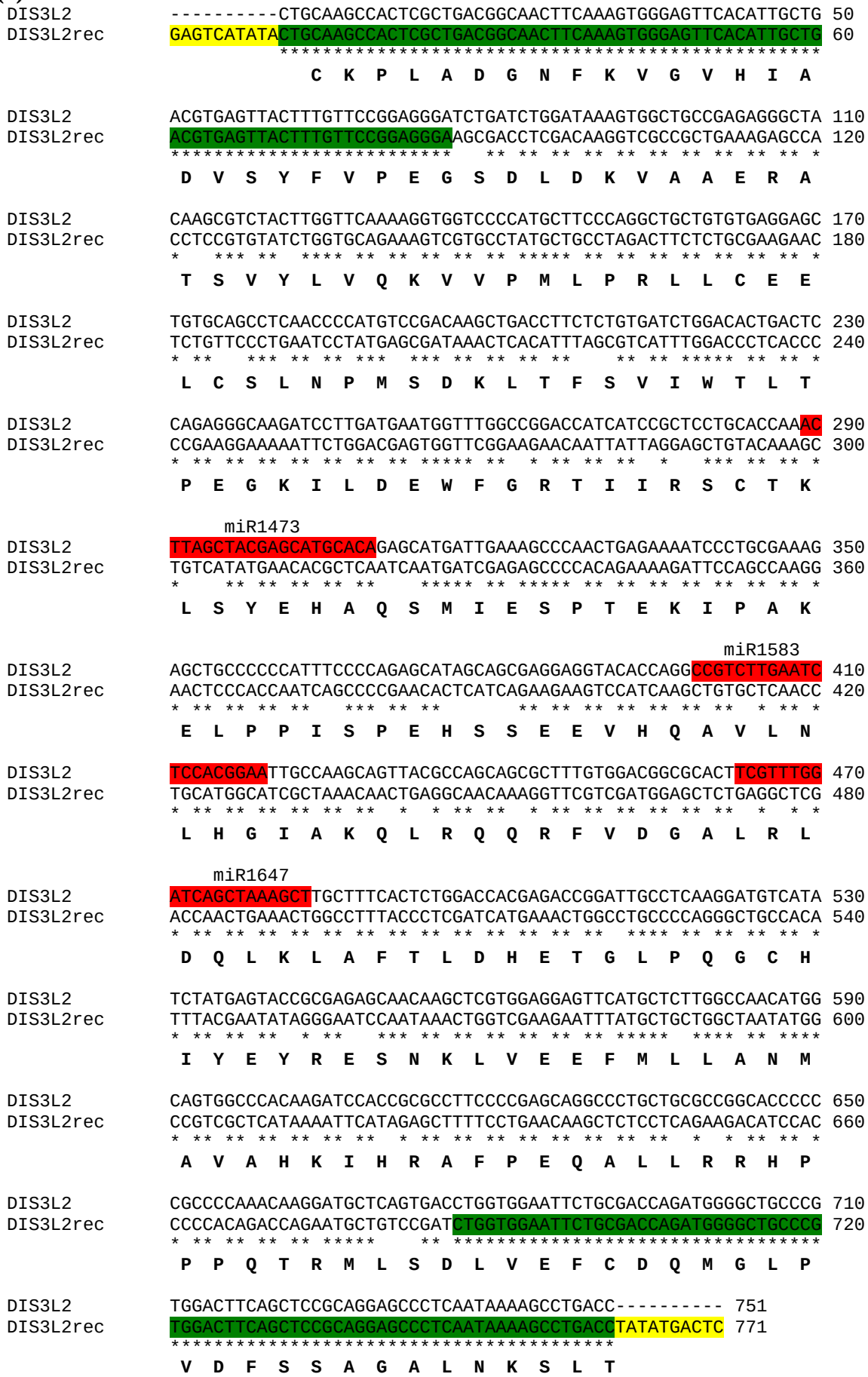
TTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGGGC
GGCAGGCCCTGCCATAGCATCGATCGCAAGCTT

Supplementary Figure S13. Cassettes with sequences encoding three sh-miRs silencing endogenous *DIS3L2/DIS3L2* genes together with flanking sites. **(a)** Cassette for production of sh-miRs silencing *DIS3L2* expression. Red, blue, green and violet letters denote EcoRI, SalI, ClaI and HindIII restriction sites, respectively, which were used during the cloning procedure; gray and black background represent 5' and 3' miR flanking regions, respectively; red background indicates 21 nt antisense target sequences (mature miRNA sequences); magenta background represents nucleotides 1-8 and 11-21 of the respective sense target sequences; green background indicates a 19 nt sequence derived from endogenous murine miR-155, and the underlined 13 nt segment can form a loop within the sh-miRNA structure; yellow background represents HSV-TK polyadenylation signal. **(b)** Same as **a**, but sh-miRs to silence *DIS3L* are shown.

(a)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C K P L A D G N F K V G V H I A
DIS3L2 ACGTGAGTTACTTTGTTCCGGAGGGATCTGATCTGGATAAAGTGGCTGCCGAGAGGGCTA 110
DIS3L2rec ACGTGAGTTACTTTGTTCCGGAGGGAAGCGACCTCGACAAGGTCGCCGCTGAAAGAGCCA 120
D V S Y F V P E G S D L D K V A A E R A
DIS3L2 CAAGCGTCTACTTGGTTCAAAAGGTGGTCCCCATGCTTCCCAGGCTGCTGTGTGAGGAGC 170
DIS3L2rec CCTCCGTGTATCTGGTGCAGAAAGTCGTGCCTATGCTGCCTAGACTTCTCTGCGAAGAAC 180
T S V Y L V Q K V V P M L P R L L C E E
DIS3L2 TGTGCAGCCTCAACCCCATGTCCGACAAGCTGACCTTCTCTGTGATCTGGACACTGACTC 230
DIS3L2rec TCTGTTCCCTGAATCCTATGAGCGATAAACTCACATTTAGCGTCATTTGGACCCTCACCC 240
L C S L N P M S D K L T F S V I W T L T
DIS3L2 CAGAGGGCAAGATCCTTGATGAATGGTTTGGCCGGACCATCATCCGCTCCTGCACCAAAC 290
DIS3L2rec CCGAAGGAAAAATTCTGGACGAGTGGTTCGGAAGAACAATTATTAGGAGCTGTACAAAGC 300
P E G K I L D E W F G R T I I R S C T K
miR1473
DIS3L2 TTAGCTACGAGCATGCACAGAGCATGATTGAAAGCCCAACTGAGAAAATCCCTGCGAAAG 350
DIS3L2rec TGTCATATGAACACGCTCAATCAATGATCGAGAGCCCCACAGAAAAGATTCCAGCCAAGG 360
L S Y E H A Q S M I E S P T E K I P A K
miR1583
DIS3L2 AGCTGCCCCCCATTTCCCCAGAGCATAGCAGCGAGGAGGTACACCAGGCCGTCTTGAATC 410
DIS3L2rec AACTCCCACCAATCAGCCCCGAACACTCATCAGAAGAAGTCCATCAAGCTGTGCTCAACC 420
E L P P I S P E H S S E E V H Q A V L N
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DIS3L2rec TGCATGGCATCGCTAAACAACTGAGGCAACAAAGGTTCGTCGATGGAGCTCTGAGGCTCG 480
L H G I A K Q L R Q Q R F V D G A L R L
miR1647
DIS3L2 ATCAGCTAAAGCTTGCTTTCACTCTGGACCACGAGACCGGATTGCCTCAAGGATGTCATA 530
DIS3L2rec ACCAACTGAAACTGGCCTTTACCCTCGATCATGAAACTGGCCTGCCCCAGGGCTGCCACA 540
D Q L K L A F T L D H E T G L P Q G C H
DIS3L2 TCTATGAGTACCGCGAGAGCAACAAGCTCGTGGAGGAGTTCATGCTCTTGGCCAACATGG 590
DIS3L2rec TTTACGAATATAGGGAATCCAATAAACTGGTCGAAGAATTTATGCTGCTGGCTAATATGG 600
I Y E Y R E S N K L V E E F M L L A N M
DIS3L2 CAGTGGCCCACAAGATCCACCGCGCCTTCCCCGAGCAGGCCCTGCTGCGCCGGCACCCCC 650
DIS3L2rec CCGTCGCTCATAAAATTCATAGAGCTTTTCCTGAACAAGCTCTCCTCAGAAGACATCCAC 660
A V A H K I H R A F P E Q A L L R R H P
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DIS3L2rec CCCCACAGACCAGAATGCTGTCCGATCTGGTGGAATTCTGCGACCAGATGGGGCTGCCCG 720
P P Q T R M L S D L V E F C D Q M G L P
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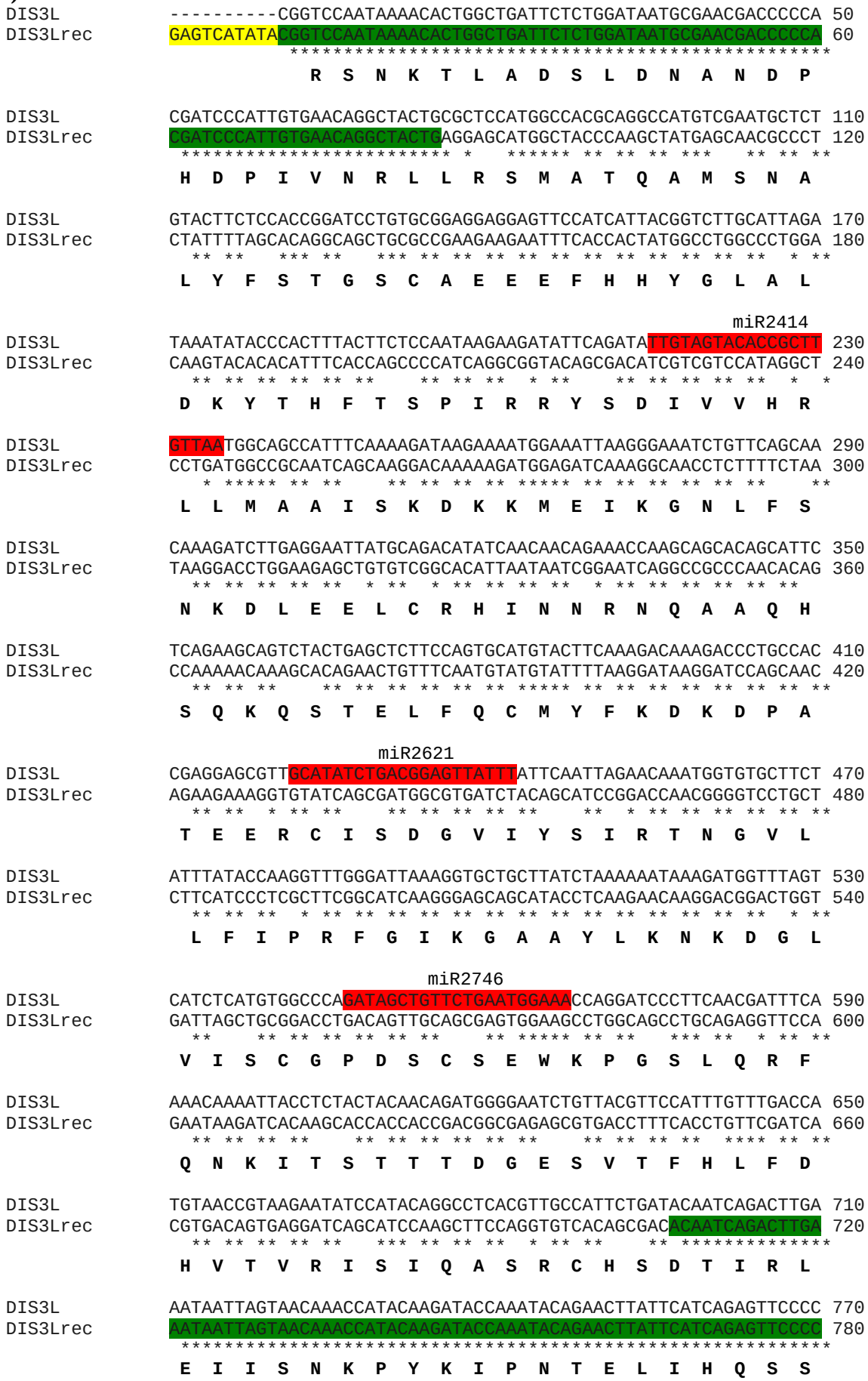
DIS3L2rec TGGACTTCAGCTCCGCAGGAGCCCTCAATAAAAGCCTGACCTATATGACTC 771
V D F S S A G A L N K S L T

(b)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DIS3Lrec GAGTCATATACGGTCCAATAAAACACTGGCTGATTCTCTGGATAATGCGAACGACCCCCA 60
R S N K T L A D S L D N A N D P
DIS3L CGATCCCATTGTGAACAGGCTACTGCGCTCCATGGCCACGCAGGCCATGTCGAATGCTCT 110
DIS3Lrec CGATCCCATTGTGAACAGGCTACTGAGGAGCATGGCTACCCAAGCTATGAGCAACGCCCT 120
H D P I V N R L L R S M A T Q A M S N A
DIS3L GTACTTCTCCACCGGATCCTGTGCGGAGGAGGAGTTCCATCATTACGGTCTTGCATTAGA 170
DIS3Lrec CTATTTTAGCACAGGCAGCTGCGCCGAAGAAGAATTTCACCACTATGGCCTGGCCCTGGA 180
L Y F S T G S C A E E E F H H Y G L A L
miR2414
DIS3L TAAATATACCCACTTTACTTCTCCAATAAGAAGATATTCAGATATTGTAGTACACCGCTT 230
DIS3Lrec CAAGTACACACATTTCACCAGCCCCATCAGGCGGTACAGCGACATCGTCGTCCATAGGCT 240
D K Y T H F T S P I R R Y S D I V V H R
DIS3L GTTAATGGCAGCCATTTCAAAAGATAAGAAAATGGAAATTAAGGGAAATCTGTTCAGCAA 290
DIS3Lrec CCTGATGGCCGCAATCAGCAAGGACAAAAAGATGGAGATCAAAGGCAACCTCTTTTCTAA 300
L L M A A I S K D K K M E I K G N L F S
DIS3L CAAAGATCTTGAGGAATTATGCAGACATATCAACAACAGAAACCAAGCAGCACAGCATTC 350
DIS3Lrec TAAGGACCTGGAAGAGCTGTGTCGGCACATTAATAATCGGAATCAGGCCGCCCAACACAG 360
N K D L E E L C R H I N N R N Q A A Q H
DIS3L TCAGAAGCAGTCTACTGAGCTCTTCCAGTGCATGTACTTCAAAGACAAAGACCCTGCCAC 410
DIS3Lrec CCAAAAACAAAGCACAGAACTGTTTCAATGTATGTATTTTAAGGATAAGGATCCAGCAAC 420
S Q K Q S T E L F Q C M Y F K D K D P A
miR2621
DIS3L CGAGGAGCGTTGCATATCTGACGGAGTTATTTATTCAATTAGAACAAATGGTGTGCTTCT 470
DIS3Lrec AGAAGAAAGGTGTATCAGCGATGGCGTGATCTACAGCATCCGGACCAACGGGGTCCTGCT 480
T E E R C I S D G V I Y S I R T N G V L
DIS3L ATTTATACCAAGGTTTGGGATTAAAGGTGCTGCTTATCTAAAAAATAAAGATGGTTTAGT 530
DIS3Lrec CTTCATCCCTCGCTTCGGCATCAAGGGAGCAGCATACCTCAAGAACAAGGACGGACTGGT 540
L F I P R F G I K G A A Y L K N K D G L
miR2746
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DIS3Lrec GATTAGCTGCGGACCTGACAGTTGCAGCGAGTGGAAGCCTGGCAGCCTGCAGAGGTTCCA 600
V I S C G P D S C S E W K P G S L Q R F
DIS3L AAACAAAATTACCTCTACTACAACAGATGGGGAATCTGTTACGTTCCATTTGTTTGACCA 650
DIS3Lrec GAATAAGATCACAAGCACCACCACCGACGGCGAGAGCGTGACCTTTCACCTGTTCGATCA 660
Q N K I T S T T T D G E S V T F H L F D
DIS3L TGTAACCGTAAGAATATCCATACAGGCCTCACGTTGCCATTCTGATACAATCAGACTTGA 710
DIS3Lrec CGTGACAGTGAGGATCAGCATCCAAGCTTCCAGGTGTCACAGCGACACAATCAGACTTGA 720
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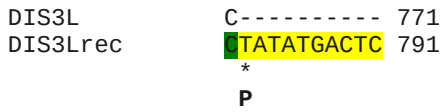

Supplementary Figure S14. Sequence alignments of endogenous and recoded DIS3L/DIS3L2. **(a)** Alignment of endogenous DIS3L2 and recoded DIS3L2rec. Unchanged nucleotides are marked with asterisks; yellow background represents SchI restriction sites used to excise the recoded DIS3L2 fragment from the ordered construct; green background shows perfectly conserved flanking regions that enabled the insert to be used as a megaprimer in overlap PCR; red background corresponds to sequences targeted by designed sh-miRs. **(b)** Same as in **a**, but the scheme represents DIS3L recoding.

Supplementary Tables

1) Supplementary Table S1

RACE-Seq results presenting the amount of vtRNA1-2 and Y4 RNA transcripts ending in the indicated number of uridines. Grey background denotes genomic uridines.

	vtRNA1-2				**Y4 RNA**			
	read counts		frequency (%)		read counts		frequency (%)	
U-tail length	WT	MUT	WT	MUT	WT	MUT	WT	MUT
0	142	309	0,35	0,45	1014	1311	1,53	1,57
1	620	608	1,52	0,88	10069	10856	15,22	12,97
2	2481	2470	6,08	3,58	25416	34701	38,43	41,46
3	15440	12512	37,84	18,12	14327	19831	21,66	23,69
4	14146	19701	34,67	28,54	10950	11115	16,56	13,28
5	3153	6056	7,73	8,77	1920	1632	2,90	1,95
6	3356	11089	8,23	16,06	1884	1712	2,85	2,05
7	711	5661	1,74	8,20	282	357	0,43	0,43
8	265	3088	0,65	4,47	75	217	0,11	0,26
9	148	1941	0,36	2,81	49	168	0,07	0,20
10	66	1124	0,16	1,63	25	145	0,04	0,17
11	27	1085	0,07	1,57	30	140	0,05	0,17
12	19	1020	0,05	1,48	27	155	0,04	0,19
13	10	620	0,02	0,90	9	157	0,01	0,19
14	6	351	0,01	0,51	10	121	0,02	0,14
15	5	227	0,01	0,33	7	120	0,01	0,14
16	3	209	0,01	0,30	9	106	0,01	0,13
17	3	167	0,01	0,24	20	141	0,03	0,17
18	1	129	0,00	0,19	8	174	0,01	0,21
19	1	62	0,00	0,09	5	173	0,01	0,21
20	1	58	0,00	0,08	2	123	0,00	0,15
21	1	23	0,00	0,03	1	73	0,00	0,09
22	3	18	0,01	0,03	1	59	0,00	0,07
23	8	16	0,02	0,02	0	48	0,00	0,06
24	12	11	0,03	0,02	1	26	0,00	0,03
25	32	24	0,08	0,03	0	16	0,00	0,02
26	123	212	0,30	0,31	0	4	0,00	0,00
27	15	177	0,04	0,26	0	7	0,00	0,01
28	0	18	0,00	0,03	0	4	0,00	0,00
29	0	15	0,00	0,02	0	1	0,00	0,00
30	0	4	0,00	0,01	0	1	0,00	0,00

2) Supplementary Table S2

Oligonucleotides used in this study

Oligonucleotide	**Sequence (5'-3')**	**Purpose**
eGFPFor	GCGGAATTCATATACCTAGGACCATGGTGAGCAAGGGCGAGGAGC	cloning of eGFP ORF into synthetic plasmid with three sh-miRs
eGFPRev	GCGCGTCGACTCACTACCTCCTCTTACTTGTACAGCTCGTCCATGC	cloning of eGFP ORF into synthetic plasmid with three sh-miRs
mutD3L2F	GCCACCTGGGGCCTGAGAAGGAGGAGGAGGAGTCTGACGG	mutagenesis od ApaI site in DIS3L2
mutD3L2R	CTTCTCAGGCCCCAGGTGGCCCTGGGTGCCTGGCCGCTTCAGGATGGCGCTG	mutagenesis od ApaI site in DIS3L2
mutDISLF	CGTGCGCATCGACTCCTGGGAGTCAACATCTGTGTATCCAAACG	mutagenesis od ClaI site in DIS3L
mutDISLR	CCCAGGAGTCGATGCGCACGACCACCCTGAAGTCCTGGAGG	mutagenesis od ClaI site in DIS3L
DIS3L2F	ATATACGCGTGCCGCCATGAGCCATCCTGACTACAGAATGAACC	cloning of DIS3L2 into BI-16 plasmid
DIS3L2R	GCGCGGGCCCTTACTTGTCGTCGTCGTCCTTGTAATCTATATCGCTGGTGCTTGAGTCCTCGG	cloning of DIS3L2 into BI-16 plasmid
DIS3LF	ATATACGCGTGCCGCCACCATGCTGCAGAAGCGGGAGAAGG	cloning of DIS3L into BI-16 plasmid
DIS3LR	GCGCGGGCCCTTACTTGTCGTCGTCGTCCTTGTAATCTATATCTATTCCATAATTGTTTGAAACATCC	cloning of DIS3L into BI-16 plasmid
BIseq_F	GATCGTCGGATCCTCTAGTC	sequencing of insert in BI-16 plasmid
BIseq_R	GCACAGTCGAGGCTGATCAG	sequencing of insert in BI-16 plasmid
seDIS3L2	CGGTGCATCACCATCCTCTC	sequencing of DIS3L2
DIS3L2_2	GGATCTGGTGGTCGTGAAAC	sequencing of DIS3L2
DIS3L2_3	ACTGTTCATCTGCCGCATTG	sequencing of DIS3L2
DIS3L2_4	ATAGCAGCGAGGAGGTACAC	sequencing of DIS3L2
DIS3L2_5	AACAGGCGGACCACTGTAAC	sequencing of DIS3L2
recDL3_3	GAGGCTCGACCAACTGAAAC	sequencing of recoded fragment of DIS3L2

E11fw	CCACTTATTATCTAGCAGATCGTCGC	sequencing of DIS3L
E13fw	GTCCAATAAAACACTGGCTG	sequencing of DIS3L
E15rv	CTTGGTATAAATAGAAGCACA	sequencing of DIS3L
E5fw	ACCTGGACAATTTCTGGCCTGAT	sequencing of DIS3L
E5rw	AGCGTCCAGATTTAATCCCA	sequencing of DIS3L
E8fw	CATACTTCAGAAGAACTGGC	sequencing of DIS3L
pucnMCSF	GATTACGCCAAGCTCCTTCC	sequencing of insert in pUCAmpMinusMCS plasmid
pucnMCSR	GCCAGTGAATTGGAGGCTAC	sequencing of insert in pUCAmpMinusMCS plasmid
egfpseqR	ACTACCTGAGCACCCAGTC	sequencing of insert in BI-16 plasmid
BISTOPR	ACAGTGGGAGTGGCACCTTC	sequencing of insert in BI-16 plasmid
L2sil_1F	CTCCAGAGGGCAAGATCCTTGATG	qPCR (endogenous DIS3L2)
L2sil_1R	TGGGCTTTCAATCATGCTCTGTG	qPCR (endogenous DIS3L2)
GAPDH_F	GTCAGCCGCATCTTCTTTTG	qPCR (endogenous GAPDH)
GAPDH_R	GCGCCCAATACGACCAAATC	qPCR (endogenous GAPDH)
smiR1473	ACTTAGCTACGAGCATGCACA	northern blot (expression of sh-miR)
smiR1583	CCGTCTTGAATCTCCACGGAA	northern blot (expression of sh-miR)
smiR1647	TCGTTTGGATCAGCTAAAGCT	northern blot (expression of sh-miR)
P0_for1	GGAGCTAGCGAGAATTTGTATTTTCAGG GTGATATCCAGCACAGTGGCGG	Generation of the p0 plasmid from the parental pcDNA5/FRT/TO plasmid
P0_rev1	CTTGGTACCGAGCTCGGATCCGAAAACC TGTACTTCCAAGGAACCGGTGGT	
P0_for2	GGTCACCTGTACAATCGATCCTGCAGGA GCTAGCGAGAATTTGTATTTTC	
P0_rev2	TCCTGCAGGATCGATTGTACAGGTGACC ACCGGTTCCTTGGAAGTACAGG	
P2_for	GGACTCTAGCGTTTAAACTTAAGCTTGG TACCATGGTGAGCAAGGGCGAGGAG	Generation of the p2 plasmid from the p0 plasmid
P2_rev	CCACCGGTTCCTTGGAAGTACAGGTTTT CGGATCCCTTGTACAGCTCGTCCATGC	
ZCCHC11_F_FRTT	GGATCCGAAAACCTGTACTTCCAAGGAA CCGGTATGGAAGAGTCTAAAACCTT	Cloning of TUT4 into the p2 plasmid
ZCCHC11_R_F	GATATCACCCTGAAAATACAAATTCTCG	

RTT_stop	CTAGCTTACTCCGACACGTTTCCTC	
ZCCHC6_F_FRTT	GGATCCGAAAACCTGTACTTCCAAGGAACCGGTATGGGAGATACAGCAAAACC	Cloning of TUT7 into the p2 plasmid
ZCCHC6_R_FRTT_stop	GATATCACCCTGAAAATACAAATTCTCGCTAGCTTATGATTCCTGCTGGGTCCTCTTC	
11_244F2	Gatttcgtgatagtgatctggctatttgtatgaccctggaagg	Pair of primers for site-directed mutagenesis of TUT4 (change of GAT into GCT codon)
11_244R1	Ccttccagggtcatacaaatagccagatcactatcacgaaatc	
11_245F2	Ggttcaaacagagtgaccttgccgtctgtatgacaattaatgg	Pair of primers for site-directed mutagenesis of TUT7 (change of GAC into GCC codon)
11_245R1	ccattaattgtcatacagacggcaaggtcactctgtttgaacc	
CGA_2F	TCCCACTCCACTAAGGTCCA	qPCR (validation)
CGA_2R	CCCCCATTACTGTGACCCTG	qPCR (validation)
PARP_1F	CAGCAACAGCCCTTGGAAAG	qPCR (validation)
PARP_1R	TAGGATGACGGCAGCGTTAG	qPCR (validation)
HIST1H2AK	CTGGGCGATGGTCACTTTAC	northern blot (validation)
HIST1H2BJ_1F	GTGTCCGAGGGTACTAAGGC	qPCR (validation)
HIST1H2BJ_1R	AGAGCCGTTAGGGTTGAGAG	qPCR (validation)
RNU5A-1 mature	GAGTTGTTCCTCTCCACGGA	northern blot (validation)
RNU5A-1 ext	ATTTCTATTGTTGGATTACCAC	northern blot (validation)
RNU1-1 mature	CAAATTATGCAGTCGAGTTTCCCACATTTG	northern blot (validation)
RNU1-1 ext	ACAAGTAACCCTCGCCGTGC	northern blot (validation)
RNU11 mature	AAAGGGCGCCGGGACCAACG	northern blot (validation)
RNU11 ext	GTTACAAAAAGCACCACTTACTCC	northern blot (validation)
RNU12 mature	CATTAGCAGTGAGGATTCGG	northern blot (validation)
RNU12 ext	CCACCCCACAGTCAGTCTAC	northern blot (validation)
RNU4atac mature	GTTGTTCAGGCGTTAGCAGT	northern blot (validation)
RNU4atac ext	TGTTTGAACTGATAAGTCTATG	northern blot (validation)
SNORD13	GGTCAGACGGGTAATGTGCCCACGTCGTAA	northern blot (validation)

5S rRNA	CATCCAAGTACTAACCAGGCCC	northern blot (loading control)
5.8S rRNA	TCCTGCAATTCACATTAATTCTCGCAGCTAGC	northern blot (loading control)
vtRNA1-1	GCTTGTTTCAATTAAAGAACTGTCG	northern blot (validation), Primer Extension
vtRNA1-2	AGGTGGTTACAATGTACTCGAAG	northern blot (validation)
Y4	AGCCAGTCAAATTTAGCAGTGGGGGGTTGTAT	northern blot (validation), Primer Extension
VTRNA1-1_PEF	GGAAAGGCTACGTCCTTGTCA	Primer Extension, cloning of VTRNA1-1 from genomic DNA to plasmid
VTRNA1-1_PER	TTGTTCTTGGTTTTTAAGCATTTCC	Primer Extension, cloning of VTRNA1-1 from genomic DNA to plasmid
Y4_PEF	AAGGACAAGAGGCTTCCATTTG	Primer Extension, cloning of RNY4 from genomic DNA to plasmid
Y4_PER	TTGAGCCCAGGAGTTTAGAGC	Primer Extension, cloning of RNY4 from genomic DNA to plasmid
Y4_RA	GTTCAGAGTTCTACAGTCCGACGATCTGGTCCGATGGTAGTGGGTTATCAG	RACE-Seq
vtRNA1-2_RA	GTTCAGAGTTCTACAGTCCGACGATCGGCTTTAGCTCAGCGGTTAC	RACE-Seq
T7_Y RNA	Agatgtaatacgactcactatagggctggtccgatggtagtgggttatcagaacttattaacattagtgtcactaaagttggtatacaacccccactgctaaatttgactggcttttt	Oligonucleotide for amplifying by PCR template for T7 transcription of Y4 RNA
T7_vault RNA 1-1	Agatgtaatacgactcactatagggctggctttagctcagcggttacttcgacagttctttaattgaaacaagcaacctgtctgggttgttcgagacccgcgggcgctctccagtcctttt	Oligonucleotide for amplifying by PCR template for T7 transcription of vtRNA1-1 RNA
T7_vault RNA 1-2	Agatgtaatacgactcactatagggctggctttagctcagcggttacttcgagtacattgtaaccacctctctgggtggttcgagacccgcgggtgctttccagctctttt	Oligonucleotide for amplifying by PCR template for T7 transcription of vtRNA1-2 RNA
T7Promun	gtgagagatgtaatacgactcactatagg	Universal forward primers for PCR generation of templates for *in vitro* transcription
YRrev	aaaaagccagtcaaatttagcagtgggg	Reverse primers for PCR generation

vR1-1rev	aaaaggactggagagcgcccgcg	of templates for T7 *in vitro* transcription of Y4 RNA, vtRNA1-1, vtRNA1-2 and the variants of the latter comprising 2, 4 and 6 additional 3’ uridines
vR1-2rev	aaaagagctggaaagcacccgcg	
vR1-2r+2	aaaaaagagctggaaagcacccgcg	
vR1-2r+4	aaaaaaaagagctggaaagcacccgcg	
vR1-2r+6	aaaaaaaaaagagctggaaagcacccgcg	
U5_RA_1	GTTCAGAGTTCTACAGTCCGACGATC ATATGTGGTAATCCAACAATAG	RACE-Seq
U4a_RA_1	GTTCAGAGTTCTACAGTCCGACGATC AAAACCTGTTTTCATAGACTTATC	RACE-Seq
U1_RA_1	GTTCAGAGTTCTACAGTCCGACGATC GGGACTGCGTTCGCGCTTTC	RACE-Seq
U11extRA	GTTCAGAGTTCTACAGTCCGACGATC TGCGGAATCGACATCAAGAGATTT	RACE-Seq
BCYRN1_P1F	CTGGGCAATATAGCGAGAC	qPCR (validation)
BCYRN1_P1R	TGCTTTGAGGGAAGTTACG	qPCR and northern blot (validation)
AluY5_1	GGGTTTCACTGGTTTAGCCA	northern blot (validation)
AluY5_2	TGCAGTACCTCGTTTATGGG	northern blot (validation)
FTL_full	GAGAGGTAGGTGTAGGAGGC	northern blot (validation)
FTL_pin3	TGGAAGAGAGTCCCCGGATC	northern blot (validation)

Supplementary Note

The enhancement of DIS3L2-mediated vtRNA degradation by uridine addition was also shown for synthetic RNA substrates with different U tail lengths.

We *in vitro* transcribed either vtRNA1-2 with 4 uridines at the 3'-end (reflecting native unmodified vtRNA1-2 species) or its variants with an additional 2, 4 or 6 uridines at the 3' termini (reflecting postranscriptionally uridylated species). After 5'-end labeling, we used those RNAs as substrates for our *in vitro* DIS3L2-mediated degradation assays. These analyses revealed enhanced degradation rates for extra-uridylated vtRNA1-2 substrates compared to their wild-type counterparts that lack an additional 3' U-tail (Supplementary Figure S6a,b). Essentially, the presence of additional 3' terminal uridines decreased the half-life of full-length substrates from 18 min (wild-type=4Us), to 9 min (wild-type+2Us) and ~4 min (wild-type+4Us/6Us). The presence of 4 additional uridines at the 3'-end of vtRNA1-2 resulted in the fastest degradation kinetics and maximal degradation efficiency in our experimental conditions, with no further increase for substrates ending with an additional 6 Us. Taken together, we demonstrated that the presence of additional uridines at the 3' end of vtRNA1-2 significantly increased its degradation rate by DIS3L2 *in vitro*.

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