SUPPLEMENTAL MATERIAL AND METHODS

Cell culture and manipulation

Undifferentiated mouse ES cells (mESC D3 cells; (Doetschman et al., 1985)) were adapted to feeder-free culture and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 15% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 100 mM non-essential amino acids (Gibco-Invitrogen), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-Invitrogen), and 1000 U/ml recombinant leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA).

Mammalian cells (HEK293T-Rex, HeLa) were maintained in DMEM supplemented with 10% fetal calf serum at 37°C in the presence of 5% CO2. For doxycycline-inducible protein expression; doxycycline was added to culture media at 10 - 50 ng/ml. For transient transfections, cells were grown to 70% confluency, and plasmid DNA was transfected using TURBOFECT (Fermentas) following the manufacturer's instructions.

Protein extracts preparation and cell fractionation

Mouse embryonic stem cells (mESC) were washed, re-suspended in buffer containing 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT and 0.2 mM PMSF and incubated 10 minutes on ice. Cells were broken in a Dounce homogenizer and nuclei were pelleted by centrifugation at 2000 rpm for 15 minutes at 4°C. The supernatant represented the cytoplasmic fraction. Nuclear pellet was then incubated with buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT and 0.2 mM PMSF. Next, the concentration of KCl was increased up to 1.2 M and extracts were incubated for additional 30 minutes at 4°C. The insoluble fraction was removed by centrifugation at 10000 rpm for 1 hour at 4°C. The cytoplasmic and nuclear extracts were subsequently dialyzed to the buffer containing 20 mM HEPES pH 7.9, 20% glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT and 0.2 mM PMSF. After dialysis extracts were centrifuged for 30 minutes at 10000 rpm at 4°C, supernatant was aliquoted and frozen in liquid nitrogen.

RNA-based protein precipitation

Affinity purifications were performed with biotin-labeled U_{30} RNA and a random 30 nt RNA. As a control for unspecific binding to RNA we used a random 30nt RNA sequence (5' GAACAUAUUUCACCAACAUUAUACUGUGUC 3'), the expression of which has not been detected in mouse and human cells (our BLAST search).
To remove unspecific binders, mESC protein extracts were first pre-cleared using 100 µl of packed Streptavidin agarose (SAg) resins (Thermo Scientific), washed twice with Low salt buffer (LSB) (20 mM HEPES pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.01% NP40, 1 mM DTT).
Fresh SAg was pre-blocked in blocking buffer (LSB containing 1 mg/ml RNase-free BSA, 20 µg glycogen, 50 µg yeast total RNA) for 1 hour at 4°C with slow rotation. One milliliter of blocking buffer was used per 100 µl of packed SAg beads. SAg were then washed twice with High salt buffer (HSB) (20 mM HEPES pH8.0, 300 mM KCl, 10 mM $MgCl_2$, 0.01% NP40, 1 mM DTT) and stored as 1:1 slurry (SAg beads : HSB).
To prepare SAg-RNA matrix, 40 µl of pre-blocked slurry of SAg beads in HSB was mixed with 5 volumes of HSB containing 10 µg biotinylated U_{30} RNA oligo (Sigma) and 50U of RNasin Plus RNase inhibitor (Promega) in HSB. The mixture was incubated for 5 hours at 4°C with rotation. SAg beads were collected by 1 minute centrifugation at 1500g and washed 3 times with 1 ml of HSB. For protein precipitation, 150 µl of pre-cleared protein extract was added and incubated for 1 hour at 30°C with rotation. SAg beads were briefly collected by centrifugation at 1500g and washed 3 times with 1 ml of HSB. Bound proteins were eluted with 20 µl of 1x SDS loading buffer. Proteins were separated on 12% polyacrylamide gels.

Preparation of stable cell lines

Plasmids (pcDNA5 FRT/TO FLAG-DIS3L2, pcDNA5/FRT/TO FLAG-D391N and pcDNA FRT/TO FLAG-AGO2) were transfected to Flp-In™ T-REx™ (Invitrogen) cell line with TURBOFECT reagent and selected for stable insertions according to the manufacturer's protocol.

LC-MS/MS analysis and database searching

The profiles of U_{30} precipitates from nuclei and cytoplasm and of proteins bound to control RNA were compared by SDS-PAGE, and bands unique to U_{30} RNA samples were identified by tandem mass spectrometry analysis (MS-MS).
1D gel areas to be analyzed were excised from the corresponding 1D gel lines. After destaining and washing procedures, each gel band was incubated with trypsin. Liquid

chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed using EASY-nLC system (Proxeon) on-line coupled with an HCTultra PTM Discovery System ion trap mass spectrometer (Bruker Daltonik). Sample volume was 10 µl. Prior to LC separation, tryptic digests were concentrated and desalted using trapping column (100 µm × 30 mm) filled with 4-µm Jupiter Proteo sorbent (Phenomenex, Torrance, CA). After washing with 0.1% formic acid, the peptides were eluted from the trapping column using an acetonitrile/water gradient (350 nL/min) onto a fused-silica capillary column (100 µm x 100 mm), on which peptides were separated. The column was filled with 3.5-µm X-Bridge BEH 130 C18 sorbent (Waters). The mobile phase A consisted of 0.1% formic acid in water and the mobile phase B consisted of 0.1% formic acid in acetonitrile. The gradient elution started at 5% of mobile phase B and increased linearly from 5% to 35% during the first 20 minutes. The gradient linearly increased to 90% of mobile phase B in the next two minutes and remained at this state for next 8 minutes. The analytical column outlet was directly connected to the nanoelectrospray ion source. Nitrogen was used as nebulizing as well as drying gas. The pressure of nebulizing gas was 8 psi. The temperature and flow rate of drying gas were set to 250ºC and 6 L/min, respectively, and the capillary voltage was 4.0 kV.

The mass spectrometer was operated in the positive ion mode in m/z range of 300 – 1500 for MS and 100-2500 for MS/MS scans. Two precursor ions per MS spectrum were selected in data dependent manner for further fragmentation. Extraction of the mass spectra from the chromatograms, mass annotation and deconvolution of the mass spectra were performed using DataAnalysis 4.0 software (Bruker Daltonik). MASCOT 2.3.02 (MatrixScience, London, UK) search engine was used for processing the MS and MS/MS data. Database searches were done against the NCBI database, taxonomy *Mus musculus* (non redundant; downloaded from ftp://ftp.ncbi.nih.gov/blast/db/FASTA/; database version 20120407; 146,059 protein sequences for taxonomy *Mus musculus*; 17,751,536 protein sequences in total). Mass tolerances of peptides and MS/MS fragments for MS/MS ion searches were 0.5 Da. Oxidation of methionine and carbamidomethylation of cysteine as optional modifications, one enzyme miscleavage and correction for one ^{13}C atom were set for all searches. Peptides with statistically significant peptide score ($p < 0.05$) were considered. Manual MS/MS spectra assignment validation was done with

identification verification using database search against the whole NCBI database without taxonomy restriction.

Cell fractionation for whole-IP LC-MS-MS analyses

D3 mESCs (10^8) were washed twice with 5 ml of ice-cold 1x PBS with protease inhibitor (PI) cocktail (Roche), harvested from dishes and resuspended in 2 ml ice-cold 1x PBS with PI. The buffer was removed by brief centrifugation and cell pellets were gently lyzed in 1 ml of ice-cold lysis buffer (0.1% NP-40, 1x PBS, 1X PI). The cytoplasmic and nuclear fractions were crudely separated by brief centrifugation 10000g for 10s in a table-top microcentrifuge. The supernatants representing the cytoplasmic fraction were collected and pellets representing the nuclear fraction were washed with 1 ml of ice-cold lysis buffer. Nuclei were broken in 1 ml of lysis buffer by sonication. The fractionation efficiency was subsequently monitored by western blot analysis with anti-Histon H1 antibodies for nuclear and anti-Tubulin alpha antibodies for cytoplasmic fractions. The extracts were the used for RNA precipitations as described above.

FASP processing

Protein pull-downs were processed by filter-aided sample preparation (FASP) method {Wisniewski, 2009 #421}{Wisniewski, 2011 #422}. The whole samples were mixed with 8 M UA buffer (8 M urea in 100 mM Tris-HCl, pH 8.5), loaded onto the Vivacon 500 device with MWCO 10 kDa (Sartorius Stedim Biotech) and centrifuged at 14,000 × g for 30 min at 20°C. The retained proteins were washed with 400 µL UA buffer. The final protein concentrates kept in the Vivacon 500 device were mixed with 100 µL of UA buffer containing 50 mM dithiothreitol and incubated for 30 min. After additional centrifugation, the samples were mixed with 100 µL of UA buffer containing 50 mM iodoacetamide and incubated in the dark for 30 min. After the next centrifugation step, the samples were washed three times with 400 µL UA buffer and three times with 200 µL of 50 mM $NaHCO_3$. Trypsin (sequencing grade, Promega) was added onto the filter and the mixture was incubated for 14 h at 37°C. The tryptic peptides were finally eluted by centrifugation followed by two additional elutions with 50 µL of 50 mM $NaHCO_3$.

LC-MS/MS analysis of peptides from FASP

LC-MS/MS analyses of peptide mixture were done using RSLCnano system connected to Orbitrap Elite hybrid spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Prior to LC separation, tryptic digests were online concentrated and

desalted using trapping column (100 µm × 30 mm) filled with 3.5-µm X-Bridge BEH 130 C18 sorbent (Waters, Milford, MA, USA). After washing of trapping column with 0.1% FA, the peptides were eluted (flow 300 nl/min) from the trapping column onto a Acclaim Pepmap100 C18 column (2 µm particles, 75 µm × 250 mm; Thermo Fisher Scientific, Waltham, MA, USA) by the following gradient program (mobile phase A: 0.1% FA in water; mobile phase B: 0.1% FA in acetonitrile): the gradient elution started at 1% of mobile phase B and increased from 1% to 45% during the first 40 min (28% in the 30^{th} and 45% in 40^{th} min), then increased linearly to 95% of mobile phase B in the next 2 min and remained at this state for the next 13 min. Equilibration of the trapping column and the column was done prior to sample injection to the sample loop. The analytical column outlet was directly connected to the Nanospray Flex Ion Source (Thermo Fisher Scientific, Waltham, MA, USA).

MS data were acquired in a data-dependent strategy selecting up to top 20 precursors based on the precursor abundance in the survey scan (350-1700 m/z). The resolution of the survey scan was 120 000 (400 m/z) with a target value of 1×10^6 ions, one microscan and maximum injection time of 200 ms. Low resolution CID MS/MS spectra were acquired with a target value of 10 000 in rapid CID scan mode with m/z range adjusted according to actual precursor mass and charge. MS/MS acquisition in the linear ion trap was carried out in parallel to the survey scan in the Orbitrap analyser by using the preview mode. The maximum injection time for MS/MS was 150 ms. Dynamic exclusion was enabled for 45 s after one MS/MS spectra acquisition and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 m/z.

The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (Thermo Fisher Scientific; version 1.3) with in-house Mascot (Matrixscience, London, UK; version 2.3.1) and Sequest search engines utilisation. Mascot MS/MS ion searches were done against UniProt protein database for mouse (downloaded from ftp://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/proteomes/ ; version 20130626; 50,818 sequences; 24,421,122 residues) with additional sequences from cRAP database (downloaded from http://www.thegpm.org/crap/). Mass tolerance for peptides and MS/MS fragments were 5 ppm and 0.5 Da, respectively. Oxidation of methionine and deamidation (N, Q) as optional modification, carbamidomethylation of C as fixed modification and two enzyme miss

cleavages were set for all searches. Percolator was used for post-processing of Mascot search results. Peptides with false discovery rate (FDR; q-value) < 1%, rank 1 and with at least 6 amino acids were considered. Label-free quantification using protein area calculation in Proteome Discoverer was used ("top 3 protein quantification" {Silva, 2006 #423}).

Two LC-MS/MS analyses in total were done for each sample with the same sample volume. The second LC-MS/MS analysis was performed with exclusion of m/z masses already assigned to peptide from target database (FDR < 1%) based on the first LC-MS/MS analysis. Mass tolerance for m/z exclusion was set to 10 ppm and retention time window to two minutes. The two resulting raw files for each sample were searched as a single data set. The results for U_{30} RNA and random RNA IP samples were compared and at least five-fold enrichment in U_{30} RNA sample over random RNA sample was set as a trash hold (labelled as oligo(U)-specific interactor in this experimental set up).

DIS3L2 constructs

Constructs for bacterial expression. The coding sequence of human DIS3L2 protein (isoform 1, 885 aminoacids, NP_689596.4) was cloned into pET28b between NheI and BamHI sites allowing expression of N-terminaly fused 6xHis-Smt3 tag. *DIS3L2* was amplified by PCR in two parts (because of XhoI internal cleavage site) using cDNA prepared from HEK293 RNA as a template. First, the 3′ end of CDS (1664 – 2658 nt) was amplified with primers (Forward 5′ AGCAGCGAGGAGGTACACCAG3′, Reverse 5′ ACCTCGAGTCAGCTGGTGCTTGAGTCCTCG 3′) and subcloned into pET28b using *Hind*III and *Xho*I sites, than the 5′ part (1 – 1663 nt) was amplified with primers (Forward 5′ CGGGATCCATGAGCCATCCTGACTACAG 3′, Reverse 5′ ATGACATCCTTGAGGCAATCC 3′) and ligated to the 3′ end via *BamH*I and *Hind*III sites. The sequence of the final clone was verified by sequencing.

To produce the mutant form, we introduced a point mutation D391N (Figure S1A), in a residue that corresponds to the catalytically essential aspartate 209 in RNase II (Frazao et al., 2006) and D551 in Rrp44p (Frazao et al., 2006; Schneider et al., 2007). D209N mutation within RNB motif allows RNA binding but prevents cleavage

(Amblar & Arraiano, 2005). The mutation was introduced by site-directed mutagenesis (Stratagene).

For the expression of the protein in human cells, the coding sequence of DIS3L2 and AGO2 were cloned in pcDNA5/FRT/TO (Invitrogen). For immunofluorescence, the coding sequence of DIS3L2 was subcloned into pEGFP-N1 (GenBank Accession #U55762) and pEGFP-C3 vectors (GenBank Accession #: U57607) to obtain N-terminal and C-terminal EGFP fused tags, respectively.

DNA construct for episomal let-7 expression was a kind gift of Prof. Narry Kim.

LIN28A coding region was amplified from the HEK293 cDNA library and the PCR product was subcloned to pcDNA4 vector to produce C-terminal Myc-6xHis fusion protein.

Purification of FLAG-DIS3L2 from human cells

FLAG-DIS3L2 was purified from stable HEK293T-Rex cell line expressing the fusion protein. Cells were lyzed in 4 ml of ice cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Triton X100 and Complete Protease Inhibitor Cocktail (Roche) and incubated rocking at 4°C for 15 min. Lysates were cleared by centrifugation (14000 rpm, 30 minutes, 4°C). For purification of FLAG-DIS3L2, 100 μl of anti-FLAG M2 beads (Sigma-Aldrich) washed with lysis buffer were incubated with cell extract for 1 hour in a cold room rotating. Beads were extensively washed with 10 volumes of wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 0.1% Triton X100). Protein elution was done with 1 volume of 3x FLAG peptide (Sigma-Aldrich) resuspended in lysis buffer or by boiling with SDS loading buffer for 5 min.

Expression and purification of recombinant wild type and mutant proteins

Recombinant DIS3L2 was expressed and purified from BL21-DE3 RIPL strain of *E. coli*. Bacterial cells were grown at 37°C and protein expression was induced at OD_{600} 0.7 with 0.5 M IPTG at 27°C for 2 hours. Cells were harvested by centrifugation and lysed by sonication in buffer containing 50 mM Tris pH 7.9, 500 mM NaCl, 10% glycerol, 2 mM 2-mercaptoethanol, and 0.1% NP-40. Lysate was cleared by centrifugation (14000 rpm, 30 minutes, 4°C). Protein was purified by Ni-NTA chromatography. SMT3 tag was removed by proteolysis with Ulp1 protease. Resulting recombinant DIS3L2 (rDIS3L2) was further purified by gel filtration on Superdex 200 column (GE Healthcare) in buffer containing 20 mM Tris pH 7.9, 300 mM NaCl, 10% glycerol, 0.01 NP-40, 2 mM 2-mercaptoethanol, 10 mM imidazole.

Immunofluorescence and image processing

Cells expressing GFP-fusion proteins were plated in dishes with cover slips coated with 0.2% gelatin. Paraformaldehyde fixed cells were permeabilized with 0.2% Triton-X100 in PBS in the presence of DAPI to stain the nucleus. Coverslips with FlouroMount reagent (Invitrogen) were mount to glass slides and fluorescent images were captured with a Leica DM 6000 B microscope. To visualize DAPI and GFP, 405 nm diode and Argon 488nm lasers were applied, respectively.

Transfected HeLa cells were fixed with 3.7% PFA in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min and blocked with 5% horse serum in PBS for 1 h. All steps were performed at RT. Samples were incubated with primary antibodies (rabbit anti-DIS3L2 or anti-GFP) diluted 1:200 in the blocking buffer overnight at 4°C. The next day, fixed cells were washed three times with 0.2% Triton X-100 in PBS (5 min) and incubated in the dark with the secondary antibody for 40 min at RT. Cy3-, or Cy5-conjugated donkey secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at dilutions of 1:2,000 or 1:200 with 2.5% horse serum in PBS buffer, respectively. The cells were mounted in FluoroMount reagent (SouthernBiotech, Birmingham, AL) and pictures were captured with a confocal microscope Leica TSC SP5.

RNA immunoprecipitation (RIP)

Empty HEK293T-REX and HEK293T-REX FLAG DIS3L2 cells were grown to 80% confluence, washed with ice cold PBS. In order to prevent unspecific protein-RNA reassociations during cell lysis (as reported in (Riley et al., 2012)), we stabilized RNA-protein contacts by UV-crosslinking (400mJ, 254nm). Cells were lysed in buffer (LB) containing 150 mM NaCl, 50 mM Tris pH 7.6, 0.5% Triton X-100, supplemented with protease inhibitors (EDTA-free Complete Protease Inhibitor Cocktail, Roche), 0.5 mM EDTA, 1 mM DTT, RNase In (Promega). Lysates were cleared by centrifugation, supernatants applied on FLAG M2 Magnetic beads (Sigma) and incubated for 60 minutes. Beads were washed twice with LB, two times with LB containing 300 mM NaCl and RNA was eluted by treating the beads with 2 mg/ml Proteinase K (New England Biolabs) for 120 min at 37 ºC. Eluted RNA was extracted using phenol/chlorophorm and precipitated with ethanol. After DNase treatment (Turbo DNase, Fermentas), equal amounts of RNA were taken for the synthesis of cDNA by Superscript III reverse transcriptase (Invitrogen). Obtained cDNA was used for PCR amplification and PCR products were resolved on 2% agarose gel.

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was isolated with TRIzol (Invitrogen) according to manufacturer's instructions, followed by RNase-free DNase (TURBO DNase, Fermentas) treatment. The total RNA concentration was measured in a Beckman Coulter DU 730. 1 ug of purified RNA was reverse transcribed using specific primers or random hexamers (as indicated for individual experiments) and SuperscriptRT III (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was performed using FastStart Universal SYBR Green Master (Roche) and gene-specific primer pairs (Table S3) on Q-PCR Light Cycler 7500 (Applied Biosystems). Each experiment was performed at least in triplicate. Transcript abundance was calculated by the ΔΔCt (delta delta Ct) method. Data were normalized to an internal control of the housekeeping gene HPRT mRNA or RNU44 for small RNA detection. Results are expressed as means and standard errors of the mean. *P*-values were calculated by Student's *t*-test; *P*- values < 0.05 were considered significant.

Supplementary figure legends

Figure S1. (**A**) Western blot analysis of the fractionated cell lysates used for the MS-MS analysis. W represents the whole cell lysate, NE is the nuclear fraction, CE is the cytoplasmic fraction. (**B**) Silver stained SDS-PAGE profile of proteins precipitated on U30 RNA and control random RNA (ctrl) from nuclear and cytoplasmic fractions of mouse ESCs.

Figure S2. (**A**) Immunofluorescence staining of HeLa cells treated with nontargeting control siRNAs (siControl) and with siRNAs targeting *DIS3L2* mRNA (siDIS3L2) was performed with DIS3L2 specific antibodies. The panel on right shows western blot analysis of DIS3L2 knock down efficiency. (**B**) Sequence alignment of RNAse type II enzymes from *Escherichia coli* (RNAse II), *Sacharomyces cerevisiae* (Rrp44p) and *Homo sapiens* (DIS3 and DIS3L2). The catalytically important aspartate residues are framed in green. In red frame is the aspartate mutated in the catalytically inactive D391N DIS3L2. (**C**) *In vitro* degradation assays with recombinant wild type (WT) and mutant (D391N) DIS3L2 with U_{30} RNA as a substrate. The migrations of input RNA and degradation products are indicated. Np stands for a control without any protein added. The left panel shows SDS-PAGE analysis of purified recombinant WT and D391N DIS3L2. The proteins were visualized by Coomassie staining. (**D**) DIS3L2 shows Mg^{++}-dependent processive activity. It generates 2 to 4 nt degradation end-products on U_{30} RNA substrate *in vitro* in manner. *In vitro* degradation assay using recombinant DIS3L2 and 5' end labeled U_{30} RNA substrate in presence of different divalent ions was performed for times indicated on top. Reactions were stopped with UREA-loading buffer and RNAs were separated on 20 % denaturing polyacrylamide gel and radioactive signal was detected by autoradiography. The RNA ladder was prepared by limited RNA hydrolysis of U_{30} RNA. np stands for no protein control, EDTA is a reaction performed in the presence of 5 mM EDTA, dp is the migration position of degradation products.

Figure S3. (**A**) D391N DIS3L2 precipitates pre-let-7 miRNA. PCR amplification of cDNAs prepared from RIP experiments by using reverse oligo specific to 3' end RNA linker ligated to the RNAs. For the PCR amplification, we used pre-let-7a specific forward oligo and 3' end linker-specific primer. Product were resolved on 2 % agarose gels and stained by ethidium bromide. Blank RIP RNA is RNA purified from IP

samples from cells with no FLAG-tagged protein expressed. PCR control is a reaction where no template DNA was added. (**B**) The sequencing results of the PCR products shown in DIS3L2 D391N RIP in (**A**). On top is the sequence of the forward oligonucleotide used for PCR amplification. In the second lane is the full-length pre-let-7a-1 sequence. The sequencing results of individual clones are aligned underneath. On the bottom is an example of the chromatograph of the sequencing reaction. (**C**) Oligouridylation stimulates DIS3L2-mediated degradation of pre-let-7 miRNA. *In vitro* degradation assays with DIS3L2 purified from HEK293T-Rex cells and pre-let-7 miRNAs with increasing number of 3'-terminal UMPs or with eight AMPs added. The substrate RNAs were prepared by *in vitro* transcription and radioactively labeled at the 5' end. The purified protein was incubated with 2 pM of labeled RNA for the indicated time periods. RNAs were separated on 20% denaturing PAGE and signals were visualized by phosphorimaging. (**D**) DIS3L2 exhibits preference for oligo(U) extended RNA substrates. The 5' end labeled pre-let-7-U8 RNA was incubated with purified DIS3L2 in presence of ten-fold excess of different unlabeled competitor RNAs (indicated on top) for times indicated. RNAs were analyzed as in (C).

Figure S4. (**A**) The catalytical mutant of DIS3L2 precipitates extended forms of pre-let-7. Northern blot analyses of RNAs precipitated with D391N DIS3L2 from HEK293T-Rex cells with or without ectopical expression of LIN28 and pri-let-7a-1. The ethidium bromide staining of the RNA isolated from the input and IP samples and separated by denaturing gel electrophoresis is shown on the bottom (EtBr). The panel on the right shows western blot analysis of protein samples from the whole cell lysates (input) and after immunoprecipitation on FLAG matrix (IP-FLAG-D391N). (B) DIS3L2 knock down causes reduced levels of mature let-7 miRNA in HeLa cells. Northern blot analysis of RNAs isolated from HeLa cells treated with control nontargeting siRNAs (siCTRL) or two different sets of siRNAs targeting *DIS3L2* mRNA. The bottom panel shows the level of DIS3L2 knock down monitored by western blot. EtBr is Ethidium bromide staining of the RNA used as a loading control. The levels of miRNAs in knock down samples were quantified relative to the signal in the control (CTRL) and are presented underneath each probe used. (C) DIS3L2 downregulation in HeLa cells leads to a decrease in the levels of mature let-7 miRNA. HeLa cells were transfected with two different 20 nM *DIS3L2*-specific or nontargeting siRNAs (siCtrl). Q-PCR analysis of total RNA purified from control and

DIS3L2 siRNA-treated cells from at least five independent biological replicates, **) p-value ≤ 0.001, n=6. *) p-value ≤ 0.031, n=5. The levels of miRNAs were normalized to the expression of RNU44 snoRNA. P-values estimated by Student's paired t-test, number of replicates as indicated. Error bars represent standard deviation. The level of DIS3L2 knockdown monitored with a DIS3L2-specific antibody by western blot is shown in (C). Tubulin served as a loading control. (D) Altered levels of DIS3L2 have no major impact on Dicer expression. Western blot analysis of HEK293T-Rex and HeLa cell lysates after siRNA mediated DIS3L2 knock down (left panel) and overexpression of WT and D391N DIS3L2.

Table S1. MS-MS results of proteins coprecipitated by U_{30} RNA from nuclear and cytoplasmic extracts of mouse embryonic stem cells, separated by SDS-PAGE and identified from the gel.

Table S2. LC-MS-MS results of whole IP samples coprecipitated by U_{30} and random RNA, respectively from nuclear and cytoplasmic extracts of mESC. The results for U_{30} and random RNA IP samples were compared and at least five-fold enrichment in U30 RNA sample over random RNA sample was set as a trash hold for U30-specific binding.

Table S3. Primers used for the qPCR analysis in this study.

Oligonucleotide name	Sequence
DIS3L2 L	GAGCCAGCACAGCAGGTC
DIS3L2 R	CAGGATGGCGCTGTACTTG
UNIVERSAL REV	GTGCAGGGTCCGAGGT
RNU44 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCAG
RNU44 F	CCTGGATGATGATAAGCAAATGCTG
HSA-LET-7A-5P RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT
HSA-LET-7A-5P 5'	GTTGCGTGAGGTAGTAGGTTG
HSA-MIR-30B-5P RV	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTGA
HSA-MIR-30B-5P F	GCAATGTAAACATCCTACACTCAG

Table S4. siRNA used in this study were purchased from Dharmacon and Sigma. Sequences of siRNA used are listed in the table below.

siRNA	Sequence
siRNA DIS3L2 (MGC42174)-1	CAAACUUAGCUACGAGCAU
siRNA DIS3L2 (MGC42174)-2	GGGAUCUGGUGGUCGUGAA
siRNA DIS3L2 (MGC42174)-3	AGGAGGAGUCUGACGGUGA
siRNA DIS3L2 (MGC42174)-4	UGGCACGGCCUAAAGAUUA
siRNA DIS3L2 (Sigma Aldrich)	GUAGUUAAACCAGAGAGCA

Table S5. DNA oligoprobes used for the northern blot analyses.

Probe name	Sequence	
miR-16	GCCAATATTTACGTGCTGCTA	
miR-30	CTTCCAGTCGAGGATGTTTACA	
let-7-a	ACTATACAACCTACTACCTCA	

Supplementary references

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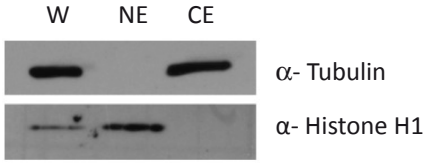
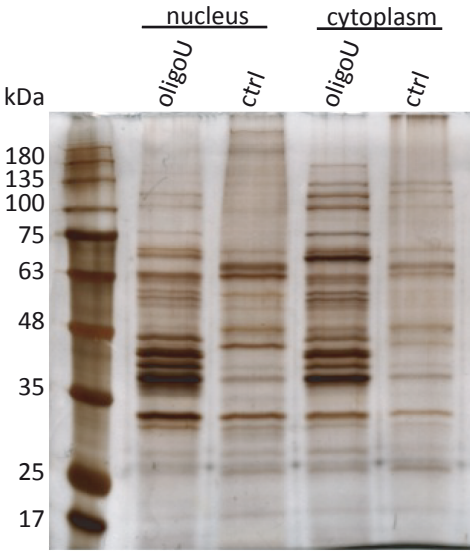
Figure S1

A


W
NE
CE
α- Tubulin
α- Histone H1

B


nucleus
cytoplasm
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oligoU
ctrl
kDa
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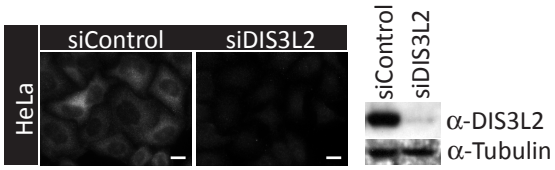
135
100
75
63
48
35
25
17

Figure S2

A



B

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RNaseII         ------------------------------------------------------------------------------- 151
Rrp44           DDDDNNESSSNTTVISDK----------------------QRRLLA--KDAMIAQRSKKIQPTAKVVYIQR-RSWRQYV 404
DIS3            NEEDV---------EKEE-----------------------ERERML--KT---AVSEKMLKPTGRVVGIIK-RNWRPYC 357
DIS3L2          DSEDGHGITQN--VLVDGVKKLSVCVSEKGREDGDAPVTKDETTCISQDTR---ALSEKSLQRSAKVVYILEKKHSRAAT 248

RNaseII         ----------------------------------------------------------------TFGDDHFVPWWVTLA 166
Rrp44           GQLAPSSVDPQSSSTQ-NVFVILMDKCLPKVRIRTRRA--------AELLDKRIVISIDSWPTTHKYPLGHFVRDLGTIE 475
DIS3            GMLSKSDIK---ESRR-HLFT-PADKRIPRIRIETRQA--------STLEGRRIIVAIDGWPRNSRYPNGHFVRNLGDVG 424
DIS3L2          GFLKLLADKNSELFRKYALFS-PSDHRVPRIYVPLKDCPQDFVARPKDYANTLFICRIVDWKEDCNFALGQLAKSLGQAG 327
                                                                                :  .::.

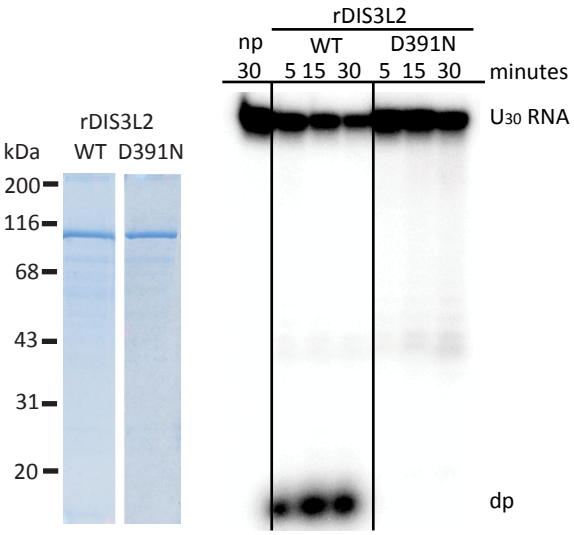
RNaseII         RHNLEKEA-------------------------PDGVATEM-------LD-EGLVREDLTALDFVTIDSASTEVDDALF 213
Rrp44           SAQAETEALLLEHDVEYRPFSKKVLECLPAEGHDWKAPTKLDDPEAVSKDPLLTKRKDLRDKLICSIDPPGCVDIDDALH 555
DIS3            EKETETEVLLLEHDVPHQPFSQAVLSFLPK--MPWSITEK-----------DMKNREDLRHLCICSVDPPGCTDIDDALH 491
DIS3L2          EIEPETEGILTEYGVDFSDFSSEVLECLPQ-GLPWTIPPE-----------EFSKRRDLRKDCIFTIDPSTARDLDDALS 395
                  : *.*                              . :              *.**    : ::*..   ******

RNaseII         AKALPDDKLQLIVAIADPTAWIAEGSKLDKAAKIRAFTNYLPGFNIPMLPRELSDDLCSLRANEVRPVLACRMTLSADGT 293
Rrp44           AKKLPNGNWEVGVHIADVTHFVKPGTALDAEGAARGTSVYLVDKRIDMLPMLLGTDLCSLKPYVDRFAFSVIWELDDSAN 635
DIS3            CRELENGNLEVGVHIADVSHFIRPGNALDQESARRGTTVYLCEKRIDMVPELLSSNLCSLQCDVDRLAFSCIWEMNHNAE 571
DIS3L2          CKPLADGNFKVGVHIADVSYFVPEGSDLDKVAAERATSVYLVQKVVPMLPRLLCEELCSLNPMSDKLTFSVIWTLTPEGK 475
                .: * :.: :: * *** : ::  *. **  .  *. : **    : *:*  *  :****.    : .::    :  ..

RNaseII         IEDNIEFFAATIESKAKLVYDQVSDWLENTGDWKP-----------ESEAIAEQVRLLAQICQRRGEWRHNHALVFKDRP 362
Rrp44           IVN-VNFMKSVIRSREAFSYEQAQLRIDDKTQ---------------NDELTMGMRALLKLSVKLKQKRLEAGALNLASP 699
DIS3            ILK-TKFTKSVINSKASLTYAEAQLRIDSANM---------------NDDITTSLRGLNKLAKILKKRRIEKGALTLSSP 635
DIS3L2          ILD-EWFGRTIIRSCTKLSYEHAQSMIESPTEKIPAKELPPISPEHSSEEVHQAVLNLHGIAKQLRQQRFVDGALRLDQL 554
                * .   *  : *.*   : * ...  ::.                .: :   :  *  :.    : *   . :

RNaseII         DYRFILG-EKGEVLDIVAEPRRIANRIVEEAMIAANICAARVLRDKLG-FGIYNVHMGFDPANADALAALLKTH-GLHVD 439
Rrp44           EVKVHMDSETSDPNEVEIKKLLATNSLVEEFMLLANISVARKIYDAFPQTAMLRRHAAPPSTNFEILNEMLNTRKNMSIS 779
DIS3            EVRFHMDSETHDPIDLQTKELRETNSMVEEFMLLANISVAKKIHEEFSEHALLRKHPAPPPSNYEILVKAARSR-NLEIK 714
DIS3L2          KLAFTLDHETGLPQGCHIYEYRESNKLVEEFMLLANMAVAHKIHRAFPEQALLRRHPPPQTRMLSDLVEFCDQM-GLPVD 633
                .  . :. *.             :* :*** *: **:..*: :   :   .: . *    .   . *        .: :.
```

C



D

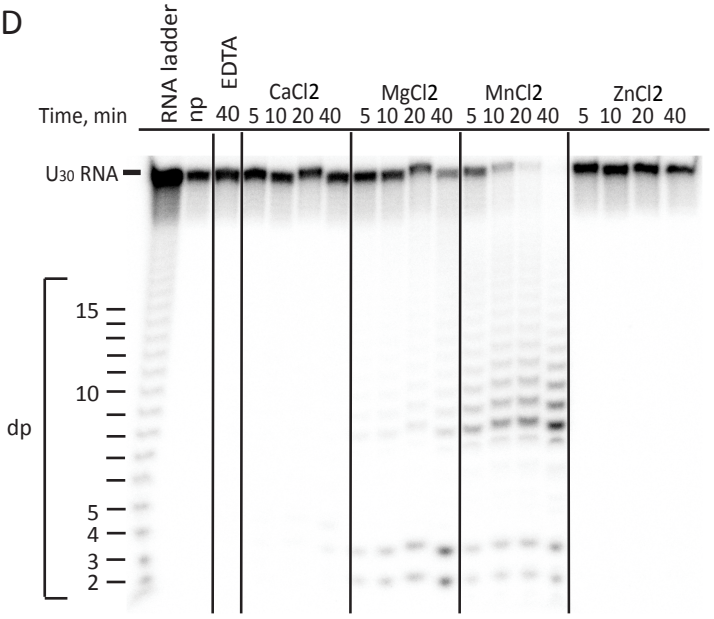
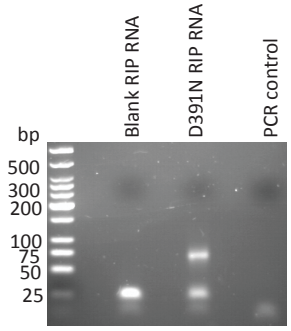


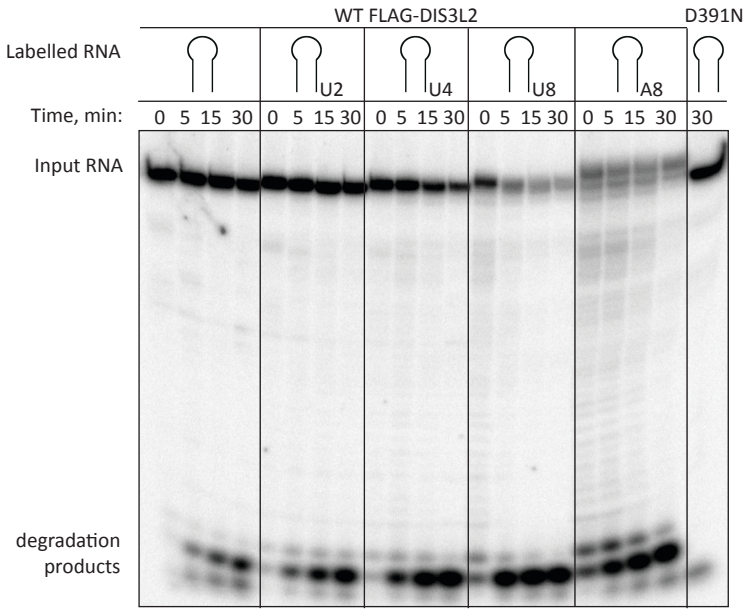
Figure S3

A



B

C



D

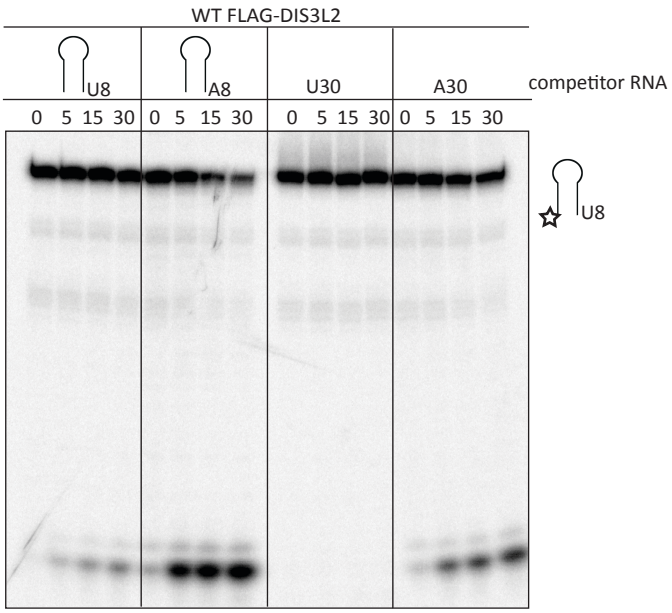
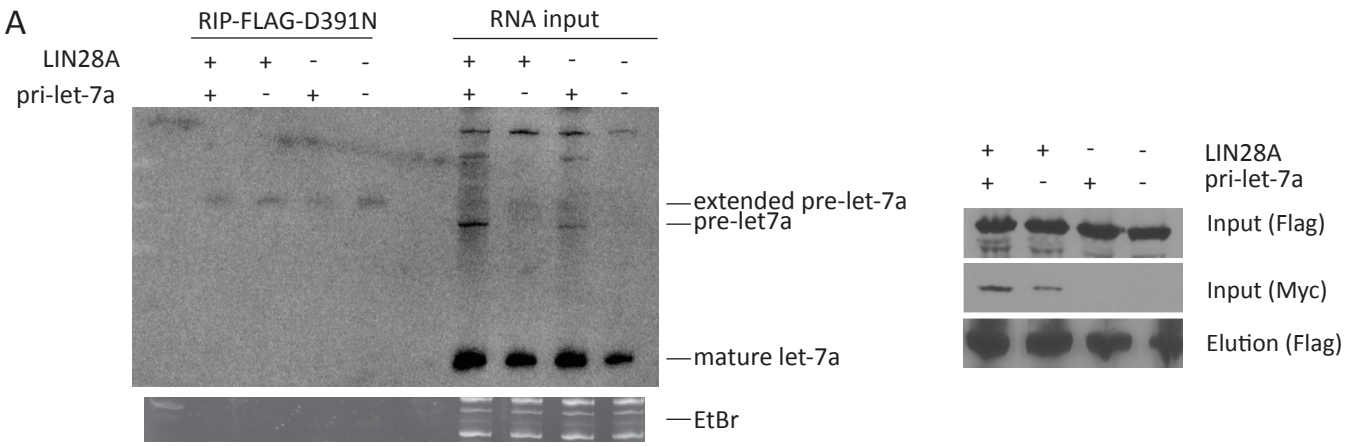
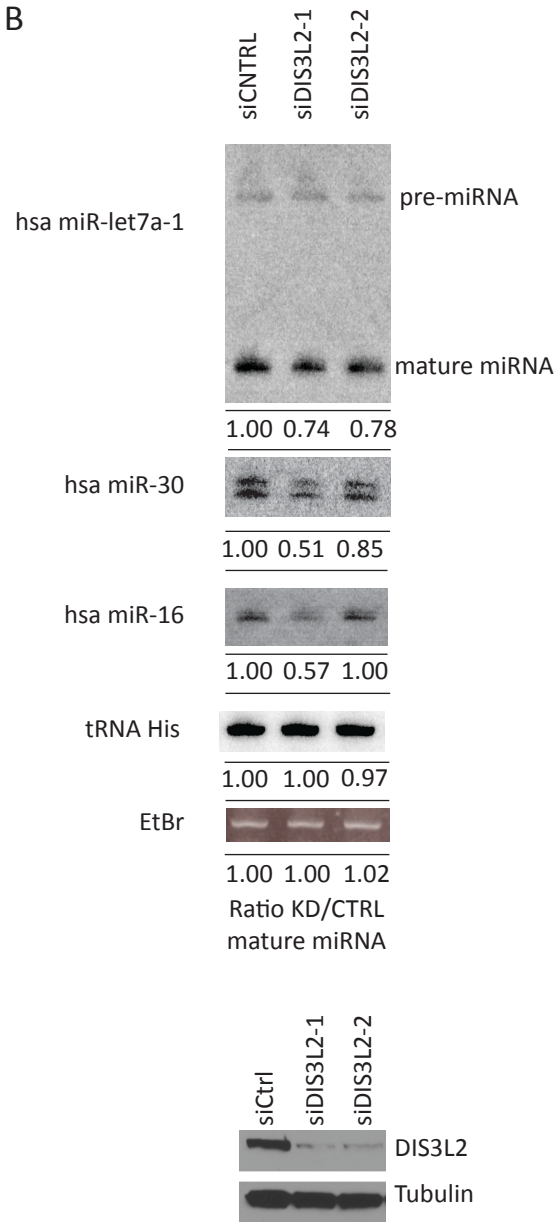
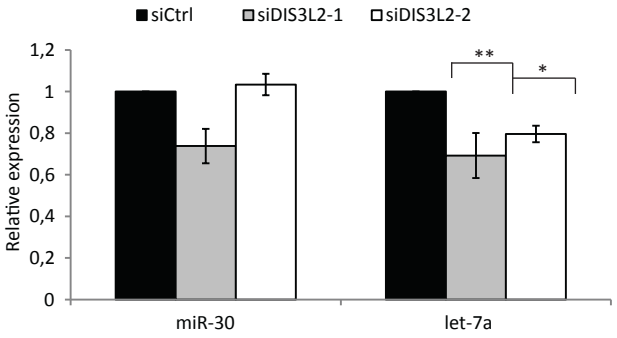
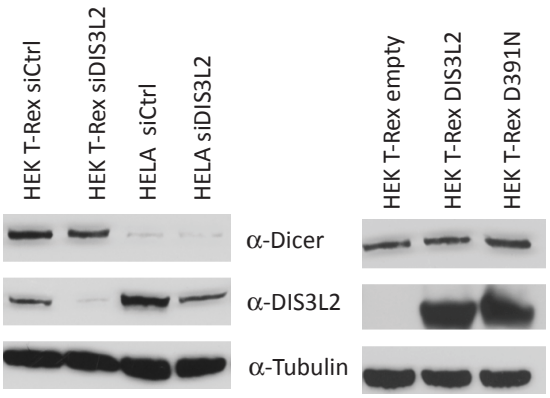


Figure S4


A
RIP-FLAG-D391N
RNA input
LIN28A
pri-let-7a
extended pre-let-7a
pre-let7a
mature let-7a
EtBr
Input (Flag)
Input (Myc)
Elution (Flag)


B
siCNTRL
siDIS3L2-1
siDIS3L2-2
hsa miR-let7a-1
pre-miRNA
mature miRNA
1.00 0.74 0.78
hsa miR-30
1.00 0.51 0.85
hsa miR-16
1.00 0.57 1.00
tRNA His
1.00 1.00 0.97
EtBr
1.00 1.00 1.02
Ratio KD/CTRL
mature miRNA
siCtrl
siDIS3L2-1
siDIS3L2-2
DIS3L2
Tubulin


C
siCtrl
siDIS3L2-1
siDIS3L2-2
Relative expression
1,2
1
0,8
0,6
0,4
0,2
0
**
*
miR-30
let-7a


D
HEK T-Rex siCtrl
HEK T-Rex siDIS3L2
HELA siCtrl
HELA siDIS3L2
HEK T-Rex empty
HEK T-Rex DIS3L2
HEK T-Rex D391N
α-Dicer
α-DIS3L2
α-Tubulin