Molecular basis for cytoplasmic RNA surveillance by uridylation-triggered decay in *Drosophila* (Appendix)

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Appendix Content:

Materials and Methods

Mass Spectrometry analysis

The nano HPLC system used was a Dionex UltiMate 3000 HPLC RSLC nano system (Thermo Fisher Scientific, Amsterdam, Netherlands) coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a Proxeon nanospray source (Thermo Fisher Scientific, Odense, Denmark). Peptides were loaded onto a trap column (Thermo Fisher Scientific, Amsterdam, Netherlands, PepMap C18, 5 mm × 300 µm ID, 5 µm particles, 100 Å pore size) at a flow rate of 25 µL min^{-1} using 0.1% TFA as mobile phase. After 10 min, the trap column was switched in line with the analytical column (Thermo Fisher Scientific, Amsterdam, Netherlands, PepMap C18, 500 mm × 75 µm ID, 2 µm, 100 Å). Peptides were eluted using a flow rate of 230 nl min^{-1}, and a binary 3h gradient, respectively 225 min.

The gradient starts with the mobile phases: 98% A (water/formic acid, 99.9/0.1, v/v) and 2% B (water/acetonitrile/formic acid, 19.92/80/0.08, v/v/v), increases to 35%B over the next 180min, followed by a gradient in 5 min to 90%B, stays there for five min and decreases in 5min back to the gradient 98%A and 2%B for equilibration at 30°C.

The Q Exactive HF mass spectrometer was operated in data-dependent mode, using a full scan (m/z range 380-1650, nominal resolution of 120 000, target value 3E6) followed by MS/MS scans of the 10 most abundant ions. MS/MS spectra were acquired using normalized collision energy 27%, isolation width of 2 and the target value was set to 1E5. Precursor ions selected for fragmentation (exclude charge state 1, 7, 8, >8) were put on a dynamic exclusion list for 40 s. Additionally, the minimum AGC target was set to 2E4 and intensity threshold was calculated to be 8E4. The peptide match feature was set to preferred and the exclude isotopes feature was enabled.

Antibody

A monoclonal antibody against CG16940/dmDis3l2 was raised by the MFPL monoclonal antibody facility (S. Schuechner and E. Ogris). Briefly, a 6 x HIS-tagged ~18KDa fragment of the N-terminus of CG16940 (-PC, aminoacids 97 to 259) was expressed in E. coli BL21 and purified under denaturing conditions by Ni-affinity chromatography. Balb/c mice were immunized subcutaneously three times (every 2-3 weeks) with 50 µg of purified antigen mixed at a ratio of 1:1 with adjuvant, before a final intravenous immunization with 30 µg purified antigen (adjuvant-free). Splenic B-cells were fused with X63- Ag8.653 mouse myeloma cells and clones were tested by Western blot analysis and immunocytochemistry for the detection of CG6940/dmDis3l2. Clone 8C2-H4 yielded the best signal-to-noise performance.

Recombinant protein interaction assays

For the Strep II pull down experiments, dmDis3l2 and dmTailor were expressed using the insect cell/baculovirus system. DNA sequences corresponding to residues 1-1032 (FL) and 234-1032 (ΔN) of dmDis3l2 were amplified from a cDNA clone from the *Drosophila* Gene Collection library and subcloned into vector 438-RGFP (developed by Scott Gradia, UC Berkeley MacroLab, Addgene #55221) in frame with N-terminal Strep II epitope tag and green fluorescent protein using the ligation-independent

cloning (LIC) method. DNA sequences encoding residues 1-560 (FL) and 209-560 (ΔN) of dmTailor were amplified from a *Drosophila* Gene Collection library cDNA clone and subcloned into vector 4B (developed by Scott Gradia, UC Berkeley MacroLab, Addgene #30115) in frame with N-terminal hexahistidine affinity tag using the LIC procedure. Recombinant baculoviruses were generated using the Bac-to-Bac kit (Invitrogen) according to manufacturer's protocol. For protein expression, 50 ml suspension cultures of Sf9 cells in SF-4 Baculo Express medium (Amimed) were infected at a density of 2.0 x 10^6 ml^{-1}. The cells expressing Strep-II-GFP-dmDis3L2 (the bait) were harvested after 72 hours, resuspended in the assay buffer (20 mM TRIS pH 8.0, 500 mM NaCl, 0.4 % Triton-X) supplemented with protease inhibitor cocktail (Roche) and lysed by sonication. 100 µl Strep-Tactin Superflow resin (IBA) were incubated with clarified cell lysates for 1 hr at 4 °C and were subsequently washed five times with the assay buffer. Cells expressing His_6-dmTailor (the prey) were harvested 72 hours post infection, resuspended in assay buffer supplemented with 5 mM imidazole and protease inhibitor cocktail (Roche), and lysed by sonication. Clarified lysates were applied to 100 µl His-Select Ni Affinity Gel (Sigma), washed five times with assay buffer supplemented with 5 mM imidazole, and proteins were eluted with the assay buffer supplemented with 250 mM imidazole. Elutions enriched in variants of dmTailor were incubated with Strep-Tactin beads loaded with Strep-II-GFP-dmDis3l2 for 1hr at 4 °C. The beads were washed five times with assay buffer and bound proteins were eluted by heating the beads in 40 µl of 1x SDS-PAGE sample loading buffer for 5 min at 95 °C. 5 µl samples were separated by SDS-PAGE and stained with Coomassie Blue.

For Glutatione S-transferase (GST) pull down experiments, GST-tagged fragments of N-terminal low complexity extensions of dmTailor and dmDis3l2 proteins (baits) were expressed in *Escherichia coli*. Sequences encoding residues 2-235, 50-235 and 2-50 of dmDis3l2 as well as the residues 8-209 and 63-123 of dmTailor were PCR amplified from cDNA clones and inserted into vector pGEX-6p-1 in frame with the N-terminal glutathione S-transferase tag using BamHI and NotI restriction sites. The proteins were expressed in 50 ml cultures of *E. coli* Rosetta II cells for 18 hrs at 18 °C. Cell pellets were resuspended in the assay buffer supplemented with 1 mM DTT and Protease Inhibitor Cocktail (Roche). The cells were lysed by sonication and clarified lysates were incubated with Glutathione Sepharose 4 Fast Flow beads (GE Healthcare) for 1 hr at 4 °C. The beads were washed five times with assay buffer supplemented with 1 mM DTT and subsequently incubated with the clarified lysates of Sf9 or *E. coli* cells expressing the prey proteins (see below). The beads were washed five times with assay buffer supplemented with 1 mM DTT and bound proteins were eluted by heating the beads in 40 µl of 1x SDS-PAGE sample loading buffer for 5 min at 95°C. 5 µl samples were separated by SDS-PAGE and stained with Coomassie.

Strep-II-GFP-tagged dmDis3l2 constructs were prepared as described above. Strep-II-GFP-tagged full-length dmTailor protein was expressed using the same procedure as for the Strep-II-GFP-dmDis3l2. To prepare His_6-MBP-tagged dmDis3l2$^{2-50}$, DNA sequence corresponding to residues 2-50 of dmDis3l2 was subcloned into vector 1M (developed by Scott Gradia, UC Berkeley MacroLab, Addgene #29656) in frame with N-terminal hexahistidine affinity tag fused to maltose-binding protein (MBP) using the LIC procedure. The protein was expressed in a 50 ml culture of *E. coli* Rosetta II cells for 18 hrs at 18 °C. Cell lysate was prepared by resuspending cells in assay buffer

supplemented with 1 mM DTT and Protease Inhibitor Cocktail (Roche) followed by sonication.

Plasmids

DmDis3l2-CDS was PCR-amplified from S2 cell cDNA, cloned by directional TOPO cloning according to manufacturer's instructions (Invitrogen). For expression in S2 cells, dmDis3l2-CDS was subcloned into pAFMW or pAGW by LR reaction (Invitrogen). A catalytic mutant point mutation was introduced by site-directed mutagenesis using the oligonucleotides CG16940_CM_FWD and CG16940_CM_REV. For truncations of tailor, specific fragments were PCR amplified and cloned by directional TOPO cloning according to the instructions of the manufacturer (Invitrogen), followed by subcloning into pAGW. For oligonucleotides see Appendix Table S1. For cloning of gRNA-expression vectors targeting the Tailor/CG1091 or CG16940/dmDis3l2 locus by CRISPR/Cas9 in *Drosophila* S2 cells, two pairs of gRNA-coding oligonucleotides (gRNA-1 to -2; see Appendix Table S1) were annealed and ligated to BspQI-digested pAc-sgRNA-Cas9 (Bassett et al., 2013). The sequence of all plasmids was verified by Sanger sequencing.

Generation of RNA substrates

Pre-miR-1003 substrates were generated by splint-ligation using the oligos miR-1003-3p or uridylated variants and miR-1003-5p+loop (see Appendix Table S1). Pre-miR-1003 substrates containing 10, 15 or 20 3´ terminal uridine, or 10 3´ terminal adenine residues were generated by three-way splint ligation using the olionucleotide miR-1003-3p, miR-1003-5p+loop and an oligonucleotide consisting of 10U, 15U, 20 U, and 10A (see Appendix Table S1). Briefly, 30 pmol miR-1003-3p variants were 5´ radiolabeled using γ -^{32}P-ATP (6000 Ci/mmol, PerkinElmer) and T4 polynucleotide kinase (NEB) and purified using G25 spin columns (GE Healthcare). 15 pmol were subjected to splint ligation: Annealing was perfomed after adding 35 pmol miR-1003-5p+loop RNA and 20 pmol miR-1003-DNA splint oligo in 1 x Lysis buffer (30 mM HEPES- KOH pH 7.4, 100 mM KOAc, 2 mM MgOAc) by heating to 70˚C for 5 min and slowly cooling to room temperature. Then the equal volume 2 x ligation buffer (132mM Tris-HCl pH7.6, 20 mM MgCl2, 2 mM DTT, 2 mM ATP, 15% PEG-8000) was added together with 1 µl T4 DNA Ligase (2,000U/ul, NEB). Ligation was performed at 25˚C for 2 h, followed by addition of 1 µl RQ1 RNase-free DNAse (Promega) and incubation for 15 min at 37˚ C. Ligation product was gel-purified on an 8% denaturing polyacrylamide gel.

Data analysis

Detailed mass spec data analysis was performed as follows: For peptide identification, the RAW-files were loaded into Proteome Discoverer (version 2.1.0.81, Thermo Scientific). All hereby created MS/MS spectra were searched using MSAmanda v1.0.0.6186 (Dorfer et al., 2014) against the *Drosophila* sequence database called flybase (version dmel_all-translation-r6.06, 22,256 sequences; 20,222,850 residues). The following search parameters were used: Beta-methylthiolation on cysteine was set as a fixed modification, oxidation on methionine, phosphorylation on serine, threonine and tyrosine, deamidation on asparagine and glutamine and acetylation on lysine were set as variable modifications. Monoisotopic masses were searched within unrestricted protein masses for tryptic enzymatic

specificity. The peptide mass tolerance was set to ±5 ppm and the fragment mass tolerance to ±30 mmu. The maximal number of missed cleavages was set to 2. The result was filtered to 1% FDR on peptide level using Percolator algorithm integrated in Thermo Proteome Discoverer. The localization of the phosphorylation sites within the peptides was performed in phosphoRS (Taus et al., 2011). Our in-house-developed tool Peakjuggler was used for the peptide and protein quantification (publication in preparation). Differentially expressed proteins were determined using limma (Smyth, 2005)
For quantification of cellular uridylation in S2 cell lysate (Figure 4 and S4), substrate S1 signals were quantified and normalized to the adenylation signal (Figure S4) for each timepoint. Signal intensities were then normalized to the signal observed at 10 min timepoint in wt lysate in each replicate and values were averaged across the indicated number of biological replicates. To test accumulation of adenylation signal over time, each sample was internally normalized to the 10 min timepoint.

Fly stocks
Generation of CG16940/dmdis3l2 (FBgn0035111) mutant flies by CRISPR/Cas9 genome engineering was performed as described (Gokcezade et al., 2014). Briefly, isogenized w^{1118} embryos were injected with the plasmid pDCC6 (Addgene) containing a gRNA sequence (Appendix Table S1) targeting the second (for obtaining KO flies) or the third exon (for obtaining a D to A mutation at the conserved protein catalytic site) of the CG16940/dmdis3l2 locus. Hatched flies were crossed to third chromosome balancer flies and F1 resulting males were screened for frameshift/missense mutations by PCR amplification of the targeted CG16940 locus using the primers CG16940-fwd and CG16940-rev or primers CG16940-fwd2 and CG16940-rev2, respectively (Appendix Table S1) followed by sanger sequencing. Progeny carrying a 5nt frameshift deletion and a D-to-A mutation were used for further experiments. Flies between 5-8 days old were used for experiments and an isogenic w^{1118} stock were used as the wild type control.

Western blotting
Lysates were separated on 8% SDS PAGE and transferred to PVDF membrane (BioRad). Antibodies were used at a dilution of 1/500 for α-Tailor (mouse), 1/500 for α-dmDis3l2 (mouse), 1/10,000 for α-M2-FLAG (mouse; Sigma) and 1/10,000 for Ago1 (mouse) and detected by secondary HRP-antibody-conjugates G21040 (Invitrogen; dilution 1/10,000). Primary antibodies were incubated at 4˚C over-night and secondary antibodies were incubated at room temperature for two hours. Images were acquired on a ChemiDoc MP Imaging System (BioRad) using ImageLab v5.1.1 (BioRad) or by Amersham Hyperfilm ECL (GE Healthcare).

Library Construction for High-Throughput Sequencing
The QuantSeq-Flex Targeted RNA-Seq Library Prep Kit (Lexogen) was used for generating libraries of RNA co-immunopurified with FLAG-dmDis3l2-CM. After immunopurification of FLAG-GFP, FLAG-Tailor, FLAG-Tailor-CM, FLAG-dmDis3l2 and FLAG-dmDis3l2-CM, the RNA bound to the IPed protein was acid-phenol extracted (ThermoFischer Scientific), cleaned with chloroform:isoamyl alcohol (BioChemica) and precipitated with 20ug glycogen carrier (Roche) and 2.5vol absolute ethanol. The precipitate was resuspended in 20µL H_2O. 2µL were subjected

to CIP treatment, phenol-extraction, and 5′ radiolabeling for resolving on a 15%PAA. For sequencing of the exonuclease-protected RNA stretch (Figure 5A), 10-20 nt RNA was purified by polyacrylamide gel electrophoresis and ligated to 3′adapter and 5′adapter containing 4 random nucleotides at the ligation interface to minimize ligation bias. Library preparation and amplification was performed as described for small RNAs (Reimão-Pinto et al., 2015).
For sequencing and identification of dmDis3l2 substrates 5µL of the FLAG-dmDis3l2-CM co-immunoprecipitated RNA was ligated to a 3′adapter; total RNA from S2 cells was subjected to identical treatment for control libraries. After ligation, RNA was phenol extracted and the RNA precipitated with 20ug glycogen carrier and 2.5vol absolute ethanol. For library preparation, first strand synthesis was performed with a custom RT primer targeting the 3′adapter (Appendix Table S1), followed by second strand synthesis using a random primer provided by QuantSeq Flex Kit (Lexogen), and library amplification according to instructions of the manufacturer. Sequencing was performed on an HiSeq 2000 instrument (Illumina) at the VBCF NGS facility.

Bioinformatics
For meta-analysis of tRNA bound to dmDis3l2CM, the following 68 tRNA loci were analyzed: FBgn0011849, FBgn0051491, FBgn0011879, FBgn0011848, FBgn0050406, FBgn0050407, FBgn0011850, FBgn0050454, FBgn0050232, FBgn0011896, FBgn0011944, FBgn0050212, FBgn0011904, FBgn0026162, FBgn0051604, FBgn0050215, FBgn0050235, FBgn0050234, FBgn0011872, FBgn0051944, FBgn0051070, FBgn0053538, FBgn0011860, FBgn0012000, FBgn0011871, FBgn0011897, FBgn0011868, FBgn0026163, FBgn0026164, FBgn0051631, FBgn0011945, FBgn0050239, FBgn0051167, FBgn0052358, FBgn0011869, FBgn0011865, FBgn0050220, FBgn0011915, FBgn0051023, FBgn0051591, FBgn0052481, FBgn0052460, FBgn0050297, FBgn0052093, FBgn0051166, FBgn0050316, FBgn0012002, FBgn0051162, FBgn0011861, FBgn0011963, FBgn0050333, FBgn0011867, FBgn0051215, FBgn0052127, FBgn0052420, FBgn0012001, FBgn0052480, FBgn0012004, FBgn0266585, FBgn0011960, FBgn0011955, FBgn0051588, FBgn0051579, FBgn0011906, FBgn0011998, FBgn0051578, FBgn0051580, FBgn0011962.

Appendix Table S1. Oligonucleotides used in this study.

Oligonucleotides for cloning and mutagenesis of dmDis3l2/CG16940 truncations for expression in *Drosophila* S2 cells (mutated nucleotides are indicated in lowercase letters).

Name	**DNA sequence (5´-3´)**
CG16940-PA-fwd	CACCATGTCGGAAACGTCAAGCGT
CG16940-rev	CTAAATTTGTTCTTCCATCTTTAAA
CG16940-CM-FWD	GATTTGGccGACGCCGTTTCTATAGAGAAG
CG16940-CM-REV	CGGCGTCggCCAAATCGCGAGCAGTCATC

Oligonucleotides for cloning of Tailor truncations for expression in *Drosophila* S2 cells.

Name	**DNA sequence (5´-3´)**
Tailor-fwd	CACCATGCGGATCGAACCTGGGG
Tailor-rev	TTAAAATGCATAGCGCTGACGC
Tailor-ΔPAPassoc-rev	TTAGAAGCCCTTGATGACGGGG
Tailor-ΔDUF1439-fwd	CACCATGGCCGCACCGGGTGAG
Tailor-ΔN-fwd	CACCCCCGAGTACGACGTTATCG
Tailor-ΔPAP/NT	TTAAATCACCTTTAACTTGTCCGCT

Oligonucleotides used for cloning of guide RNA expression vectors in CRISPR/Cas9 genome engineering of Drosophila S2 cells (capital letters indicate targeting sequence; lower case letters indicate restriction-site compatible overhangs).

Name	**DNA sequence (5´-3´)**
Tailor-gRNA-1-fwd	ttcgGCCAGGTGCGGGTCTAGCTG
Tailor-gRNA-1-fwd	aacCAGCTAGACCCGCACCTGGCc
Tailor-gRNA-2-fwd	ttcgCCACCATAATGCGCTCCAGC
Tailor-gRNA-2-fwd	aacGCTGGAGCGCATTATGGTGGc
dmDis3l2/CG16940-gRNA-1-fwd	ttcgGCAGCCACTAATGCGCTGGG
dmDis3l2/CG16940-gRNA-1-fwd	AACCCCAGCGCATTAGTGGCTGCc
dmDis3l2/CG16940-gRNA-2-fwd	ttcgGATTGGTTGGGATGTGGCCG
dmDis3l2/CG16940-gRNA-2-fwd	AACCGGCCACATCCCAACCAATCc

Oligonucleotides used for CRISPR/Cas9 genome editing in flies.

Name	**DNA sequence (5´-3´)**
gRNA CG16940/dmDis3l2 KO	CAAGATTGTCCAACGTCACG
gRNA CG16940/dmDis3l2 CM	TCCGATGACTGCTCGCGATT

DNA Oligonucleotides used for PCR amplification of CRISPR-targeted genomic cg16940/dmdis3l2 locus followed by Sanger Sequencing.

Name	**DNA sequence (5´-3´)**
CG16940/dmDis3l2 KO fwd	TCGAGTGGAGTCAGACCAGG
CG16940/dmDis3l2 KO rev	TTCCAGGTCGGAGAAGTTACC
CG16940/dmDis3l2 CM fwd	GCATCGCTGAACTAATCCAGCC
CG16940/dmDis3l2 CM rev	AGACTGAGGCAGCATGTGGATT

RNA oligonucleotides for HTP biochemical characterization.

Name	**RNA sequence (5´-3´)**
Substrate RNA	ACACUCUUUCCCUACACGACGCUCUUCCGAUCUNNNN

Oligonucleotides used for RNA library preparation (ddN, 2´-3´-dideoxynucleotide modification; hexamer-barcode for multiplexing is shown in red).

Name	**Sequence (5´-3´)**
3´ linker (DNA)	/5rApp/NNNNAGATCGGAAGAGCACACGTCT/3ddC/
5´ linker (RNA)	ACACUCUUUCCCUACACGACGCUCUUCCGAUCUNNNN
RT primer (DNA)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Library PCR fwd (DNA)	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCT
Library PCR rev (DNA)	CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGA CGTGTGCTCTTCCGATCT

RNA oligonucleotides used for in vitro exonuclease assays.

Name	**DNA sequence (5´-3´)**
miR-1003-5p+loop	GUGGGUAUCUGGAUGUGGUUGGCUCUGGCGGUCC
miR-1003-3p WT	UCUCACAUUUACAUAUUCACAG
miR-1003-3p-U	UCUCACAUUUACAUAUUCACAGU
miR-1003-3p-$(U)_2$	UCUCACAUUUACAUAUUCACAGUU
miR-1003-3p-$(U)_3$	UCUCACAUUUACAUAUUCACAGUUU
miR-1003-3p-$(U)_5$	UCUCACAUUUACAUAUUCACAGUUUUU
10 A	AAAAAAAAAA
10 U	UUUUUUUUUU
15 U	UUUUUUUUUUUUUUU
20 U	UUUUUUUUUUUUUUUUUUUU

DNA oligonucleotides used for splint ligation to generate pre-miR-1003 variants.

Name	**DNA sequence (5´-3´)**
miR-1003 splint	GTGAATATGTAAATGTGAGAGGACCGCCAGAGCCAACCACATC CAGATACC
miR-1003 U splint	AAAAAAAAAACTGTGAATATGTAAATGTGAGAGGACCGCCAGA GCCAACCACATCCAGATACC
miR-1003 A splint	TTTTTTTTTTCTGTGAATATGTAAATGTGAGAGGACCGCCAGAG CCAACCACATCCAGATACC

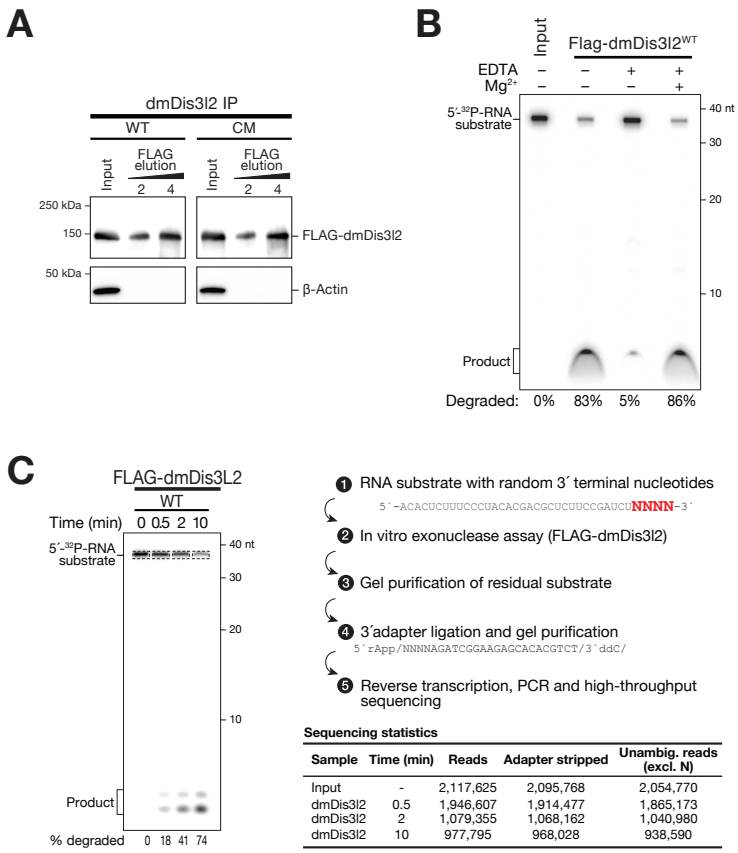
Oligonucleotides used for in vitro transcription of tRNA by T7 RNA polymerase (2´ O-methyl modification is indicated by mN).

Name	**DNA sequence (5´-3´)**
T7-tRNA(Ala)TGC fwd	GCTAATACGACTCACTATAGGGGATGTAGCTCAGTGGTAG
tRNA(Ala)TGC 3Trailer rev	mAmAAAAATTGTACTTGGAGATGCC
tRNA(Ala)TGC mat CCA rev	mUmGGTGGAGATGCCGGGGATTGAA

Probes for Northern hybridization assays.

Name	**DNA sequence (5´-3´)**
5S rRNA	CGAGAACCGATGTATTCAGCGTG
RNaseMRP RNA	TCCTACCAACTCGTCCATATCTAAG
tRNA-Ala(TGC) 3Trailer	AAAAAATTGTACTTGGAGATGCCG
tRNA-Ala(TGC) mature	GCCTCTCACATGCAAAGCGAGCG
2S rRNA	TACAACCCTCAACCATATGTAGTCCAAGCA
RNase MRP 5´ cleavage product	ACACCTTGATACTTTCTATCCTA
RNase MRP 3´ cleavage product	CTGTGTAGGCGGTTTCTCAGACG

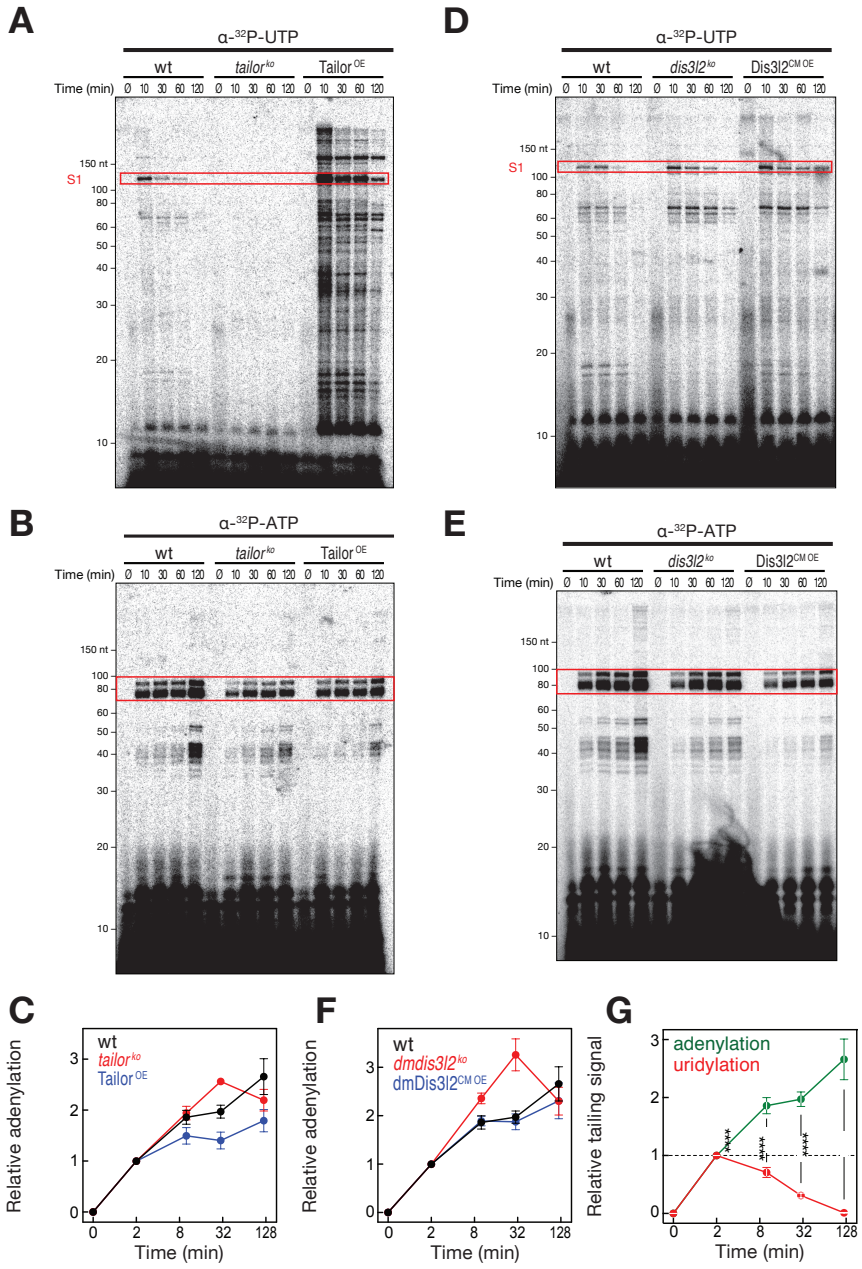
Appendix Figures



Sequencing statistics

Sample	Time (min)	Reads	Adapter stripped	Unambig. reads (excl. N)
Input	-	2,117,625	2,095,768	2,054,770
dmDis3l2	0.5	1,946,607	1,914,477	1,865,173
dmDis3l2	2	1,079,355	1,068,162	1,040,980
dmDis3l2	10	977,795	968,028	938,590

Appendix Figure S1 – DmDis3l2/CG16940 is a terminal-uridylation-triggered, processive 3′-to-5′ exoribonuclease.
(A) Western blot analysis of immunopurified FLAG-dmDis3l2^{WT} and FLAG-dmDis3l2^{CM}. Beta-actin is shown as control.
(B) DmDis3l2 is a Mg^{2+}-dependent exoribonuclease. RNA decay assay using 5′ radiolabeled substrate RNA (see Figure 2A) and immunopurified FLAG-dmDis3l2. RNA decay was assayed without or with 5 mM EDTA and excess Mg^{2+}, as indicated. Quantification of fraction degraded is indicated.
(C) Overview of experimental setup for high-throughput biochemical characterization of dmDis3l2. 5′ radiolabeled RNA substrate containing four random nucleotides at the 3′ end (see also Figure 2A) was subjected to treatment with dmDis3l2 for the indicated time. Residual substrate RNA was gel-purified and subjected to 3′ adapter ligation, followed by reverse transcription and PCR amplification to generate cDNA libraries that were subjected to high-throughput sequencing. Sequencing statistics of resulting libraries are shown.



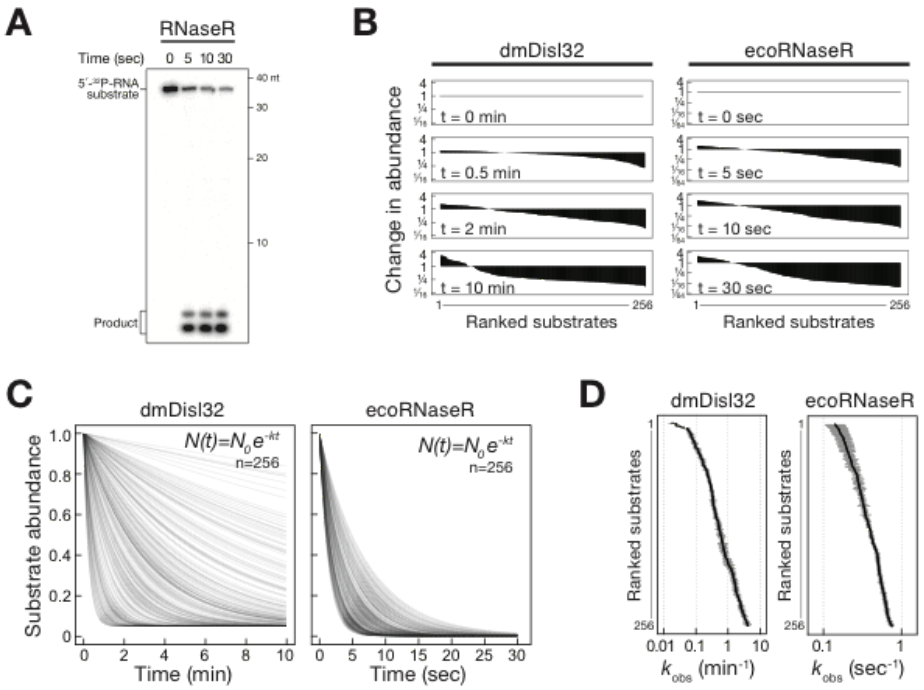
Appendix Figure S2 – In vitro uridylation assay in S2 cell lysate recapitulates uridylation-triggered RNA decay of cellular RNA transcripts.

(A) and (D) Gel images shown in Figure 4B and F, respectively. Substrates used to quantify relative uridylation intensities are indicated by red box.

(B) and **(E)** Gel images shown in Figure 4C and G. Substrate used to quantify relative adenylation intensities are indicated in red box.

(C) and **(F)** Quantification of adenylation signal in (B) and (E). The indicated area (red box) was used for quantification. Each sample was internally normalized to the 2 min timepoint. Plots show mean±SEM of at least five (C) or seven (F) independent biological replicates.

(G) Quantification of relative tailing signal in adenylation and uridylation assay using wild-type Drosophila S2 cell lysate. Plots show mean±SEM of twelve independent biological replicates. Statistical analysis was performed using Student's t-test. ****, $p<10^{-4}$.



Appendix Figure S3 – High-throughput biochemical comparison of *E. coli* RNase R and *D. melanogaster* Dis3l2.

(A) In vitro exoribonuclease assay using 5´ radiolabeled RNA substrate and recombinant RNaseR, incubated for the indicated time and separated by 15 % polyacrylamide gel electrophoresis followed by phosphorimaging.

(B) Change in abundance of 256 different substrate RNAs as determined by high-throughput sequencing of substrates in experiment shown in (A) (ecoRNaseR) and independent experimental replicate of experiment shown in Figure 2 (dmDis3l2).

(C) Decay rates of 256 different substrate RNAs. Data was normalized to overall decrease in substrate abundance as determined by phosphorimaging (A) and fit to the indicated model for exponential decay.

(D) Overview of decay rates (k_{obs}) of all 256 different substrate RNAs, as determined in (C). Error of curve fit is indicated as SEM.

Appendix References

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