Supplementary Material for

Title: Spatial organization of single mRNPs at different stages along the gene expression pathway

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MATERIALS AND METHODS

Reagents used, stock concentrations, working concentrations and treatment conditions

Puromycin dihydrochloride (Sigma P8833) – stock at 5 mg/ml in water, Cycloheximide (Sigma C7698-1G) – stock 5 mg/ml in ethanol, Sodium Arsenite (Sigma 35000-1L-R) – stock 50 mM in water, Homoharringtonine (Sigma SML1091-10MG) – stock 10mg/ml in DMSO. The drugs were diluted in warm media to get final working concentrations and cells were treated prior to fixation as follows: Puromycin (100 μg/ml for 10 min), Cycloheximide (100 μg/ml for 10 min), Homoharringtonine – 100μg/ml for 10 mins or 1hr and Sodium Arsenite (2 mM for 1 hour).

Cell culture and drug treatment

HEK293 and U2OS osteosarcoma cell lines were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Wisent, 319-005-CL) supplemented with 10% fetal bovine serum (FBS) (Wisent, 080-150) and passaged every 2-3 days with Trypsin (Wisent 325-043-EL). Cells were plated on poly-L-Lysine (Sigma, P8920) coated coverslips the day before treatment and fixation. On the day of the experiment, media was replaced with fresh warm media containing drug in indicated concentrations and placed back in the incubator. After treatment, the cells were briefly washed with 1xPBS, fixed with 4% paraformaldehyde in 1xPBS (pH 7.4) for 10 minutes at room temperature, washed three times with 1xPBS and stored overnight in 70% ethanol at -20°C for permeabilization. Alternatively, the cells were permeabilized using 0.1% TritonX-100 + 0.5%BSA in 1x PBS for 15mins after which they were washed 2 times with 1x PBS for 5 mins each immediately before using the samples for smFISH.

Plasmid Preparation: The phage-ubc-flag-24xSunTag-Fluc-oxBFP-AID-baUTR-24xMS2 plasmid was prepared as described in (*1*).

Generation and screening of EIF4G1 and PABPC1 mutant cell lines

Mutant cell lines were generated using CRISPR-Cas9. To produce sgRNAs targeting either eIF4G1 or PABPC1, annealed DNA oligos (Table S1) were ligated into the BbsI site of plasmid pX330 (Ran 2013). Homology repair constructs containing the intended mutations and upstream and downstream homology arms (~1 kb in total) were ligated into the plasmid Lox-Stop-Lox-TOPO-Δstop (Rakheja 2014), in which homology arms are cloned surrounding a puromycin resistance cassette flanked by loxP sites (Table S1).

HEK293 cells (5 x 105 cells in one well of a 6-well plate) were transfected with 250 ng of the pX330-sgRNA construct and 1 μg of the repair construct using Lipofectamine 2000 according to the manufacturer instructions, and then incubated in EMEM supplemented with 10% FBS in a humidified incubator at 37° C with 5% CO2. Two days following transfection, cells were trypsinized and 10% of the cells were moved into a 15-cm dish. After 24 h, puromycin was added to a final concentration of 3 μg/mL, and the media was changed daily for the next 3 days. The following day, single cells were seeded into each well of a 96-well plate on a MoFlo Astrios cell sorter (Beckman Coulter) at the Flow and Mass Cytometry Facility at SickKids Hospital, Toronto. Following expansion of single colonies, cells were harvested and screened by PCR using primers that anneal to the genome outside of the homology arm region (Table S1). To excise the puromycin resistance cassette from positive clones, the cells were transfected with 1μg of pgk-Cre (*2*) and incubated for 3 days before single-cell seeding, expansion, and screening for loss of the puromycin

resistance gene by PCR as described above. The PCR products were analyzed by Sanger sequencing to ensure that the intended mutations were present.

Cell viability assays

Cell viability was measured using PrestoBlue Cell Viability Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in triplicate in 96-well plates at 1000 cells per well in 90 µL of EMEM supplemented with 10% FBS, and then incubated at 37° C with 5% CO_2. At 24 h, 48 h, and 72 h after seeding, 10 µL of PrestoBlue reagent was added to each well. After a further 6.5-h incubation at 37°C with 5% CO_2, the fluorescence of each well was read on a SpectraMax M2 microplate reader (Molecular Devices).

Polysome profiling

To generate polysome profiles, cycloheximide was added to cells in a 10-cm dish to a final concentration of 100 µg/mL, and the cells were incubated for 10 min at 37°C. The cells were then placed on ice and washed twice with ice-cold PBS containing 100 µg/mL cycloheximide. Cells were lysed by shearing four times through a 26-gauge needle in 500 µL of lysis buffer (10 mM Tris-HCl pH 7.4, 5 mM MgCl2, 100 mM KCl, 1% Triton X-100, 2 mM DTT, 500 U/ml Rnasin (Promega), EDTA-free protease inhibitor cocktail (Sigma), 100 µg/mL cycloheximide). Following centrifugation at 1300 × g for 10 min at 4°C, the supernatant was collected, flash frozen in liquid nitrogen, and stored at -80°C until further processing.

Lysates were separated by loading 300 µL onto a 10-50% (w/v) sucrose gradient prepared with a Gradient Master (BioComp Instruments) and centrifuging for 2 h at 36,000 rpm in a SW41Ti rotor (Beckman Coulter) at 4° C. Gradients were fractionated on a Piston Gradient Fractionator

(BioComp) coupled to a EM-1 Econo UV detector (Bio-Rad). UV profile data were recorded using Gradient Profiler software v 2.07 (BioComp).

smRNA FISH

Custom DNA probe sets were designed using Stellaris® Probe Designer, synthetized by Biosearch Technologies containing 3' amine reactive group and labeled with far red dye Cy5 (GEPA25001), red dyes Cy3 (GEPA23001) from Sigma or Dylight 550 (Thermo Scientific 62263) or green dye Dy488 (Thermo Scientific 46403) as described in (*3*). Probe sequences are shown in Table S2. Probe combination used are shown in Table S3. smFISH was done as described in (*4*). Prior to hybridization, cells were rehydrated in 1xPBS, then washed with 10% formamide/2xSSC for 10 minutes at room temperature. The cells were hybridized with 10-20 ng of each probe mix plus 40 μg of ssDNA/tRNA resuspended in the hybridization solution (10% dextran sulfate/10% formamide/2xSSC/2 mM VRC/0.1 mg/ml BSA) for 3 hours in the dark at 37ºC. Post hybridization washes (2x 30 min) were carried out at 37ºC with 10% formamide/2xSSC. Samples were then rinsed with 1xPBS and mounted with ProLong Gold antifade reagent with DAPI (P36935, Invitrogen).

Image Acquisition and pixel shift correction

Images were acquired with a 63x NA 1.46 oil objective on a Zeiss Elyra PS.1 system equipped with an Andor EMCCD iXon3 DU-885 CSO VP461 camera (1004x1002 pixels), the following filter sets: DAPI: BP420-480 + LP750 (Zeiss SR cube 07), Cy2: BP495-590+LP750 (Zeiss SR cube 13), Cy3: LP570 (Zeiss SR cube 14), Cy5: LP655 (Zeiss SR cube 10) and the following lasers: 50 mW 405 nm HR diode, 100 mW 488 nm HR diode, 100 mW 561 nm HR DPSS, 150 mW 642 nm HR diode. Each image was acquired using 3 rotations and a grid size of 42 μm for all

channels. The microscope was located in a temperature-controlled room and samples were kept in the room for at least an hour before imaging to minimize thermal fluctuations. To correct for pixel shifts between channels, 0.1 μm TetraSpec beads (Invitrogen T-7279) were imaged in all channels, and the channel shift values and chromatic aberration were calculated and corrected using the built-in channel alignment tool in ZEN 2012 SP5 which uses an affine image alignment algorithm and later applied to the images. This correction was calculated for each day of imaging.

RNA spot detection, spot assignment and distance measurements

For image analysis, 3D datasets were reduced to 2D data using maximum projections in FiJi. Spot detection was done by 2D Gaussian fitting as described in (*5*, *6*). For 3D analysis, the spots were detected using AIRLOCALIZE as described in (7). To separate cytoplasmic and nuclear mRNPs, masks were created in FiJi by manual segmentation using DAPI stained nuclei as reference, while ensuring that regions with overlapping spots within the same channel were not included. Assignment of the 5', 3' and/or the mid spots to either the cytoplasmic or the nuclear masks was done using MATLAB (MathWorks). To measure distances between different regions of mRNPs, spots from different channels were first grouped to assign neighboring spots corresponding a single RNA. This was achieved by using spots from one channel as a reference and finding spots from the other channels within a defined radius using the coordinates from 2D Gaussian fitting or 3D Gaussian fitting using a custom MATLAB script. 300nm for 2D analysis and 400nm for 3D analysis were chosen as radii to limit assigning signals from neighboring RNAs. Groups with more than one spot from each channel, which could correspond to overlapping mRNPs or mRNPs close together in space, were discarded. For 2 color imaging, the 5' signal was taken as reference and for 3 color imaging, the middle was taken as reference. Switching references yielded comparable

results (not shown). 2D or 3D distances between different regions of the mRNPs were then calculated for each signal within a group.

Combined smFISH and Immunofluorescence for simultaneous detection of mRNA conformation and nascent translation

Human U2OS osteoscarcoma cell line (American Type Culture Collection HTB-96) expressing stdMCP-Halotag , phR-scFV-GCN4-sfGFP-GB1-NLS-dWPRE, and pBabe-TIR1-9myc was prepared as described in (1). Single-molecule FISH immunofluorescence was performed as described in (1, 8). In brief, cells were plated on 18mm diameter, 0.13mm thick collagen coated coverslips (Fisher) in a 12-well dish. Cells were then transfected with 250 ng of the phage-ubc-flag-24xSunTag-Fluc-oxBFP-AID-baUTR-24xMS2 construct using X-tremeGENE 9 transfection reagent (XTG9-RO ROCHE). Six hours after transfection, IAA (Sigma-Aldrich) was added to a final concentration of 250 μM. 20 hours after transfection, fresh IAA was added to a final concentration of 250 μM. 24 hours after transfection, cells were fixed for 10 minutes in PBS + 5 mM $MgCl_2$ (PBSM), permeabilized for 15 minutes in PBSM + 0.1% Triton-X and 0.5 % BSA, and incubated with 100 nM MS2v5-Cy5 and 50 nM SunTagV4-Qusar 570 smFISH probe sets (Table S2) and primary antibody against GFP (GFP-1010, Aves labs, Inc.) and incubated for three hours at 37°C. After washing, cells were incubated with Alexa Fluor 488 labeled secondary antibody (ThermoFischer) and mounted in ProLong Diamond antifade reagent with DAPI (Life Technologies). Images were acquired on a custom inverted wide-field Nikon Eclipse Ti-E microscope equipped with three Andor iXon DU897 EMCCD cameras (512x512 pixels) , Apochromatic TIRF 100X Oil Immersion Objective Lens/1.49 NA (Nikon MRD01991), encoded Stage with 150 micron Piezo Z (ASI), and LU-n4 four laser unit with solid state 405 nm, 488 nm, 561 nm, and 640 nm lasers (Nikon), a TRF89901-EM ET-405/488/561/640nm Laser Quad Band

Filter Set for TIRF applications (Chroma), and Nikon H-TIRF system. Images were acquired using in-unit intermediate 1.5x magnification changer for a final magnification of 150x and independent, epi-illumination from the 488, 561, and 640 nm lasers. Image pixel size: XY, 106.7 nm; Z-step, 200 nm. A total of 29 cells without drug treatment (total of individual 397 mRNAs) and 40 cells (98 individual mRNAs) upon puromycin treatment we analyzed.

Combined smFISH and Immunofluorescence Data Analysis

All image analysis was performed using existing or custom build packages in MatLab (MathWorks). Gaussian fitting of smFISH and immunofluorescence spot intensities was performed using FISH-quant (*9*). Briefly, cytoplasmic FISH spots were fit to a 3D Gaussian to determine the mRNA and translation site coordinates in each color. Both 5'-end, 3'-end, and translation site intensities were detected independently by this method. Image registration was performed by imaging 100 nm TetraSpeck Microspheres (ThermoFisher) and calibrating the field correction based on an affine transformation in a custom built MatLab package. The transformation matrix was first verified for reproducibility on other microsphere samples and then applied to mRNA samples (data not shown). Only 2D distances were considered for this analysis. To determine the end-to-end mRNA distance, we first assigned the Quasar 570 channel (SunTag Probes) to FITC channel (Alexa 488 labeled translation site) by setting a colocalization threshold of 300 nm after image correction. We then assigned the Quasar 570 to Cy5 (MS2 Probes), again with a colocalization threshold of 300 nm. We first grouped mRNA with both Cy3 and Cy5 colocalization, and then determined if there was also a colocalized translation site signal. We then binned two-color mRNA based on the presence (translating) or absence (non-translating) of translation site signal. We then determined the end-to-end distance, and, in the case of the translating mRNAs, the associated translation site intensity.

Data Plotting

All measurements were made for at least 2 independent biological replicates and the data plotted are representative from one of the replicates. For each measurement, at least 5 different fields were imaged with each image containing a minimum of 10 cells to make a total of at least 50 cells. A minimum of 593 RNAs were considered for cytoplasmic plots and a minimum of 430 RNAs were considered for the nuclear plots, unless mentioned otherwise. The center of mass plots in Fig. 1G, 2D, 4D were made using R. The center of mass was calculated as the mean of the coordinates of the three regions. The different conformations were then aligned using their center of masses. For the 3-color scatter plot in Fig. 2E, 4E, S5 and S7, to get a pair of co-localization precision values, two values were chosen randomly from our data. These values were taken as the X and Y coordinates for the scatter plot. The values that served as the X and Y coordinates were used to get density plots in the same figure. The mean Radius of gyration ($<R_g>$) was calculated using:

$$< R_g >= \sqrt{\frac{1}{3}\sum_{k=1}^{3}(r_k - r_{mean})^2}$$

where k represents one of the three regions of the mRNP and r_k the position of the corresponding position in space as determined by 2D Gaussian fitting.

Immunoprecipitations and western blotting

Cells were washed with 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4) and then lysed with 1 ml ice-cold lysis buffer A (100 mM KCl, 0.1 mM EDTA, 20 mM Hepes, pH 7.6, 0.4% NP-40, 10% glycerol, with freshly added 1 mM DTT and complete

mini EDTA-free protease inhibitors [Roche; one tablet per 25 ml lysis buffer]) per 2.5 million cells. 50 μl was saved as the input sample. Cells were incubated with antibody (diluted according to manufacturer's instructions) for 2 hours, rotating at 4°C. α-PABPC1 antibody was purchased from Abcam (ab21060), and α-eIF4G1 from MBL International. EZ view protein G Sepharose (Sigma) was washed twice with lysis buffer and added to lysate with 40 μl slurry used per ml of lysate. The beads and lysate were incubated with the lysate for an additional hour rotating at 4°C. The beads were washed 3X with cold lysis buffer. After the first wash, the beads were transferred to a new tube. The beads were then resuspended in protein loading dye (Life Technologies) with freshly added reducing agent, according to manufacturer's instructions, and boiled for 3 min. 2% lysate and 10% immunoprecipitants were loaded onto an SDS-PAGE gel and probed for PABPC1 and eIF4G1. α-PABPC1 and α-eIF4G1 were used at 1:1000, and α-rabbit IgG HRP (at 1:10,000) was used as the secondary antibody.

Supplementary Figures

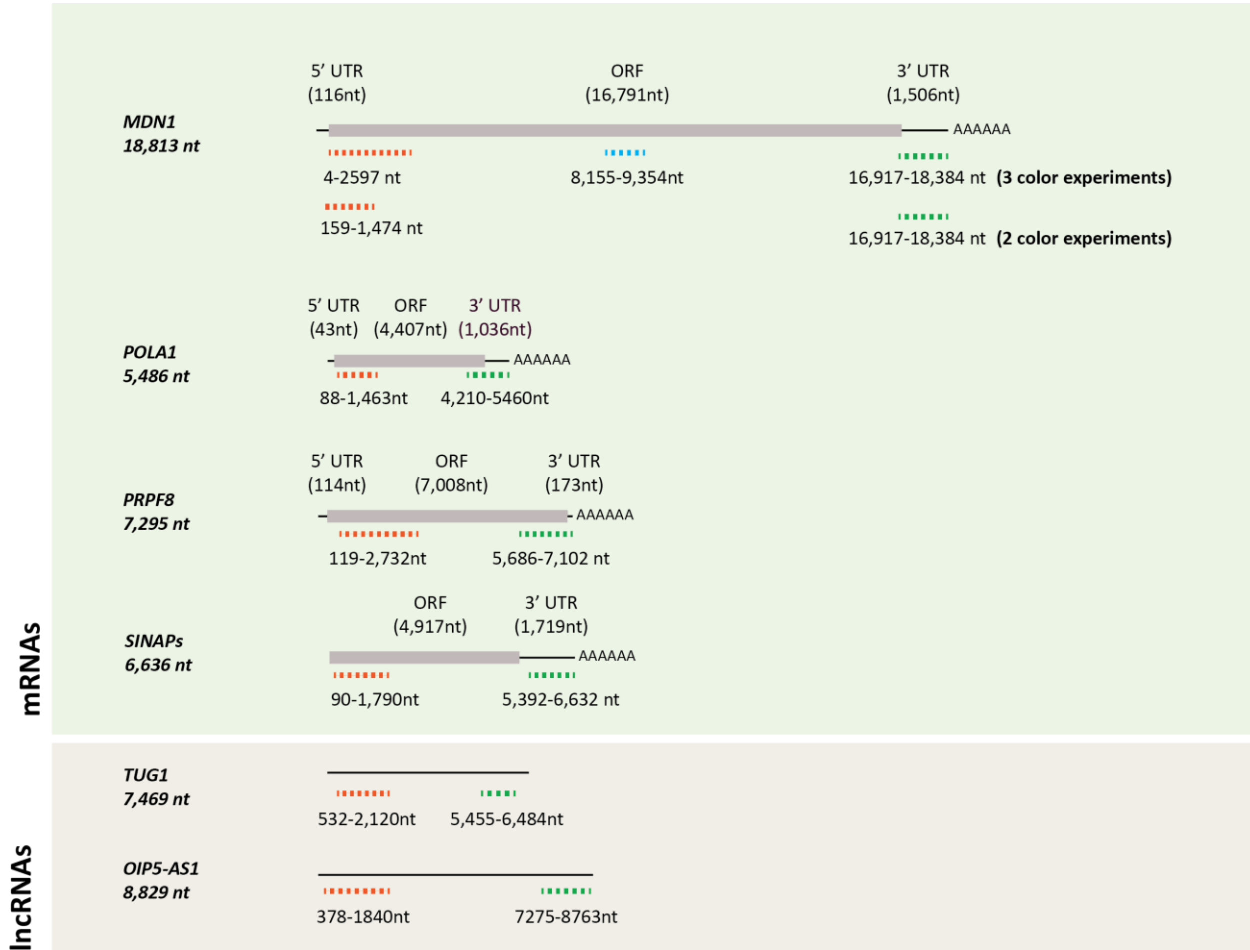

Figure S1: Positions of smFISH probes used in this study. Cartoons illustrating the positions of the probes used for the different genes used. See Supplemental Table 1 for probe sequences. The transcripts sequences were obtained from ensembl – MDN1 (ENST00000369393), POLA1 (ENST00000379068), PRPF8 (ENST00000304992), TUG1 (ENST00000644773) and OIP5-AS1 (ENST00000500949)

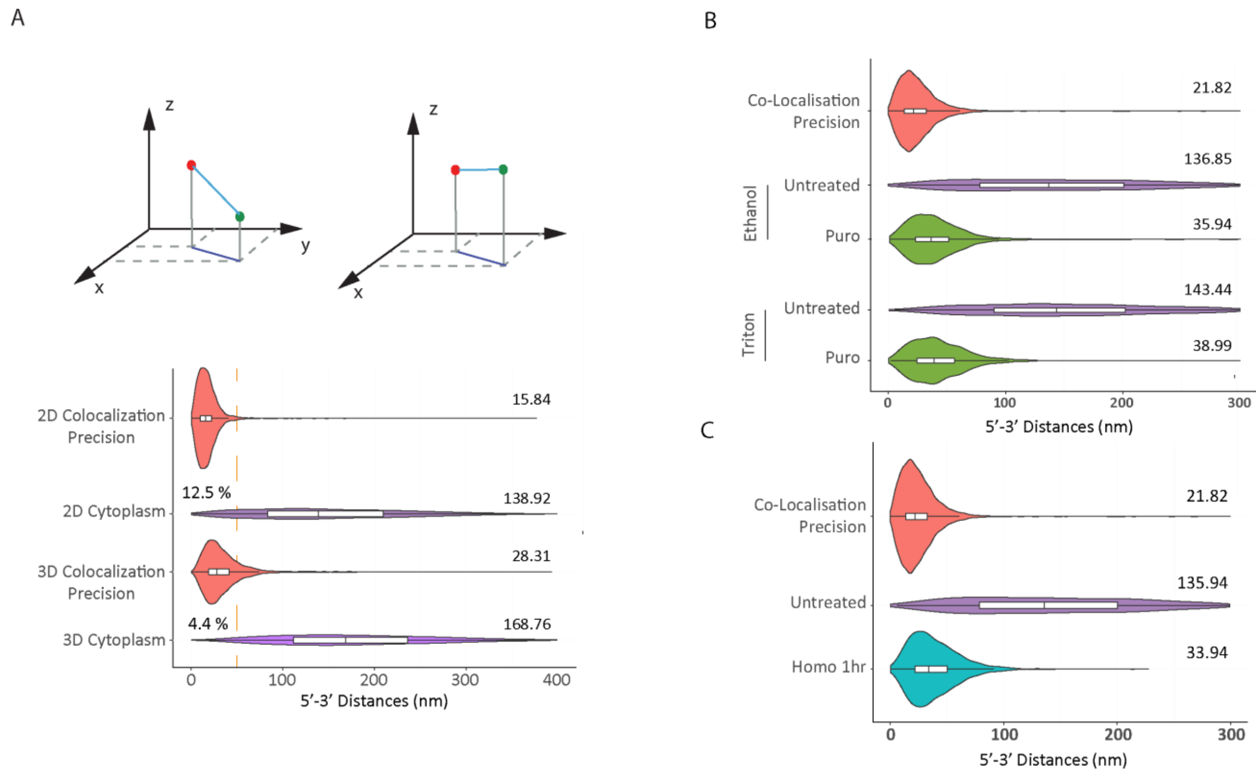

Figure S2: 5'-3' distances measured in 2D and 3D and upon treatment with translation inhibitors (A) Cartoon illustrating how 2D projection alters 5'-3' distances measured (above) and violin plots showing distance distribution of co-localization precision and 5'-3' distances for MDN1 mRNAs calculated in 2D and 3D. Dotted line delineates the percentage of MDN1 mRNA with 5'-3' distances less than 50nm. **(B)** Violin plots showing distance distribution of co-localization precision and 5'-3' distances for MDN1 mRNAs in cells permeabilized with either Ethanol or TritonX-100, **(C)** Violin plots showing distance distribution of co-localization precision and 5'-3' distances for MDN1 mRNAs determined by Gaussian fitting from untreated and homoharringtonine (1hr) treated cells.

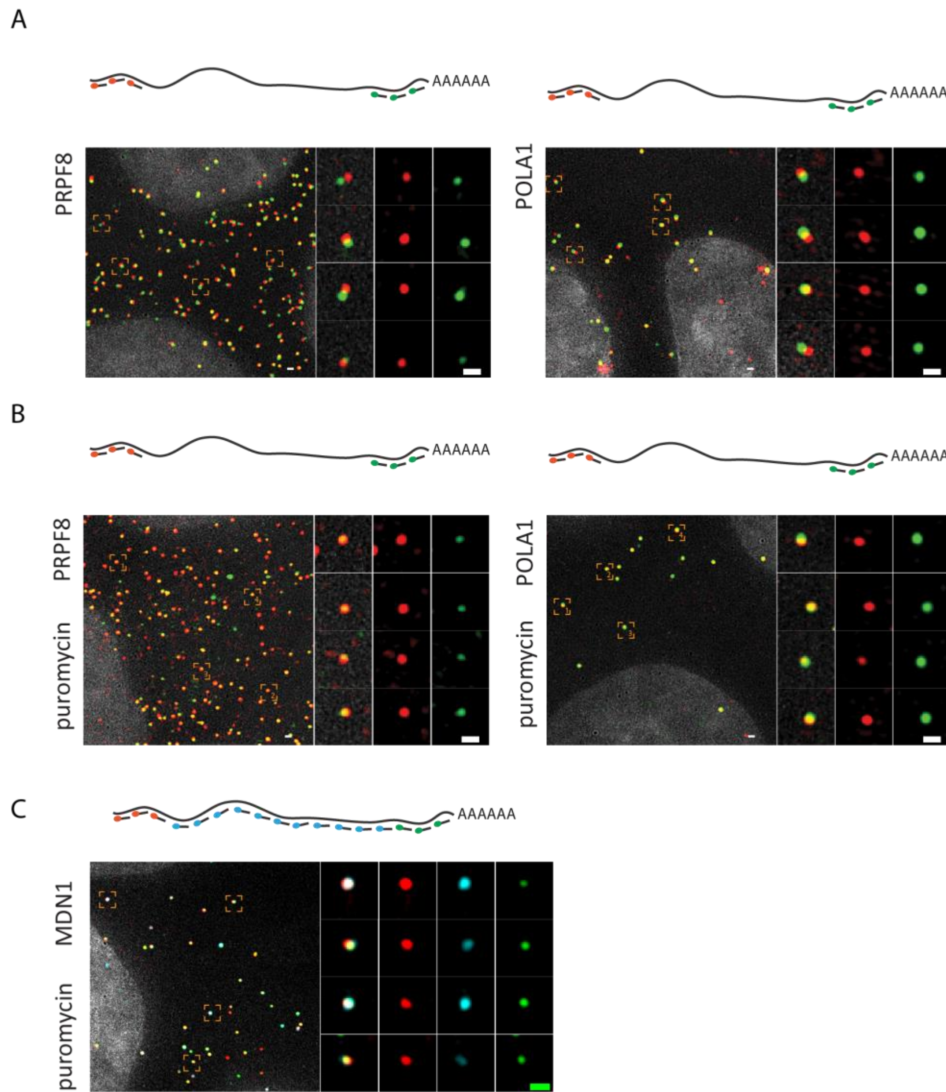

Figure S3: Visualizing mRNP conformation of single POLA1, PRPF8 and MDN1 mRNAs. **(A and B)** smFISH images using probes hybridizing to the 5' and 3' ends of PRPF8 and POLA1 mRNAs in paraformaldehyde fixed HEK293 cells, either untreated (A) or treated with puromycin (10 min, 100 µg/ml) (B). **(C)** smFISH using 5' (red), 3' (green), and tiling (cyan) for MDN1 mRNA in paraformaldehyde fixed HEK293 cells treated with puromycin (10 min, 100 µg/ml). Nuclei are visualized by DAPI staining (grey). Magnified images of individual RNAs marked by dashed squares are shown on the right. Schematic position of probes shown on top. Scale bars, 500 nm.

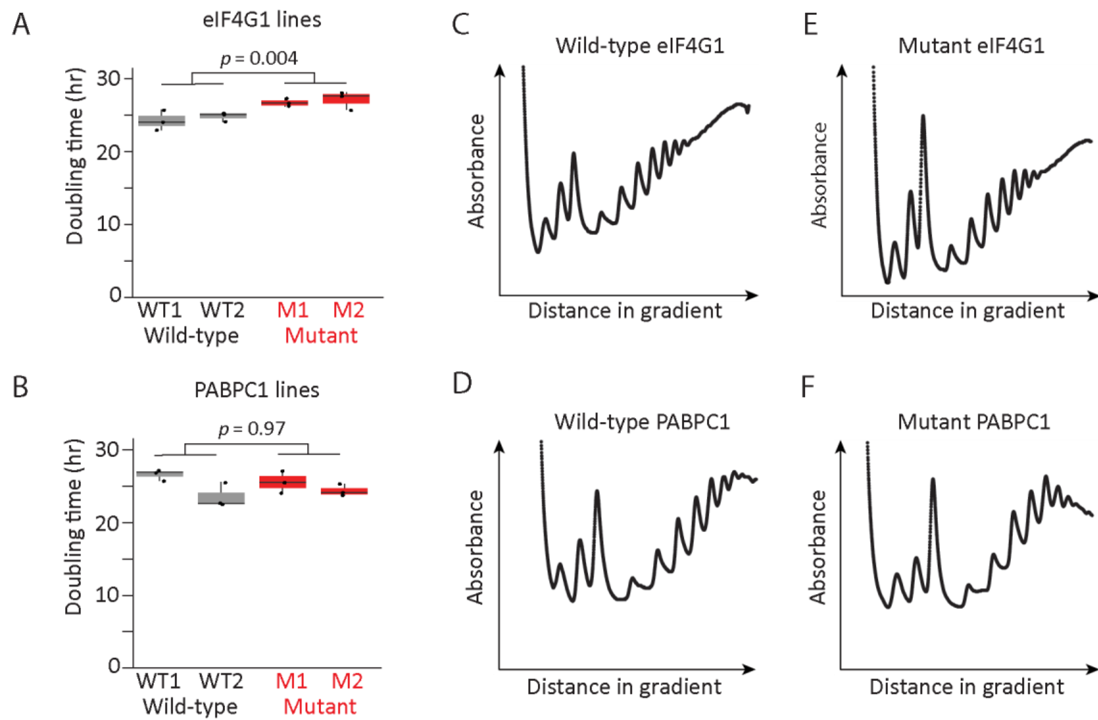

Figure S4: Close fraction of mRNP 5'-3' distances are not a result of eIF4G-PABPC1 interaction. (A) Doubling time for eIF4G1 CRISPR-edited lines. Shown are the doubling times calculated for three independent biological replicates for two independent wild-type and mutant eIF4G1 lines. (B) As in (A), except for PABPC1 CRISPR-edited lines. (C–F) Polysome profiles for wild-type eIF4G1 (C), wild-type PABPC1 (D), mutant eIF4G1 (E) , and mutant PABPC1 (F) lines.

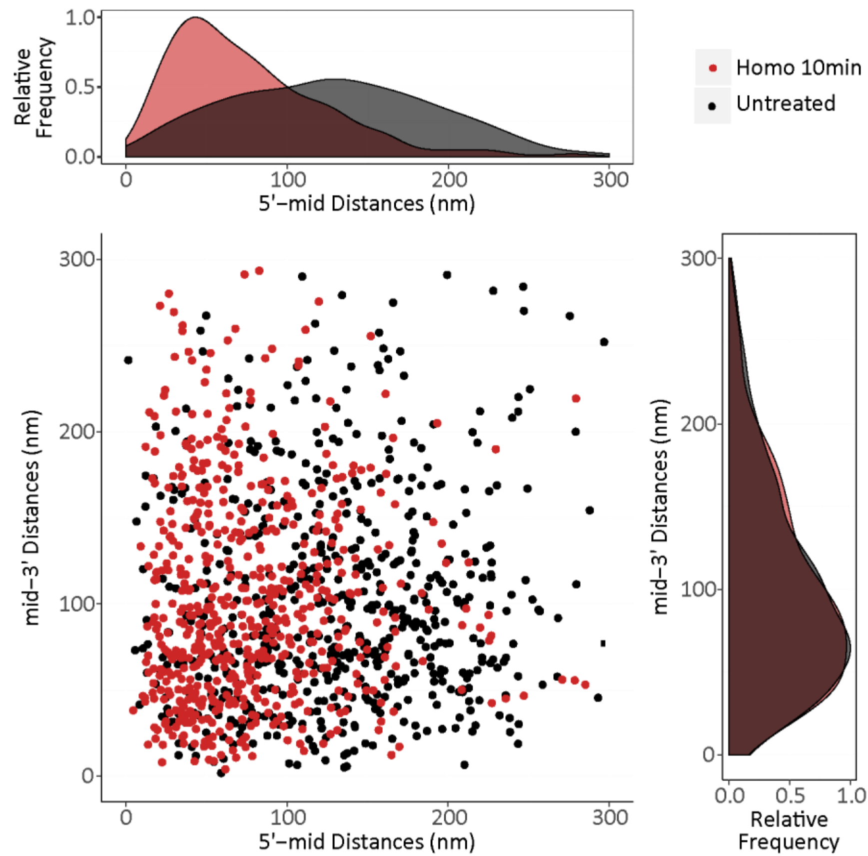

Figure S5: Compaction of the 5' end is altered upon a pulsed homoharringtonine treatment for 10min. Scatter plot showing 5'- mid and mid-3' distances for individual cytoplasmic MDN1 mRNAs from untreated cells (black) and cells treated with homoharringtonine (100µg/ml, 10min) (red). Frequency distribution are shown on top and on the right.

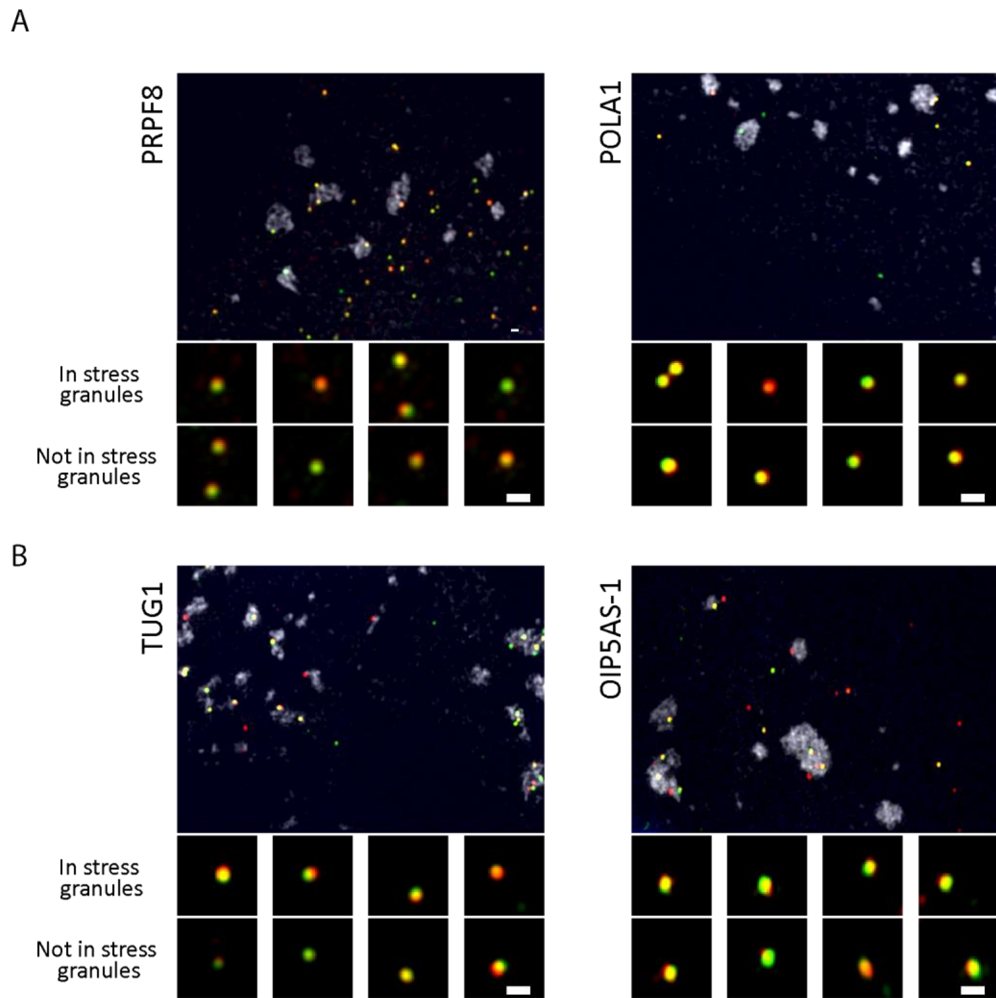

Figure S6: mRNA and lncRNA compaction and accumulation in stress granules. smFISH visualizing 5' and 3' ends of PRPF8 and POLA1 mRNAs (A) or TUG1 and OIP5-AS1 lncRNAs (B) in U2OS cells treated with arsenite (1 hour, 2 mM). Only a selected cytoplasmic region of cells is shown. Stress granules are visualized using an oligo dT probe (grey). Magnified images of individual RNAs localized inside or outside of stress granules are shown on the bottom of the images. For POLA1 and OIP5-AS1, not all magnified single RNAs shown in the bottom are from the corresponding image above. Scale bars, 500 nm.

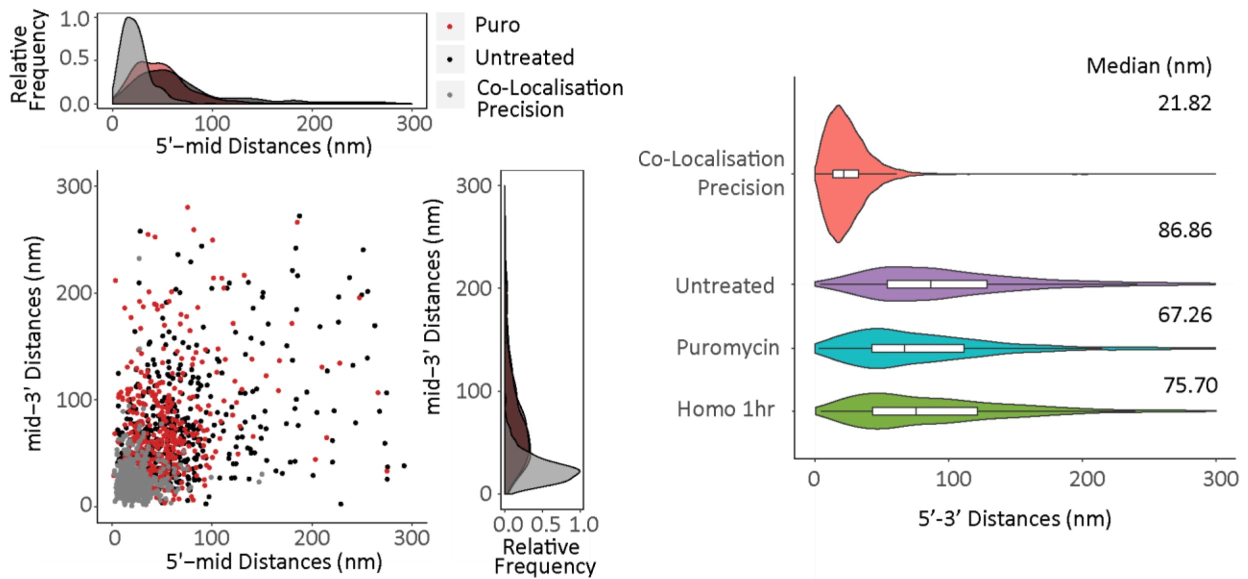

Figure S7: Compaction of nuclear MDN1 mRNA is not altered upon puromycin or homoharringtonine treatment. (Left) Scatter plot showing 5'- mid and mid-3' distances for individual nuclear MDN1 mRNAs from untreated cells (black) and cells treated with puromycin (100µg/ml, 10min) (red). Co-localization precision is shown in grey. Frequency distribution are shown on top and on the right. **(Right)** Violin plots showing distance distribution of co-localization precision and 5'-3' distances for MDN1 mRNAs determined by Gaussian fitting from untreated, puromycin (100µg/ml, 10min) or homoharringtonine (100µg/ml, 1 hour) treated HEK293 cells. White box plot inside the violin plot shows first quartile, median and third quartile. Median distances are shown on the right.

Tables

Table S1: Primers used for making CRISPR/Cas9 cells lines

Name	Sequence (5'-3')	Purpose
PABPC1 M161A gRNA 1 Fw	CACCGAATCTGTTAGCCATCTAACC	Guide RNA targeting PABPC1; annealed and ligated into pX330
PABPC1 M161A gRNA 1 Rev	AAACGGTTAGATGGCTAACAGATT C	
PABPC1 region Fw NotI	GCGCACTAGCGGCCGCGAGGAAGCGTTCAACTGTGA	To amplify 5' arm region of PABPC1
PABPC1 Not I 5' arm Rev guide 1 XhoI	GCGCACTAGCGGCCGCCTCGAGACCTGGATATTTGTGAAATAAAG	
PABPC1 BamHI 3' arm Fw guide 1	CGCGGATCCTAGATGGCTAACAGATTGTCTCTC	To amplify 3' arm region of PABPC1
PABPC1 region Rev BamHI	CGCGGATCCTTGGTCAGGCTGGTCTCAAA	
PABPC1 mutation Fw	GAAAGAGCTATTGAAAAAATGAATGGAGCGCTCCTAAATGATCGCAAAGTATTTGTTGG	Internal primers for stitch PCR to make PABPC1 mutation
PABPC1 mutation Rev	CCAACAAATACTTTGCGATCATTTAGGAGCGCTCCATTCATTTTTTCAATAGCTCTTTC	
PABPC1 region Fw	GGCGAGAGATTGCGTCAAGAA	Screening for puromycin cassette insertion/excision
PABPC1 region Rev	CCCTGGTAACAGGCATTTGTGAG	
PABP seq Fw1	GCAATATGGAATTCTTTTATATG	To sequence PCR products from PABPC1 mutant cell line screening
PABP seq Fw2	GGAACTGTGCAGTAATGGATATC	
PABP seq Rev1	CAATCTTGTCGCCCAGACTGG	
eIF4G1 mutant gRNA 1 Fw	CACCGTGCTGCTGGGACATTGTGC	Guide RNA targeting eIF4G1; annealed and ligated into pX330
eIF4G1 mutant gRNA 1 Rev	AAACGCACAATGTCCCAGCAGCAC	
eIF4G1 5' HR Arm Fw NotI	GCGCACTAGCGGCCGC GAGACAGGAACTAGACTCAAG	To amplify 5' arm region of eIF4G1
eIF4G1 5' HR Arm Rev NotI XhoI	GCGCACTAGCGGCCGC CTCGAG CAATGTCCCAGCAGCACCTGACC	
eIF4G1 3' HR Arm Fw BamHI	CGC GGATCC TGCCGGAAAGAGCAGTGACTTG	To amplify 3' arm region of eIF4G1
eIF4G1 3' HR Arm Rev BamHI	CGC GGATCC GGCACCCTATTCTGGGCACC	
eIF4G1 mutation Fw	TGCAGCCGCTGCCGCGGGAGCAATCTGGGGTGGCTGGTTC	Internal primers for stitch PCR to make eIF4G1 mutation
eIF4G1 mutation Rev	CGCCAGTGGGAAACTGCTGCACCCCTTGGGCTGGATAGTAGG	

eIF4G1 region Fw	GTAGTCGCACAGTCTTGGCTC	Screening for puromycin cassette insertion/excision
eIF4G1 region Rev	GAGTCCAGGGCAGAACAGAC	
4G seq Rev1	CACCCCTCGTAGGCAGGCACTC	To sequence PCR products from eIF4G mutant cell line screening
4G seq Fw 1	CAGAGTATGTGTGTACATGTTG	
4G seq Rev2	CTTCCTCGCTAGGCACTTCAG	
4G seq Rev 3	CCAGCAGTCCCCAAGTCAGTGG	

Table S2: List of probes used

MDN1 5'	tcgttcttggctgcgattaa	POLA1 3'	tgtacagggacttgtcagaa
MDN1 5'	taaggtactcaggacacact	POLA1 3'	taccggtaaaagcacagctg
MDN1 5'	cacagtacagtccttatcca	POLA1 3'	gtgcacactccgcatcaaaa
MDN1 5'	agcaaatccaaaaggagagg	POLA1 3'	tctcatgatcggtagtaagt
MDN1 5'	ttgaaagactggggatgtgt	POLA1 3'	ctgtagtcctgcagaacttt
MDN1 5'	gcatctgaactctctaggaa	POLA1 3'	ctctgctgtgttcttgagtt
MDN1 5'	gtgctcttcattcatacagg	POLA1 3'	tagccacttcgggacaagaa
MDN1 5'	cctcaacctgaaatggatca	POLA1 3'	tttgctcagattcacttcgg
MDN1 5'	aaaaccaaggccttctccaa	POLA1 3'	ttaggatttcacggcacaac
MDN1 5'	aaagggagacttctggattg	POLA1 3'	cttggttactcctgggattc
MDN1 5'	tcagacgaaacaagatgtcc	POLA1 3'	ggaagctgggatttttcaac
MDN1 5'	accagcacataagacctaag	POLA1 3'	caagggagaaacagatgctg
MDN1 5'	gaagacttttgcagacagac	POLA1 3'	acacaaacatgagacacagt
MDN1 5'	acagcattctgagaagcaac	POLA1 3'	gactcaacatttttgcagcc
MDN1 5'	tcctattggtccttccaaca	POLA1 3'	ctcagaaaccgggtcttcag
MDN1 5'	cctgtcactgcagctaaata	POLA1 3'	gctactctcaatccaagtag
MDN1 5'	aagctggactttgagaagct	POLA1 3'	gcctggggtcacttacattc
MDN1 5'	acatctgtgcagcgatacat	POLA1 3'	cataggctaaaggccctgag
MDN1 5'	atatcctccagaaggatcca	POLA1 3'	ttcagtcaggctctgagaag
MDN1 5'	aagagctctccattctccaa	POLA1 3'	tgaaaaagcaaaacgtcagc
MDN1 5'	aaatccaggtgccactttca	POLA1 3'	ttagaccgggtttaattggc
MDN1 5'	caactacgcccgcaggaaag	POLA1 3'	actcctggatggctggagaa
MDN1 5'	agcaagaagtgctccatgac	POLA1 3'	agacaagactgaaaaggaca
MDN1 5'	caagaacctgcccaactcac	POLA1 3'	agtgcaaggcttctaaatct
MDN1 5'	cgatcttgaggtgtccacac	POLA1 3'	gagcaattcaacaaacaagc
MDN1 5'	aatggcttcggcattccttt	POLA1 3'	cagtgtgtgtctgttggact
MDN1 5'	gttcatgcagatcatggttg	POLA1 3'	atgtgagtgtaaaacacctg
MDN1 5'	gtttgctcatcgacacacat	POLA1 3'	gcactttctatttaaggggc
MDN1 5'	gacatcaggatggttaccaa	POLA1 3'	ccctacacatgttaatggat
MDN1 5'	aatatctcagggcaaacggg	POLA1 3'	tgaatacaacacagtgatcc
MDN1 5'	tacgtccatagcgtactgga	POLA1 3'	ggccaattaagcatccctct
MDN1 5'	gcagaaacttgaaggctgct	POLA1 3'	agaaaaaaatagcaagcgcc
MDN1 5'	cggaacacagactgctcctg	POLA1 3'	agacggtcctattgtgaaga
MDN1 5'	aaggtgtcatggcttctgag	POLA1 3'	aaatacattttgctgctgcc
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MDN1 5'	acaccacaaacagctgtcac	POLA1 3'	cagcataattgtacaagggg
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MDN1 tiling additional	cgttcttggctgcgattaac	PRPF8 3’	gttgtcagccatgatcttgg
MDN1 tiling additional	tgaaggctgcttccattagg	PRPF8 3’	ataatggtcttctcgccatc
MDN1 tiling additional	aaggtgtcatggcttctgag	PRPF8 3’	cttgtaggccgtcagtgtac
MDN1 tiling additional	cagcattctgagaagcaacc	PRPF8 3’	cccattcgtagccactgggg
MDN1 tiling additional	cctattggtccttccaacaa	PRPF8 3’	ttgcccttgtctgtgttctg
MDN1 tiling additional	aagcaggtgatcaaccactg	PRPF8 3’	ctctcatagtgtgaaggcag
MDN1 tiling additional	ccaacagaactatcactcca	PRPF8 3’	gtccgacagcagcatctgca
MDN1 tiling additional	tgagaacagaggacggccgt	PRPF8 3’	ccatgaagaagccaaggaaa
MDN1 tiling additional	acacacactgcaagctgctc	PRPF8 3’	gaagttgtagttccacgagg
MDN1 5’ additional	tcgttcttggctgcgattaa	PRPF8 3’	agctcatatttcatgttggg
MDN1 5’ additional	taaggtactcaggacacact	PRPF8 3’	ctctttggggttcgccagct
MDN1 5’ additional	cacagtacagtccttatcca	PRPF8 3’	gcctgtgcacctcgtggtag
MDN1 5’ additional	agcaaatccaaaaggagagg	PRPF8 3’	ctgcaggagagcaaagttga
MDN1 5’ additional	ttgaaagactggggatgtgt	PRPF8 3’	gatccgcagagtaaacctcc
MDN1 5’ additional	gcatctgaactctctaggaa	SunTagV4	ccacttcgttctcaagatga
MDN1 5’ additional	gtgctcttcattcatacagg	SunTagV4	cccttttcagtctagctac
MDN1 5’ additional	cctcaacctgaaatggatca	SunTagV4	aatttttgctcagcaactcc
MDN1 5’ additional	aaaaccaaggccttctccaa	SunTagV4	ttctttagtcgtgctacttc
MDN1 5’ additional	aaagggagacttctggattg	SunTagV4	tttcgagagtaactcctcac
MDN1 5’ additional	tcagacgaaacaagatgtcc	SunTagV4	ccacttcgttttcgagatga
MDN1 5’ additional	accagcacataagacctaag	SunTagV4	acttcccttttttaagcgtg
MDN1 5’ additional	gaagacttttgcagacagac	SunTagV4	tcttggatagtagctcttca

MDN1 5’ additional	acagcattctgagaagcaac	SunTagV4	acctcgttctcaagatgata
MDN1 5’ additional	tcctattggtccttccaaca	SunTagV4	cggaacccttcttcaaacgc
MDN1 5’ additional	cctgtcactgcagctaaata	SunTagV4	agttcttcgagagcagttcc
MDN1 5’ additional	aagctggactttgagaagct	SunTagV4	gatcccttttttaatcgagc
MDN1 5’ additional	acatctgtgcagcgatacat	SunTagV4	tgaaagtagttcctcaccac
MDN1 5’ additional	atatcctccagaaggatcca	SunTagV4	cttcgttttcgaggtggtaa
MDN1 5’ additional	aagagctctccattctccaa	SunTagV4	ccctgaacctttctttaatc
MDN1 5’ additional	aaatccaggtgccactttca	SunTagV4	tactcagtaattcttcaccc
MDN1 5’ additional	caatttcctccacagctcaa	SunTagV4	tttcgatagcaactcttcgc
MS2V5	tgattgtgaagtgtcgggtg	SunTagV4	tttttgagcctagcaacttc
MS2V5	tccacccttgtgtattgtac	SunTagV4	ttttcgagagcaactcctcg
MS2V5	tgtaatgtgtctggagggtg	SunTagV4	acctcattttccaagtggta
MS2V5	gcttctgtttgattggattt	SunTagV4	tttgctcaataactcctcgc
MS2V5	gatggtgattccttgttgta	SunTagV4	cgcgacttcgttctctaaat
MS2V5	gtatattgcacagggaatcc	SunTagV4	ttcgataagagttcttcgcc
MS2V5	gatattcgggaggcgtgatc	SunTagV4	ctcattttcgaggtggtagt
MS2V5	acgcactgaattcgaaagcc	SunTagV4	agtggtagttcttgctcaag
MS2V5	attcgactctgattggctgc	SunTagV4	ttcaatctcgcgacctcatt
MS2V5	ctcttcgcgaaagtcgactt	SunTagV4	attcttgctgagcaattcct
MS2V5	taagaatggcgcgaaggctg	SunTagV4	cgacttcgttctccaaatga
MS2V5	gtaggggagagtgtggtttg	SunTagV4	cgacttcattttccaagtgg
MS2V5	caggaacgctgatgctgttc	SunTagV4	ttgctcaataactcttcgcc
MS2V5	ttttcttgagttgggtactg	SunTagV4	ttcgttctccaagtggtaat
MS2V5	tgatgctgcatggggacata	SunTagV4	agttcttcgataagagctcc
MS2V5	ttggggatgtattcttgggg	SunTagV4	gcgacttcattctctaagtg
MS2V5	ttggtgctcggatgtgattt	SunTagV4	ttcttgctcaagagctcttc
MS2V5	aagaaacaacactccgagcc	SunTagV4	cacctcattttccaagtggt
MS2V5	atggagggtttgtccagttg	SunTagV4	ttagatagtaactcttcccc
MS2V5	tttgtcttgttggtgagagt	SunTagV4	cctcgttctcgagatgataa
MS2V5	ctgatgctgcttcgagaaga	SunTagV4	gatagttcttcgacaggagt
MS2V5	gtatgctcgagtgtttcgaa	SunTagV4	cctttttaagtcttgcaacc
MS2V5	gatcgtccacccaagaaata	SunTagV4	ttactgagtagttcctcacc
MS2V5	aattcgtgagagcatgggtg	SunTagV4	ttcgttttccaggtggtaat
MS2V5	tcgtattggacgtggaacga	SunTagV4	tcctgatcctttcttcaaac
MS2V5	tcgtgatcccgaaaggtaag	SunTagV4	cttttgagagcagttcttcg
MS2V5	atcgtgcatgcttgaatgtc	SunTagV4	gcaacctcattttccaaatg
MS2V5	gttgagacttgtggagcatg	SunTagV4	tgccacttcccttttttaaa
MS2V5	tgaacccatttggtagtttc	SunTagV4	tttcgacagaagttcctcac
MS2V5	tttgaggtaggagtgggttc	SunTagV4	gctacttcattctcgagatg
MS2V5	ttgccagttttgtgggaaga	SunTagV4	gagccagaaccctttttaag
MS2V5	tttggtatgttggaatgggc		
MS2V5	gatgctgtaccagtaattgt		
MS2V5	tagtagtgagagatgtgggc		
MS2V5	tgctgaacggtttggttttt		
MS2V5	ttgatttttccgtgtgtacc		
MS2V5	gtctttcgtatttgtaaacc		
MS2V5	ttgcgctggacgaaagcgtg		
MS2V5	ccgtcggatgtttttcgtaa		

MS2V5	ggttgtaagtttgtgggttg		
MS2V5	ctgaggtgtttgatgtacgg		

Table S3: Probe and antibody combinations used

Experiment	Combination of probes/antibodies and dyes used (From Table S1)
MDN1 5’-3’	MDN1 5’ -Cy5 MDN1 3’- Dy550
MDN1 5’-tiling-3’	MDN1 5’+ MDN1 5’ additional – Dy488 MDN1 tiling – Cy5 MDN 3’ – Dy550
MDN1 5’-middle-3’	MDN1 5’+ MDN1 5’ additional – Dy488 MDN1 middle – Cy5 MDN 3’ – Dy550
PRPF8 5’-3’	PRPF8 5’ -Cy5 PRPF8 3’- Cy3
POLA1 5’-3’	POLA1 5’ -Cy3 POLA1 3’- Cy5
TUG1 5’-3’	TUG1 5’ -Cy5 TUG1 3’- Cy3
OIP5-AS1 5’-3’	OIP5-AS1 5’ -Cy5 OIP5-AS1 3’- Cy3
MDN1 tiling-3’	MDN1 tiling + MDN1 tiling additional– Cy5 MDN1 3’– Dy550
MDN1 5’-3’-dT	MDN1 5’ -Cy5 MDN1 3’- Dy550 dT – Cy2
MDN1 tiling-3’-dT	MDN1 tiling + MDN1 tiling additional– Cy5 MDN1 3’– Dy550 dT- Cy2
PRPF8 5’-3’-dT	PRPF8 5’ -Cy5 PRPF8 3’- Cy3 dT – Cy2
POLA1 5’-3’-dT	POLA1 5’ -Cy3 POLA1 3’- Cy5 dT – Cy2
TUG1 5’-3’-dT	TUG1 5’ -Cy5 TUG1 3’- Cy3 dT – Cy2
OIP5-AS1 5’-3’-dT	OIP5-AS1 5’ -Cy5 OIP5-AS1 3’- Cy3 dT – Cy2
SINAPs 5’-3’	SuntagV4 5’ – Quasar 570 MS2v5 3’ – Cy5 Chicken anti-GFP Antibody Goat-Anti Chicken Alexa 488

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