Supplementary Materials for

Title: mRNA structure determines specificity of a polyQ-driven phase separation

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Caption for Movie S1

References (19-22)

Other Suplementary Materials for this manuscript includes the following:

Movies S1

Materials and Methods

Plasmid and Strain Construction

To create *pcln3sm::GEN*; cln3Δ::NAT1 construct, under the control of its own promoter, the entire plasmid AGB237 (p*CLN3*- GFP::GEN3, AMP) was amplified using primers Phusion polymerase (New England Biolabs) and AGO2308 (GGGCTGTTA ATATCTCATACCCGTTGTGGTTTGCATATTATACACATATTCG) and AGO2309 (CGAATATGTGTATAATATGCAAACCACAACGGGTATGAGATATTAACAGCCC). This mutated a region 31 base pairs upstream of the ORF from AACACC to TTGTGG. The reaction was digested with DpnI (New England Biolabs). The 5'UTR, ORF, and 3'UTR were sequenced by Genewiz, confirming that TTGTGG was the only mutation. This plasmid was transformed into *A. gossypii* AG612 (*cln3Δ::NAT1*) via electroporation and selection on AFM+G418 plates.

To create *pcln3sm::GEN* under control of a T7 promoter, the entire plasmid AGB690 (*CLN3*-GFP) was amplified with the primers AGO2308 and AGO 2309 using the QuickChange XL Site-Directed Mutagenesis kit (Agilent Technologies) following the instructions. The 5'UTR, ORF, and 3'UTR were sequenced by Genewiz, confirming that TTGTGG was the only mutation. This plasmid was then digested and used as a template to transcribe RNA using the T7 HiScribe in vitro transcription kit (NEB).

To create the p*cln3scr2*; ΔlΔt a geneblock containing the scrambled coding sequence for *CLN3* was ordered from GenScript and amplified with primers 5' TAAACGAAG GCAAAGAGCTCGGTACCCGGGGACGTTCACTAATCTTAATACG 3' and 5'GTGAAAAGTTCTTCTCCTTTACTCATCTCAGTACGCGGCCGCTCGAGAGATC

TTGTAATTAATCCTGC 3'. AGB392, a plasmid containing GFP under the control of the *S. cerevisiae HIS3* promoter, was cut with BamHI and SpeI restriction enzymes to linearize the plasmid. The *CLN3 scr2* amplified coding sequence was cloned into the cut AGB392 vector using NEBuilder HiFi DNA Assembly (New England Biolabs). Cloning was confirmed via sequencing (Genewiz) using 5' TGGTTATGGCGCCCTCACAG 3' and 5' GCCCATTAACATCACCATCTAATTC 3'. This plasmid was transformed into WT *A. gossypii* (AG416) via electroporation and selection on AFM+G418 plates.

Cell culture and Imaging

Ashbya cells were grown in 10 mL Ashbya full media (AFM) under selection of either G418 (200 ug/ml) or Clonat (100 ug/ml) in a 125 ml baffled flask shaking at 30°C for 16 hours. The cultures were then transferred to 15 ml conical tubes (VWR) for centrifugation at 350 rpm for 2 min. AFM was removed and cells were suspended in 10 ml 2X Low Fluorescence media. Cells were then placed on a 2X Low Fluorescence media gel-pad containing 1% agarose embedded in a depression slides, sealed with valap and imaged. For dye incorporation experiments, *Ashbya* cells encoding a Whi3 HaloTag were spun down, washed with 2X Low Fluorescence Media and then incubated with 62.5 nM JF649 Halo dye (Lavis Lab, Janelia Farms Research Campus) for 20 min. The cells were then washed twice before being placed on gel pads and imaged at indicated time intervals. Image intensity for each droplet was quantified by finding the highest intensity pixel within the droplet and then taking the average intensity of a circle of about 9 pixels around the highest intensity pixel. All image processing and analysis for *in vivo*

experiments was carried out in either Fiji or MATLAB (*19, 20*). For Whi3-tomato measurements, N=2 independent experiments with n=4 cells, 73 perinuclear droplets and 27 tip droplets were measured and for Whi3-Halo, N=2 independent experiments n=7 cells, 40 perinuclear droplets and 15 tip droplets were measured.

Single molecule RNA F.I.S.H and Imaging

RNA smFISH labeling was performed as previously described (*12*). Briefly, cells were grown overnight at 30 °C in AFM and were fixed using 3.7% (vol/vol) formaldehyde (Fisher Scientific) and then washed twice with cold buffer B. Cells were resuspended in 1mL of spheroplasting buffer and digested using 1.5 mg/ml Zymolyase (MP Biomedicals) for 30 min at 37 °C. Cells were washed twice with buffer B, and then treated with Proteinase K for 5 min to digest proteins and unmask protein-bound RNAs. Cells were then washed twice before being resuspended in 70% ethanol, and incubated overnight at 4 °C. Cells were washed twice with SSC wash buffer before being resuspended in hybridization buffer containing Tetraethyl rhodamine (TAMARA) or cy5-conjugated RNA FISH probes (Stellaris LGC Biosearch Technologies) complementary to each transcript of interest. Cells were incubated in the dark overnight at 37 °C. After incubation, cells were washed once with wash buffer, incubated with 5 ug/ml Hoechst (Invitrogen) and incubated at room temperature for 30 min before being washed a final time with wash buffer, mounted on glass slides with 20 ul Prolong Gold mounting medium (Invitrogen), sealed with nail polish, and then imaged. Images were de-

convolved using 29 iterations of the Lucy-Richardson algorithm on Nikon Elements software and then processed in Fiji.

Analysis of mRNA fraction co-localized

A 8 X 10 ROI was generated at the tips of hyphae or around individual nuclei in merged images. Percent of co-localization was determined by counting the presence of overlapping signal from each of the individual mRNA channels. A total of 40 tips and nuclei were used for analysis and taken across at least 2 biological replicates. mRNAs inside the nucleus were counted and co-localization events inside the nucleus were counted as well.

Recombinant Protein Expression and Purification

Protein purification was performed as previously described (*3*). In brief, full length Whi3 or whi3-Y610-F653A was tagged with an N-terminal 6-Histidine tag and expressed in BL21 *E. coli* (New England Biolabs). For labeled protein, full length Whi3 was tagged with a C-terminal GFP. Cells were lysed in lysis buffer (1.5M KCl, 20 mM Tris pH 8.0, 20 mM Imidazole pH 8, 1 mM DTT, 1 tablet of Roche protease inhibitor cocktail). The supernatant was incubated and passed over a Ni-NTA resin (Qiagen) in gravity columns. The resin was then washed with 10CV lysis buffer and protein was eluted with 6CV elution buffer (150 mM KCl, 20 mM Tris pH 8.0, 200 mM Imidazole pH 8.0,1 mM DTT). The fractions containing Whi3 protein were dialyzed into fresh droplet buffer (150

mM KCl, 20 mM Tris pH 8.0, 1 mM DTT) and measured with Bradford reagent. Aliquots of protein were flash frozen and stored at -80 ºC.

RNA Transcription

The T7 promoter 5' TAATACGACTCACTATAGGG 3' was cloned into the 5' end of *CLN3* (AGB 690), *BNI1* (AGB 691), and *SPA2* (AGB 692). The *cln3*-scr plasmid was synthesized with the T7 promoter (IDT). Plasmid DNA was digested with restriction enzymes to obtain a linear DNA template that was then transcribed using T7 Hiscribe *in vitro* transcription kit (NEB) according to manufacturer's instructions. To obtain labeled RNA for imaging, 0.1 ul of 5 mM cy3-UTP or cy5-UTP (GE Healthcare) was added into the transcription reaction. Transcribed RNAs were then treated with DNase, precipitated with LiCl, and washed with 85% ethanol before being re-suspended in TE buffer and stored at -80 ºC.

Droplet Assembly and Imaging

Whi3 used in experiments contain 10:1 unlabeled to GFP-labeled protein. For recruitment experiments, Whi3 protein and cy5 labeled *BNI1* RNA were mixed and incubated for four hours in glass chambers (Grace Bio-Labs) at room temperature to desired concentrations in droplet buffer. The chambers were pre-treated with 30 mg/mL BSA (Sigma) for 30 minutes. After four hours, cy3-labeled RNA of interest was added to the wells, mixed, and incubated for 30 min. Imaging of droplets was done either on a

spinning disc confocal microscope (Nikon CSU-W1) with VC Plan Apo 60X/1.49 NA oil immersion objective and an sCMOS 85% QE camera (Photometrics) or a custom spinning disk confocal microscope (Nikon Ti-Eclipse equipped with a Yokogawa CSU-X spinning disk module) using a 60X 1.49 NA oil immersion objective and an air-cooled EM-CCD camera. For the RNA melting experiments, RNA was denatured at 95 ºC for three minutes and then immediately added to preformed droplets. For the RNA refolding experiments, RNA was denatured at 95 ºC for three minutes and then cooled down at 1-4 ºC per minute to 37 ºC final temperature in a thermocycler, added to preformed droplets, and imaged immediately. For the *BNI1* melted experiments, *BNI1* mRNA was heated to 95 ºC for 3 minutes before being mixed with Whi3 and incubated for 2 hours. *CLN3* mRNA was then added and immediately imaged. For the *CLN3* oligonucleotide experiments, 5 nM *CLN3* was mixed with a 100 nM mixture of the 5 oligo nucleotides (Supplemental Table 2). This mixture was then melted to 95 ºC for 5 min then cooled to 70 ºC (average melting temperature for the 5 oligos) for 10 min in a thermocycler before being added to *BNI1* droplets and imaged. For the *SPA2* oligonucleotide experiments, 5 nM of *SPA2* was mixed with a 100 nM mixture of the 4 oligo nucleotides (Supplemental Table 2). This mixture was then incubated at 25 ºC for 10 min in a thermocycler before being added to *BNI1* droplets and imaged. At least five independent imaging areas were analyzed for each condition for each replicate. Data shown are representative of three or more independent replicates, across at least two RNA and protein preparations.

RNA Spermine experiments and analysis

For RNA spermine experiments, 10 nM *in vitro* transcribed mRNAs were incubated for 4 hours in Spermine buffer (10 mM Spermine tetrahydrochlroide (Sigma), 20 mM KCl, 10 mM $MgCl_2$) as previously described (*14*) and then imaged on a custom spinning disk confocal microscope (Nikon Ti-Eclipse equipped with a Yokogawa CSU-X spinning disk module) using a 60X 1.49 NA oil immersion objective and an air-cooled EM-CCD camera. For the RNA melting experiments, both *BNI1* and *CLN3* were melted for 3 minutes at 95 ºC before being mixed together and incubated for four hours. For the *CLN3* oligonucleotide experiments, 10 nM *CLN3* was mixed with a 100 nM mixture of the 5 oligo nucleotides. This mixture was then melted to 95 ºC for 5 min then cooled to 70 ºC for 10 min in a thermocycler before being added to wells to incubate for four hours. For the *SPA2* oligonucleotide experiments, 10 nM of *SPA2* was mixed with a 100 nM mixture of the 4 oligo nucleotides. This mixture was then incubated at 25 ºC for 10 min in a thermocycler before being added to wells to incubate for four hours. Images were cropped to the middle 11 z stacks. Cy3 and cy5 channels, representing the different RNAs, were split and each channel was background subtracted with a 100-pixel rolling ball. Data shown are representative of three or more independent replicates, across at least two RNA preparations. The ImageJ plugin "JACoP" (https://imagej.nih.gov/ij/plugins/track/jacop.html) was used to calculate the Pearson's Correlation Coefficient between the two background-subtracted channels. The Wilcoxon Rank Sum Test was used to test for statistical significance between experimental conditions.

The DNA oligonucleotide sequences (IDT) used are as follows.

CLN3 to *BNI1*:

30 mer 461-479:	5'tcaaagTGCGCATCTCCACGGTAAGttccg 3'
30 mer 748-758:	5'cagagcacaCAGACCAGTTAaggctcttga 3'
30 mer 969-992:	5'ctaTGAACTCGGAGAGCTCAGCGAGTCgga3'
30 mer 1225-1248:	5'gcaCTGTCCGGTGCtGAGCCTGCACATggc 3'

SPA2 to *BNI1*:

30 mer *55*-85:	5' GGGTCGAGCTTagacctgggcgcaggcatt 3'
30 mer 1266-1296:	5' gtcgaaAGTGATGATATCAACAGaggatgg 3'
30 mer 4173-4203:	5'tcgggcTGCTTCTCCAGGATCATtggtgtc 3'
30 mer 4294-4324:	5' tcgcctTTTAATTTTGAGACTTCatcaggg 3'
30 mer 10018-10048:	5' tcgaatataAGCCGCACCGCCtcccactct 3'

Analysis of RNA Recruitment

We calculated a Recruitment Coefficient (RC) for RNAs added into preformed Whi3-*BNI1* droplets which is defined by the following equation:

$$\mathrm{RC} = \frac{[\mathrm{RNA}]_{\mathrm{PD}}}{[\mathrm{RNA}]_{\mathrm{O}}}$$

where RC is the recruitment coefficient, $[RNA]_{PD}$ is the concentration of added RNA (cy3) within the preformed droplets, $[RNA]_{O}$ is the concentration of added RNA (cy3) outside the preformed droplets. A higher RC indicates that the RNA is more concentrated

in preformed droplets. All image analysis was performed using Fiji. To obtain the concentration of RNA, image channels were split into preformed droplets (cy5) and added RNA (cy3). A mask was created on the preformed droplet channel after background subtraction. Droplets were identified using the 3D objects Counter plugin, with intensity threshold of 1480 for data acquired on the Yokogawa CSU-X and 100 for data acquired on the Nikon CSU-W1. This method was used to obtain the integrated density of the cy3 channel and the area of the preformed droplets. Average mean fluorescent intensity (MFI) of preformed droplets was then calculated as the sum of integrated density across the z-stack volume divided by sum of area across the z-stack volume. Then, per z-stack volume, inverse selection of preformed droplets is measured for integrated density and area. Average MFI outside preformed droplets calculated as above. MFI values are converted to dye concentration using linear fit of a concentration curve of cy3 dye (one for each instrument). RNA concentrations were obtained by normalizing the dye concentration by the average number of dye molecules incorporated into each different RNA. To calculate significance between different RNAs, unpaired t tests were performed on log-transformed RC values between experimental conditions in R version 3.4 (*21*).

Analysis of Whi3 to RNA ratios

Whi3-RNA droplets were imaged, as previously described, over the course of four hours, starting thirty minutes after the addition of the RNA to Whi3. Image analysis was performed in Fiji. For each time point, the RNA and Whi3 channels were split,

background subtracted, and 3D object counter was used to measure the MFI of both RNA and Whi3 channels separately. MFI values for RNA and Whi3 were then converted to concentrations based on a linear GFP or RNA dye curve. Whi3 concentrations were corrected for the unlabeled protein present. For each time point, the average concentration was calculated and then the ratio of Whi3 to RNA was calculated as the average Whi3 concentration divided by the average RNA concentration. The standard deviation of the ratio was calculated based on the following equation:

$$SD = \sqrt{\frac{Var(Whi3)}{Whi3^2} - 2 \times \frac{Whi3}{RNA^3} \times Cov(Whi3,RNA) + \frac{Whi3^2}{RNA^4} \times Var(RNA)}$$

where Whi3 is the concentration of Whi3, RNA is the concentration of RNA, Var(x) is the variance of variable x, and Cov (x, y) is the covariance of variables x and y.

SHAPE-MaP experiments and Analysis

SHAPE-MaP was performed as previously described (*15*). In brief, 500 ng of *in vitro* transcribed RNA was added to one-ninth volume of 1M7 or neat DMSO (10 mM final concentration) and incubated at room temperature for 10 minutes. RNAs were exchanged into TE buffer (10 mM Tris-HCl, 1 mM EDTA) (Ambion) using a desalting column (GE Life Sciences). For *CLN3*, *CLN3* refolded, *cln3 scr*, *cln3 sm*, *BNI1* and *SPA2* mRNAs, modified (1M7) and untreated (DMSO) RNAs were subjected to mutational profiling (MaP) reverse transcription with a random primer (200 ng/ ul R9, NEB). The resulting

cDNA was purified (Agencourt RNACLean XP beads, Beckman Coulter) and prepared for library construction by second strand synthesis module (NEB). These cDNAs were then used in amplicon library preparation using Nextera XT indices 1 and 2 and tagmentation and PCR protocol. *CLN3* that was modified with 1M7 or incubated with DMSO in the presence of Whi3 protein (2 uM Whi3, 27 nM *CLN3*), was heated to 95 °C to denature the proteins and RNA was recovered and purified with RNAClean XP beads. The RNAs were subjected to mutational profiling (MaP) reverse transcription with a gene specific primer (5' CGGCTGGGACTGGAAGC 3'; IDT) to amplify the first 500 nucleotides of the mRNA. Sequencing libraries were created with CLN3 and Whi3 + CLN3 amplicons using IDT gene specific primers F1 (5' GACTGGAGTTCAGACGTGTGCTCTTCCGATCT NNNNNGTCTGCATACCAAGGATCAGC 3') and R1 (5' CCCTACACGACGCTCTTCCGATCTNNNNNCGGCTGGGACTGGAAGC 3'). Libraries were purified using DNA beads (Agencourt) and sequencing libraries were pooled and then sequenced on an Illumina MiSeq instrument. SHAPE reactivity profiles were created by aligning the RNA reference sequence using ShapeMapper v 0.15 (www.chem.unc.edu/rna/software.html). Default parameters were used and the folding module was not used. RNA structural modeling was created by Superfold with 1M7 reactivities under default parameters and deltaSHAPE was calculated and plotted using deltaSHAPE software (www.chem.unc.edu/rna/software.html). RNA secondary structure arc plots were drawn with recently developed tools implemented in the Integrative Genomics Viewer (*22*).

Median read depths are reported in **Table S1**.

RNA	Modified read depth	Untreated read depth
CLN3	88,775	31,645
CLN3 refold	15,989	15,380
cln3 scr	38,418	32,906
cln3 sm	101,590	98,472
BNI1	27,147	38,362
SPA2	20,936	9,314
CLN3 (amplicon)	233,978	219,741
CLN3 + WHI3 (amplicon)	37, 236	128,531

Table S1. Median read depth for modified and untreated SHAPE-MaP experiments.

Single Molecule FRET experiments and Analysis.

A custom-built total internal reflection fluorescence (TIRF) microscope was used for all single molecule fluorescence assays. Lasers of different wavelengths were used to excite different fluorescent dyes. For FRET experiments involving Cy3 (donor) and Cy5 (acceptor) dyes, a solid state 532nm laser (75mW, Coherent CUBE) was used to excite the donor dye. For the counting of GFP photobleaching steps, a 488nm laser (50mW, Coherent Sapphire) was used. For FRET imaging, the emitted signals were separated by dichroic mirrors with a wavelength cutoff of 630nm to separate the Cy3 and Cy5 emissions. The signals were then detected by an EMCCD camera (iXon DU-897ECS0-#BV; Andor Technology). The camera was controlled via a custom C++ program, and single molecule traces extracted recorded data using IDL software. Single molecule traces were then displayed and analyzed using Matlab and Origin software. All code can be found in the single molecule FRET (smFRET) package available at the Center for the Physics of Living Cells (https://cplc.illinois.edu/software/, Biophysics Department, University of Illinois at Urbana-Champaign).

RNA substrate prep

Single strand RNAs were ordered from Integrated DNA Technologies (Coralville, IA, USA) containing amino modifier at the labeling site. The RNAs were labeled using Cy3/Cy5 monofunctional NHS esters (GE Healthcare, Princeton, NJ, USA). Then, 10 nmol of amino modified oligonucleotides in 50 ml of ddH_2O and 100 nmol of Cy3/Cy5 NHS ester dissolved in DMSO was added and incubated with rotation overnight at room temperature in the dark. The labeled oligonucleotides were purified by ethanol precipitation.

For FRET experiments, *CLN3* and *BNI1* FRET RNA partial duplex RNA substrates were composed of dsRNA consisting of 18 bps of random CG-rich sequences and ssRNA consisting of truncated versions of *CLN3* or *BNI1* RNA. A Cy5-Cy3 FRET pair was placed at the junction and the 3' end of the ssRNA, respectively (Fig. 4C).

The RNA sequences used are as follows.

18mer for *CLN3*: 5' – Biotin – UGG CGA CGG CAG CGA GGC – Cy5 – 3'

CLN3 RNA: 5' – Cy3 – UAC CUG CAC GCG GUC GAG ACG UCU GCA UAC CAA GGA UCA GCC GCU UGC AUU AAA GGG GAC GAA CCG GGG C GCC UCG CUG CCG UCG CCA – 3'

18mer for *BNI1*: 5' – Biotin – ACC GCU GCC GUC GCU CCG – Cy5 – 3'

RNA: 5' – Cy3 – AUA UUC UAC AUG AUU AUG AUG CAU UAG AGA AGG AAA ACG CCU ACU AUA AGU GUU UGA GAG UCC AUA UUC UAC AUG AU CGG AGC GAC GGC AGC GGU – 3'

RNA substrates were annealed by mixing the biotinylated and non-biotinylated oligonucleotides in a 1:2 molar ratio in T50 buffer (50mM NaCl, 10mM Tris-HCl (pH 8.0)). The final concentration of the mixture was 10uM. The mixture was then incubated at 95°C for 2 minutes followed by slow cooling to room temperature to complete the annealing reaction in just under two hours. The annealed RNAs were diluted to 10nM single molecule stock concentration and stored at -20°C.

All experiments were performed in Whi3 Reaction Buffer (150mM KCl, 50mM Tris-HCl (pH 8.0), 1mM DTT) with an oxygen scavenging system containing 0.8% v/v dextrose, 1 mg/ml glucose oxidase, 0.03 mg/ml catalase[1], and 10mM Trolox. All chemicals were purchased from Sigma Aldrich (St. Louis, MO). The experiments were all performed at room temperature. Fifty to 100pM of biotinylated FRET RNA were immobilized on polyethylene glycol (PEG)-coated quartz surface via biotin-neutravidin linkage.

Whi3-RNA FRET experiment

Whi3 proteins were prepared as described above. Then, 0.5 to 5uM of Whi3 or whi3-Y610-F653A proteins in droplet buffer was added to immobilized RNA substrates, and 10-20 short movies (10 seconds) and 3-4 long movies (2 minutes) were then taken to

monitor the Cy3 and Cy5 emission intensities over time. These are then analyzed to produce the FRET histograms and trajectories shown in Fig. 4D.

Dip-to-dip FRET fluctuation dwell times were collected between each successive dip in the FRET trace. These time intervals were then fitted to a single exponential decay function using Origin (OriginLab Corporation, Northampton, MA) to extract the half-life for each protein-RNA combination.

Fig. S1. Whi3, a polyQ-containing protein, forms LLPS droplets in vitro.

A. Cartoon schematic of Whi3 protein sequence, in *A. gossypii*, depicting the disordered (polyQ, blue) and RNA binding domains (RRM, red).

B. Whi3-*BNI1* droplets (green) incorporate more Whi3 protein over time *in vitro* compared to Whi3-*CLN3* droplets (pink). Data are mean ± SD. n ≥ 100 droplets for N= 2 biological replicates.

C. Fluorescence microscopy images showing *CLN3* (pink*)* is excluded from Whi3-*BNI1* (green) droplets in a variety of protein and RNA concentrations. Top, Whi3 2 μM, RNA 1.25 nM; Bottom, Whi3 20 μM, RNA 5 nM. Scale bar is 10 μm.

D. Fluorescence microscopy images show cy3 labeled *CLN3* mRNA (pink) is not efficiently recruited into Whi3-*SPA2* droplets (green) (8 μM Whi3, 5 nM RNA) Images are representative of observations from three independent experiments. Scale bar is 10 μm.

E. Fluorescence microscopy images showing the recruitment of *cln3 scrambled* mRNA (5 nM, pink) into droplets with Whi3 (8 μM, green). Scale bar is 10 μm.

Supplemental Figure 1 Langdon et al.

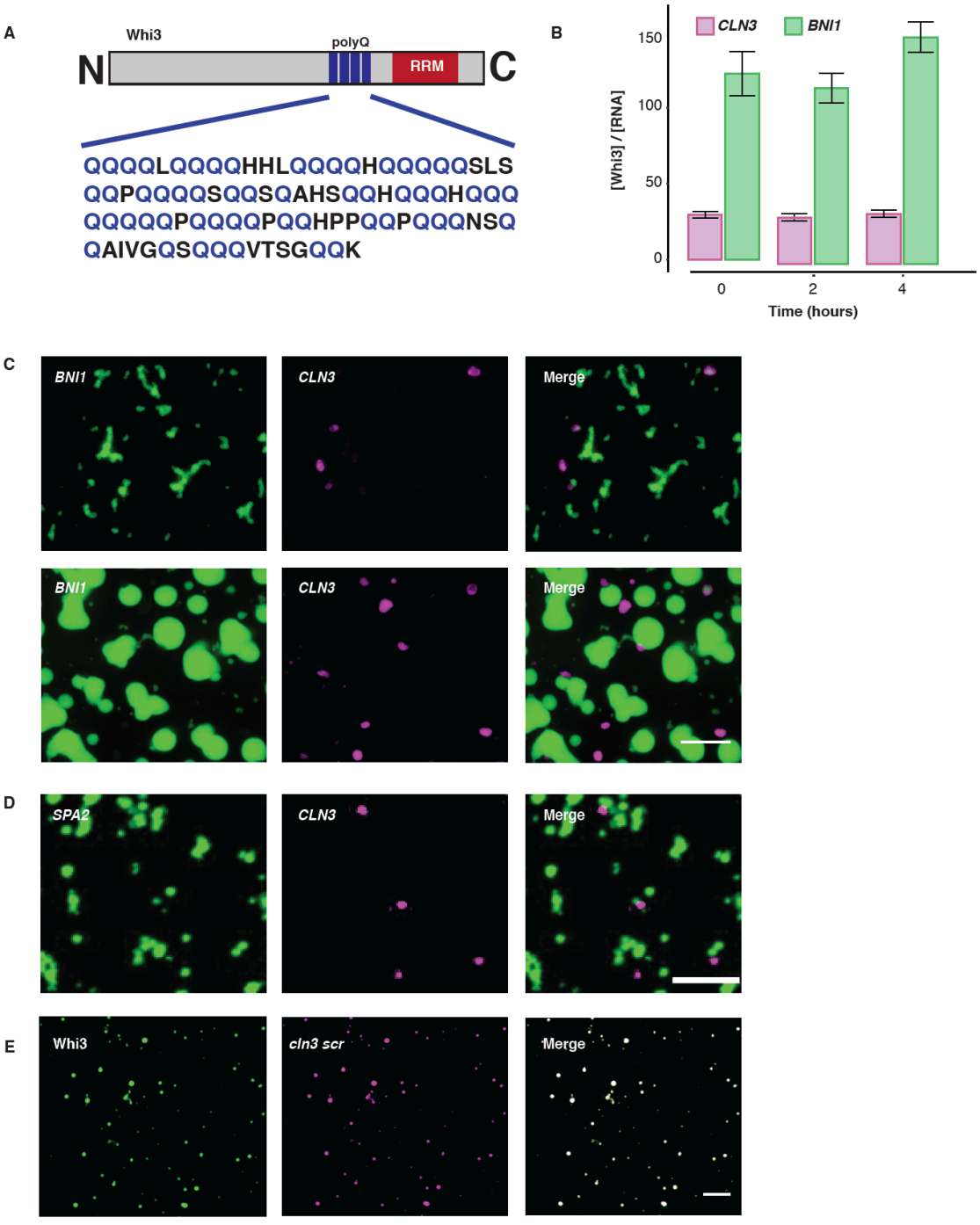


Fig. S2. *CLN3* and *BNI1* co-assemble when regions of complementarity between the two RNAs are exposed through melting.

A. Fluorescence microscopy images and recruitment coefficients show *CLN3* mRNA (5 nM, pink) is modestly recruited into preformed Whi3 droplets made with melted *BNI1* (8 μM Whi3, 5 nM *BNI1*, green) unless complement regions of *CLN3* are also exposed through melting (*CLN3* ml). In contrast, recruitment of *CLN3* ml is lost when the mRNA is mixed with oligos targeting the sequence on *CLN3* with complementarity to *BNI1* mRNA (*CLN3* ml + oligos). Outliers are indicated with (•) marks. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (unpaired t test). $n \geq 500$ droplets for $N \geq 3$ biological replicates.

B. Fluorescence microscopy images and quantification (Pearson's correlation coefficient) show *CLN3* (10 nM, pink) RNA only droplets co-localize with *BNI1* (10 nM, green) droplets only when melted (*CLN3* ml) and recruitment is lost when the mRNA is mixed with oligos targeting the sequence on *CLN3* with complementarity to *BNI1* mRNA (*CLN3* ml + oligos) Outliers are indicated with (•) marks. *, $p<0.05$, **, $p < 0.01$; ***, $p < 0.001$ as determined by Wilcoxon rank-sum test. $n \geq 200$ droplets for $N \geq 3$ biological replicates.

Supplemental Figure 2 Langdon et al.

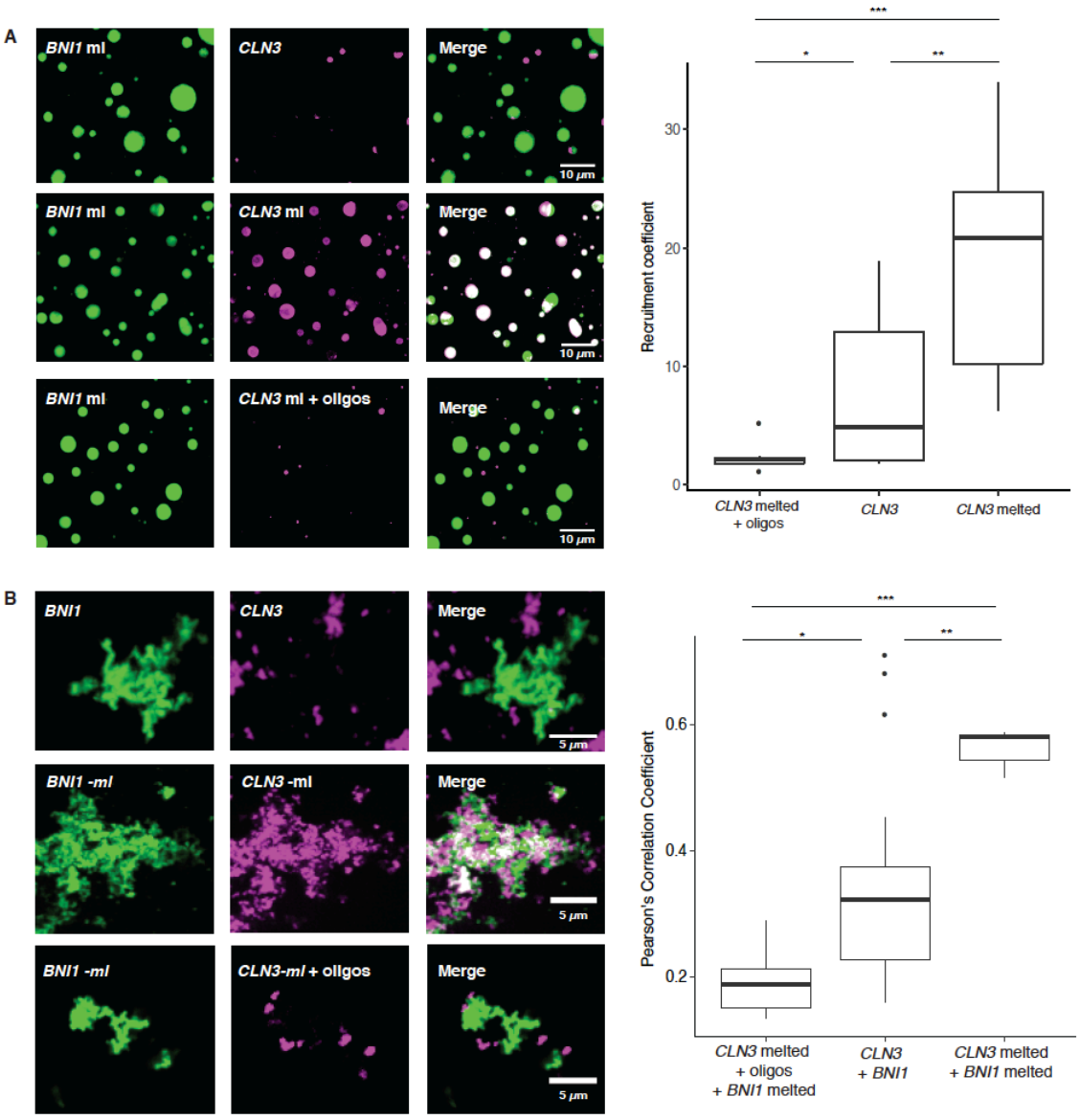


Fig. S3. *CLN3* mRNA secondary structure is altered when the mRNA sequence is scrambled or refolded.

A. SHAPE reactivity profiles for *CLN3* and *CLN3* refolded. High values indicate unstructured regions or many possible structures, while low values suggest a single well-defined structure. Data are mean ± SD

B. SHAPE reactivity profiles for *cln3 scr* indicates it has a completely different secondary structure than *CLN3* native sequence. Data are mean ± SD.

C. SHAPE reactivity and Shannon entropy values for the first 400 nucleotides of the RNAs shown as the median reactivity smoothed over a 19-nt sliding window relative to the global median. Reactivity values for *CLN3* in this region are significantly lower than reactivity values for *CLN3* refolded (median of 0.19 vs 0.32 respectively; Wilcoxon rank sum test, $p = 0.001$), suggesting that the *CLN3* refolded mRNA contains less well-defined structures. Purple shaded regions show
low-SHAPE/low-entropy regions. These regions transition to high-SHAPE/high-entropy regions when the *CLN3* sequence is melted and refolded.

Supplemental Figure 3 Langdon et al.

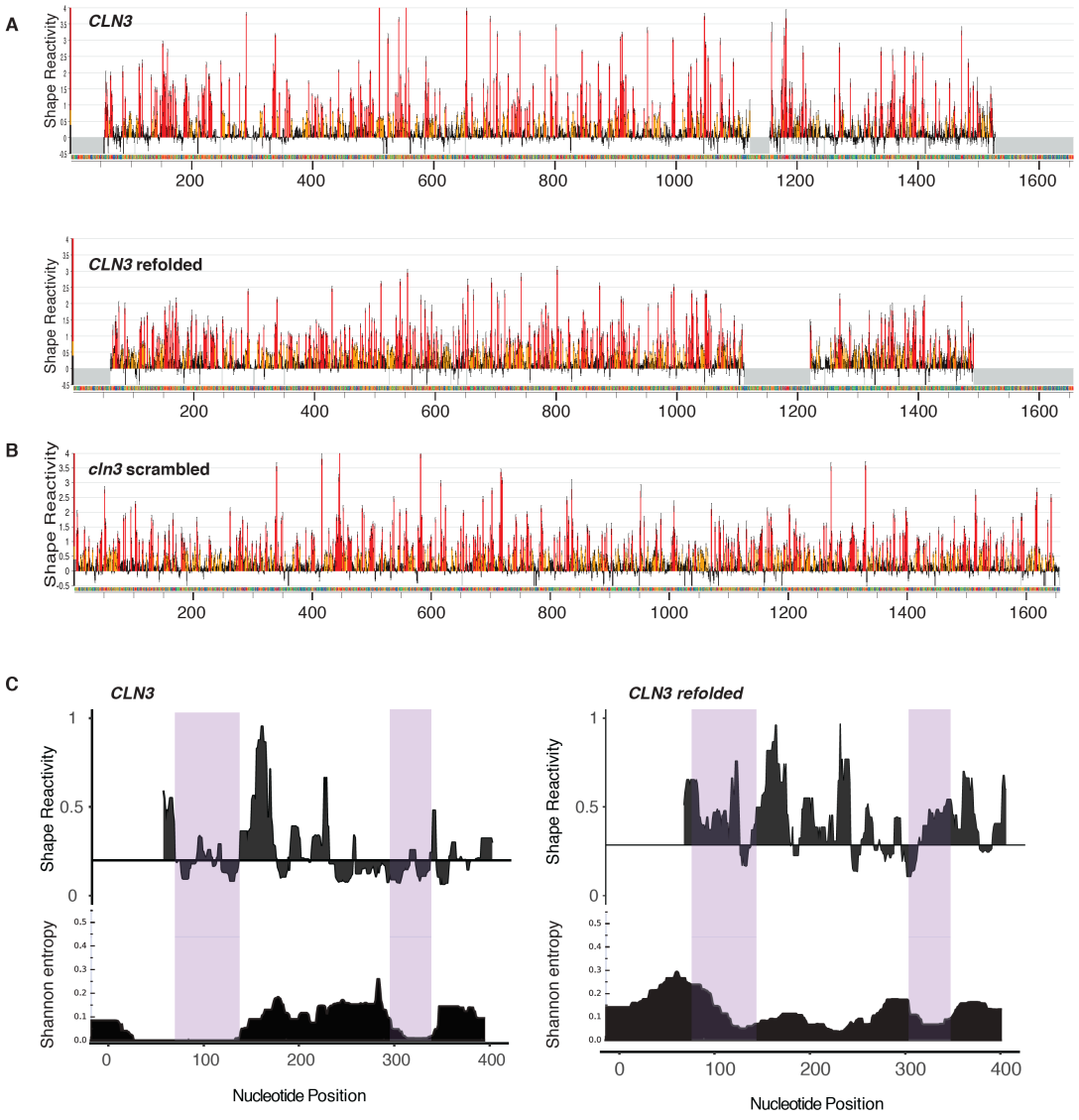


Fig. S4. Regions of Complementarity between *CLN3* and *BNI1*.

A. Cartoon schematic of *CLN3* and *BNI1* RNA depicting the location of Whi3 binding sites (orange) and the regions of complementarity (numbered) between the two RNAs. RNAs are drawn to scale.

B. Shape reactivity and arc plots for the five regions along *CLN3* that can base-pair with *BNI1* and regions on *BNI1* that can base pair with *CLN3.* The blue shaded regions indicate the exact complement sequence. Most of these regions on both *CLN3* and *BNI1* are located in low SHAPE, high structured regions, indicating that these regions are likely not exposed and unable to interact when properly folded. The median SHAPE reactivity per region is displayed above its respective region. Data are mean ± SD.

Supplemental Figure 4 Langdon et al.

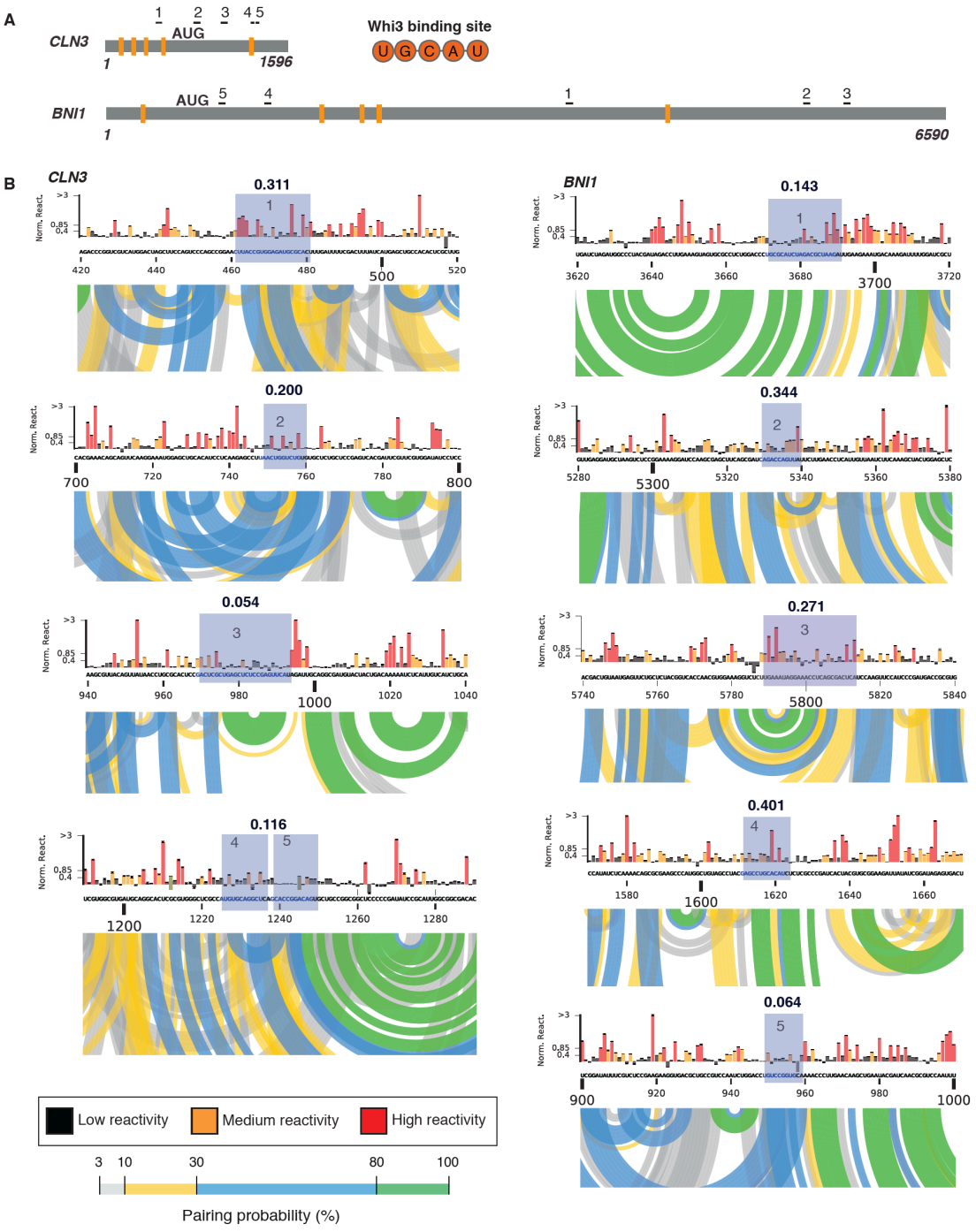


Fig. S5. *CLN3* mRNA secondary structure is altered with point mutations to target stem loops.

A. Shape reactivity profiles for *CLN3* structure mutant *(cln3 sm)*. High values indicate unstructured regions or many possible structures, while low values suggest a single well-defined structure. Data are mean ± SD.

B. Base pairing probability compared among *CLN3* and *cln3 sm* show differences in the secondary structure between the two mRNAs. Arcs connect base pairs and are color coded by probability.

C. Secondary structure models for the first 400 nucleotides of *CLN3* and *cln3 sm*. Whi3 binding sites are denoted in orange. The sequence targeted for mutation in *cln3 sm* is denoted in purple.

Supplemental Figure 5 Langdon et al.

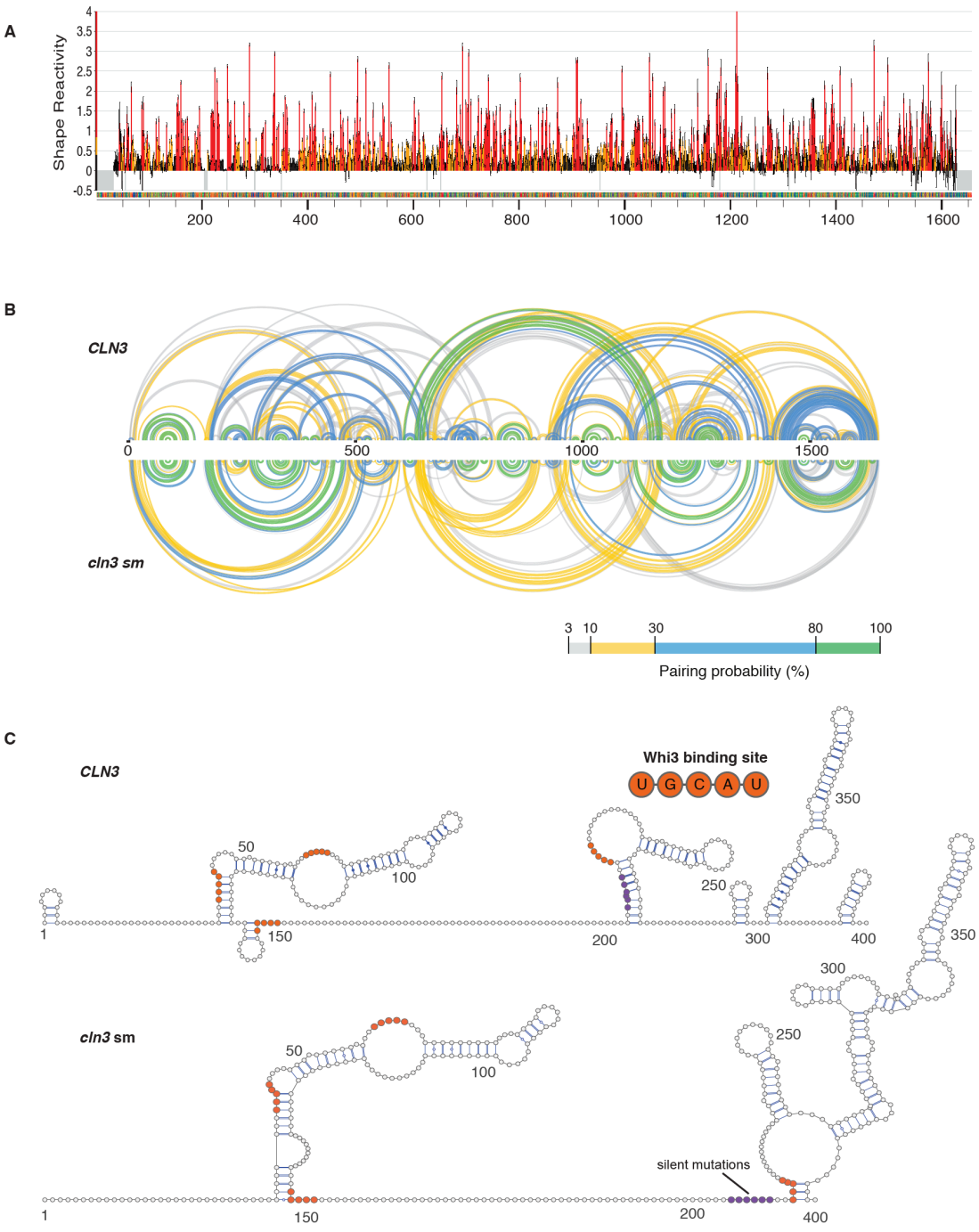


Fig. S6. Regions of complementarity between *BNI1* and *SPA2*.

A. Cartoon schematic of *BNI1* and *SPA2* RNA depicting the location of Whi3 binding sites (orange) and the regions of complementarity between the two RNAs. RNAs are drawn to scale.

B. Shape reactivity and arc plots for the five regions along *BNI1* that can base-pair with *SPA2* and regions on *SPA2* that can base pair with *BNI1*. The blue shaded regions indicate the exact complement sequence. Most of these regions are located in high SHAPE, low structured regions, indicating that these regions are likely exposed and able to interact. The median SHAPE reactivity per region is displayed above its respective region. Data are mean ± SD.

Supplemental Figure 6 Langdon et al.

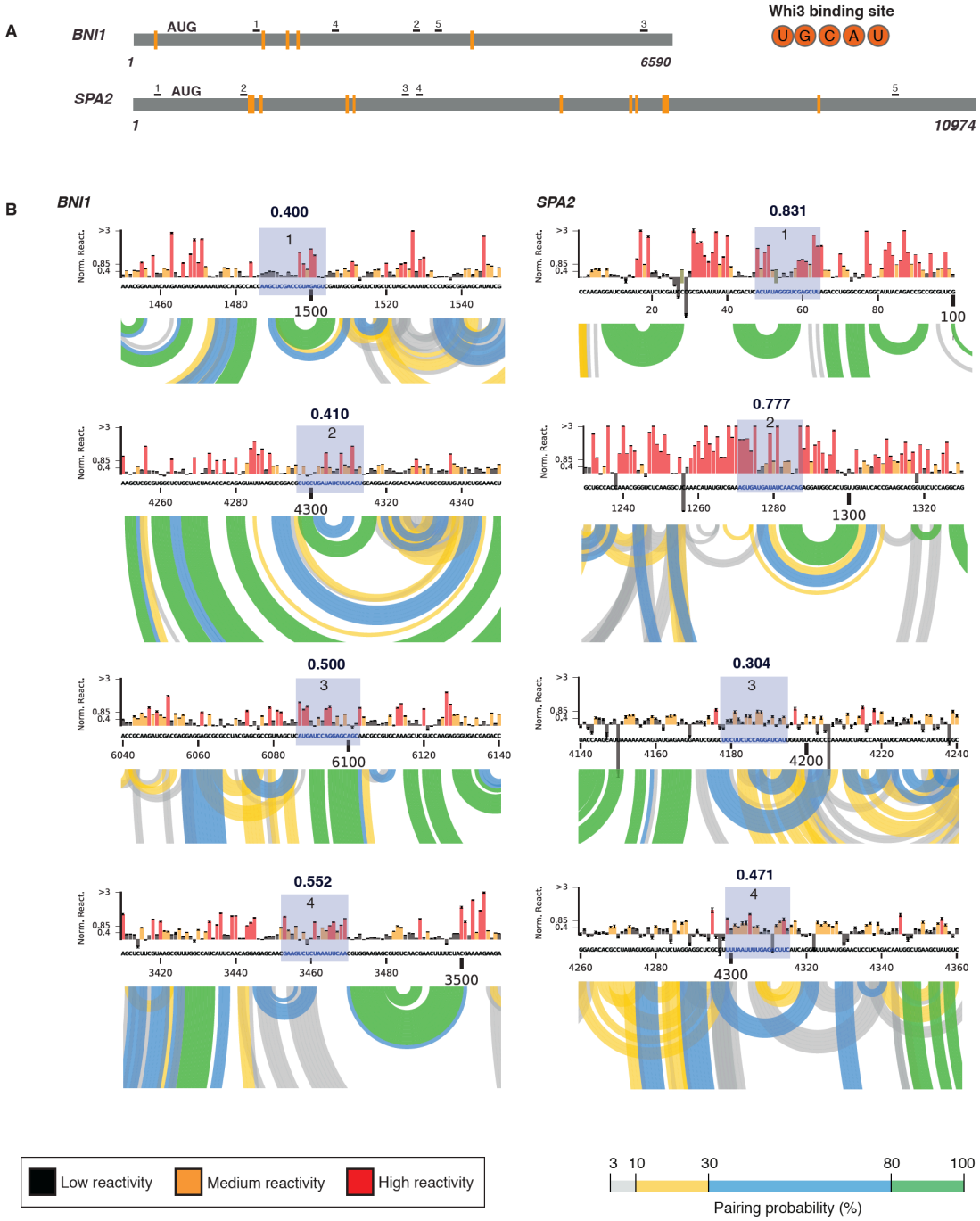


Fig. S7. Disruption of regions of complementarity interferes with co-assembly between two RNAs.

A. Fluorescence microscopy images and quantification show *SPA2* mRNA incubated with oligos to target the complement nucleotide regions of *BNI1* (5 nM, pink) is less recruited into preformed Whi3-*BNI1* droplets (8 μM Whi3, 5 nM *BNI1*, green) compared to native *SPA2* alone. The line at the center of the box indicates the median value, and boxes indicate interquartile range. Whiskers contain data points within 3 times the interquartile range, and outliers are indicated with (•) marks. *, $p < 0.05$ (unpaired t test). Scale bar is 10 μm. n≥500 droplets for N≥ 3 biological replicates.

B. Fluorescence microscopy images and quantification (Pearson's correlation coefficient) showing *SPA2* mRNA (10 nM, pink) incubated with oligos to target the complement nucleotide regions to *BNI1* (5 nM, pink) are excluded from *BNI1* mRNA (10 nM, green) in the presence of Spermine. ***, $p < 0.001$ (Wilcoxon rank-sum test). Scale bar is 5 μm. n≥200 droplets for N≥ 3 biological replicates.

C. Single molecule mRNA FISH images show that a codon randomized *CLN3* (*cln3 codon*, green) mRNA does not co-localize with endogenous *CLN3* mRNA (pink) *in vivo*. Scale bar is 5 μm.

Supplemental Figure 7 Langdon et al.

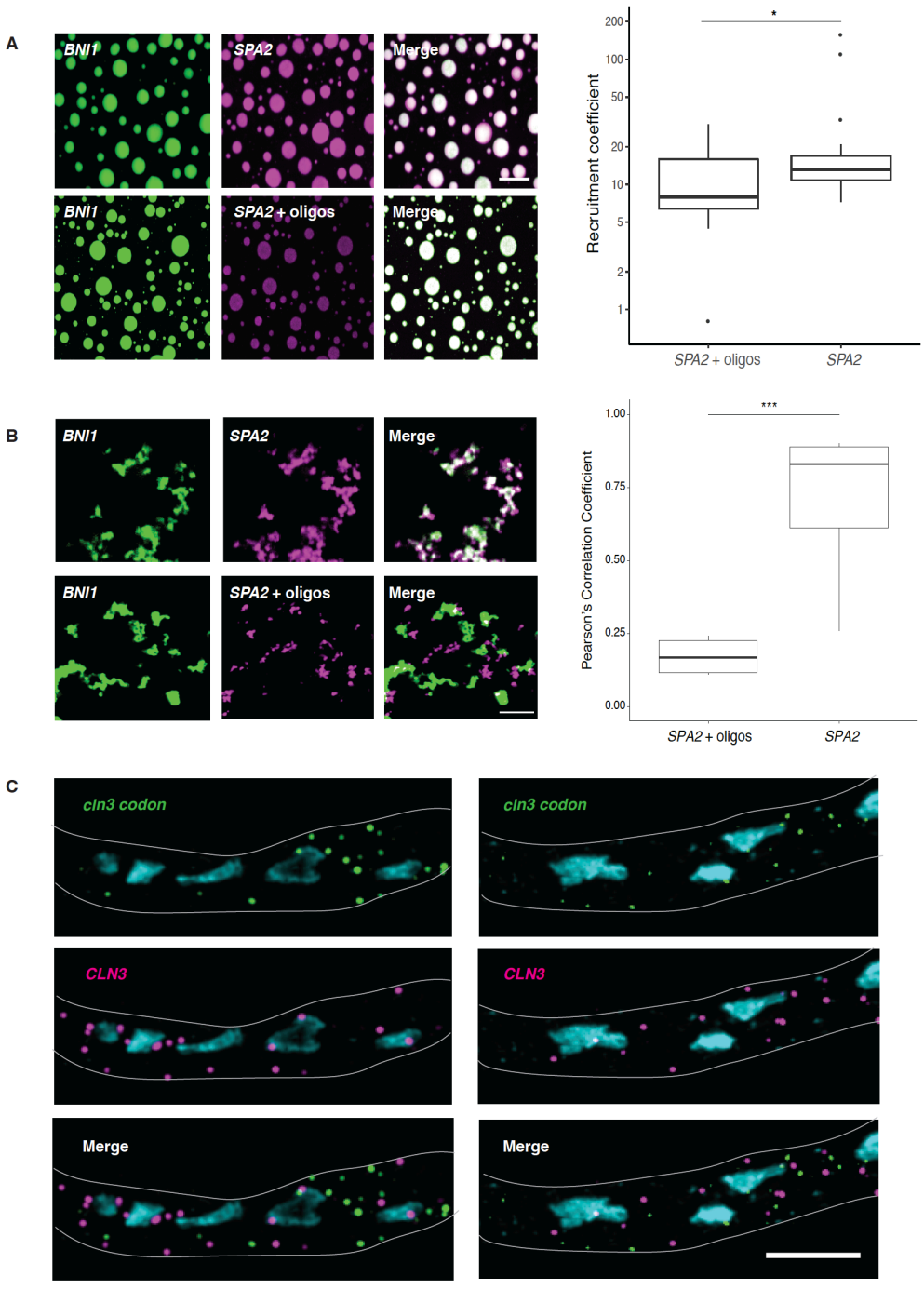


Fig. S8. Whi3 binding sites are located in highly structured region along *BNI1* mRNA.

A. Cartoon schematic of *BNI1* RNA depicting the location of the five Whi3 binding sites (orange). RNA is drawn to scale.

B. Secondary structure plots show regions of *BNI1* mRNA surrounding Whi3 binding sites

Supplemental Figure 8 Langdon et al.

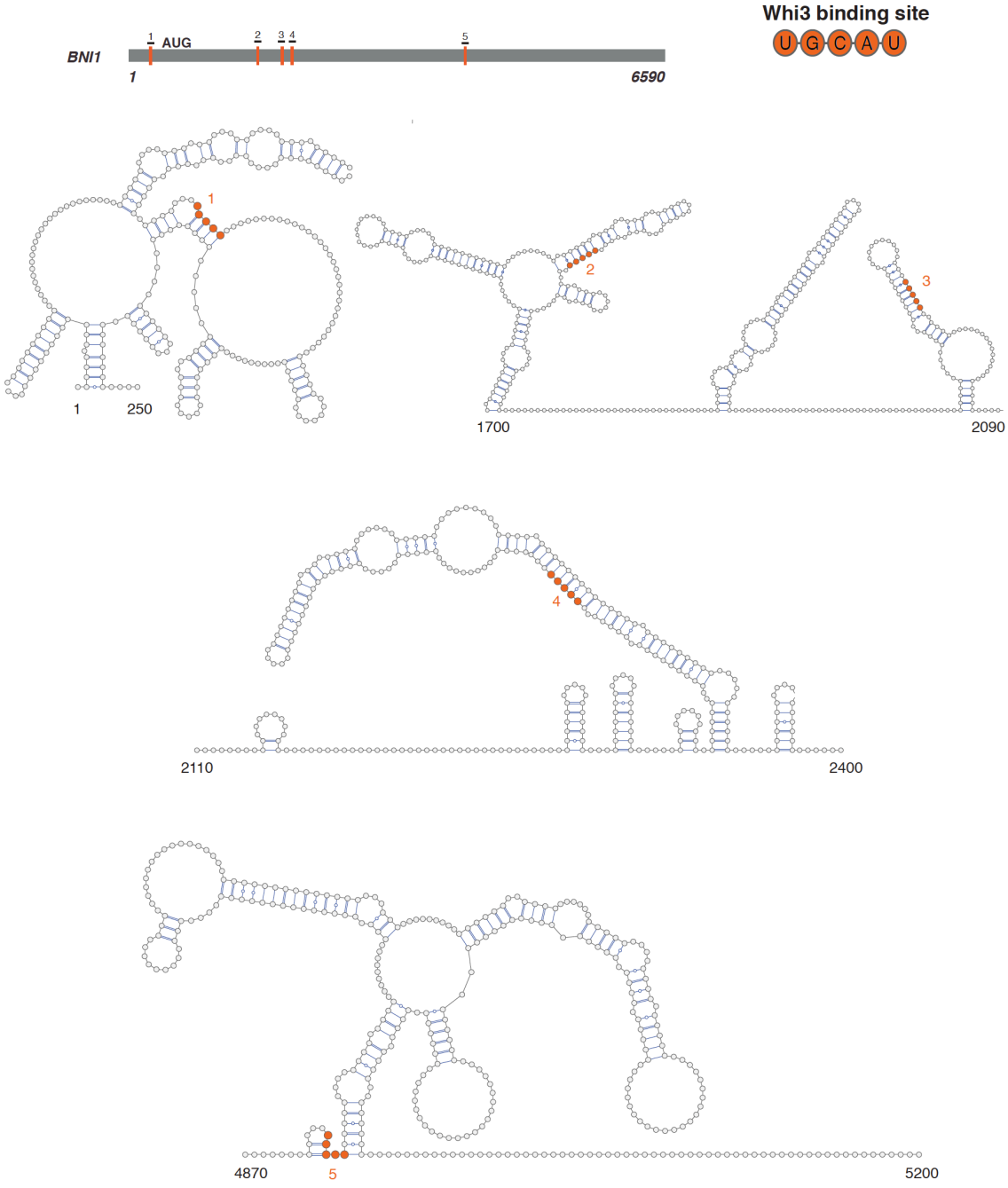


Fig. S9. The majority of Whi3 binding sites are located in highly structured region along *SPA2* mRNA.

A. Cartoon schematic of *SPA2* RNA depicting the location of the eleven Whi3 binding sites (orange). RNA is drawn to scale.

B. Secondary structure plots show regions of *SPA2* mRNA surrounding Whi3 binding sites.

Supplemental Figure 9 Langdon et al.

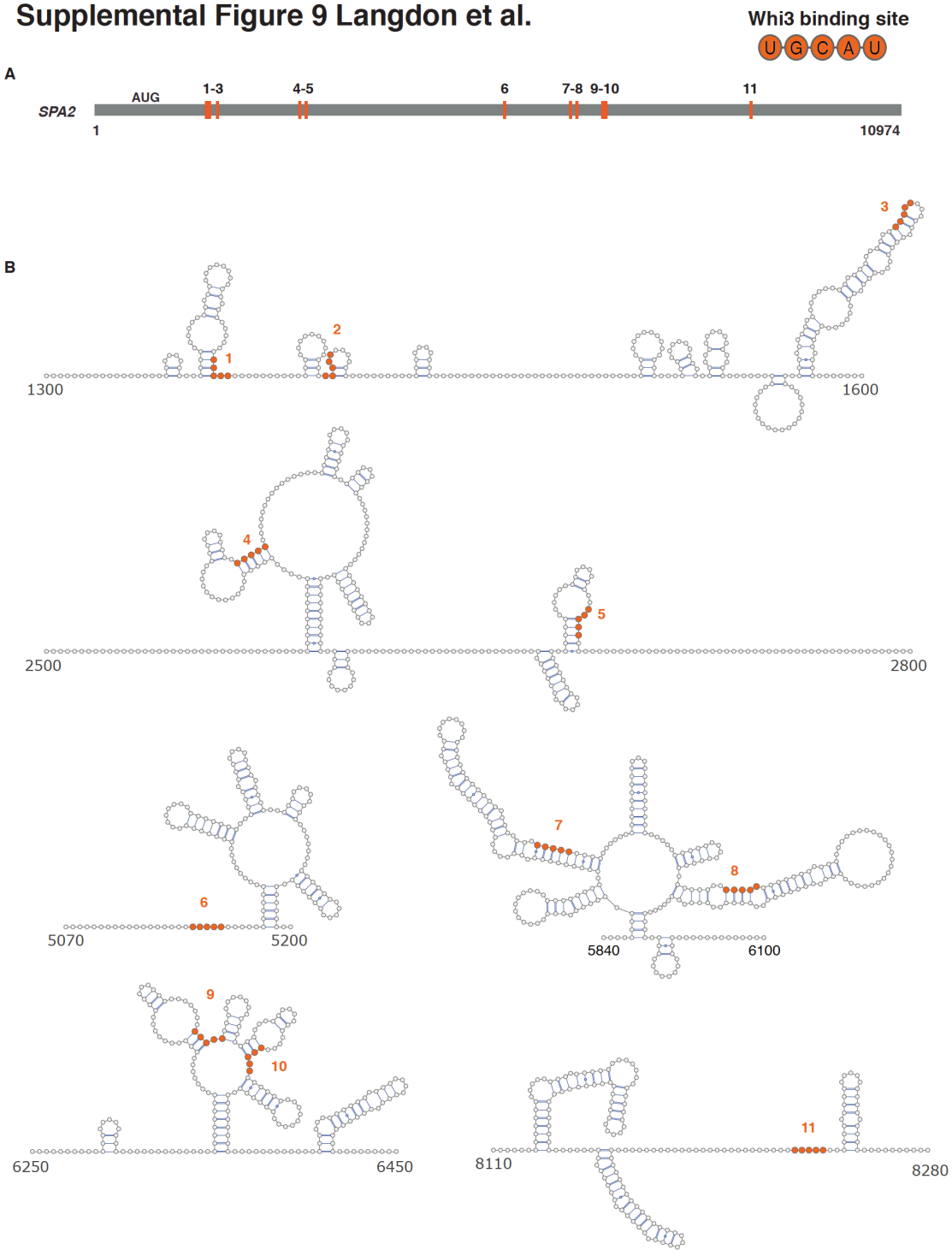


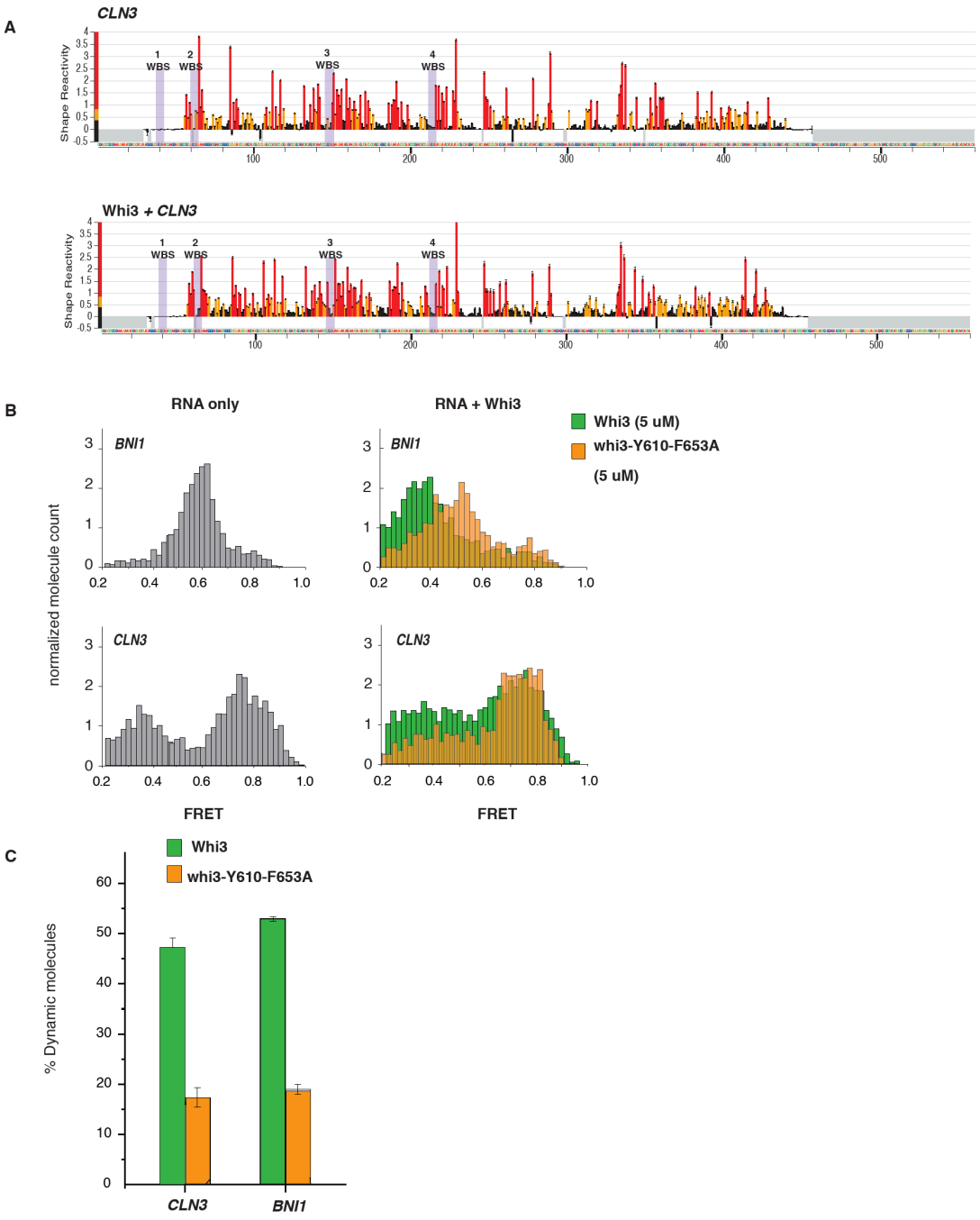
Fig. S10. Whi3 binding is essential to induce dynamic fluctuations to its target mRNAs, *CLN3* and *BNI1*.

A. Shape reactivity profiles for *CLN3* and *CLN3* in the presence of Whi3 protein. High values indicate unstructured regions and many possible structures, while low values suggest a single well-defined structure. The four Whi3 binding sites (WBS) are shaded in purple and show the differences in SHAPE reactivity along *CLN3* in the presence or absence of Whi3. Data are mean ± SD.

B. FRET histograms show RNA only (gray) or addition of Whi3 (green) or a Whi3 RNA binding mutant (whi3-Y610-F653A, orange).

C. The mutant exhibits much lower dynamics with both *CLN3* and *BNI1* indicating that Whi3 binding is needed to induce dynamic fluctuations to the two RNAs.

Supplemental Figure 10 Langdon et al.



Movie S1. Whi3-droplets in *Ashbya* cells exhibit dynamic movement and fusion events around nuclei and at tips.

References and Notes:

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22. S. Busan and K.M. Weeks, Accurate detection of chemical modifications in RNA by mutational profiling (MaP) with Shapemapper 2. *RNA* 23 (2017).