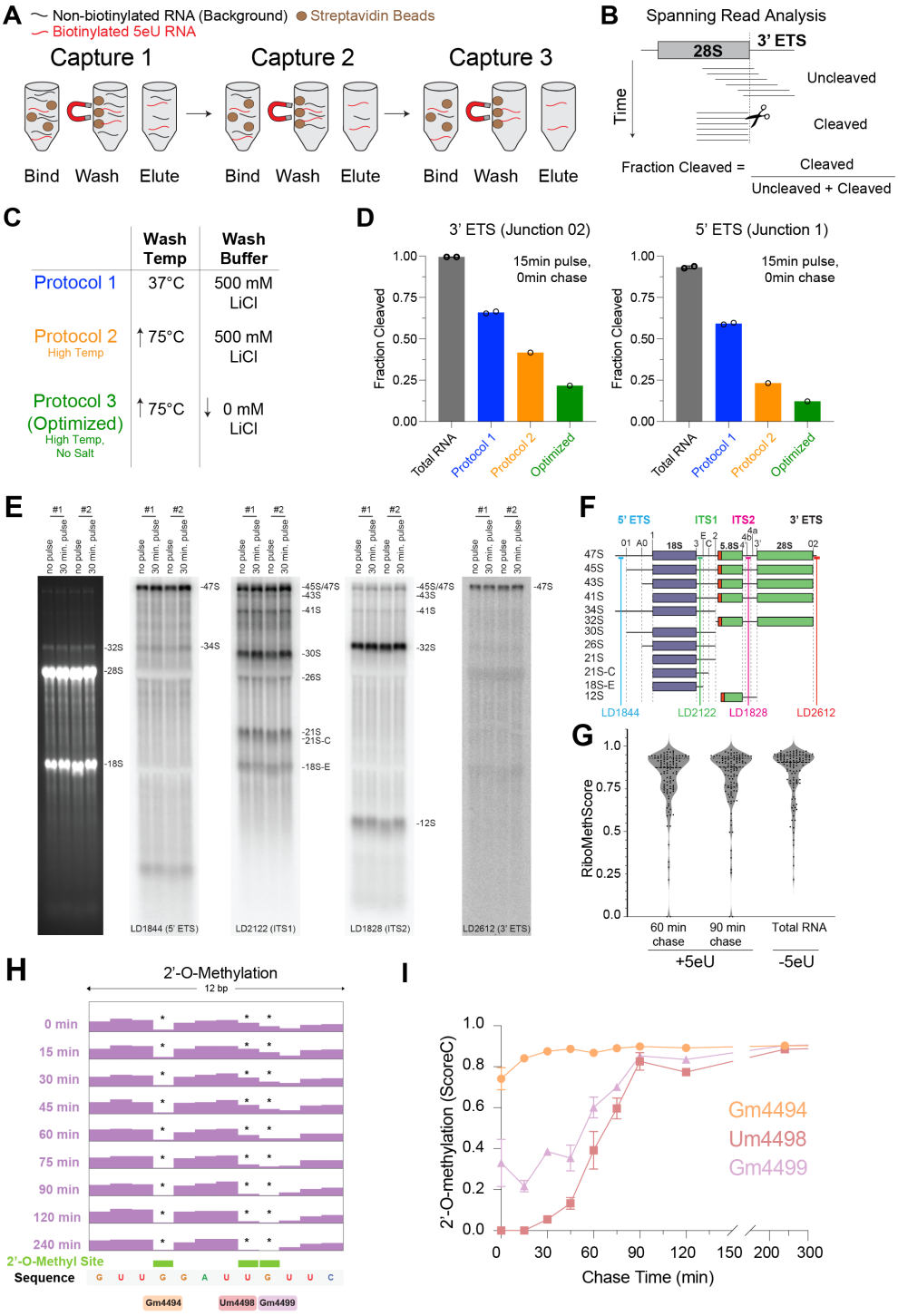
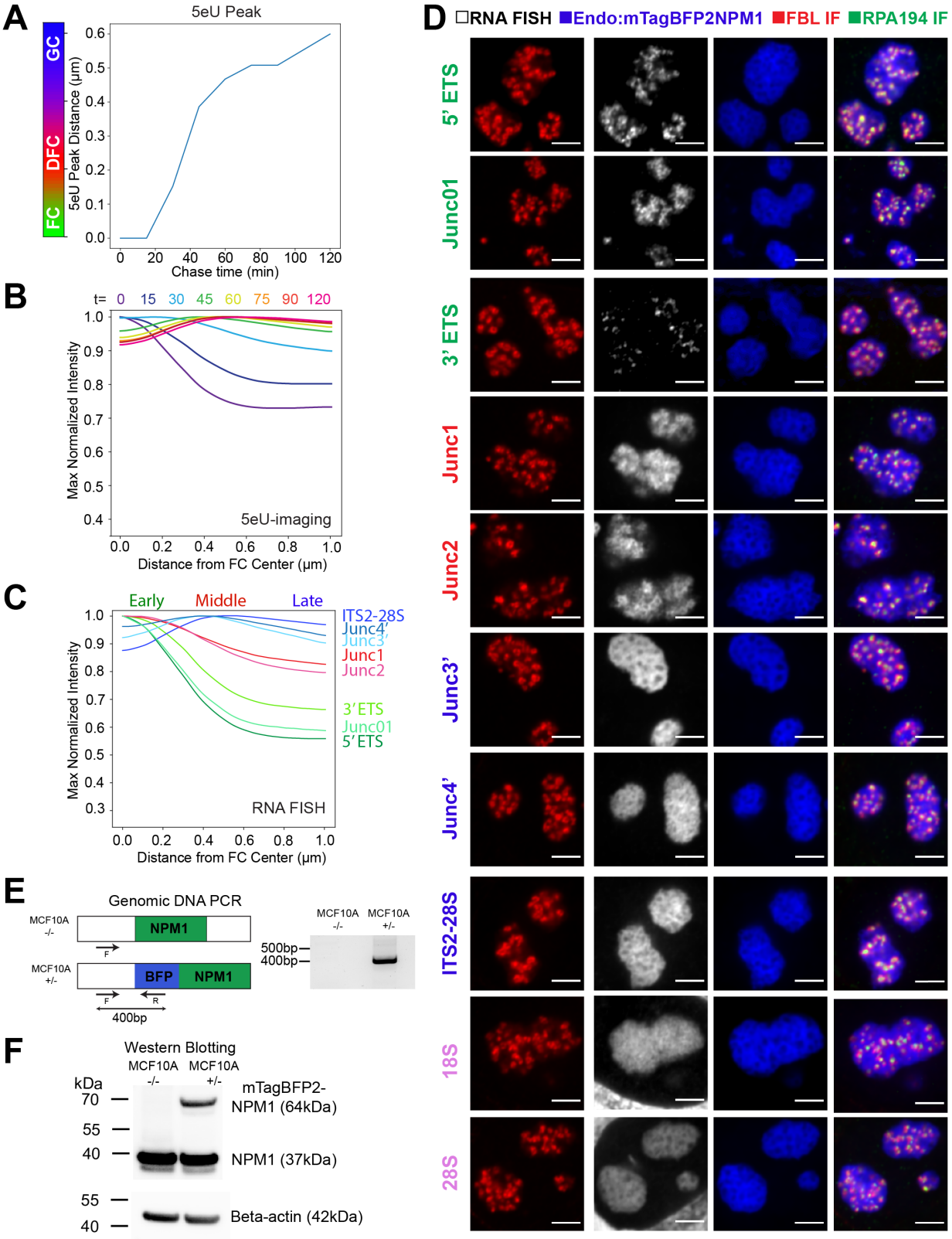
Supplementary Figure 1: 5eU-seq method description and validation.



(A) 5eU-sequencing protocol is performed using 3 rounds of sequential captures, where biotinylated 5eU-labeled RNA is captured to magnetic streptavidin beads, washed, and eluted, as described previously[33].
(B) The "fraction cleaved" metric is calculated by measuring the number of "cleaved" reads, which end exactly at a cleavage junction (dashed line), divided by the total number of reads at the same position (uncleaved + cleaved). "Uncleaved" reads are reads that span a cleavage junction.

(C) The 5eU-seq protocol[33] (Protocol 1), which performs washes at 37° C and with 500 mM LiCl, was further optimized to reduce background from highly abundant mature rRNA, which causes the fraction cleaved metric to appear artificially high. We found that both higher temperature washes at 75° C (Protocol 2) and washing in a buffer lacking salt (0 mM LiCl) significantly reduces background (Protocol 3, Optimized).
(D) The fraction of reads cleaved at Junctions 02 and 1. 5eU pulse-labeled material (15 min pulse, 0 min chase) from HEK293T cells was captured with streptavidin beads and washed under the Protocols 1, 2, or 3 conditions. Protocol 3 is the optimized protocol used for all datasets in this paper. Total RNA is provided as a reference for background from mature rRNA.
(E) Northern blot analysis of 5eU pulse-labeled material (30 min) and unlabelled material as a control (No pulse). Two replicates were performed.
(F) Northern blot probes used in E.
(G) RiboMethScore of 2'-*O*-Methylation for 5eU 30 min pulse labeled material (+5eU, 60 or 90 min chase) and unlabelled material as a control (-5eU).
(H) Zoom-in on 5' end read counts of 15 min 5eU pulse-labeled material from MCF10A cells over 0-240 min chase timepoints in a region on 28S rRNA showing characteristic dips at fast (Gm4494) and slow (Um4498, Gm4499) 2'-*O*-methylation sites.
(I) Quantification of 2'-*O*-methylation levels (ScoreC) over different chase timepoints at 28S Gm4494, Um4498, Gm4499 2'-*O*-methylation sites shown in h. Error bars are s.e.m.

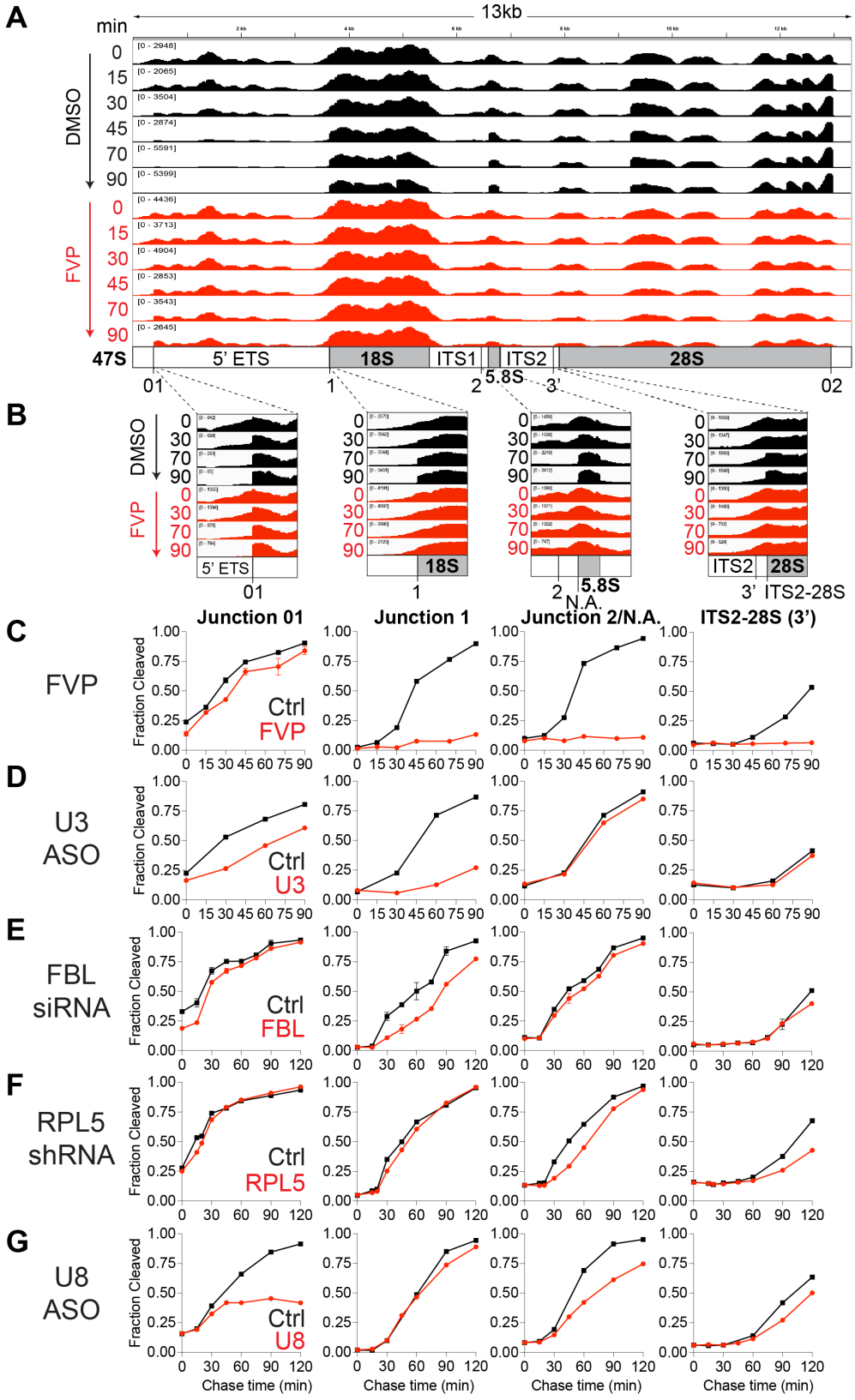
Supplementary Figure 2: RNA FISH and 5eU-imaging of rRNA flux as well as validation of endogenously tagged mTagBFP2-NPM1 cells.



(A) Peak of 5eU signal (distance from FC center) over chase time quantified for images in Figure 1D.

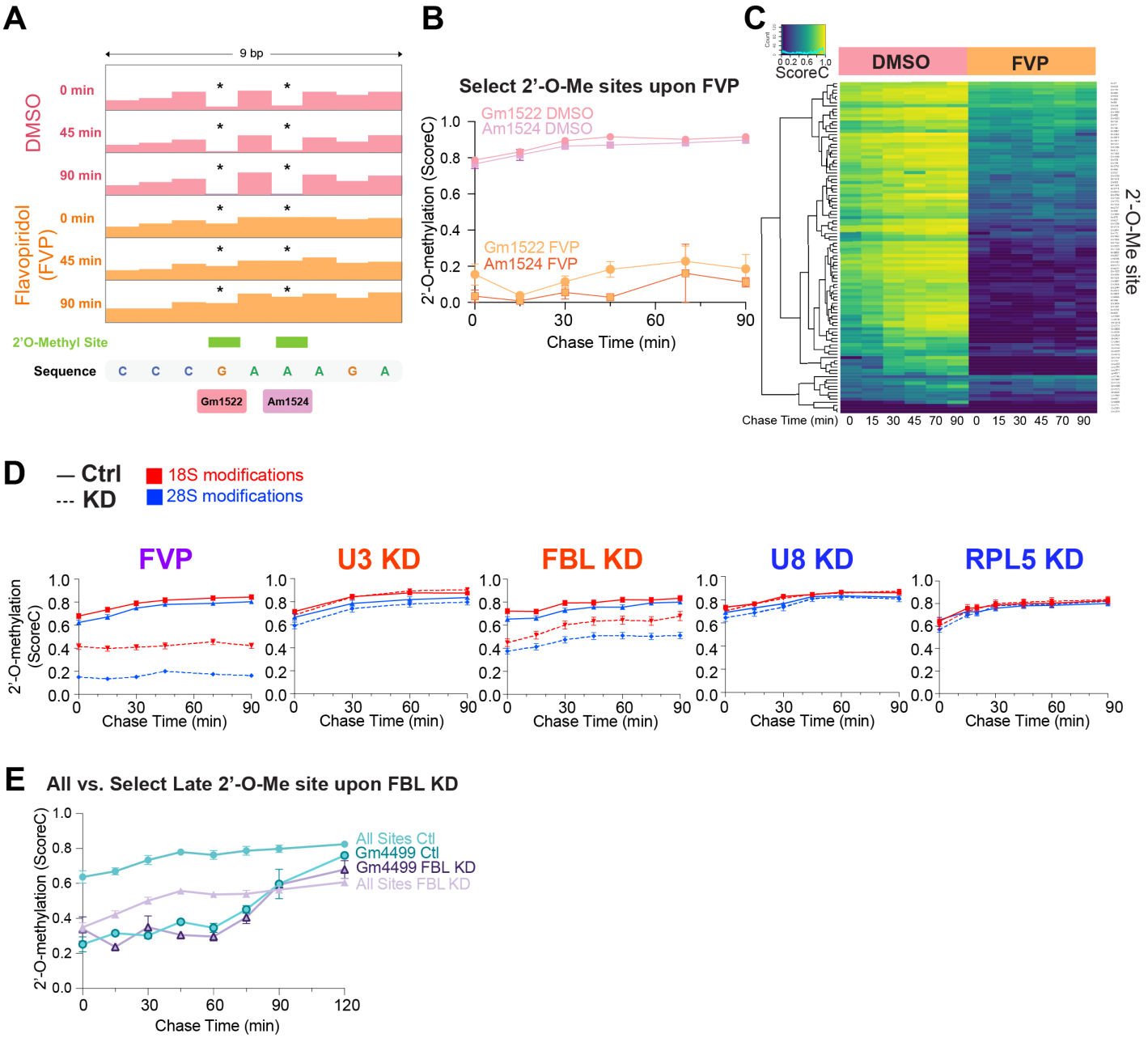
(B) Max normalized 5eU intensity over distance from FC center over time, quantified from images in Figure 1D.
(C) Max normalized FISH intensity over distance from FC center, quantified from images in D.
(D) Example images of RNA FISH probes from Figure 1I-J, with FC (RPA194 IF), DFC (FBL IF), and GC (mTagBFP2-NPM1) shown. Scale bar = 3 μm.
(E) Junction PCR of 400bp region of genomic locus spanning the inserted mTagBFP2 in MCF10A -/- (parental) and MCF10A +/- (one copy of NPM1 tagged with mTagBFP2-NPM1) cells.
(F) Western blot for NPM1 in MCF10A -/- and MCF10A +/- cells with beta-actin as loading control.

Supplementary Figure 3: Example 5eU-seq reads and altered pre-rRNA cleavage kinetics measured by 5eU-seq upon all perturbations.



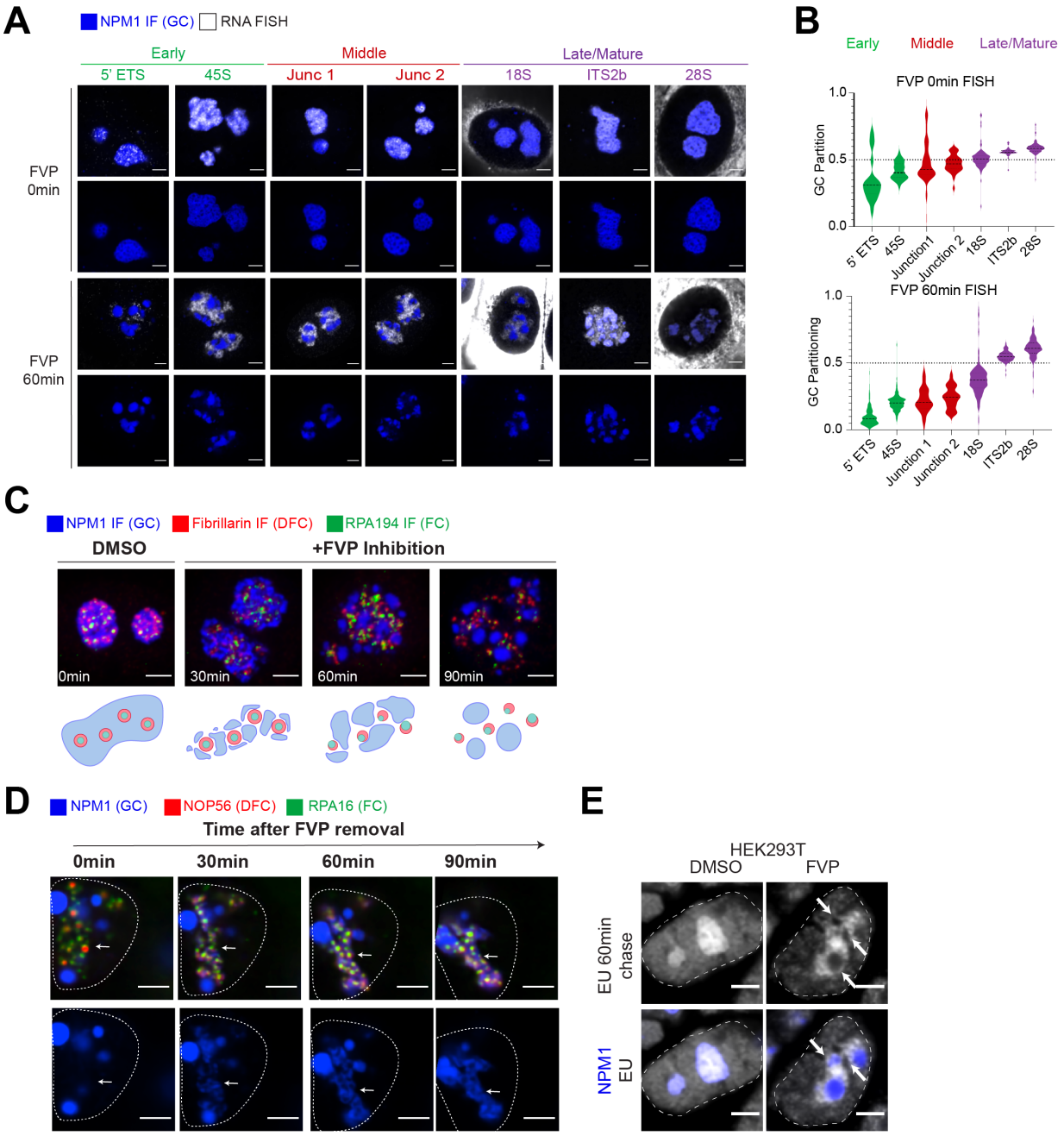
(A) 5eU-seq reads over 47S pre-rRNA for 15 min pulse labeled material over 0-90 min chase timepoints in DMSO-treated (black) and FVP-treated (red) MCF10A cells. FVP-treated cells were pretreated with 2 μM FVP for 1 hr prior to 5eU pulse-chase and throughout the time course.
(B) Zoom-in examples of 5eU-seq reads at 01, 1, 2/NA, and 3'/ITS2-28S regions in A.
(C-G) Quantification of the fraction of reads cleaved at each cleavage junction displayed in B for MCF10A cells upon multiple perturbations to rRNA processing (red): FVP-treatment or knockdown of U3 snoRNA (U3 ASO), Fibrillarin (FBL siRNA), RPL5 (RPL5 shRNA), and U8 snoRNA (U8 ASO) compared to their respective controls (black; DMSO for FVP, or scramble control for ASO, siRNA and shRNA treatments). All error bars are s.e.m.

Supplementary Figure 4: Altered pre-rRNA modification kinetics measured by 5eU-seq upon all perturbations.



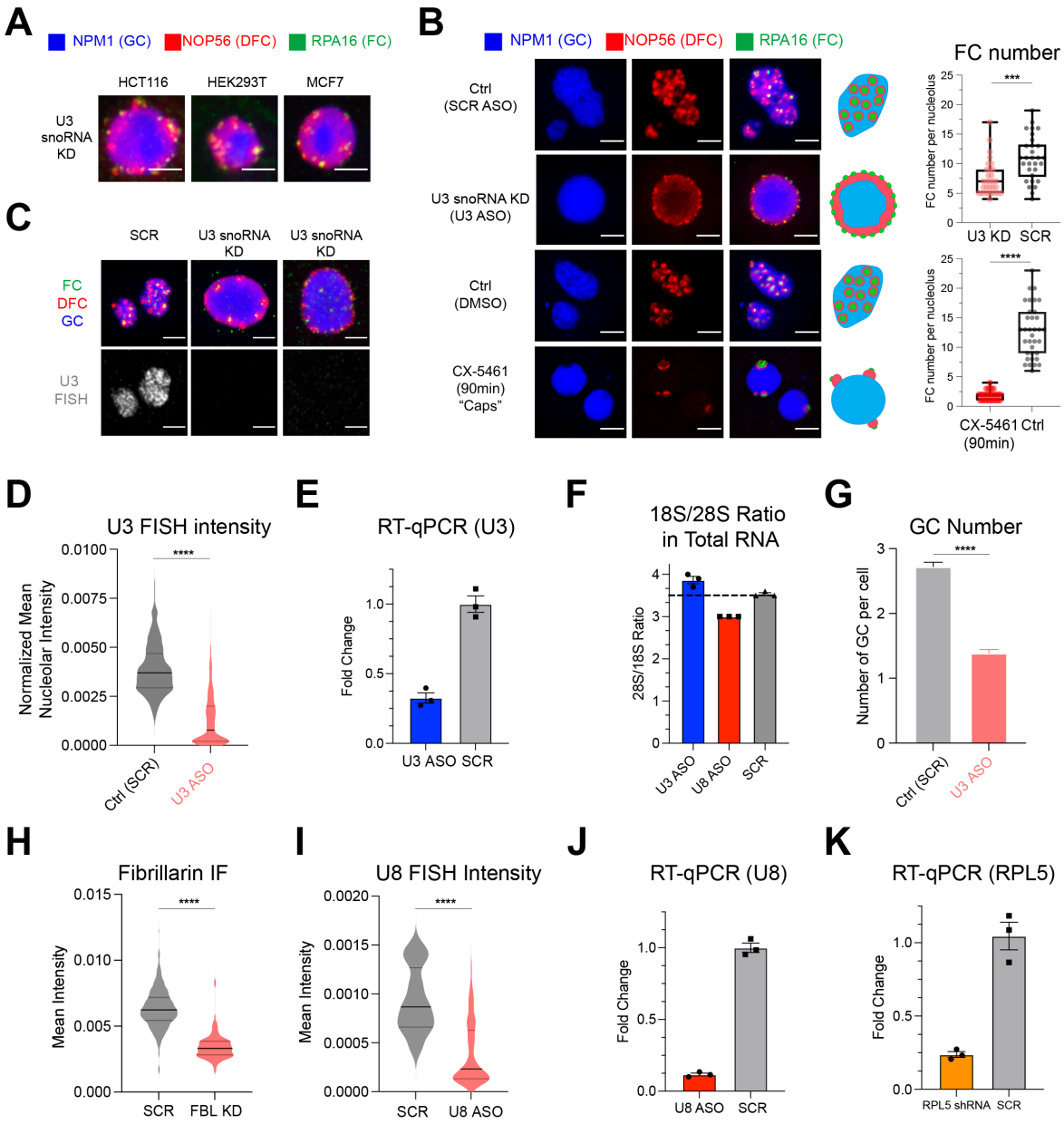
(A) Zoom-in on 5' end read counts in control (DMSO; pink) and 2 μM FVP treatment (orange) conditions 0-90 min after transcription in 28S rRNA region at Gm1522 and Am1524 2'-*O*-methylation sites showing impairment of characteristic dips at 2'-*O*-methylation sites.
(B) Quantification of 2'-*O*-Me levels (ScoreC) at the 28S Gm1522 and 28S Am1524 sites shown upon DMSO and FVP treatment.
(C) Heatmap of 2'-*O*-Me levels (ScoreC) at all 18S and 28S rRNA sites in control (DMSO) and 2 μM FVP treatment 0-90 min after transcription.
(D) Average 2'-*O*-Me levels (ScoreC) on 18S (red) and 28S (blue) rRNA in perturbations (dashed line) and control conditions (solid line) over 0 to 90 min from transcription.
(E) 2'-*O*-Me levels (ScoreC) at 28S Gm4499 and all other 18S and 28S (average) sites in control (SCR) and FBL KD treatment conditions 0-120 min after transcription. All error bars are s.e.m.

Supplementary Figure 5: Altered localization of RNA species and nucleolar morphology upon FVP treatment.



(A) RNA FISH for early, middle, and late rRNA cleavage sites, and mature rRNA species shown with GC (mTagBFP2-NPM1) upon 2 μM FVP treatment for 0 min and 60 min.
(B) Quantification of GC partitioning of early, middle and late/mature RNA FISH probes upon 0 min or 60 min 2 μM FVP treatment. Violin plots are centered by median.
(C) Nucleolar morphology showing FC (RPA194 IF), DFC (FBL IF), and GC (NPM1 IF) in DMSO and 30-90 min of 2 μM FVP treatment. Schematics show progressive GC detachment.
(D) Nucleolar morphology showing FC (RPA16-GFP), DFC (NOP56-mCherry), and GC (mTagBFP2-NPM1) 0-90 min after wash-out of 2 μM FVP. Dashed lines demarcate nuclei. Arrows point to sites of GC reattachment to FC/DFC over time.
(E) Example images of 5eU labeled RNA (white, 30 min pulse,60 min chase) with GC (mTagBFP2-NPM1) upon DMSO or 2 μM FVP treatment in HEK293T cells. Scale bars = 3 μm.

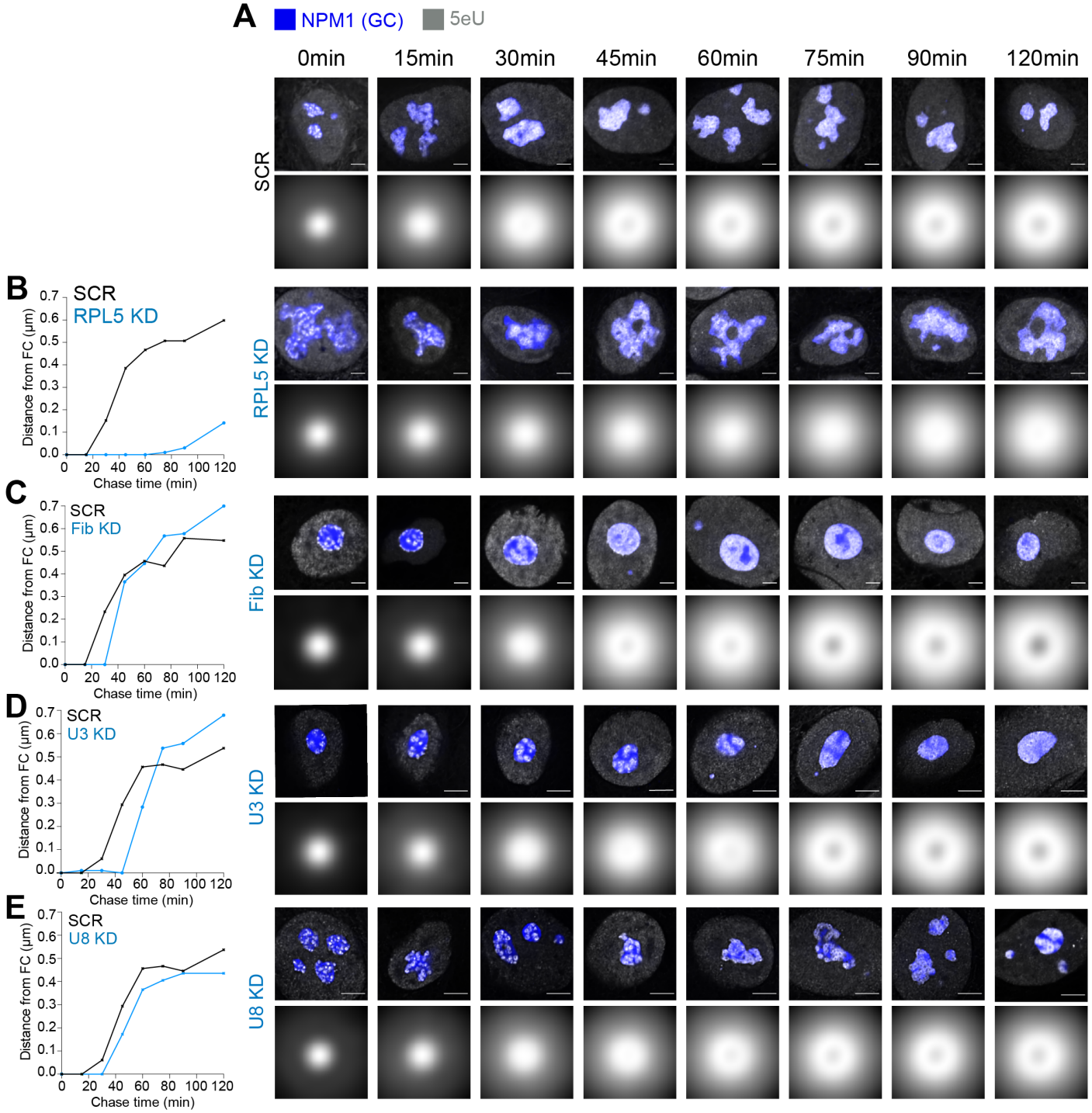
Supplementary Figure 6: Morphology comparison between U3 snoRNA KD and CX-5461 treatment and validation of all knockdowns performed in this study.



(A) Representative images of inverted nucleolar morphology upon U3 snoRNA KD in HCT116, HEK293T, and MCF7 cells, visualized with GC (mTagBFP2-NPM1), DFC (NOP56-mCherry) and FC (RPA16-GFP).
(B) Left, representative images of nucleolar morphology in U3 snoRNA KD, CX-5461 treatment, and corresponding control conditions in MCF10A cells with FC, DFC, GC labeled the same as A. Right, quantification of the number of FCs per nucleolus in U3 snoRNA (n=30) and SCR (n=30) ASO (top), and CX-5461 (n=35) and control (n=31) treatment. *** p-value = 0.0002, **** p-value < 0.0001 (two-tailed Mann Whitney test).
(C) Normal and inverted nucleolar morphology in control (SCR) and U3 snoRNA KD conditions in MCF10A cells with IF staining for NPM1 (GC), FBL (DFC) and RPA194 (FC) and RNA FISH for U3 snoRNA.
(D) Quantification of normalized mean nucleolar intensity of U3 snoRNA FISH from C. For scramble (n=479) and U3 ASO (n=329) nucleoli.
(E) Fold change of U3 snoRNA levels (RT-qPCR) in MCF10A cells treated with U3 snoRNA ASO and scramble (SCR) ASO for 72 hrs (n=3).

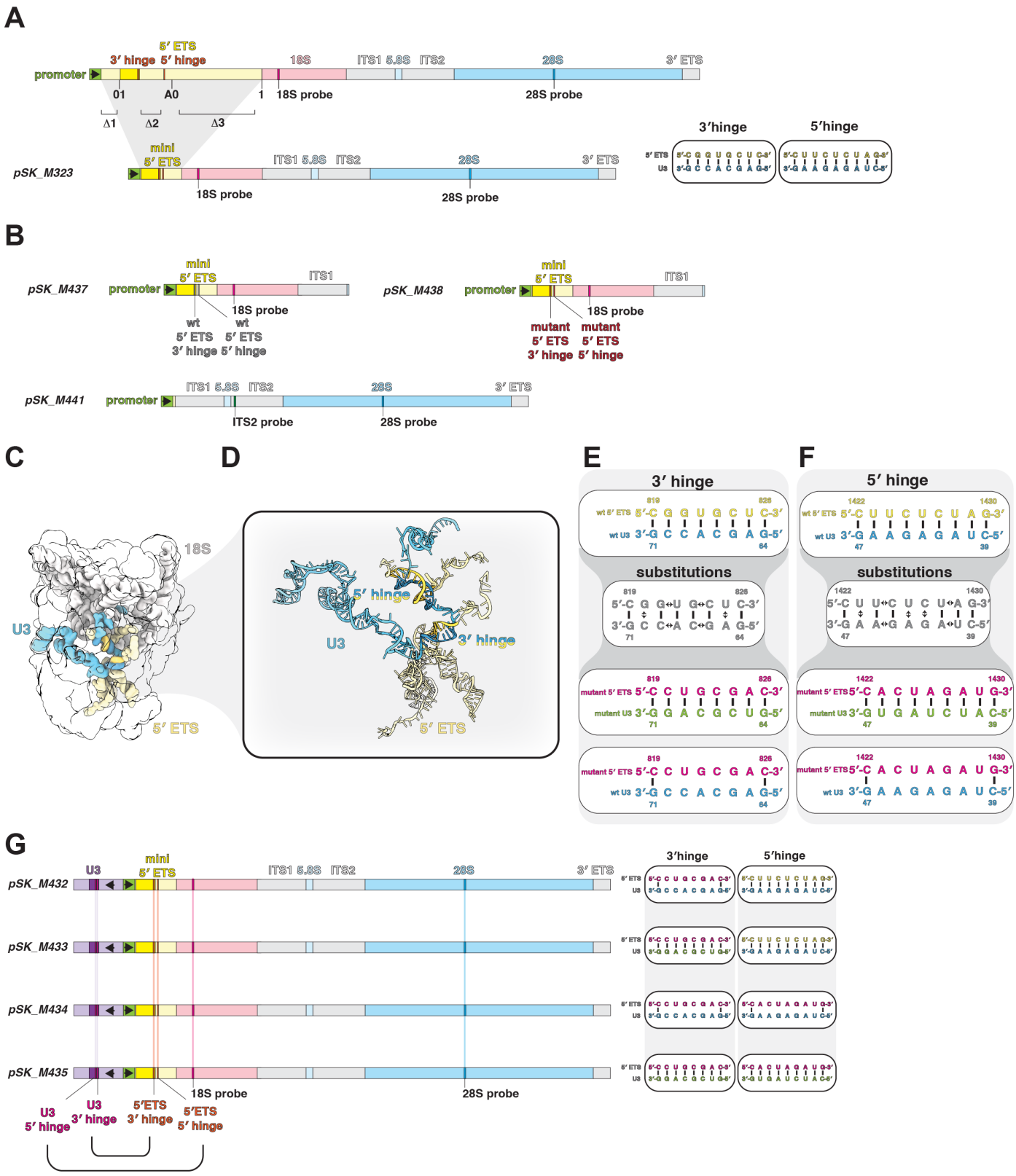
(F) RNA electrophoresis measured 18S to 28S ratio in total RNA from MCF10A cells treated with U3 or U8 snoRNA ASO, scramble (SCR) ASO for 72 hrs (n=3 per condition).
(G) Number of GC per cell quantified from U3 ASO (n=177) or SCR (n=384) ASO treated cells.
(H-K) Quantification of mean nucleolar intensity of FBL (IF; n=541 SCR, 278 Fib KD) and U8 snoRNA (FISH; n=174 SCR, 40 U8 ASO), as well as fold change of U8 snoRNA (RT-qPCR) and RPL5 mRNA (RT-qPCR), respectively, under each perturbation condition (n=3 per condition). All scale bars = 3 μm. Box and Whisker Plots: median plotted, boxes span 25th to 75th percentiles, whiskers span min-max values. Violin plots are centered by median and quartiles are shown. All error bars are s.e.m. **** P-value < 0.0001 (two-tailed Mann Whitney test).

Supplementary Figure 7: 5eU imaging examples for all perturbations.



(A) Example images of 5eU with GC (mTagBFP2-NPM1) and averaged 5eU intensity around FC in control (SCR) and all perturbation conditions over 0-120 min from transcription. Scale bars = 3 µm.
(B-E) Quantification of 5eU peak distance from FC center over time for multiple perturbation conditions compared to corresponding controls.

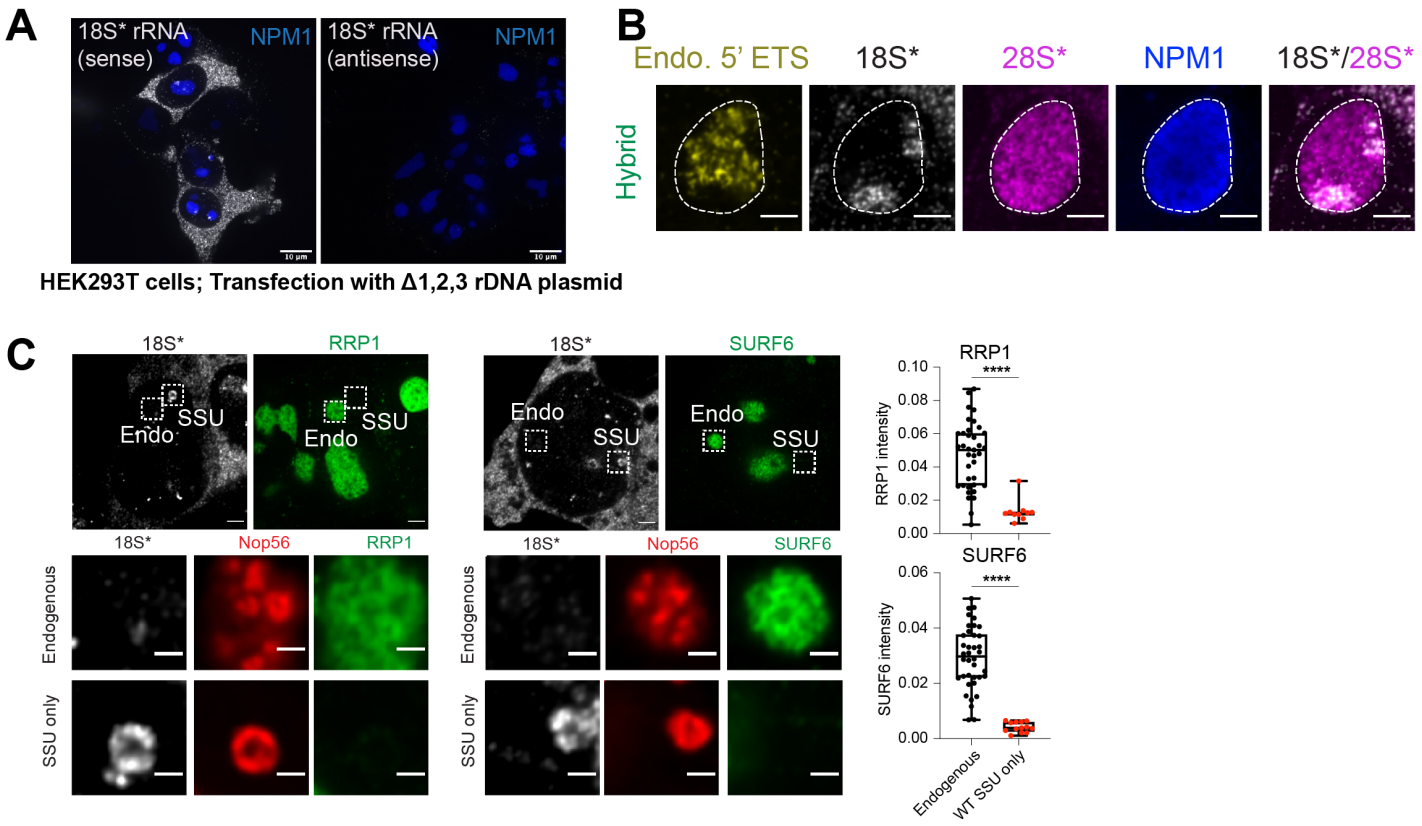
Supplementary Figure 8: rDNA plasmid designs used in this study.



(A) Schematics of endogenous (top) 47S rDNA and synthetic (bottom, pSK_M323) rDNA plasmid with minimized (mini) 5' ETS. Sequences of 3' and 5' hinge regions of 5' ETS-U3 snoRNA base pairing are shown.

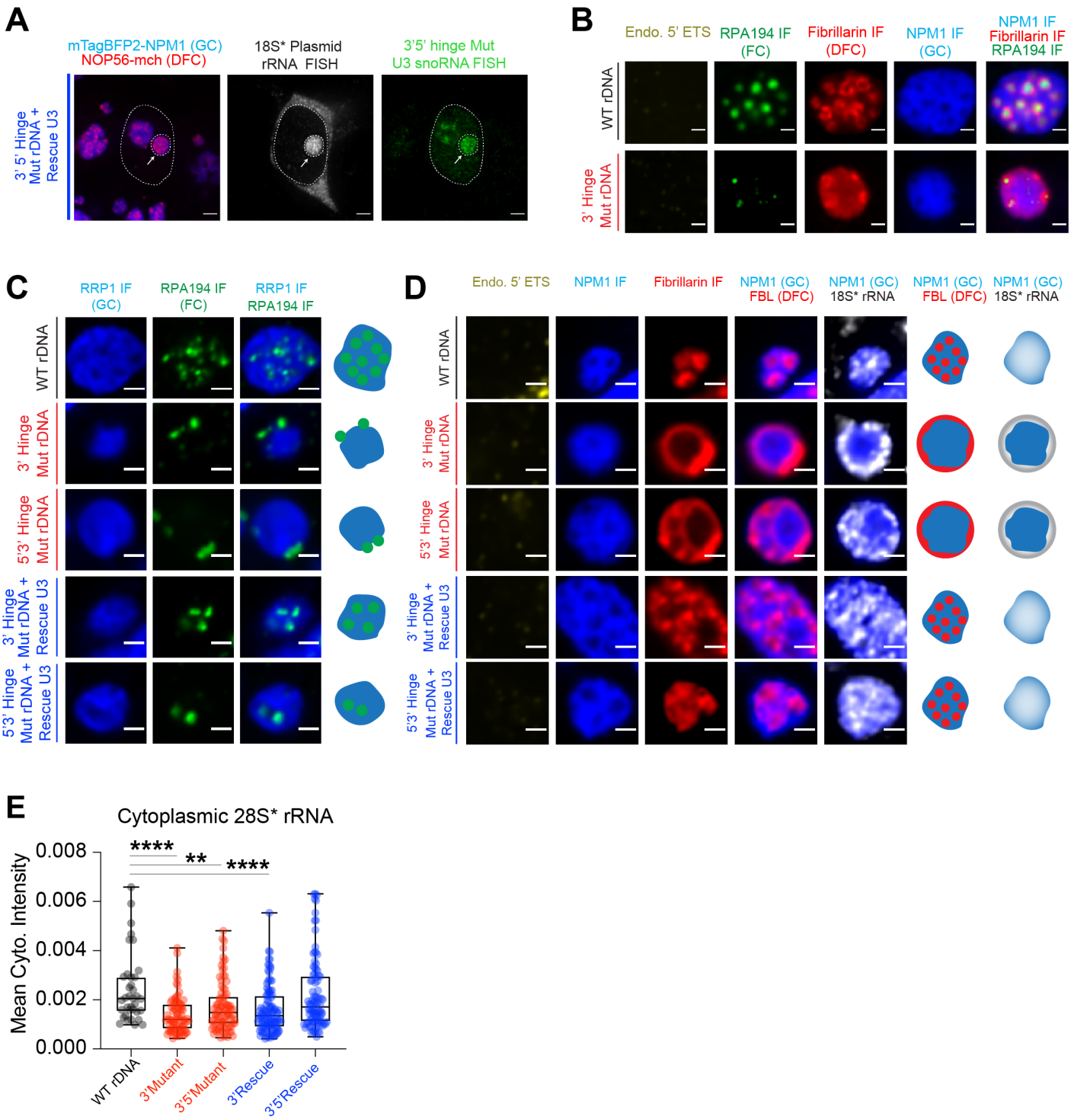
(B) Schematics of wildtype (WT) synthetic SSU only, 5' ETS 3' hinge mutant SSU only, and wildtype LSU only rDNA plasmids.
(C) Structure of SSU processome in state pre-A1 (PDB: 7mq8).
(D) Zoom-in on 5' and 3' hinge RNA duplexes between the U3 snoRNA and 5' ETS in C.
(E-F) Sequences of 3' (E) and 5' (F) hinges of 5' ETS-U3 snoRNA base pairing in wildtype (WT), mutant 5' ETS with mutant U3, and mutant 5' ETS with WT U3 conditions. Sequence substitutions for mutants are marked by double-sided arrows.
(G) Schematics of complete U3 snoRNA gene combined with rDNA plasmids with 5' ETS and U3 snoRNA 5' hinge and 3' hinge mutations.

Supplementary Figure 9: GC factors do not localize to SSU only nucleoli.



(A) Antisense FISH probes to show RNA FISH is specific to RNA not DNA. Localization of sense and antisense 18S* RNA FISH probes with GC (mTagBFP2-NPM1) shown in HEK293T cells transfected with Δ1,2,3 rDNA plasmid. Scale bar = 10 μm.
(B) A "Hybrid" nucleolus, as defined by signal from both endo. 5' ETS probes and plasmid 18S*/28S* probes. 18S* labeled SSU precursors have a territory-like structure in GC (labeled with NPM1 IF) while 28S* labeled LSU precursors (transcribed from the same rDNA plasmids as 18S*) localize everywhere in GC.
(C) Localization of RRP1 (IF) and SURF6 (IF) in endogenous and SSU only nucleoli labeled with DFC (NOP56-mCherry) and 18S* RNA FISH. Right, quantification of RRP1 (n=37, 10) and SURF6 (n=38, 13) fluorescent signal intensity in endogenous and SSU only nucleoli, respectively. Scale bars in a = 10 μm, in B and top row of C = 3 μm, in the rest of C = 1 μm. Box and Whisker Plots: median plotted, boxes span 25th to 75th percentiles, whiskers span min-max values. *** P-value < 0.0001 (two-tailed Mann-Whitney test).

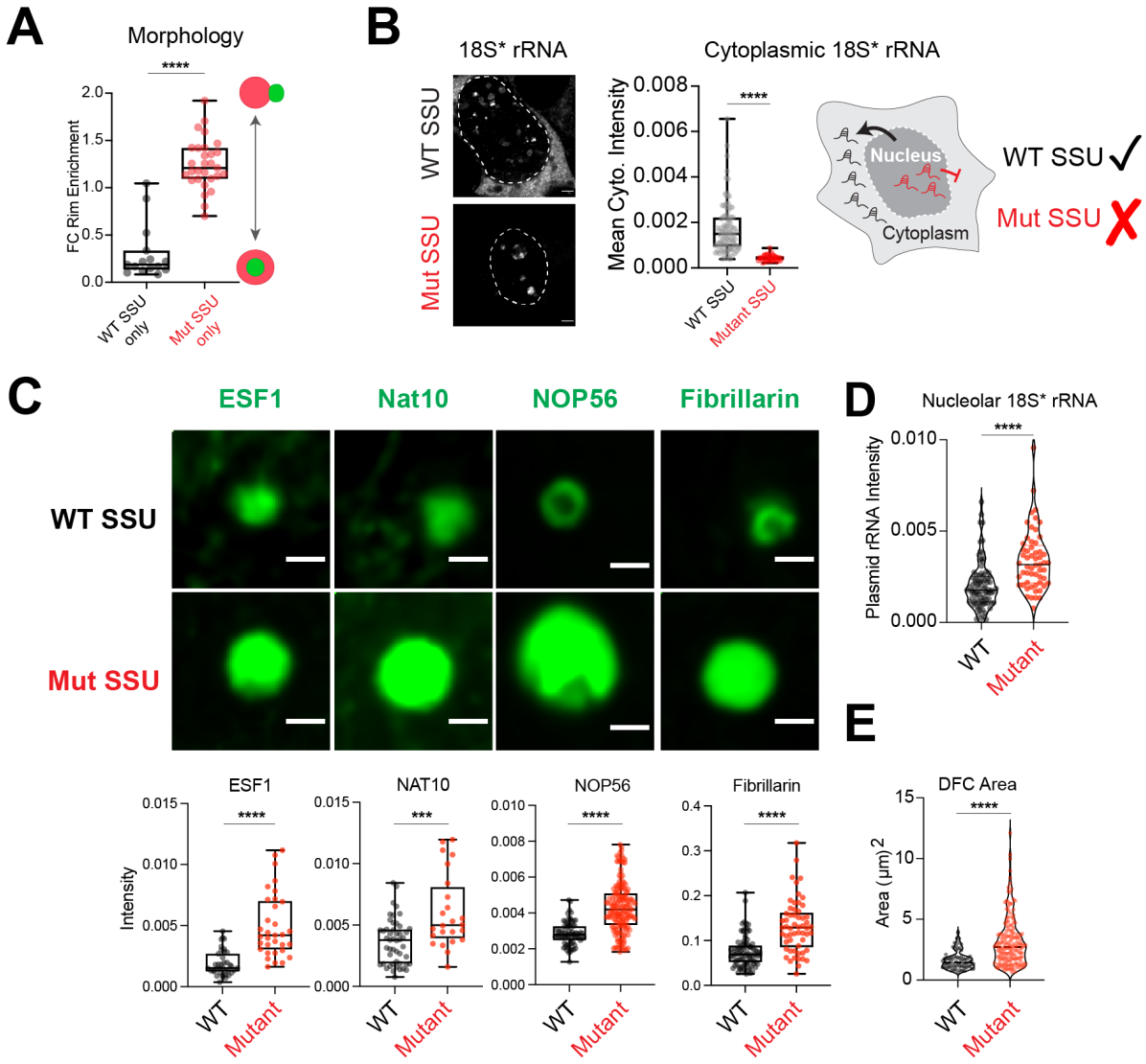
Supplementary Figure 10: Nucleolar morphology changes upon SSU processing inhibition



(A) Localization of 3' 5' hinge mutant U3 snoRNA and 18S* plasmid rRNA by RNA FISH in cells transfected with a plasmid expressing 3' 5' hinge mutant rDNA and rescue U3 snoRNA. GC (mTagBFP2-NPM1) and DFC (NOP56-mCherry) are shown. Arrow points to the nucleolus with plasmid rRNA (18S*).
(B) *De novo* nucleolus (FC labeled by RPA194 IF, DFC labeled by FBL IF, GC labeled by NPM1 IF) in cells transfected with WT or 3' hinge mutant rDNA plasmids. Endogenous 5' ETS is labeled by RNA FISH.
(C) Localization of FCs (RPA194 IF) with GC (RRP1 IF) shown in cells transfected with plasmids including various mutations in U3 snoRNA binding sites. Schematics of FC and GC localization in each transfection condition.

(D) Nucleolar morphology labeled with IF staining for DFC (FBL), GC (NPM1), and RNA FISH for 18S* rRNA and endogenous 5' ETS in cells transfected with plasmids including various mutations in U3 snoRNA binding sites. Schematics of nucleolar morphology (DFC and GC) and 18S* rRNA localization in GC in all conditions.
(E) Quantification of cytoplasmic plasmid 28S rRNA signal from Figure 4B. n = 36, 74, 84, 103, 92 cells. ** P-value = 0.0026; **** P-value < 0.0001 (two-tailed Mann-Whitney test). Scale bar = 3 μm for A and 1 μm for all the rest. Box and Whisker Plots: Median plotted, Boxes span 25th to 75th percentiles, Whiskers span min-max values.

Supplementary Figure 11: SSU only mutants have impaired cytoplasmic export and growing DFCs with increased recruitment of early SSU processing factors.



(A) Quantification of their FC rim enrichment score from SSU WT (n=15) and SSU mutant (n=29) nucleoli shown in Figure 4F. **** p-value < 0.0001

(B) Localization of 18S* rRNA by RNA FISH in cells transfected with WT and mutant SSU only rDNA plasmids; 18S* rRNA mean cytoplasmic intensity is quantified in both conditions. Schematic showing normal and impaired cytoplasmic export of 18S* rRNA in WT (n=90) and mutant SSU (n=34) conditions, respectively. Scale bar = 3 μm. **** P-value < 0.0001

(C) Top, Localization of early SSU processing factors ESF1, NAT10, FBL (labeled by IF) and NOP56-mcherry in WT and mutant SSU conditions; bottom, mean nucleolar intensity of each is quantified in both conditions. Scale bar = 1 μm. WT SSU only n=38, 49, 74, 87; Mut SSU only n=33, 24, 164, 57 for ESF1, Nat10, Nop56, and Fib, respectively. *** P-value = 0.0005; **** P-value < 0.0001

(D) Quantification of mean nucleolar 18S* rRNA intensity in wildtype (WT; n=87) and mutant SSU (n=57) conditions. **** P-value < 0.0001

(E) DFC area (μm^2) (labeled with NOP56-mCherry) is quantified in WT (n=74) and mutant SSU (n=164) conditions. **** P-value < 0.0001. Violin plots are centered by median. Box and Whisker Plots: Median plotted, Boxes span 25th to 75th percentiles, Whiskers span min-max values. All statistical comparisons are two-tailed Mann-Whitney tests.