Supplementary Information for:

Stalled translation on transcripts cleaved by RNase L activates signaling important for innate immunity

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spike in	sequence
18-mer-1	UCGAACCUUAAGGUUAAU
18-mer-2	AACUACCGACUCAUCCCA
18-mer-3	CUAAUACUUACGAACCAG
30-mer-1	AAUACCACCCCCAUGAACGCUGCACACACG
30-mer-2	AACUACCGACUCAUCCCAUCUUGCCAGUAC
30-mer-3	CUAAUACUUACGAACCAGACGAAUCCCUUG

Table S1. Oligonucleotide sequences utilized for spike-in normalization for 15-34 nt ribosome profiling experiments.

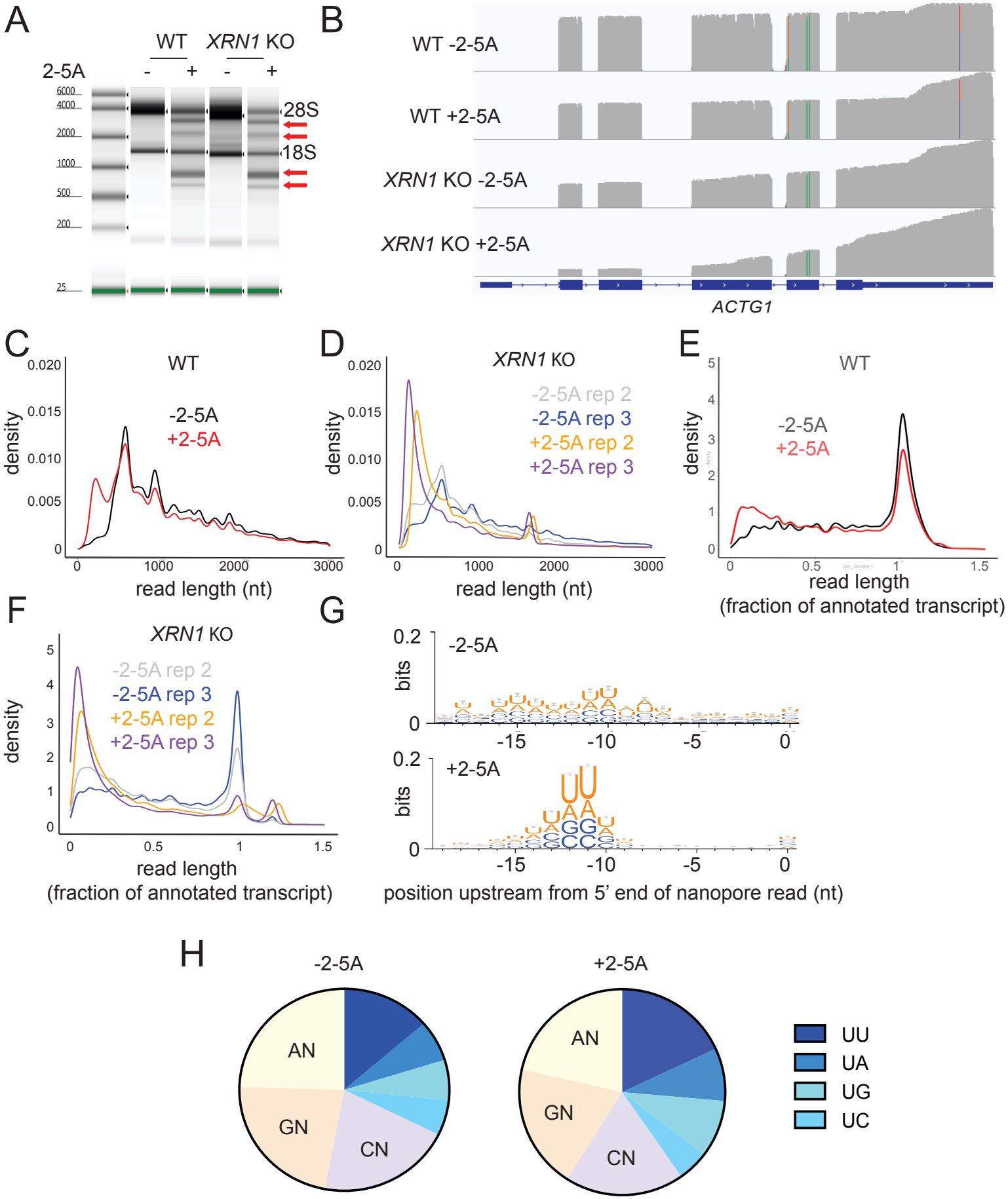
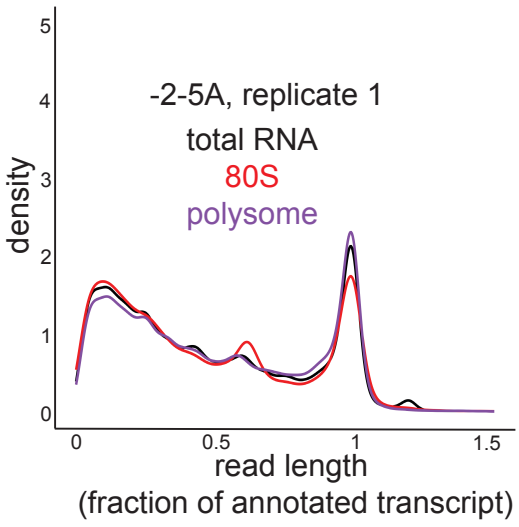
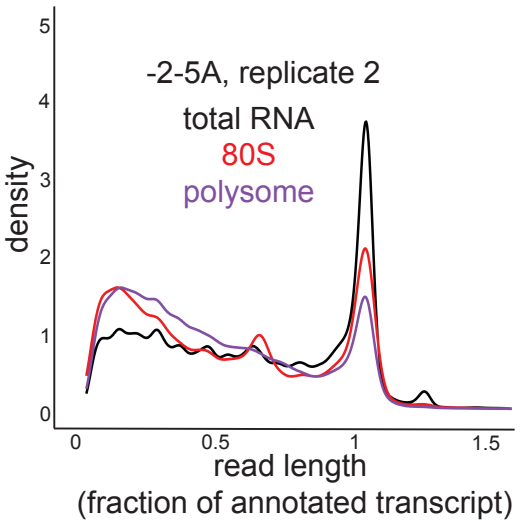

A
WT
XRN1 KO
2-5A
-
+
-
+
6000
4000
2000
1000
500
200
25
28S
18S
B
WT -2-5A
WT +2-5A
XRN1 KO -2-5A
XRN1 KO +2-5A
ACTG1
C
WT
-2-5A
+2-5A
density
read length (nt)
D
XRN1 KO
-2-5A rep 2
-2-5A rep 3
+2-5A rep 2
+2-5A rep 3
density
read length (nt)
E
WT
-2-5A
+2-5A
density
read length
(fraction of annotated transcript)
F
XRN1 KO
-2-5A rep 2
-2-5A rep 3
+2-5A rep 2
+2-5A rep 3
density
read length
(fraction of annotated transcript)
G
-2-5A
+2-5A
bits
position upstream from 5' end of nanopore read (nt)
H
-2-5A
+2-5A
AN
GN
CN
UU
UA
UG
UC

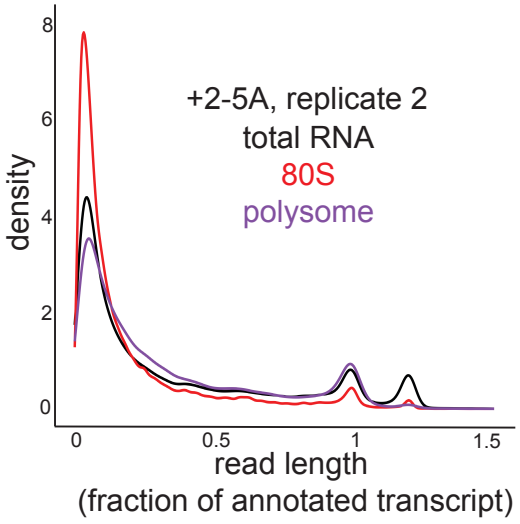
Figure S1. A rRNA cleavage assays showing RNase L activity in treated (+2-5A) but not in untreated (−2-5A) cells. Red arrows indicate RNase L cleavage products. rRNA cleavage is comparable in WT and *XRN1* KO cells. Image output by Agilent TapeStation software with ladder shown on left. **B** Example gene model (*ACTG1*, oriented 5' to 3') showing a "pile up" view of nanopore raw (not normalized) reads. Image taken from IGV and colored bars indicate mismatches. **C** Length distribution of nanopore direct sequencing reads in 2-5A treated (+2-5A) and untreated (−2-5A) samples in WT cells. **D** Length distribution of nanopore direct sequencing reads in replicate 2-5A treated (+2-5A) and untreated (−2-5A) samples in *XRN1* KO cells. **E** Normalized length distribution of nanopore direct sequencing reads to their respective annotated reference transcript in 2-5A treated (+2-5A) and untreated (−2-5A) samples in WT cells. **F** Normalized length distribution of replicate nanopore direct sequencing reads to their respective annotated reference transcript in 2-5A treated (+2-5A) and untreated (−2-5A) samples in *XRN1* KO cells. **G** Weblogo analysis shows enrichment of U bases in transcriptome positions just upstream of where nanopore sequencing stopped in ±2-5A treated WT cells. **H** Di-nucleotide motif distribution near the 5' end of 3' fragments in ±2-5A treated WT cells (−11-−12 positions as shown in G to account for RNA left in pore after sequencing stops). Total number of fragments in each sample were: 110,068 (-2-5A), 619,964 (+2-5A). In both G and H, 3' fragments were defined as reads that were shorter than a third of their respective annotated transcript.

A


-2-5A, replicate 1
total RNA
80S
polysome
density
read length
(fraction of annotated transcript)


-2-5A, replicate 2
total RNA
80S
polysome
density
read length
(fraction of annotated transcript)

B


+2-5A, replicate 2
total RNA
80S
polysome
density
read length
(fraction of annotated transcript)

C

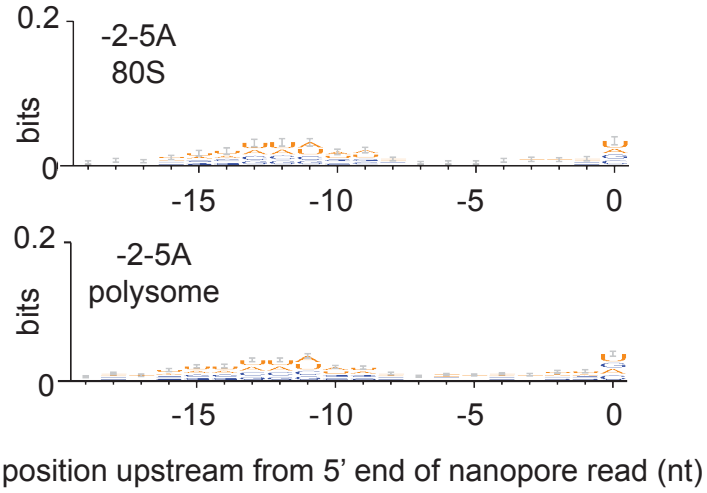

-2-5A
80S
bits
-2-5A
polysome
bits
position upstream from 5' end of nanopore read (nt)

Figure S2. A Normalized length distribution of nanopore direct sequencing reads to the annotated reference transcript in total RNA, monosomes and polysomes in untreated (−2-5A) *XRN1* KO cells (replicate 1 and 2). **B** Replicate experiments for Figure 2C. **C** Weblogo analysis shows enrichment of U bases in transcriptome positions just upstream of where nanopore sequencing stopped in the 80S and polysome fractions of sucrose gradient sedimentation experiments in untreated (−2-5A) *XRN1* KO cells. 3' fragments were defined as reads that were shorter than a third of their respective annotated transcript that mapped to the 3' end.

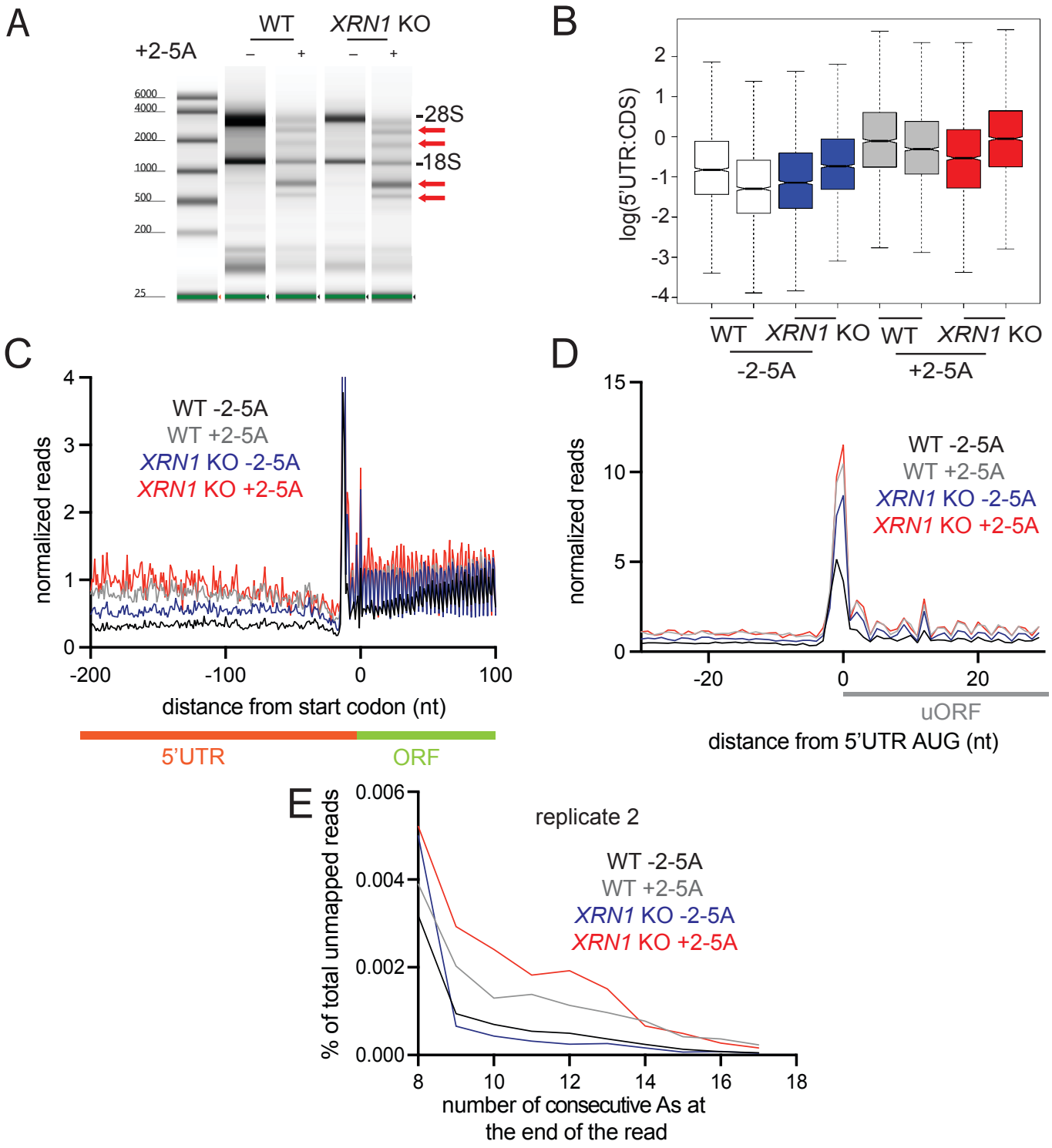

A
WT
XRN1 KO
+2-5A
–
+
–
+
6000
4000
2000
1000
500
200
25
-28S
-18S
B
log(5'UTR:CDS)
2
1
0
-1
-2
-3
-4
WT
XRN1 KO
WT
XRN1 KO
-2-5A
+2-5A
C
normalized reads
4
3
2
1
0
WT -2-5A
WT +2-5A
XRN1 KO -2-5A
XRN1 KO +2-5A
-200
-100
0
100
distance from start codon (nt)
5'UTR
ORF
D
normalized reads
15
10
5
0
WT -2-5A
WT +2-5A
XRN1 KO -2-5A
XRN1 KO +2-5A
-20
0
20
uORF
distance from 5'UTR AUG (nt)
E
% of total unmapped reads
0.006
0.004
0.002
0.000
replicate 2
WT -2-5A
WT +2-5A
XRN1 KO -2-5A
XRN1 KO +2-5A
8
10
12
14
16
18
number of consecutive As at
the end of the read

Figure S3. A rRNA cleavage assays in lysates used in ribosome profiling showing RNase L activity in treated (+2-5A) but not in untreated (−2-5A) cells (replicate 1). Red arrows indicate RNase L cleavage products. rRNA cleavage is comparable in WT and *XRN1* KO cells. Image output by Agilent TapeStation software with ladder shown on left. **B** 5'UTR:CDS ratios do not increase in treated (+2-5A) and control (−2-5A) *XRN1* KO as compared to WT cells. Boxes represent the interquartile range (IQR) and horizontal line is the median. Whiskers show most extreme data point no more than 1.5*IQR and notches are 1.58*IQR/$\sqrt{N}$. 2114 genes included. **C** Normalized average ribosome footprint occupancy (metagene plot) around the start codon of main ORFs (CDSs) reveals increased relative ribosome footprint levels in 5' UTRs when RNase L is active vs −2-5A control. XRN1 KO cells don't exhibit increase in 5'UTR footprints as compared to WT. Ribosome footprints are plotted by 5' assignment without any shift. **D** Normalized average ribosome footprint occupancy around the start codon of upstream ORFs in the 5'UTR reveal increased uORF translation during RNase L activation. This is not further increased in *XRN1* KO cells as compared to WT cells. Ribosome footprints are plotted by 5' assignment shifted by 12 nt (~P site). **E** Replicate experiments of Figure 3E. Percentage of ribosome profiling footprints in samples prior to mapping that contained given length of consecutive poly(A) sequences at their 3' end in WT and *XRN1* KO cells. Total read counts >1000 for all samples. These represent ribosomes that run into the poly(A) tail.

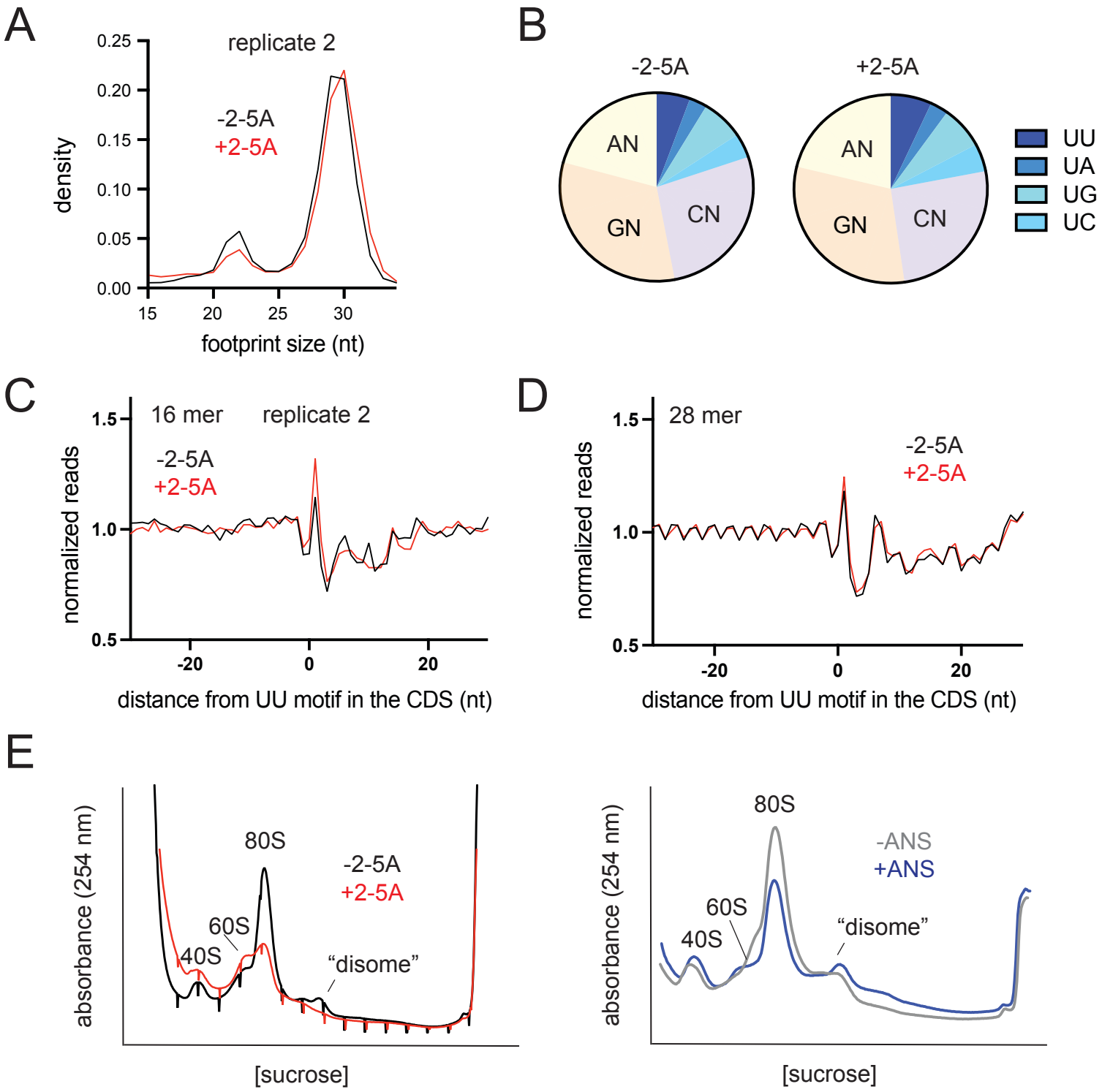
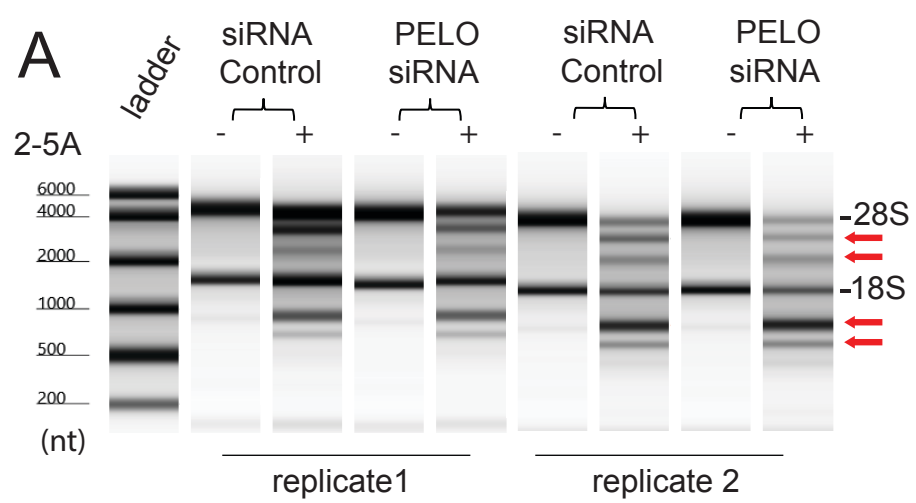
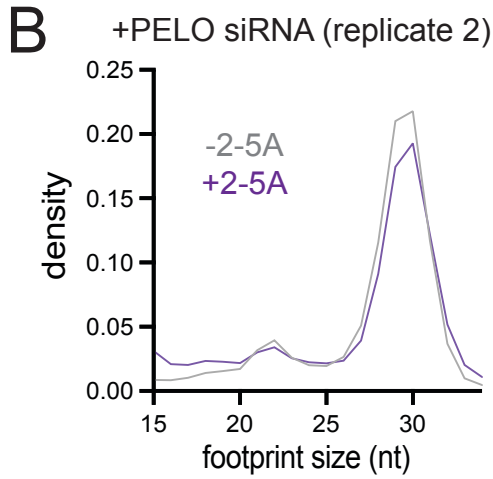
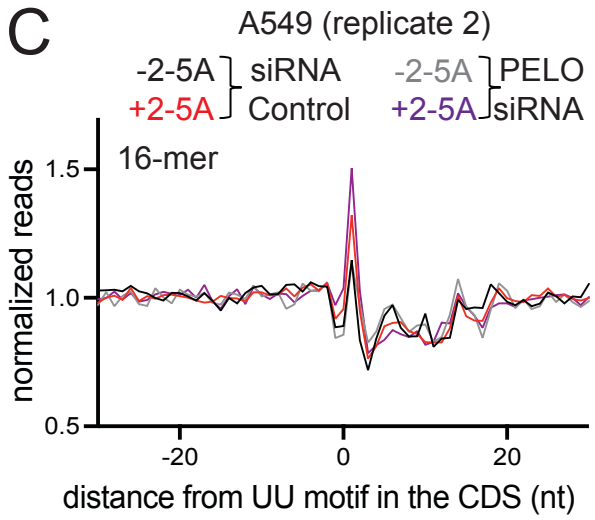

A
replicate 2
-2-5A
+2-5A
density
0.25
0.20
0.15
0.10
0.05
0.00
15
20
25
30
footprint size (nt)
B
-2-5A
+2-5A
AN
GN
CN
UU
UA
UG
UC
C
16 mer
replicate 2
-2-5A
+2-5A
normalized reads
1.5
1.0
0.5
-20
0
20
distance from UU motif in the CDS (nt)
D
28 mer
-2-5A
+2-5A
normalized reads
1.5
1.0
0.5
-20
0
20
distance from UU motif in the CDS (nt)
E
absorbance (254 nm)
80S
-2-5A
+2-5A
60S
40S
"disome"
[sucrose]
absorbance (254 nm)
80S
-ANS
+ANS
60S
40S
"disome"
[sucrose]

Figure S4. A Normalized length distribution of transcriptome mapped ribosome profiling reads (15-34 nt) for replicate experiments (replicate 2). 11,819,637 reads for −2-5A and 19,314,584 reads for +2-5A. **B** 3’ end di-nucleotide motif distribution in 16-mer reads. Proportion of short footprints (15-18 nt) containing UN motif at their 3’ end is modestly increased in 2-5A treated cells (replicate 2). **C** and **D** Position average plot of 3’ ends of 16-mers and 28-mers at RNase L cleavage sites (UU) for replicate experiments. Ribosome footprints are plotted by 3’ assignment. **E** RNase A treatment of the lysates combined with 10-35% sucrose gradient ultracentrifugation serves as an assay for ribosome collisions with ANS serving as a positive control. The level of RNA was monitored by absorbance at 254 nm during fractionation. Data in all panels from A549 cells and **A**-**D** were treated with control siRNA for comparison to data in Figure S5.


A
ladder
siRNA Control
PELO siRNA
siRNA Control
PELO siRNA
2-5A
- + - + - + - +
6000
4000
2000
1000
500
200
(nt)
-28S
-18S
replicate1
replicate 2


B
+PELO siRNA (replicate 2)
density
0.25
0.20
0.15
0.10
0.05
0.00
-2-5A
+2-5A
15
20
25
30
footprint size (nt)


C
A549 (replicate 2)
-2-5A
+2-5A
siRNA Control
-2-5A
+2-5A
PELO siRNA
16-mer
normalized reads
1.5
1.0
0.5
-20
0
20
distance from UU motif in the CDS (nt)

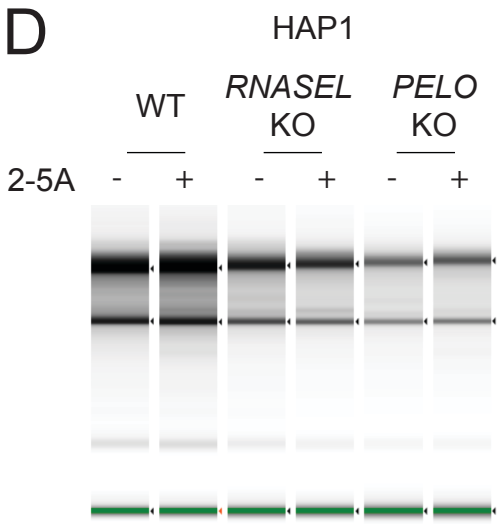

D
HAP1
WT
RNASEL KO
PELO KO
2-5A
- + - + - +

Figure S5. A rRNA cleavage assays from lysates used in the 15-34 nt ribosome profiling experiments where siRNA was used show RNase L activity in treated (+2-5A) but not in untreated (−2-5A) cells. Red arrows indicate RNase L cleavage products. rRNA cleavage is comparable across samples. Image output by Agilent TapeStation software with ladder shown on left. **B** Normalized length distribution of transcriptome mapped ribosome profiling reads (15-34 nt) in PELO siRNA treated cells (replicate 2). 8,112,399 reads for −2-5A and 7,295,879 reads for +2-5A. **C** Position average plot of 3' ends of 16-mers near RNase L cleavage motif (UU) in PELO KD A549 cells (replicate 2). Control data same as in Fig. S4. **D** rRNA cleavage assays in HAP1 WT and PELO KO cells shows little RNase L activity.