Phosphorothioate oligonucleotides can displace *NEAT1 RNA* to form nuclear paraspeckle-like structures

SUPPLEMENTARY DATA

SUPPLEMENTAL MATERIALS AND METHODS

siRNAs:

P54nrb (s9612 and s9614), PSPC1 (s30594), hnRNPK (s6737), LRPPRC (HSS115403), and KHSRP (HSS112552) siRNAs were purchased from Life Technologies. NCL1 siRNA (S9312) was purchased from Ambion.

Antibodies:

Anti-PSPC1 (ab104238), anti-hnRNP K (ab32969), anti-Fibrillarin (ab5821), anti-TCP1-β (ab92746), embryonic stem cell marker panel (ab109884) antibodies were purchased from Abcam. Anti-P54nrb (sc-376865) antibody for western analysis and IF was purchased from Santa Cruz Biotechnology. Anti-P54nrb (05-950) antibody for RIP (Fig.6 H) was purchased from EMD Millipore. Anti-PSF (P2860) and anti-GAPDH (G8795) were purchased from Sigma. Anti-RNase H1 antibody was kindly provided by Hongjiang Wu. Anti-rabbit secondary antibody conjugated to HRP (170-6515) and anti-mouse secondary antibody conjugated to HRP (170-6516) were purchased from Bio-Rad. Anti-rabbit secondary antibodies conjugated to AF488 (ab150077), AF555 (ab150078), and AF647 (ab150079), as well as anti-mouse secondary antibodies conjugated to AF488 (ab150113), AF555 (ab150114), and AF647 (ab150115) were purchased from Abcam.

Plasmids:

GFP-tagged P54nrb (RG226567) plasmid was purchased from ORIGENE. Plasmid transfection was performed using Effectene Transfection Reagent (QIAGEN), according to manufacturer's instructions.

ASOs:

In the tables below, deoxyribonucleotides are in plain font, ribonucleotides are underlined, and 2'-modified ribonucleotides are indicated in bold. Phosphorothioate (PS) backbone residues are indicated in italic.

Table 1. ASOs for protein isolation

Figure	ASO	Sequence	2′ Modification
1A, B, and C	Capture	ISIS386652: 5′- biotin – ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	MOE
	Elution	ISIS116847: 5′- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	MOE
1D	Capture	ISIS586183: 5′- biotin – ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	cEt
	Elution	ISIS404130: 5′- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	2′-α-fluoro
		XL168D: 5′- *CTGCTAGCCTCTGGATTTGA* -3′	DNA
		ISIS582801: 5′- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	cEt
		ISIS390896: 5′- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	LNA
		XL169R: 5′- ***CTGCTAGCCTCTGGATTTGA*** -3′	2′-O Methyl
		ISIS116847: 5′- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	MOE
		XL209-1: 5′- *CUGCUAGCCUCUGGAUUUGA* -3′	RNA
		XL183: 5′- CUGCUAGCCUCUGGAUUUGA -3′	RNA
1E	Capture	ISIS586183: 5′- biotin – ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	cEt
	Elution	ISIS622870: 5′- **CTGCT**AGCCTCTGGA -3′	cEt
		ISIS622869: 5′- ***CTGCT***AGCCTCTGGA -3′	cEt
		ISIS622865: 5′- **CTGCT***AGCCTCTGGA* -3′	cEt
		ISIS617421: 5′- ***CTGCT****AGCCTCTGGA* -3′	cEt
1F	Capture	ISIS586183: 5′- biotin – ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	cEt
	Elution	ISIS617421: 5′- ***CTGCT****AGCCTCTGGA* -3′	cEt
		ISIS622866: 5′- ***CTGCT****AGCCTCTG* -3′	cEt
		ISIS622867: 5′- ***CTGCT****AGCCTC* -3′	cEt
		ISIS622868: 5′- ***CTGCT****AGCC* -3′	cEt

Table 2. ASOs for localization

Figure	ASO Sequence	2′ Modification
3A, 3B, 3C, 4A, 4E, 5A, 5B, 5C, S2, S3, S4, S5, and S6	ISIS446654: 5′- Cy3- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	MOE
6A, 7 and S9	ISIS454395: 5′-Fluorescein- ***GCTGA****TTAGA-GAGAG****GTCCC*** -3′ (mixed backbone with 1 PO, indicated by -)	MOE
S8	ISIS652721: 5′- Cy3- ***ATGGG****CTCTGGAACA****AGCAT*** -3′	MOE

Sequences and modifications of ASOs tested in Fig. S3A, S4A, and S6B are listed in the Figures.

Table 3. Other ASOs

Figure	Target	ASO Sequence	2′ Modification
2B, 6B, 6C, and 6H	NCL1	ISIS110074: 5′- ***GTCAT****CGTCATCCTC****ATCAT*** -3′	MOE
2B, 6B, and 6C	U16 snoRNA	ISIS462026: 5′- ***CAGCA****GGCAACTGTC****GCTGA*** -3′	MOE
2D	*Malat1* lncRNA	ISIS395254: 5′-***GGCAT****ATGCAGATAA****TGTTC***-3′	MOE
6B, 6D, 6E, 6G, 6H and S6D	*NEAT1* lncRNA	ISIS407248: 5′- ***ATGGG****CTCTGGAACA****AGCAT*** -3′	MOE
6H	*NEAT1* lncRNA	ISIS407279: 5′- ***TTAGG****TCTTGTTTTC****CAAAC*** -3′	MOE

6B, 6D, 6E, 6G, 6H,6I and 6J	no target control	ISIS141923: 5′- ***CCTTC****CCTGAAGGTT****CCTCC*** -3′	MOE
S1	Drosha mRNA	ISIS25690: 5′- ***ATCCC****TTTCTTCCGC****ATGTG*** -3′	MOE
S1	PTEN mRNA	ISIS116847: 5′- ***CTGCT****AGCCTCTGGA****TTTGA*** -3′	MOE

Oligonucleotides and primers:

Table 4. RNA sequences for ASO/RNA duplex–binding protein isolation

In the table below, "m" indicates 2'-O-methyl modification, "r" indicates ribose, and underlined sequence indicates RNase I cleavage linker.

RNA	Sequence
XL180	5′- biotin- rArGrCrArGrUrUrCmUmCmAmAmAmUmCmCmAmGmAmGmGmCmUmAmGmCmAmG -3′
XL181	5′- biotin- rArGrCrArGrUrUrCmGmUmGmAmUmGmGmGmUmUmUmGmUmCmUmGmCmGmCmU -3′

Table 5. Primer-probe sets for qRT-PCR

Target	Sequence
P54nrb	Forward: 5′- GATTTGGCTTTATCCGCTTGG -3′ Reverse: 5′- ACACATACTGAGGAAGGTTTCG -3′ Probe: 5′- TTGGCAATCTCCGCTAGGGTTCG -3′
LRPPRC	Forward: 5′- AGGACCGACGGAAGCTGTT -3′ Reverse: 5′- AATGCTCCTCCTTGGCCTGTA -3′ Probe: 5′- TTCTTTATAACTTGATTGACAGCATGAG -3′
NCL1	Forward: 5′- GCTTGGCTTCTTCTGGACTCA -3′ Reverse: 5′- TCGCGAGCTTCACCATGA -3′ Probe: 5′- CGCCACTTGTCCGCTTCACACTCC -3′
U16 snoRNA	Forward: 5′- CTTGCAATGATGTCGTAATTTGC -3′ Reverse: 5′- TCGTCAACCTTCTGTACCAGCTT -3′ Probe: 5′- TTACTCTGTTCTCAGCGACAGTTGCCTGC -3′
MALAT1 LncRNA	Forward: 5′- AAAGCAAGGTCTCCCCACAAG -3′ Reverse: 5′- TGAAGGGTCTGTGCTAGATCAAAA -3′ Probe: 5′- TGCCACATCGCCACCCCGT -3′
5'-*hNEAT1* (*NEAT1_1&1_2*)	Forward: 5′- TGGTAAGCCCGGGACAGTAA -3′ Reverse: 5′- CAGCGGGAAGGCCTCTCT -3′ Probe: 5′- CCGAGTGGCTGTTGGAGTCGGTATTG -3′
3'-*hNEAT1* (*NEAT1_2*)	Forward: 5′- AGTACCCTGAGAGCCAGTATTGGT -3′ Reverse: 5′- GGCAGCTGAGTCAATCTCCTTT -3′ Probe: 5′- CAGCACTGAGAACCGCAGGAACGG -3′
5'-*mNEAT1* (*NEAT1_1&1_2*)	Forward: 5′- TGAGCCATGCCTTCAAAACAC -3′ Reverse: 5′- TTAAATAAGCAAACCCTGTTGTCTTC -3′

	Probe: 5´- TCTTGAATTTCTGGAATTGGCC -3´
3'-*mNEAT1* (*NEAT1_2*)	Forward: 5´- ACGGACTTACATTTCCAAGCC -3´ Reverse: 5´- ACACATACTGAGGAAGGTTTCG -3´ Probe: 5´- AGCAGGAGTCTTCCAGGGCAC -3´
Pre-hNEAT1	Forward: 5'- TGCTTTTCACCTTCCCATCTG -3' Reverse: 5'- CAACACTGCGGGGACTCG -3' Probe: 5'- CGCCTTTTGCTTTTTCCTGCTCACTC -3'
PTEN mRNA	Forward: 5'-AATGGCTAAGTGAAGATGACAATCAT Reverse: 5'-TGCACATATCATTACACCAGTTCGT Probe: TTGCAGCAATTCACTGTAAAGCTGGAAAGG
Drosha mRNA	Forward: 5'- CAAGCTCTGTCCGTATCGATCA Reverse: 5'- TGGACGATAATCGGAAAAGTAATCA Probe: 5'-CTGGATCGTGAACAGTTCAACCCCGAT
KHSRP mRNA	Forward: 5'-TTCTCAACTTGGACCCATCC-3' Reverse: 5'-CCACCGCAATCTGTACTTTG-3' Probe: 5'-TGTTAATTTGTTCACCTCCTCTGCCCC-3'

Table 6. Primers for FISH probe sub-cloning

In the table below, underlined italics indicate T7 promoter sequence

Target	Sequence
5'-*hNEAT1* (*NEAT1_1&1_2*)	Forward: 5´- TAGTTGTGGGGGAGGAAGTG -3´ Reverse: 5´- *<u>TAATACGACTCACTATAGGG</u>*TGGCATGGACAAGTTGAAGA -3´
3'-*hNEAT1* (*NEAT1_2*)	Forward: 5´- GTCTTTCCATCCACTCACGTCTATTT -3´ Reverse: 5´- *<u>TAATACGACTCACTATAGGG</u>*CACCCTAACTCATCTTACAGACCACCAG -3´
5'-*mNEAT1* (*NEAT1_1&1_2*)	Forward: 5´- CTGCTGTCTGCTGGCACTTG -3´ Reverse: 5´- *<u>TAATACGACTCACTATAGGG</u>*CCAAGACTTGCGCCTTCAAT -3´
3'-*mNEAT1* (*NEAT1_2*)	Forward: 5´- GCTACTAACACTGAGATCATG -3´ Reverse: 5´- *<u>TAATACGACTCACTATAGGG</u>*CTGAGGTTTGTGGACTGCCAGG -3´

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Depletion of nuclear-localized PS-ASO-associated proteins NCL1 and KHSRP has no effect on antisense activity of ASOs. A. Reduction of NCL1 and KHSRP mRNAs by corresponding siRNAs as determined by qRT-PCR analysis. UTC, mock treated control cells. ***B.*** Depletion of NCL1 or KHSRP has no effect on ASO-mediated cleavage of Drosha mRNA as determined by qRT-PCR analysis. ***C.*** Depletion of NCL1 or KHSRP has no effect on ASO-mediated cleavage of U16 snoRNA as determined by qRT-PCR

analysis. ***D.*** Depletion of NCL1 or KHSRP has no effect on ASO-mediated cleavage of PTEN mRNA as determined by qRT-PCR analysis.

Figure S2. PS-ASOs can co-localize with transiently expressed exogenous GFP-P54nrb. HeLa cells transiently expressing GFP-P54nrb (RG226567, ORIGENE) were transfected with 60 nM fluorescently tagged PS-ASO (ISIS446654). Live-cell images were acquired 4 hrs post ASO transfection. Red and yellow arrows indicate PS bodies and paraspeckles, respectively.

Figure S3. ASOs with different sequences and chemical modifications localize to paraspeckles or paraspeckle-like foci in multiple tested cell lines. A. Co-localization of different ASOs with P54nrb to paraspeckles/paraspeckle-like foci in HeLa cells. Backbone modification, 2'-moiety, and sequences of the tested ASOs are indicated. PS-ASOs were transfected at a final concentration of 60 nM for 6hrs. ***B.*** Co-localization of PS-ASO ISIS446654 with P54nrb (arrows) to nuclear paraspeckles/paraspeckle-like foci was observed in different mouse cell lines including hepatocytes, MEF, bEND, and MHT.

Figure S4. ASOs with different sequences and chemical modifications localize to perinucleolar caps in multiple tested cell lines. A. Co-localization of different ASOs with P54nrb to perinucleolar caps in HeLa cells in the presence of actinomycin D (1.5 µg/mL, 1.5 h). Backbone modification, 2'-moiety, and sequences of the tested ASOs are indicated. PS-ASOs were transfected at a final concentration of 60 nM for 6hrs. ***B.*** PS-ASO ISIS446654 co-localized with P54nrb (arrows) to perinucleolar caps upon actinomycin D treatment (1.5 µg/mL, 1.5 h) was observed in multiple tested cell lines including mouse MHT, human A431, and mouse ESCs. Oct-4 served as a marker for mESCs.

Figure S5. Transcriptional inhibition has no effect on PS body integrity. IF staining was performed for P54nrb and TCP1-β in HeLa cells transfected with 60 nM PS-ASO (ISIS446654) and then treated with actinomycin D (1.5 µg/mL, 1.5 h). DNA was stained with DAPI. White and yellow arrows indicate perinucleolar caps and PS bodies, respectively.

Figure S6. Co-localization of PS-ASOs with P54nrb in nuclear ASO-induced filaments was observed for ASOs with different sequences and chemical modifications in multiple tested cell lines. A. PS-ASO ISIS446654, delivered to mESCs by electroporation, also formed nuclear filament structure, as exemplified by three different cells. ***B.*** Co-localization of different ASOs with P54nrb to nuclear

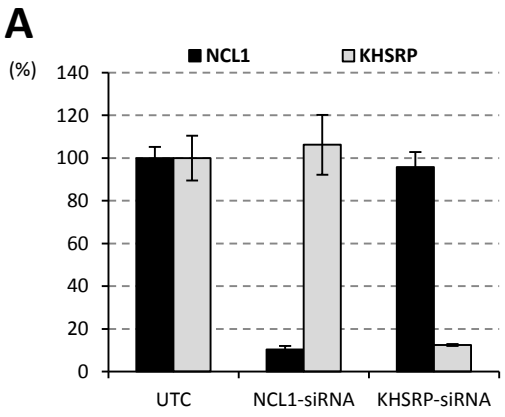
filaments in HeLa cells. Backbone modification, 2'-modification, and sequences of the tested ASOs are indicated. PS-ASOs were transfected at a final concentration of 60 nM for 6 hrs. ***C.*** Co-localization of PS-ASO ISIS446654 with P54nrb (arrows) to nuclear filaments was observed in different tested mouse cell lines including hepatocytes, MHT, and bEND. Paraspeckle protein PSPC1 also localizes to ASO-filaments as exemplified in mouse bEND cells. ***D.*** Formation of P54nrb-containing nuclear filaments in HeLa cells transfected with 60 nM *NEAT1*-specific ASO ISIS407248 for 6 hrs.

Figure S7. An average of 3 to 10 paraspeckles per HeLa cells were observed. A population view of co-IF-staining of P54nrb and RNA-FISH of *NEAT1* RNA (5'-probe) in untreated HeLa cells. An average of 3-10 paraspeckles were observed.

Figure S8. A NEAT1-specific ASO co-localized with P54nrb in distinct nuclear foci in HeLa cells. IF-staining for P54nrb in HeLa cells transfected with 60 nM Cy3-labeled 5-10-5 PS/MOE-modified *NEAT1*-specific ASOs ISIS625751 showed the co-localization of paraspeckle protein P54nrb with *NEAT1*-ASOs in distinct nuclear foci (yellow arrows).

Figure S9. Localization of PS-ASOs to paraspeckle-like structures was not affected by the depletion of a essential paraspeckle protein P54nrb. A. IF-staining for PSF in HeLa cells transfected with 60 nM PS-ASO ISIS454395. Co-localization of PS-ASOs with PSF paraspeckle-like structures was observed in a well-transfected cell, while in the other cell transfected with little or no PS-ASOs, enrichment of PSF in distinct foci (canonical paraspeckles) was observed. ***B.*** IF-staining for PSF in (-) P54nrb-depleted HeLa cells transfected with 60 nM PS-ASO ISIS454395. Co-localization of PS-ASOs with PSF in paraspeckle-like structures was observed in a well-transfected cell, while in the other cell transfected with little or no PS-ASOs, no localization of PSF in canonical paraspeckles was observed.

Figure S1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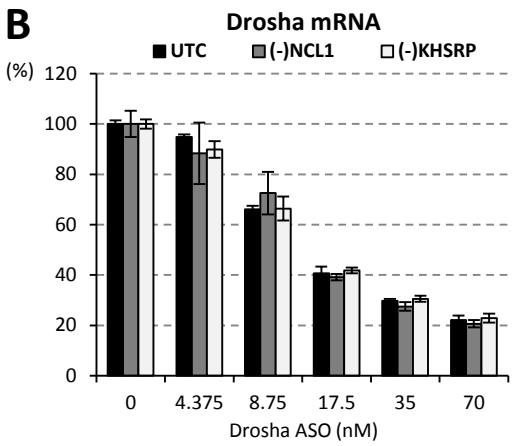
UTC
NCL1-siRNA
KHSRP-siRNA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(-)KHSRP
(%)
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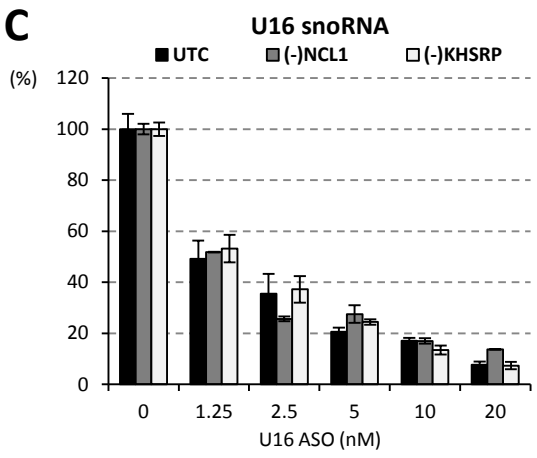
8.75
17.5
35
70
Drosha ASO (nM)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(%)
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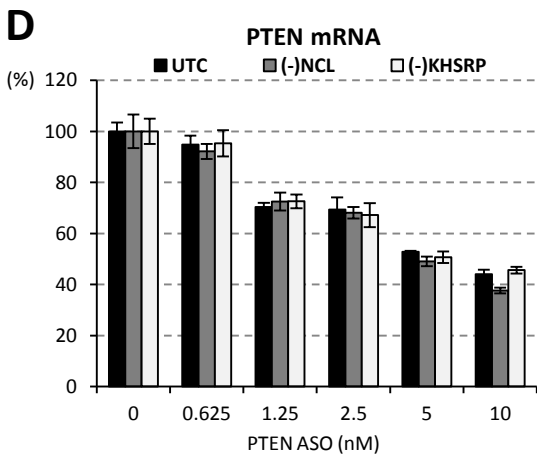
5
10
20
U16 ASO (nM)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(%)
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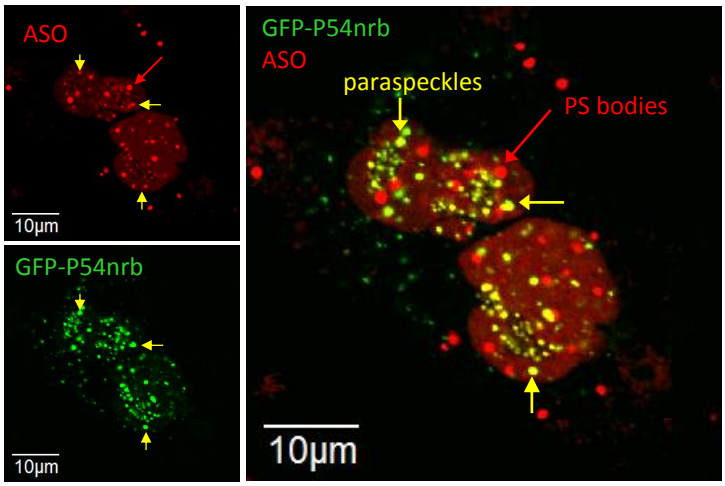
5
10
PTEN ASO (nM)

Figure S2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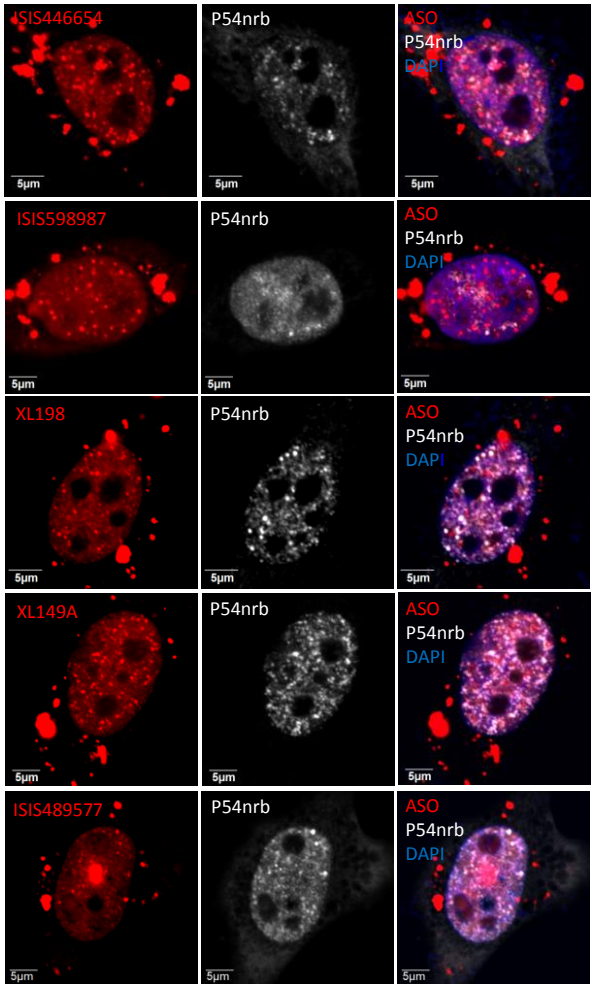
GFP-P54nrb
10μm
GFP-P54nrb
ASO
paraspeckles
PS bodies
10μm

Figure S3

A



	Backbone modification	2'-moiety	Sequence
ISIS446654	P=S	2'-Moe	5'-CTGCTAGCCTCTGGATTTGA-3'
ISIS598987	P=S	2'-cEt	5'-CTGCTAGCCTCTGGATTTGA-3'
XL198	P=S	2'-H	5'-CTGCTAGCCTCTGGATTTGA-3'
XL149A	P=S	2'-OH	5'-UUGUCUCUGGUCCUUACUU-3'
ISIS489577	P=S P=O	Moe 2'-α-Flouro 2'-O-Methyl	5'-TTGTCTCTGGTCCTTACTTAA-3'

B

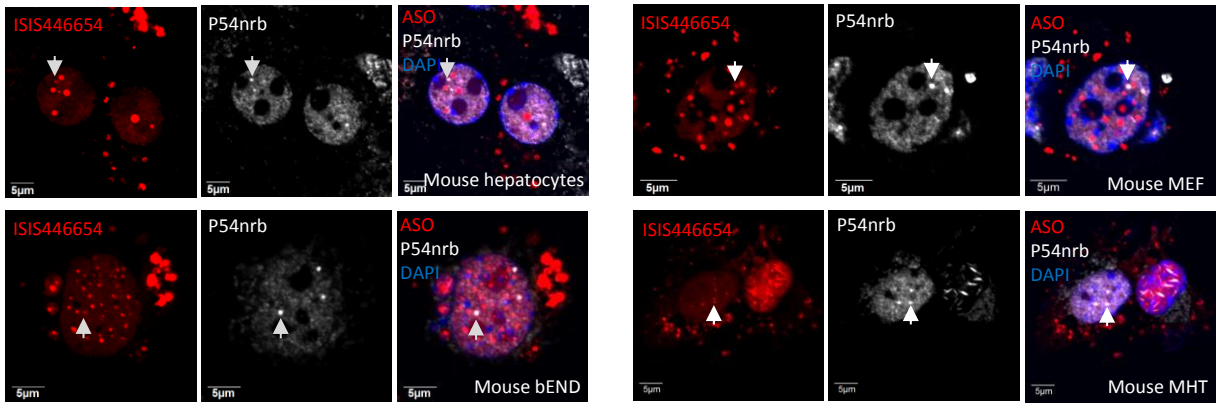


Figure S4

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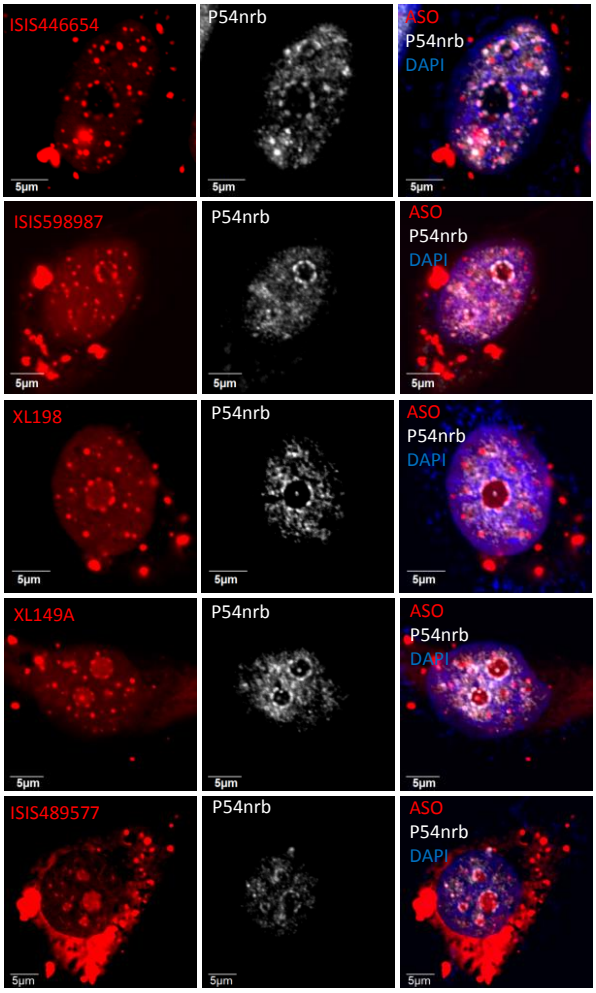


	Backbone modification	2’-moiety	Sequence
ISIS446654	P=S	2’-Moe	5’-CTGCTAGCCTCTGGATTTGA-3’
ISIS598987	P=S	2’-cEt	5’-CTGCTAGCCTCTGGATTTGA-3’
XL198	P=S	2’-H	5’-CTGCTAGCCTCTGGATTTGA-3’
XL149A	P=S	2’-OH	5’-UUGUCUCUGGUCCUUACUU-3’
ISIS489577	P=S P=O	Moe 2’-α-Flouro 2’-O-Methyl	5’-TTGTCTCTGGTCCTTACTTAA-3’

B

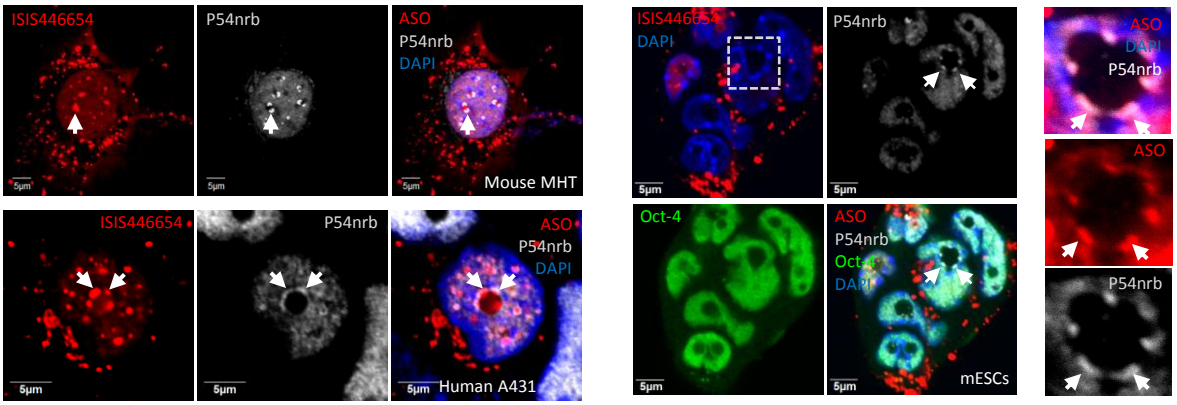
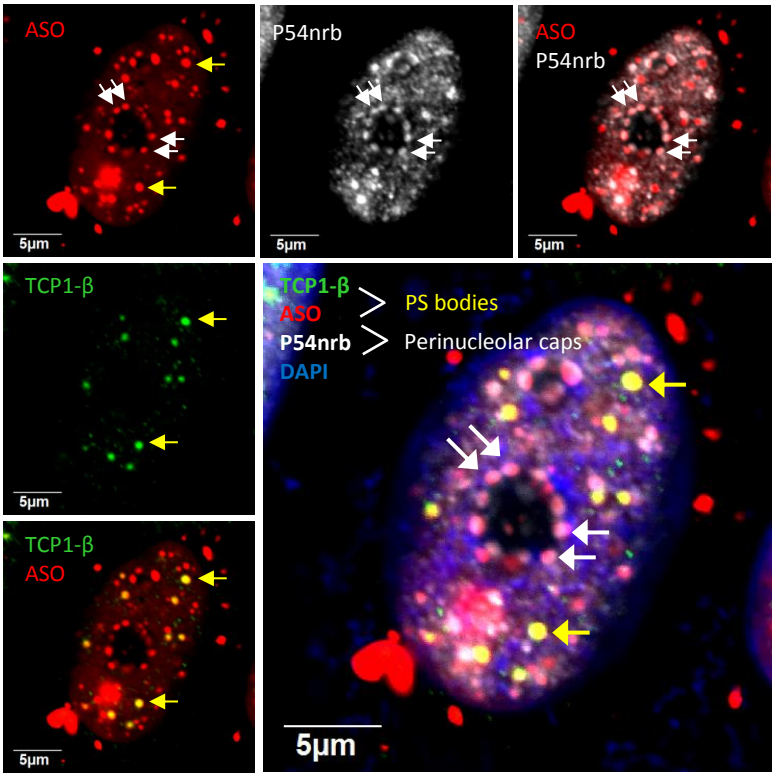


Figure S5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P54nrb
5μm
ASO
P54nrb
5μm
TCP1-β
5μm
TCP1-β
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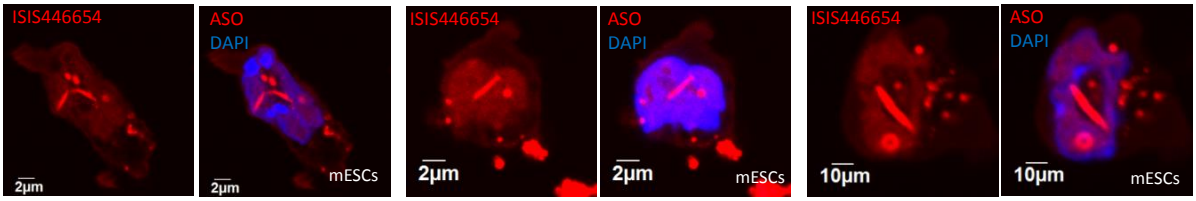
5μm
TCP1-β
ASO
P54nrb
DAPI
PS bodies
Perinucleolar caps
5μm

Figure S6

A



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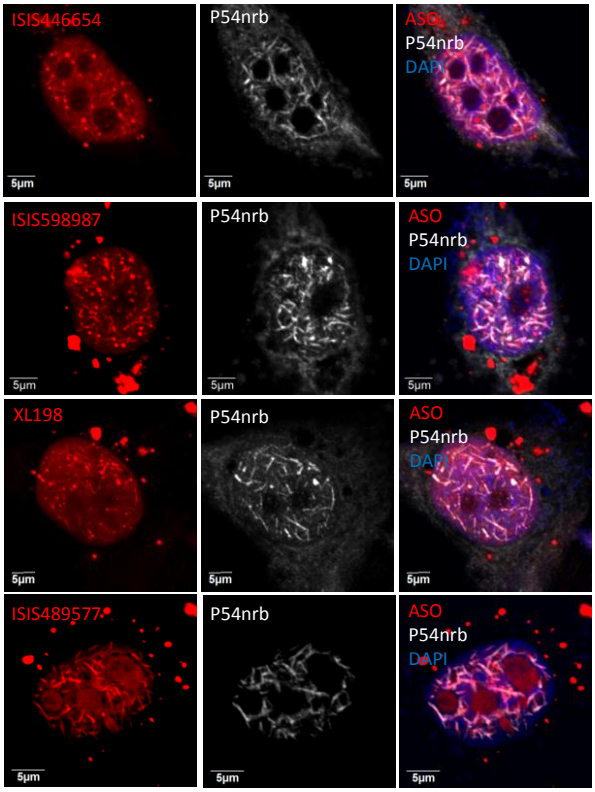

Backbone modification	2'-moiety	Sequence
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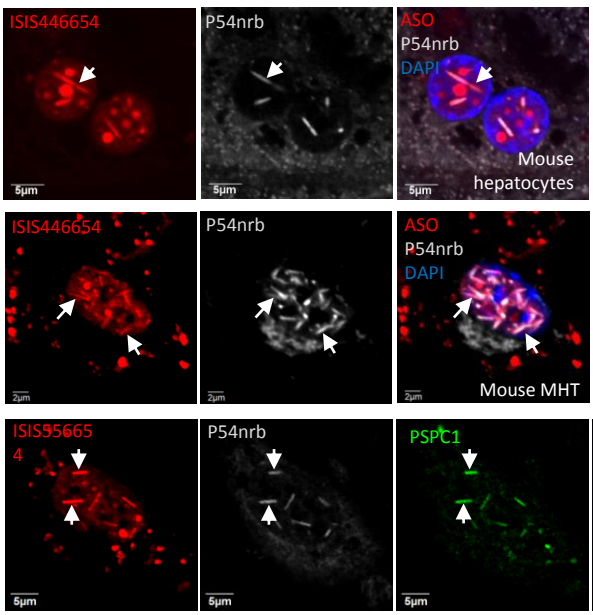
P=S	2'-cEt	5'-CTGCTAGCCTCTGGATTTGA-3'
P=S	2'-H	5'-CTGCTAGCCTCTGGATTTGA-3'
P=S P=O	Moe 2'-α-Flouro 2'-O-Methyl	5'-TTGTCTCTGGTCCTTACTTAA-3'

C



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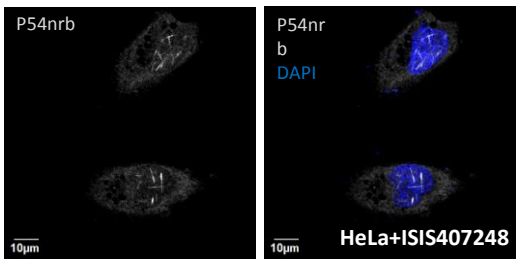
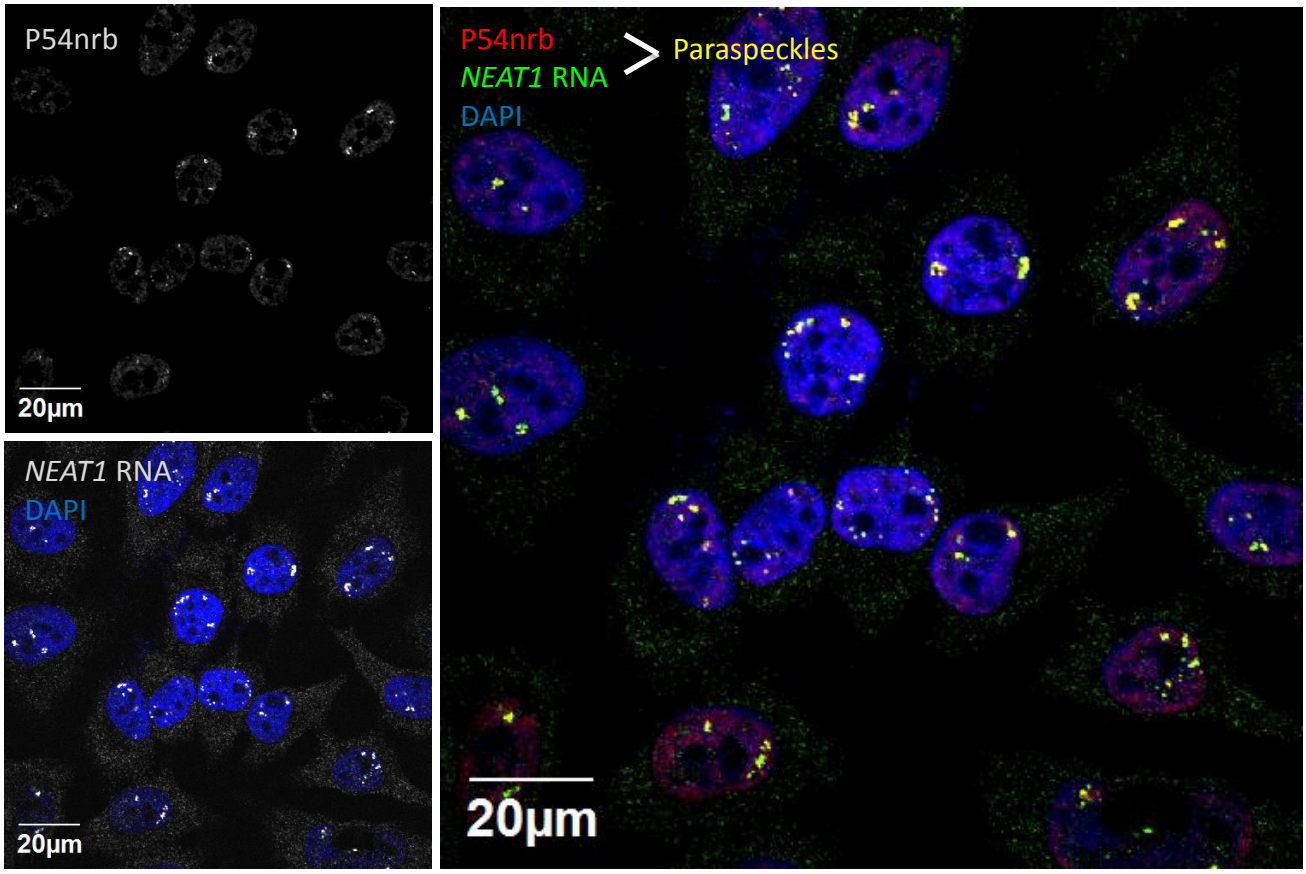


Figure S7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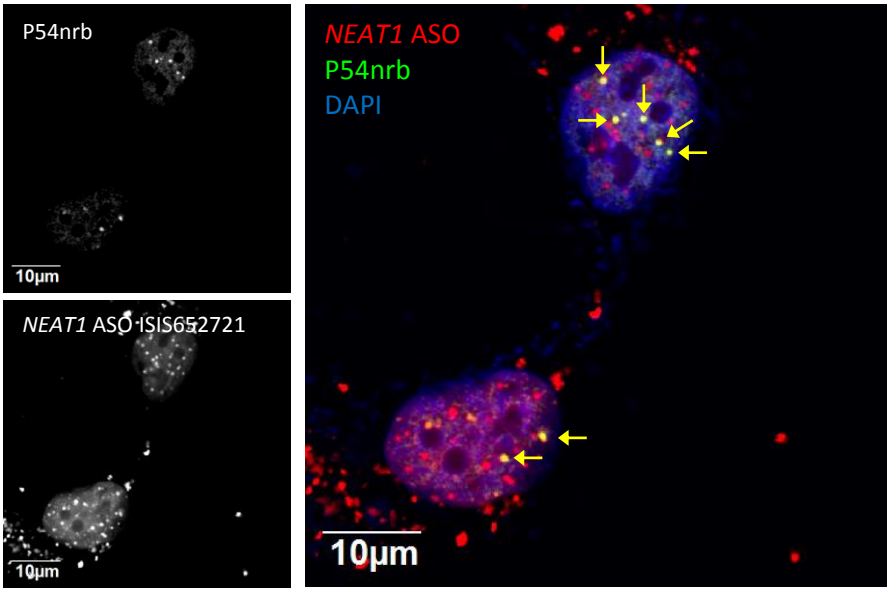
NEAT1 RNA
DAPI
20μm
P54nrb
NEAT1 RNA
DAPI
Paraspeckles
20μm

Figure S8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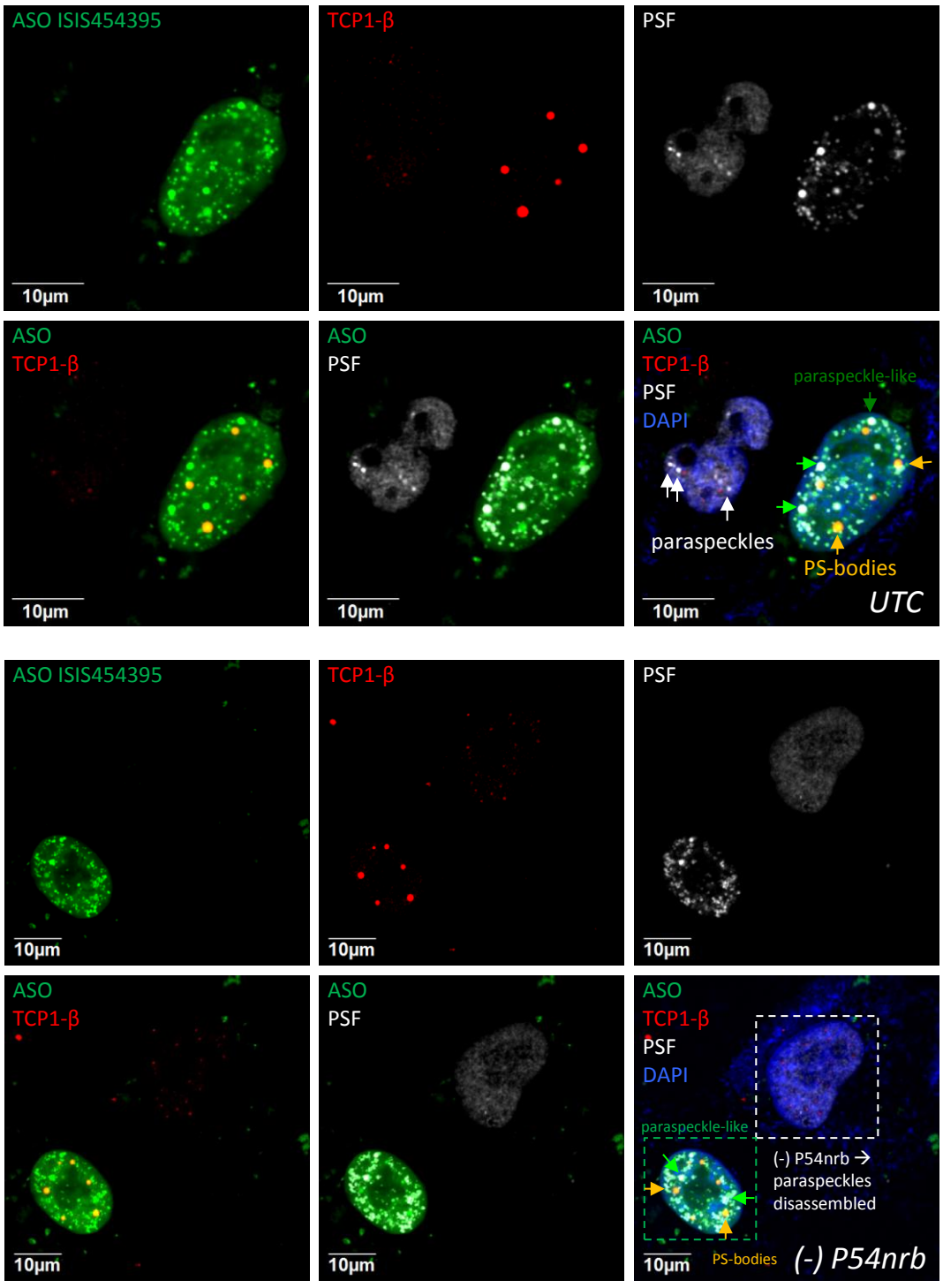
NEAT1 ASO ISIS652721
10μm
NEAT1 ASO
P54nrb
DAPI
10μm

Figure S9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PSF
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ASO
TCP1-β
ASO
PSF
ASO
TCP1-β
PSF
DAPI
paraspeckle-like
paraspeckles
PS-bodies
UTC
B
ASO ISIS454395
TCP1-β
PSF
ASO
TCP1-β
ASO
PSF
ASO
TCP1-β
PSF
DAPI
paraspeckle-like
(-) P54nrb →
paraspeckles
disassembled
PS-bodies
(-) P54nrb