Supplementary Data

TCP1 complex proteins interact with phosphorothioate oligonucleotides and can co-localize in oligonucleotide-induced nuclear bodies in mammalian cells

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Supplemental Materials

Oligonucleotides. Unless indicated, all ASOs used in this study were 5-10-5 RNA/DNA chimeric oligonucleotides with phosphorothioate backbones, with ten deoxyribonucleotides flanked by five 2'-modified ribonucleotides at each end. The sequences of PS-ASOs, siRNAs, and primer probe sets for qRT-PCR are listed below.

ASOs

116847 (PTEN), 5'-C*T*G*C*T*A*G*C*C*T*C*T*G*G*A*T*T*T*G*A-3', the underlined are 2'-MOE modified.

386652 (PTEN), same as 116847, with a 5' biotin.

557442 (PTEN), same as 116847, with a 3' biotin.

446654 (PTEN), same as 116847, labeled at 5' with Cy3.

256903 (PTEN), same as 116847, labeled at 5' with FITC.

582801 (PTEN), 5'-**C*T*G*C*T***A*G*C*C*T*C*T*G*G*A***T*T*T*G*A**-3', the underlined bold nucleotides are 2'-cEt modified.

586183 (PTEN), same as 582801, with a 5' biotin.

XL168D, 5'- C*T*G*C*T*A*G*C*C*T*C*T*G*G*A*T*T*T*G*A-3', PS-DNA oligonucleotides.

XL168, same as XL168D, with a 5' biotin.

XL169R, 5'- C*U*G*C*U*A*G*C*C*U*C*U*G*G*A*U*U*U*G*A, PS-RNA oligonucleotides with 2-O-methyl modification.

XL169, same as XL169R, with a 5' biotin.

XL183, 5'- rCrUrGrCrUrArGrCrCrUrCrUrGrGrArUrUrUrGrA-3', PO-RNA oligonucleotides.

XL209-1, 5'-rC*rU*rG*rC*rU*rA*rG*rC*rC*rU*C*rU*rG*rG*rA*rU*rU*rU*rG*rA-3', PS-RNA oligonucleotide.

XL273, 5'-mCmUmGmCmUmAmGmCmCmUmCmUmGmGmAmUmUmUmGmA-3', 2'-methyl PO-RNA oligonucleotide.

390896, 5'-**C*T*G*C*T***A*G*C*C*T*C*T*G*G*A***T*T*T*G*A-3'**, underlined are locked nucleic acids (LNA).

404130, 5'-C*T*G*C*T*A*G*C*C*T*C*T*G*G*A*T*T*T*G*A-3', the red nucleotides are 2'-fluorine modified.

617422, 5'-**C*T*G*C*T** , the underlined bold nucleotides are 2'-cEt modified.

622868, 5'-**C*T*G*C*T** ***A*G*C*C**, the underlined bold nucleotides are 2'-cEt modified.

622867, 5'- **C*T*G*C*T** ***A*G*C*C*C*C*T*C**, the underlined bold nucleotides are 2'-cEt modified.

622866, 5'- **C*T*G*C*T*** **A*** **G*C*C*C*C*T*C*T*G**, the underlined bold nucleotides are 2'-cEt modified.

617421, 5'- **C*T*G*C*T*** **A*G*C*C*C*C*T*C*T*G*G*A**, the underlined bold nucleotides are 2'-cEt modified.

622869, 5'- **C*T*G*C*T*** **AGCCCTCTGGA**, the underlined bold nucleotides are 2'-cEt modified.

622865, 5'- **CTGCT*** **A*G*C*C*C*C*T*C*T*G*G*A**, the underlined bold nucleotides are 2'-cEt modified.

622870, 5'- **CTGCT** **AGCCCTCTGGA**, the underlined bold nucleotides are 2'-cEt modified.

367070 (P53), 5'-A*G*C*G*C*A*G*A*C*A*A*A*C*C*C*A*T*C*A*C, the underlined are 2'-MOE modified.

110074 (NCL1), 5'- G*T*C*A*T*C*G*T*C*A*T*C*C*T*C*A*T*C*A*T, the underlined are 2'-MOE modified.

395254(Malat1), 5'- G*G*C*A*T*A*T*G*C*A*G*A*T*A*A*T*G*T*T*C, the underlined are 2'-MOE modified.

siRNAs:

TCP1-α, ID No: S224715, from Ambion
TCP1-β, ID No: S20756, S20757, from Ambion
TCP1-ε, ID No: 19566 and 136414, from Ambion.
LRPPRC, ID No: HSS115402, HSS115403, from Life Technologies.
RAN, ID No: SC-36382, from Santa Cruz Biotechnologies.
KHSRP, ID No: HSS112552, HSS189434, from Life Technologies.

shRNA lentiviral particles:

Control shRNA, sc-108080, from Santa Cruz Biotechnologies.
TCP1- β shRNA, sc-36622-V, from Santa Cruz Biotechnologies.

Primer probe sets for qRT-PCR

TCP1-α:

Forward: 5'- TTCTCTTGGTCCAGTTGGCTTGGA-3'
Reverse: 5'-TCTTGCAGATCAGCCAGCTCACAA -3'
Probe: 5'- ACTGGAGGTAGAACATCCTGCAGCTA -3'

TCP1-β:

Forward: 5’-TTCTAAGCAGTGGACGAGATGCCT -3’
Reverse: 5’-ACGGTAACAGAGGTAGTGCCATCA -3’
Probe: 5’-ACATTGGTGTTGACAATCCAGCAGCT -3’

TCP1-ε:
Forward: 5’-TGGTCAACAGTTGTCACCGACAGA-3’
Reverse: 5’-TCCTTGTCCACAATCACGCCCTTA-3’
Probe: 5’-ATTGCTGTGAATGCCGTCCTCACTGT -3’

LRPPRC:
Forward: 5’-TCTCACCAACTGATTTCCTGG-3’
Reverse: 5’-GAATACTGCCTCTGTAACTGGG-3’
Probe: 5’-CACTCGATTTGGTTGAATGTTTGCTTCC-3’

RAN:
Forward: 5’- GAGCCCCAGGTCCAGTTCA-3’
Reverse: 5’- TGACGTTTCACGAAGGTCGTT-3’
Probe: 5’- CTTGTATTGGTTGGTGATGGTGGTACTGGAA-3’

KHSRP:
Forward: 5’-TTCTCAACTTGGACCCATCC-3’
Reverse: 5’-CCACCGCAATCTGTACTTTG-3’
Probe: 5’-TGTTAATTTGTTCACCTCCTCTGCCCC-3’

NCL1:
Forward: 5’- GCTTGGCTTCTTCTGGACTCA -3’
Reverse: 5’- TCGCGAGCTTCACCATGA -3’
Probe: 5’- CGCCACTTGTCCGCTTCACACTCC-3’

Malat1:
Forward: 5’- GCTTGGCTTCTTCTGGACTCA -3’
Reverse: 5’- TCGCGAGCTTCACCATGA -3’
Probe: 5’- CGCCACTTGTCCGCTTCACACTCC-3’

Antibodies. Primary antibodies used for immunofluorescence staining or western analysis were: TCP1-α (ab90357 and ab109126 from Abcam, and NBP1-40822 from Novus), TCP1-β (ab92746, Abcam; NBP1-40823, Novus), TCP1-ε (SC-376188, SCBT), TCP1-η (SC-271439, SCBT), TCP1-θ (ab96321 and ab88223, Abcam), VPS28 (Novus, NBP1-03506; sc-166514, SCBT), Ku70 (ab3114, Abcam), Ku80 (ab119935, Abcam), hnRNP K (ab32969, Abcam), RAN (ab155103, Abcam), Lamp1 (ab25630, Abcam), GAPDH (G8795,Sigma; SC-25778, SCBT), ACTB (A5316, Sigma), α-tubulin (T5168, Sigma), and γ-tubulin (T6557, Sigma). RNase H1 (Ab56560, ms, Abcam). In addition, a rabbit RNase H1 antibody was

kindly provided by Hongjiang Wu. Secondary antibodies conjugated with either HRP (for western) or different fluorophores (for immunofluorescence staining) were purchased from Abcam.

Supplemental Figure legends

Figure S1. TCP1-β binds PS- but not PO-ASOs and is not present in the same ASO-protein complex with RNase H1. **A**) ASO-binding proteins were isolated using a PS-cEt ASO, and bound proteins were eluted by competition using ASOs containing different modifications, as indicated. Two third of eluted material was analyzed for Ku70 and TCP1β proteins by western analysis. The remaining material was separated on an 8%, 7M urea PAGE gel, and the presence of elution ASOs was determined by ethidium bromide staining. **B**) ASO-binding proteins (ASO-protein) were first enriched using a two-step affinity selection approach with PS-cEt-ASOs, and immunoprecipitation was performed from the ASO-protein pools using an RNaseH1 antibody (raised from rabbit, a gift kindly provided by Hongjiang Wu), or a GAPDH antibody (SC-25778, SCBT) as a negative control. The immunoprecipitated proteins were analyzed by western analysis for TCP1-ε (SC-376188, SCBT), TCP1-β (Ab119923, Abcam), and RNase H1 (Ab56560, Abcam). WC, 0.5% of whole cell extract used as input for ASO affinity selection. ASO-Protein, 10% of ASO-protein pool as used for immunoprecipitation.

Figure S2. **Only TCP1-β subunit appears to be present in the PS-bodies**. **A**) HeLa cells transfected with 60 nM ASO ISIS446654 for 4 hr were fixed, and stained individually for four different subunits of the TCP1 complex (FITC). **B**) TCP1-β is detected in the nuclear PS-bodies with a different antibody. HeLa cells transfected with PS-ASOs as in panel A were fixed and stained for TCP1-β (FITC) using antibody NBP1-40823 (Novus), rather than ab92746 (Abcam) as shown in other figures.

Figure S3. **TCP1-β stained PS-body formation depends on the concentration but not sequence of PS-ASOs. A)** HeLa cells were transfected for 4 hr with PS-ASO ISIS116847 at different concentrations as indicated above lanes. **B**) HeLa cells were transfected for 4 hr with 60 nM PS-ASOs with different sequences as indicated by the ASO numbers. Cells were fixed and stained for TCP1-β (AF647). The nuclei exhibiting PS bodies are indicated by arrows. Scale bars: 10 and 20 μm for panels A and B, respectively.

Figure S4. **The effect of reduction of TCP1 proteins on ASO activity is moderate but highly reproducible**. A) Reduction of expression of different TCP1 subunits can decrease ASO antisense activity. Left panel, qRT-PCR analyses for mRNA levels in cells treated or not treated with siRNAs targeting different TCP1 subunits, as described in Figure 3. Right panel, qRT-PCR for NCL1 mRNA

levels in different test cells treated with ASOs, as in Figure 3. B) Similar experiment was performed as in panel A. In all cases, moderately reduced ASO activity was observed. The error bars represent standard deviation of three independent experiments.

Figure S5. The influence of TCP1 reduction on ASO activity was not due to non-specific effects. A) TCP1-β levels were reduced by treatment of cells with two different siRNAs. HeLa cells were treated with TCP1- β siRNAs for 24 hr. Total RNA was prepared and the level of TCP1-β mRNA was determined by qRT-PCR. **B**) Reduction of TCP1-β by two different siRNAs led to similar effect on the activity of PS-ASOs. HeLa cells treated or not treated with TCP1-β siRNAs were transfected for 4 hr with different concentrations of PS-ASOs targeting NCL1 mRNA. Total RNA was prepared and the levels of NCL1 mRNA were determined by qRT-PCR. **C**) HeLa cells treated with 5 nM siRNAs targeting LRPPRC, KHSRP, or U16 for 24 hr were transfected again for 4 hours with PS-ASOs targeting NCL1 mRNA. The siRNA-mediated reduction of LRPPRC and KHSRP mRNAs were analyzed by qRT-PCR. The levels of TCP1 mRNAs in the test cells were also analyzed. **D**) Treatment by siRNAs targeting LRPPRC or KHSRP, or U16 snoRNA had no significant effect on the activity of PS-ASOs targeting NCL1 mRNA, as determined by qRT-PCR. **E**) qRT-PCR analysis for the reduction of TCP1β using lentiviral shRNA particles. HeLa cells were transducted with lentiviral particles expressing control shRNAs or shRNAs targeting TCP1-β for 48 hr, followed by transfection of ASOs targeting different mRNAs for 4 hr. The level of TCP1β mRNA was determined by qRT-PCR. The effect of reducing TCP1-β on ASO activity was determined by analyzing the mRNA levels targeted by ASOs specific to Drosha (panel **F**), NCL1 (panel **G**), or PTEN (panel **H**). The error bars represent standard deviation of three independent experiments for the above described panels. **I**) Reduction of TCP1-β by siRNA treatment had no significant effect on actin network. HeLa cells treated or not treated with TCP1-β siRNA as used in Fig. 2 were fixed, and stained for ACTB protein (FITC). Scale bars: 20 μm. **J**) TCP1-β-depleted cells as used in panel E were fixed and stained for α-tubulin (FITC). UTC, untreated control cells. Scale bars: 10 μm.

Figure S6. PS-bodies form in a time-dependent manner. HeLa cells were transfected with 60 nM PS-ASO ISIS446654 for different times, fixed, and stained for TCP1-β (AF647). Representative cells are shown and the PS-bodies are indicated by arrows. PS-bodies start to form around 2 hr after transfection and majority of cells already contain PS-bodies at 4 hr post-transfection. Scale bars: 10 μm.

Figure S7. **RAN depletion reduced nuclear ASO levels but did not completely block PS-body formation.** HeLa cells treated with RAN specific siRNAs for 24 hr were transfected again with 60 nM ISIS446654 for 5 hr, fixed, and stained for TCP1-β (AF647). PS-bodies were observed in RAN-depleted cells even with low levels of diffuse nuclear ASOs. Scale bars: 20 μm.

Figure S8. 3D analysis for co-localization of TCP1-β and ASOs with LAMP1 in the cytoplasm upon free uptake. HeLa cells were incubated with 1.5 μM ASO ISIS446654 for 24 hr, fixed, and stained for TCP1-β and LAMP1. A Z-section imaging was performed with 0.26 μM thickness per slide for 12 slides. A) A single Z-section was shown for different channels, as well as overlaps between channels. The co-localization of TCP1-β, ASO, and LAMP1 was exemplified using arrows. Scale bars: 10 μm. B) A 3-D image of an amplified area, as shown in a dash box in the lower right panel of Panel A. The DAPI stained nucleus was pseudocolored to pink. The two arrows indicate the two co-localized loci within the box as marked in Panel A.

Figure S9. The cytoplasmic PS-body-like structures do not co-localize with LAMP1 in RAN reduced cells upon PS-ASO transfection. RAN-depleted and control cells described in Fig. 6 were transfected with 60 nM PS-ASO ISIS446654 for 12 hr, and co-stained for TCP1-β (FITC) and LAMP1(AF647). The cytoplasmic PS-body-like structures contain TCP1-β and PS-ASOs, but not LAMP1 protein, as indicated by blue arrows. LAMP1 largely co-localizes with PS-ASOs (white colored dots in the merge panel), but not with TCP1-β protein (gray arrow head). The white arrow indicates a nuclear PS-body. Scale bars: 10 μm.

Fig. S1

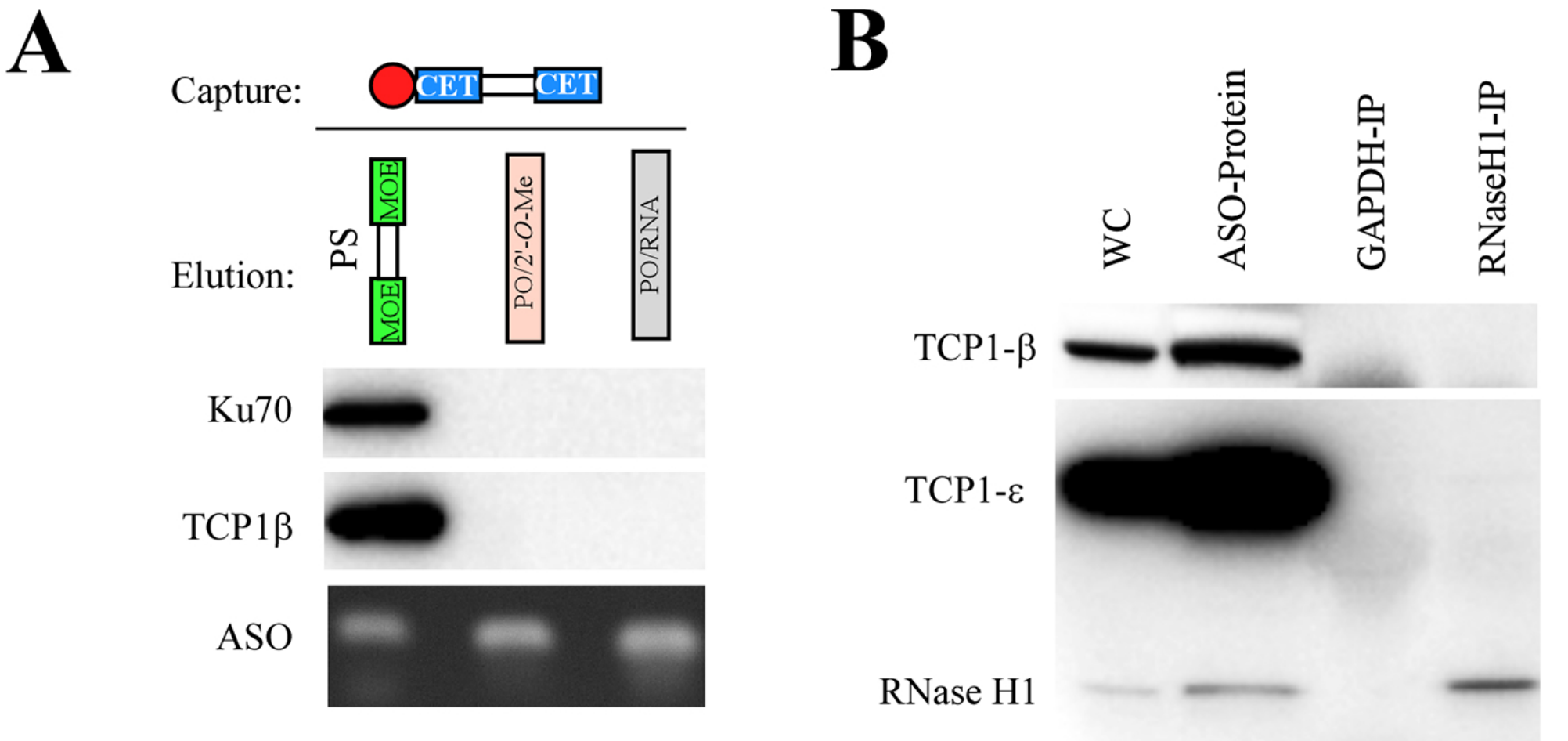
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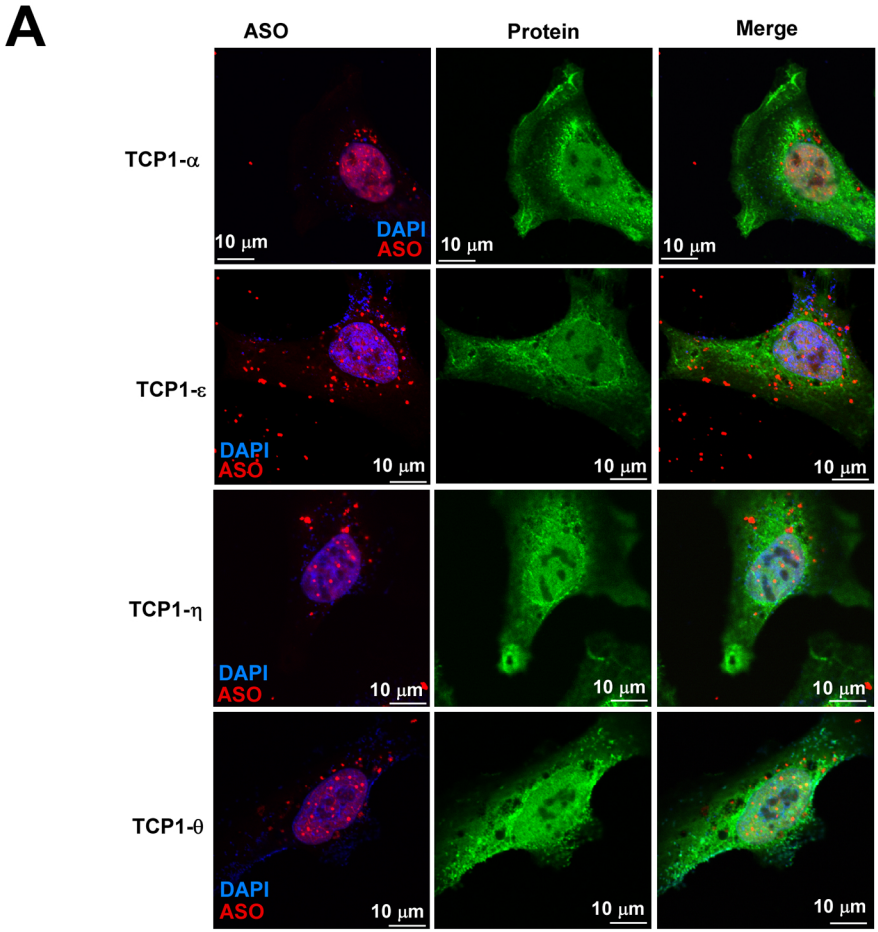
Fig. S2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TCP1-θ
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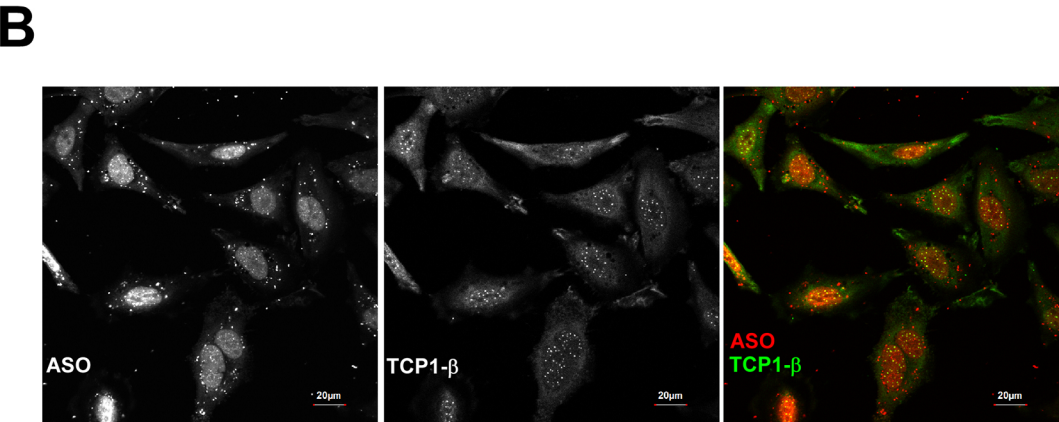
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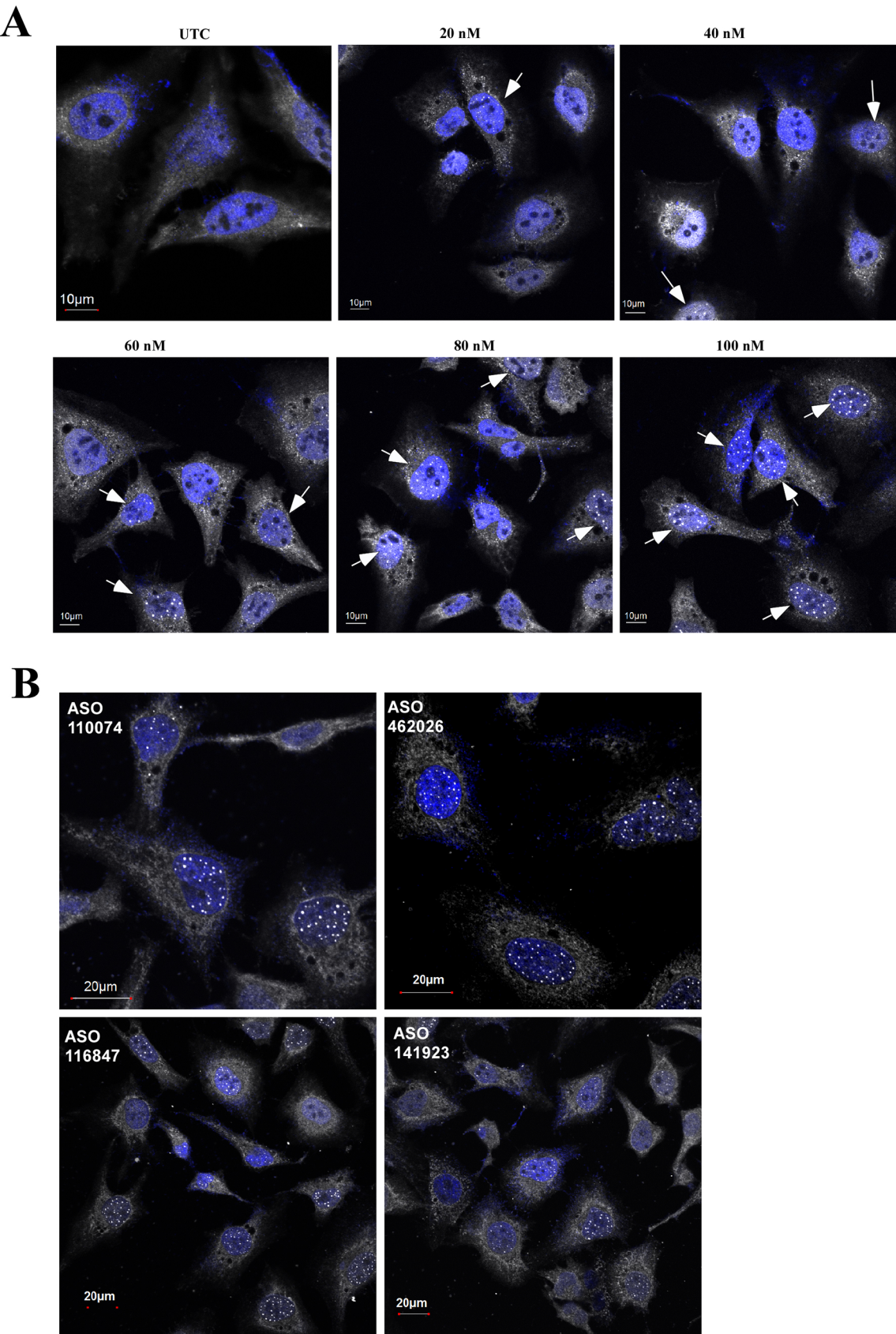
ASO
TCP1-β
ASO
TCP1-β
20μm

Fig. S3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40 nM
60 nM
80 nM
100 nM
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ASO
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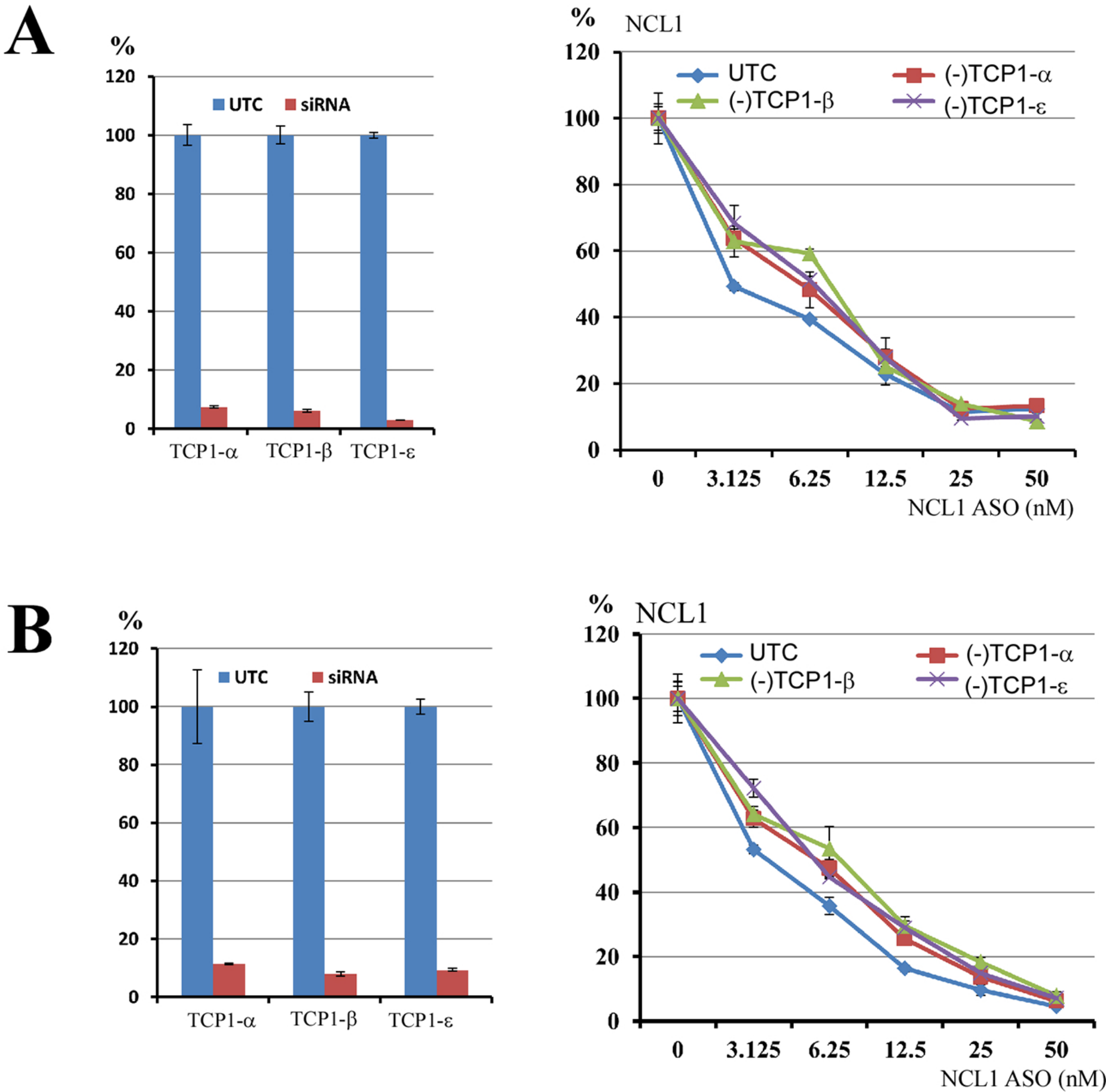
ASO
141923
20μm

Fig. S4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80
60
40
20
0
UTC
(-)TCP1-α
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3.125
6.25
12.5
25
50
NCL1 ASO (nM)
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120
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40
20
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UTC
siRNA
TCP1-α
TCP1-β
TCP1-ε
%
NCL1
120
100
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20
0
UTC
(-)TCP1-α
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25
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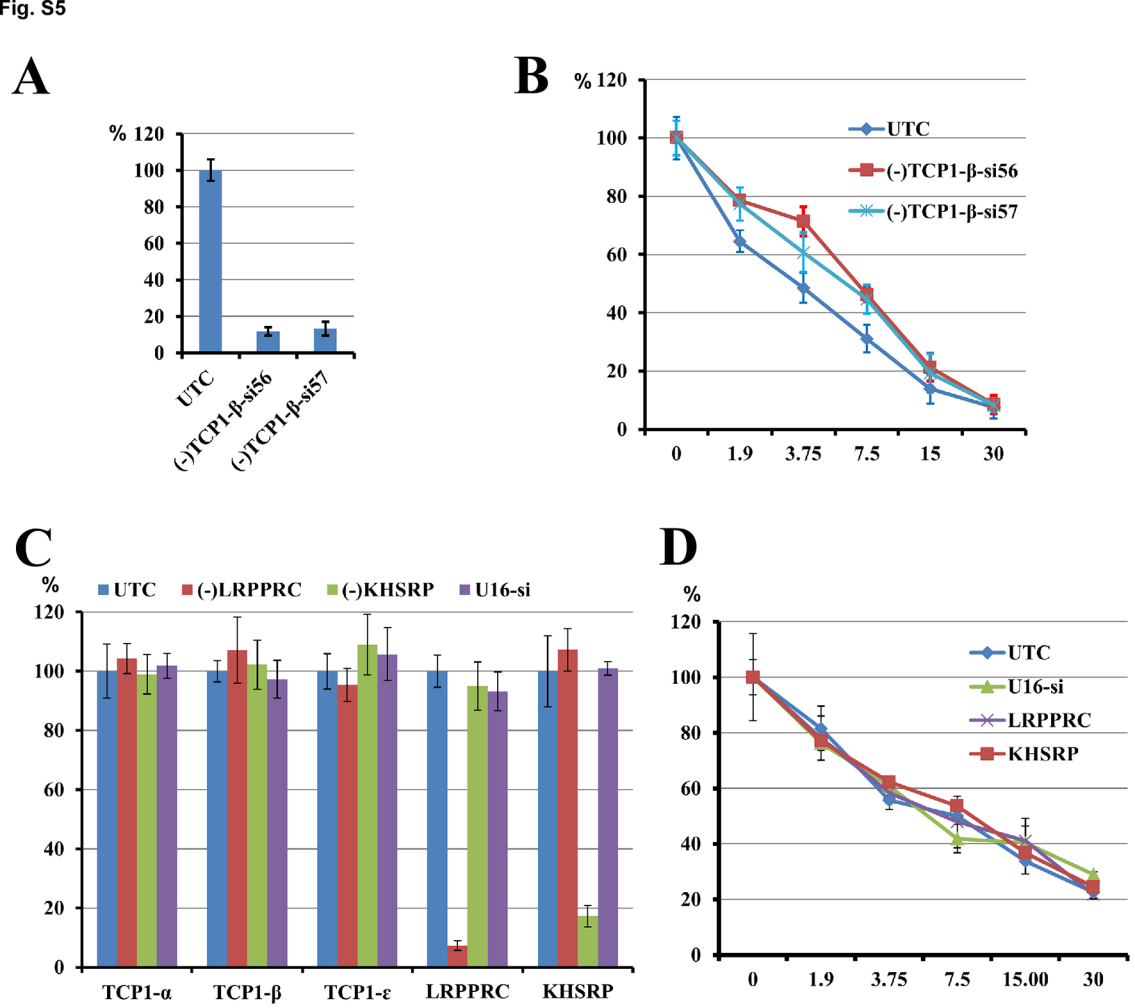
NCL1 ASO (nM)

Fig. S5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7.5
15
30
C
%
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100
80
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KHSRP
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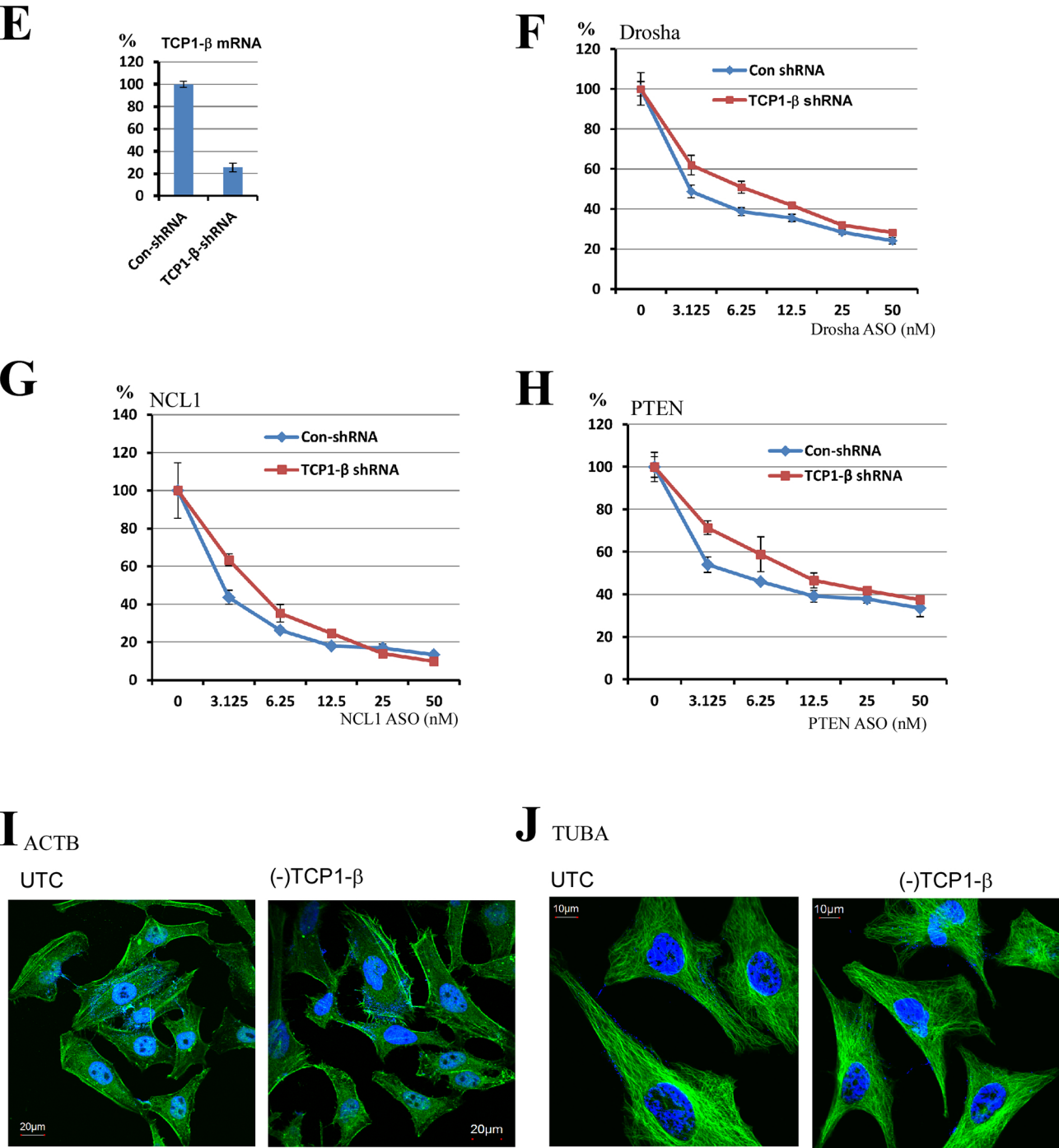
Fig. S5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Drosha ASO (nM)
G
% NCL1
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120
100
80
60
40
20
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Con-shRNA
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NCL1 ASO (nM)
H
% PTEN
Con-shRNA
TCP1-β shRNA
0 3.125 6.25 12.5 25 50
PTEN ASO (nM)
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20μm
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10μm
10μm

Fig. S6

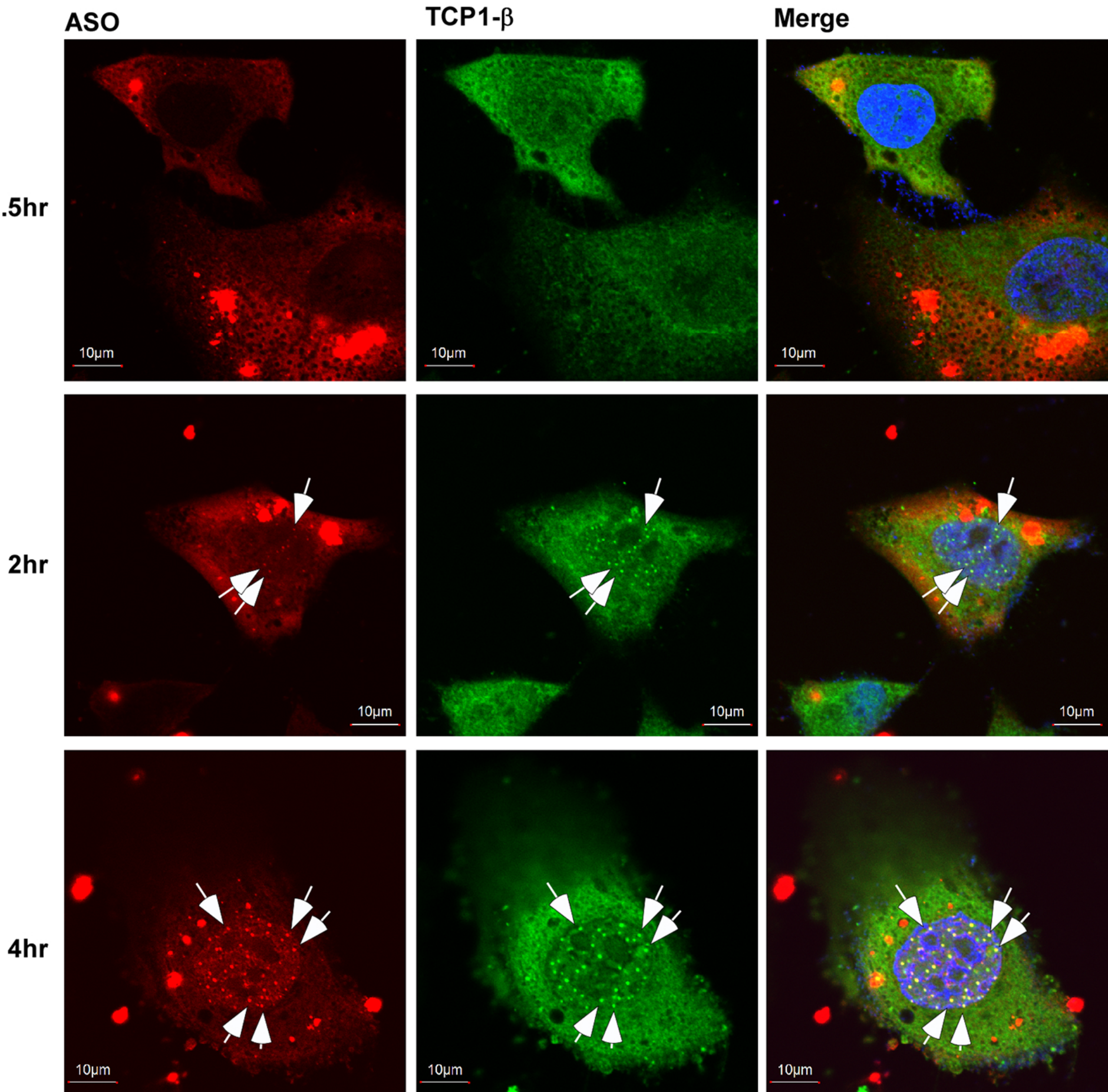

Fig. S7

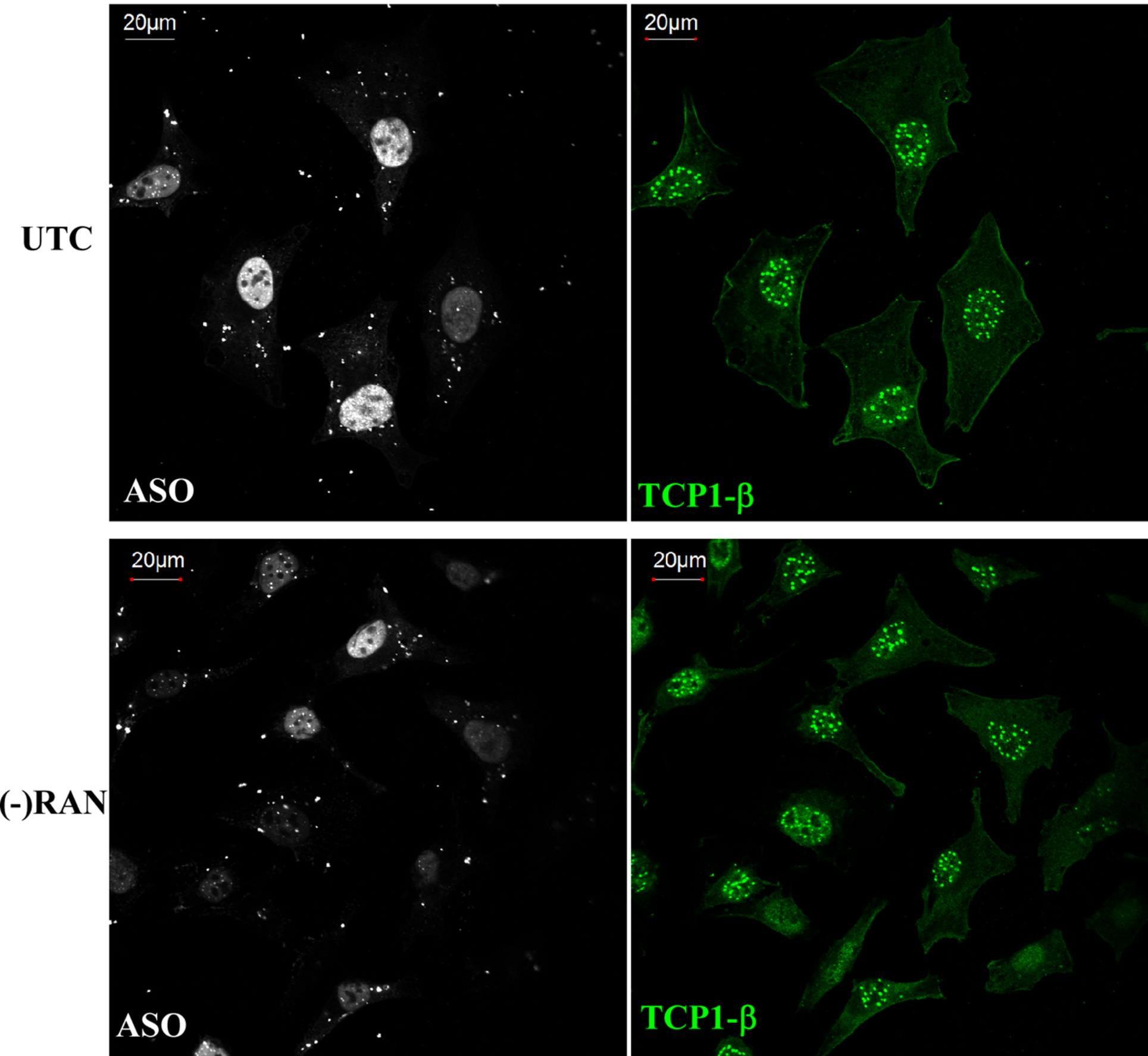

Fig. S8

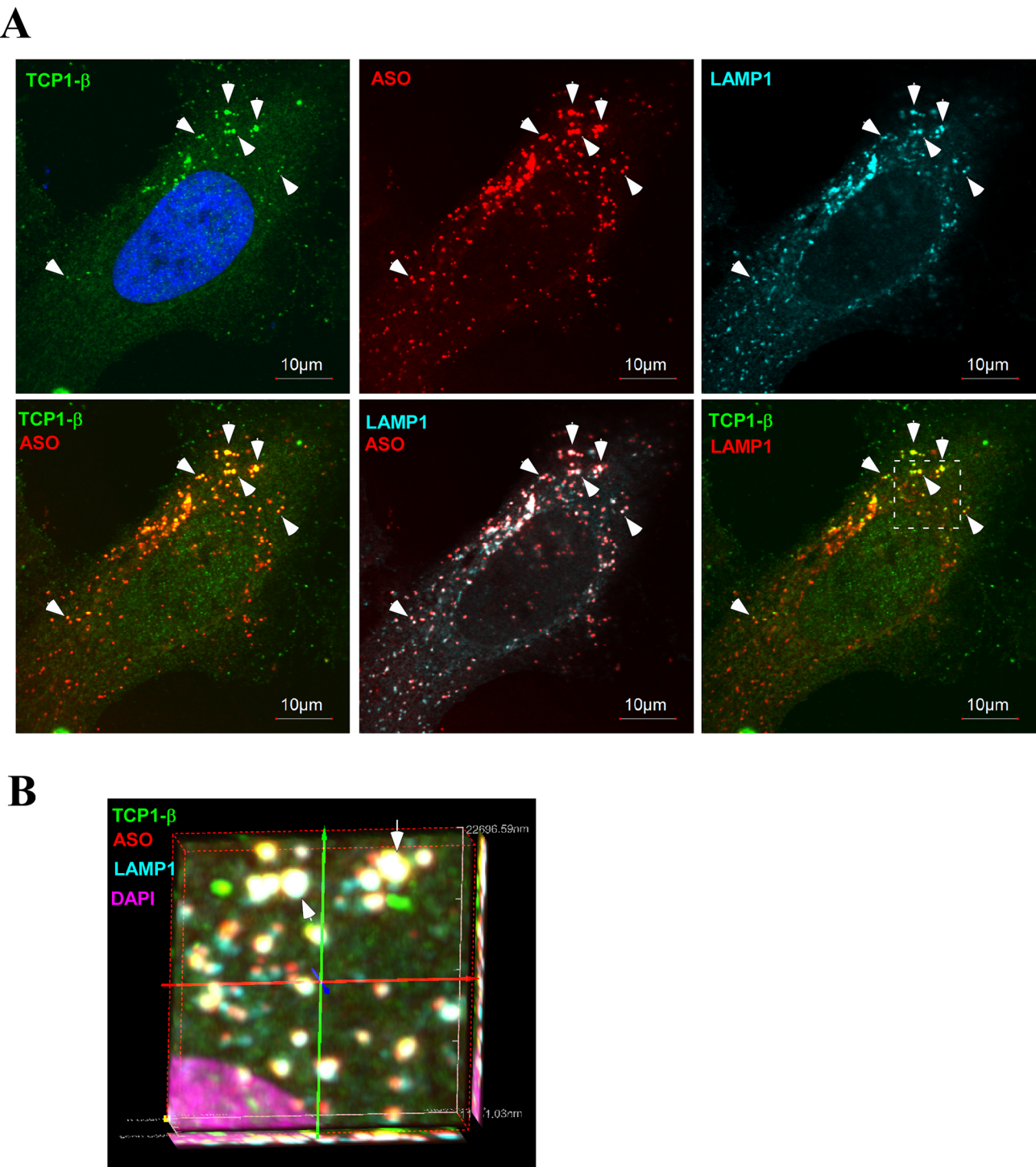
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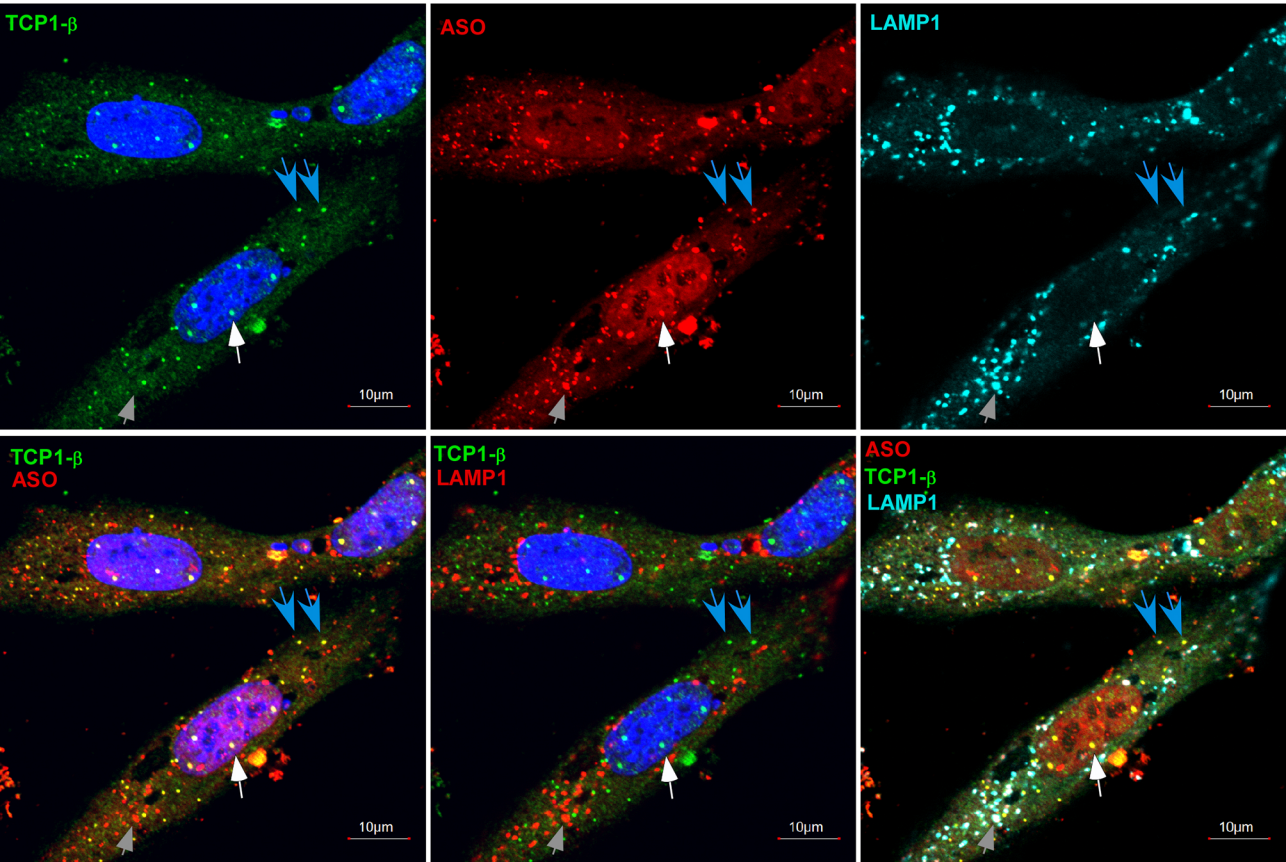
Fig. S9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10μm
10μm