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Supplemental Information

Topoisomerase 3α Is Required for Decatenation and Segregation of Human mtDNA

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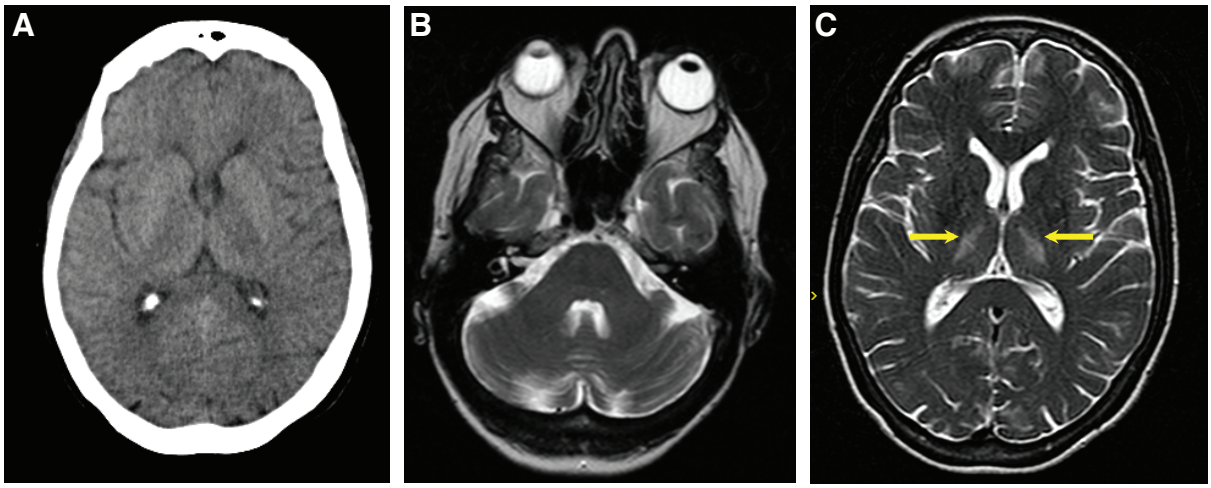
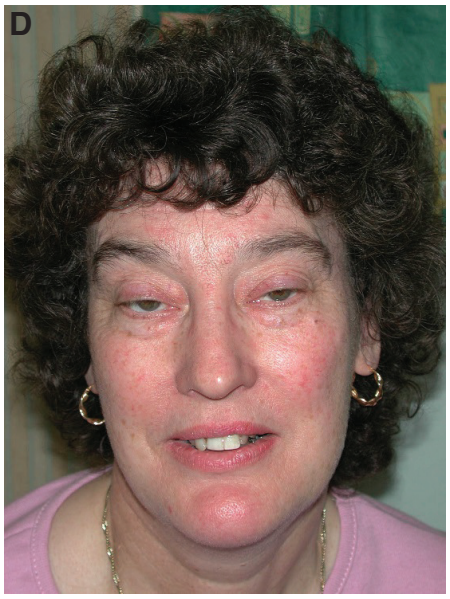
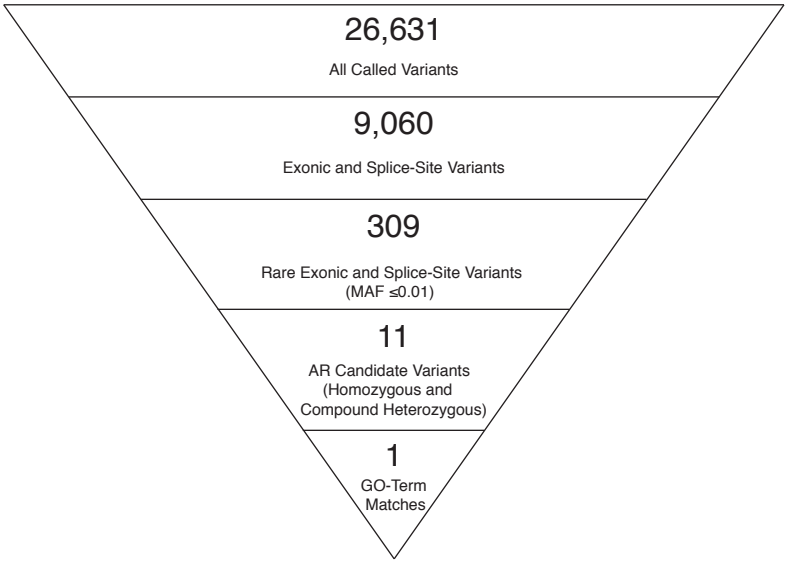
Fig. S1 - Related to Figure 2.





F



E

Mean Depth per CCDS (bp)	51.78
Min Depth	0
Max Depth	2415
# CCDS Bases Covered 30-fold	21431786
% CCDS Bases Covered 30-fold	65.05
# CCDS Bases Covered 20-fold	24682166
% CCDS Bases Covered 20-fold	74.91
# CCDS Bases Covered 10-fold	27360019
% CCDS Bases Covered 10-fold	83.04
# CCDS Bases Covered 5-fold	28781235
% CCDS Bases Covered 5-fold	87.35
# CCDS Bases Covered 1-fold	30571544
% CCDS Bases Covered 1-fold	92.79

G

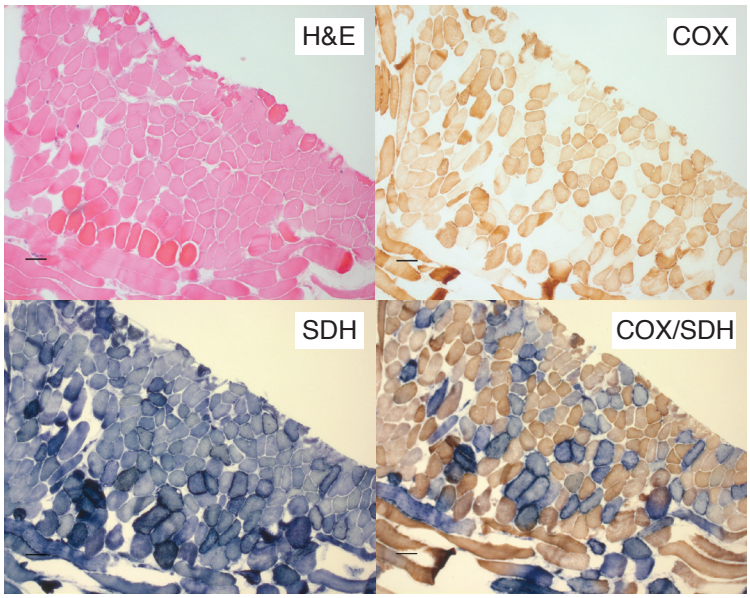


Figure S1 - Related to Figure 2.

(A-C) Neuroimaging of patient. **(A)** CT head demonstrated no abnormality, however **(B)** MRI head (T2 axial view) demonstrated mild cerebellar atrophy and bilateral, symmetrical hyperintensities within the thalami (highlighted by arrows) **(C)**. **(D)** Clinical features of the patient with *TOP3A* variants, highlighting marked bilateral ptosis and use of frontalis muscle to elevate the eyelids. (**E**) Whole exome sequencing read coverage and depth statistics for the TOP3A patient. '#' - number, 'CCDS' – exome consensus coding sequence. **(F)** WES analysis and filtering for rare, autosomal recessive variants in nuclear genes involved in mitochondrial function, DNA repair, transcription and replication identified only compound heterozygous *TOP3A* variants. **(G)** Muscle histopathology reveals numerous COX-deficient, ragged-red fibers. Skeletal muscle sections were stained with hematoxylin and eosin (H&E), or investigated by enzymatic histochemical reactions for SDH and COX activities. In the sequential COX-SDH reaction, cells with normal COX activity are brown whilst those deficient in COX activity are blue (COX-deficient, SDH-positive).

Fig. S2 - Related to Figure 3.

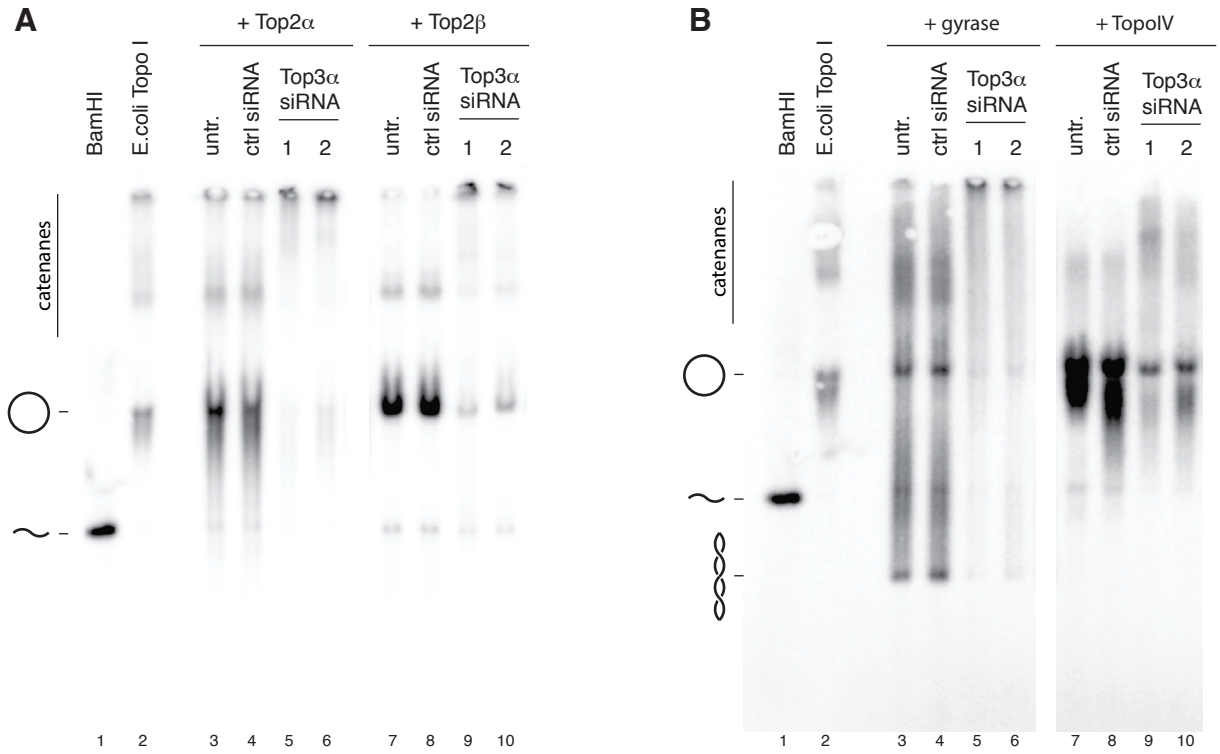


Figure S2 - Related to Figure 3.

(A) Uncut total DNA (3 μg) from control siRNA or Top3α siRNA treated cells was treated with Top2α or Top2β prior to separation on low-percentage agarose gels, then detected using probe (a). BamHI-treated DNA marks the migration of linear mtDNA and *E. coli* TopoI-treated DNA marks the migration of relaxed open circular form mtDNA. **(B)** Uncut total DNA (3 μg) from control siRNA or Top3α siRNA treated cells was treated with *E. coli* gyrase or *E. coli* TopoIV prior to separation on low-percentage agarose gels as in (A).

Fig. S3 - Related to Figure 4.

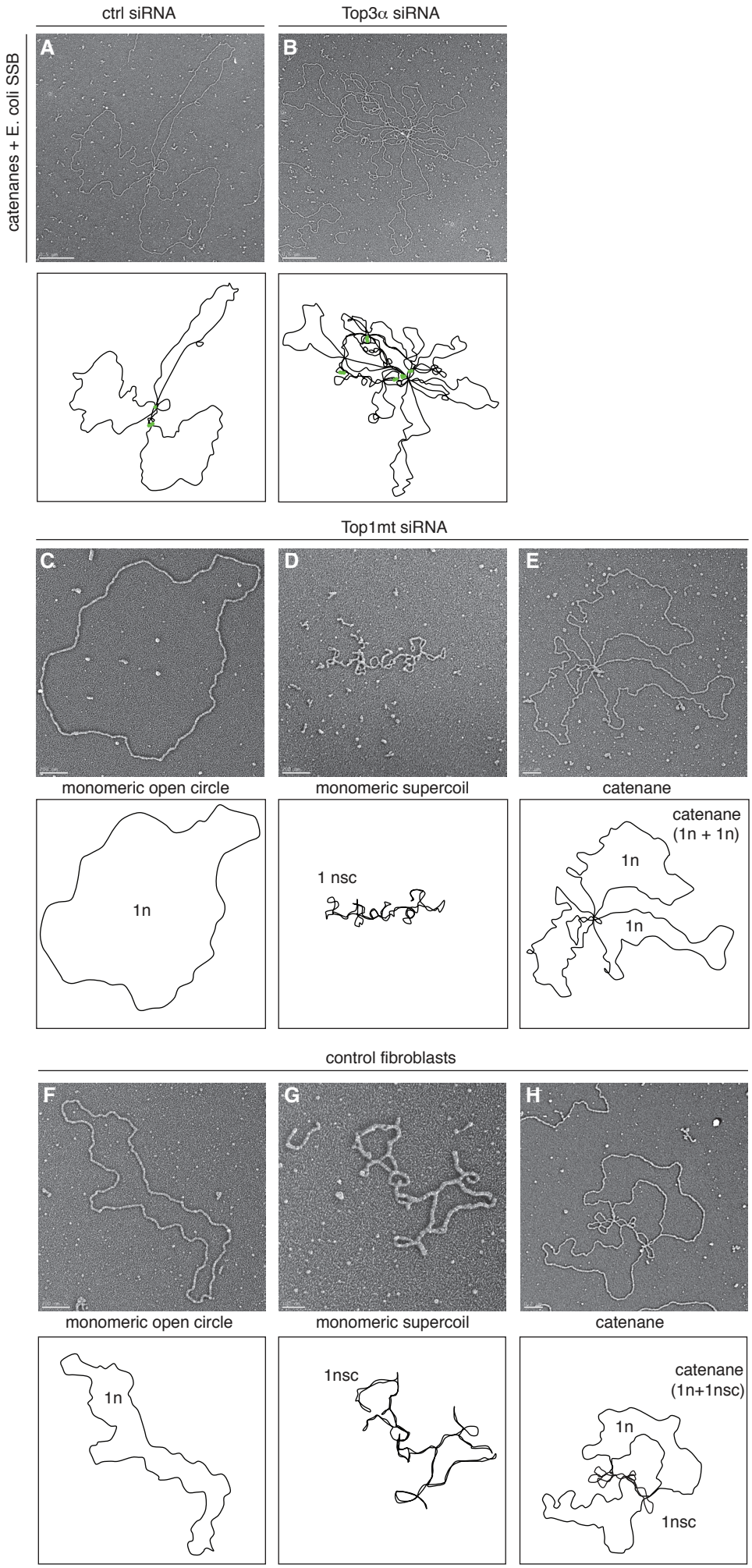


Figure S3 - Related to Figure 4.

(A+B) Representative electron microscopy images of catenated mtDNA from control **(A)** and Top3α **(B)** siRNA-treated cells bound to *E. coli* SSB protein, with interpretations. Only few molecules of *E. coli* SSB protein (represented by green circles in the lower explanatory diagrams) were found bound to mtDNA. Scale bars, 0.5 μm. **(C-E)** Representative EM images of mtDNA from Top1mt depleted cells, with interpretations. **(C)** Monomeric open circle (1n). **(D)** Supercoiled monomeric mtDNA circle (1nsc). **(E)** Catenane consisting of dimeric circles. All scale bars, 200 nm. **(F-H)** Representative EM images of mtDNA from control human fibroblasts, with interpretations. **(F)** Monomeric open circle (1n). Scale bar, 200 nm. **(G)** Supercoiled monomeric mtDNA circle (1nsc). Scale bar, 100 nm. **(H)** Catenane consisting of dimeric circles. Scale bar, 200 nm.

Fig. S4 - Related to Figure 4.

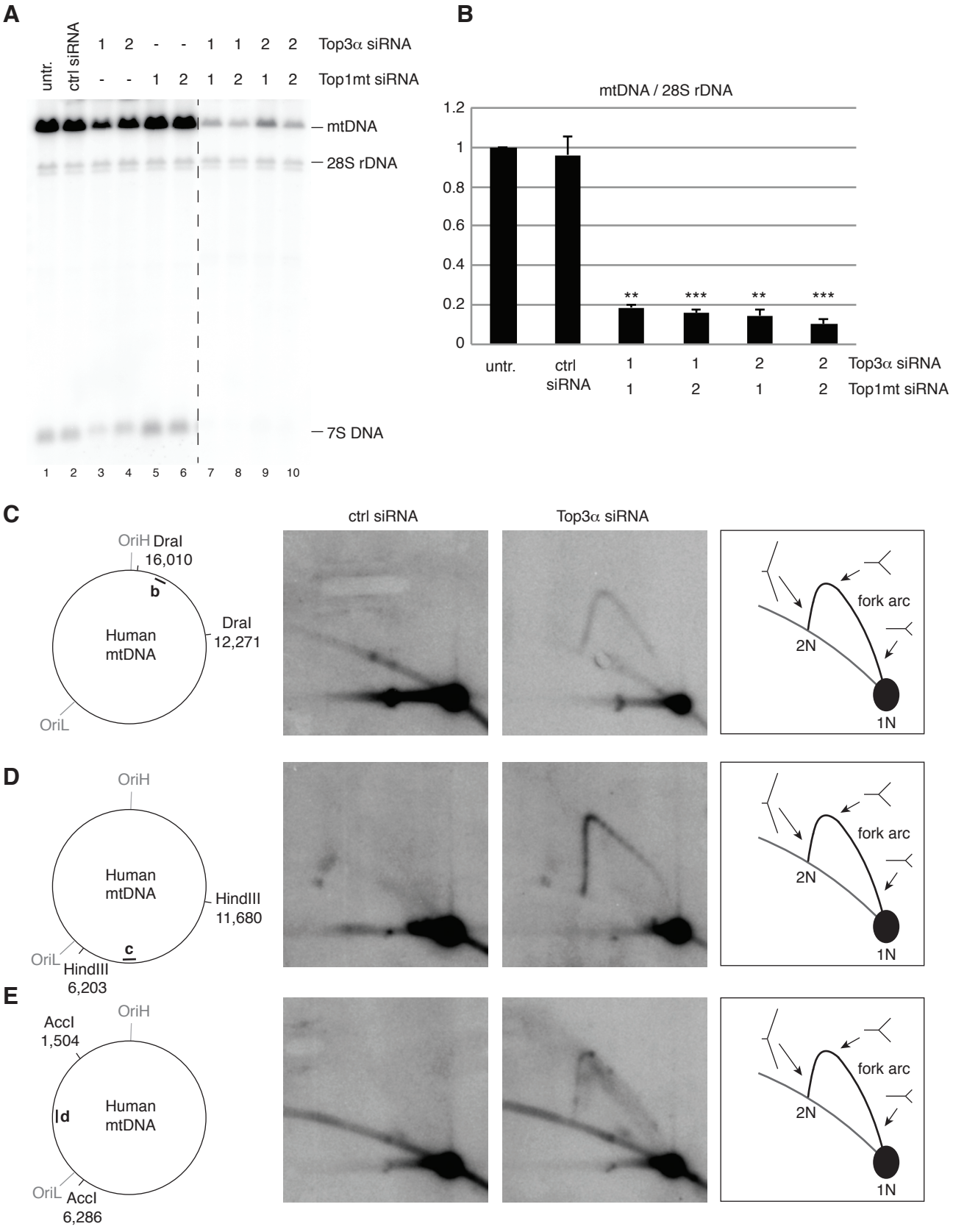


Figure S4 - Related to Figure 4.

(A) Southern blot of mtDNA from HeLa cells depleted of Top3α, Top1mt or both proteins simultaneously using all four different possible combinations of siRNA oligonucleotides. Samples were restricted using BamHI, separated on agarose and blotted using probe (a). 28S rDNA is used as a loading control. **(B)** Quantification of mtDNA copy number from cells depleted of Top3α and Top1mt as in (A). Data represent the mean of three independent experiments, normalised to 28S rDNA levels. Error bars represent SEM.

(**C-E**) DNA from control siRNA-treated or Top3α-depleted HeLa cells was restricted using DraI **(C)**, HindIII **(D)** or AccI **(E)** and separated by 2-dimensional agarose gel electrophoresis (2D-AGE) to analyse replication intermediates. Restriction maps (left) show the location of the probe (indicated by black bars). Interpretations of the structures observed are given on the right panels. The increase in signal from all points on the fork arc in Top3α depleted cells relative to control, in conjunction with the depletion of mtDNA copy number in these cells, is consistent with a genome-wide replication stalling phenotype.

Fig. S5 - Related to Figure 5.

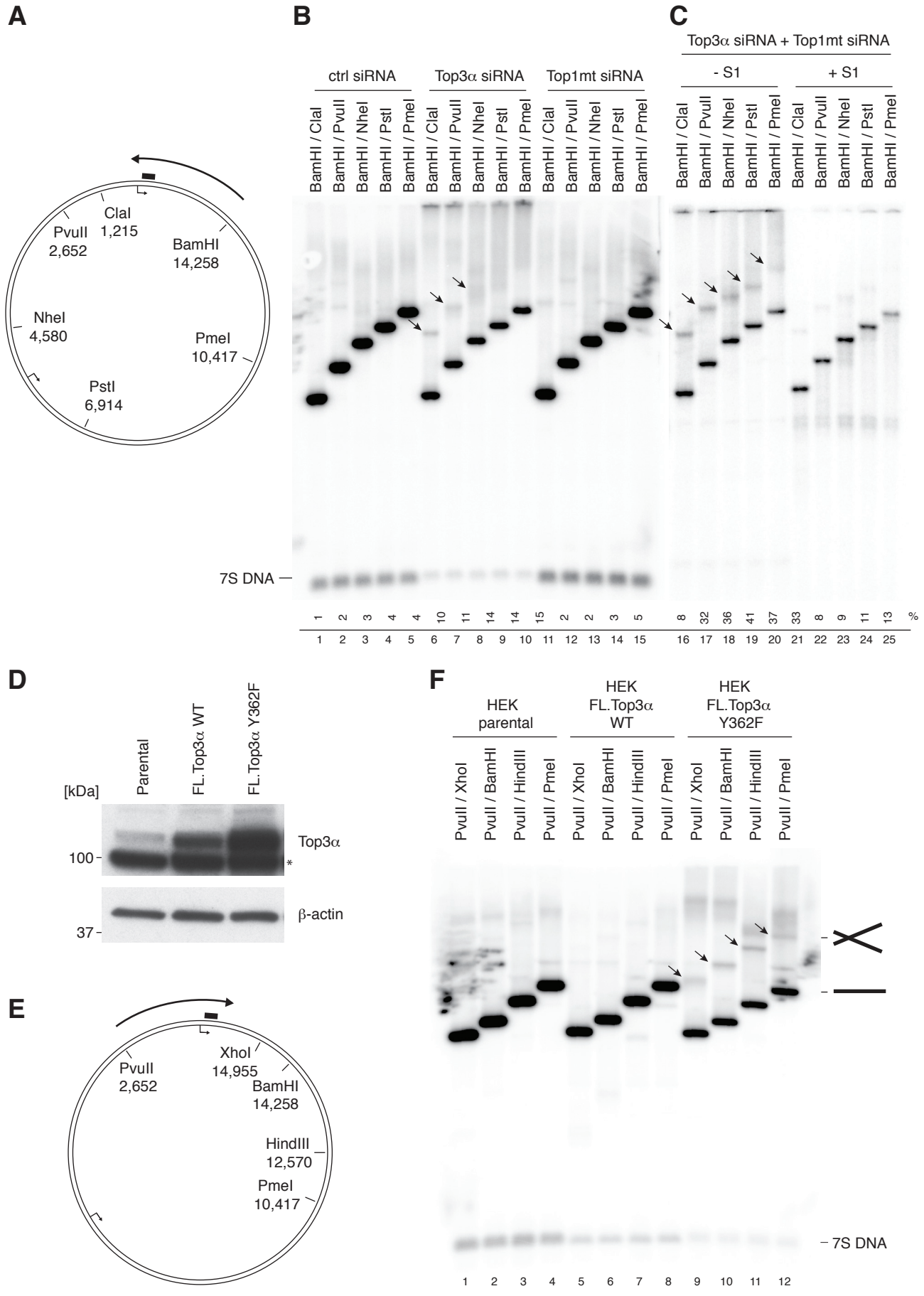


Figure S5 - Related to Figure 5.

(A) Restriction map of human mtDNA indicating sites of restriction enzymes used. Black bar indicates the probe. **(B)** Total DNA from cells treated with control siRNA, Top3α siRNA or Top1mt siRNA was restricted with the indicated enzymes, separated on agarose and blotted. Additional species present in Top3α depleted cells are indicated with arrows. **(C)** Total DNA from cells simultaneously depleted of both Top3α and Top1mt was restricted and blotted as in (B). Where indicated, samples were treated with S1 nuclease immediately before separation on gel. The proportion of X-shaped species is indicated below the lanes, expressed as a percentage of the linear restriction fragment. (**D-F**) Generation of X-shaped mtDNA intermediates from high-level expression of full-length, catalytically-inactive Top3α. **(D)** Western blot of wild-type (WT) and catalytically inactive (Y362F) Top3α expression induced using 10 ng/ml tetracycline. β-actin is used as a loading control. **(E)** Restriction map of human mtDNA indicating sites of restriction enzymes used. Black bar indicates the probe. **(F)** Total DNA from parental Flp-In T-REx 293 cells, or cells expressing full-length wild-type (WT) or catalytically inactive (Y362F) Top3α was restricted with the indicated enzymes, separated on agarose, blotted and detected using probe (a). Additional species present in Top3α Y362F expressing cells are indicated with arrows.

Fig. S6 - Related to Figure 5.

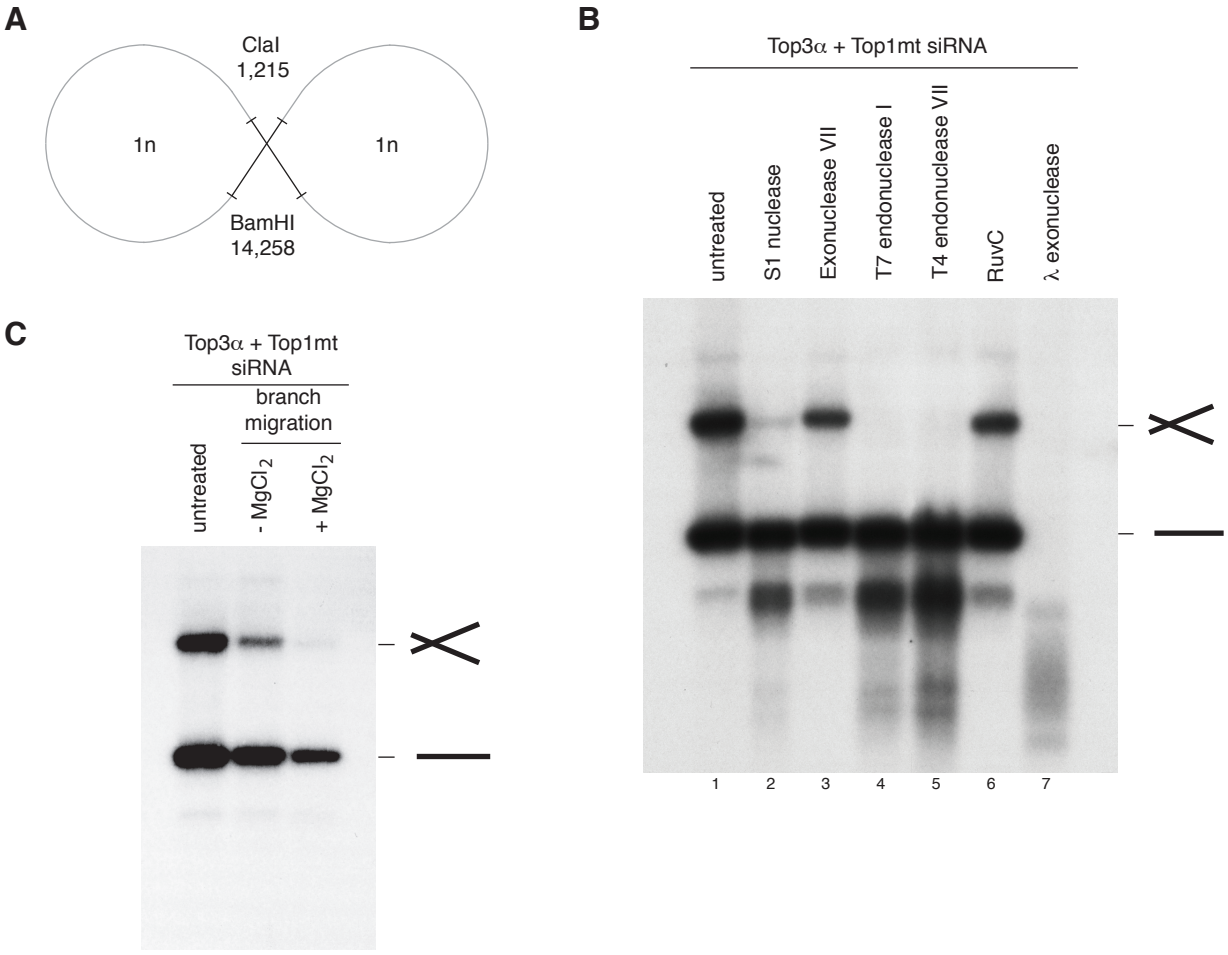


Figure S6 - Related to Figure 5.

Characterisation of the catenane junction in cells depleted of Top3α and Top1mt. (**A**) Diagram indicating cleavage sites of the restriction enzymes used and the expected structure of X-shaped molecules generated from cleavage of two mtDNA molecules joined through the NCR region. (**B**) Total DNA (3 μg) from cells depleted of Top3α and Top1mt was double digested with BamHI and ClaI, precipitated, treated with the indicated enzymes and then separated on agarose and blotted using probe (a). The migration of linear DNA and X-shaped DNA species is indicated. **(C)** Total DNA (5 μg) from cells depleted of Top3α and Top1mt was double digested with BamHI and ClaI, precipitated and then incubated in branch migration buffer in the presence or absence of magnesium for 2 hours at 65 °C, then separated on agarose and blotted using probe (a).

Fig. S7 - Related to Figure 7.

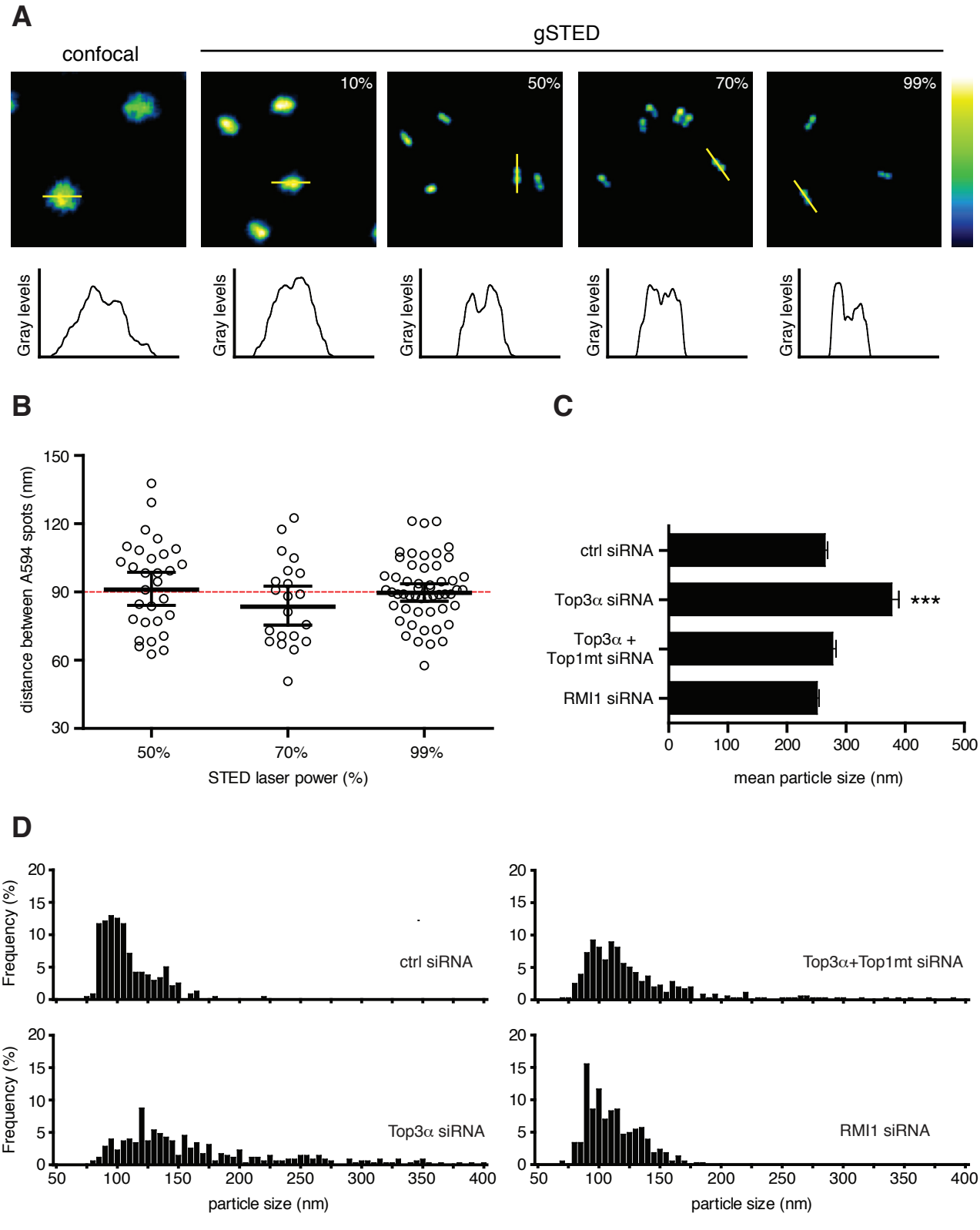


Figure S7 - Related to Figure 7.

(A) Representative images of A594 spots (GreenFireLUT) imaged with confocal and g-STED with different depletion lasers powers. A Gaussian Blur filter (radius 2.0) was applied to the final images. For each picture, pixel intensity profiles of a selected ROI (yellow bar, 640 nm) have been generated to show the different capability to resolve the two A594 spots. **(B)** Quantification of the distance between the two A594 spots, measured at the indicated depletion laser powers. The dotted red line corresponds to the value provided by the manufacturer. Numbers of A594 spots analyzed per condition: 50 % = 30; 70 % = 21; 99 % = 53. Bars indicate geometric mean + 95 % Cl. **(C)** Mean size of nucleoids determined by confocal microscopy under the indicated siRNA conditions. Error bars represent SEM. **(D)** Frequency (%) histogram profiles of the diameter of nucleoids analyzed in g-STED as in Fig. 4D.

Table S1 – Related to STAR Methods. Sequences for oligonucleotides used.

Primer sequences for probe synthesis		
Oligo name	**Sequence (5'-3')**	**mtDNA position**
Probe (a) Fwd	CTC ACC CAC TAG GAT ACC AAC	16,262 - 16,282
Probe (a) Rev	GAT ACT GCG ACA TAG GGT GC	128 - 109
Probe (b) Fwd	CCA CTC ATT CAT CGA CCT CCC	14,791 - 14,811
Probe (b) Rev	CCT AGG GGG TTG TTT GAT CCC	15,375 - 15,355
Probe (c) Fwd	GGC ACA TGC AGC GCA AGT AGG	7,588 - 7,608
Probe (c) Rev	GAC CGT AGT ATA CCC CCG GTC	8,168 - 8,148
Probe (d) Fwd	GCC ACA TCT ACC ATC ACC CTC	3,496 - 3,516
Probe (d) Rev	TAG AGT TCA GGG GAG AGT GCG	4,070 - 4,050
28S Fwd	GCC TAG CAG CCG ACT TAG AAC TGG	-
28S Rev	GGG CCT CCC ACT TAT TCT ACA CCT C	-
Sequences of siRNA oligonucleotides		
siRNA name	**Sequence (5'-3')**	**Cat#; PubChem SID**
Top1mt siRNA 1	Sense: CCC UGU AUU UCA UCG AUA Att; Antisense: UAU CGA UGA AAU ACA GGG cc	s42017; 160755427
Top1mt siRNA 2	Sense: CAA AGG AGG UUU UCC GGA Att; Antisense: UUC CGG AAA ACC UCC UUU Gtt	s42018; 160755428
Top3α siRNA 1	Sense: CGG CUU GCC UAG UUC UCU Att; Antisense: UAG AGA ACU AGG CAA GCC Gtg	s14310; 160718251
Top3α siRNA 2	Sense: CAG GUU AAA GUU AAA GUU Utt; Antisense: AAA CUU UAA CUU UAA CCU Gta	s14311; N/A
Top2α siRNA 1	Sense: GGA UUC UGC UAG UCC ACG Att; Antisense: UCG UGG ACU AGC AGA AUC Ctt	s14307; 160718247
Top2α siRNA 2	Sense: GAG UCA CAA UUG AUC CGG Att; Antisense: UCC GGA UCA AUU GUG ACU Cta	s14308; 160718248
Top2β siRNA 1	Sense: CGA UUA AGU UAU UAC GGU Utt; Antisense: AAC CGU AAU AAC UUA AUC Gta	s106; 160714417
Top2β siRNA 2	Sense: GGG UGA UCU UGA UAC UGC Att; Antisense: UGC AGU AUC AAG AUC ACC Ctt	s107; 160714515
BLM siRNA 1	Sense: CCC ACU ACU UUG CAA GUA Att; Antisense: UUA CUU GCA AAG UAG UGG Gaa	s1997; 160727423
BLM siRNA 2	Sense: GGA UGU UCU UAG CAC AUC Att; Antisense: UGA UGU GCU AAG AAC AUC Ctc	s1998; 160727458
RMI1 siRNA 1	Sense: GAG UUG UUC UUU AAG AUC Att; Antisense: UGA UCU UAA AGA ACA ACU Ctg	s36811; 160749950
RMI1 siRNA 2	Sense: GCU AAC UGA UGG AAU CGU Att; Antisense: UAC GAU UCC AUC AGU UAG Ctg	s36812; 160749951
RMI2 siRNA 1	Sense: GCA ACU ACC AUA ACC AGU Utt; Antisense: AAC UGG UUA UGG UAG UUG Ctg	s41904; 160755307
RMI2 siRNA 2	Sense: ACA UGG UUG UAG ACU UUC Att; Antisense: UGA AAG UCU ACA ACC AUG Uca	s41905; 160755308
Primer sequences for Sanger sequencing of *TOP3A* variants		
Oligo name	**Sequence (5'-3')**	**Chr. position**
TOP3A_Ex3_Fwd	TCA ACA TGC CTC AAA ATT CCT	Chr17:18211587-18211823
TOP3A_Ex3_Rev	GAA GCT CCT CCC TCT CAT TTT	
TOP3A_Ex5_Fwd	GCA GCA CTT ACC AGC CTT ACA	Chr17:18208415-18208646
TOP3A_Ex5_Rev	GTC ACA CTC CAA GAG CAG CA	
Substrate oligo for EMSA (5'-3')		
ACC ACA TTA ACA ACA TAA AAC CCT CAT TCA CAC GAG AAAA CAC CCT CAT GTT CAT ACA CCT ATC CCC CAT TCT CCT CCT A		