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Supplementary Information for

The human mitochondrial degradosome prevents harmful mitochondrial R loops and mitochondrial genome instability

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Supplementary Material and Methods

Cell Culture and transfection

HeLa and U2OS cells were grown at 37°C (5% CO_2) in DMEM (Gibco) supplemented with 10% foetal bovine serum (heat-inactivated), 1% glutamine and 1% Pen-Strep (Biowest). For immunofluorescence experiments, protein extracts and RNA samples, cells were transfected in 6-well plates at 25-30% confluence with siRNAs using DharmaFECT1 (Dharmacon) according to instructions and processed for analysis after 48h or 72h. In experiments with RNase H1 *in vivo*, cells were transfected a second time after 48h of incubation with the siRNAs with 3µg of either the control plasmid (pCDNA3) or RNase H1 overexpressing plasmid (pCDNA3-RNaseH1) using Lipofectamin (Invitrogen) according to instructions, and examined after additional 24h. For DRIP experiments, cells were seeded in 10 cm dishes and immediately processed for DNA extraction 72h after transfection with the siRNAs. siRNA sequences are presented in Table S1.

Immunofluorescence (IF) and Microscopy

For visualization of RNA-DNA hybrids, dsRNA and specific proteins of interest, cells were fixed with ice-cold methanol or 4% FA, blocked and subsequently incubated with the appropriate primary and secondary antibodies. For staining mitochondria cells were incubated with MitoTracker Orange CMTMRos (M7510, Molecular Probes) or MitoTracker Deep Red FM (M22426, Molecular Probes) at a final concentration of 200nM or 500nM for 30-45 min at 37°C. For RNase treatments cells were incubated in their respective commercial buffers at 1x containing 40 U/ml RNase III (AM2290, Ambion) or 60 U/ml RNase H (M0297S, NEB), for 30 min at 37°C. Double treatments were done sequentially. Samples were then blocked, incubated with primary antibodies at room temperature for 1h, followed by incubation with anti-mouse AlexaFluor 488 (A11029; Life Technologies) secondary antibody for 1 hr at room temperature. DNA was stained with 40 ,60 -diamidino-2-phenylindole (DAPI). Immunofluorescence images were acquired using a Leica DM6000 wide-field microscope or a Leica TCS SP5 confocal microscope. Data acquisition and image processing were performed using the LAS AF software (Leica). Microscopy data analysis was performed using the Metamorph v7.5.1.0 software (Molecular Probes).

Mitochondrial morphology analysis

Analysis of mitochondrial network morphology in individual cells was done using the MiNa tool for the opensource software Fiji (ImageJ) (1, 2). 2D microscopy images were minimally processed using the "unsharp mask" included in the Fiji package to enhance sharpness and then binarized to allow running the remaining features of the MiNa script, namely skeletonizing followed by measurement of networks (branched structures), individuals, mitochondrial coverage area (mitochondrial footprint).

DNA-RNA immunoprecipitarion (DRIP)

DRIP assays were performed essentially as described (3), with minor modifications. In brief, HeLa cells were gently lysed after 72h of transfection with the control and SUPV3L siRNAs. Following enzymatic digestion and treatment or not with RNaseH1, RNA-DNA hybrids were immunoprecipitated using the S9.6 antibody. Quantitative PCR was performed at the indicated regions of interest (Figure S2) using a 7500 Fast Real-Time PCR system (Applied Biosystems). Changes in the abundance of RNA-DNA hybrids in each region were determined as the percentage of input recovered for each immunoprecipitated sample using the equation $2^{[\,Ct_{IP} - (Ct_{INPUT} - \log_2 df)\,]}$ (df - dilution factor). Primers for detecting the constitutive R loop downstream of the LSP at the control region have previously been described (4). All other primer pairs were designed for this study using the Primer Express® Software v3.0.1 (Applied Biosystems) in order to coincide or not with regions expected to form native R loops in the human mitochondrial genome, according to previously published DRIPseq data (5). Primer sequences used for this analysis are described in Table S2.

Mitochondrial DNA replication analysis

For analysing mitochondrial replication, cells were first incubated with 6μM Aphidicolin (Sigma) for 4 hours followed by 2 hours incubation with the nucleotide analogue 5-ethynyl-29-deoxyuridine (EdU). Click-iT 5-ethynyl-29-deoxyuridine Alexa Fluor 555 Imaging Kit (Life Technologies) was used to detect incorporated EdU into mtDNA according to instructions.

Protein, mtRNA and mtDNA content analysis

Protein samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, GE Helthcare). Membranes were probed with primary antibodies

diluted 1:2000 against human SUV3 (PA5-32082; Invitrogen), PNPase (PA5-22397, Invitrogen) and α-actin (A3853, Sigma) followed by secondary antibodies IRDye 800CW goat anti-rabbit IgG (LI-COR P/N 926-32231) and IRDye 680RD goat anti-mouse IgG (LI-COR P/N 926-68070) at 1:5000, scanned and quantified using the Odyssey Imaging System (LiCor Inc., Lincoln, NE). Total RNA was obtained using the RNeasy kit (Qiagen) and used for cDNA synthesis with the QuantiTect Reverse Transcription Kit with random primers (Qiagen) according to instructions. Changes in mtRNA levels were analysed by quantitative PCR. Relative mitochondrial DNA content was estimated from total DNA extracts by comparing the abundance of the mitochondrial *COX2* and *CYTB* genes to that of the nuclear *RPL13A* and *APOE* genes, using the formula $2\times2^{\Delta CT}$, according to (6). Primer sequences used for this analysis are described in Table S3.

In vitro enzyme substrate specificity assay

RNA and DNA oligonucleotides purchased from Integrated DNA Technologies (IDT) were end-labelled with [γ-$_{32}$P]ATP using T4 polynucleotide kinase (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Oligonucleotide sequences used are described in Table S3. Linear substrates (RNA-RNA, DNA-DNA, RNA-DNA and R loop) were prepared by annealing labelled oligonucleotides with complementary cold oligonucleotides as previously described (7). The R loop substrate was produced by annealing two partially complementary DNA molecules with the RNA-25 oligonucleotide, complementary to a region within DNA-RL-75c. With the exception of the dsDNA, the RNA chain was the labelled moiety used to yield all the other substrates. Digestion reactions were performed with the indicated nucleic acid species at 30–50 nM final concentration and incubated with 1U of RNase H (NEB) or 1U of RNase III (Invitrogen) in the respective 1x reaction buffer for 30 min at 37°C. Samples were resolved in 6% PAGE and 0.5× TBE run for 45 minutes at 15 mA to separate the displaced digestion products derived from the labelled strand. Gels were dried, and images were taken with a Fujifilm Life Science FLA-5100 imaging system.

Supplementary Figures

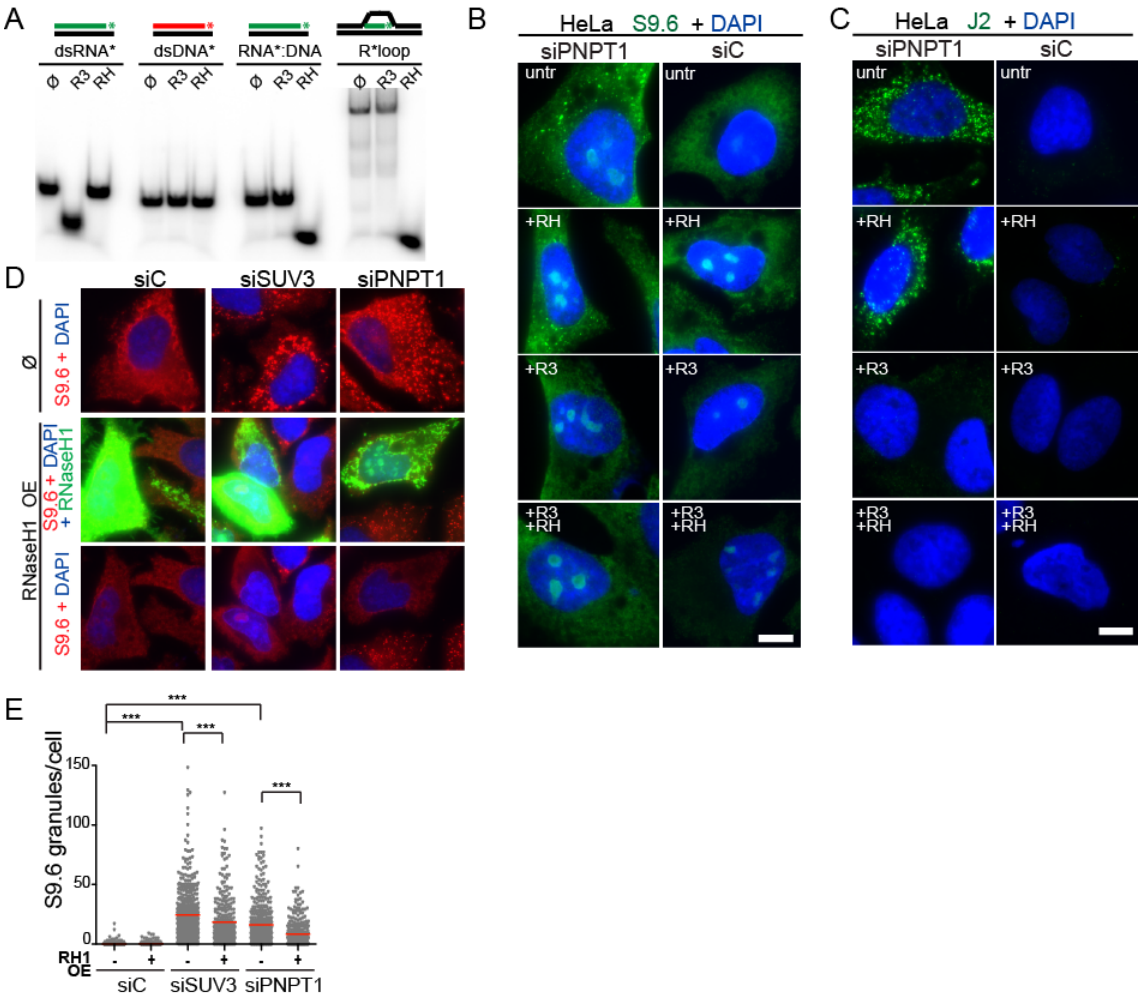

Figure S1.

Accumulation of S9.6 and J2 granules in mitochondria of HeLa cells that are sensitive to treatment with RNase H and RNase III in cells depleted of SUV3 and PNPase. **(**A) Comparison of efficiency of degradation of different nucleic acid substrates by RNases H (RH) and RNase III (R3) compared with a buffer treatment control (∅). The substrates and released degradation products from the labelled strand were separated in a 6% PAGE and visualized with a Fujifilm Life Science FLA-5100 imaging system. (B) IF of RNA-DNA hybrids using the S9.6 antibody and (C) dsRNA using the J2 antibody in HeLa cells transfected with siC (control) and siPNPT1 in the same conditions as data in Figure 2. (D) RNA-DNA hybrids (S9.6, in red) in HeLa cells transfected with siC (control), siSUV3 and siPNPT1 with or without overexpression of RNase H1 (in green) *in vivo*. Cells were transfected with pCDNA3 (control ø) or pcDNA3-RNaseH1 24 hours prior to fixation and immunostaining. (E) Quantification of S9.6 granules per cell in all conditions. Red bar represents the mean value for at least three independent experiments for each condition. Differences were analysed using the Mann-Whitney test (*** refers to $p<0.0001$).

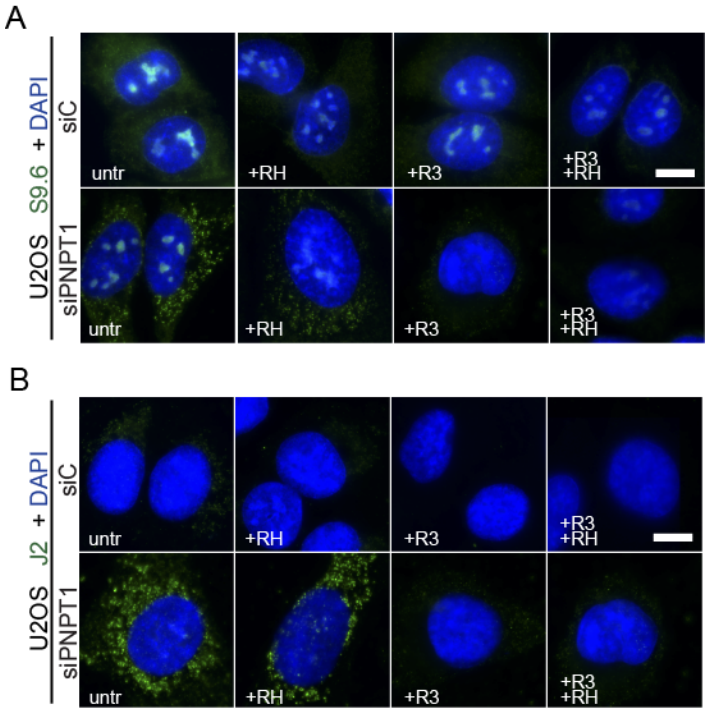

Figure S2.
Representative IF images for immunostaining with S9.6 (A) J2 (B) antibodies in siC and siPNPT1 transfected U2OS cells as in Figure 3, in control and treatment conditions with RNase H, RNase III or double treatment *in vitro*. S9.6 and J2 signals in green and nuclei stained with DAPI. Scale bar, 10 μm.

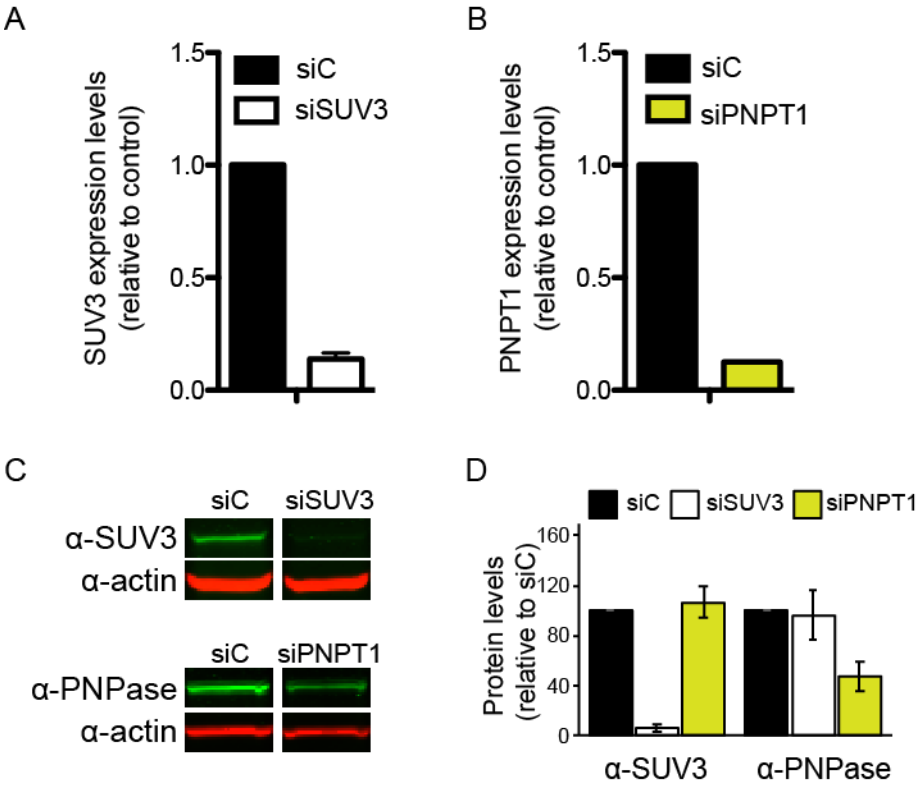

Figure S3.

Accumulation of the R loop-forming RNA species after *SUV3* and *PNPT1* silencing. (A-B) Confirmation of silencing of *SUV3* (A) and *PNPT1* (B) by siRNA transfection at the mRNA level. Values were normalized with respect to the *HPRT1* levels of control cells. (C-D) Quantification of SUV3 and PNPase protein levels in siSUV3 and siPNPT1-transfected HeLa cells. Detection and quantification was performed using the Odyssey Imaging System (LiCor Inc., Lincoln, NE). (E-F) Expression levels of different mtRNA species in siC (control) SUV3- and PNPase-depleted cells relative to nuclear transcription levels. Values represent the mean and SEM in logarithmic scale (log_2) of at least 4 independent experiments. Statistical significance was analysed using *t*-test (* refers to $p<0.05$).

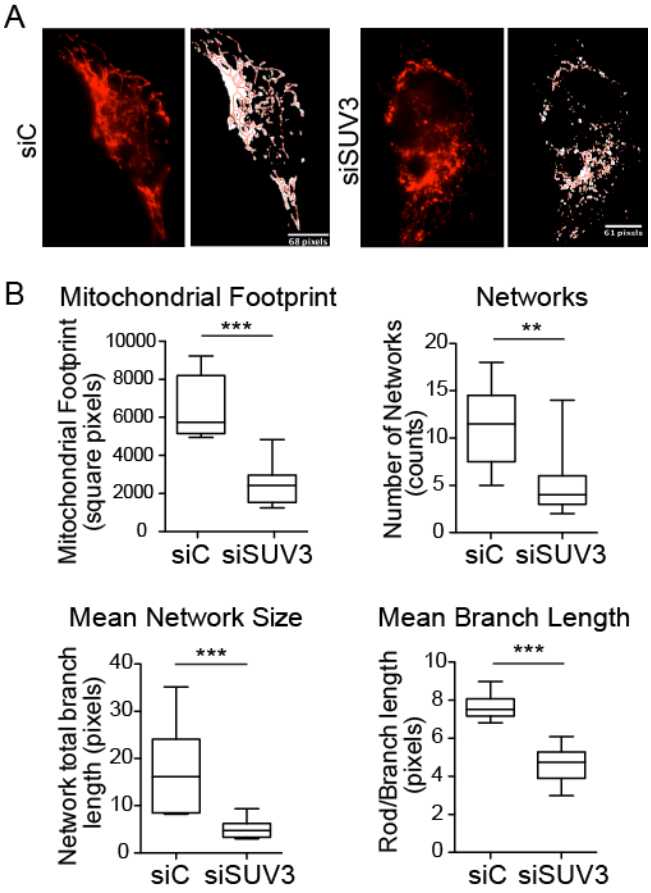

Figure S4.
Analysis of mitochondrial network morphology changes in mtEXO-deficient HeLa cells using the MiNa macro for ImageJ. (A) Representative images for siC and siSUV3 HeLa cells generated by applying the MiNa algorithm on the mitochondria stained with MitoTracker. (B) Box plots with median and quartile levels for morphology parameters obtained from the analysis of >10 individual cells for control and SUV3-depleted cells (*** refers to $p<0.0001$; ** refers to $p<0.001$).

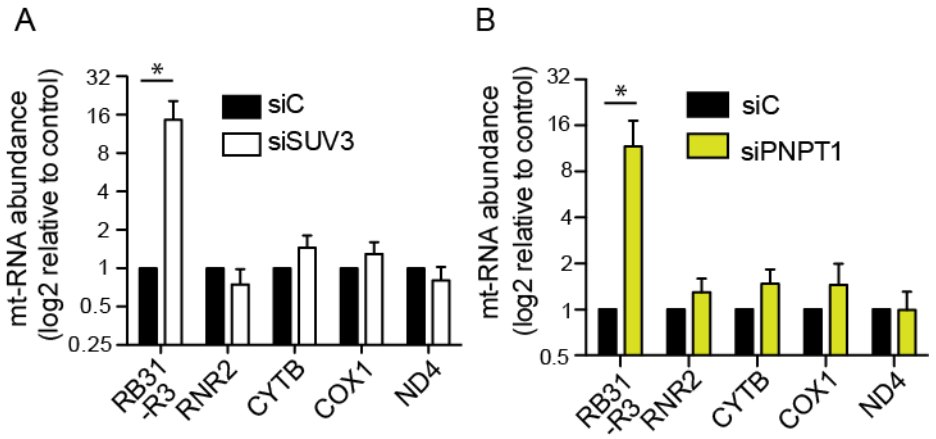

Figure S5.
Expression analysis of different mtRNA species in siSUV3 (A) and siPNPT1 (B) compared to siC (control) transfected HeLa cells. Values represent the mean and SEM in logarithmic scale (log_2) of expression relative to nuclear *HPRT1* transcription levels in control cells, of at least 4 independent experiments. Statistical significance was assessed using *t*-test (* refers to $p<0.05$).

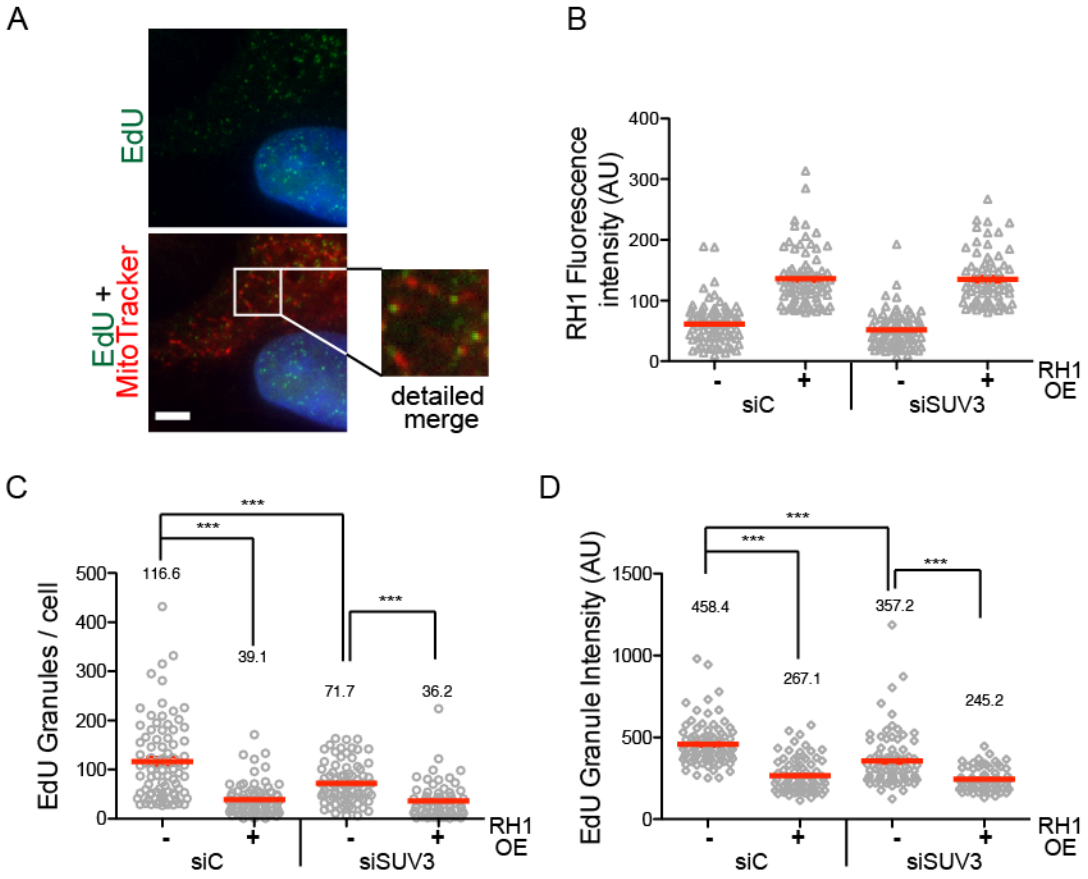

Figure S6.

Analysis of hybrid-associated replication failure of mitochondrial genomes in cells lacking mtEXO function. (A) IF detail showing the co-localization of MitoTracker with EdU incorporated into mitochondrial DNA in control cells. Scale bar, 5 µm. (B) Levels of RNase H1 in HeLa cells transfected with pCDNA3-RNaseH1 plasmid by measuring fluorescence intensity. Control and SUV3-depleted cells were transfected with the plasmid for RNase H1 overexpression 24 hours prior to incubation with EdU under the same conditions as results presented in Figure 5. (C) Quantification of total number of EdU granules per cell. (D) Quantification of EdU granule intensity in cells over-expressing or not RNase H1 in siC and siSUV3 transfected cells. All graphs present the mean value (red line) for at least three independent experiments for each condition. Differences were analysed using the Mann-Whitney test (*** refers to $p<0.0001$; AU, arbitrary units).

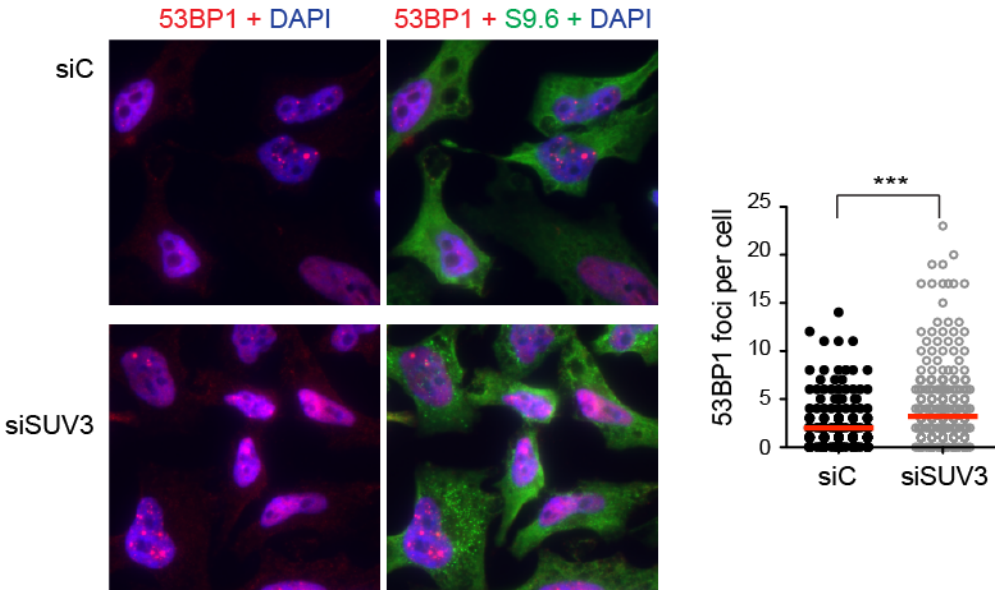

Figure S7. Analysis of nuclear DNA damage after *SUV3* silencing. Visualization and quantification of nuclear DNA damage in HeLa cells transfected with siC (control) or siSUV3 was assessed by the accumulation of 53BP1 foci. Mean values (red line) obtained from 3 independent experiments are presented. Statistical significance was analysed using *t*-test (*** refers to $p<0.0001$).

Supplementary Tables

Table S1. siRNAs used in this study

siRNA	Target sequence
siC ON-TARGET plus Non-targeting pool D-001810-10-50	
siSUV3 ON-TARGET plus human SUPV3L1 (6832) SMARTpool L-017841-01-0005	J-017841-09 UGGCUAAGCUACCGAUUUA J-017841-10 GUAAGGAUGAUCUACGUAA J-017841-11 CGGUGCAGCUCAUGCGGAU J-017841-12 GGAAAGACUUAUCACGCAA
siPNPT1 ON-TARGET plus human PNPT1 (87178) SMARTpool L-019454-01-0005	J-019454-09 GACAGAAGUAGUAUUGUAA J-019454-10 ACAGAAAGAUUAUUGGCUA J-019454-11 GAAUGUAAGUUGUGAGGUA J-019454-12 AAUCAGAGAUACUGGUGU

Table S2. Oligonucleotides used for qPCR in this study

Oligo name	Sequence (5' - 3')
RB31-R3 fw	GGTTTGGTGGAAATTTTTTGTT
RB31-R3 rv	TCTTTGATTCCTGCCTCATTCT
CYTB 5' fw	GCCAATGGCGCCTCAATAT
CYTB 5' rv	AGGCCTCGCCCGATGT
RNR2 fw	GCGCAATCCTATTCTAGAGTCCAT
RNR2 rv	TCGGGATGTCCTGATCCAA
CYTB out fw	GACGCCCTCGGCTTACTTC
CYTB out rv	GGAGGTCTGGTGAGAATAGTGTTAATG
ND4 fw	AGCTCCATCTGCCTACGACAA
ND4 rv	TGTGGCTGATTGAAGAGTATGCA
COX2 fw	CTGAAATCTGTGGAGCAAACCA
COX2 rv	CGGGCCCATTTTCAAAGATTTT
COX1 fw	CGCCGACCGTTGACTATTCT
COX1 rv	GCGCCGAATAATAGGTATAGTGTTC
HPRT1 fw	GGACTAATTATGGACAGGAC TG
HPRT1 rv	TCCAGCAGGTCAGCAAAGAA
APOE fw	GGGAGCCCTATAATTGGACAAGT
APOE rv	CCCGACTGCGCTTCTCA
RPL13A fw	GCTTCCAGCACAGGACAGGTAT
RPL13A rv	CACCCACTACCCGAGTTCAAG

Table S3. Oligonucleotides used for RNase H and RNase III *in vitro* digestions

Oligo name	Sequence (5' - 3')	Reference
DNA25C	AGCGTGCCGTGCAACAACATTACAC	This study
RNA-25C	GUGUAAUGUUGUUGCACGGCACGCU	(7)
DNA-25	GTGTAATGTTGTTGCACGGCACGCT	(7)
RNA-25	AGCGUGCCGUGCAACAACAUUACAC	(7)
DNA-RL-75c	CGATGACATGTTGCATCGTATCGATAGCGT GCCGTGCAACAACATTACACTGCTGATCAG TCTAGATTATCAGTC	This study
DNA-RL-75nc	CGATGACATGTTGCATCGTATCGATGATAC AATACATGGTGGTGCAGTGTTGCTGATCAG TCTAGATTATCAGTC	This study

Supplementary References

1. Valente AJ, Maddalena LA, Robb EL, Moradi F, & Stuart JA (2017) A simple ImageJ macro tool for analyzing mitochondrial network morphology in mammalian cell culture. *Acta Histochem* 119(3):315-326.
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3. Herrera-Moyano E, Mergui X, Garcia-Rubio ML, Barroso S, & Aguilera A (2014) The yeast and human FACT chromatin-reorganizing complexes solve R-loop-mediated transcription-replication conflicts. *Genes Dev* 28(7):735-748.
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5. Nadel J, *et al.* (2015) RNA:DNA hybrids in the human genome have distinctive nucleotide characteristics, chromatin composition, and transcriptional relationships. *Epigenetics Chromatin* 8:46.
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7. Garcia-Rubio M, *et al.* (2018) Yra1-bound RNA-DNA hybrids cause orientation-independent transcription-replication collisions and telomere instability. *Genes Dev* 32(13-14):965-977.