Integrity of the human centromere DNA repeats is protected by CENP-A, CENP-C and CENP-T

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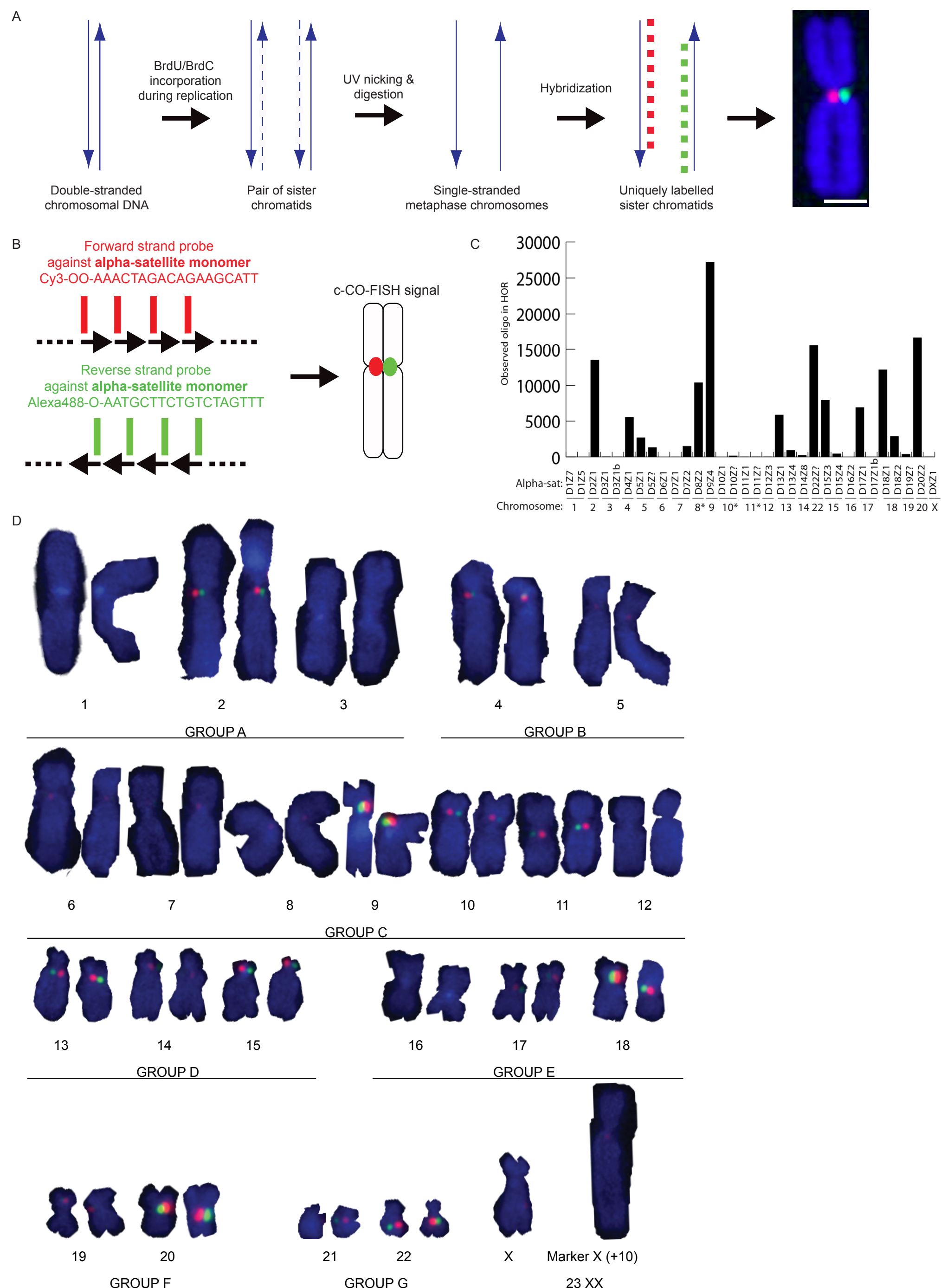

Figure S1. c-CO-FISH using PNA probe set 1 against alpha-satellite repeat in primary hTERT-RPE1 cells
(A) Schematic of CO-FISH procedure. UV, ultraviolet. Right panel: centromere CO-FISH on a human mitotic chromosome. Red and green probes label a single sister chromatid, stained by DAPI. Scale bar, 1 μm. (B) c-CO-FISH using PNA probe set 1 against a 18 bp sequence within the 171 bp alpha-satellite monomer (black arrows). (C) Representation of the abundance of sequence corresponding to the probe set 1 within the centromere HOR (higher order repeats) of human chromosomes in the HuRef reference genome (41). Asterisks indicate chromosomes with HOR arrays varying from the HuRef reference database. (D) Karyotype of human RPE1 line using the probe set 1.

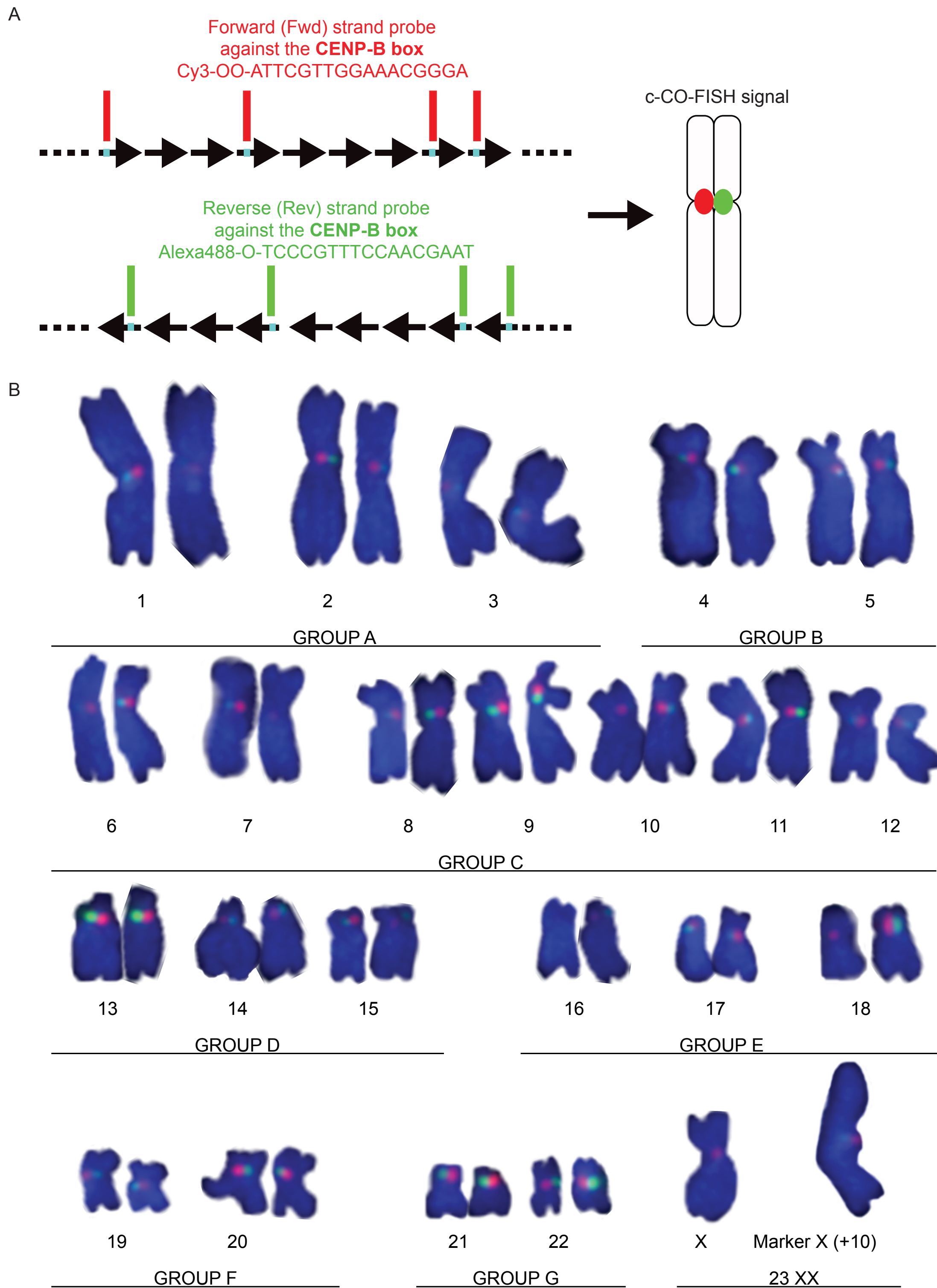

Figure S2. c-CO-FISH using PNA probe set 2 against the CENP-B box motif in primary hTERT-RPE1 cells
(A) Centromeric CO-FISH using PNA probe set 2 targeting the 17 bp CENP-B box (teal boxes), a sequence repeated in a subset of the monomers throughout the core alpha-satellite HOR. (B) Karyotype of human hTERT-RPE1 using the probe set 2 shows representation in most chromosomes.

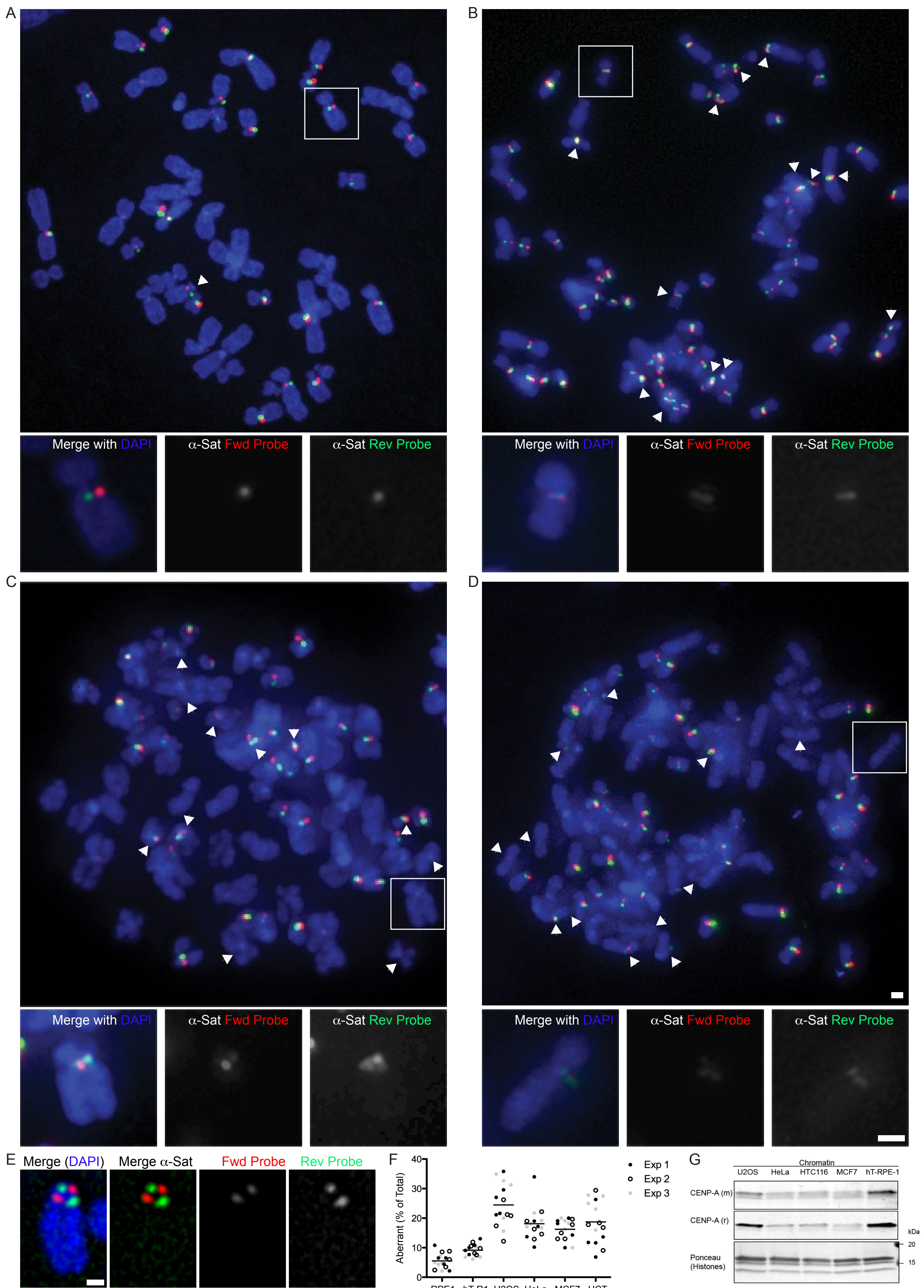

Figure S3.

Figure S3. Metaphase spread showing c-CO-FISH in various human cell lines (A) c-CO-FISH (probe set 1) analysis on primary RPE1, and cancer lines: (B) bone osteosarcoma epithelial cells (U2OS), (C) cervix epithelial adenocarcinoma cells (HeLa Kyoto) and (D) metastatic breast carcinoma cells (MCF-7). Arrows indicate chromosomes with aberrant c-CO-FISH patterns. The white boxes mark insert area which were magnified 2.5x, levels and intensities adjusted and presented into 3 panels below each image, from the left merged with DAPI, forward or reverse-only strand probe of set 1 hybridized to alpha-satellite centromere DNA (E) An example of apparent chromosome fusion in U2-OS cell line stained by c-CO-FISH displaying two sets of centromeres separated by DAPI signal. (F) Cancer cell lines c-CO-FISH quantification of aberrant centromeres using probe set 1. Data from three independent experiments are compiled. Bars indicate the median across the three experiments. Cells lines (left to right): RPE-1, hTERT-RPE1 (hT-R1), U2-OS, Hela Kyoto, MCF-7 and HCT116 (HCT). (G) Western blotting using two anti-CENP-A antibody (mouse and rabbit) and ponceau staining of chromatin fractions for the cell lines as indicated. Scale bars 1 μm.

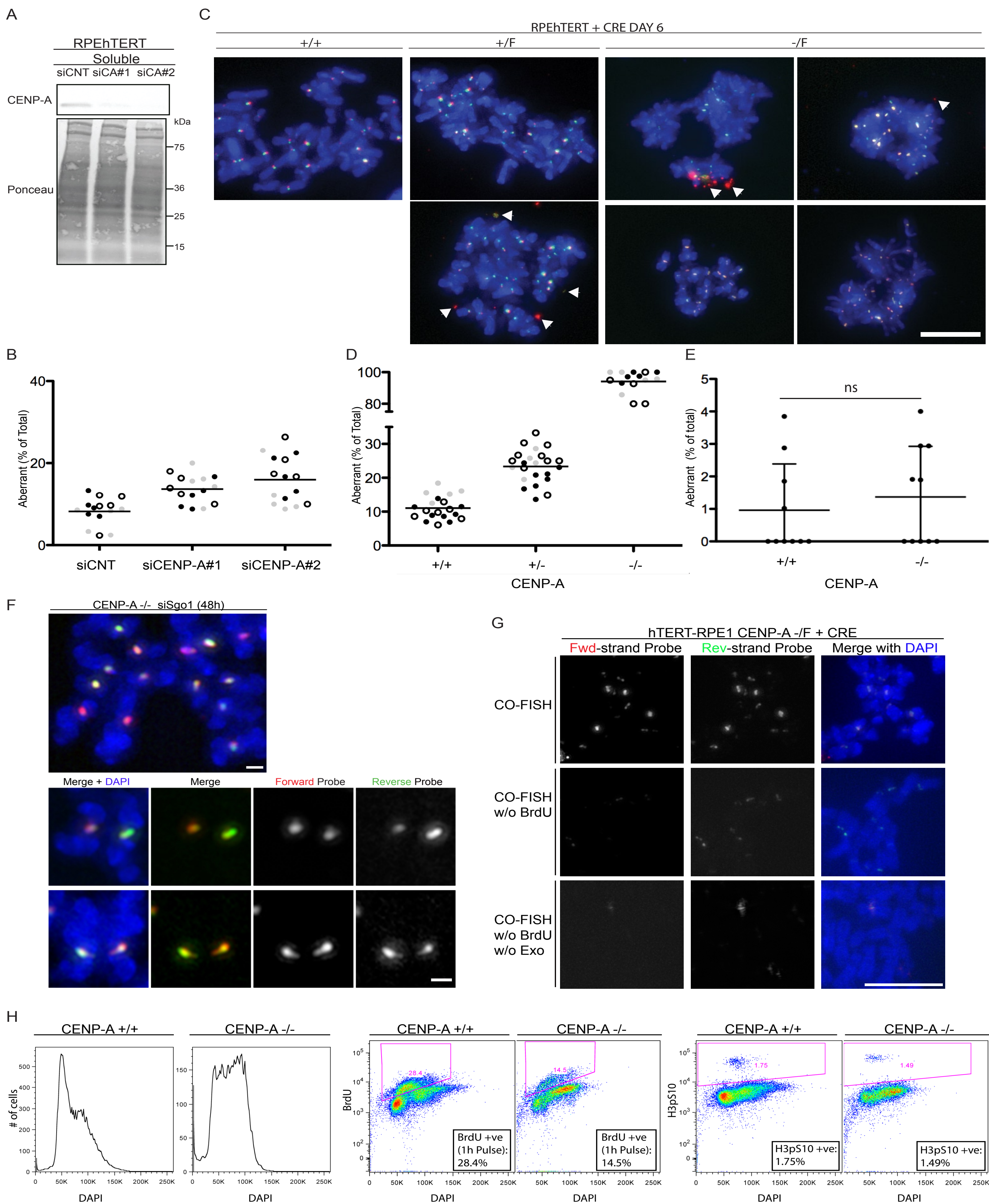

Figure S4. CENP-A is required to maintain the integrity of the alpha-satellite repeats in human cells

(A) Western blotting using anti-CENP-A antibody and ponceau staining of soluble fraction derived from the samples in Fig. 2A. (B) Down-regulation of CENP-A by two siRNAs for 72 h followed by c-CO-FISH quantification of aberrant centromeres using probe set 1. Data from 3

independent experiments were compiled. Bars indicate the median across the 3 experiments. (C) Examples of c-CO-FISH (probe set 1) in hTERT-RPE1 lines as in Fig. 3D after infection with Adeno-CRE for 6 days. White arrows indicate examples of excised centromeres. (D) c-CO-FISH quantification of aberrant c-CO-FISH patterns using probe set 1 in 6 days Cre-treated cell lines as indicated. Data from 3 independent experiments were compiled. Bars indicate the median across the 3experiments. (E) Quantification of aberrant telomeres by CO-FISH of cells as in (A). ns, not significant. (F) Metaphase spread of siRNA-mediated down-regulation of Sgo1 after 48 h in hTERT-RPE1 CENP-A F/- cells treated with Cre for 6 days. Panels below display two examples of aberrant c-CO-FISH signal of prematurely separated sister chromatids. Scale bar is 1 μm. (G) c-CO-FISH of CENP-A knockout cells omitting different steps as indicated. Scale bar is 5 μm. (H) Flow cytometry analysis of cell cycle distribution in CENP-A +/+ and CENP-A -/F cells treated with CRE for 6 days. DAPI fluorescence intensity was plotted in a histogram, or against BrdU and H3pS10 signal, as indicated. The percentage of positive cells found in the gated quadrants (in purple) is shown.

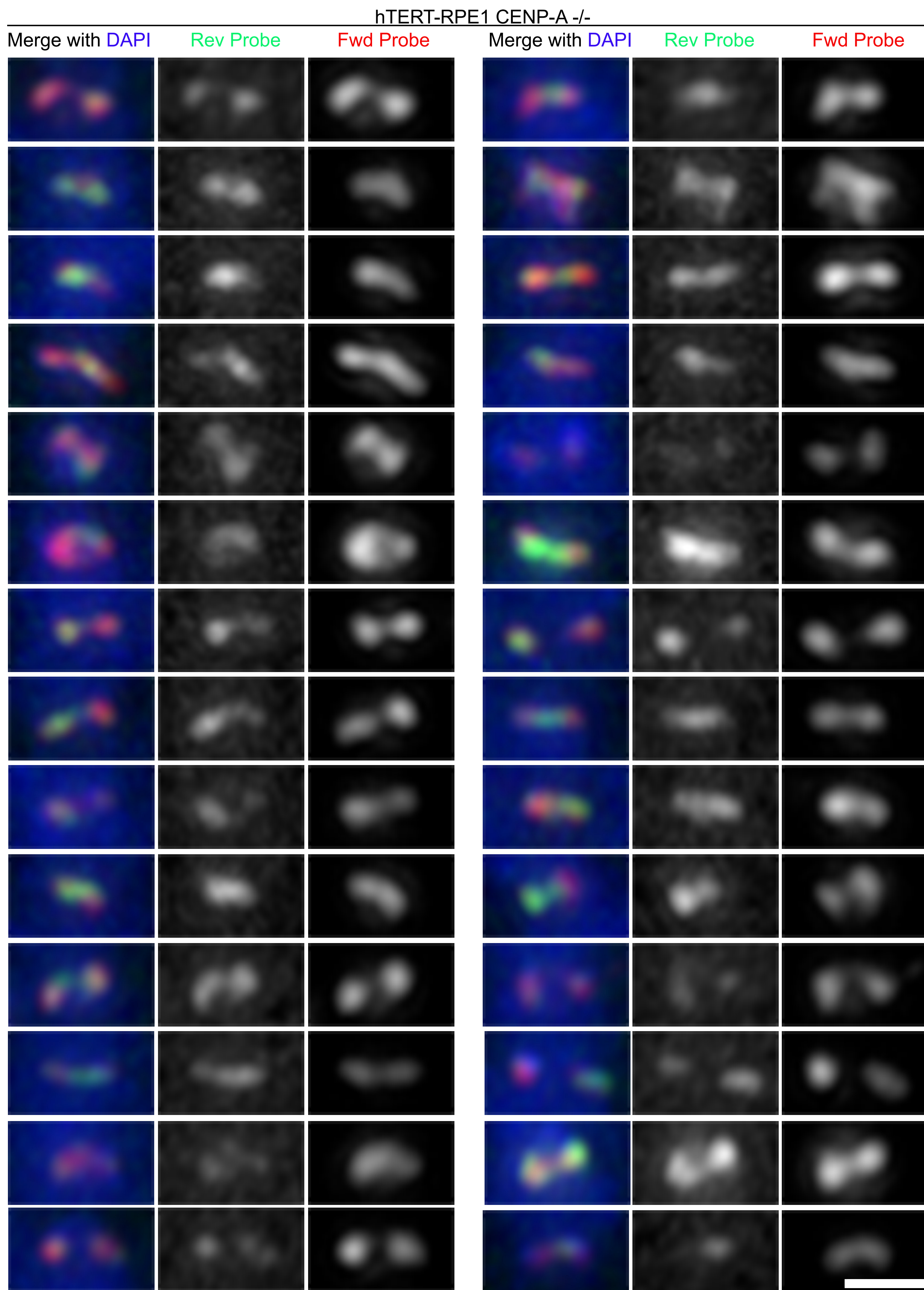

Figure S5. Aberrant c-CO-FISH rearrangements in CENP-A knockout cells Examples of aberrant c-CO-FISH patterns found in a single metaphase spread in hTERT-RPE1 CENP-A -/F cells 6 days after Cre induction. Probe set #2 was used. Scale bar is 0.5 μm.

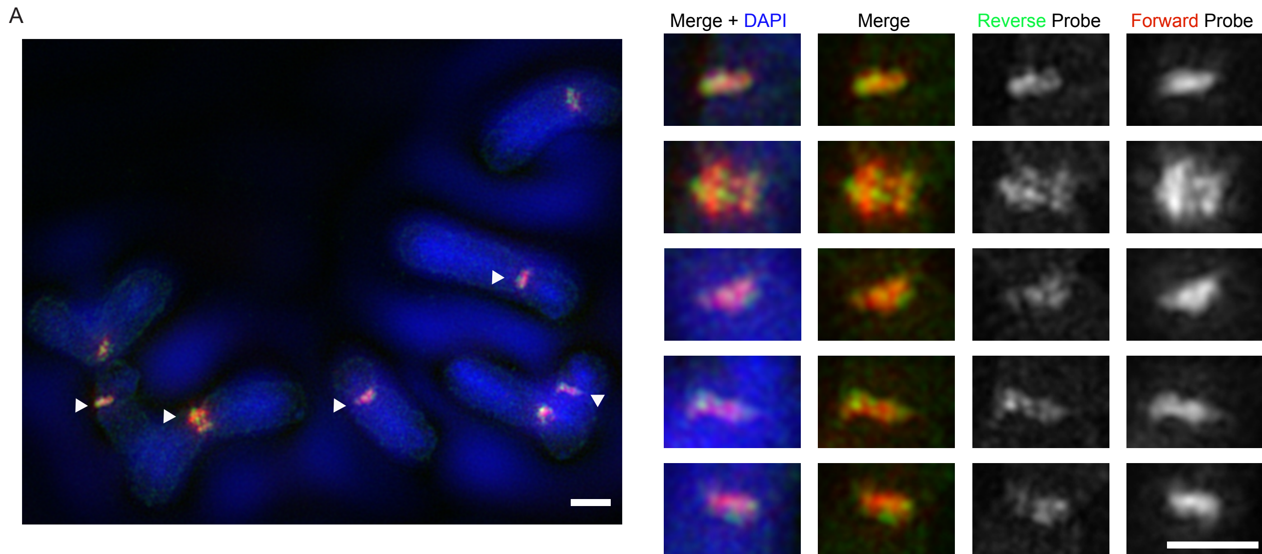

Figure S6. Super-resolution imaging of human centromeres in CENP-A knockout cells

(A) CO-FISH (probe set 1) 3D-SIM image of metaphase chromosomes in CENP-A -/F cells 6 days after Cre induction. Centromeres marked by white arrowheads were magnified 2.3x and displayed on the right. Scale bars are 1 μm.

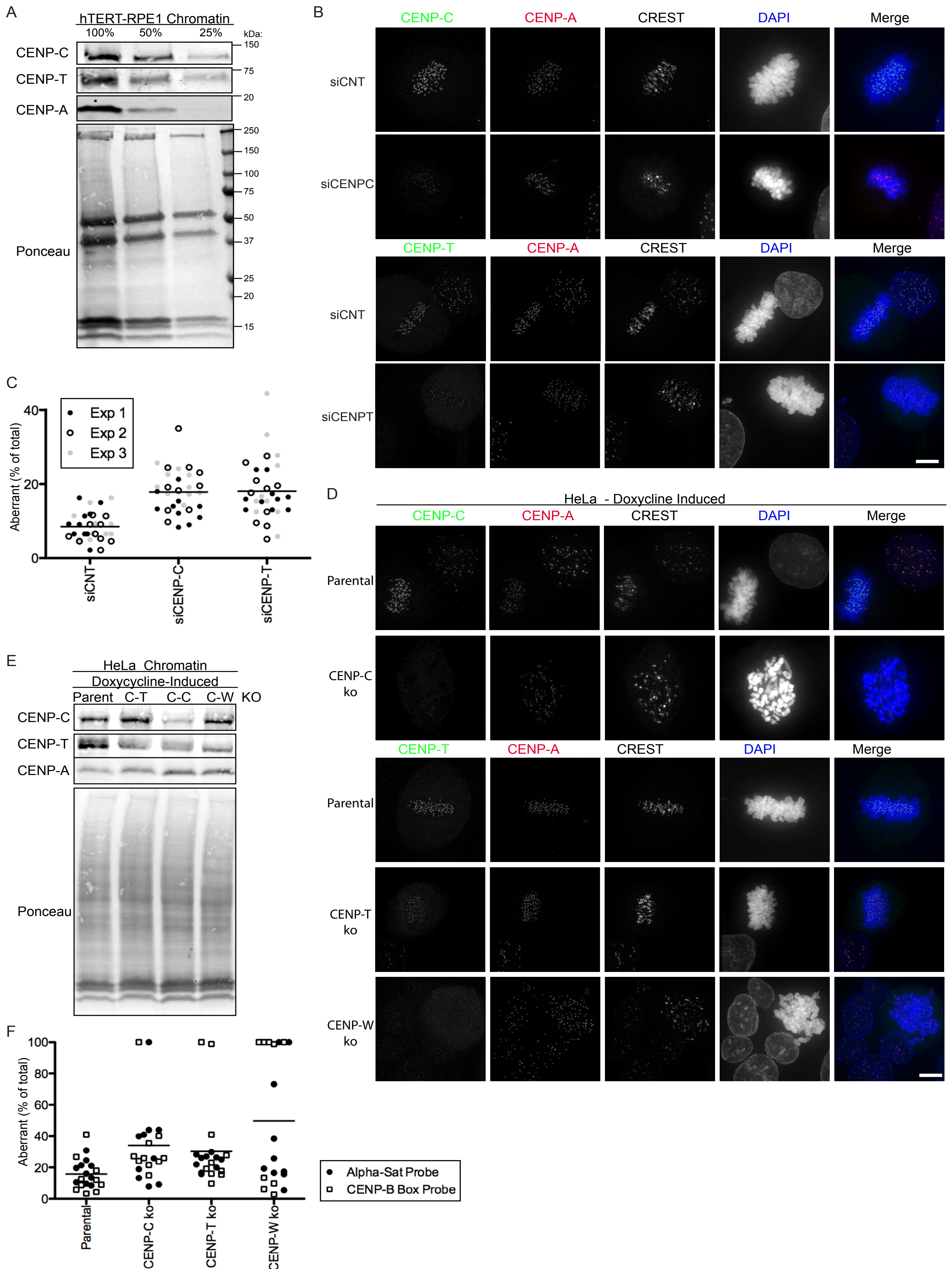

Figure S7.

Figure S7. Removal of CCAN components leads to centromere rearrangements in human cells

(A) Western blotting of chromatin fractions of hTERT-RPE1 cells titrated from 100% of chromatin extract and stained as indicated. (B) hTERT-RPE1 cells were treated with control siRNA (siCNT) or siRNA targeting to CENP-C and CENP-T for 72 h and stained for IF as indicated. The merge shows CENP-C or CENP-T (green), CENP-A (red) and DAPI (blue). (C) c-CO-FISH quantification of centromere rearrangements using probe set 1 in cells as in (A). Data from three independent experiments were compiled. Bars indicate the median across the three experiments. (D) IF and (E) WB of Inducible knockout HeLa cells with Cas9 under control of a stably-integrated Tet-On promoter. Doxycycline was added for 5 days for CENP-C and CENP-T knockouts and 3 days for CENP-W knockout, cells were then harvested for c-CO-FISH and (F) the aberrant centromeres was quantified using both probes set 1 and set 2. Bars indicate the median between the two experiments using either probe set. Scale bars, 5 μm.

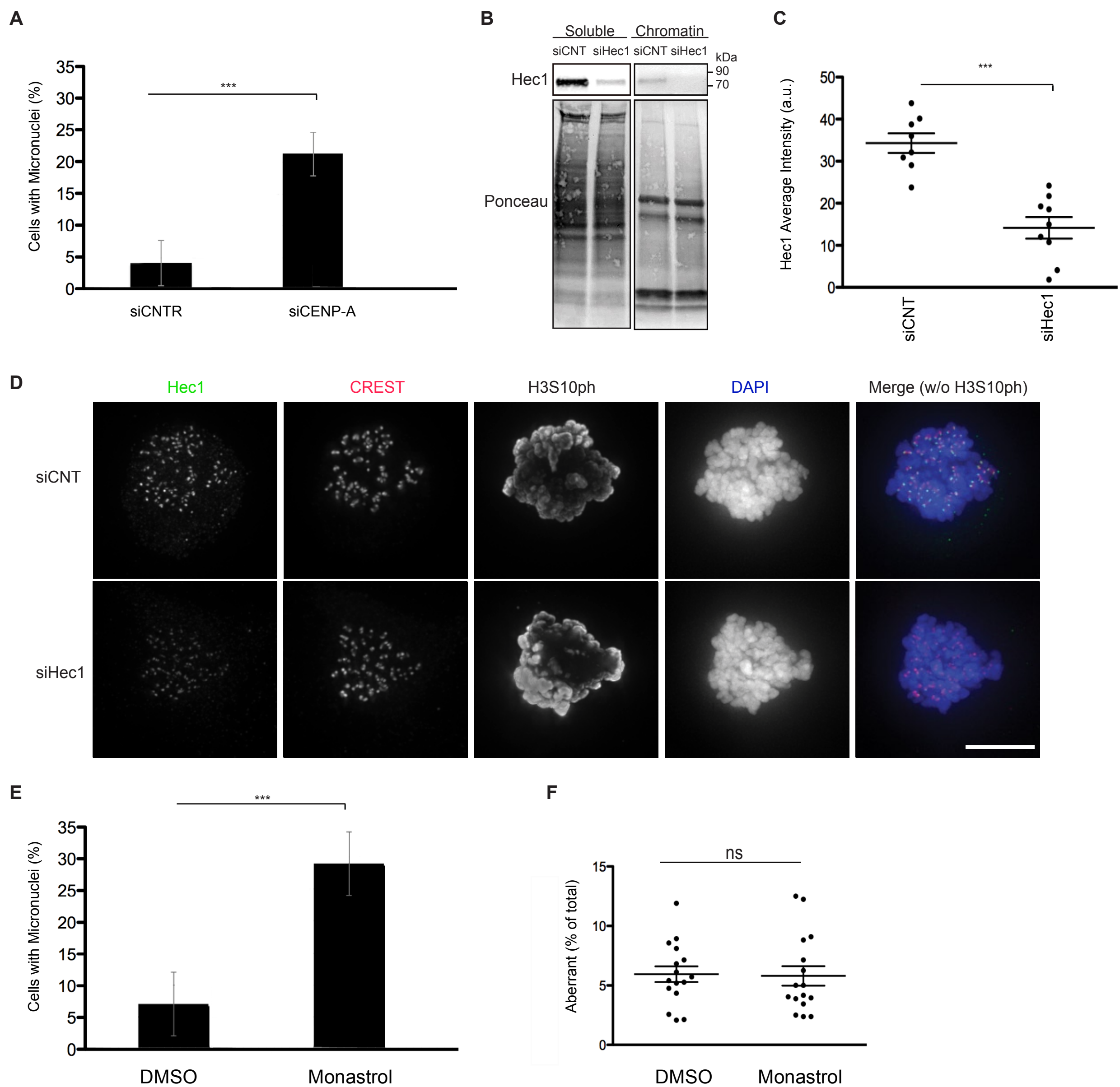

Figure S8. Chromosome missegregation induced by monastrol wash-out is not correlated with alpha-satellite repeats instability

(A) Quantification of micronuclei in hTERT-RPE1 cells treated with control siRNA (siCNT) and siRNA (#1) targeting to CENP-A (siCENP-A). Error bars, ± SD. ***, $p \leq 0.0005$. (B) Western blotting using anti-Hec1 antibody (top) and ponceau staining of chromatin and soluble fractions of cells. (C) Quantification of Hec1 fluorescence intensity. a.u. = arbitrary units. Error bars, ± SD. ***, $p \leq 0.0005$. (D) Representative images of DAPI staining and immunofluorescence in nocodazole-arrested cells using anti-Hec1, CREST, and the mitotic marker H3 phospho-serine 10 (H3S10ph). Scale bar, 10 μm. (E) Quantification of micronuclei in hTERT-RPE1 cells transiently treated with Monastrol or DMSO (control). Error bars, ± SD. ***, $p \leq 0.0005$. (C) Quantification of aberrant c-CO-FISH patterns in cells as in (F). Error bars, ± SD. Ns, not significant.

Supplemental Materials and Methods

Cell cultures

All cells were maintained at 37°C in a 5% CO_2 atmosphere with 21% oxygen. Human primary retinal pigmented epithelial RPE1 cells were grown in Dulbecco Modified Minimal Essential medium DMEM/F-12 media (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals) and 100 U/ml Penicillin-Streptomycin (P/S; Life Technologies). hTERT-RPE1 were obtained from parental RPE1 line infected with pLVX-hTERT-IRES-Hygro lentiviral plasmid and selected for hygromycin resistance, and grown in DMEM/F-12, 10% FBS, P/S. U2OS and HeLa cancer lines were cultured in standard DMEM with 10% FSB and P/S. MCF7 were cultured in DMEM, 10% FBS and P/S with addition of 1 mM sodium pyruvate, 2 mM L-glutamine and 0.01 mg/ml porcine insulin. HCT116 were grown in McCoy's 5A medium with 10% FBS and P/S. Floxed CENP-A lines, a gift of D. Fachinetti and D. Cleveland, were grown as described in (1). Briefly, Adeno-CMV-Cre (VectorBiolabs) was used as previously described (2) and cells harvested 6 days post-infection. HeLa inducible knockout cells (3), a gift of I. Cheeseman, were induced by doxycycline for 5 days for CENP-C and CENP-T knockout and for 3 days for CENP-W knockout before harvesting. Monastrol (Tocris Biosciences) was used at 100 µM for 15 h, followed by shake-off of mitotic cells and release for CO-FISH and other assays.

RNAi

On-TARGETplus RNAi (Dharmacon) were used for the down-regulation of CENP-A (RNAi #1: GGACUCUCCAGAGCCAUGAUU or RNAi #2: AGACAAGGUUGGCUAAAGG (1)), of siHec1 (GTTCAAAAGCTGGATGATC; (4)), siCENP-C (siGenome SMARTpool # NC0752121), siCENP-T (On-TARGETplus SMARTpool NC0752122) or non-targerting siCONTROL (D001810). Cells were transfected with the RNAi using Lipofectamine/RNAiMAX (Thermofisher Scientific) and harvested after 72 h post-transfection.

CO-FISH

CO-FISH at centromere was adapted from (5). Cells were grown in 10 µM BrdU:BrdC (3:1) for 16–20 h before 0.1 µg/mL colcemid (Roche) was added for 2 h. Cells were harvested and swollen in pre-warmed 0.075 M KCl at 37ºC for 30 min. Cells were then fresh fixed in 3:1 methanol/acetic acid and dropped onto glass slides in a Thermotron Cycler (20ºC, 50% humidity) and left in the dark overnight. The slides were re-hydrated in 1X PBS for 5 min and treated with RNase A (0.5 µg/ml in PBS, Sigma R5000) for 10 min at 37ºC, stained with 0.5 µg/ml Hoechst 33258 (Invitrogen) in 2XSSC for 10 min at RT and exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 30 min. The BrdU/dC labelled DNA strand was digested with 10 U/ml Exonuclease III (Promega M1811) for two rounds of 10 min at RT, followed by consecutive incubation with 75%, 95% and 100% ethanol. The slides were allowed to air dry for 30 min before applying Hybridizing Solution (70% formamide, 1 mg/ml blocking reagent (Roche), 10 mM Tris-HCl pH 7.2) containing the PNA probe (PNABio) and hybridized at RT for 2 h. The slides were washed briefly in wash buffer 1 (70% formamide/10 mM Tris-HCl) before the second 2 h hybridization with the complementary PNA probe (PNABio). Slides were then washed twice in wash buffer 1 for 15 min each wash, followed by three washes in 0.1 M Tris-HCl, pH 7.0/0.15 M NaCl/0.08% Tween-20 (5 min each). DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma D-9542) added to the second wash. Slides were mounted in antifade reagent (ProLong Gold, Invitrogen) and imaged.

Chromatin extracts, immunoblotting and antibodies

Cells were washed once with 5 ml cold PBS, drain to get all the liquid out and the dish was placed on ice. The cells were harvested by scraping into Lysis Buffer (250 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 0.5% NP-40) supplemented with protease inhibitors (Roche, cat. # 1 697 498), phosphatase, Protein Phosphatase Inhibitor 2 (New England Biolabs) and Trichostatin A HDAC inhibitor (TSA; Sigma) into cold microcentrifuge tube and kept on ice for lysing for 15 min. Tubes were spun 10 min, 14000 RPM at 4°C to pellet chromatin. The soluble fraction in the supernant was moved to a new tube. Chromatin was resuspended in equal volume lysis buffer and sonicated at 4°C using Bioruptor ® (Denville, NJ) for 45 min. Concentration was measured using using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and samples were prepared into 2X Loading Buffer, and resolved by SDS-PAGE, transferred onto nitrocellulose membranes. Ponceau staining was used to assess loading. Membranes were blocked with Odyssey Blocking Buffer (Li-Cor 927-40000), primary antibody incubated in 5% BSA TBS with 0.1% Tween 20 and detected with IRDye 800CW and 680LT secondary antibodies using the Odyssey Infrared Imaging System (Li-Cor). CENP-A mouse antibody was kindly provided by G. Goshima (7), CENP-T rabbit a kind gift from I. Cheeseman (8) and CENP-C rabbit (serum 554) was a kind gift of W. Earnshaw (9). Titration of the antibodies used for WB can be found in Suppl. Fig. 6A.

Immunofluorescence

Cells were grown on poly-L-lysine-coated coverslips. All the following procedures were performed at room temperature. Cells were fixed with 2% paraformaldehyde for 20 min, washed three times with PBS, permeabilised with 0.2% Triton X-100 in PBS for 5 min and blocked with 5% BSA in PBS/0.1%Tween-20 for 10 min. The incubation with primary antibodies was for 45 min followed by three washes with PBS and a 30 min incubation with the corresponding secondary antibodies: Alexa-Fluor 488 (green), 555 (red) and 647 (far red) at 1:1000 (Alexa). Coverslips were washed three times with PBS and mounted using Vectashield mounting medium containing 4,6 diamidino-2-phenylindole (DAPI; Vector Laboratories).

Beta-galactosidase assay

Senescence-associated β-Gal activity was evaluated using a cellular senescence assay staining kit (Millipore) according to the manufacturer's instructions. Briefly, cells were fixed in 1× fixative solution for 15 min at room temperature. After two washes with PBS, cells were incubated overnight at 37°C in 1× staining solution containing isopropyl β-D-1-thiogalactopyranoside as the substrate. Thereafter, cells were washed with PBS once, and the number of SA-β-Gal–positive cells (blue staining) was monitored under bright-field illumination microscopy and imaged.

Microscopy and image analysis

3D-SIM was performed as described by (10). All microscopy data were acquired using a DeltaVision OMX V4/Blaze 3D-SIM super-resolution microscope (GE Healthcare) at the Rockefeller University Bio-Imaging Resource Center (BIRC). This OMX system is fitted with a 100x/1.40 NA UPLSAPO oil objective (Olympus) and three Evolve EMCCD cameras (Photometrics) that were used in EM gain mode fixed at 170 electrons per count. Immersion oils ranging in refractive index from 1.512 to 1.518 were used depending on the ambient temperature and fluorochromes used. 405, 488 and 568 nm laser lines were used for excitation and the corresponding emission filter sets were 436/31, 528/48 and 609/37 nm respectively. Two identical stacks of optical sections with 125 nm spacing were collected for each dataset, first using conventional wide-field illumination of all channels, and then using structured illumination of selected channels. The system produces an effective xy pixel size of 40 nm in the 3D-SIM model. Following

acquisition, all datasets were processed using SoftWoRx v. 6.1 software (GE Healthcare) using Optical Transfer Functions (OTFs) generated from Point Spread Functions acquired with 100 nm (green and red) or 170 nm (blue) FluoSpheres (Invitrogen/Life Technologies). Wide-field datasets were deconvolved using constrained iterative deconvolution and structured illumination datasets were reconstructed as described (10) using channel-specific k0 values, custom OTFs, and Wiener filters of 0.005, 0.002, and 0.002 for the blue, green, and red channels, respectively. Image registration was performed with parameters refined using 100 nm TetraSpeck beads (Invitrogen/Life Technologies). Imaris software (Bitplane), Metamorph and ImageJ were used for 3D visualization and generating projection images for visualization and for quantification measurements of individual centromeres from each metaphase. Image J was used for line scan analysis. Quantification of structured illumination (SI) images at centromeres was performed using Imaris software (Bitplane) surface fitting function and extracting data on each centromere volume and sphericity. All images presented were imported and processed in Photoshop (Adobe Systems, Inc).

Supplemental References

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