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CENP-A chromatin prevents replication stress at centromeres to avoid structural aneuploidy

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Main Text

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Abstract

Chromosome segregation relies on centromeres, yet their repetitive DNA is often prone to aberrant rearrangements under pathological conditions. Factors that maintain centromere integrity to prevent centromere-associated chromosome translocations are unknown. Here, we demonstrate the importance of the centromere-specific histone H3 variant CENP-A in safeguarding DNA replication of alpha-satellite repeats to prevent structural aneuploidy. Rapid removal of CENP-A in S-phase, but not other cell cycle stages, caused accumulation of R-loops with increased centromeric transcripts, and interfered with replication fork progression. Replication without CENP-A causes recombination at alpha-satellites in an R-loop-dependent manner, unfinished replication and anaphase bridges. In turn, chromosome breakage and translocations arise specifically at centromeric regions. Our findings provide insights into how specialized centromeric chromatin maintains the integrity of transcribed noncoding repetitive DNA during S-phase.

Significance statement

CENP-A, the histone H3 variant that forms a unique centromeric chromatin, is essential for faithful chromosome segregation during mitosis. Inability to connect the centromere to the mitotic spindle causes aneuploidy, a hallmark of many cancers. In addition to chromosome missegregation, chromosome fusions at (peri-)centromeres are prevalent in cancers, but how such rearrangements arise remains unclear. Here, we identified a novel role for CENP-A in maintaining the integrity of centromere-associated repetitive sequences by ensuring their effective replication in human cells. In the absence of CENP-A, generation of DNA-RNA hybrids due to transcription-replication conflicts causes delayed DNA replication, centromere breakage, recombination and chromosome translocations at centromeres. Centromeres thus possess a special mechanism to facilitate their replication and suppress chromosome translocations.

Introduction

Centromeres hold a paradox: their essential function to support accurate chromosome segregation is evolutionarily conserved yet their DNA sequence is rapidly evolving (1–3). Human centromeres span ~0.3-5 Mb with repetitive alpha-satellite DNA arranged in tandem and then reiterated as homogenous higher order repeat (HOR) blocks (4). Evolution of centromere repeats has been modelled by short and long-range recombination events such as gene conversion, break-induced replication, crossing over and other mutagenic processes (2, 3, 5, 6). Changes in centromere size may contribute to meiotic drive (2), but may also elicit genome instability (7) by increasing the rate of chromosome mis-segregation (8). While recombination at centromeres is suppressed in meiosis (9), it is present in somatic cells and enhanced in cancerous cells (10) where whole-arm chromosome translocations are prevalent (11, 12). The mechanism(s) that prevent(s) breakage and translocations at these highly repetitive centromere sequences remain elusive.

Human centromeres are epigenetically specified by the histone H3 variant CENP-A (13, 14), which acts as a locus-specifying seed to assemble kinetochores for mitotic functions (15). While pericentromeric regions form heterochromatin, the core centromere harbors euchromatic characteristics with active transcription throughout the cell cycle, where long non-coding RNAs act *in cis* to contribute to centromere functions (16, 17). To date, it is unknown how a) transcription (17), b) recombination (10), c) late replication (18, 19) and d) propensity to form non-B-DNA and secondary structures (20–22), all features commonly associated with human fragile sites (23), are regulated to maintain the integrity of centromeric repeats. Here we identified a critical role for CENP-A in the maintenance of centromeric DNA repeats by repressing R-loop formation during DNA replication, thereby avoiding DNA replication stress and suppressing abortive chromosome translocation at the centromeres.

Results

We previously demonstrated that long-term CENP-A loss promotes alpha-satellite recombination events (10), suggesting a potential role for CENP-A in the maintenance of the integrity of centromere repeats, beyond its role in kinetochore formation and spindle stability (14, 24). As telomeres and rDNA possess specialized mechanisms that prevent their repetitive sequences from instability in S-phase (25, 26), we hypothesized that CENP-A plays a role in suppressing

alpha-satellite fragility during DNA replication. To test this, we used a system that allows rapid removal of endogenous CENP-A-containing nucleosomes using an Auxin Inducible Degrader (AID) (Figures 1A and S1A) in hTERT-immortalized, non-transformed, diploid Retinal Pigment Epithelial (hTERT-RPE-1) cells (27). We first monitored consequences of CENP-A removal on nucleosome stability using stably expressed SNAP-tagged H4 (Figure S1B). In untreated RPE-1 cells, when SNAP-H4 is labeled with tetramethylrhodamine (TMR) in G1, the majority of signals accumulate at centromeres (Figure S1C), as previously reported (28). This is likely caused by the fact that CENP-A nucleosomes assemble during late M/early G1 (29), whereas canonical nucleosomes assembly is coupled to DNA replication. Since the SNAP-tagged H4 transgene expression is not cell-cycle regulated while endogenous H4 levels peak in S-phase, this favors incorporation together with CENP-A at centromeric nucleosomes in G1. Auxin (IAA) addition led to the disassembly of CENP-A-containing nucleosomes as shown by rapid loss of CENP-A and of previously incorporated SNAP-tagged H4 selectively at centromeres but not globally at bulk chromatin (Figure S1B-E). However, new incorporation of H4 at centromeres at any phases of the cell cycle was not altered by CENP-A removal in G1 (Figure S1F, G), as assessed by H4^{SNAP} quenching/release and H4K5Ac CUT&RUN-qPCR (30). Considering that human centromere segments are occupied mostly with H3 nucleosomes with ~200 interspersed CENP-A nucleosomes (31), we expect the impact of short-term CENP-A depletion on the physical property of centromeric chromatin to be minimal.

To establish the critical time when CENP-A disruption causes alpha-satellite instability, we induced CENP-A depletion at different stages of the cell cycle (Figure S1H, I) and monitored recombination at centromeres in the first mitosis using centromeric chromosome-orientation fluorescence *in situ* hybridization (hereafter referred as Cen-CO-FISH; Figure 1B) (32). CENP-A depletion upon release from G1 arrest (by the CDK4/6 inhibitor Palbociclib), 8 h after G1 release corresponding to S-phase entry, or release from early S-phase arrest (by thymidine block), but not in G2 or metaphase-arrested cells, triggered severe centromere rearrangements (Figures 1C-F and S1J). These data indicate that absence of CENP-A in S-phase increases recombination at centromeres. We noticed that CENP-A depletion upon release from the early S-phase arrest by thymidine caused more robust aberrations in the Cen-CO-FISH pattern than CENP-A depletion upon G1 arrest by Palbociclib. This suggests that centromere integrity is sensitive to pre-induced DNA replication stress and further enhanced by CENP-A depletion.

Since DNA replication stress can induce recombination, we assessed if replication fork dynamics were altered by CENP-A depletion using DNA combing for single molecule analysis (33). We performed sequential labeling of nascent DNA with nucleotide analogs, CldU and IdU (Figure 2A) during early S (3 h) when mostly euchromatin is replicated (34), and in late S (7 h) when centromeres are replicated (18, 19) as confirmed by BrdU-Immunoprecipitation (Figure S2A). A change in CldU + IdU tracks length was used as indicator of a change in replication fork progression. Removal of CENP-A did not alter replication fork speed at 3 h after thymidine-release (Figure 2B, C). However, at 7 h, when fork speed accelerated in untreated cells as previously observed (35), fork speed was reduced upon CENP-A depletion (Figures 2B, C and S2B, C). These phenotypes were not due to perturbations in cell cycle progression, as CENP-A-depleted cells entered and progressed through S-phase with the same kinetics as untreated cells (Figure S2D, E).

To directly assess replication fork dynamics at centromeric repetitive DNA, alpha-satellites were labelled by two different FISH probes on combed DNA. Among these FISH-positive segments of fibers ranging 50 – 200 kb, the overall frequency of IdU and/or CldU labelled fibers at 7 h from thymidine release was reduced upon CENP-A depletion (Figure 2D), suggesting that replication at alpha-satellite repeats was impaired. To cover longer and more diverse stretches of alpha-satellites, we used a mix of 6 different ~50 bp probes that hybridized to alpha-satellites DNA ranging 50 – 1300 kb (median of ~250 kb) (Figure S2F). This analysis revealed that replication of centromeric DNA occurs in clusters (Figure 2E, F) leading to many converging forks at 7 h after thymidine release. As termination promotes topological burdens (36), this could contribute to slower replication at centromeres compared to the bulk genome (37). In CENP-A-depleted cells, replication fork speed at fibers labelled with the probes mix was reduced compared to untreated conditions (median 0.72 kb/min to 0.60 kb/min) (Figures 2G and S2G). Within replication clusters seen in late S-phase at centromeres (Figure 2F), we observed an increase in the number of ongoing forks/100 kb (median 1.60 vs. 2.01) upon CENP-A depletion (Figure 2H), indicating dormant origin firing, possibly reflecting compensation for the diminished fork velocity. However, dormant origin firing was not sufficient to fully rescue timely replication of entire segments of centromeres in late S-phase (Figure 2D, F). Altogether, our data suggest that CENP-A is needed for efficient replication fork progression through alpha-satellite repeats.

As a candidate for the impediments that induce replicative stress at centromeres upon CENP-A depletion, we monitored the occurrence of DNA-RNA hybrids (R-loops), known to obstruct DNA replication fork progression (Figure 3A) (38). To detect R-loops at centromeres we used DNA-RNA immunoprecipitation (DRIP) analysis, which captures DNA-RNA hybrids in their native chromosomal context (39), followed by qPCR amplification of centromere X-specific alpha-satellites (Figure 3B) or pan-centromeric alpha-satellites (Figure 3C). We found a modest but reproducible increase of centromere-associated R-loops upon CENP-A removal particularly in late S-phase (Figures 3B, C and S3A), the time when most centromeres are replicated (Figure S2A). The signals were specific to DNA-RNA hybrid, since they were not detectable with the control IgG, and were eliminated by treatment with RNase H (Figure 3C). DNA-RNA hybrids were induced upon IAA treatment at centromeres, but not within the hybrids-rich β -actin terminator (Figure S3A). Induction of centromere-associated DNA-RNA hybrids upon acute CENP-A depletion during thymidine release was also confirmed using immunofluorescence-based detection of the S9.6 antibody (Figure S3B-D), where such induction at centromeres is only seen in late S-phase and absent when CENP-A was depleted during G2 or M phase (Figure S3D). Taken together, these data show that CENP-A depletion causes impaired DNA replication progression and R-loop formation at centromeres.

R-loops, which form co-transcriptionally, are prevalent at replication-transcription conflicts (38, 40). Centromeres are actively transcribed throughout the cell cycle (17, 41), raising the hypothesis that CENP-A depletion causes R-loop formation due to convergence of replication and transcription machineries. Upon release from thymidine-mediated arrest, CENP-A depletion did not affect the levels of centromere transcripts during early S-phase, but when cells progressed to late S-phase, the RNA levels increased (Figure 3A, D). This increase was reproducible using different centromeric probes and in two other cell lines U2OS and DLD-1 (Figure 3E). We also confirmed that *de novo* centromere transcription is indeed active in late S-phase by immunoprecipitation followed by RT-qPCR of 5-fluorouridine (FU) (Figure S3E-G). Finally, while the large majority of active RNA polymerase II dissociates from mitotic chromosomes (42, 43), an increase in centromere-associated active polymerase after CENP-A depletion was detected during mitosis (Figure S3H, I). This increase was concomitant with the accumulation of the DNA damage kinase ATR (21), consistent with persistent stress at centromeric regions, but with no detectable increase in R-loops (Figure S3I).

Consistent with the notion that R-loops derive from transcription-replication conflicts (44), levels of γ H2AX at centromeres increased in late S-phase after CENP-A depletion (Figure S4A-C), with γ H2AX foci occupying a region within and at the periphery of the centromere (Figure S4B), perhaps reflecting previously reported peripheral replication of centromeres (45). To test if R-loops could cause centromere damage and alpha-satellite repeat instability, we stably integrated RNase H1 under a doxycycline-inducible promoter in the hTERT-RPE-1^{EYFP-AID}CENP-A cell line (Figure 3F) (21). Expression of RNase H1 was able to rescue phenotypes observed in CENP-A-depleted cells such as enhanced centromere-associated γ H2AX (Figure S4C) and aberrant Cen-CO-FISH patterns indicative of centromere recombination (Figure 3G). Similar results were obtained by targeting RNase H1 to the alpha-satellite repeats via the inducible expression of the RNase H1 fused to the CENP-B DNA binding domain (Figure S4D-F). These data demonstrate that the centromere instability in the absence of CENP-A is caused by the generation of centromeric R-loops during alpha-satellite DNA synthesis.

Replicative stress is a main driver of genomic instability (46, 47). It has been shown that persistent replication intermediates in mitosis are processed *via* the error-prone mitotic DNA synthesis (MiDAS), prominently observed at common fragile sites (CFS) (48). To test if the replication stress imposed by CENP-A depletion in S-phase results in MiDAS, we depleted CENP-A in asynchronous cell culture for 10 h and monitored incorporation of EdU during the subsequent first mitosis (Figure 4A), assuming that these mitotic cells had gone through S-phase without CENP-A. We noticed an increase in cells containing EdU foci following CENP-A depletion, to a similar level of the DNA replication slowdown induced with low dose of aphidicolin (48) (Figure 4B). The EdU foci in the IAA-treated sample were found to be enriched at centromeric regions with Proximity Ligation Assay (PLA), which was not observed with aphidicolin treatment only (Figure 4C). This suggests that removal of CENP-A causes a subset of centromeres to continue replication into mitosis.

Mitotic entry with under-replicated or unresolved recombination intermediates causes anaphase bridges (49). To monitor if replication stress induced by CENP-A depletion generates such mitotic perturbations, we used live cell imaging to follow chromosome separation in RPE-1 cells. CENP-A depletion in G1/early S (Figure 4A) indeed increased frequency of chromosome bridges threaded between the segregating DNA masses (Figure 4D and Suppl. Movie 1). In contrast, the number of ultrafine-bridges (UFBs) marked with PICH, often seen at centromeres

(50), was not affected by CENP-A depletion (Figure S5A), implying different underlying molecular origins (51, 52) and substantiating that most centromeric UFBs are not originating from under-replication but instead through DNA decatenation impairment (53). Moreover, we detected a significant increase in regions with no or reduced DAPI/Giemsa staining – possibly due to chromosome breakage, under-replicated or decondensed regions – affecting specifically one or more metaphase centromeres (Figures 4E and S5B) and resembling cytological abnormalities seen at CFS after replicative stress. We also found co-localization between the DNA double strand break factor 53BP1 and the centromeric sequence specific DNA binding protein CENP-B by PLA in the following cell cycle after CENP-A depletion (Figure 4F), suggesting that DNA breaks persist at the centromere in interphase. In summary, our data show that upon entry into mitosis, replication impairments caused by CENP-A depletion lead to centromere instability, therefore mimicking fragile sites and under-replicated regions of the genome.

DNA damage at centromere segments may induce chromosome translocations between chromosomes with homologous centromere sequences (Figure 5A). To score them, we combined multicolor FISH (mFISH) followed by CENP-B boxes FISH staining to check the status of centromeric DNA (Figure 5B). Within two cell cycles following CENP-A depletion, centromeric abnormalities and rearrangements were induced at one or more chromosomes, beyond numerical aneuploidy. These abnormalities spanned from centromere breakage and fragmentation, isochromosomes and a high proportion of whole-arm chromosome translocations involving both acrocentric and metacentric chromosomes (Figures 5C, D and S5C-E). No chromosomal translocations were detected in cells arrested in the first mitosis without CENP-A (10 h IAA) (Figure 5D), indicating that anaphase bridge breakage is likely a necessary step for centromere instability to trigger overall genome instability, as recently shown for mitotic chromosome breakage-fusion-bridge cycle (54). To ensure that this phenotype was not due to whole chromosome mis-segregation resulting from CENP-A depletion (27), we generated mitotic defects independently of centromeric dysfunction by centrosome depletion with a Plk4 inhibitor (48 h centrinone) (55). In this case, we still detected an increase in chromosomal rearrangements, but such alterations occurred mainly outside the centromeric regions (e.g. telomere fusions; Figures 5D and S5C-D) confirming the specific effect of CENP-A depletion on centromere DNA fragility and chromosome integrity. These results imply that chromosome translocations at

centromeres are not the primary consequence of chromosome missegregation, but are generated by mitotic DNA breakages due to replication stress at centromeres.

5 **Discussion**

Here we demonstrate that CENP-A is critical in the maintenance of centromeric DNA repeats by repressing R-loop formation during DNA replication (Figure 5E). While impaired histone assembly onto newly replicated DNA impedes replication (56), our CENP-A depletion did not interfere with histone assembly during S-phase (Figure S1F, G). Instead, CENP-A nucleosomes removal on parental DNA slows replication fork progression at centromeres, likely due to replication-transcription conflicts, which stabilize R-loops. Intriguingly, slowed DNA replication upon CENP-A depletion was observed at genomic levels during late S (Figure 2B, C), raising a possibility that CENP-A removal also perturbs replication of non-centromeric regions. This may be related to the observation that CENP-A accumulates outside centromeres (31) at DNA damage sites (57) or at transcriptionally active regions before being removed in a replication-dependent manner (58). However, the effect of CENP-A depletion in the replication slowdown is specific to late S-phase – a time when centromeres are actively replicated (Figure S2A), and, importantly, the subsequent increase in fragility and rearrangements were specifically found at centromeres (Figures 5D and S5B). Together, our data highlight the role of CENP-A in S-phase to prevent DNA instability at the centromeres.

While nucleosomes generally suppress transcriptional initiation (59) and compete with R-loop formation (40), CENP-A depletion increased R-loop and centromeric RNA levels only during centromere replication. Thus, we propose that CENP-A chromatin holds a specialized function to facilitate fork progression and suppress R-loop formation at centromeres during DNA replication. Since a subset of alpha-satellite RNAs stay associated with the centromere from which they were transcribed throughout the cell cycle (17), CENP-A chromatin may sequester these RNAs to minimize R-loop formation upon replication-transcription conflicts. This is consistent with the report that CENP-C and other centromeric proteins can bind to RNA (17), and depletion of Constitutive Centromere Associated Network components (as CENP-C, CENP-T, CENP-W) induces aberrant centromere recombination without obvious displacement of CENP-A from chromatin (10). It is also possible that CENP-A is important for recruitment of

proteins, such as helicases that remove DNA-RNA hybrids (38). Altogether, our results suggest that centromeric DNA regions are intrinsically difficult to replicate to a similar extent as other repetitive sequences where specialized proteins act to facilitate fork progression (60, 61).

Mitosis with under-replicated DNA and/or recombination caused by CENP-A depletion can lead to DNA damage in a subset of centromeres (Figure 4), potentially promoting non-allelic exchanges between near-identical repeats on different chromosomes (Figure 5) (7, 11). Only a small fraction of CENP-A-depleted centromeres undergo such instability, possibly due to low level of transcription (17, 41), compensation by dormant origins firing (Figure 2H) and MiDAS (Figure 4A-C), which minimize centromeric under-replication ahead of cell division. In addition to sequence homology, centromere clustering may facilitate chromosome translocation at centromeres (62). Notably, inter-centromeric rearrangements following centromere instability increased preferentially in acrocentric chromosomes, which are frequently linked together at their rDNA loci forming the nucleoli (63). As CENP-A levels are reduced in senescent cells (64) and in certain type of organismal aging in human (65), centromere-induced structural aneuploidy may represent a key mechanism underlying aging-associated tumorigenesis. Senescent cells present hypomethylated and highly transcribed centromeres (66, 67) that show altered nucleolar association (68), which in turn might impact the regulation of alpha-satellite expression (41) and favor rearrangements at acrocentric chromosomes. Finally, in agreement with a role of CENP-A in protecting centromeric regions, loss of CENP-A from the original alpha-satellite locus during neocentromere formation can be accompanied by erosion at the repeats (69). Our data point toward the possibility that the high prevalence of centromeric transcripts overexpression and whole-arm chromosome translocations seen in cancer are caused by compromised functionalities of centromeric chromatin that suppress DNA replication stress (11, 12).

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Authors contribution. D.F., H.F., S.G. and S.H. designed the study, made figures and wrote the manuscript. A. Scelfo. and R.G. designed the centromere probe mix. R.W., T.W. and S.G. conducted the single molecule replication assays. M.D., G.R., C.K.W. and D.F. performed the mFISH experiments. S.G. and S.H. performed and analyzed all other experiments. A. Smogorzewska supervised R.W. A. Smogorzewska, R.W., G.R. and T.W. commented on the manuscript. D.F. and H.F. oversaw the entire project and provided financial support.

Figure Legends

Figure 1. Rapid removal of CENP-A during S-phase increases centromere recombination.

(A) Schematic of the inducible degradation system to deplete endogenous CENP-A after addition of Auxin (IAA) in RPE-1 cells. (B) Schematic illustration of the Cen-CO-FISH centromeric DNA probes. Hybridization by unidirectional PNA probes differentially labels the forward or reverse strands of each sister chromatid. Each black arrow symbolizes a HOR in the alpha-satellite array. (C) Quantification of the percentage of aberrant Cen-CO-FISH patterns/cell with or without CENP-A depletion in the different phases of the cell cycle as indicated. For synchronization strategy see figure S1H, I. Cells from two or three independent experiments are depicted with a different shape with $n = 15$ cells per condition and per experiment. Red lines represent the median. A Mann-Whitney test was performed on the pooled single cell data of the two or three independent experiments: **** $p < 0.0001$. (D) Synchronization strategy for the Cen-CO-FISH experiment shown in E-F. (E) (Left) Representative Cen-CO-FISH on metaphase chromosomes after CENP-A depletion in the previous S-phase. Scale bar: $5 \mu\text{m}$. (Right) Schematic of the resulted Cen-CO-FISH staining patterns in normal and abnormal centromeres, with visible SCE due to recombination and cross over events. (F) Quantification of the percentage of aberrant Cen-CO-FISH patterns/cell after CENP-A depletion in the previous S-phase. The bar graph is the average of three independent experiments (depicted with different shapes) with $n = 15$ cells per condition. Error bars: standard error of the mean. A Mann-Whitney test was performed on pooled single cell data of three independent experiments: **** $p < 0.0001$.

Figure 2. CENP-A removal leads to impaired replication fork progression through centromere alpha-satellites in late S-phase.

(A) Schematic illustration of the single molecule DNA replication experiments shown in B-D. (B) Quantification of the replication fork speed in $n > 100$ tracks/condition. The red bars represent the median. Mann-Whitney test: **** $p < 0.0001$. (C) Summary of the quantification of the replication fork speed in the bulk genome at 3 h or 7 h thymidine release in 3 or 4 independent experimental replicates represented by different symbols. The bars represent the standard deviation of the mean. Mann-Whitney test: * $p = 0.0286$. (D) Quantification of the percentage of actively replicating centromeres in the DNA combing assay (IdU and/or CldU-positive). Two different oligo PNA probes were used to target alpha-satellites. Each dot represents one experiment with $n > 30$ centromeric tracks per condition. The

bars represent the standard error of the mean. χ^2 test: **p=0.005, ****p<0.0001. (E) Schematic illustration of the single molecule DNA replication experiments at centromeric region shown in F-H, where 6 RNA probes were used to target alpha-satellites. (F) Representative images of single molecule alpha-satellite DNA combing at 7 h thymidine release. A probe mix recognizing alpha-satellite repeats was used to specifically label centromeres. Scale bar: 10 μ m. (G) Quantification of the centromeric replication fork speed at 7 h thymidine release. n>110 tracks/condition. The lines represent the median with interquartile range. Mann-Whitney test: ***p=0.0006. (H) Quantification of the number of ongoing forks/100 kb at centromeres at 7h thymidine release. n>90 tracks/condition. The lines represent the median with interquartile range. Mann-Whitney test: *p=0.0411.

Figure 3. Accumulation of R-loops following CENP-A depletion causes increased centromere recombination. (A) Schematic illustration of the experiments shown in B-E. (B) DRIP-qPCR quantification of the R-loops levels at the centromere of chromosome X as a measure of the percentage of input retrieved at different timepoints in S-phase. n = 1. Each dot shows the mean of technical replicates. (C) DRIP-qPCR of R-loops levels at the centromeres as a measure of the percentage of input retrieved using alpha-satellite primers at 7 h post-thymidine release. Each dot shows the mean of three technical replicates of six independent experiments represented by different symbols. A *t*-test was performed including the technical replicates. *p =0.019; ****p<0.0001 (D) Quantification of centromere transcripts levels using alpha-satellite primers by RT-qPCR after thymidine release. IAA data were normalized over the corresponding NT. Each dot represents the mean of 2-8 independent experiments. The bars represent the standard error of the mean. One sample Wilcoxon Signed Rank Test: *p=0.0312 (E) Quantification of the transcript levels at the centromeres using alpha-satellite and Cen X primers and by RT-qPCR after 7 h thymidine release in DLD-1 and U2OS cells. IAA data were normalized over the corresponding NT. Each dot represents the mean of three technical replicates. The bars represent the standard error of the mean. U2OS cells: One sample Wilcoxon Signed Rank Test: *p=0.0312. DLD-1 cells: One sample t test: *p=0.0273. (F) Schematic illustration of the rescue experiment shown in G. (G) Quantification of the percentage of aberrant Cen-CO-FISH patterns/cell after RNase H1 overexpression, and subsequent CENP-A depletion during the prior S-phase. Cells from two independent experiments are depicted with a different

shape with $n = 15$ cells per condition and per experiment. The red lines represent the median. A Mann-Whitney test was performed on the pooled single cell data of the two independent experiments: **** $p < 0.0001$.

5 **Figure 4. CENP-A removal in S-phase causes centromere fragility in mitosis.** (A) Schematic illustration of the experiments shown in B-C. (B) Quantification of the percentage of EdU-positive metaphases after 10 h IAA or 24 h aphidicolin ($0.2 \mu\text{M}$). Each dot represents one experiment. The bars represent the standard error of the mean. χ^2 test: * $p = 0.0435$, ** $p = 0.0044$. (C) Quantification of EdU-positive centromeres in metaphase cells by measuring mean
10 fluorescence intensity signal from PLA between CENP-B and EdU-biotin after 10 h IAA or 24 h aphidicolin ($0.2 \mu\text{M}$). $n > 25$ cells/condition. The lines represent the median with interquartile range. Mann-Whitney test: **** $p = 0.0009$. (D) (Left) Representative still images of H2B-mCherry RPE-1 live-cell imaging showing the presence of an anaphase bridge after 10 h IAA. Scale bar: 10 μm . (Right) Quantification of the percentage of RPE-1 cells showing at least one anaphase
15 bridge after 10 h IAA. Each dot represents one of four independent experiments depicted by different symbols. The bars represent the standard error of the mean. χ^2 test: **** $p < 0.0001$. (E) (Left) Representative FISH images of a chromosome with reduced DAPI/Giemsa staining at the centromere, identified with a CENP-B box FISH probe. (Right) Quantification of the percentage of metaphase spreads showing reduced DAPI/Giemsa staining at the centromere after 24 h IAA
20 (square) or after thymidine release (circle and inverted triangle). Each dot represents one experiment with $n > 70$ cells. The bars represent the standard error of the mean. χ^2 test: * $p = 0.0113$. Scale bar: 3 μm . (F) (Left) Representative immunofluorescence images of Proximity Ligation Assay (PLA) between CENP-B and 53BP1. (Right) Quantification of centromeric DNA damage in RPE-1 cells by measuring PLA mean fluorescence intensity signal between CENP-B
25 and 53BP1 after 24 h IAA. $n > 65$ cells/condition. The bars represent the standard error of the mean. Mann-Whitney test: **** $p < 0.0001$. Scale bar: 5 μm .

Figure 5. Centromere instability caused by CENP-A depletion during DNA replication initiates chromosome rearrangements and translocations in the ensuing cell cycles.

30 (A) Illustration of the formation of centromeric chromosomal rearrangements after CENP-A depletion due to breaks generated during the previous cell cycle and repaired by non-allelic

homologous recombination. (B) Schematic representation of the multicolor-FISH (mFISH) experiments shown in C and D. (C) Representative images of mFISH and centromeric FISH (CENP-B box probe) of DLD-1 chromosome spreads showing chromosomal aberrations occurring at centromeres after 48 h IAA. Schematics of the observed events are also shown. Scale bar: 2 μ m. (D) Quantification of the percentage of metaphase spreads showing chromosomal rearrangements by multicolor-FISH after CENP-A depletion or centrosome depletion in DLD-1 cells. n=3 (NT & 48 h IAA) or 1 (10 h IAA & 48 h centrinone 0.2 μ M). The bars represent the standard error of the mean. χ^2 test: ****p<0.0001. (E) Model for maintenance of centromeric DNA stability mediated by CENP-A-containing chromatin. See text for details.

10

Methods

Cell synchronization & treatments

15 In preparation to the experiment, hTERT RPE-1 cells were split at low confluency ($\sim 3 \times 10^5$ cells/10 cm dish) approximately 24 h prior to the treatment. Indole-3-acetic acid (IAA, auxin) (I5148; Sigma) was dissolved in ddH₂O and used at a final concentration of 500 μ M. RNase H1-GFP expression was induced using 200 ng/ μ l of doxycycline. RNase H treatment on fixed cells was performed using 5 U RNase H (M0297, NEB) for 3 h at 37 °C, or mock for no RNase H digestion control in 1X RNase H digestion buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT) without the enzyme, before proceeding to immunofluorescence. Thymidine synchronization and release experiment was performed by incubating cells with 2-5 mM thymidine for 22-28 h; cells were then treated with IAA for 1 h, washed three times with Dulbecco sterile phosphate-buffered saline (PBS) and released into fresh medium containing IAA. For G1 synchronization, cells were treated for 24 h with 150 nM Palbociclib (S1116; Selleck), a CDK4/6 inhibitor, before washing out and releasing from the arrest (70). G2 arrest was induced by RO-3306 CDK1 inhibitor (ALX-270-463, Enzo) at 10 μ M final concentration for 20 h. Mitotic arrest was achieved using 0.1 μ g/mL colcemid (Roche) for 5 h, followed by mitotic shake off to obtain a pure mitotic population for processing. Centrinone (Clinisciences, #HY-18682) treatment to induce centrosome loss was performed at 0.2 μ M for 48 h.

30

Cen-CO-FISH

CO-FISH at centromere was performed as previously described (32). Detailed protocol available in the Supplementary Methods.

5 Microscopy, Live-Cell Microscopy and Image Analyses

Images were acquired on a Fluorescent microscope DeltaVision Core system (Applied Precision) with 100X Olympus UPlanSApo 100 oil-immersion objective (NA 1.4), 250W Xenon light source equipped with a Photometrics CoolSNAP_HQ2 Camera. ~ 4 μm Z-stacks were acquired (Z step size: 0.2 μm). Imaris software (Bitplane) was used to quantify fluorescence intensity in the deconvolved 3D images using centromere surfaces automatically determined by ACA staining detection. Quantification of gamma-H2AX and S9.6 R-loops at centromeres was performed using Imaris software (Bitplane) surface fitting function and extracting mean fluorescence intensity for each centromere. All images presented were imported and processed in Photoshop (Adobe Systems, Inc S.J.). Movies of live cells were acquired using an Inverted Eclipse Ti-2 (Nikon) full motorized + Spinning disk CSU-W1 (Yokogawa) microscope. Cells were grown on high optical quality plastic slides (ibidi) for this purpose.

Multicolor FISH (mFISH) karyotyping

Cells grown to ~ 75–80 % confluency were treated with colcemid (0.1 $\mu\text{g}/\text{mL}$) for 3 h and prepared as previously described (8) for the mFISH karyotyping. The Metafer imaging platform (MetaSystems) and the Isis software were used for automated acquisition of the chromosome spread and mFISH image analysis.

CUT&RUN-qPCR

CUT&RUN was performed according to the procedure previously reported (71) starting from 1 million cells and using anti-H4K5Ac (Abcam, ab51997, 1/1000) antibody. Rabbit IgG isotype control antibody (ThermoFisher, 10500C, 1/100) was used for background detection. qPCR was performed using the LightCycler 480 (Roche) system with primers reported below. Fold enrichment at centromeres was calculated using alpha-satellite primers, with the $\Delta\Delta\text{Ct}$ method. The rabbit IgG isotype sample was used as control sample and normalization was performed to the Ct values from the Alu repeat primers.

Statistical testing

5 Statistical analysis of all the graphs was performed using GraphPad Prism 8. A two-sided χ^2 test was performed on categorical datasets. *t*-tests and Mann-Whitney tests were performed on quantitative datasets depending normal distribution. When data from the untreated samples were normalized to 1, a one sample Wilcoxon signed rank test or a one sample *t*-test were performed and compared to a theoretical value of 1.

Additional Methods

10 Detailed description of the following materials and methods can be found in the Supplementary Materials file:

- Cell culture
- Fluorescence in situ hybridization (FISH)
- Cen-CO-FISH
- 15 • DNA combing
- Chromatin extraction and immunoblotting
- Histone deposition monitoring with SNAP-labelling
- Generation of Stable Cell Lines
- Flow cytometry
- 20 • EdU staining in metaphase cells
- Immunofluorescence, Proximity Ligation Assay (PLA) and Antibodies
- Ultrafine bridges IF staining
- Metaphase spreads centromere quantification
- Unfixed chromosome spreads
- 25 • BrdU Immunoprecipitation for nascent DNA & DNA-RNA hybrids Immunoprecipitation (DRIP)
- 5-Fluouridine Immunoprecipitation for nascent RNA
- RT-qPCR
- qPCR primers

Supplementary Information in a separate File includes:

Supplementary Methods:

Supplementary Figures S1-S5

Movies S1

5 **References**

1. T. Fukagawa, Centromere DNA, proteins and kinetochore assembly in vertebrate cells. *Chromosome Res* **12**, 557–567 (2004).
2. S. Henikoff, K. Ahmad, H. S. Malik, The Centromere Paradox: Stable Inheritance with Rapidly Evolving DNA. *Science* **293**, 1098–1102 (2001).
- 10 3. P. B. Talbert, S. Henikoff, Centromeres Convert but Don't Cross. *PLoS Biol* **8**, e1000326 (2010).
4. M. G. Schueler, A. W. Higgins, M. K. Rudd, K. Gustashaw, H. F. Willard, Genomic and Genetic Definition of a Functional Human Centromere. *Science* **294**, 109–115 (2001).
- 15 5. W. R. Rice, “A Game of Thrones at Human Centromeres II. A new molecular/evolutionary model” (Evolutionary Biology, 2019) <https://doi.org/10.1101/731471> (May 8, 2020).
6. G. P. Smith, Evolution of repeated DNA sequences by unequal crossover. *Science* **191**, 528–535 (1976).
7. E. M. Black, S. Giunta, Repetitive Fragile Sites: Centromere Satellite DNA as a Source of Genome Instability in Human Diseases. *Genes* **9**, 615 (2018).
- 20 8. M. Dumont, *et al.*, Human chromosome-specific aneuploidy is influenced by DNA-dependent centromeric features. *EMBO J.* **39**, e102924 (2020).
9. M. Nambiar, G. R. Smith, Repression of harmful meiotic recombination in centromeric regions. *Semin Cell Dev Biol* **54**, 188–197 (2016).
- 25 10. S. Giunta, H. Funabiki, Integrity of the human centromere DNA repeats is protected by CENP-A, CENP-C, and CENP-T. *Proceedings of the National Academy of Sciences*, 201615133 (2017).
11. V. Barra, D. Fachinetti, The dark side of centromeres: types, causes and consequences of structural abnormalities implicating centromeric DNA. *Nature Communications* **9**, 4340 (2018).
- 30 12. R. Beroukhi, *et al.*, The landscape of somatic copy-number alteration across human cancers. *Nature* **463**, 899–905 (2010).

13. D. Fachinetti, *et al.*, A two-step mechanism for epigenetic specification of centromere identity and function. *Nat Cell Biol* **15**, 1056–1066 (2013).
14. K. L. McKinley, I. M. Cheeseman, The molecular basis for centromere identity and function. *Nat Rev Mol Cell Biol* **17**, 16–29 (2016).
- 5 15. S. Santaguida, A. Musacchio, The life and miracles of kinetochores. *The EMBO Journal* **28**, 2511–2531 (2009).
16. S. Corless, S. Höcker, S. Erhardt, Centromeric RNA and Its Function at and Beyond Centromeric Chromatin. *Journal of Molecular Biology* **432**, 4257–4269 (2020).
- 10 17. S. M. McNulty, L. L. Sullivan, B. A. Sullivan, Human Centromeres Produce Chromosome-Specific and Array-Specific Alpha Satellite Transcripts that Are Complexed with CENP-A and CENP-C. *Developmental Cell* **42**, 226–240.e6 (2017).
18. K. G. T. Hagen, D. M. Gilbert, H. F. Willard, S. N. Cohen, Replication timing of DNA sequences associated with human centromeres and telomeres. *Molecular and Cellular Biology* **10**, 6348–6355 (1990).
- 15 19. D. J. Massey, D. Kim, K. E. Brooks, M. B. Smolka, A. Koren, Next-Generation Sequencing Enables Spatiotemporal Resolution of Human Centromere Replication Timing. *Genes* **10**, 269 (2019).
- 20 20. A. Aze, V. Sannino, P. Soffientini, A. Bachi, V. Costanzo, Centromeric DNA replication reconstitution reveals DNA loops and ATR checkpoint suppression. *Nature Cell Biology* **18**, 684–691 (2016).
21. L. Kabeche, H. D. Nguyen, R. Buisson, L. Zou, A mitosis-specific and R loop–driven ATR pathway promotes faithful chromosome segregation. *Science*, eaan6490 (2017).
22. S. Kasinathan, S. Henikoff, Non-B-Form DNA Is Enriched at Centromeres. *Mol Biol Evol* **35**, 949–962 (2018).
- 25 23. B. Le Tallec, *et al.*, Updating the mechanisms of common fragile site instability: how to reconcile the different views? *Cell. Mol. Life Sci.* **71**, 4489–4494 (2014).
24. S. Gemble, *et al.*, Centromere Dysfunction Compromises Mitotic Spindle Pole Integrity. *Current Biology* **29**, 3072–3080.e5 (2019).
- 30 25. M. Higa, M. Fujita, K. Yoshida, DNA Replication Origins and Fork Progression at Mammalian Telomeres. *Genes* **8**, 112 (2017).
26. M. S. Lindström, *et al.*, Nucleolus as an emerging hub in maintenance of genome stability and cancer pathogenesis. *Oncogene* **37**, 2351–2366 (2018).
27. S. Hoffmann, *et al.*, CENP-A Is Dispensable for Mitotic Centromere Function after Initial Centromere/Kinetochores Assembly. *Cell Reports* **17**, 2394–2404 (2016).

28. D. L. Bodor, L. P. Valente, J. F. Mata, B. E. Black, L. E. T. Jansen, Assembly in G1 phase and long-term stability are unique intrinsic features of CENP-A nucleosomes. *Mol. Biol. Cell* **24**, 923–932 (2013).
- 5 29. L. E. T. Jansen, B. E. Black, D. R. Foltz, D. W. Cleveland, Propagation of centromeric chromatin requires exit from mitosis. *The Journal of Cell Biology* **176**, 795–805 (2007).
30. R. E. Sobel, R. G. Cook, C. A. Perry, A. T. Annunziato, C. D. Allis, Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *PNAS* **92**, 1237–1241 (1995).
- 10 31. D. L. Bodor, *et al.*, The quantitative architecture of centromeric chromatin. *eLife* **3**, e02137 (2014).
32. S. Giunta, Centromere Chromosome Orientation Fluorescent in situ Hybridization (Cen-CO-FISH) Detects Sister Chromatid Exchange at the Centromere in Human Cells. *Bio Protoc* **8** (2018).
- 15 33. P. Norio, C. L. Schildkraut, Visualization of DNA Replication on Individual Epstein-Barr Virus Episomes. *Science* **294**, 2361–2364 (2001).
34. C. Marchal, J. Sima, D. M. Gilbert, Control of DNA replication timing in the 3D genome. *Nature Reviews Molecular Cell Biology* **20**, 721–737 (2019).
35. D. Housman, J. A. Huberman, Changes in the rate of DNA replication fork movement during S phase in mammalian cells. *Journal of Molecular Biology* **94**, 173–181 (1975).
- 20 36. D. Fachinetti, *et al.*, Replication termination at eukaryotic chromosomes is mediated by Top2 and occurs at genomic loci containing pausing elements. *Mol Cell* **39**, 595–605 (2010).
37. A. Mendez-Bermudez, *et al.*, Genome-wide Control of Heterochromatin Replication by the Telomere Capping Protein TRF2. *Molecular Cell* **70**, 449–461.e5 (2018).
- 25 38. T. García-Muse, A. Aguilera, R Loops: From Physiological to Pathological Roles. *Cell* **179**, 604–618 (2019).
39. S. J. Boguslawski, *et al.*, Characterization of monoclonal antibody to DNA · RNA and its application to immunodetection of hybrids. *Journal of Immunological Methods* **89**, 123–130 (1986).
- 30 40. F. Chedin, C. J. Benham, Emerging roles for R-loop structures in the management of topological stress. *J. Biol. Chem.* **295**, 4684–4695 (2020).
41. L. Bury, *et al.*, Alpha-satellite RNA transcripts are repressed by centromere–nucleolus associations. *eLife* **9**, e59770 (2020).
42. F. L. Chan, *et al.*, Active transcription and essential role of RNA polymerase II at the centromere during mitosis. *PNAS* **109**, 1979–1984 (2012).

43. C. Perea-Resa, L. Bury, I. M. Cheeseman, M. D. Blower, Cohesin Removal Reprograms Gene Expression upon Mitotic Entry. *Molecular Cell* **78**, 127-140.e7 (2020).
44. S. Hamperl, M. J. Bocek, J. C. Saldivar, T. Swigut, K. A. Cimprich, Transcription-replication conflict orientation modulates R-loop levels and activates distinct DNA damage responses. *Cell* **170**, 774-786.e19 (2017).
45. J.-P. Quivy, *et al.*, A CAF-1 dependent pool of HP1 during heterochromatin duplication. *The EMBO Journal* **23**, 3516–3526 (2004).
46. M. K. Zeman, K. A. Cimprich, Causes and consequences of replication stress. *Nature Cell Biology* **16**, 2–9 (2014).
47. R. A. Burrell, *et al.*, Replication stress links structural and numerical cancer chromosomal instability. *Nature* **494**, 492–496 (2013).
48. S. Minocherhomji, *et al.*, Replication stress activates DNA repair synthesis in mitosis. *Nature* **528**, 286–290 (2015).
49. C. Gelot, I. Magdalou, B. S. Lopez, Replication Stress in Mammalian Cells and Its Consequences for Mitosis. *Genes* **6**, 267–298 (2015).
50. C. Baumann, R. Körner, K. Hofmann, E. A. Nigg, PICH, a Centromere-Associated SNF2 Family ATPase, Is Regulated by Plk1 and Required for the Spindle Checkpoint. *Cell* **128**, 101–114 (2007).
51. S. M. Germann, *et al.*, TopBP1/Dpb11 binds DNA anaphase bridges to prevent genome instability. *J Cell Biol* **204**, 45–59 (2014).
52. M. Kaulich, F. Cubizolles, E. A. Nigg, On the regulation, function, and localization of the DNA-dependent ATPase PICH. *Chromosoma* **121**, 395–408 (2012).
53. S. Gemble, *et al.*, Topoisomerase II α prevents ultrafine anaphase bridges by two mechanisms. *Open Biol* **10** (2020).
54. N. T. Umbreit, *et al.*, Mechanisms generating cancer genome complexity from a single cell division error. *Science* **368** (2020).
55. Y. L. Wong, *et al.*, Reversible centriole depletion with an inhibitor of Polo-like kinase 4. *Science* **348**, 1155–1160 (2015).
56. J. Mejlvang, *et al.*, New histone supply regulates replication fork speed and PCNA unloading. *J Cell Biol* **204**, 29–43 (2014).
57. S. G. Zeitlin, *et al.*, Double-strand DNA breaks recruit the centromeric histone CENP-A. *Proc Natl Acad Sci U S A* **106**, 15762–15767 (2009).

58. Y. Nechemia-Arbely, *et al.*, DNA replication acts as an error correction mechanism to maintain centromere identity by restricting CENP-A to centromeres. *Nat Cell Biol* **21**, 743–754 (2019).
59. S. L. Klemm, Z. Shipony, W. J. Greenleaf, Chromatin accessibility and the regulatory epigenome. *Nature Reviews Genetics* **20**, 207–220 (2019).
60. A. Sfeir, *et al.*, Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. *Cell* **138**, 90–103 (2009).
61. A. Madireddy, *et al.*, FANCD2 Facilitates Replication through Common Fragile Sites. *Molecular Cell* **64**, 388–404 (2016).
- 10 62. H. Muller, J. Gil, I. A. Drinnenberg, The Impact of Centromeres on Spatial Genome Architecture. *Trends in Genetics* **35**, 565–578 (2019).
63. T. A. Potapova, *et al.*, Superresolution microscopy reveals linkages between ribosomal DNA on heterologous chromosomes. *J Cell Biol* **218**, 2492–2513 (2019).
- 15 64. K. Maehara, K. Takahashi, S. Saitoh, CENP-A Reduction Induces a p53-Dependent Cellular Senescence Response To Protect Cells from Executing Defective Mitoses. *Molecular and Cellular Biology* **30**, 2090–2104 (2010).
65. S.-H. Lee, P. Itkin-Ansari, F. Levine, CENP-A, a protein required for chromosome segregation in mitosis, declines with age in islet but not exocrine cells. *Aging (Albany NY)* **2**, 785–790 (2010).
- 20 66. M. D. Cecco, *et al.*, Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. *Aging Cell* **12**, 247–256 (2013).
67. E. C. Swanson, B. Manning, H. Zhang, J. B. Lawrence, Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. *J Cell Biol* **203**, 929–942 (2013).
- 25 68. S. Dillinger, T. Straub, A. Németh, Nucleolus association of chromosomal domains is largely maintained in cellular senescence despite massive nuclear reorganisation. *PLOS ONE* **12**, e0178821 (2017).
69. D. J. Amor, *et al.*, Human centromere repositioning “in progress.” *PNAS* **101**, 6542–6547 (2004).
- 30 70. E. W. Trotter, I. M. Hagan, Release from cell cycle arrest with Cdk4/6 inhibitors generates highly synchronized cell cycle progression in human cell culture. *Open Biol* **10** (2020).
71. P. J. Skene, J. G. Henikoff, S. Henikoff, Targeted *in situ* genome-wide profiling with high efficiency for low cell numbers. *Nature Protocols* **13**, 1006–1019 (2018).









