Selective Y centromere inactivation triggers chromosome shattering in micronuclei and repair by non-homologous end joining

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Chromosome missegregation into a micronucleus can cause complex and localized genomic rearrangements1,2 known as chromothripsis3, but the underlying mechanisms remain unresolved. Here we developed an inducible Y centromere-selective inactivation strategy by exploiting a CENP-A/histone H3 chimaera to directly examine the fate of missegregated chromosomes in otherwise diploid human cells. Using this approach, we identified a temporal cascade of events that are initiated following centromere inactivation involving chromosome missegregation, fragmentation, and re-ligation that span three consecutive cell cycles. Following centromere inactivation, a micronucleus harbouring the Y chromosome is formed in the first cell cycle. Chromosome shattering, producing up to 53 dispersed fragments from a single chromosome, is triggered by premature micronuclear condensation prior to or during mitotic entry of the second cycle. Lastly, canonical non-homologous end joining (NHEJ), but not homology-dependent repair, is shown to facilitate re-ligation of chromosomal fragments in the third cycle. Thus, initial errors in cell division can provoke further genomic instability through fragmentation of micronuclear DNAs coupled to NHEJ-mediated reassembly in the subsequent interphase.

Chromosome segregation errors during mitosis are a well-recognized cause of numerical aneuploidy and have been implicated in the formation of chromosomal translocations4, both hallmark features of cancer genomes. Whether—and if so, how—such mitotic errors also contribute to the development of complex structural rearrangements are poorly understood. Chromothripsis can be detected in a broad spectrum of human cancers5,5 and is predicted to occur during a single, catastrophic event that contemporaneously generates multiple genomic rearrangements onto one or a few chromosome(s)3. While features of chromothripsis have been recapitulated from experimental systems involving dicentric bridge formation6 or exogenous DNA damage induction7, sequencing evidence from plant models1 and human cells2, and genetics in yeast8, has suggested that complex and localized rearrangements can be triggered by chromosome missegregation into aberrant compartments called micronuclei. The exact cellular mechanism(s) for how missegregated chromosomes become chromothriptic remains unknown, although an attractive hypothesis9,10 involves chromosome pulverization in micronuclei—an observation initially made almost half a century ago11—followed by incorrect reassembly of fragments through DNA repair.

Attempts to directly address these underlying mechanisms have been lacking due to experimental limitations of conventional cell-based methods for producing micronuclei by chemically induced prolonged mitotic arrest to generate random chromosome segregation errors. This approach precludes discriminating the micronucleated chromosome from normal, nuclear chromosomes in mitosis and prevents monitoring the fate of the initially missegregated chromosome for more than one cell generation. Moreover, mitotic arrest can itself produce unwanted DNA damage and/or activate an apoptotic degradation event12-15. We sought to bypass these limitations by inducing the missegregation of a specific chromosome-of-interest into micronuclei that can be examined over successive cell cycles without mitotic perturbation. To do so, we exploited a unique feature of the human Y chromosome centromere that permitted conditional, centromere-selective control for assembly of the kinetochore—the spindle microtubule-attachment complex that is required for chromosome segregation.

Kinetochores are assembled on the outer surface of centromeric chromatin through nucleation by centromere protein C (CENP-C)16, which is recruited and stably attached at centromeres through its...
Dox-induced synthesis of rescue CENP-A\(^{\text{C-H3}}\) (red circles). Doxycycline (dox) induces transcriptional synthesis of the rescue CENP-A\(^{\text{C-H3}}\) chimaera and auxin (IAA) triggers rapid degradation of the endogenous, auxin-inducible degron (AID)-tagged CENP-A protein.

(c) Immuno-FISH images of DLD-1 cells rescued with CENP-A\(^{\text{C-H3}}\) following 24 h dox/IAA treatment. DNA FISH was used to spatially identify the locations of the X (green) and Y (red) centromeres in DAPI-stained interphase nuclei combined with immunofluorescence for detection of CENP-A or CENP-C. Images are representative of two independent experiments. Scale bar, 5 μm.

**Figure 1** An inducible CENP-A replacement strategy enables functional and selective inactivation of the Y chromosome centromere in human cells. (a) A schematic depicting the strategy used to functionally inactivate the CENP-B-deficient Y centromere with a CENP-A chimaera containing the carboxy-terminal tail of histone H3 (CENP-A\(^{\text{C-H3}}\)) that cannot directly recruit CENP-C. All other chromosomes assemble kinetochores through the CENP-B-dependent pathway. (b) A schematic depicting the approach for inducible replacement of endogenous CENP-A (green circles) at all centromeres with CENP-A\(^{\text{C-H3}}\) (red circles). Doxycycline (dox) induces transcriptional synthesis of the rescue CENP-A\(^{\text{C-H3}}\) chimaera and auxin (IAA) triggers rapid degradation of the endogenous, auxin-inducible degron (AID)-tagged CENP-A protein.

This was followed by TAL effector核酸-mediated gene editing to disrupt one endogenous CENP-A allele and to amino-terminally tag the CENP-A encoded by the second allele with an enhanced yellow fluorescent protein fused to an auxin-inducible degron (CENP-A\(^{\text{EYFP-AID}}\)), the latter of which permits rapid and complete degradation\(^{26,27}\) of AID-tagged CENP-A within ~90 min after addition of the plant hormone auxin (indole-3-acetic acid, IAA) (Supplementary Fig. 1c). Finally, we stably integrated a single copy, doxycycline (dox)-inducible gene encoding the CENP-A\(^{\text{C-H3}}\) chimaera (or CENP-A\(^{\text{WT}}\) (wild-type) as a control) whose basal level of transcription produced CENP-A\(^{\text{C-H3}}\) at ~10% of normal CENP-A levels (Supplementary Fig. 1d–f).

Induced destruction of CENP-A\(^{\text{EYFP-AID}}\) simultaneous with induced transcription of CENP-A\(^{\text{C-H3}}\) following dox/IAA addition led to rapid loss of endogenous CENP-A and its replacement with CENP-A\(^{\text{C-H3}}\) within one cell cycle\(^{28}\) at every centromere without loss of centromere identity (as indicated by co-localization with anti-centromere antibodies—Supplementary Fig. 1e). Whereas CENP-A\(^{\text{EYFP-AID}}\) depletion alone resulted in complete lethality, its replacement with CENP-A\(^{\text{C-H3}}\) rescued broad centromere function and cell viability without affecting clonogenic growth (Supplementary Fig. 1g). Proliferation rate (~25–27 h per doubling—Supplementary Fig. 1h-i) or cell cycle distribution (Supplementary Fig. 1j)—all of which were
Figure 2 Y centromere inactivation triggers Y chromosome missegregation and accumulation into micronuclei. (a) Percentage of Y chromosome-positive signals in the primary nucleus measured by centromere FISH. Lines represent the mean ± s.e.m. of n = 6 (0 d, 1,758 cells) and n = 3 independent experiments (3–8 d, 672–1,605 cells per condition). The right schematic depicts how Y loss is perpetuated over repeated doublings within a population. (b) Percentage of micronucleated cells measured by DAPI staining. Lines represent the mean ± s.e.m. of n = 3 independent experiments (2,045–3,153 cells per condition). Asterisks in a, b indicate significance by two-tailed Student’s t-test compared with untreated control cells. **P < 0.001; ***P < 0.0001; ****P < 0.01; NS, not significant. (c) 2 d CENP-Awt-rescued cells stably encoding histone H2B-mRFP were filmed by time-lapse microscopy for chromosome segregation errors. Data represent the mean of n = 3 independent experiments. Scale bar, 5 μm. (d) Cells rescued with CENP-Awt or CENP-Aht for 5 d were analysed by DAPI staining to quantify the percentage of micronucleated cells (x axis) and cross-plotted against the percentage of micronuclei containing either chromosome Y or 4 (y axis) as measured by centromere FISH. Data represent the mean ± s.e.m. of n = 3 independent experiments per axis, and statistical analyses and sample sizes for each axis are provided in Supplementary Table 1. Source data for a, b, c, g are provided in Supplementary Table 1.

As expected, after induced degradation of CENP-Apyfpp–AID and its replacement with CENP-Aht, the essential kinetochore-nucleating protein CENP-C was selectively lost from the Y centromere (Fig. 1c) despite continued marking of Y centromere position by CENP-Aht. Correspondingly, the Y chromosome underwent rapid population-wide loss from primary nuclei at a rate of ∼30% per cell division (as measured by a Y centromere-bound fluorescent in situ hybridization (FISH) probe, Fig. 2a). Almost a quarter (23 ± 1%) of CENP-Aht-rescued cells contained micronuclei within 48 h of CENP-A replacement (Fig. 2b), whereas elevated micronucleation was not observed in control cells or those rescued with CENP-Awt (Supplementary Fig. 2a). By the second day after CENP-Aht replacement, more than half of mitoses (57 ± 5%) developed chromosome segregation errors, the large majority (87 ± 5%) of which were accompanied by defects in initial chromosome alignment (Fig. 2c).

FISH probes targeting the Y centromere and the centromere of a control autosome (chromosome 4) were used to establish that most micronuclei (527/754 micronuclei examined, or 70 ± 10%, P = 0.008) contained the Y chromosome (Fig. 2d and Supplementary Fig. 2b), while micronucleated chromosome 4 was rarely observed (<3%). A ∼35-fold enrichment in CENP-Aht cells with Y-containing micronuclei (Supplementary Fig. 2c) was measured compared with comparable to untreated parental cells or those rescued by CENP-Awt. CENP-Aht-rescued cells had normal mitotic duration (41 ± 11 min, n = 50 H2B-mRFP-labelled mitoses filmed) and were also capable of sustaining mitotic arrest when challenged with the microtubule inhibitor nocodazole (Supplementary Fig. 1f).
untreated CENP-A\textsuperscript{WT}-rescued cells, with a proportion (~49%) of micronuclei containing two or three Y centromere signals per micronucleus (Fig. 2e and Supplementary Fig. 2d). These micronuclei were often larger in size ($R^2 = 0.70$, Supplementary Fig. 2e), probably indicating replication of an initial micronuclear chromosome\textsuperscript{29} and/or the entrapment of multiple Y chromosomes into the same micronucleus—perhaps through nondisjunction of sister chromatids. To further confirm selective enrichment of the Y chromosome, micronuclei were purified using differential centrifugation\textsuperscript{30} and analysed by FISH with a Y chromosome painting probe (Fig. 2f). FISH analysis revealed that 52 ± 4% of purified micronuclei contained Y-specific sequences (Fig. 2g) without detectable contamination of primary nuclei, and the remainder were suspected to entrap a distribution of randomly missegregated chromosomes. We conclude that selective inactivation of the Y centromere efficiently produces micronuclei that are specifically
enriched for the human Y chromosome without compromising other major cellular characteristics (summarized in Supplementary Fig. 2f).

As nuclear envelope disruption is a common fate for micronuclei, a fluorescent protein fused to a nuclear localization signal (2xRFP-NLS) was used to measure compartmentalization between induced Y chromosome micronuclei and those spontaneously generated from a low basal rate of segregation errors entrapping mostly non-Y chromosomes. Both sources of micronuclei underwent disruption at comparable frequency (~26%, Supplementary Fig. 3a), indicative of similar nuclear membrane integrity in the Y and non-Y micronuclei. Moreover, using immunofluorescence for γ-H2AX as a marker for DNA double-strand breaks, a spectrum of micronucleus-specific DNA damage was identified in ~30% of micronuclei that ranged from a single focus to extensive damage (Supplementary Fig. 3b-c).

To directly test if and when micronucleated chromosomes underwent fragmentation, a dual-coloured FISH assay was performed on mitotic spreads using DNA probes spanning the entire Y chromosome. Y chromosome fragmentation was monitored for 4 days following centromere inactivation (Fig. 3a). One day later (day three), abundant fragmentation (24 ± 3% of Y chromosome-positive spreads, \( P = 0.0003 \)) appeared (Fig. 3a,b and Supplementary Fig. 4a,b), indicating that shattering requires at least one complete cell cycle following initial micronucleus formation.

Following three-day centromere inactivation, copy number analysis of > 300 metaphases with a detectable Y chromosome (Fig. 3c) revealed frequent Y chromosome aneuploidy (Fig. 3d), although additional copies of intact Y chromosome(s) rarely accompanied spreads with Y fragmentation (Fig. 3e). Sixty shattering events were examined in detail; each generating between 3 and 57 chromosomal FISH fragments large enough to be detected microscopically with an average of 18 fragments per event (Fig. 3f). Approximately 35% of fragmented metaphases contained a single centromere focus (with up to 53 Y chromosome fragments), whereas others harboured ≥2 foci (Fig. 3g), indicative of either a break within the centromeric region and/or co-fragmentation of multiple Y chromosomes. There was a weak correlation \( (R^2 = 0.22) \) between the number of fragments generated and the number of centromere signals present (Supplementary Fig. 4c), consistent with the majority of events produced from one Y chromosome. Additionally, mitoses with overt fragmentation of a non-Y chromosome were rare (4/643 spreads examined).

Micronuclei are prone to nuclear envelope disruption during interphase that terminates normal nuclear function—a defect that can provoke delayed or stalled replication in S-phase and/or the acquisition of DNA damage throughout interphase—suggesting that one mechanism for chromosome fragmentation is the sudden compaction following mitotic entry of incompletely replicated or unrepaird micronuclear DNAs. To test this, the PP1/PP2A phosphatase inhibitor calyculin A was added to interphase cells containing micronuclei prior to shattering (2 d dox/IAA treatment—Fig. 2b and Fig. 3b) to drive abrupt, premature chromosome condensation accompanied by Ser10 phosphorylation of histone H3 (Fig. 3h and Supplementary Fig. 4d). Calyculin A alone was insufficient to fragment the Y chromosome in non-micronucleated control cells regardless of cell cycle position (Fig. 3i). In contrast, addition of calyculin A to micronucleated cells in interphase produced fragmented Y chromosomes prematurely (Fig. 3i), consistent with mitotic entry as the trigger for shattering or detection of shattering that had occurred during interphase. Use of cell synchronization (Supplementary Fig. 4e) revealed that calyculin A promoted fragmentation of the Y chromosome in cells in G2, but not in G1 (Fig. 3i). Thus, condensation-induced fragmentation was dependent on passage into or through S-phase, consistent with the hypothesis that disruption during interphase ‘primes’ the micronuclear chromosome(s) for shattering in mitosis.

An alternative shattering mechanism could be cytoplasmic accumulation of active cyclin B1-Cdk1 complexes in late G2 that may prematurely shuttle into disrupted micronuclei to initiate micronuclear chromosome condensation and fragmentation in interphase prior to mitotic entry.

Despite continued cell proliferation (Supplementary Fig. 1h), the frequency of Y chromosome fragmentation remained constant between days 3–5 after centromere inactivation (Fig. 3b), suggesting possible cycles of shattering coupled with reassembly of fragments into intact chromosomes. To test this hypothesis and to identify the potential repair pathway(s), we assessed whether Y chromosome fragments from the first mitotic cycle (day 3) would persist into the second mitosis (day 4) (Fig. 4a) following inhibition of each of the three mammalian DNA double-strand break repair mechanisms: canonical non-homologous end joining (NHEJ), homologous recombination (HR), and microhomology-mediated end joining (MMEJ). Two essential components from each distinct pathway were individually suppressed using small interfering RNAs (siRNAs) (Fig. 4b,c). Inhibition of NHEJ by reductions in DNA Ligase IV (LIG4) or DNA-PKcs resulted in a twofold increase in Y fragmentation frequency in the second mitotic cycle (Fig. 4d,e), but neither interfered with cell proliferation (Fig. 4f) nor affected fragmentation in the first mitotic cycle with or without centromere inactivation (Fig. 4g). Pharmacological inhibition of LIG4 or DNA-PK similarly prevented reassembly of chromosomal fragments (Fig. 4h).

In contrast to NHEJ, inhibition of HR or MMEJ by depletion of BRCA2/RAD51 or LIG3/PARP-1, respectively, or treatment with a RAD51 inhibitor, had no effect on fragment reassembly (Fig. 4e,h). Given that suppression of NHEJ alone is sufficient to prevent fragment repair (Fig. 4d,e,h), shattered micronuclear DNAs may be poor substrates for recognition and/or processing by components of HR and MMEJ. Thus, canonical NHEJ is the predominant DNA repair mechanism that facilitates the re-joining of micronuclei-derived chromosome fragments, an outcome consistent with the sequence junctions of chromothriptic events identified in human cancer genomes, experimentally derived breakpoints, and the majority of which lack stretches of homology or microhomology.

Prior sequencing efforts had suggested that chromothriptic-like reassembly in the primary nucleus is surprisingly efficient, although a proportion of micronuclei persist throughout mitosis and fail to reincorporate into the nucleus. Whether—and if so, to what extent—micronucleus-specific DNA repair activity contributes to fragment re-ligation remains unknown. To determine whether fragment reassembly occurs within micronuclei, DNAs extracted from purified micronuclei (following 4 d dox/IAA treatment, Fig. 2f,g)
Figure 4 Shattered chromosomal fragments are re-ligated by canonical non-homologous end joining. (a) A schematic depicting hypothesis of turnover between fragmentation and reassembly. (b) Depletion of target protein as confirmed by immunoblotting 3 d post-siRNA transfection in DLD-1 cells. The asterisk indicates a nonspecific band from the anti-LIG4 antibody. Blots are representative of two independent experiments, and unprocessed original scans are shown in Supplementary Fig. 6. (c) Experimental schematic used for the indicated panels. A detailed timeline for each experiment is provided in Supplementary Fig. 4f. (d) Representative metaphase FISH images of Y chromosome fragmentation events derived from 4 d CENP-A rescued cells 3 d post-transfection with the indicated siRNAs. (e) Quantification of Y chromosome fragmentation events (which accounts for ~0.05% of total sequencing pairs) can be estimated as a polynomial function of chromosome concentration within a given sample (a second-order rate reaction, Supplementary Fig. 5c). For the Y chromosome, discordant pairs were detected slightly below an expected frequency—a reduction that may reflect the complex sequence features of the human Y chromosome. As sequencing of micronuclear fractions excludes chromosomes that were subjected to repair after nuclear incorporation, these data suggest that NHEJ-dependent re-ligation spatially occurs primarily in the main nucleus following fragment reincorporation. Thus, consistent with terminated nuclear function within disrupted micronuclei, repair activity in micronuclei either does not occur or occurs at an efficiency too low to be detected by sequencing.

The approach reported here can in principle be used to inactive any CENP-B-deficient centromere, in particular rare chromosomes containing neocentromeres. Applying this strategy for the Y chromosome, we have determined the fate of missegregated chromosomes over subsequent cell generations and have reconstructed the sequence of major events underlying chromothripsis involving chromatid-like micronucleation, fragmentation, and reassembly. Our results support a multi-cell cycle mechanism for chromothripsis (Fig. 5c) in which...
Figure 5 Repair by non-homologous end joining does not occur efficiently within micronuclei. (a) Enrichment in the number of paired-end sequencing reads mapping to each chromosome. Each data point represents an independent DNA sample, and micronuclear DNAs were obtained from the purified fractions shown in Fig. 2f,g. The total numbers of reads are provided in Statistics Source Data (Supplementary Table 1). (b) Discordant pairs (with read-ends mapping >100 kilobases apart on the same chromosome) were quantified and plotted against an expected number of discordant pairs produced from random ligation events during library construction that were extrapolated from the graphs in Supplementary Fig. 5c. Each dot represents a single chromosome from n = 3 independent samples derived from the indicated DNA source, and the red dots represent the Y chromosome. (c) A model depicting how micronucleation, shattering, and NHEJ-mediated repair facilitates chromothripsis on initially missegregated chromosomes within three consecutive cell cycles.

a missegregated chromosome entrapped into a micronucleus first undergoes shattering induced through mitotic entry-driven premature micronuclear chromosome condensation. In the next cell cycle, the resulting fragments are incorporated into one or both newly formed daughter nuclei—perhaps through one or more undetermined DNA tethering mechanism(s)—and efficiently re-ligated in random order by LIG4 in a manner analogous to translocation formation in human cells. We propose that canonical NHEJ, which joins DNA breaks independent of sequence homology, produces structural rearrangements on chromosomes initially missegregated from two cell cycles earlier.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

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Cell culture, generation of stable cell lines, and reagents. T-REX Flp-In DLD-1 cells (provided by S. Taylor, University of Manchester, UK) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin–streptomycin. Cells were maintained at 37°C under 5% CO₂ and atmospheric oxygen. CENPA alleles were genetically modified in TIR1-expressing T-REX Flp-In DLD-1 cells by co-transfection with pCDNA3.1 plasmids (Invitrogen) encoding TAL effector nucleases, as previously described, and an EF1α-AID donor construct targeting the translation start codon of CENPA. Single EFYF cells were isolated by fluorescence-activated cell sorting (Sony SH800) and screened by immunoblotting and PCR for CENP-A39Δ stop codons. CENP-A39Δ and CENP-AΔ rescue tDNAs were cloned into pCDNA/FRT/TO plasmids and co-transfected with pOG44 into TIR1-DLD-1 CENP-AΔ EFYF cells using X-tremeGENE 9 (Roche). Cells that underwent stable Flp recombine–mediated transgene integration at the FRT locus were selected with 100 µg ml⁻¹ hygromycin (Thermo Fisher), and Y chromosome-positive clones were confirmed by FISH.

To generate stable cell lines expressing fluorescent reporter interests, H2B-mRFP and mCherry-NLS TagRFP (annotated as zEBFP-NLS, a gift from E. Hatch and M. Hetzer, Salk Institute, USA) open reading frames were cloned into pBABE retroviral vectors and packaged in 293GP cells by co-transfection with pVSV-G using X-tremeGENE 9. Viral supernatants after 48- or 72-h transfection were filtered (0.45 µm) and used to transduce cells infected in the presence of 5 µg ml⁻¹ Polybrene (Santa Cruz) for 16 h. Fluorescent cells were isolated by fluorescence-activated cell sorting (Sony SH800).

Doxycycline and the auxin plant hormone indole-3-acetic acid (IAA) was used for mitotic arrest, 1 µM of the CDK4/6 inhibitor PD-0332991 (Sigma) was used for mitotic arrest, 1 µM of the CDK1 inhibitor RO-3306 (Sigma) was used for G2 arrest, all of which were dissolved in dimethylsulfoxide (DMSO). The following DNA damage repair inhibitors were dissolved in DMSO and used at the indicated concentrations: 250 µM SCR7 (LIG4 inhibitor), 25 µM RI-1 (RAD51 inhibitor, both provided by A. Shiau, Ludwig Institute for Cancer Research, USA), and 10 µM NUT7026 (DNA-PK inhibitor, Abcam).

All cell lines were tested for mycoplasma and confirmed free of contamination. The cell lines used in this study were not authenticated and are not found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI BioSample.

Cell growth and clonogenic assays. For cell doubling time measurements, cells were plated into six-well dishes in triplicate and counted at three-day intervals. For cell cycle analysis, ethanol-fixed cells were stained with 10 µg ml⁻¹ propidium iodide and 50 µg ml⁻¹ RNAse A and analysed for DNA content by flow cytometry on a BD LSRII instrument (BD Biosciences). For clonogenic growth assays, 100 cells were plated into six-well dishes in triplicate for two weeks. Methanol-fixed colonies were stained with a 0.5% crystal violet, 25% methanol solution and manually quantified.

Mitotic spread preparation and DNA fluorescence in situ hybridization (FISH). For interphase FISH, cells plated in chambered slides were fixed in methanol/acetic acid (3:1) for 15 min and dehydrated with 80% ethanol. For metaphase spreads, cells were arrested for 4 h with 100 µg ml⁻¹ colcemide (KaryoMAX, Ther mofisher) and incubated at 37°C. After 1 h, 9 ml cold PBS was added, centrifuged, and cells were processed as described for metaphase spreads.

For DNA FISH, centromere enumeration and/or whole chromosome painting probes (MetaSystems) combined at equal ratio were applied to slides, sealed with a coverslip, co-denatured at 75°C for 2 min, and hybridized overnight at 37°C in a humidified chamber. Slides were subsequently washed with 0.4 x SSC at 72°C for 2 min and rinsed in 2 x SSC, 0.05% Tween-20 at room temperature for 30 s. Slides were then rinsed in water, counterstained with DAPI and mounted in antifade solution. FISH images were acquired on a DeltaVision Core system (Applied Precision) at x60 magnification (5 x 1 µm z-sections) and maximum intensity projections were generated using the softWoRx program.

Quantification of fragmentation FISH. After Y chromosome paint and centromere FISH on metaphase spreads, Y chromosome-positive spreads were manually scored for fragmentation on the basis of the following criteria: Y chromosome paint signal must be DAPI-positive; Y centromere signal must be DAPI-positive and overlap with paint signal; each fragmentation event must generate a minimum of three Y chromosome fragments; and at least one acentric Y fragment must be generated. For caliculin A experiments, only ‘metaphase-like’ spreads that yielded distinct and normal appearing single (G1) or double chromatids (G2) were scored.

Immunofluorescence and immuno-FISH. For indirect immunofluorescence, cells plated onto poly-L-lysine-coated coverslips were fixed in 4% formaldehyde for 10 min. Cells were pre-extracted with 0.3% Triton X-100 in PBS for 5 min and incubated in Triton Block (0.2 M glycine, 2.5% FBS, 0.1% Triton X-100, PBS). The following primary antibodies were used at 1:1,000 dilution (unless noted) in Triton Block and washed with 0.1% Triton X-100 in PBS: anti-CENP-A (Abcam, ab13939), anti-CENP-C (MBL, PD030), 1:400 anti-centromere antibodies (Antibodies Incorporated, 15–230–0001), and anti-H2AX (Clone SJW301, EMD Millipore, 0.05–0.636), and 1:200 anti-Lamin B1 (ProteinGroup Tech, 12987-1-AP). Immunofluorescent images were acquired on a DeltaVision Core System with ×40–60 magnification (30 x 0.2 µm z-sections) and deconvolved maximum intensity projections were generated using the softWoRx program. ImageJ was used to quantify fluorescent intensity. For immunofluorescence combined with DNA FISH (immuno-FISH), the immunofluorescence procedure was performed first followed by methanol/acetic acid (3:1) fixation and the described FISH procedure.

Immunoblotting. Whole-cell extracts were collected in SDS sample buffer and boiled for 10 min. Samples were resolved by SDS-PAGE, transferred to PVDF, and blocked with 5% milk in PBST (PBS, 0.1% Tween-20). The following primary antibodies were used at 1:1,000 dilution (unless noted) in PBST: anti-CENP-A (Abcam, ab13939), anti-CENP-C (MBL, PD030), 1:400 anti-centromere antibodies (Antibodies Incorporated, 15–230–0001), anti-phospho H2AX (S139) clone ICB103 (EMD Millipore, 0.05–0.636), anti-phospho histone H3 (Ser10) (Cell Signaling, 9706), 1:4,000 anti-histone H3 (Sigma H0164), anti-LIG4 (Genetex, GTX10100), anti-DNA-PKcs (Bethyl, A300-516A), anti-LI G3 (Bethyl, A301-637A), anti-PARP (BD Pharmingen, 56362, provided by X. Wu, The Scripps Research Institute, USA), anti-BRCA2 (Bethyl, A303-434A), anti-RAD51 (Abgent, AM8421b), and 1:2,000 anti-GAPDH (Cell Signaling, 14C10). Blots were probed with 1:4,000 dilutions of HRP-conjugated secondary antibodies (GE Healthcare) and exposed to film. All unprocessed film scans with the appropriate size markers are provided in Supplementary Fig. 6.

siRNA transfection. The following SMARTpool ON-TARGETplus siRNAs were purchased from GE Dharmacon and used: LIG4 (L-004254-00-0005), DNA-PK (L-005300-00-0005), BRCA2 (L-003462-00-0005), RAD51 (L-003530-00-0005), LIG3 (L-009227-00-0005) and PARP1 (L-006566-03-0005). Transfections were conducted using Lipofectamine RNAiMAX (Thermo Fisher) at a final siRNA concentration of 20 nM. Non-targeting siRNAs (D-001810-04-05) were used for experimental controls, and siRNAs targeting ubiquitin B (UBB, L-013382-00-0005) were used to measure transfection efficiency by cell lethality (>99% cell death following 48 h transfection).

Live-cell imaging. DLD-1 cells expressing retrovirus-integrated H2B-mRFP were plated in chambered slides (Ibidi) and switched to CO₂-independent medium (Thermo Fisher) supplemented with 10% fetal bovine serum prior to firming by time-lapse microscopy. Images were acquired on a DeltaVision Core system in a controlled 37°C environment at 4-min intervals for 12 h using x40 magnification (10 x 4 µm z-sections) and low-powered exposures. Maximum intensity projections were generated using the softWoRx program and videos were analysed in ImageJ. Mitotic timing is defined as the duration from nuclear envelope breakdown to anaphase onset.

Micronuclei purification and sequencing. The procedure described in ref. 30 was closely followed for micronuclei purification with the exception of hydroxyurea treatment. Briefly, ~200 million cells were collected, resuspended, and incubated in DMEM containing 10 µg ml⁻¹ cytochalasin B (Sigma) for 30 min at 37°C, pelleted, and gently Dounce homogenized in cold lysis buffer (10 mM Tris-HCl, 2 mM magnesium acetate, 3 mM CaCl₂, 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 0.15 mM spermine, 0.75 mM spermidine, 10 µg ml⁻¹ cytochalasin B, pH 8.5, 4°C) with ten slow strokes of a loose-fitting pestle. Release of nuclei was confirmed by DAPI staining and microscopy. The homogenate was collected from the top of the gradient and examined for purity by DAPI staining. Fractions containing pure micronuclei free of primary nuclear contamination were diluted and pooled in BME. Five per cent of the final fraction was fixed in methanol/acetic acid (3:1) and spotted onto a glass slide for FISH analysis. Genomic
Discordant sequencing analysis. Sequencing read quality for all samples was confirmed with FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), and adapter sequences were trimmed on both ends using cutadapt\(^\text{41}\). Reads were mapped to the hg38 human reference genome using Bowtie2\(^\text{35}\) with maximum valid fragment length set to 2,000 bp. Alignment files were sorted and merged using SAMtools\(^\text{42}\) to generate individual files for each sample. Duplicate reads were removed using Picard’s MarkDuplicates (http://broadinstitute.github.io/picard).

DNA fragment sizes were recalculated using 1,000 mapped reads per chromosome, with mapping quality \(\geq 35\) and no clipped bases. Fragment size was defined as the distance between the first and last bases on the reference genome to which either read in the pair mapped. As extreme fragment size outliers can skew these calculations, and are probably the result of chimaeric fragments or true rearrangements rather than representing extremely large fragments, the top and bottom 2% of fragment sizes were removed before calculating the mean and variance. Read pairs with mapping quality \(< 35\) were filtered out for the discordant pair analysis. Repeats such as LINEs and SINEs were not masked, as differences in the locations of such repeats are unlikely given that both genomic and micronuclear DNA samples were derived from the same cell line. Discordant read pairs caused by differences between the reference genome and the genome of the cell line should therefore be present at equal proportions in both samples. Discordant pairs were defined as paired sequencing reads in which ends mapped at least 100,000 bp apart on the same chromosome.

To measure whether discordant pairs were enriched in the micronuclear samples, we considered how discordant read pairs arise in the absence of de novo rearrangements. Discordant pairs are present in sequencing data sets as a result of random ligation of short DNA fragments during library preparation. As this formation is random, discordant pairs can be viewed as a second-order reaction in which two fragments of DNA from the same chromosome randomly collide and ligate. Therefore, the number of such pairs should be proportional to the square of the fraction of total reads from that chromosome—a prediction that is supported by the genomic and micronuclear DNA sequencing data sets shown in Supplementary Fig. 5c. The predicted fraction of discordant pairs for each chromosome was calculated as \(f^2 \times n \times 18.34\), where \(f\) is the fraction of total reads from the chromosome, \(n\) is the total number of discordant pairs in the data set, and 18.34 is a normalization factor equal to 1 divided by the sum of the squares of the fraction of total reads from each chromosome.

Statistical analysis and reproducibility. No statistical method was used to predetermine sample size and experiments were not randomized. Investigators were not blinded to group allocation during experiments or outcome assessment. GraphPad Prism 5.0 software was used to calculate statistical significance as specified in the figure legends. Graphs represent mean \(\pm\) standard error (unless noted) and a \(P\) value of \(< 0.05\) derived from at least three independent experiments was considered to be statistically significant. Figures with representative images were repeated independently at least twice (except for Fig. 3h, which was performed once to identify optimal treatment time for downstream experiments). Data points for each quantitative experiment can be found in the Statistics Source Data (Supplementary Table 1). Unprocessed film scans from all immunoblotting experiments are shown in Supplementary Fig. 6 with the relevant size markers indicated.

Data availability. DNA sequencing data reported in this study have been deposited in NCBI Sequence Read Archive with the primary accession code SRP074439, and source data for Fig. 5a,b and Supplementary Fig. 5a,b have been provided in Supplementary Table 1. All other data that support the findings of this study are available from the corresponding author on request.
Correction notice

Nature Cell Biology http://dx.doi.org/10.1038/ncb3450 (2016).

Selective Y centromere inactivation triggers chromosome shattering in micronuclei and repair by non-homologous end joining

Peter Ly, Levi S. Teitz, Dong H. Kim, Ofer Shoshani, Helen Skaletsky, Daniele Fachinetti, David C. Page and Don W. Cleveland

The original version of this file contained a loss of resolution in the Supplementary Figures and a loss of superscript text formatting. These errors were introduced during the production of the file and were corrected on 22 December 2016.
**Supplementary Figure 1**

**a** Human Centromere Protein A (CENP-A):

- amino tail
  - DVQLARRIRGEEGLG
- carboxy tail
  - DVQLARRIRGERA

**b** T-REx Flp-In DLD-1 cells

- Retroviral TiR1
- Puromycin selection
- Co-transfect with CENP-A TALENs
- EYFP-AID targeting vector
- Single-cell sorting
- Integrate CENP-A<sup>WT</sup> clone
- Isolate CENP-A<sup>WT</sup> clone
- Integrate doxycycline-inducible CENP-A<sup>WT</sup> rescue into Flp-In locus
- Hygromycin selection
- DLD-1 CENP-A<sup>WT</sup> with doxycycline-inducible CENP-A<sup>WT</sup> or C-H3

**c** Histone H2B<sup>EYFP</sup> / CENP-A<sup>EYFP-AID</sup>

**d** No rescue  CENP-A<sup>WT</sup>  CENP-A<sup>C-H3</sup>

**e** No rescue  CENP-A<sup>WT</sup>  CENP-A<sup>C-H3</sup>

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Supplementary Figure 1 Construction of human DLD-1 cells with auxin-degradable CENP-A<sup>AITD</sup> and a doxycycline-inducible CENP-A<sup>CH-3</sup> rescue that is capable of maintaining centromere identity and function. (a) Amino acid sequence of wild-type CENP-A (WT) and the carboxy-terminal tail chimera (CH-3) swapped with the corresponding tail of histone H3. Schematic not drawn to scale. CATD; centromere targeting domain. (b) Schematic for the construction of DLD-1 cell lines used in all experiments. (c) Unfixed DLD-1 CENP-A<sup>EYFP-AID</sup> cells stably expressing H2B-mRFP were imaged 2d after IAA addition. Scale bar, 5 μm. (d) DLD-1 cells as in (b) were treated with combinations of dox and IAA for 24h and whole-cell extracts were analyzed by immunoblotting for CENP-A. The predicted molecular weight of CENP-A fused to an EYFP-AID tag is ~66 kDa. (e) Representative immunofluorescent images of engineered DLD-1 cells treated with combinations of dox and IAA for 24h. Both CENP-A<sup>WT</sup> and CENP-A<sup>CH-3</sup> rescues correctly localized to centromeres. Enlarged images of CENP-A staining following dox/IAA addition is shown below. ACA; anti-centromere antibodies. Scale bar, 5 μm. (f) Dox-inducible CENP-A is expressed at low basal levels without supplemented doxycycline, allowing for the simultaneous addition of dox and IAA without epigenetic loss of centromere identity. (g) CENP-A<sup>CH-3</sup>-rescued cells are capable of sustaining long-term clonal growth and viability using a 2-week colony formation assay. Data were normalized to untreated cells and represent the mean ± SEM of n = 3 independent experiments each performed in biological triplicate. Asterisks indicate significance by two-tailed Student’s t-test compared to untreated cells. **P=0.0019, NS = not significant. (h) Quantification of DLD-1 CENP-A<sup>CH-3</sup> cell growth rate with or without dox/IAA over a 9d period performed in biological triplicate. Line represents linear regression analysis. (i) Estimated doubling time calculated from h. (j) 5d CENP-A<sup>CH-3</sup>-rescued cells were subjected to propidium iodine staining followed by flow cytometry analysis for DNA content with and without 6h treatment with 100 ng/ml nocodazole. Source data for g and h have been provided in Supplementary Table 1.
Supplementary Figure 2 Induced Y centromere inactivation provokes Y chromosome missegregation into micronuclei. (a) DLD-1 cells were rescued with CENP-AWT or CENP-A<sup>C-H3</sup> for 5d and the percentage of micronucleated cells were quantified by DAPI staining. Data represent the mean ± SEM of n = 3 independent experiments (1,453–1,945 cells per condition). P-values indicate significance by two-tailed Student’s t-test compared to untreated cells. (b) DLD-1 cells were rescued with CENP-AWT and CENP-A<sup>C-H3</sup> for 5d and micronuclei were quantified for the percentage harboring centromere Y or centromere 4 signal(s). Data represent the mean ± SEM of n = 3 independent experiments (380–754 micronuclei) or the mean of 2 independent experiments (CENP-AWT, dox/IAA; 290 micronuclei). P-values indicate significance by two-tailed Student’s t-test compared as denoted. (c) Comparison between the frequency of cells with the specified chromosome in micronuclei when treated as indicated by extrapolating the percentage of micronucleated cells and the percentage of micronuclei containing either chromosome Y or 4. (d) Quantification of the number of Y centromere foci observed in spontaneously-derived or induced micronuclei following 5d CENP-A<sup>C-H3</sup> rescue. Data represent the mean ± SEM of n = 65 (WT, -dox/IAA), 76 (WT, +dox/IAA), 67 (C-H3, -dox/IAA), 102 (C-H3, +dox/IAA) micronuclei. (e) Comparison between the number of Y centromere foci per micronucleus and micronuclear diameter from 5d CENP-A<sup>C-H3</sup>-rescued cells. Data were compiled from n = 150 micronuclei pooled from 3 independent experiments, and means are indicated by the line. R<sup>2</sup>-value represents correlation of size and foci number by linear regression analysis. (f) Summary of cellular characteristics comparing untreated (CENP-A<sup>EYFP-AID</sup>) cells with dox/IAA-treated (CENP-A<sup>C-H3</sup>) cells. Source data for a and b have been provided in Supplementary Table 1.
Supplementary Figure 3  Induced Y chromosome micronuclei share common features of spontaneously derived micronuclei including micronuclear envelope disruption and the acquisition of DNA damage. (a) DLD-1 CENP-A<sup>C-H3</sup> cells stably expressing 2xRFP-NLS treated with or without 5d dox/IAA (experimentally vs. spontaneously derived micronuclei, respectively) were fixed and DAPI-stained. Representative images from dox/IAA-treated cells and quantifications for micronuclear RFP compartmentalization are shown on the left. Data on the right panel represent the mean of 2 independent experiments (166–294 total micronuclei). Scale bar, 5 μm. (b) DLD-1 CENP-A<sup>C-H3</sup> cells treated with 5d dox/IAA were immunostained for the DNA damage marker γH2AX and nuclear envelopes with Lamin B1. Representative images (scale bar, 5 μm) of micronuclei without and with varying degrees of detectable DNA damage signals are shown. (c) γH2AX fluorescent signal intensities from b were measured from 200 micronuclei (pooled from 3 independent experiments) and individually plotted. a.u., arbitrary units. Source data for a and c have been provided in Supplementary Table 1.
Supplementary Figure 4 Characterization of chromosome fragmentation events and induction of premature chromosome condensation using calyculin A. (a) Representative example of interphase cells hybridized to Y chromosome paint (green) and Y centromere (red) FISH probes following 3d CENP-AC-H3 rescue. Scale bar, 10 μm. (b) Additional example of Y chromosome fragmentation event derived from 3d CENP-AC-H3-rescued cells. Scale bar, 10 μm. (c) Centromere and fragment counts from Fig. 3f-g were cross-plotted per mitotic shattering event. Red line indicates linear regression analysis. (d) Representative image of DAPI-stained, metaphase-like spreads induced by 1h treatment with calyculin A, showing examples for G1-, S-, and G2-phase spreads. G1-phase chromosomes appear as single chromatids, S-phase appears as highly pulverized and abnormal nuclei (and excluded from quantitative analyses), and G2-phase appears as normal mitotic chromosomes with two distinguishable sister chromatids. Scale bar, 25 μm. (e) DLD-1 cells treated with 1 μM of the CDK4/6 inhibitor PD-0332991 (also known as Palbociclib) or 10 μM of the CDK1 inhibitor RO-3306 for 24h were subjected to propidium iodine staining followed by flow cytometry analysis for DNA content. (f) Experimental schematic for panels shown in Fig. 4b, d, e, f, g, and h.
Supplementary Figure 5 Paired-end sequencing information for each source of DNA. (a) Base pair sizes (mean ± SD, n=1,000 reads) of sequencing fragments for each sample. (b) Percentage of sequencing reads in which both ends of a pair mapped to the reference genome following removal of duplicate and mitochondrial reads. (c) The concentration of discordant sequencing reads for each chromosome follows a second order reaction and rises as the square of the concentration of total sequencing reads (see Methods). Each dot represents a single chromosome from three independent genomic or micronuclear DNA samples, and the green dot indicates the Y chromosome. The curved line shows a predictive model of discordant pairs that is described under the methods section. Source data for a and b have been provided in Supplementary Table 1.
Supplementary Figure 6 Unprocessed film scans of all immunoblots with corresponding protein size markers.
Supplementary Table Legends

Supplementary Table 1  Statistics source data.