DNA breaks and chromosome pulverization from errors in mitosis

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The involvement of whole-chromosome aneuploidy in tumorigenesis is the subject of debate, in large part because of the lack of insight into underlying mechanisms. Here we identify a mechanism by which errors in mitotic chromosome segregation generate DNA breaks via the formation of structures called micronuclei. Whole-chromosome-containing micronuclei form when mitotic errors produce lagging chromosomes. We tracked the fate of newly generated micronuclei and found that they undergo defective and asynchronous DNA replication, resulting in DNA damage and often extensive fragmentation of the chromosome in the micronucleus. Micronuclei can persist in cells over several generations but the chromosome in the micronucleus can also be distributed to daughter nuclei. Thus, chromosome segregation errors potentially lead to mutations and chromosome rearrangements that can integrate into the genome. Pulverization of chromosomes in micronuclei may also be one explanation for ‘chromothripsis’ in cancer and developmental disorders, where isolated chromosomes or chromosome arms undergo massive local DNA breakage and rearrangement.

Whole-chromosome aneuploidy is a major feature of cancer genomes, yet its role in tumour development remains controversial1,2. This contrasts with chromosome breaks and rearrangements, which are known to produce cancer-causing mutations. Recent genetic evidence demonstrates that increased rates of whole-chromosome mis-segregation can accelerate oncogenesis3,4; however, the only established mechanism by which whole-chromosome segregation errors promote tumorigenesis is by facilitating the loss of heterozygosity for tumour suppressors5. Intriguingly, two animal models where whole-chromosome segregation errors result in robust tumour development also display extensive structural alterations in chromosomes6,4. This raises the question of whether errors in mitosis can predispose to DNA damage. We considered the possibility that segregation of chromosomes into micronuclei might produce DNA damage. Whole-chromosome-containing micronuclei form from anaphase lagging chromosomes9–13; micronuclei can also be generated from acentric fragments of chromosomes11. Micronuclei have many features of primary nuclei, but much controversy surrounds their actual composition and functional properties. Studies differ on whether micronuclei are transcriptionally active, replicate DNA, mount a normal DNA damage response, or assemble normal nuclear envelopes; moreover, the ultimate fate of chromosomes trapped within micronuclei remains unclear1,14,15.

DNA damage in micronuclei

To determine if newly formed whole-chromosome micronuclei develop DNA damage, we generated micronuclei in synchronized cells and tracked them through the cell cycle. As a first synchronization approach, micronuclei were generated in non-transformed RPE-1 and transformed U2OS cells by release from nocodazole-induced microtubule depolymerization. When mitotic cells are released from nocodazole, spindles reassemble abnormally, producing merotelic kinetochore attachments (one kinetochore attached to two opposite spindle poles), lagging chromosomes, and ~10% of cells with micronuclei16. Because prolonged mitotic arrest causes DNA damage17–19, RPE-1 cells were arrested for a short (6 h) interval with nocodazole. After release from the 6 h nocodazole block, neither the primary nuclei nor the newly formed kinetochore-positive micronuclei showed significant DNA damage during the subsequent G1 phase as measured by damage-dependent phosphorylation of the histone variant H2AX20 (γ-H2AX foci formation; Fig. 1a–c and Supplementary Fig. 2a–f). TdT-mediated dUTP nick end labelling (TUNEL) to detect DNA breaks21 (Fig. 1d and Supplementary Fig. 2e, f), and visualization of DNA breaks with the comet assay22 (Supplementary Fig. 3d, e).

Because aneuploidy can produce a p53-dependent G1 cell-cycle arrest23,24, p53 was silenced by RNA interference (RNAi) to allow us to monitor the fate of micronucleated RPE-1 cells at later stages of the cell cycle. As expected, after S-phase entry, low-level DNA damage was detected in both the micronuclei and the primary nucleus25; however, in G2 phase cells, most micronuclei showed DNA damage whereas almost none was detected in the primary nucleus (Fig. 1a–d). Similar results were observed in U2OS cells (Supplementary Fig. 3) and in cells where merotelic kinetochore–microtubule attachments were generated by knockdown of the kinetochore-associated microtubule depolymerase MCAK26 or the kinetochore protein NUF2 (ref. 27) (Supplementary Fig. 4a–c). MCAK knockdown does not delay cells in mitosis, demonstrating that the acquisition of DNA damage in micronuclei is independent of mitotic arrest17–19. This damage did not represent activation of apoptosis because it was not accompanied by caspase-3 activation, and it was not suppressed by a pan-caspase inhibitor (Supplementary Fig. 5).

As a completely independent method for generating micronuclei, we used a human cell line (HT1080) carrying a human artificial chromosome (HAC) with a kinetochore that could be conditionally inactivated28. In this system, kinetochore assembly on the HAC is blocked by washout of doxycycline from the medium; consequently, the HAC is unable to attach to the mitotic spindle and is left behind at anaphase,

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DNA replication in micronuclei is inefficient and asynchronous with the primary nucleus. This is suggested because micronuclei in G2 cells that had mitotic shake-off, S (6 h), G2 (22 h) and irradiated RPE-1 cells (IR) labelled for DNA (blue), centromeres (ACA, red) and γ-H2AX (green). Insets show enlarged images of micronuclei. Scale bars, 10 μm. c, d, Percentage of primary nuclei (PN, blue bars) and centric micronuclei (MN, red bars) with γ-H2AX foci (c) and TUNEL labelling (d) (3 experiments, n = 100). Errors bars indicate s.e.m.

Defective DNA replication in micronuclei

To determine directly whether acquisition of DNA damage in micronuclei requires DNA replication, synchronized micronucleated cells were released into medium containing thymidine to block DNA replication. Blocking DNA replication with thymidine abolished the acquisition of DNA damage (Fig. 3a–c), demonstrating that the breaks in micronuclei occur in a replication-dependent manner.

We next tested whether the acquisition of DNA damage in micronuclei is mediated by aberrant DNA replication. This possibility was suggested because micronuclei in G2 cells that had γ-H2AX foci were almost always additionally labelled for phosphorylated RPA2 (Ser 33)29, a marker of DNA replication stress (Supplementary Fig. 4d). Moreover, the characteristic S-bromodeoxyuridine (BrdU) labelling patterns of early, mid and late S-phase cells30 suggested that DNA replication in micronuclei is inefficient and asynchronous with the primary nucleus (Fig. 3d). By measuring the fluorescence intensity of BrdU relative to DNA area from single focal plane confocal images, DNA replication in micronuclei was detected at a markedly reduced level throughout S phase when compared to the primary nuclei (Fig. 3e). Notably, pulse-labelling with BrdU demonstrated asynchronous DNA replication of the micronuclei relative to the primary nucleus, with ~30% of micronuclei replicating DNA in G2 cells (22 h; Fig. 3d).

The compromised DNA replication observed in newly formed micronuclei led us to test whether complexes necessary to license DNA replication origins are recruited to chromosomes in micronuclei. The first step in assembling replication origins is the loading of the origin recognition complex (ORC) after chromosome decondensation during telophase31. Quantitative fluorescence imaging suggested that ORC recruitment is equally efficient in the micronuclei as in the primary nucleus (Supplementary Fig. 6a). By contrast, newly generated micronuclei showed significant reduction in the recruitment of replicative DNA helicase components MCM2 and MCM3 (refs 32, 33) as well as the replication initiation factor CDT1 (ref. 31) (Fig. 4a, b and Supplementary Fig. 6b, c). We observed an increased recruitment of MCM subunits into micronuclei as cells progressed through the cell cycle, but the levels of MCM subunits never exceeded 20% of that observed in the primary nucleus (Fig. 4b).

To examine whether the DNA damage response in micronuclei was also abnormal, cells were treated with replication inhibitor (aphidicolin) or exposed to 2 Gy irradiation to induce damage and then labelled to detect components of the DNA damage response. After aphidicolin treatment, micronuclei showed clear signal for γ-H2AX and its kinase ATR (ataxia telangectasia mutated-related), but downstream constituents of the DNA damage response such as 53BP1 were not efficiently recruited, as previously reported34 (Supplementary Fig. 7). A similar defect in the recruitment of DNA-damage-response factors into micronuclei was also observed after irradiation (Supplementary Fig. 8a). This recruitment defect had clear consequences for DNA damage repair: whereas γ-H2AX foci were resolved in primary nuclei by 6 h after 2 Gy irradiation, γ-H2AX foci persisted in micronuclei for >24 h (Supplementary Fig. 8b). The failure of micronuclei to recruit MCM subunits and components of the DNA repair machinery may be due, at least in part, to a defect in nucleocytoplasmic transport. Newly formed micronuclei have a marked reduction in the density of nuclear pores11,14,15 (Supplementary Fig. 9) and exhibit strongly reduced nuclear import (Fig. 4c, d and Supplementary Fig. 10).

Micronuclei and chromosome pulverization

Next we tested the prediction that abnormal DNA replication in micronuclei could generate chromosome breaks. Chromosome spreads were prepared from non-transformed cells in the first cell cycle after release from nocodazole or from DMSO-treated controls (Fig. 5a–f and Supplementary Fig. 11a). Notably, 7.6% of chromosome spreads prepared from micronucleated cells released from nocodazole block (n = 722) exhibited chromosomes with a ‘pulverized’ appearance, characterized by fragments of 4’,6-diamidino-2-phenylindole (DAPI)-stained material that were often clustered into a discrete location on the spread35 (Fig. 5a, d (yellow arrows), i and Supplementary Fig. 11b). By contrast, pulverization was exceedingly rare (0.14%) in controls. Multicolour fluorescence chromosome painting (SKY) confirmed that 34 of 47 spreads with pulverized chromosomes were composed of fragments from a single chromosome and another eight were from two chromosomes (Fig. 5a–i and Supplementary Fig. 11). Despite this marked disruption to chromosomal structure, only one cell harboured non-reciprocal chromosome translocations 24 h after nocodazole release (n = 101), whereas no translocations were observed in control cells (data not shown). At 72 h after nocodazole release (n = 100), we still did not detect chromosome translocations, but we did detect truncated or derivative single chromosomes in three cells. Although our experiments show that whole-chromosome mis-segregation does not produce frequent non-reciprocal translocations, we do not exclude the possibility that the DNA damage acquired in micronuclei could result in translocations, albeit at a frequency lower than ~1/100 per generation.
Chromosome pulverization has been observed in cell-fusion experiments where chromosomes from an S-phase cell are pulverized because of exposure to signals from mitotic cytoplasm. It has also been observed when aberrant late-replicating chromosome translocations are generated. The mechanism of pulverization involves compaction of partially replicated chromosomes induced by mitotic cyclin-dependent kinase activity, termed premature chromosome compaction.

Figure 2 | DNA breaks in a HAC targeted to a micronucleus. a, Right: schematic; left: fluorescence in situ hybridization images of HAC (red) in a primary nucleus (+Dox) or micronucleus (−Dox). b, Images of micronucleated cells as in Fig. 1b (enlarged and brightened in insets). Scale bars, 10 μm. c, d, Percentage of primary nuclei (blue bars) and centric micronuclei (red bars) with γ-H2AX foci (c) and TUNEL labelling (d) (3 experiments, n = 100). Errors bars indicate s.e.m.

Figure 3 | DNA damage in micronuclei results from aberrant DNA replication. a–c, DNA replication requirement for acquisition of DNA damage in micronuclei. a, RPE-1 cells were synchronized as in Fig. 1a and released into media with (+TH) or without (−TH) 2 mM thymidine. Cells were co-labelled for TUNEL (green) and cyclin B1 (red). Shown are images from the 22 h sample. Scale bar, 10 μm. b, c, Percentage of TUNEL-positive primary nuclei (blue bars) and micronuclei (red bars) with (c) or without (b) thymidine treatment; (3 experiments, n = 100). Errors bars indicate s.e.m. d, Inefficient and asynchronous DNA replication in micronuclei. RPE-1 cells as in Fig. 1a were pulse-labelled with BrdU and labelled: DNA (white) and BrdU (red). e, The ratio of BrdU incorporation in micronuclei relative to primary nuclei after a 30-min pulse label. Normalized fluorescence intensity (FI) measurements are as shown in the box and whisker plots (2 experiments, n = 50). Boxes represent upper and lower quartiles, lines within boxes represent median, and the error bars comprise the whiskers which extend to the maximum and minimum value data sets.
Figure 4 | Defective MCM2-7 complex recruitment, DNA damage response and nucleocytoplasmic transport in micronuclei. a, Impaired MCM2 recruitment into micronuclei. G1 RPE-1 cells were synchronized as in Fig. 1a and stained for chromatin-bound MCM2 and DNA. Scale bar, 10 μm. b, Relative MCM2 fluorescence intensity (FI) as in Fig. 3e. Approximate cell cycle stage of time points: 6 h, G1; 10 h, early S phase; 16 h, mid S phase; 20 h, late S phase; (2 experiments, n = 50). c, d, Micronuclei are partially defective for nuclear import of NFATc1–EGFP. c, Representative images of micronucleated U2OS cells stably expressing NFATc1–EGFP in G1 and G2 with or without treatment with 0.2 μM thapsigargin for 10 min. d, Ratio of NFATc1–EGFP fluorescence intensity between micronuclei and primary nuclei; (3 experiments, n = 30). For b and d, error bars comprise the whiskers which extend to the maximum and minimum value data sets.

Figure 5 | The fate of chromosomes in micronuclei. Micronuclei were induced in RPE-1 cells as described in Fig. 1a (after p53 knockdown) and chromosome spreads were prepared 24 h after nocodazole release. a–c, Pulverization of chromosome 1 demonstrated by DAPI staining (chromosome fragments, brightened in inset, yellow arrows) (a), SKY probes (pseudo-coloured) (b), and aligned SKY karyotype (c). d–f, Pulverization of chromosome 16, viewed as in a–c. g, A BrdU-positive micronuclei-containing G2 RPE-1 cell. 2 h BrdU pulse label. DNA, white; BrdU, red. h, Selective BrdU labelling of a pulverized chromosome. i, The percentage of cells with intact micronuclei (blue bars) or pulverized chromosomes (PC, red bars) from control or nocodazole-released (NOC) RPE-1 cells. Interphase is 18-h sample and metaphase is 24 h. j, Images from live-cell experiment showing a pre-converted green fluorescent micronucleus (white arrows) photo-converted to a red-fluorescent micronucleus (yellow arrows) imaged through mitosis. After anaphase, the micronucleus either reincorporated with the primary nucleus or failed to reincorporate and reformed as a micronucleus. Top row: reincorporation of micronuclei into primary nucleus; bottom row: no reincorporation. Time is shown as h:min.
that lacked micronuclei (Supplementary Table 1 and Supplementary Movies 1 and 2).

To test directly whether micronuclei reincorporate during mitosis, we generated a U2OS cell line stably expressing H2B fused to the photoactivatable fluorescent protein Kaede \(^{36}\), which converts from green to red fluorescence emission after ultraviolet illumination. This enabled us to photoactivate selectively and subsequently track a single chromosome encapsulated in a micronucleus throughout mitosis (Fig. 5j and Supplementary Movies 3 and 4). This experiment demonstrated that chromosomes within micronuclei reincorporate into daughter nuclei at a significant frequency (11 of 29; 38%). The remaining micronuclei persisted in cells well into the second generation.

Discussion

Together, our findings indicate a novel mechanism by which whole-chromosome segregation errors can cause chromosome breaks and potentially cancer-causing mutations. Mitotic errors can lead to lagging chromosomes which in turn can be partitioned into micronuclei \(^{13}\). This can produce DNA damage in two ways. First, DNA damage can result as a direct consequence of aberrant DNA replication, potentially due to a reduced density of replication origins. Second, chromosome pulverization due to premature chromosome compaction can occur if a micronucleated cell enters mitosis with micronuclei still undergoing DNA replication. This may not be the only mechanism by which whole-chromosome mis-segregation can cause DNA damage, as it was recently proposed that lagging chromosomes can be damaged by the cytokinetic furrow \(^{41}\). In contrast to previous studies that have suggested that most micronuclei are inactive, discarded or degraded \(^{11}\), we have recently proposed that lagging chromosomes can be damaged by the cytokinetic furrow \(^{41}\). In contrast to previous studies that have suggested that most micronuclei are inactive, discarded or degraded \(^{11}\), we find that micronuclei persist over several generations but that the chromosomes within the micronuclei can be segregated at a significant frequency into daughter cell nuclei. Thus, DNA rearrangements and mutations acquired in micronuclei could be incorporated into the genome of a developing cancer cell.

Our experiments suggest a mechanism that could explain the recently discovered phenomenon of chromothripsis \(^{42,43}\). Chromothripsis was discovered by cancer genome sequencing and DNA copy number analysis \(^{42}\). It is defined by small-scale DNA copy number changes and extensive intrachromosomal rearrangements that are restricted to a single chromosome or chromosome arm. Two non-exclusive models have been proposed for chromothripsis are: (1) fragmentation of a chromosome followed by stitching together of the resulting fragments by non-homologous end joining \(^{42}\); and (2) aberrant DNA replication resulting in fork stalling and template switching or microhomology-mediated break-induced replication \(^{43,44}\). Partitioning of a chromosome into a spatially isolated micronucleus can explain why a single chromosome would be subject to aberrant DNA replication and can explain how extensive DNA damage can be restricted to a single chromosome. Because micronuclei may persist as discrete entities over several generations, damage to the chromosome need not occur all at once \(^{42,43}\), but could accrue from multiple failed attempts at DNA replication. Chromothripsis restricted to a chromosome arm could be generated fromacentric chromosome fragments, as chromosome pulverization was previously correlated with the presence of micronuclei in cancer cells. However, chromothripsis seems to be relatively rare, DNA damage in micronuclei could be more common. We propose that chromothripsis may be an extreme outcome of a mutagenesis mechanism that could be widespread in human cancer.

METHODS SUMMARY

All cell lines were maintained at 37°C with 5% CO₂ atmosphere. To generate micro-nuclei, cells were treated with nocodazole (100 ng ml⁻¹) for 6 h followed by mitotic shake-off and nocodazole washout. Alternatively, micronuclei were generated by depletion of MCAD or NTF2 by short interfering RNA (siRNA). Micronuclei were also generated in HT1080 cells by inactivation of the kinasechore of the human artificial chromosome by washout of doxycycline. Detailed descriptions of immunofluorescence, RNAi, BrDU labelling, comet assays, inactivation of the human artificial chromosomes (HAC), flow cytometry, quantitative analysis of nuclear import, chromosome spreads, spectral karyotyping, antibodies used, photoconversion of H2B-Kaede and long-term live-cell imaging experiments can be found in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.
32. Dimitrova, D. S., Prokhorova, T. A., Blow, J. J., Todorov, I. T. & Gilbert, D. M. Mammalian nuclei become licensed for DNA replication during late telophase. *J. Cell Sci.* **115**, 51–59 (2002).

33. Mendez, J. & Stillman, B. Chromatin association of human origin recognition complex,cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol. Cell. Biol.* **20**, 8602–8612 (2000).

34. Terradas, M., Martin, M., Tusell, L. & Genesca, A. DNA lesions sequestered in micronuclei induce a local defective-damage response. *DNA Repair* **8**, 1225–1234 (2009).

35. Obe, G., Beek, B. & Vaidya, V. G. The human leukocyte test system. III. Premature chromosome condensation from chemically and x-ray induced micronuclei. *Mutat. Res.* **27**, 89–101 (1975).

36. Johnson, R. T. & Rao, P. N. Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. *Nature* **226**, 717–722 (1970).

37. Smith, L., Plug, A. & Thayer, M. Delayed replication timing leads to delayed mitotic chromosome condensation and chromosomal instability of chromosome translocations. *Proc. Natl Acad. Sci. USA* **98**, 13300–13305 (2001).

38. Nichols, W. W., Levau, A., Aula, P. & Norrby, E. Chromosome damage associated with the measles virus in vitro. *Hereditas* **54**, 101–118 (1965).

39. Kato, H. & Sandberg, A. A. Chromosome pulverization in human binucleate cells following colcemid treatment. *J. Cell Biol.* **34**, 35–45 (1967).

40. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl Acad. Sci. USA* **99**, 12651–12656 (2002).

41. Janssen, A., van der Burg, M., Suzhai, H., Kops, G. J. & Medema, R. H. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. *Science* **333**, 1895–1898 (2011).

42. Stephens, P. J. et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell* **144**, 27–40 (2011).

43. Liu, P. et al. Chromosome catastrophes involve replication mechanisms generating complex genomic rearrangements. *Cell* **146**, 889–903 (2011).

44. Hastings, P. J., Lupski, J. R., Rosenberg, S. M. & Ira, G. Mechanisms of change in gene copy number. *Nature Rev. Genet.* **10**, 551–564 (2009).

45. Sen, S., Hittelman, W. N., Teeter, L. D. & Ku, M. T. Model for the formation of double minutes from prematurely condensed chromosomes of replicating micronuclei in drug-treated Chinese hamster ovary cells undergoing DNA amplification. *Cancer Res.* **49**, 6731–6737 (1989).

**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** D.P. conceived the project; K.C., N.J.G., R.D., A.B.L., D.C. and D.P. designed the experiments; D.P., K.C. and N.J.G. wrote the manuscript with edits from all authors; K.C. contributed Figs. 1–3, 4a, b and Supplementary Figs 2–8 with help from R.D.; N.J.G. contributed Fig. 5j, Supplementary Fig. 12, Supplementary Table 1 and Supplementary Movies. R.D., E.V.I. and A.P. contributed Fig. 5a–i and Supplementary Figs 1 and 11; A.B.L. contributed Fig. 4c, d and Supplementary Figs 9 and 10; Y.P. and D.C. contributed Supplementary Fig. 3d; L.N. contributed Fig. 2a.

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Methods

Cell culture. All cell lines were maintained at 37°C with 5% CO2, in Dulbecco’s Modified Eagle’s Medium (DMEM) (U2OS, HT1080), DMEM:F12 (HTERT-RPE-1), or DMEM:F12 medium without phenol red (H2B–GFP and H2B–mRFP U2OS, H2B–GFP and H2B–mRFP RPE-1, H2B–GFP Caco2, H2B–GFP SCC-114, H2B–GFP HeLa, H2B–mRFP MDA-231). All media were supplemented with 10% FBS, 100 IU mL–1 penicillin and 100 μg mL–1 streptomycin. HT1080 cells bearing the aliphodio human artificial chromosome 2 and expressing the tetracycline repressor TetR fused to the transcriptional silencer TTS (a gift from V. Larijani) were also supplemented with 1 μg mL–1 doxycycline (Sigma), 0.5 μg mL–1 G418 (geneticin, Invitrogen) and 4 μg mL–1 blasticidin S (Invitrogen). U2OS cells stably expressing human p53/cell fused to the C terminus of eGFP (Thermo Scientific; R04-017-01) were supplemented with 0.5 μg mL–1 G418.

Generation of whole-chromosome micronuclei. To generate cells with whole-chromosome micronuclei, HTERT-RPE-1 and U2OS cells were treated with 100 ng mL–1 nocodazole (Sigma) for 6 h. Mitotic cells were collected, washed twice with fresh medium containing 10% FBS, and then plated into medium containing 20% FBS where they completed cell division. To overcome the aneuploidy-induced p53-dependent GI arrest associated with nocodazole washout experiments, RPE-1 cells were transfected with 50 nmol p53 siRNA (Smartpool, Dharmacon) using Lipofectamine RNAi Max (Invitrogen) 12 h before the nocodazole treatment, allowing cell-cycle progression of the subsequent aneuploid daughter cell with micronuclei. For U2OS, M and MCAK knockdown, U2OS cells were serum starved for 2 days before transfection with 50 nmol U2OS or MCAK siRNAs (Smartpool, Dharmacon). Cells were then washed in fresh medium containing 10% FBS and 24 h later treated with 4 μm dihydrocytochalasin B (DCB; Sigma) for 16 h to inhibit cytokinesis. The subsequent arrested tetraploid cells depleted of U2OS or MCAK (many of which harboured micronuclei) were then treated for 6 h with 10 μM SB203580 (Sigma), a p38 inhibitor that promotes cell-cycle progression of tetraploids. Sequence information of the small interference RNA (siRNA) pools used from Dharmacon are as follows: human TP53 ON-TARGETplus SMARTpool siRNA L-003329-00-0005, (J-003329-14) 5'-GAAAUUGUGGUGGAGUA-3', (J-003329-15) 5'-GUGACGAGUGUGGUAGUAA-3', (J-003329-16) 5'-GCAAGCAAGACCCUAGGGU-3', (J-003329-17) 5'-GGAGAAUAAUUCCCCUUC-3', human KIF2C/MCAK ON-TARGETplus SMARTpool siRNA L-004955-00-0005, (J-004955-06) 5'-GGCAAGAUGUCGUGCUA-3', (J-004955-07) 5'-CCAAACGAGAAUUGUUA-3', (J-004955-08) 5'-GCAAGCAAGACCCUAGGGU-3', (J-004955-09) 5'-UGAUGAGCAGUCAUGAAGA-3', human CDC41/NUF2 ON-TARGETplus SMARTpool siRNA L-005289-00-0005, (J-005289-06) 5'-GAGCAAGACCCUAGGGU-3', (J-005289-07) 5'-GUGACGAGUGUGGUAGUAA-3', (J-005289-08) 5'-GUGAAGUAAUUGUUA-3', (J-005289-09) 5'-GCAAGCAAGACCCUAGGGU-3', human NUF2 ON-TARGETplus SMARTpool siRNA L-005289-00-0005, (J-005289-06) 5'-GAGCAAGACCCUAGGGU-3', (J-005289-07) 5'-GUGACGAGUGUGGUAGUAA-3', (J-005289-08) 5'-GUGAAGUAAUUGUUA-3', (J-005289-09) 5'-GCAAGCAAGACCCUAGGGU-3'.

Generation of micronuclei containing the HAC. HT1080 cells carrying the HAC culture were cultured in doxycycline-free medium and treated with 100 ng mL–1 nocodazole for 6 h to synchronize cells in mitosis. The absence of doxycycline enables TetR binding to TetO, which induces inactivation of the HAC centromere by TTS and the subsequent formation of a micronucleus after release from mitotic arrest. Cells released from mitotic arrest were synchronized at G0/G1 by serum starvation for 24 h, then released into the cell cycle with fresh medium containing 10% serum for further analysis.

Flow cytometry. Cells were fixed with 70% ethanol at 4°C followed by incubation with 250 μg mL–1 RNase A and 10 μg mL–1 propidium iodide at 37°C for 30 min. FACS analysis was performed with a FACScalibur flow cytometer (Becton Dickinson) and data analysed with CellQuest software.

Indirect immunofluorescence microscopy. For most experiments, cells were seeded on glass coverslips, washed in CSK buffer (10 mM PIPES, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2), pre-extracted with CSK buffer containing 0.3% Triton X-100 for 5 min at 4°C, and fixed in PBS containing 4% paraformaldehyde for 15 min. After fixation, cells were permeabilized in PBS, 0.2% Triton X-100 for 5 min, blocked in blocking buffer (PBS containing 5% FBS, 2% BSA and 0.1% Triton X-100) for 30 min, and then incubated with primary antibodies at room temperature for 1 h or overnight at 4°C. Cells were washed with PBS, 0.1% Triton X-100 and incubated with fluorescein-conjugated secondary antibodies (1:10,000, Molecular Probes) at room temperature for 1 h. Cells were also stained for DNA with Hoechst 33342 (1:5,000, Invitrogen) in PBS. More specific immunostaining protocols were as follows: for γ-H2AX and other DNA damage-response proteins, as in ref. 46; for ELYS and mAb414, ref. 47; for cyclin B1/γ-H2AX/Brdu, ref. 48; and for MCM2, CDT1 and ORC2, refs 49, 50. Images for most experiments were collected with a 0.3 NA objective with pinhole at 1 Airy unit. Photomultipliers were calibrated to ensure linear range. For quantification of MCM2 and BrdU signals, mean fluorescence intensity within the primary nuclei or micronuclei was measured using ImageJ software. For quantification of γ-H2AX, primary nuclei were classified as positive when five or more foci were detected, with the threshold of foci intensity set against the irradiated sample. The same threshold was applied to score γ-H2AX foci in micronuclei. Acquisition parameters, shutters, filter positions and focus were controlled by Slidebook software (Intelligent Imaging Innovations). Images presented in figures are maximum intensity projections of entire z-stacks, unless otherwise stated.

Antibodies for immunofluorescence. Samples were incubated with primary antibodies for human anti-centromere ACA (1:1,000, Antibodies, Inc.), rabbit γ-H2AX-Ser139 (1:500, Cell Signaling), mouse γ-H2AX (clone JBW301, 1:500, Upstate), rabbit 53BP1 (1:500, Cell Signaling), rabbit phospho-33BP1-Ser1778 (1:500, Cell Signaling), rabbit phospho-CHK1-Ser317 (1:500, Cell Signaling), rabbit phospho-ATM-Ser1981 (1:500, Cell Signaling), rabbit ATR (1:500, Cell Signaling), rabbit MRE11 (1:300, Abcam), rabbit cyclin B1 (1:300, Santa Cruz), mouse Brca1 (1:300, Santa Cruz), mouse RPA2 (1:500, Abcam), rabbit phospho-RPA2-S33 (1:500, Abcam), mouse monoclonal antibody 414 (1:5,000, Abcam), ELYS/MEL28 (1:1,000, gift from I. Mattila), mouse lamin A/C (1:300, Abcam), rabbit cyclin A (1:300, Santa Cruz), rabbit cyclin D1 (1:300; Santa Cruz) and mouse BrdU (Sigma). Secondary antibodies used were Alexa Fluor 488 (green), 594 (red) and 647 (far red) from Molecular Probes.

TUNEL assay. TUNEL assay was performed according to the manufacturer’s instructions (In situ cell death detection kit, Roche).

BrdU labelling. Cells were pre-labelled with 10 μM BrdU for 30 min and subsequently fixed and permeabilized according to the manufacturers’ conditions (5-bromo-2-deoxyuridine labelling and detection kit 1, Roche). Cells were then stained with anti-BrdU (1h, diluted 1:300) and processed for immunofluorescence. In the box and whiskers plot, the box represents upper and lower quartiles, line within box represents median and the whiskers extend to the highest and lowest value data sets.

Long-term live-cell imaging. Labelled cells were grown on glass-bottom 12-well tissue culture dishes (MatTek) and imaged on either a Nikon TE2000-E2 or Nikon Ti-E inverted microscope. Both microscopes were equipped with a cooled CCD camera (TE2000, Orca ER, Hamamatsu; Ti-E, CoolSNAP HQ2, Photometrics), a precision motorized stage (Bioprecission, LuId), and Nikon Perfect Focus, and both were enclosed within an atmosphere of 37°C and 3–5% humidified CO2, GFP or RFP images were captured at multiple points every 5–10 min for 3–5 days with either ×10 (0.3 NA) or ×20 (0.5, 0.75 NA) objectives, and exposure to fluorescent light was minimized to the greatest extent possible (all image acquisition used neutral density filters and/or 2 × 2 binning). Cell viability was confirmed by the continuous observation of mitotic cells throughout the duration of experiments. Captured images from each experiment were analysed using ImageJ (U.S. National Institutes of Health).
on interphase and mitotic samples (from both DMSO- and nocodazole-treated samples) according to the SkyPaint DNA kit H-5 for human chromosomess procedure (Applied Spectral Imaging, SKY00029) and imaged on a Nikon Eclipse E6000 microscope equipped with the SD300 Spectracle and Spectral Imaging acquisition software. To determine if pulverized chromosomes were derived from late-replicating chromosomes in micronuclei, the above procedure was also performed with addition of BrdU labelling reagent (Roche Applied Science) only in the last 2 h of colcemid treatment before fixation. Mitotic samples from the BrdU samples were treated according to the 5-bromo-2'-deoxy-uridine labelling and detection kit J protocol (Roche Applied Science, 11296736001), except that the anti-BrdU antibody was diluted 1:50.

Single-cell gel electrophoresis (comet) assay. Single-cell comet assays were performed according to the manufacturer’s instructions (Trevigen). Briefly, U2OS cells were collected at G1, S and G2 phases of the cell cycle after nocodazole washout was used to induce micronuclei. As controls, G1 cells were irradiated at 10 Gy. Cells were re-suspended in cold PBS at 2 × 10^5 cells ml^-1, mixed with low-melt agarose (1:10 ratio) and spread on frosted glass slides. After the agarose solidified, the slides were sequentially placed in lysis and alkaline solutions (Trevigen). Slides were then subjected to electrophoresis at 12 V for 10 min in 1× TBE buffer, fixed with 70% ethanol, and stained with DAPI. Nuclei were visualized using epifluorescent illumination on a Zeiss microscope and images were analysed with the NIH Image J program. DNA damage was quantified for 50 cells with micronuclei for each experimental condition by determining the tail DNA percentage using Comet Score (TriTek) software.

Quantitative analysis of nuclear pore complexes. To determine nuclear pore complex density in primary nuclei and micronuclei, cells were pre-extracted, fixed, permeabilized and immunostained with monoclonal antibody 414 and ELYS antibodies. Confocal images were collected on a Leica SPS laser scanning confocal with both a 405-nm and white light laser (at 488nm and 568nm) using a 1.4 NA oil objective. Z-stacks were collected with a 0.3 μm step size with pinhole at 1 Airy unit. Photomultipliers were calibrated to ensure linear range. Single layers of the Z-stack showing optimal nuclear pore staining for the primary nucleus and the micronuclei were chosen for quantitative analysis using MetaMorph. The areas of primary nuclei and micronuclei were judged from Hoechst staining and used to determine the perinuclear rim area. Total fluorescence intensity of monoclonal antibody 414 and ELYS was measured in the perinuclear rim area. To compare nuclear pore densities between primary nuclei and micronuclei, the total nuclear pore fluorescence was divided by the perinuclear rim area of primary nuclei and micronuclei, resulting in an average intensity ratio.

Nuclear import. To measure nuclear import, we used U2OS cells stably expressing the fusion protein NFATc1–EGFP (Thermo-Fisher Scientific). The inactive transcription factor NFATc1 resides in the cytosol. Elevated calcium levels in the cytosol lead to the dephosphorylation of NFATc1 by calcineurin, thereby inducing its rapid translocation into the nucleus. To increase the calcium level in the cytoplasm, U2OS cells released from nocodazole washout were treated with 0.2 μM thapsigargin, an inhibitor of the sarco-endoplasmic reticulum Ca^{2+} ATPase. Nuclear import of NFATc1 was then measured in the primary nuclei and micronuclei of U2OS cells at both 6 h and 20 h after nocodazole release. Cells were subsequently fixed with 2% paraformaldehyde and import was quantified based on nuclear fluorescence. To measure nuclear import of IBB-DiHcRed50, U2OS cells were transiently transfected with the IBB-DiHcRed-plasmid (gift from J. Ellenberg) using Lipofectamine 2000 (Invitrogen). Cells were then synchronized for 10 h with nocodazole, washed as previously described, and released into the cell cycle. Cells were fixed with 2% paraformaldehyde both 6 h and 20 h later, and import was quantified based on nuclear fluorescence.

46. Bekker-Jensen, S. et al. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J. Cell Biol. 173, 195–206 (2006).
47. Franz, C. et al. MEL-28/ELYS is required for the recruitment of nucleoporins to chromatin and postmitotic nuclear pore complex assembly. EMBO Rep. 8, 165–172 (2007).
48. Peterson-Roth, E., Reynolds, M., Quievryn, G. & Zhitkovich, A.Mismatch repair proteins are activators of toxic responses to chromium-DNA damage. Mol. Cell. Biol. 25, 3596–3607 (2005).
49. Prasanth, S. G., Mendez, J., Prasanth, K. V. & Stillman, B. Dynamics of pre-replication complex proteins during the cell division cycle. Phil. Trans. R. Soc. Lond. B 359, 7–16 (2004).
50. Dultz, E. et al. Systematic kinetic analysis of mitotic dis- and reassembly of the nuclear pore in living cells. J. Cell Biol. 180, 857–865 (2008).