Interference in DNA Replication Can Cause Mitotic Chromosomal Breakage Unassociated with Double-Strand Breaks

Mari Fujita¹, Hiroyuki Sasanuma¹, Kimiyo N. Yamamoto¹, Hiroshi Harada², Aya Kurosawa³, Noritaka Adachi³, Masato Omura¹, Masahiro Hiraoka⁴, Shunichi Takeda¹, Kouji Hirota¹

¹Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Yoshidakonoe, Sakyo-ku, Kyoto, Japan, ²Group of Radiation and Tumor Biology, Career-Path Promotion Unit for Young Life Scientists, Kyoto University, Yoshidakonoe, Sakyo-ku, Kyoto, Japan, ³International Graduate School of Arts and Sciences, Yokohama City University, Yokohama, Japan, ⁴Radiation Oncology and Image-Applied Therapy, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

Morphological analysis of mitotic chromosomes is used to detect mutagenic chemical compounds and to estimate the dose of ionizing radiation to be administered. It has long been believed that chromosomal breaks are always associated with double-strand breaks (DSBs). We here provide compelling evidence against this canonical theory. We employed a genetic approach using two cell lines, chicken DT40 and human Nalm-6. We measured the number of chromosomal breaks induced by three replication-blocking agents (aphidicolin, 5-fluorouracil, and hydroxyurea) in DSB-repair-proficient wild-type cells and cells deficient in both homologous recombination and nonhomologous end-joining (the two major DSB-repair pathways). Exposure of cells to the three replication-blocking agents for at least two cell cycles resulted in comparable numbers of chromosomal breaks for RAD54+/−/KU70−/− DT40 clones and wild-type cells. Likewise, the numbers of chromosomal breaks induced in RAD54+/−/LIG4−/− Nalm-6 clones and wild-type cells were also comparable. These data indicate that the replication-blocking agents can cause chromosomal breaks unassociated with DSBs. In contrast with DSB-repair-deficient cells, chicken DT40 cells deficient in PIF1 or ATRIP, which molecules contribute to the completion of DNA replication, displayed higher numbers of mitotic chromosomal breaks induced by aphidicolin than did wild-type cells, suggesting that single-strand gaps left unreplicated may result in mitotic chromosomal breaks.

Citation: Fujita M, Sasanuma H, Yamamoto KN, Harada H, Kurosawa A, et al. (2013) Interference in DNA Replication Can Cause Mitotic Chromosomal Breakage Unassociated with Double-Strand Breaks. PLoS ONE 8(4): e60043. doi:10.1371/journal.pone.0060043

Editor: Kerstin Borgmann, University Medical Center Hamburg-Eppendorf, Germany

Received December 20, 2012; Accepted February 20, 2013; Published April 3, 2013

Copyright: © 2013 Fujita et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Environment Research and Technology Development Fund (RF-1005) of the Ministry of the Environment, Japan and a research grant from CERI. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: khirota@rg.med.kyoto-u.ac.jp

Introduction

Morphological analysis of chromosomal aberrations in mitotic cells is widely used for the diagnosis of leukemia and the identification of mutagenic chemical agents [1,2]. Chromosomal aberrations include chromosomal breakage, fusion, and translocation [3]. According to the International System for Human Cytogenetic Nomenclature (ISCN), chromosomal breakage, i.e., the discontinuity of sister chromatids, is classified into two types: chromatid-type breaks, which involve discontinuity in one of the sister chromatids, and isochromatid-type breaks, which involve discontinuity in both sister chromatids at the same location [4] (Figure S1). Chromosomal breaks are induced by a variety of mutagenic agents, such as ionizing radiation [5–8]. It is generally believed that virtually all chromosomal breaks are associated with DSBs at the site of the break. This idea is supported by experimental data. DSBs induced by restriction endonucleases indeed induce chromosomal breakage, as well as translocation [9–13]. Additionally, chromosomal breaks and subsequent chromosomal translocation are frequently observed at genes encoding antigenic receptors in lymphocytes derived from patients with Ataxia Telangiectasia Mutated (ATM) dysfunction and lymphocytes deficient in DSB repair [8,14–17].

Chromosomal breaks are caused not only by DSB-inducing agents such as ionizing radiation, but by chemical agents that repress DNA replication [18]. Such agents include aphidicolin, 5-fluorouracil (5-FU), and hydroxyurea (HU). Aphidicolin is a reversible inhibitor of replicative DNA polymerases [19,20]. 5-FU, when metabolized to fluorodeoxyuridine, is a potent inhibitor of thymidylate synthase, and thereby depletes TTP pools and promotes dUTP incorporation into chromosomal DNA [21]. HU reduces dNTP levels by inhibiting the ribonucleotide reductase enzyme [22]. Although these drugs, as well as ionizing radiation, are capable of inducing chromosomal breaks, it has not previously been determined whether or not they induce chromosomal breaks by generating DSBs.

DSBs are repaired by two major pathways: homologous recombination (HR) and nonhomologous end-joining (NHEJ) [23,24]. The RAD54 protein significantly promotes HR-mediated DSB repair [7,25,26], while the KU70/KU80 proteins and ligase IV (LIG4) are all essential for NHEJ [27]. HR and NHEJ play a substantially overlapping role in DSB repair, as evidenced by the fact that cells deficient in both RAD54 and KU70 are considerably...
more sensitive to ionizing radiation than are cells deficient in either RAD54 or KU70 [7,20,29]. Accordingly, DSB-inducing chemical agents can be identified by detecting reduced cell viability and an increase in the frequency of chromosomal breakage in a DSB-repair-deficient mutant, compared to wild-type cells [30].

We here employ a genetic approach to analyze the cause of mitotic chromosomal breaks induced by three replication-blocking agents: aphidicolin, 5-fluorouracil, and hydroxyurea. We compared the number of induced chromosomal breaks in wild-type cells and in cells deficient in both HR and NHEJ. Interestingly, the agents induced comparable numbers of chromosomal breaks in both human Nalm6 and chicken DT40 cell lines [31,32], indicating that interference with DNA replication can cause mitotic chromosomal breakage that does not result from DSB. To gain an insight into the nature of aphidicolin-induced mitotic chromosomal breaks, we analyzed chicken DT40 cells deficient in PIF1 or ATRIP. PIF1 facilitates DNA-replication-fork progression when forks slow down and encounter barriers on template strands [33–35]. ATR kinase also contributes to the completion of DNA replication by preventing replication-fork collapse when replication forks are stalled. The absence of PIF1 or ATR causes marked increases in the number of aphidicolin-induced mitotic chromosomal breaks. The data suggest that single-strand gaps due to incomplete DNA replication may represent mitotic chromosomal breaks.

Materials and Methods

Cell Lines and Culture Conditions

DT40 cell line is derived from chicken B lymphoma [32] and was cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (AusgeneX, QLD 4210, Australia), 1% chicken serum (GIBCO-BRL, Grand Island, NY, USA), 50 μM mercaptoethanol (Invitrogen), L-glutamin (Nacalai Tesque), 50 U/mL penicillin, and 50 μg/mL streptomycin (Nacalai Tesque). The cell lines were maintained at 39.5°C in a humidified atmosphere and 10% CO2. Isogenic DNA-repair-deficient cell lines with disruption constructs were generated from genomic PCR products combined with neoR and bsrR selection-marker cassettes. Genomic DNA sequences were amplified using primers 5'- GGTCTGACATCGAACAAATTTCTTCTCATAAAAGTGG-3' and 5'- GGACTAGTACACCAACTTTGATTCAACA CACTGAAA-3', and the amplified 1.2 kb fragment was cloned into the SalI-SpeI sites of a pBlueScript SK vector. Marker-gene cassettes, neoR and bsrR selection-marker genes, flanked by loxP sequences, were inserted into the BamHI site of the amplified fragment to generate PIF1-neoR and PIF1-bsrR. To generate PIF1-Δ cells, wild-type DT40 cells were transfected sequentially with PIF1-neoR and PIF1-bsrR. The 0.5 kb PCR fragment from genomic DNA, using primers 5'- TCTCGGAGCTCCTCATGCTTACAGT-3' and 5'- CTTCACAGGCAATGAGAAGAAGAC-3', was used as a probe for Southern blot analysis to screen gene-targeting events. Targeting efficiency for the first and second allele was 78% (18/23) and 42% (5/12), respectively.

Chromosomal Aberration Analysis

For chromosomal aberration analysis of the DT40 cells, we prepared chromosome samples as previously reported [5]. The chicken karyotype consists of 80 chromosomes: 11 major autosomal macrochromosomes, the 2 ZW sex chromosomes, and 67 microchromosomes [37]. Giemsa-stained metaphase cells were scored at 1000× magnification, with scoring limited to the 11 major macrochromosomes and the Z chromosome [37]. Chromosomal aberrations (CAs) were classified as isochromatid or chromatid gaps, breaks, and exchanges (fusions including triradial, quadriradial, ring, dicentric, or other) according to the ISCN system [4]. We used a different protocol for chromosomal aberration analysis of the Nalm-6 cells. Briefly, compound-treated wild-type and DNA-repair-deficient clones were incubated at 37°C for 48 h. To arrest cells in the metaphase, 0.06% colcemid (GIBCO-BRL) was added 2 h before harvest. Cells were pelleted by centrifugation, resuspended in 5 mL of 0.6% sodium citrate for 20 min at room temperature, and fixed in 2 mL of a freshly prepared 3:1 mixture of methanol:acetic acid (i.e., Carnoy’s solution). The pelleted cells were then resuspended in 1 mL of Carnoy’s solution and dropped onto clean glass slides and air dried. The slides were stained with a 5% HARLECO Giemsa stain solution (Nacalai Tesque) for 10 min, rinsed with water, and air dried. All chromosomes in each mitotic cell were scored at 1000× magnification.

Flow-cytometric Analysis to Measure Cell Viability

Annexin V (BioVision, Mountain View, CA, USA) assays were performed after treatment with drugs or irradiation with γ-ray. Flow-cytometric analysis was performed with a FACS Calibur flow cytometer (Beckton Dickinson, Mountain View, CA). Data were acquired and analyzed using CellQuest software.

Cell Counting and Cell-cycle Analysis

Cell numbers were determined by mixing a PI-stained sample with a fixed number of 25 μm microspheres (Polysciences Inc., Warrington, PA), which can be distinguished from cells by forward and side-scatter characteristics during flow-cytometric analysis. Beads and living cells were counted simultaneously as gated events, and cell numbers were calculated. We analyzed the growth curve of each clone at least three times.

For cell-cycle analysis, cells were labeled for 10 min with 20 μM bromodeoxyuridine (BrdU; Amersham, Buckinghamshire, UK). Harvested cells were fixed overnight with 70% ethanol at 4°C and successively incubated as follows: (i) in 2 N HCl and 0.5% Triton X-100 for 30 min at room temperature; (ii) in FITC-conjugated anti-BrdU antibody (Pharmingen, San Diego, CA) for 1 h at room temperature; and (iii) in 5 μg/mL propidium iodide (PI) in PBS. Cells were washed with PBS containing 2% FBS and 0.1% sodium azide between each incubation. Subsequent flow-cytometric analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA, USA). Fluorescence data were displayed as dot plots using Cell Quest software (Becton Dickinson).
Measurement of Cellular Sensitivity

$10^4$ cells were seeded into 24-well plates containing 1 mL of culture medium and incubated at 39.5°C. ATP assays were carried out with 96-well plates using a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega Corp., Madison, WI, USA) at 48 h after chemical exposure. Briefly, we transferred 100 µL cell suspensions to the individual wells of the 96-well plates, held the plates at room temperature for approximately 30 min, added 100 µL of CellTiter-Glo reagent, and mixed the contents for 2 min on an orbital shaker to induce cell lysis. The plate was then incubated at room temperature for 10 min to stabilize the luminescent signal. We measured luminescence using a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Results

DSB-repair-proficient and -deficient Cells Show Similar Sensitivity to Three Replication-blocking Agents: Aphidicolin, 5-FU, and HU

To investigate whether or not DSBs are induced by aphidicolin, 5-FU, and HU, we measured the viability of wild-type DT40 cells and the KU70<sup>-/-</sup>, RAD54<sup>-/-</sup> and KU70<sup>-/-</sup>/RAD54<sup>-/-</sup> clones exposed to these agents. Remarkably, cytotoxicity resulting from the three agents was comparable for these clones (Figure 1A and S2). This is in marked contrast to the effects of ionizing irradiation, which killed KU70<sup>-/-</sup>/RAD54<sup>-/-</sup> cells to a significantly higher degree than it did wild-type cells (Figure 1A and S2). Given the fact that unrepaired DSBs effectively trigger apoptosis, the comparable sensitivity between DSB-repair-deficient and -proficient cells indicates that cytotoxicity caused by the three agents probably does not result from DSBs.

We next analyzed the effect of each of the three replication-blocking agents on the cell cycle. To assess DNA replication, we measured the uptake of BrdU, a thymidine analog, after 4 and 24 h exposure to the three agents (Figure 1B). Treatment with 45 µM 5-FU did not affect BrdU uptake at 4 h and partially repressed uptake at 24 h. Treatment with 25 µM HU and 0.25 µM aphidicolin partially repressed BrdU uptake at 4 h but did not affect uptake at 24 h. Treatment with aphidicolin, 5-FU, and HU delayed cellular proliferation only transiently (Figure 1C). In summary, cells exposed to the three replication-blocking agents were capable of continuously proliferating despite the strong cytotoxicity of these agents.

Aphidicolin, 5-FU, and HU Induce Comparable Numbers of Visible Chromosomal Breaks for Both DSB-repair-proficient and -deficient Chicken DT40 Cell Lines

The three replication-blocking agents all induce chromosomal breaks. To explore the cause of these induced chromosomal breaks, we measured the number of visible chromosomal breaks in wild-type and RAD54<sup>-/-</sup>/KU70<sup>-/-</sup> cells exposed to the three agents and ionizing-radiation (Figure 2A). RAD54<sup>-/-</sup>/KU70<sup>-/-</sup> cells exposed to 0.3 Gy ionizing radiation had eight times more chromosomal breaks than did the wild-type cells (Figure 2A). Thus these chromosomal breaks indeed represent unrepaired DSBs. In marked contrast, the number of visible chromosomal breaks induced by the three replication-blocking agents was comparable for wild-type and RAD54<sup>-/-</sup>/KU70<sup>-/-</sup> cells (Figure 2B-D, Figure S1). Likewise, viability was also comparable for wild-type and RAD54<sup>-/-</sup>/KU70<sup>-/-</sup> cells (Figure S3A-D). We therefore conclude that a large percentage of chromosomal breaks produced by the three replication-blocking agents did not result from DSBs.

Figure 1. Comparable sensitivity to 5-FU, HU, and aphidicolin for wild-type and RAD54<sup>-/-</sup>/KU70<sup>-/-</sup> DT40 cells. (A) Indicated cells were either irradiated with γ-rays and cultured for 48 h or continuously incubated with aphidicolin for 72 h or, with 5-FU or HU for 48 h. Living cells were measured in terms of level of cellular ATP. The average for three independent experiments is shown. Error bars show the standard deviation for three independent experiments. (B) Cell-cycle analysis of wild-type DT40 cells after treatment for 4 h and 24 h with 45 µM 5-FU,
5-FU and HU Induce Similar Numbers of Chromosomal Breaks in Both DSB-repair-proficient and -deficient Human Nalm-6 Cell Lines

To confirm that the results were reproducible in human cells, we conducted chromosomal analysis using the human Nalm-6 pre-B cell line [31]. RAD54<sup>−/−</sup>/LIG4<sup>−/−</sup> cells consistently showed more than two times higher number of chromosomal breaks than did wild-type cells at 8 h after treatment with 0.1 Gy ionizing radiation (Figure 2E). We also measured the number of visible chromosomal breaks after 48 h exposure to 5-FU or HU.

---

**Figure 2.** Replication-blocking agents induce comparable numbers of chromosome breaks in both DSB-repair-proficient and -deficient chicken DT40 and human Nalm-6 cell lines. (A–D) Frequency of chromosomal aberrations (CAs) in wild-type and RAD54<sup>−/−</sup>/KU70<sup>−/−</sup> DT40 cells before (0) and after treatment with (A) γ-irradiation, (B) 5-FU, (C) HU, and (D) aphidicolin (APH). Cells were analyzed at 3 h after irradiation (A). Cells were incubated with 5-FU or HU for 24 h, or with aphidicolin for 48 h at the indicated concentrations (B–D). In each case, cells were treated with colcemid for the last 3 h. More than 100 cells were analyzed in each case. (E–H) Frequency of chromosomal aberrations (CAs) in wild-type and RAD54<sup>−/−</sup>/LIG4<sup>−/−</sup> Nalm-6 cells before (0) and after treatment with (E) γ-irradiation, (F) 5-FU, and (G) HU. Cells were incubated with 5-FU or HU for 48 h at the indicated concentrations (F, G). (H) The number of induced CAs was calculated by subtracting the number of non-treated cells from the number of cells treated with γ-rays or chemicals. More than 50 cells were analyzed at 8 h after irradiation at 0.1 Gy. More than 100 cells were analyzed for 5-FU and HU. Error bars show standard error, based on the Poisson distribution of spontaneous chromosomal aberrations observed previously [37,52].

doi:10.1371/journal.pone.0060043.g002
(Figure 2F, G). By contrast, 5-FU and HU induced comparable numbers of chromosomal breaks in wild-type and RAD54<sup>+/−</sup>LG4<sup>−/−</sup> cells (Figure 2H). These results are consistent with those found for DT40 cells.

To further understand the occurrence of DSBs, we counted the number of subnuclear foci carrying phosphorylation of H2AX (γH2AX foci) after 48 h exposure to aphidicolin. The number of γH2AX foci found in both wild-type and KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> cells was comparable (Figure S4). Thus, as with mitotic chromosomal breaks, γH2AX may represent not only DSBs but other lesions as well, which idea agrees with the fact that phosphorylation of H2AX occurs at single-stranded DNA regions and other lesions as well, which idea agrees with the fact that phosphorylation of H2AX occurs at single-stranded DNA regions that arise during replication [38–40]. We therefore decided not to analyze γH2AX foci in subsequent studies.

Analysis of Chromosomal Breakage after Removal of Replication-blocking Agents

We next addressed the possibility that the inhibition of DNA replication interfered with DSB repair, since DSB repair is associated with DNA synthesis [41]. To explore this possibility, we analyzed chromosomal breaks and quantified cell viability under conditions that provide cells with additional time to repair DNA damage in aphidicolin-free medium after exposure to cells at 0.25 μM aphidicolin for 48 h (Figure S5A). It should be noted that only aphidicolin was suitable for this experiment, because elimination of aphidicolin but not of 5-FU or HU leads to immediate restoration of DNA replication. Wild-type cells and RAD54<sup>+/−</sup>/KU70<sup>+/−</sup> clones displayed comparable viabilities at both 12 and 24 h during incubation in aphidicolin-free medium, which stands in remarkable contrast to the fact that viability of the KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> cells was three times lower than that of the wild-type cells after ionizing radiation (Figure S5B).

We next measured visible chromosomal breaks at 3 and 6 h after release from the 48 h treatment with aphidicolin (Figure 3). The number of chromosomal aberrations decreased over time, with similar kinetics for both wild-type and KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> clones. The data as well as the comparable viability of the two clones confirmed our conclusion that visible chromosomal breaks do not always occur as a consequence of DSBs.

Complete Replication Blockage by Aphidicolin Followed by Release from the Blockage May Induce DSBs

Previous papers have suggested that DSBs are induced when DNA replication is completely blocked by exposing cells to a higher concentration (2 mM) of HU than the concentrations shown in Figure 2C [42,43]. The data shown in Figure 2C indicate that the majority of visible chromosomal breaks induced by lower concentrations of HU do not result from DSBs. To investigate whether the results reported in these previous papers could be duplicated in DT40 cells, we treated wild-type cells and KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> clones with 2 mM HU for 2 h, which treatment blocked DNA replication completely (Figure 4A). At 3 and 6 h after release from the replication blockage, we measured the number of mitotic chromosomal breaks (Figure 4B). The KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> mutant exhibited more visible chromosomal breaks than did the wild-type cells. This result suggests that a portion of the chromated breaks resulted from DSBs. We repeated the experiment using 0.5 μM aphidicolin instead of 2 mM HU. We blocked DNA replication by exposing cells to 0.5 μM aphidicolin (Figure 4C) for 2 h, then cultured cells with aphidicolin-free media and measured the number of mitotic chromosomal breaks at 3 and 6 h after the removal of aphidicolin (Figure 4D). Again, the KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> mutant exhibited more visible chromosomal breaks than did the wild-type cells. Thus the release from the complete replication blockage by aphidicolin and HU may induce DSBs.

The Inactivation of PIF1 Causes Hypersensitivity to Replication-blocking Agents

To gain insight into the molecular mechanisms underlying chromosomal breakage not resulting from DSBs, we analyzed DT40 cells deficient in PIF1 helicase or ATRIP, the essential component of the ATR kinase. We generated PIFI<sup>−/−</sup> DT40 cells by inserting a marker gene in exon9, which results in the deletion of the essential helicase domain (Figure S6A and B). The resulting PIFI<sup>−/−</sup> DT40 cells were able to proliferate with normal kinetics (Figure S6C). Remarkably, the PIFI<sup>−/−</sup> cells were hypersensitive to the replication-blocking agents (aphidicolin and HU) but not to any other DNA-damaging agents (Figure 5A). We then measured chromosomal breakage. The PIFI<sup>−/−</sup> cells exhibited significant increases in the number of chromosomal breaks after exposure to 0.1 μM aphidicolin (Figure 5B), which concentration had no effect on the cell cycle of either wild-type or KU70<sup>−/−</sup>/RAD54<sup>−/−</sup> cells. Thus, the chromosomal breaks observed in the PIFI<sup>−/−</sup> cells may represent unreplicated DNA sequences, since PIFI is required for the completion of DNA replication when forks slow down [33–35]. A possible scenario is that the unreplicated DNA sequences might interfere with local chromosomal condensation, and thereby cause isochromatid-type breaks, cytologically visible discontinuity in both sister chromatids at the same location, shown in Figure 5B.

We next measured chromosomal breakage induced by aphidicolin in ATRIP-deficient cells, where the ATRIP gene is excised by the Cre recombinase upon addition of tamoxifen to the culture medium [44]. Taking into account the fact that ATRIP<sup>−/−</sup> cells stop proliferating at 72 h after addition of tamoxifen, prior to cell death due to genome instability, we analyzed the mitotic chromosomes at 48 h after addition of tamoxifen. We exposed the ATRIP<sup>−/−</sup> cells to aphidicolin for 24 h prior to mitotic chromosome analysis. Surprisingly, exposure to 0.1 μM aphidicolin induced extensive chromosome breakage without any detectable suppression of DNA replication (Figure 1, 5C). Over 40% of the mitotic ATRIP<sup>−/−</sup> cells showed five or more mitotic

![Figure 3. Comparable chromosomal breakage after removal of replication-blocking agents.](image-url)
chromosomal breaks (Figure 5C). These data again support the notion that aphidicolin-induced mitotic chromosomal breaks can result from single-strand gaps due to defective completion of DNA replication, since ATR/ATRIP prevents replication fork collapse under replication stress and thereby contributes to the re-start and completion of DNA replication.

**Discussion**

We here show that the partial repression of DNA replication by aphidicolin, 5-FU, and HU induces chromosomal breaks in mitotic cells during the cell cycle. Remarkably, the partial repression induced a comparable number of chromosomal breaks in both the DSB-repair-deficient cells (human $RAD54^{-/-}/LIG4^{-/-}$ and chicken $RAD54^{-/-}/KU70^{-/-}$ cell lines) and their wild-type controls (Figure 2). This result is in marked contrast with the finding that the number of chromosomal breaks induced by $\gamma$-rays in $RAD54^{-/-}/KU70^{-/-}$ DT40 cells was more than eight times

Figure 4. Release from complete replication blockage by a high concentration of aphidicolin or HU induces DSBs. (A) Cell-cycle analysis after treatment with 2 mM HU for 2 h. The BrdU-positive fraction was quantified as in Figure 1. (B) Frequency of chromosomal aberrations (CAs) for wild-type DT40 cells and $RAD54^{-/-}/KU70^{-/-}$ cells. Cells were exposed to 2 mM HU for 2 h and then released in a drug-free medium for 3 or 6 h. (C) Cell-cycle analysis after treatment for 2 h with 0.5 $\mu$M aphidicolin (APH). The BrdU-positive fraction was quantified as in Figure 1. (D) Frequency of chromosomal aberrations (CAs) for wild-type DT40 cells and $RAD54^{-/-}/KU70^{-/-}$ cells. Cells were exposed with 0.5 $\mu$M aphidicolin (APH) for 2 h and released in a drug-free medium for 3 or 6 h. More than 50 cells were analyzed in each case. Error bars show standard error for the number of CAs in 50 mitotic cells, calculated as in Figure 2. Asterisk and double asterisk: significant difference compared with wild-type cells ($P<0.05$). doi:10.1371/journal.pone.0060043.g004

Figure 5. Contribution of PIF1 and ATRIP to the prevention of chromosomal breakage. (A) Cells with the indicated genotype were exposed to the indicated replication-blocking agents and DNA damage agents. The dose of the agents is displayed on the x-axis on a linear scale, while the percent fraction of surviving cells is displayed on the y-axis on a logarithmic scale. Error bars show standard deviation of mean for three independent assays. (B) Frequency of chromosomal aberrations (CAs) in wild-type, PIF1$^{-/-}$, and $RAD54^{-/-}/KU70^{-/-}$ DT40 cells before (0) and after treatment with aphidicolin at indicated concentration for 48 h. (C) Percentage of the cells carrying the indicated number of chromosomal breaks is indicated as a histogram. Indicated cells were treated with 0.1 $\mu$M aphidicolin (APH) for 24 h. More than 50 cells were analyzed in each case. Error bars show standard error for the number of CAs in 50 mitotic cells, calculated as in Figure 2. doi:10.1371/journal.pone.0060043.g005
Mitotic Chromosome Breaks Unassociating with DSB

larger than the number found in the wild-type control (Figure 2A). We therefore conclude that, unlike γ-ray-induced chromosomal breaks, aphidicolin, 5-FU, and HU can induce chromosomal breaks that are not subject to the major DSB repair pathways: RAD54-dependent HR and KU70- or LIG4-dependent NHEJ. This shows that there are two types of mitotic chromosomal breaks: those that result from DSBs and those that do not.

5-FU and HU are widely used for chemotherapy. Our data suggest that there are two distinct mechanisms underlying the cytotoxic effects of these agents. First, high concentrations of these agents stall replication, leading to replication collapse and DSB formation. Indeed, treatment with 2 mM HU for 2 h followed by incubation of the cells in drug-free media induced a higher number of mitotic chromosomal breaks in ku70+/−/rad54−/− cells than in wild-type cells (Figure 4B), indicating that a portion of the induced chromosomal breaks may result from DSBs. A recent report showed that prolonged treatment (~24 h) with a high concentration of HU (2 mM) resulted in replication fork collapse and formation of DSBs that were repaired by HR [42]. Second, treatment with lower concentrations of HU, in which replication fork progression was slowed but not completely inhibited (Figure 1B), induced mitotic chromosomal breaks that were not associated with DSBs (Figure 2). In summary, replication stress induces two different types of mitotic chromosomal breaks, depending on the concentration of the replication-blocking agent. It should be noted that the concentrations of HU employed for the experiments illustrated by Figure 1C were similar to the serum concentrations of HU used for chemotherapeutic treatment (100–300 μM) [45]. Thus, the chemotherapeutic effects of 5-FU and HU may not result from DSB formation, even though chemotherapy by these agents efficiently induces chromosomal breaks in mitotic cells. An unanswered question is, how do these agents have therapeutic effects on malignant cells when they neither stop DNA replication nor induce DSBs? One possible scenario is that chronic replication stress induces senescence manifested by cell-cycle arrest [46], collisions between replication forks and transcription [47], or mis-segregation of sister chromatids during mitosis [48].

Another pressing question is, what is the molecular mechanism for the generation of chromosomal breaks without associating DSBs? One possible answer is that even when bulk chromosomal replication is not compromised (Figure 1B), replication might not be completed at regions with low origin-density and replication barriers such as DNA sequences prone to secondary structure formation, which correspond to common fragile chromosome sites [49,50]. The resulting unreplicated single-strand DNA gaps might interfere with local chromosome condensation and thereby induce cytogenetically visible break sites. This scenario is supported by the fact that PIF1, which facilitates replication-fork progression, repressed the formation of mitotic chromosomal breakage (Figure 5B). Moreover, a previous study showed that premature condensation of chromosomes indeed induces chromosomal breaks in mitotic cells [51]. In summary, we posit that replication stress caused by aphidicolin and therapeutic concentrations of 5-FU and HU can induce chromosomal breakage that is not associated with DSBs. The molecular mechanism for the cytotoxicity of these chemotherapeutic agents is a topic for future experimentation.

Supporting Information

Figure S1 Representative image of the cytogenetically visible chromosome breaks. Wild-type DT40 cells were treated with HU as in Figure 2. Representative chromatid-type break and isochromatid-type break was indicated by arrowhead and arrow respectively.

Figure S2 Comparable sensitivity to 5-FU, HU, and aphidicolin for wild-type, rad54−/−, ku70−/−, and rad54−/−ku70−/− DT40 cells. Indicated cells were either irradiated with γ-rays and cultured for 48 h or continuously incubated with aphidicolin for 72 h or, with 5-FU or HU for 48 h. Living cells were measured in terms of level of cellular ATP. The average for three independent experiments is shown. Error bars show the standard deviation for three independent experiments.

Figure S3 Quantitative analysis of cell viability after 24 h treatment with aphidicolin, 5-FU, and HU. (A) Dot plots represent the intensity of Annexin V fluorescent staining on the x axis (logarithmic scale) and the intensity of propidium-iodine (PI) staining on the y axis (logarithmic scale). (B–D) Numbers indicate the percentages of live, preapoptotic, and dead cells defined by Annexin V+/PI−, Annexin V+/PI+, and PI+ staining, respectively after (B) 5-FU, (C) HU, and (D) aphidicolin (APH) treatment. The average for three separate experiments is shown. Error bars show the standard deviation for three independent experiments.

Figure S4 Comparable number of γH2AX foci following replication stress. Percentage of cells carrying the indicated number of γH2AX foci is shown as histogram. Indicated cells were treated with aphidicolin (APH) for 48 h.

Figure S5 Cell viability after removal of replication-blocking agents. (A, B) Cells were exposed to 0.25 μM aphidicolin (APH) for 24 h (A) or were irradiated with 2 Gy of γ-ray (B) and released in a drug-free medium for 12 or 24 h. Numbers indicate the percentages of live, preapoptotic, and dead cells, as in Fig. S1.

Figure S6 PIF1 disruption in DT40 cells. (A) A neo or bsr selection-marker gene was inserted in the wild-type chicken PIF1 locus exon 7. The targeting construct is shown and compared with the relevant chicken PIF1 genomic sequences (top). Open boxes indicate the position of the exons. Relevant StuI sites and the position of the probe used in the Southern blot analysis are indicated. (B) Disruption of PIF1 was confirmed by Southern blot. (C) Relative growth rate plotted for the indicated genotypes. Error bars show the standard deviation of mean for three independent experiments.

Acknowledgments

We thank J. Sale (Cambridge, UK) for his critical reading of the manuscript. We thank the members of the Takeda laboratory for their help and support. Special thanks go to Ms. Noguchi for technical support.

Author Contributions

Conceived and designed the experiments: KH ST. Performed the experiments: KS KN HH AK NA MO. Analyzed the data: MF KH. Contributed reagents/materials/analysis tools: HH MH AK NA. Wrote the paper: MF ST KH.
26. Kanaar R, Troelstra C, Swagemakers SM, Essers J, Smit B, et al. (1996) Human
25. Eppink B, Tafel AA, Hanada K, van Drunen E, Hickson ID, et al. (2011) The
23. Sonoda E, Hochegger H, Saberi A, Taniguchi Y, Takeda S (2006) Differential
22. Koc A, Wheeler LJ, Mathews CK, Merrill GF (2004) Hydroxyurea arrests DNA
19. Ikegami S, Taguchi T, Ohashi M, Oguro M, Nagano H, et al. (1978)
17. Wang JH, Gostissa M, Yan CT, Goff P, Hickernell T, et al. (2009) Mechanisms
16. van Gent DC, Hoeijmakers JH, Kanaar R (2001) Chromosomal stability and the
14. Cohen MM, Shaham M, Dagan J, Shmueli E, Kohn G (1975) Cytogenetic
6. Gebhart E (1981) Sister chromatid exchange (SCE) and structural chromosome
5. Yamamoto KN, Hirota K, Kono K, Takeda S, Sakamuru S, et al. (2011)
3. Sachs RK, Hlatky LR, Trask BJ (2000) Radiation-produced chromosome
7. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, et al. (1998)
6. Gebhart E (1981) Sister chromatid exchange (SCE) and structural chromosome
231–236.
36. Wlodarska I, Aventin A, Ingles-Esteve J, Falzetti D, Criel A, et al. (1997) A new
35. Pinter SF, Aubert SD, Zakian VA (2008) The Schizosaccharomyces pombe
338–356.
34. Anand RP, Shah KA, Niu H, Sung P, Mirkin SM, et al. (2012) Overcoming
282–285.
28. Couedel C, Mills KD, Barchi M, Shen L, Olshen A, et al. (2004) Collaboration
5336–5351.
40: 1091–1105.
27. Lieber MR (2010) The mechanism of double-strand DNA break repair by the
24. Weinstock DM, Richardson CA, Elliott B, Jasin M (2006) Modeling oncogenic
223–230.
18. Wang JH, Gostissa M, Yan CT, Goff P, Hickernell T, et al. (2009) Mechanisms
22. Koc A, Wheeler LJ, Mathews CK, Merrill GF (2004) Hydroxyurea arrests DNA
21. Suki RB, Costello JF, Li J, Diehl JF, Revet I, et al. (2011) Homologous recombination and nonhomologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J 17: 5497–5508.
20. Yang H, Liu Y, Good MA, Sasaki M, Sonoda E, et al. (2004) Homologous recombination and nonhomologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J 17: 5497–5508.
19. Ikegami S, Taguchi T, Ohashi M, Oguro M, Nagano H, et al. (1978) Mitotic Chromosome Breaks Unassociating with DSB.