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RESEARCH BRIEF

DNA-Damage Response during Mitosis Induces Whole-Chromosome Missegregation

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ABSTRACT Many cancers display both structural (s-CIN) and numerical (w-CIN) chromosomal instabilities. Defective chromosome segregation during mitosis has been shown to cause DNA damage that induces structural rearrangements of chromosomes (s-CIN). In contrast, whether DNA damage can disrupt mitotic processes to generate whole chromosomal instability (w-CIN) is unknown. Here, we show that activation of the DNA-damage response (DDR) during mitosis selectively stabilizes kinetochore–microtubule (k-MT) attachments to chromosomes through Aurora-A and PLK1 kinases, thereby increasing the frequency of lagging chromosomes during anaphase. Inhibition of DDR proteins, ATM or CHK2, abolishes the effect of DNA damage on k-MTs and chromosome segregation, whereas activation of the DDR in the absence of DNA damage is sufficient to induce chromosome segregation errors. Finally, inhibiting the DDR during mitosis in cancer cells with persistent DNA damage suppresses inherent chromosome segregation defects. Thus, the DDR during mitosis inappropriately stabilizes k-MTs, creating a link between s-CIN and w-CIN.

SIGNIFICANCE: The genome-protective role of the DDR depends on its ability to delay cell division until damaged DNA can be fully repaired. Here, we show that when DNA damage is induced during mitosis, the DDR unexpectedly induces errors in the segregation of entire chromosomes, thus linking structural and numerical chromosomal instabilities. *Cancer Discov; 4(11); 1281–9. ©2014 AACR.*

See related commentary by Jelluma and Kops, p. 1256.

INTRODUCTION

DNA damage elicits a complex signaling cascade that leads to cell-cycle arrest. DNA-damage response (DDR) signaling comprises two arms, ATR/CHK1 and ATM/CHK2, which respond to damage induced by replication stress and double-strand DNA breaks, respectively (1). Upon DNA damage, CHK1 and CHK2 inhibit mitotic entry by deregulating Polo-like kinase 1 (PLK1) whose subsequent activation by Aurora-A is required for checkpoint recovery (1, 2). This provides sufficient time for DNA repair before cells enter mitosis and commit to chromosome segregation. However, cancer cells often encounter DNA damage during mitosis secondary to checkpoint slippage with persistence of premitotic damage (3)

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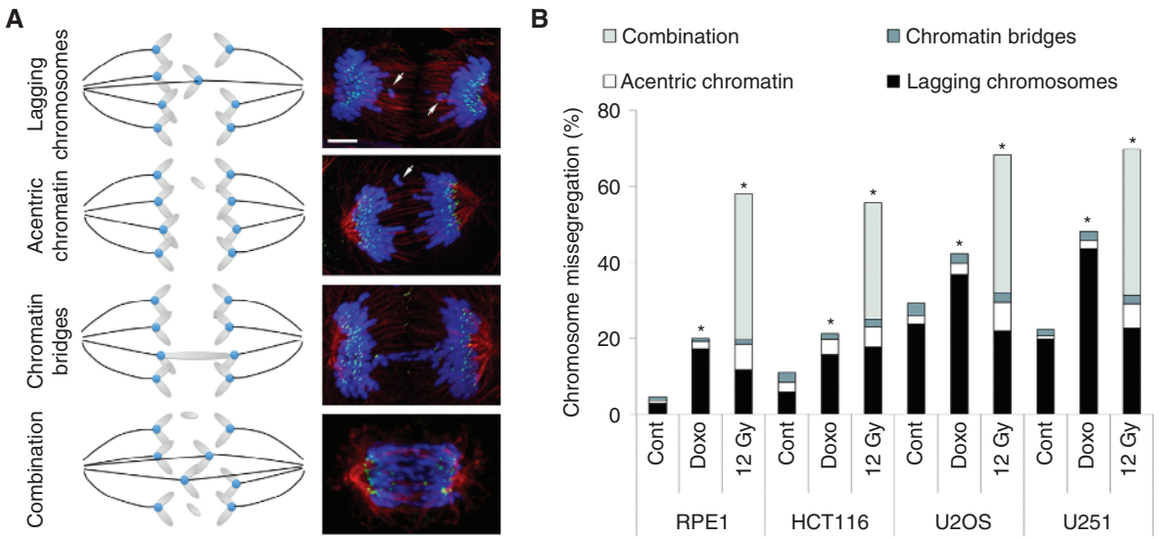

Figure 1. DNA damage during mitosis induces whole-chromosome missegregation. **A,** anaphase spindles of U251 cells containing lagging chromosomes, acentric chromatin fragments, chromatin bridges, and a combination of lagging chromosomes with acentric chromatin (from top to bottom). Cells were stained for HEC1/kinetochores (green), DNA (blue), and microtubules (red). Scale bar, 5 μm. **B,** percentage of anaphase spindles with chromosome segregation defects 25 minutes after exposure to doxorubicin (doxo) or 12 Gy of IR. Cont, control. Bars, mean; $n = 150$ cells, three experiments; *, $P < 0.01$; error bars were omitted for clarity.

or due to *de novo* induction of DNA breaks by therapeutic agents such as ionizing radiation (4). Although the compact chromatin structure of mitotic chromosomes may confer protective properties from DNA-damaging agents (5), mitosis has long been recognized, for unknown reasons, as the most sensitive phase of the cell cycle to DNA damage (4, 6). Mitotic cells do not possess the capacity to repair DNA breaks, and ectopic activation of DNA repair can lead to deleterious consequences (7). Nonetheless, DNA damage during mitosis results in a partial DDR (8), whose consequences, at a time when the cell is chiefly preoccupied by the process of chromosome segregation, remain elusive.

Chromosome segregation errors during anaphase fall into three subtypes that arise from distinct mechanisms. Errors in mitotic spindle function spawn lagging chromosomes (9), whereby an entire chromosome fails to segregate properly by virtue of its attachment to microtubules emanating from opposite spindle poles (Fig. 1A). Lagging chromosomes are a hallmark of whole chromosomal instability (w-CIN; ref. 10). Anaphase spindles can also exhibit bridged chromatin (3, 11) such that DNA, from the same chromosome or from nondisjoined sister chromatids, is stretched toward opposite spindle poles (Fig. 1A). The third class of segregation errors consists of acentric chromatin fragments that are devoid of centromeres and thus cannot establish canonical kinetochore–microtubule (k-MT) attachments to the mitotic spindle (Fig. 1A). Chromatin bridges and acentric chromatin fragments are hallmarks of structural chromosomal instability (s-CIN; ref. 3).

The relationship between DNA damage and s-CIN has long been established. Premitotic DNA damage can lead to the formation of acentric chromosome arms as well as dicentric chromosomes that undergo successive breakage–fusion–bridge cycles, a defining feature of s-CIN (11). However, it was recently shown that premitotic replication stress, which leads to DNA damage that can persist into mitosis, is also a feature of colorectal cell lines with w-CIN (3). This suggests that s-CIN and w-CIN may coexist in an interdependent relationship. Yet, a causative relationship between DNA damage and whole-chromosome missegregation (w-CIN) is unclear, and whether the mechanisms that lead to s-CIN can also directly engender w-CIN is unknown. Here, we use high-resolution immunofluorescence microscopy and live quantitative single-cell imaging to directly investigate the consequences of inducing DNA damage during mitosis. We then use genetic and short-term pharmacologic interventions to determine the role of the DDR signaling on the fidelity of the process of whole-chromosome segregation during anaphase.

RESULTS

DNA Damage Leads to Chromosome Segregation Errors

To investigate the effect of DNA damage on chromosome segregation during mitosis, we used four human cell lines: two were near-diploid and chromosomally stable (RPE1 and HCT116), and two were aneuploid and exhibited w-CIN (U251 and U2OS). Cells were derived from either normal epithelium (RPE1), colorectal (HCT116), glioblastoma (U251), or osteosarcoma (U2OS) tumors. We induced DNA damage during mitosis through two independent mechanisms: by exposing cells to 0.5 μmol/L of doxorubicin, a DNA-intercalating agent, or varying doses of ionizing radiation (IR). We then examined anaphase spindles for evidence of chromosome missegregation 25 minutes later. This provides sufficient time for many of the cells that were in mitosis during DNA-damage induction to enter anaphase, but not sufficient time

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for cells that were in G_2 to proceed through to anaphase (12). Exposure to doxorubicin or IR led to a significant increase in anaphase spindles containing lagging chromosomes and in the average number of lagging chromosomes per anaphase spindle, where single kinetochores were attached to microtubules emanating from opposite spindle poles (Fig. 1, Supplementary Fig. S1A). There was no increase in the frequency of chromatin bridges during anaphase (Fig. 1B), and the majority of anaphase spindles exhibited normal bipolar, and not multipolar, geometry (Supplementary Fig. S1B). Furthermore, there was only a slight increase in anaphase spindles containing only acentric chromatin fragments (Fig. 1B and Supplementary Fig. S1C), although our ability to resolve chromatin fragments in spindles that contained >3 lagging chromosomes was limited, as was the case with many cells irradiated with 12 Gy. We thus termed these "combination": cells containing multiple lagging chromosomes as well as chromatin fragments (Fig. 1). Thus, *de novo* induction of DNA damage during mitosis leads to chromosome segregation errors in otherwise normal-appearing mitotic spindles.

DNA Damage Increases k-MT Stability

Multiple mitotic defects can increase the frequency of lagging chromosomes in anaphase, including pathways that perturb spindle geometry, the spindle assembly checkpoint (SAC), sister-chromatid cohesion, and k-MT attachment stability (9). Exposing mitotic cells to IR did not substantially alter pre-anaphase spindle geometry, as evidenced by the paucity of monopolar and multipolar spindles 25 minutes after irradiation (Supplementary Fig. S2A–S2B). To test whether cohesion was perturbed due to DNA damage, we assessed mitotic chromosome spreads after exposure to IR or doxorubicin (Supplementary Fig. S3A–S3C). We first irradiated mitotic cells that were arrested in the presence of nocodazole for 6 hours and examined mitotic chromosome spreads for defects in sister-chromatid cohesion 1 hour later. We found no significant increase in the frequency of mitotic spreads with uncohesed sister chromatids between irradiated and control mitotic cells (Supplementary Fig. S3A and S3C). We also examined sister-chromatid cohesion in mitotic cells that were arrested in nocodazole for up to 6 hours after being exposed to either nocodazole alone or doxorubicin with nocodazole and found no disparity in sister-chromatid cohesion upon doxorubicin exposure (Supplementary Fig. S3B). To examine the effect of DNA damage on the ability of cells to maintain SAC signaling, we again exposed mitotic cells, arrested in the presence of nocodazole for 3 hours, to varying doses of IR and counted the mitotic index 1 hour later. All cell lines exhibited equivalent mitotic index when exposed to 0 Gy or 12 Gy of IR (Supplementary Fig. S4A). We then obtained 5×10^4 mitotic cells using mitotic shakeoff 1 hour after treatment with either nocodazole alone or nocodazole and doxorubicin (Supplementary Fig. S4B) and assessed the number of mitotic cells that were able to maintain SAC signaling when further challenged with nocodazole alone for up to 6 hours, and found no difference between cells that were exposed to only nocodazole and those exposed to nocodazole and doxorubicin (Supplementary Fig. S4B). As a control, when nocodazole-arrested nonirradiated mitotic cells were placed in a medium devoid of nocodazole, they satisfied the SAC and rapidly exited mitosis (Supplementary Fig. S4B). Collectively, these data show that induction of DNA damage during mitosis does not significantly alter spindle geometry, sister-chromatid cohesion, or the ability of cells to maintain SAC signaling. Thus, these mechanisms are unlikely to account for the observed increase in lagging chromosomes.

To test whether k-MT attachment stability changes in response to mitotic DNA damage, we exposed RPE1 cells expressing photoactivatable GFP-tubulin to doxorubicin or 12 Gy of IR. We then photoactivated a linear region on the mitotic spindle and quantified the rate of fluorescence dissipation of the photoactivated region as previously described (13). Control and irradiated cells were treated in 5 μmol/L of MG132 to prevent anaphase onset, which by itself did not alter k-MT attachment stability (14). Quantitative measurements of fluorescence decay fit a double-exponential curve ($r^2 > 0.99$), where slow-decaying fluorescence corresponded to the more stable k-MT population and fast-decaying fluorescence corresponded to the less stable, non–kinetochore bound, spindle microtubules (Fig. 2A–B). Interestingly, the half-life of k-MT fluorescence in metaphase spindles was significantly increased when mitotic cells were exposed to doxorubicin (5.6 ± 0.4 and 6.19 ± 0.4 minutes for 0.5 and 2 μmol/L concentrations, respectively) or 12 Gy of IR (6.0 ± 0.6 minutes) compared with control cells (3.8 ± 0.2 minutes), corresponding to an approximately 50% to 60% rise in k-MT stability (Fig. 2C). Prometaphase cells exhibited a similar increase in k-MT stability when exposed to doxorubicin (Fig. 2C). Neither doxorubicin nor IR influenced the fraction of stable spindle microtubules (not shown) or the stability of the population of microtubules not attached to kinetochores (Supplementary Fig. S5A). Furthermore, exposure of U2OS cells to doxorubicin also led to an increase in k-MT stability, and this effect was dose dependent (Supplementary Fig. S5B). In addition, irradiated metaphase spindles exhibited unperturbed poleward microtubule flux (Supplementary Fig. S5C), suggesting that induction of mitotic DNA damage selectively increases the stability of the k-MT attachments without compromising other microtubule-based spindle functions.

To confirm that the generation of lagging chromosomes in response to mitotic DNA damage was mediated by increased k-MT stability, we overexpressed GFP-tagged microtubule-depolymerizing kinesin-13 protein, KIF2B, in RPE1 cells (Fig. 2D). KIF2B localizes to kinetochores and selectively destabilizes k-MT attachments to chromosomes (13). Overexpression of GFP–KIF2B led to a significant, but not complete, reduction in lagging chromosomes in RPE1 cells exposed to 12 Gy (Fig. 2E), suggesting that DNA damage leads to lagging chromosomes, in part, by excessively stabilizing k-MT attachments.

The DDR Links DNA Damage to Chromosome Missegregation

Induction of double-stranded DNA breaks during mitosis leads to a partial DDR through the phosphorylation and activation of CHK2 (8), but not CHK1 (15). We used short-term pharmacologic inhibition of the CHK2 arm of the DDR pathway and measured k-MT stability shortly after the induction of DNA damage. KU55933, an inhibitor of activated ATM kinase (16), completely abolished the effects of doxorubicin on k-MT stability in metaphase cells (Fig. 3A, with controls

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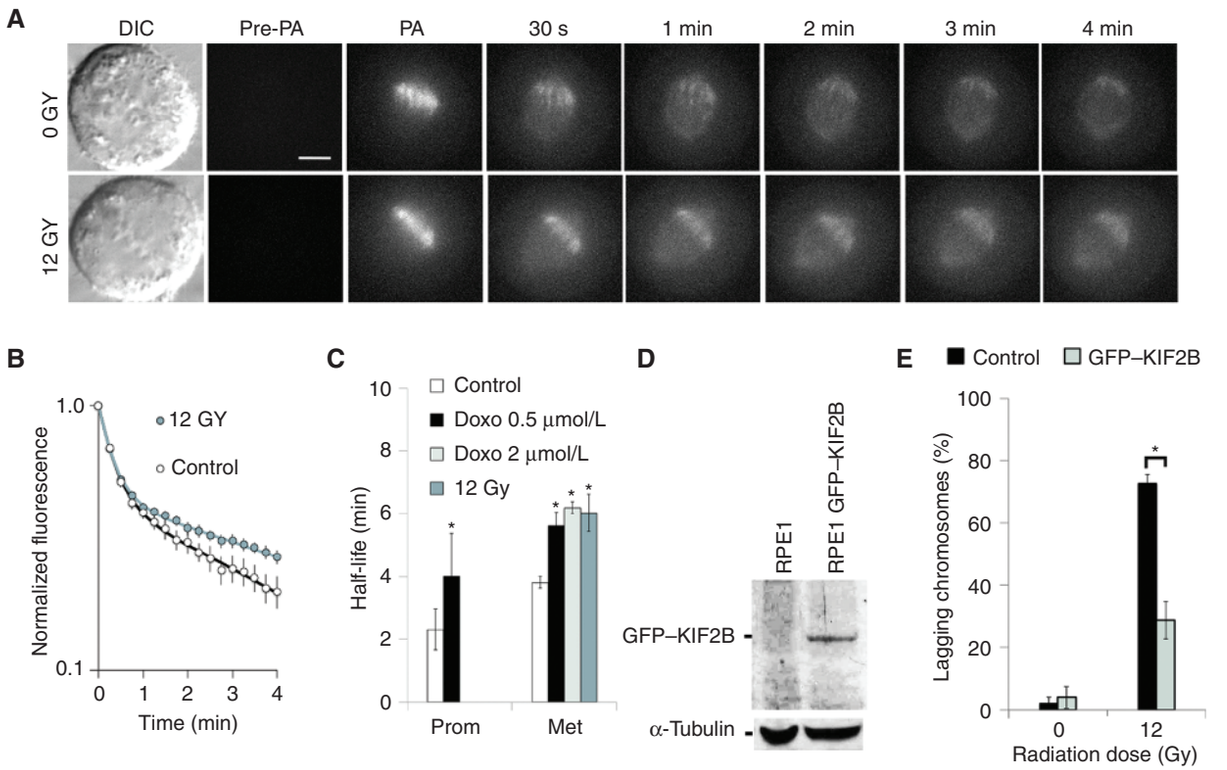

Figure 2. DDR pathway selectively increases k-MT stability during mitosis. **A,** examples of differential interference contrast (DIC) and time-lapse fluorescence images of spindles of RPE1 cells before (Pre-PA) and at the indicated times after activation (PA) of GFP–tubulin fluorescence. Scale bar, 5 μm. **B,** normalized fluorescence intensity over time after photoactivating RPE1 metaphase spindles. Data points, mean ± SE; $n \geq 10$ cells, $r^2 > 0.99$ for all conditions. **C,** half-life of kinetochore-associated microtubules (k-MTs) in RPE1 cells in prometaphase (Prom) and metaphase (Met) cells exposed to doxorubicin (doxo) or 12 Gy. Bars, mean ± SE; *, $P < 0.01$, two-tailed *t* test. **D,** immunoblots of RPE1 cells expressing GFP and GFP–KIF2B, stained using anti-GFP antibodies. DM1-α antibody was used to blot for α-tubulin as a loading control. **E,** percentage of anaphase spindles containing lagging chromosomes as a function of IR dose in control RPE1 cells and cells expressing GFP–KIF2B. Bars, mean ± SEM; $n = 150$ cells, three experiments; *, $P < 0.05$, two-tailed *t* test.

depicted in Fig. 2C). To confirm that this effect is mediated by DDR-specific activity of ATM, we used 2-arylbenzimadazole, a selective inhibitor of activated CHK2 kinase (17), which similarly abolished any increase in k-MT stability in the presence of either doxorubicin or IR (Fig. 3A). These inhibitors were also effective at suppressing lagging chromosomes in the presence of doxorubicin (Fig. 3B).

We then asked whether constitutive activation of the DDR in the absence of DNA damage was sufficient to induce chromosome segregation defects. To this end, we exposed cells to chloroquine, an independent activator of ATM kinase (18). Interestingly, exposure of mitotic cells to chloroquine for 25 minutes was sufficient to induce the formation of lagging chromosomes to levels comparable to mitotic cells exposed to DNA damage (Fig. 3C). Chloroquine did not alter the levels of γ-H2AX in mitotic cells compared with control cells, as evidenced by immunofluorescence and immunoblotting (Fig. 3D–E). However, it led to a significant increase in phosphorylated (p) Chk2-S19 levels in mitotic cells (Fig. 3E), indicating that it can lead to DDR activation without causing DNA damage in mitotic cells. This chloroquine-induced phenotype was significantly suppressed with the CHK2 inhibitor 2-arylbenzimadazole (Fig. 3C), confirming its specificity to the DDR pathway. Furthermore, chloroquine exposure did not increase the frequencies of either acentric chromatin fragments or chromatin bridges (Fig. 3C), thus influencing only the process of whole-chromosome segregation.

We then genetically abrogated the DDR through two independent means. First, we depleted cells of the CHK2 protein, using siRNA, and found an observable increase in both lagging chromosomes and chromatin bridges during anaphase, as previously reported (19). However, we observed no further increase in chromosome missegregation when cells were exposed to doxorubicin during mitosis (Supplementary Fig. S6A–S6B). We then used AT22IJE-T human fibroblasts containing the Ataxia-Telangiectasia–disrupting frameshift mutation at codon 762 of the ATM gene, rendering it highly unstable (20). These cells failed to exhibit an increase in lagging chromosomes in the presence of doxorubicin or chloroquine. Interestingly, expression of FLAG-tagged wild-type recombinant human ATM (20) in AT22IJE-T fibroblasts rescued this phenotype, as there was a >3-fold increase in cells containing lagging chromosomes upon the addition of chloroquine or doxorubicin (Supplementary Fig. S6C). Collectively, these results suggest that the DDR during mitosis induces errors in whole-chromosome missegregation by excessively stabilizing k-MT attachments.

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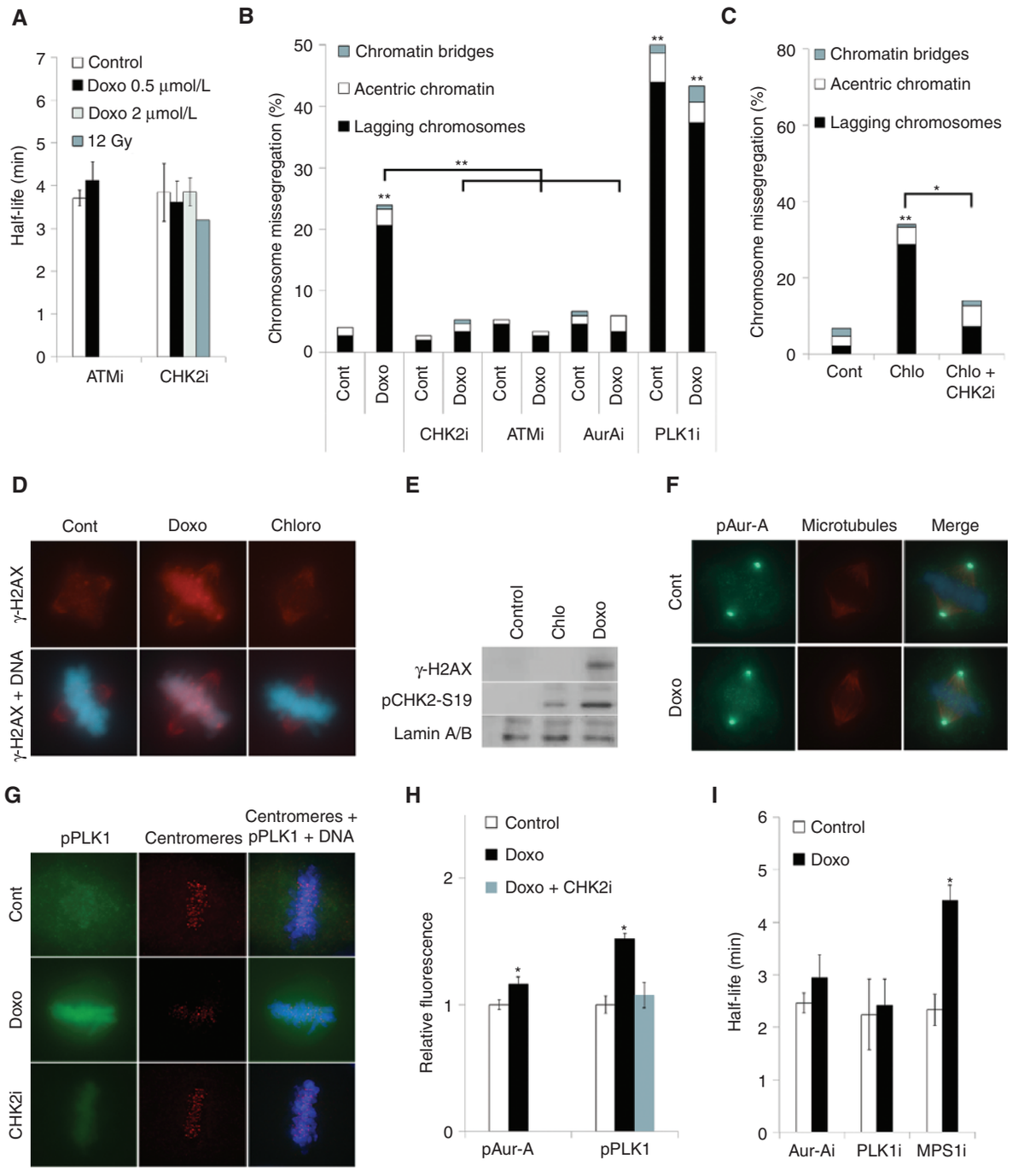

Figure 3. DDR mediates chromosome segregation errors in response to DNA damage. **A,** half-life of k-MTs in metaphase RPE1 cells and cells exposed to doxorubicin (doxo) or to 12 Gy of IR in the presence of either CHK2 inhibitor (i) or ATM inhibitor. Control for **A** is depicted in Fig. 2C. Bars, mean ± SE. **B,** chromosome segregation defects in control RPE1 cells (cont) and cells exposed to doxorubicin in the presence of ATM, CHK2, Aurora-A, or PLK1 inhibitors. Bars, mean; $n = 150$ cells, three experiments; **, $P < 0.01$. Error bars were omitted for clarity. **C,** chromosome missegregation in control RPE1 cells and cells exposed to chloroquine (chlo) or chloroquine and the CHK2 inhibitor. Bars, mean; $n = 150$ cells, three experiments; *, $P < 0.05$; **, $P < 0.01$. **D,** RPE1 cells stained for γ-H2AX, and DNA in the presence of doxorubicin or chloroquine. **E,** immunoblots of control RPE1 cells and cells exposed to doxorubicin or chloroquine stained using anti–γ-H2AX and anti–pCHK2-S19 antibodies. Lamin A/B (L A/B) antibody was used as a loading control. **F** and **G,** RPE1 cells stained for pAurora-A and pPLK1 in the presence of doxorubicin or doxorubicin and the CHK2 inhibitor. **H,** relative fluorescence intensity of pAurora-A and pPLK1 in mitotic RPE1 cells exposed to doxorubicin or doxorubicin and the CHK2 inhibitor. Bars, mean ± SEM; $n = 20$ cells, three experiments; *, $P < 0.05$. **I,** half-life of k-MTs in RPE1 cells and cells exposed to doxorubicin in the presence of either Aurora-A inhibitor or pPLK1 inhibitor. Control for **I** is depicted in Fig. 2C. Bars, mean ± SE; $n > 10$ cells. *, $P < 0.05$.

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The DDR Acts through Aurora-A and PLK1 Kinases

Mitotic entry following recovery from DNA damage requires activation of PLK1 by Aurora-A (2). Aurora-A localizes to the centrosomes where it activates PLK1, which in turn regulates the function of Aurora-A (21), and both kinases have been shown to modulate k-MT attachments during mitosis (22, 23). We used immunofluorescence microscopy to examine the behavior of these two mitotic kinases in response to DNA damage. There was a 1.5-fold increase in overall levels of pPLK1 and a smaller but significant increase in pAurora-A levels after doxorubicin treatment compared with control cells (Fig. 3F–H). Inhibition of CHK2 did not prevent the localization of pPLK1 to chromosomes; however, it reduced chromosome-associated pPLK1 levels to control levels. This is interesting, given what is known about the regulation of PLK1 by the DDR before the G_2–M checkpoint, and suggests that the wiring of the DDR may differ once cells proceed past the G_2–M checkpoint. To test whether inhibition of Aurora-A or PLK1 alters the effect of the DDR on k-MT attachments, we measured k-MT attachment stability following inhibition of Aurora-A and PLK1 with MLN8237 and BI2526, respectively (22). Inhibition of Aurora-A caused an approximately 30% decrease in k-MT attachment stability relative to untreated cells in metaphase (compare Fig. 3I with Fig. 2C), as expected from its role in establishing k-MT attachments (23). However, there were no differences in k-MT stability between cells treated with the Aurora-A inhibitor alone or with both the Aurora-A inhibitor and doxorubicin (Fig. 3I). Accordingly, inhibition of Aurora-A suppressed the rise in lagging chromosomes in cells exposed to doxorubicin during mitosis (Fig. 3B). Inhibition of PLK1 caused monopolar spindles and decreased the percentage of microtubules in the stable population (22). However, the half-lives of k-MT between cells treated with the PLK1 inhibitor alone or with the PLK1 inhibitor and doxorubicin were equivalent (Fig. 3I). The few cells that escaped mitotic arrest from short-term PLK1 inhibition and proceeded to anaphase exhibited a substantial increase in lagging chromosomes; however, no further increase was found upon the addition of doxorubicin (Fig. 3B). Finally, inhibition of another DDR-responsive k-MT–regulating mitotic kinase, MPS1, with reversine (24) did not prevent the stabilization of k-MT attachments induced by doxorubicin (Fig. 3I). We then tested the effect of DNA-damage induction on Aurora-B kinase and its kinetochore substrates. We found that 12 Gy of IR influenced neither the centromeric localization of Aurora-B nor the levels of Aurora-B substrates HEC1 and phospho-histone H3. Yet, there was an increase in the levels of phospho–Centromere protein A (pCENP-A), which is also phosphorylated by Aurora-A kinase (Supplementary Fig. S7A–S7G). Collectively, these results suggest that the DDR signals through Aurora-A and PLK1 to increase k-MT stability in response to mitotic DNA damage.

Inhibition of the DDR Suppresses Chromosome Segregation Defects in Cancer Cells

We asked whether inhibition of the DDR could alter w-CIN in cell lines that naturally exhibit DNA damage during mitosis, a feature particularly prominent in human colorectal cancers. We pharmacologically inhibited CHK2 in RPE1 cells and in cells derived from colorectal (HCT116, HT29, SW480, and SW620), cervical (HeLa), brain (U251 and U87), and bone (U2OS) human cancers and assessed chromosome missegregation 25 minutes later. Strikingly, CHK2 inhibition during mitosis led to significant suppression of inherent lagging chromosomes in five of nine cell lines surveyed (U2OS, U87, HT29, SW480, and SW620), but it did not influence the frequencies of chromatin bridges or acentric chromatin fragments (Fig. 4A). Many of the assayed cell lines exhibited elevated levels of γ-H2AX, as well as phosphorylated CHK2, compared with chromosomally stable RPE1 cells, as assessed by semi-quantitative immunofluorescence (Fig. 4B). This suggests that they exhibit some level of DNA damage during mitosis with associated activation of the DDR. Interestingly, the extent to which CHK2 inhibition suppressed lagging chromosomes was directly proportional to the relative fluorescence of γ-H2AX during mitosis (Fig. 4C). This semi-quantitative correlation indicates that CHK2 inhibition suppresses whole-chromosome missegregation associated with w-CIN discriminately in cells with elevated levels of DNA damage during mitosis.

DISCUSSION

Our work uncovers an unexpected consequence of partial DDR activation during mitosis, namely, the collateral stabilization of k-MTs leading to whole-chromosome missegregation (Fig. 4D). It has long been known that premitotic DNA damage propagates s-CIN by generating chromatin bridges and acentric chromatin fragments during anaphase (11). However, it remained unclear whether (and how) DNA damage in mitosis can lead to w-CIN. Here, we showed that the activation of ATM and CHK2 in response to DNA damage during mitosis leads to excessive stabilization of k-MT attachments through Aurora-A and PLK1 kinases, prompting the generation of lagging chromosomes during anaphase (Fig. 4D). Suppression of k-MT stability, however, did not fully restore chromosome missegregation (Fig. 2E), suggesting other potential mechanisms such as damage to centromeric chromatin or other spindle function. Our data also concur with prior studies showing that unlike short-term pharmacologic inhibition of ATM and CHK2, their genetic depletion induces chromosome missegregation during mitosis in the absence of DNA damage (19). Yet, in these cells, DNA damage during mitosis fails to further increase chromosome missegregation, suggesting that the enzymatic activities of ATM and CHK2 during mitosis are required to link DNA damage to whole-chromosome missegregation. Genetic depletion differs from pharmacologic inhibition in that it abrogates the enzymatic and nonenzymatic activities of DDR proteins and depletes cells of ATM and CHK2 before mitosis. Thus, the observed increase in chromosome segregation in ATM and CHK2-depleted cells not exposed to DNA damage may be attributed to defects leading up to mitosis that interfere with the establishment of a functional mitotic spindle.

Many tumor types simultaneously exhibit s-CIN and w-CIN. w-CIN can generate micronuclei, predisposing chromosomes to pulverization (s-CIN; ref. 25). This damage persists until the subsequent mitosis. We propose that this would in turn generate whole-chromosome missegregation in a codependent relationship whereby w-CIN generates s-CIN, which subsequently leads to w-CIN. This feed-forward

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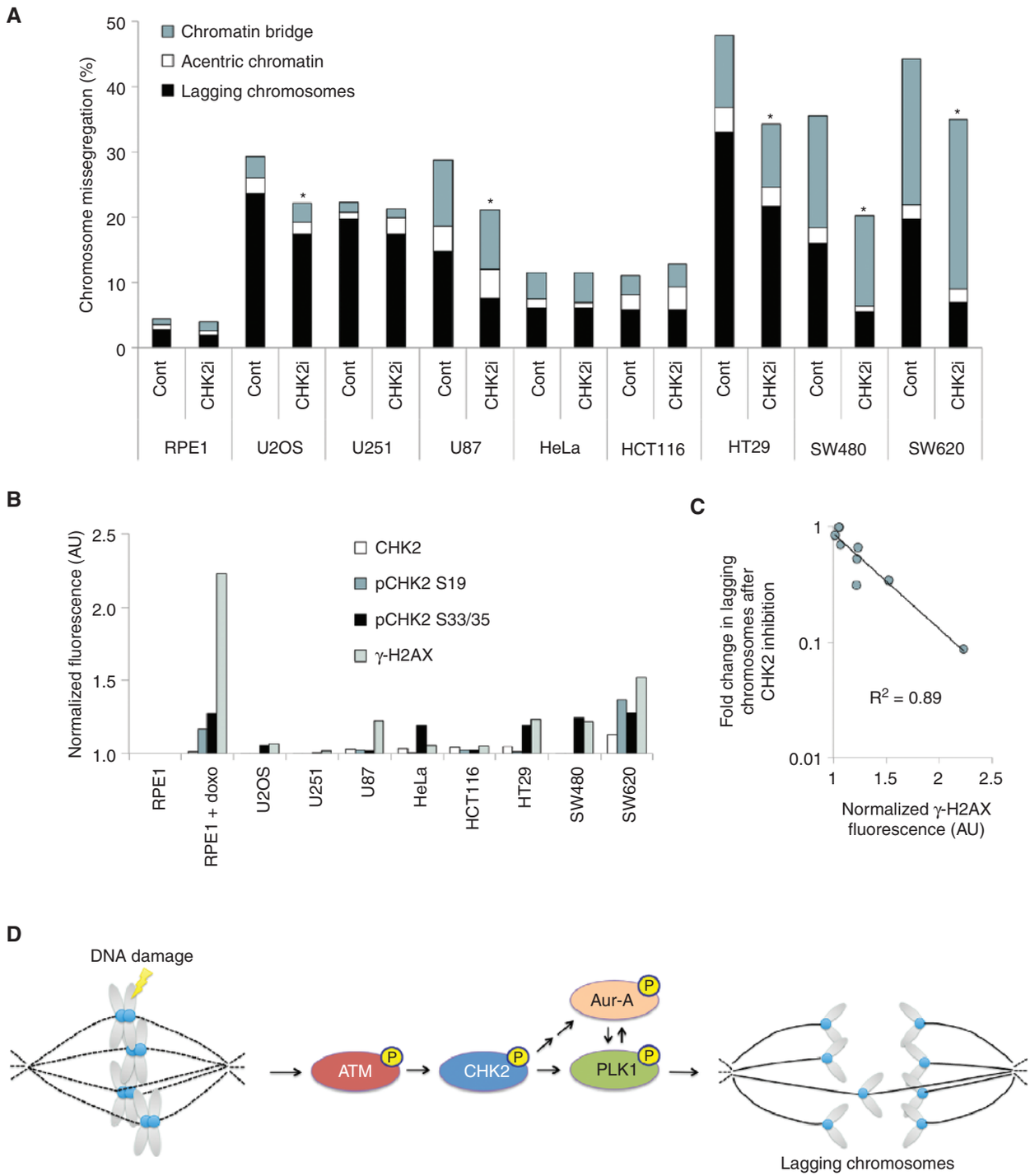

Figure 4. Inhibition of the DDR pathway suppresses chromosome missegregation in cancer cells. **A,** chromosome missegregation frequencies in a panel of cell lines derived from normal epithelium (RPE1), bone (U2OS), brain (U251 and U87), ovarian (HeLa), and colorectal (HCT116, HT29, SW480, and SW620) cancers with and without the CHK2 kinase inhibitor. Bars, mean; $n = 150$ cells, three experiments; *, $P < 0.01$. **B,** fluorescence intensity of CHK2, pCHK2 S19, pCHK2 S33/35, and γ-H2AX during mitosis in different cancer cell lines normalized to RPE1 cells. AU, arbitrary units. Bars, mean; $n = 150$ cells, three experiments; *, $P < 0.01$. **C,** fold change in frequencies of lagging chromosomes after CHK2 inhibition as a function of γ-H2AX during mitosis. **D,** schematic pathway linking DNA damage during mitosis to the formation of chromosome missegregation.

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relationship provides one explanation for the frequent co-occurrence of s-CIN and w-CIN in cancer and the self-propagating nature of chromosomal instability.

METHODS

Cell Culture and Irradiation

Cells were maintained at 37°C in a 5% CO_2 in DMEM or McCoy's medium (HCT116) with 10% FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin and in 0.5 to 1.0 mg/mL of G418 (geneticin) for plasmid selection. U251 cells were obtained as a gift from the Israel Lab (Dartmouth College, Hanover, NH). AT22IJE-T and FLAG-ATM AT22IJE-T were kindly provided by Alan Eastman (Dartmouth College, Hanover, NH). No authentication was done by the authors. Cells were γ-irradiated with ^{137}Cs-irradiator (2.38 Gy/min) or external beam radiation (6 MeV) delivered by a linear accelerator.

Inhibitors and Small Molecules

The following were used: 2-arylbenzimadazole (5 μmol/L; Alan Eastman, Dartmouth College, Hanover, NH), KU-55933 (10 μmol/L; Santa Cruz Biotechnology), MLN8054 (500 nmol/L; Selleckchem), BI2536 (100 nmol/L; Selleckchem), reversine (5 μmol/L), and chloroquine (31 μg/mL; T.Y. Chang, Dartmouth College, Hanover, NH).

Antibodies

Tubulin-specific DM1α (Sigma-Aldrich), anti-centromere (CREST; Dartmouth College, Hanover, NH), HEC1/NDC80-specific (Novus Biologicals), anti–γ-H2AX (Novus Biologicals), GFP-specific (William Wickner, Dartmouth College, Hanover, NH), anti-pCHK2 S19 and S33/35 (Cell Signaling Technology), anti-CHK2 (Cell Signaling Technology), anti–pPLK1-Thr210 (Cell Signaling Technology), anti–p-Aurora-A antibodies were used at dilutions of 1:1,000 (1:10,000 for anti-GFP antibody).

Immunoblots

Membranes were blocked with 0.5% milk-TBS with 0.1% Tween 20, and then blotted at 4°C overnight with antibodies at 1:1,000. Secondary HRP-conjugated anti-mouse/rabbit (Bio-Rad) antibodies were used at 1:3,000.

Immunofluorescence Imaging

Cells were fixed with 3.5% paraformaldehyde or methanol (−20°C) for 15 minutes, as previously described (14). Images were acquired with an Orca-ER Hamamatsu cooled CCD camera mounted on an Eclipse TE2000-E Nikon microscope. Image acquisition and iterative restoration were performed using Phylum-Live software (Improvision), as previously described (14). For phospho-antibodies, wash buffers were supplemented with 80 nmol/L okadaic acid and 40 nmol/L microcystin.

Photoactivation and Measurement of k-MT Stability

Images were acquired using the Quorum WaveFX-X1 spinning-disk confocal system (Quorum Technologies) equipped with a Mosaic digital mirror for photoactivation (Andor Technology) and a Hamamatsu ImageEM camera, as previously described (14).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.F. Bakhoum, L. Kabeche, J.P. Murnane, D.A. Compton
Development of methodology: S.F. Bakhoum, L. Kabeche
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.F. Bakhoum, L. Kabeche, B.I. Zaki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.F. Bakhoum, L. Kabeche, D.A. Compton
Writing, review, and/or revision of the manuscript: S.F. Bakhoum, L. Kabeche, B.I. Zaki, D.A. Compton
Study supervision: S.F. Bakhoum, D.A. Compton

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