

Role of ATM and the Damage Response Mediator Proteins 53BP1 and MDC1 in the Maintenance of G₂/M Checkpoint Arrest^{∇†}

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ATM-dependent initiation of the radiation-induced G₂/M checkpoint arrest is well established. Recent results have shown that the majority of DNA double-strand breaks (DSBs) in G₂ phase are repaired by DNA nonhomologous end joining (NHEJ), while ~15% of DSBs are slowly repaired by homologous recombination. Here, we evaluate how the G₂/M checkpoint is maintained in irradiated G₂ cells, in light of our current understanding of G₂ phase DSB repair. We show that ATM-dependent resection at a subset of DSBs leads to ATR-dependent Chk1 activation. ATR-Seckel syndrome cells, which fail to efficiently activate Chk1, and small interfering RNA (siRNA) Chk1-treated cells show premature mitotic entry. Thus, Chk1 significantly contributes to maintaining checkpoint arrest. Second, sustained ATM signaling to Chk2 contributes, particularly when NHEJ is impaired by XLF deficiency. We also show that cells lacking the mediator proteins 53BP1 and MDC1 initially arrest following radiation doses greater than 3 Gy but are subsequently released prematurely. Thus, 53BP1^{-/-} and MDC1^{-/-} cells manifest a checkpoint defect at high doses. This failure to maintain arrest is due to diminished Chk1 activation and a decreased ability to sustain ATM-Chk2 signaling. The combined repair and checkpoint defects conferred by 53BP1 and MDC1 deficiency act synergistically to enhance chromosome breakage.

DNA double-strand breaks (DSBs) activate the DNA damage response (DDR), a coordinated process that functions to enhance survival and maintain genomic stability. The DDR includes pathways of DSB repair and a signal transduction response that activates apoptosis and cell cycle checkpoint arrest and influences DSB repair (15). DNA nonhomologous end joining (NHEJ) and homologous recombination (HR) represent the major DSB repair mechanisms, NHEJ being the major mechanism in G₀/G₁, while both processes function in G₂ (9, 32). Ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) are related phosphoinositol 3-kinase-like kinases (PIKKs) that regulate the DNA damage signaling response. ATM is activated by DSBs, while ATR is activated at single-strand (ss) regions of DNA via a process that involves ATRIP-replication protein A (RPA)-ssDNA association. Ionizing radiation (IR) induces DSBs, base damage, and ss nicks. Since neither base damage nor ss nicks activate ATR, IR-induced signaling in the G₁ and G₂ phases is predominantly ATM dependent (3, 29). In S phase, ATR can be activated by both endogenous and exogenously induced lesions following replication fork stalling/collapse (8).

Recent work has shown that in G₂ phase, DSBs can undergo resection via an ATM-dependent process generating ssDNA

regions that can activate ATR following RPA association (11). ATR activation at resected DSBs is coupled to loss of ATM activation (11). Although ATM and ATR share overlapping substrates, there is specificity in their signaling to the transducer kinases; ATM uniquely phosphorylates Chk2, while ATR phosphorylates Chk1. Phosphorylation of either Chk1 or Chk2 causes their activation. Critical targets of Chk1/Chk2 are the Cdc25 phosphatases, which regulate the cyclin-dependent kinases (Cdks), including Cdk1, the regulator of mitotic entry (18). Collectively, these studies suggest that two components of ATM-dependent signaling to the G₂/M checkpoint machinery can occur: ATM-Chk2 signaling at unresected DSBs and ATM-ATR-Chk1 signaling at resected DSBs.

Although much is known about the mechanism leading to G₂/M checkpoint activation, few studies have addressed how arrest is maintained and how release coordinates with the status of DSB repair. We examine here the maintenance of checkpoint arrest during the immediate phase of DSB repair. We do not address the issue of checkpoint adaptation, a distinct phenomenon which occurs after prolonged checkpoint arrest (22). Further, we focus on the process maintaining arrest in irradiated G₂-phase cells and do not consider how arrest is maintained in irradiated S-phase cells that progress into G₂ phase. (Previous studies have shown that while G₂/M arrest is ATM dependent at early times post-IR, at later times it becomes ATR dependent as S-phase cells progress into G₂ phase [2, 33].) To focus on mechanisms maintaining ATM-dependent signaling in G₂-phase cells, we use aphidicolin (APH) to prevent S-phase cells from progressing into G₂ during analysis. We, thus, examine checkpoint maintenance in cells irradiated in G₂ phase and do not evaluate arrest regulated by ATR following replication fork stalling. The basis for our work stems from two recent advances. First, we evaluate the impact of

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ATM-mediated ATR activation in the light of recent findings that resection occurs in G₂ phase (11). Second, we consider the finding that NHEJ represents the major DSB repair mechanism in G₂ and that a 15 to 20% subset of DSBs, representing those that are rejoined with slow kinetics in an ATM-dependent manner, undergo resection and repair by HR (3, 25). Thus, contrary to the notion that HR represents the major DSB repair pathway in G₂ phase, it repairs only 15 to 20% of X- or gamma-ray-induced DSBs and represents the slow component of DSB repair in G₂ phase. Given these findings, several potential models for how checkpoint arrest is maintained in G₂ can be envisaged. A simple model is that the initial signal generated by IR is maintained for a defined time to allow for DSB repair. Such a model appears to explain the kinetics of checkpoint signaling in fission yeast after moderate IR (17). In mammalian cells, the duration of arrest depends on dose and DSB repair capacity (6). Thus, it is possible that the status of ongoing repair is communicated to the checkpoint machinery to coordinate timely release with the process of DSB repair. Here, we consider the impact of resection leading to ATM-ATR-Chk1 signaling versus ATM-Chk2 signaling from nonresected DSBs and how they interplay to maintain rather than initiate checkpoint arrest.

Mediator proteins, including 53BP1 and MDC1, assemble at DSBs in an ATM-dependent manner, but their roles in the DDR are unclear. Cells lacking 53BP1 or MDC1 are proficient in checkpoint initiation after moderate IR doses, leading to the suggestion that these proteins are required for amplification of the ATM signal after exposure to low doses but are dispensable after high doses, when a robust signal is generated, even in their absence (7, 16, 28, 31). Despite their apparent subtle role in ATM signaling, cells lacking these mediator proteins display significant genomic instability (19). We thus also examine whether the mediator proteins contribute to the maintenance of checkpoint arrest.

We identify two ATM-dependent processes that contribute to the maintenance of checkpoint arrest in G₂-phase cells: (i) ATR-Chk1 activation at resected DSBs and (ii) a process that involves sustained signaling from ATM to Chk2 at unrepaired DSBs. Further, we show that 53BP1 and MDC1 are required for maintaining checkpoint arrest, even following exposure to high radiation doses due to roles in ATR-Chk1 activation and sustained ATM-Chk2 signaling, and that this contributes to their elevated genomic instability.

MATERIALS AND METHODS

Cell culture, irradiation, and drug treatment. 1BR3 (wild-type [WT]) hTERT, ATR-Seckel (ATR-SS) hTERT, and 2BN (XLF^{-/-}) hTERT are immortalized human fibroblasts from normal, ATR-defective, and XLF-defective individuals, respectively. *MDC1*^{-/-} and *53BP1*^{-/-} mouse embryo fibroblasts (MEFs) were a gift from J. Chen. All fibroblast cells were cultured in minimal essential medium (MEM) or Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LBLs) were cultured in RPMI with 15% FCS. GM2188 and DK0064 are wild-type and ATR-defective Seckel LBLs, respectively. Gamma irradiation was from a ¹³⁷Cs source at a dose rate of 7.5 Gy/min. X-irradiation was carried out at a dose rate of 2 Gy/min. The ATM inhibitor (ATMi) KU55933 and the DNA-PK inhibitor NU7441 were gifts from KuDOS Pharmaceuticals (Cambridge, United Kingdom). A total of 10 μM KU55933 (ATM inhibitor) and/or 10 μM NU7441 (DNA-PK inhibitor) was added at the times indicated. A total of 2.5 μM SB218078 (Chk1/Chk2 inhibitor) was added 30 min post-IR.

siRNA knockdown. Small interfering RNA (siRNA) transfection of A549, 1BR3 (WT) hTERT, and 2BN (XLF^{-/-}) hTERT cells was carried out using HiPerFect (Qiagen, Hilden, Germany). siRNA oligonucleotides against scrambled control, Chk1, Chk2, 53BP1, and XLF were obtained from the Dharmacon SMARTpool siRNA (these oligonucleotides are labeled "#1" in figures). The sequence of siRNA oligonucleotides against Chk1 was 5'-AAU CGU GAG CGU UUG AAC TT-3', and Chk2 was obtained from Qiagen (Hilden, Germany) (these oligonucleotides are labeled #2 in figures).

Antibodies for immunofluorescence and immunoblotting. Procedures used were as described previously (6) using antibodies against γ-H2AX (Upstate Biotechnology, Buckingham, United Kingdom), CENP-F (Santa Cruz, Santa Cruz, CA), pSer 10 histone H3 (Upstate Biotechnology, Buckingham, United Kingdom), Chk2 pThr68 (Cell Signaling Technology, Beverly, MA), Chk2 (Abcam, Cambridge, United Kingdom), Chk1 pSer317 (Abcam, Cambridge, United Kingdom), and β-tubulin (Abcam, Cambridge, United Kingdom). Slides were visualized using a Zeiss Axioplan microscope, and image processing was performed on Simple PCI software. Signal intensity following immunofluorescence (IF) or immunoblotting was analyzed using NIH Image-J. IR-induced intensity was calculated by subtracting the signal in nuclei without damage from that in IR-treated nuclei.

G₂/M checkpoint analysis. For G₂/M checkpoint analysis, exponentially growing cells were irradiated on glass coverslips. Cells were stained with pSer10-histone H3 and DAPI (4',6-diamidino-2-phenylindole), and pSer10-histone H3-positive and condensed chromatin cells were counted as mitotic cells. A total of 3 μM aphidicolin was routinely added to block entry of irradiated S-phase cells into G₂ during analysis.

Analysis of chromosome and chromatid breaks in mitotic cells. Exponentially growing MEFs were irradiated with 3 Gy IR, and colcemid (100 ng/ml) was added after 2 h (Sigma-Aldrich, Poole, United Kingdom). Cells were fixed for metaphase preparation 12 h post-IR using standard protocols. Slides were stained with 3% Giemsa for 3 min. Chromosome spreads were captured using a Zeiss Axioplan2 microscope and Isis software (MetaSystems, Altlußheim, Germany), and chromatid breaks were counted.

Premature chromosome condensation (PCC) analysis. Cells were treated with 50 ng/ml calyculin A (Calbiochem) for 20 to 30 min before harvesting. Chromatid breaks and excess fragments (counted as two chromatid breaks) were scored in 100-chromosome spreads from at least three independent experiments per data point.

RESULTS

Chk1/Chk2 regulates the initiation and maintenance of checkpoint arrest. First, we verified that ATM and Chk1/Chk2 function in initiating checkpoint arrest by adding the ATM inhibitor KU55933 or the Chk1/Chk2 inhibitor SB218078 (here called ATM or Chk1/Chk2 inhibitor, respectively) 30 min prior to exposure to 3 Gy IR in 1BR3 (WT) hTERT cells. Both treatments abolished G₂/M checkpoint arrest at 1 and 2 h post-IR (Fig. 1A), demonstrating that ATM and Chk1/Chk2 are required for checkpoint initiation.

Next, we examined whether Chk1 and Chk2 are required for checkpoint maintenance (the role of ATM is examined below). In trial experiments, we observed that neither p-Chk1 nor p-Chk2 levels showed any further increase 30 min after IR, i.e., maximal levels were reached within the first 30 min (Fig. 1B and C). To assess the combined role of Chk1/Chk2 in checkpoint maintenance, we added the Chk1/Chk2 inhibitor 30 min post-IR (i.e., following maximal Chk1/Chk2 activation). Although arrest was maintained at 1 h post-IR (i.e., 30 min after Chk1/Chk2 inhibitor addition), mitotic entry commences by 2 h (Fig. 1D). This shows that Chk1 and -2 are the major components regulating checkpoint arrest and release rather than any downstream proteins, such as Cdc25. The rapid mitotic entry following Chk1/Chk2 inhibitor addition was subsequently used as a benchmark to monitor factors required for maintaining checkpoint arrest.

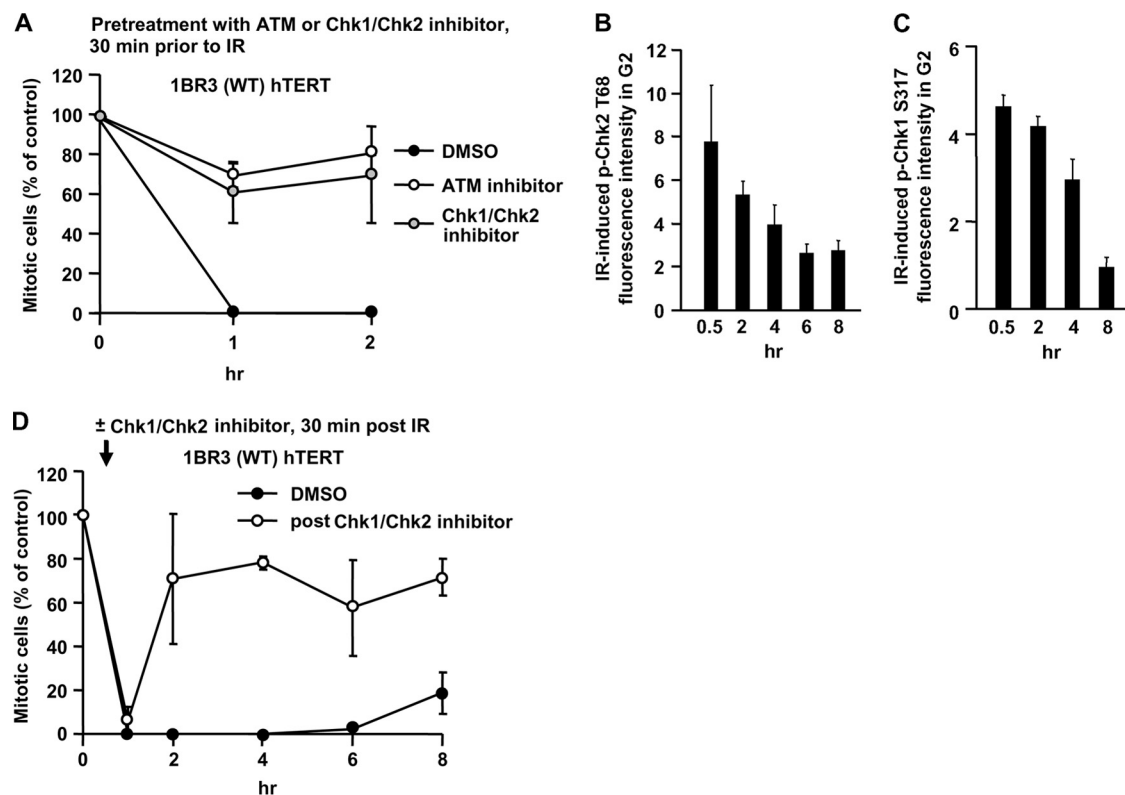


FIG. 1. Chk1/Chk2 regulates the initiation and maintenance of checkpoint arrest. (A) Checkpoint initiation requires ATM and Chk1/Chk2. Mitotic entry was examined in 1BR3 hTERT cells following 3 Gy IR in untreated cells or cells treated with the ATM inhibitor or Chk1/Chk2 inhibitor 30 min prior to IR. Cells, >400, were scored for mitotic index. Results represent the mitotic index relative to untreated cells. Maximal levels of p-Chk1 and p-Chk2 are reached within 30 min after 3 Gy. 1BR hTERT and A549 cells were exposed to 3 Gy IR, and p-Chk2 (B) and p-Chk1 (C) levels assessed by IF at various times post-IR. (D) Checkpoint maintenance requires Chk1/Chk2. The time of mitotic entry following exposure to 3 Gy IR in cells either untreated or treated with the Chk1/Chk2 inhibitor 30 min post-IR was examined. Error bars represent the standard error of the mean (SEM) from 3 experiments.

ATR-Chk1 activation at resected DSBs contributes to checkpoint maintenance. G₂-phase DSBs can undergo ATM-dependent resection, leading to ATR-dependent Chk1 activation and loss of ATM activation (11). We recently observed that, contrary to the notion that HR represents the major DSB repair process in G₂ phase, only 15 to 20% of IR-induced DSBs undergo resection in G₂ phase (3). Therefore, since Chk1 is activated only at a fraction of IR-induced DSBs, we examined whether ATR-Chk1 contributes to IR-induced G₂/M arrest. To examine checkpoint maintenance in irradiated G₂-phase cells and to prevent progression of S-phase cells into G₂ during analysis, we added aphidicolin (APH), an inhibitor of the replicative polymerase. Control experiments showing that APH inhibits progression of S-phase cells into late S/G₂ phase are shown in Fig. S1A in the supplemental material. Additional controls showing that APH does not impact DSB repair in G₂ phase are described in references 3 and 6. Moreover, IR-induced sister chromatid exchanges (SCEs) in G₂ phase, an established marker for HR, are unaffected by APH treatment (see Fig. S1B in the supplemental material). To directly examine the role of Chk1 in G₂/M checkpoint arrest, we used two distinct oligonucleotides for Chk1 siRNA and found that arrest was initiated normally but was not efficiently maintained (Fig. 2A). We also observed that treatment with UCN-01, a Chk1-specific inhibitor at the concentration used, impairs checkpoint

maintenance and does not impact checkpoint initiation (data not shown). We also examined mitotic entry in ATR-Seckel (ATR-SS) hTERT cells, which have impaired ATR activity (21). Strikingly, although ATR-SS hTERT cells activate G₂/M arrest normally following 3 Gy IR, they enter mitosis earlier (4 to 6 h) than control cells (6 to 8 h) (Fig. 2B). We show, as a control, that ATR loss reduces p-Chk1 levels but does not affect resection or p-Chk2 in G₂ using CENP-F to identify G₂ cells and quantifying p-Chk1 and p-Chk2 levels by IF (Fig. 2C). The specificity of the anti-p-Chk1 and anti-p-Chk2 antibodies for IF is shown in Fig. S2A to F in the supplemental material. As a further approach, we used ATR siRNA to deplete ATR in 1BR3 hTERT (WT) and ATR-SS hTERT cells. ATR siRNA-treated control cells showed a pattern of checkpoint arrest and maintenance similar to that observed with ATR-SS cells (Fig. 2B). Further, although ATR siRNA in ATR-SS cells reduced ATR expression levels, the kinetics of checkpoint entry remained similar to that observed with ATR-SS cells, suggesting that residual ATR activity in ATR-SS cells does not appreciably contribute to the arrest observed. Finally, we also employed ATR-SS lymphoblastoid cells (LBLs) for complementation analysis. Like ATR-SS hTERT cells, ATR-SS LBLs initiate checkpoint arrest normally but show premature mitotic entry (Fig. 2D). Importantly, introduction of ATR cDNA into

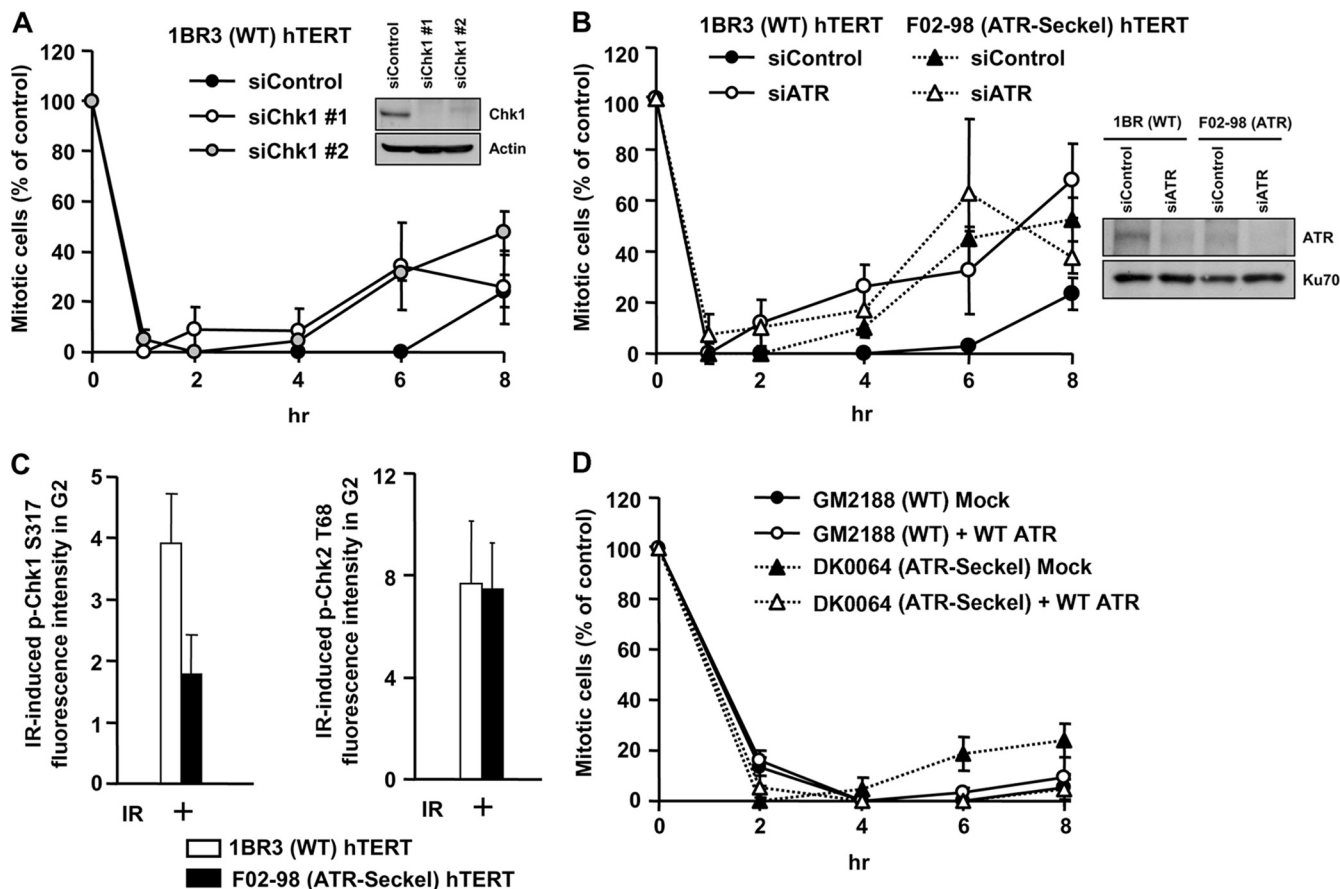


FIG. 2. ATR-Chk1 signaling contributes to checkpoint maintenance. (A) Mitotic entry in 1BR3 hTERT cells following treatment with control and Chk1 siRNA and 3 Gy IR was examined. Chk1 knockdown did not significantly compromise G_2 proportion based on fluorescence-activated cell sorting (FACS) analysis (data not shown). (B) Mitotic entry in 1BR3 hTERT and ATR-SS hTERT cells following treatment with control or ATR siRNA and 3 Gy IR. The right panel shows ATR expression levels. (C) ATR-SS hTERT cells show impaired Chk1 phosphorylation but normal Chk2 phosphorylation. The levels of p-Chk1 Ser317 and p-Chk2 Thr68 were quantified by IF in ATR-SS hTERT G_2 cells at 30 min after IR exposure. (D) Mitotic entry in control and ATR-SS LBL cell lines following mock and ATR cDNA transfection. To verify the impact of Chk1 siRNA on Chk1 activity, we examined hydroxyurea (HU)-induced 53BP1 focus formation, which is a characterized Chk1-dependent response (26). 53BP1 foci failed to form after HU treatment in ATR-SS hTERT cells and following Chk1 siRNA, consistent with previous findings (data not shown) (30). Error bars represent the SEM from 3 experiments.

ATR-SS LBLs conferred prolonged checkpoint arrest similar to that observed with control cells.

Collectively, these findings provide strong evidence that ATR-Chk1 contributes to checkpoint maintenance after 3 Gy IR. They also distinguish the initiation of G_2/M checkpoint arrest, which has either no or a less stringent requirement for ATR-Chk1, from the maintenance of arrest, which is compromised when either ATR or Chk1 activity is impaired. A greater role for ATR-Chk1 in maintaining arrest is consistent with our finding that HR represents the slow component of DSB repair in G_2 phase (3). Thus, while only 15 to 20% of induced DSBs undergo resection and activate Chk1, at late times post-IR, the resected DSBs represent a much higher percentage of the unrepaired DSBs.

Sustained ATM-Chk2 signaling also contributes to the maintenance of checkpoint arrest. Next, we considered the contribution of Chk2 to maintaining G_2/M arrest and examined whether sustained ATM-Chk2 signaling might contribute—i.e., whether unrepaired DSBs might lead to the prolongation of Chk2 activation. To investigate this, we examined the

impact of the ATM inhibitor added 30 min after 3 Gy IR—i.e., when checkpoint arrest had been initiated and maximal phosphorylated Chk1/Chk2 levels had been attained. In control experiments, we show that ATM inhibitor addition 5 or 15 min prior to IR completely inhibits Chk2 phosphorylation and checkpoint arrest (Fig. 3A), indicating that ATM inhibitor addition inhibits ATM activity within 5 min. Following ATM inhibitor addition 30 min post-IR to 1BR3 (WT) hTERT cells, mitotic entry commenced at 4 to 6 h post-IR (Fig. 3B). Thus, mitotic entry occurred earlier than in control (dimethyl sulfoxide [DMSO]-treated) cells (which enter mitosis at 6 to 8 h), implicating ATM signaling not only in initiating the G_2/M checkpoint but also in its full maintenance.

We also examined the impact of ATM inhibitor addition 30 min post-IR on ATR-dependent Chk1 activation. Two hours after ATM inhibitor addition, the levels of RPA foci and p-Chk1 were similar to that of control cells, but by 8 h both were elevated (see Fig. S2G and H in the supplemental material). Thus, maximal resection and Chk1 activation occur within the first 30 min post-IR, but subsequent ATM inhibitor addition

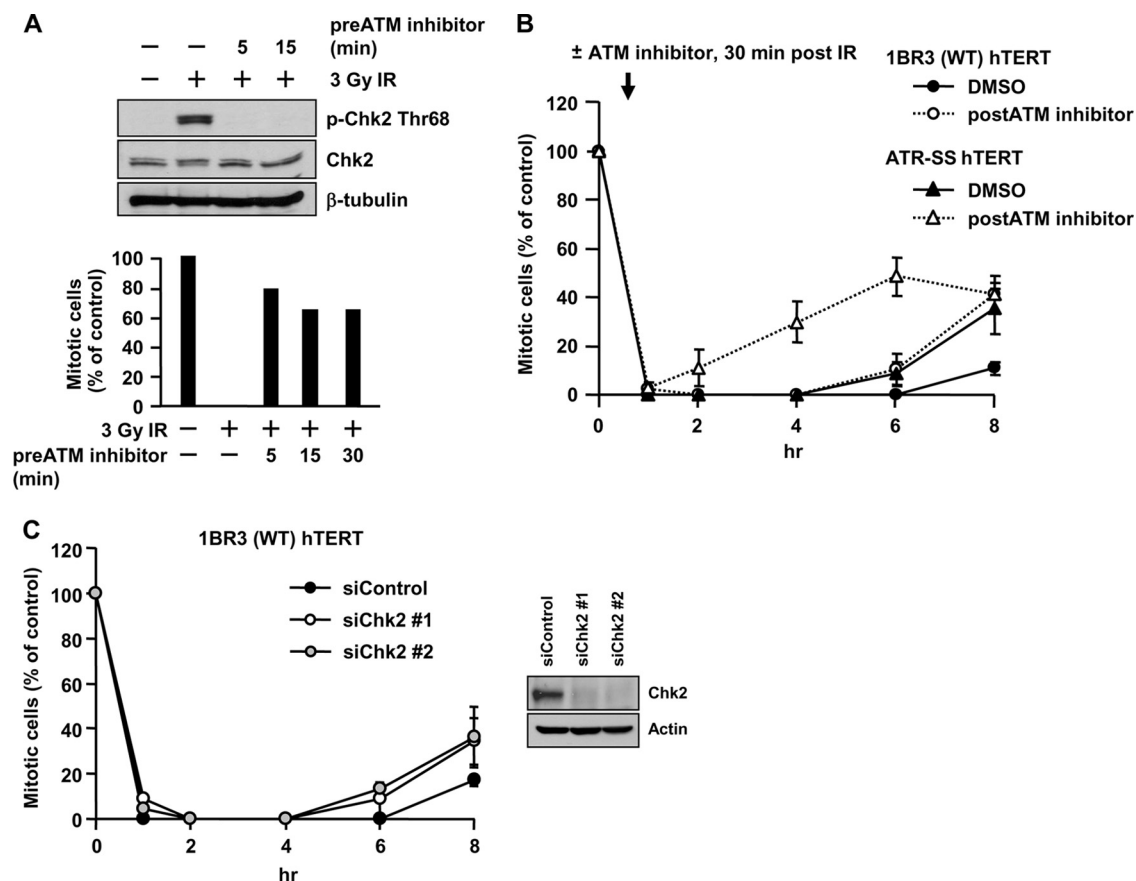


FIG. 3. Sustained ATM signaling also contributes to checkpoint maintenance. (A) ATM inhibitor addition impairs checkpoint arrest and signaling within 5 min. 1BR3 hTERT cells were untreated or treated with ATM inhibitor 5, 15, or 30 min prior to IR. Cells were examined by immunoblotting using anti-Thr68-p-Chk2 at 30 min (top) and for mitotic entry at 1 h post-IR (bottom). Chk2 and β -tubulin antibodies were loading controls. (B) ATM inhibitor addition 30 min post-IR diminishes the duration of checkpoint arrest after 3 Gy IR in 1BR3 hTERT and in ATR-SS hTERT cells. (C) siRNA-Chk2 diminishes the duration of checkpoint arrest after 3 Gy IR. Error bars represent the SEM of 3 experiments.

impairs RPA focus loss. However, despite the elevated level of p-Chk1 at 8 h, ATM inhibitor-treated cells were released prematurely from checkpoint arrest (Fig. 3B). This strongly implies that Chk2 contributes to checkpoint maintenance and that elevated Chk1 activity cannot fully substitute. As expected, ATM inhibitor addition to ATR-SS hTERT cells resulted in very early checkpoint release (at 2 to 4 h) (Fig. 3B) since this reduces ATR-Chk1 and ATM-Chk2 signaling.

We further examined the impact of Chk2 using Chk2 siRNA. Following 3 Gy IR, checkpoint arrest was initiated normally but released prematurely compared to control cells (Fig. 3C), suggesting that there is redundancy between Chk1 and Chk2 in checkpoint initiation, but both Chk1 and Chk2 contribute to its maintenance. In summary, these findings provide preliminary evidence that sustained ATM signaling to Chk2 represents an additional process that contributes to checkpoint maintenance.

Sustained ATM signaling enhances the duration of checkpoint arrest by maintaining p-Chk2 levels in control and, more strikingly, in NHEJ-defective cells. We reasoned that sustained ATM signaling might be optimally examined with a cell line in which elevated levels of unrepaired and nonresected DSBs persist. We examined 2BN hTERT cells, which are de-

fective in the NHEJ factor XLF (1, 4, 5). 2BN (XLF^{-/-}) hTERT cells show elevated calyculin-induced premature chromosome condensation (PCC), a procedure that monitors DSB repair in G₂ phase (Fig. 4A). Thus, 2BN (XLF^{-/-}) hTERT cells show substantially reduced DSB rejoining in G₂ phase, consistent with the notion that NHEJ is the major DSB repair process in G₁ and G₂. Further, 2BN (XLF^{-/-}) hTERT cells, unlike Ku-defective cells, do not show significantly increased RPA or Rad51 foci at 2 to 4 h post-IR, suggesting that they do not undergo excessively high resection (data not shown).

First, we demonstrated that ATM or Chk1/Chk2 inhibitor addition prior to IR abolished checkpoint arrest in 2BN (XLF^{-/-}) hTERT cells (Fig. 4B). Next, we examined the duration of checkpoint arrest in 2BN (XLF^{-/-}) hTERT cells. Following 3 Gy IR, 1BR3 (WT) hTERT cells enter mitosis at ~8 h, while 2BN (XLF^{-/-}) hTERT cells arrest for >12 h (Fig. 4C). 2BN (XLF^{-/-}) hTERT cells exposed to 6 or 9 Gy IR arrest for >24 h (data not shown). Given the characterized role of XLF in DSB repair, these findings show that the duration of checkpoint arrest is dependent upon dose and DSB repair capacity, indicating that unrepaired DSBs cause prolonged arrest. Thus, the status of DSB repair is continuously monitored and communicated to the checkpoint machinery. We next

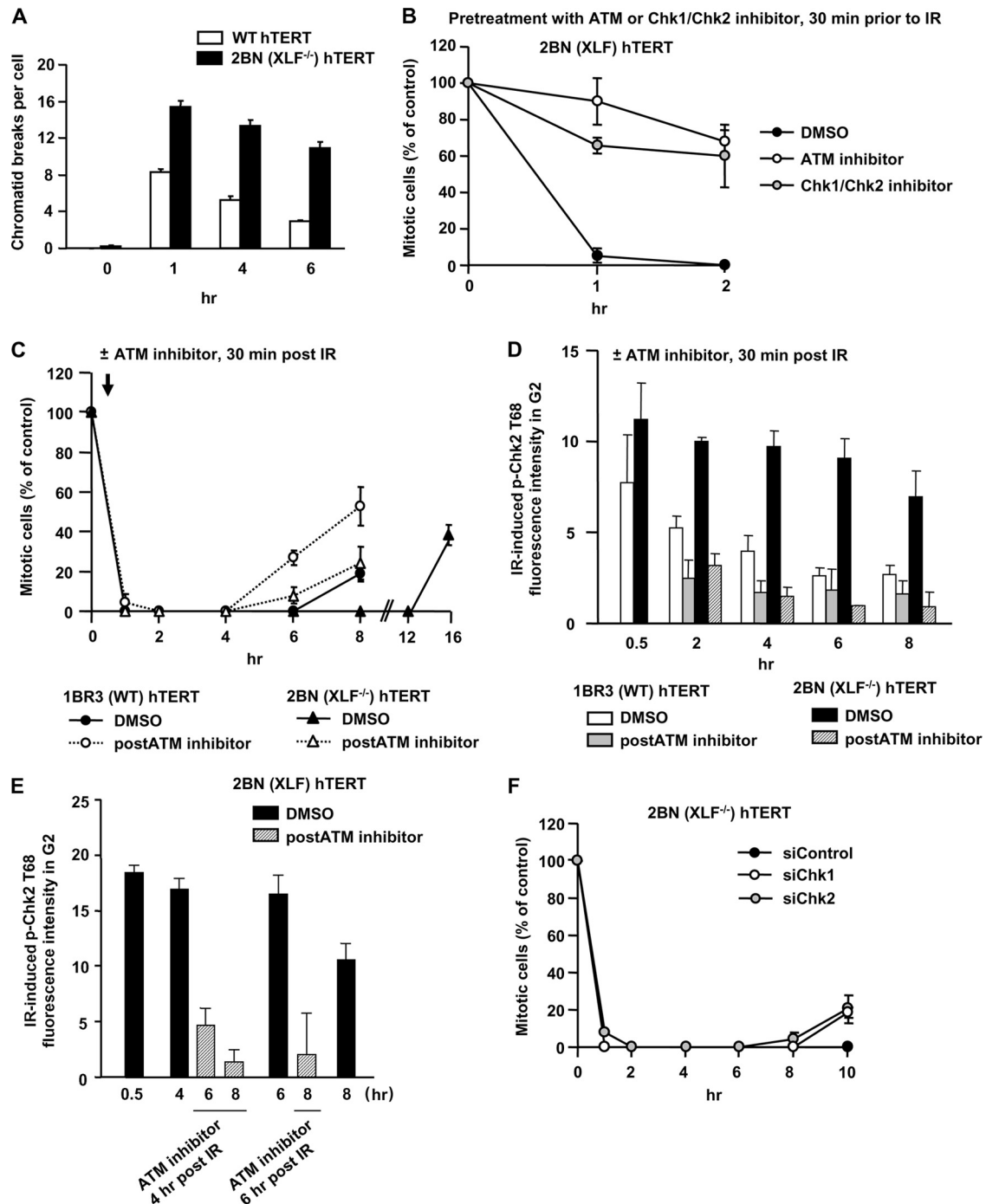


FIG. 4. Sustained ATM signaling contributes to prolonged checkpoint arrest in NHEJ-defective cells. (A) Cells were exposed to 1 Gy IR, and APH was added. A total of 50 ng/ml calyculin A was added 30 min before fixation, and PCCs were scored. (B) ATM and Chk1/Chk2 are required for checkpoint initiation in 2BN (XLF^{-/-}) hTERT cells. Mitotic entry was examined following 3 Gy IR in 2BN (XLF^{-/-}) hTERT cells either untreated or treated with the ATM or Chk1/Chk2 inhibitor 30 min prior to IR. (C) Prolonged checkpoint arrest in 2BN (XLF^{-/-}) hTERT cells requires sustained ATM signaling. Mitotic entry after 3 Gy IR was examined in 1BR3 and 2BN (XLF^{-/-}) hTERT cells either with or without ATM inhibitor 30 min post-IR. (D) 1BR3 and 2BN (XLF^{-/-}) hTERT cells were exposed to 3 Gy IR with or without ATM inhibitor addition 30 min post-IR. p-Chk2 levels were quantified in CENP-F⁺ (G₂) cells by IF using anti-Thr68-p-Chk2 antibodies. The background signal in unirradiated cells was subtracted. At <1 h post-IR, 2BN (XLF^{-/-}) hTERT cells harbor elevated DSBs compared to 1BR3 hTERT cells (as shown in panel A), consistent with their elevated p-Chk2 signal. By 8 h the p-Chk2 level decreased by >50% in 1BR3 hTERT cells compared with 20% in 2BN (XLF^{-/-}) hTERT cells. (E) 2BN (XLF^{-/-}) hTERT cells were exposed to 3 Gy IR with or without ATM inhibitor addition 4 or 6 h post-IR. p-Chk2 levels were quantified in CENP-F⁺ (G₂) cells by IF using anti-Thr68-p-Chk2 antibodies. (F) The maintenance of checkpoint arrest in 2BN (XLF^{-/-}) hTERT cells requires Chk1 and Chk2. Mitotic entry was examined in 2BN (XLF^{-/-}) hTERT cells treated with control, Chk1, or Chk2 siRNA. Error bars represent the SEM of 3 experiments.

added ATM inhibitor 30 min post-IR to 2BN (XLF^{-/-}) hTERT cells and observed premature release at 6 to 8 h (Fig. 4C), demonstrating that sustained ATM signaling plays a significant role in maintaining arrest in a repair-defective background.

The process of sustained ATM signaling to Chk2, although arguably expected, has not been examined previously. Therefore, we determined whether sustained ATM signaling maintains p-Chk2 levels. We examined p-Chk2 levels in G₂-phase cells since Chk2 activation might differ in S phase and since G₁-phase cells do not undergo detectable resection (11). We achieved this by quantifying p-Chk2 by IF in G₂ cells identified by CENP-F staining. 1BR3 (WT) hTERT cells were irradiated with 3 Gy IR, and ATM inhibitor was added 30 min post-IR. We observed elevated p-Chk2 following IR, which by 2 and 4 h had decayed to a greater extent in the presence of ATM inhibitor (Fig. 4D). At later times the assay was too insensitive to reliably assess p-Chk2 levels in WT cells. Nonetheless, the results demonstrate that ATM inhibitor addition after initial Chk2 activation results in diminished p-Chk2 levels, confirming that sustained ATM-to-Chk2 signaling helps to maintain p-Chk2 levels.

As anticipated, p-Chk2 levels remain elevated in 2BN (XLF^{-/-}) hTERT compared to control cells, reflecting sustained signaling from the elevated level of unrepaired DSBs (Fig. 4D). Addition of ATM inhibitor at 30 min post-IR to 2BN (XLF^{-/-}) hTERT cells resulted in dramatically decreased p-Chk2 levels. These findings provide strong evidence that sustained ATM signaling maintains p-Chk2 in control cells and, more strikingly, in an NHEJ-deficient background. The level of p-Chk2 at 30 min post-IR was greater in 2BN (XLF^{-/-}) hTERT compared to control cells, which we attribute to XLF-dependent DSB repair during the first 30 min post-IR. To verify that the sustained p-Chk2 levels are not a consequence of the level of initially activated Chk2, we treated 2BN (XLF^{-/-}) hTERT cells with ATM inhibitor at 4 or 6 h post-IR (Fig. 4E). p-Chk2 was dramatically reduced 2 h later in stark contrast to its maintenance in the absence of ATM inhibitor, demonstrating that p-Chk2 is lost rapidly when ATM signaling is abrogated.

Finally, to verify that p-Chk1 and p-Chk2 contribute to the maintenance of checkpoint arrest in a repair-deficient background, we subjected 2BN (XLF^{-/-}) hTERT cells to Chk1 or Chk2 siRNA treatment and observed premature release compared to control siRNA treatment (Fig. 4F). We conclude that sustained ATM signaling to Chk2 represents a second process that maintains G₂/M checkpoint arrest.

53BP1^{-/-} and MDC1^{-/-} MEFs show premature release from checkpoint arrest. 53BP1 has been reported to amplify ATM signaling, a suggestion based on the finding that it is required for the initiation of checkpoint arrest following exposure to low IR doses, when the signal is low, but is dispensable for checkpoint arrest after high doses, when the signal is more robust (7, 31). MDC1 is also required for initiation of G₂/M arrest after low doses (7, 28, 31). Here, we examine whether 53BP1 and MDC1 are required for checkpoint maintenance. In 53BP1^{-/-} and MDC1^{-/-} MEFs, >3 Gy IR activates G₂/M checkpoint arrest, but mitotic entry occurs prematurely compared to WT MEFs (Fig. 5A and B). Thus, 53BP1 and MDC1 have roles in maintaining checkpoint arrest while being dis-

pensable for checkpoint initiation after exposure to 3 or 6 Gy IR.

53BP1^{-/-} cells show diminished Chk1 activation and reduced sustained ATM signaling to Chk2. To evaluate the mechanism by which 53BP1 functions in checkpoint maintenance, we first examined whether 53BP1 is required for Chk1 activation in irradiated G₂ cells by IF. We examined, as one approach, synchronized cells. Eight hours after release from thymidine block, ~75% of the cells were in G₂ phase (Fig. 5C). Examination of p-Chk1 levels by immunoblotting, 1 h after exposure to IR at this time point, revealed an ~50% decrease in p-Chk1 levels following treatment with 53BP1 siRNA (Fig. 5D). We also observed reduced IR-induced p-Chk1 in unsynchronized G₂ cells (identified by CENP-F staining) following treatment with 53BP1 siRNA (Fig. 5E). Thus, 53BP1 is required for efficient Chk1 activation in G₂ cells after IR, which likely contributes to the impaired checkpoint maintenance in 53BP1^{-/-} MEFs.

We also examined the requirement for 53BP1 in maintaining ATM-Chk2 signaling. In Fig. 4D and E, we show that sustained signaling maintains p-Chk2 levels and prolonged checkpoint arrest in XLF^{-/-} cells. To evaluate the impact of 53BP1 on ATM-Chk2 signaling, we examined the duration of arrest following treatment with siRNA of either 53BP1 or XLF alone or combined (Fig. 6A). Similar to our findings with 2BN (XLF^{-/-}) hTERT cells, XLF siRNA conferred prolonged arrest (>8 h) compared to cells subjected to control siRNA (released at 6 to 8 h). 53BP1 siRNA-treated cells were released prematurely, consistent with our findings with 53BP1^{-/-} MEFs. Strikingly, cells subjected to combined 53BP1 and XLF siRNA showed prolonged checkpoint arrest compared to 53BP1 siRNA alone, but release occurred earlier than in cells treated with XLF siRNA (Fig. 6A). This suggests that 53BP1 contributes to, but is not essential for, the ability of ATM signaling to respond to the status of DSB repair.

Since 53BP1 siRNA may not fully deplete 53BP1 activity, we verified this finding using 53BP1^{-/-} MEFs. To inhibit DSB repair, we treated MEFs with an inhibitor of DNA-PK, an NHEJ component (Fig. 6B). The DNA-PK inhibitor was added 30 min post-IR to ensure that we monitor the impact of impaired DSB repair on sustained versus initial signaling. We observed similar findings to those shown in Fig. 6A, namely, that DNA-PK inhibitor-treated 53BP1^{-/-} MEFs show prolonged arrest compared to untreated cells but are released earlier than DNA-PK inhibitor-treated control cells (Fig. 6B).

Since MDC1 is required for 53BP1 focus formation after IR, we also examined whether MDC1 functions in checkpoint maintenance. While MDC1 siRNA-treated cells were released by 6 h post-IR, cells treated with MDC1 siRNA plus DNA-PK inhibitor remained arrested for 10 h (data not shown). Thus, both 53BP1- and MDC1-defective cells undergo prolonged arrest in a repair-defective background, providing strong evidence that neither 53BP1 nor MDC1 is essential for ATM-Chk2 signaling.

To substantiate that sustained signaling occurs via Chk2, we monitored p-Chk2 levels in G₂-phase cells subjected to siRNA of 53BP1 or 53BP1 plus XLF. Quantification of the total cellular p-Chk2 signal revealed that, although the signal at 30 min post-IR was not significantly reduced following 53BP1 siRNA treatment, it was reduced by 2 h post-IR (Fig. 6C and D). At

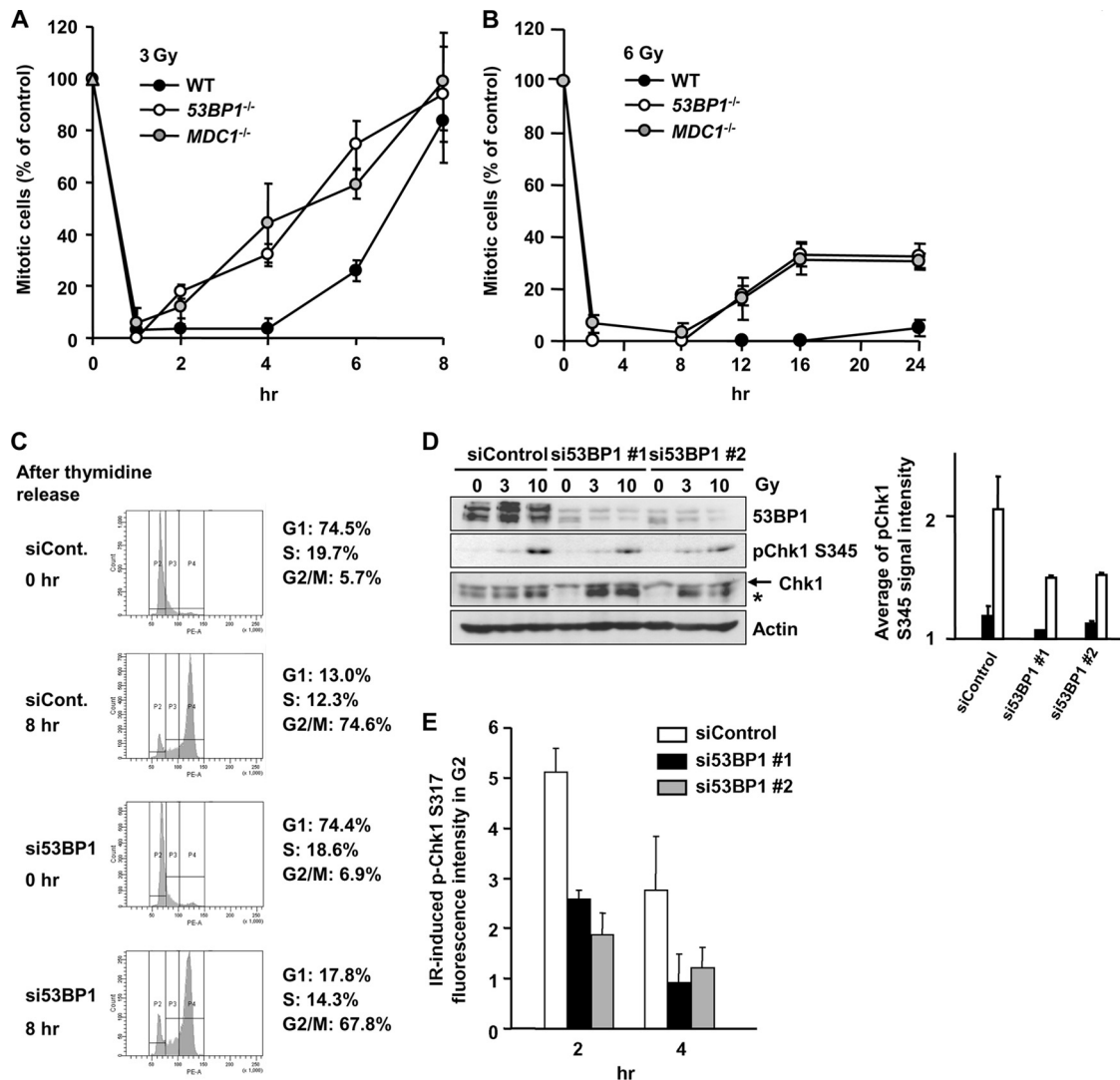


FIG. 5. *MDC1*^{-/-} and *53BP1*^{-/-} MEFs show impaired G₂/M checkpoint maintenance and reduced Chk1 phosphorylation. (A and B) Mitotic entry in WT, *MDC1*^{-/-}, and *53BP1*^{-/-} MEFs after 3 Gy and 6 Gy IR was evaluated. APH was added after IR. (C) Cell cycle profile in control and 53BP1 siRNA cells after double thymidine block. (D) p-Chk1 is reduced in synchronized G₂ 53BP1 siRNA cells post-IR. Cells were examined by immunoblotting using anti-pChk1 Ser345 antibody. The asterisk represents a nonspecific band. Quantification of p-Chk1 from 2 experiments is shown. (E) 53BP1 siRNA cells show impaired Chk1 activation. The p-Ser317 Chk1 signal was quantified by IF in CENP-F⁺ (G₂) A549 cells treated 3 Gy IR and 53BP1 or control siRNA. The signal in undamaged nuclei is subtracted. Error bars represent the SEM of 3 experiments.

later times, the low signal precluded accurate assessment. Greater sensitivity was provided following XLF siRNA treatment. At 30 min post-IR, the levels of p-Chk2 were similar with or without 53BP1 siRNA but were elevated at later times in the presence of 53BP1 (Fig. 6E), demonstrating that sustained ATM-Chk2 signaling is diminished in the absence of 53BP1. However, the p-Chk2 levels remained higher following treatment with 53BP1 plus XLF siRNA compared to 53BP1 siRNA alone (compare Fig. 6D and E), confirming that 53BP1 is dispensable for ATM-Chk2 signaling. Thus, consistent with our studies on checkpoint release, ATM-Chk2 signaling occurs in the absence of 53BP1, although it is substantially diminished in magnitude compared to control cells. This implies that ATM signaling can be sustained at persistent DSBs in the absence of 53BP1.

Collectively, these findings suggest that 53BP1 has a role in both ATR-Chk1 and sustained ATM-Chk2 signaling. The requirement of 53BP1 for both arms of the signaling response is consistent with the observation that loss of 53BP1 confers earlier checkpoint release than that observed with ATR-SS hTERT cells (compare Fig. 2B and 5A), although both show similarly diminished p-Chk1 levels (compare Fig. 2C and 5E). Indeed, checkpoint release in the absence of 53BP1 was similar to that observed in ATM inhibitor-treated ATR-SS hTERT cells, which are also impaired in ATR-Chk1 and ATM-Chk2 signaling (compare Fig. 3B and 5A).

53BP1^{-/-} and MDC1^{-/-} MEFs show elevated chromosome breakage compared to Artemis^{-/-} MEFs. We have previously shown that 53BP1 and MDC1 are required for ATM-dependent DSB repair in G₁ (20, 24). Using calyculin A-induced

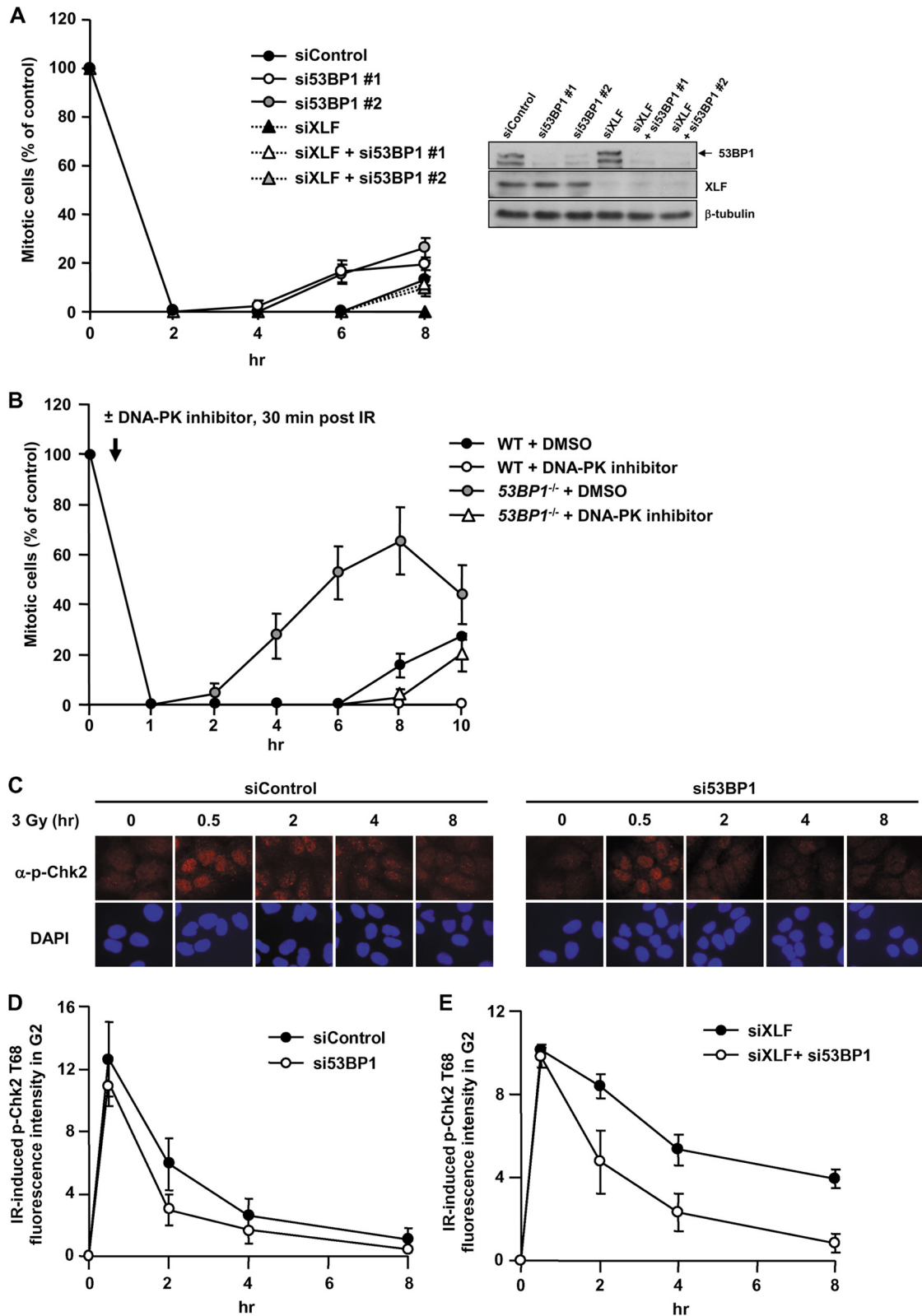


FIG. 6. 53BP1-defective cells show impaired sustained ATM-Chk2 signaling. (A) A549 cells treated with 53BP1 siRNA, XLF siRNA, or both were exposed to 3 Gy IR, and the mitotic entry was evaluated. 53BP1 and XLF siRNA causes prolonged arrest compared to 53BP1 siRNA alone. The knockdown efficiency is shown in right panel. (B) WT or 53BP1^{-/-} MEFs were treated with or without DNA-PK inhibitor addition 30 min after 3 Gy IR, and mitotic entry was examined. (C) Typical picture of p-Chk2 Thr68 in 53BP1 siRNA A549 cells after 3 Gy IR. α , anti. (D) Quantification of p-Chk2 levels in 53BP1 siRNA-treated A549 cells after 3 Gy IR. p-Chk2 levels were assessed by IF quantification in CENP-F⁺ (G₂) cells. (E) Quantification of the p-Chk2 in G₂ cells following XLF or 53BP1/XLF siRNA after 3 Gy IR.

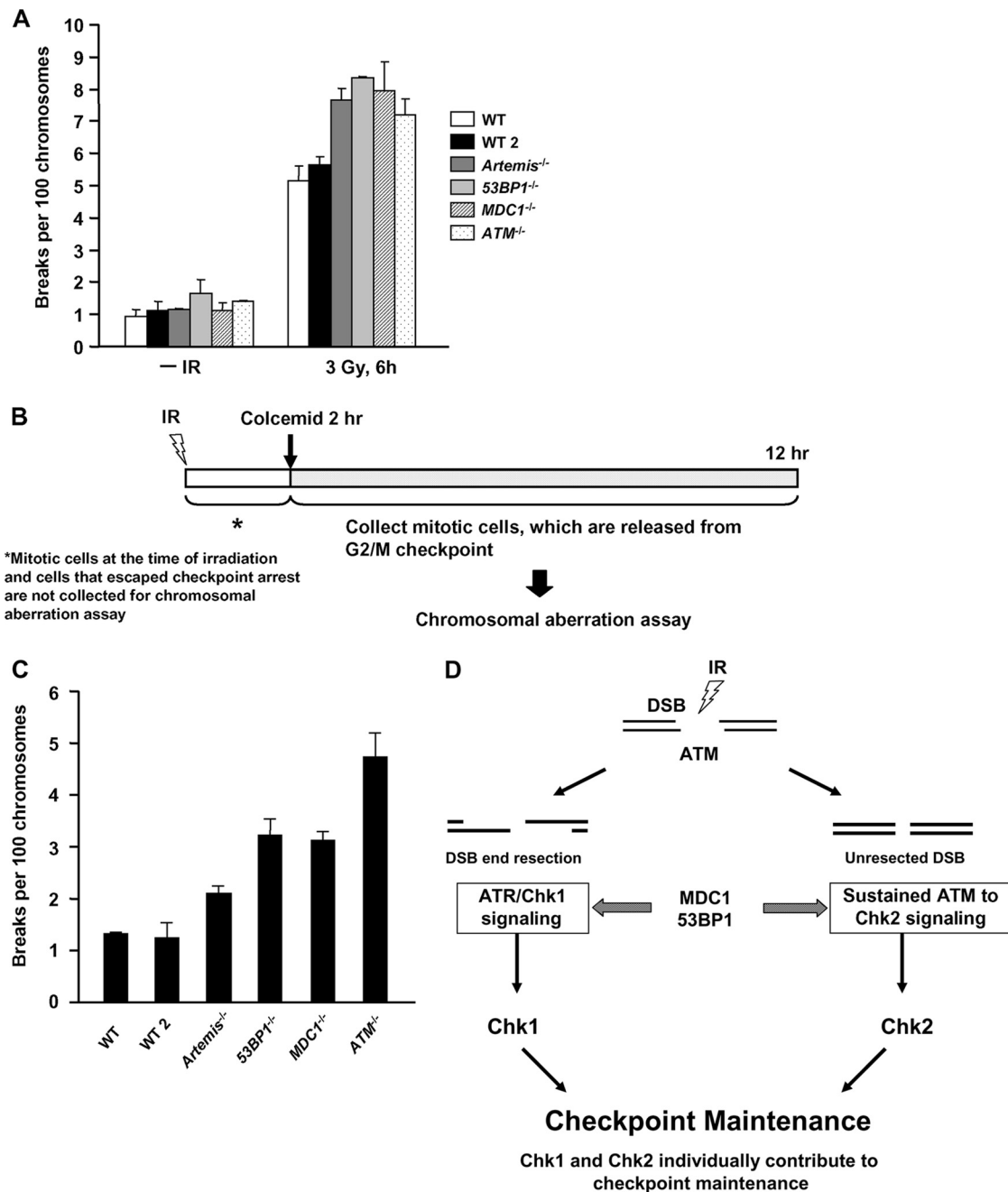


FIG. 7. The combined DSB repair and signaling defects in *MDC1*^{-/-} and *53BP1*^{-/-} MEFs contribute to chromosome breakage. (A) Cells were exposed to 3 Gy IR. A total of 50 ng/ml calyculin A was added 20 min before fixation. PCCs are scored per 100 chromosomes. (B) Diagram showing the procedure used. Colcemid was added from 2 to 12 h after 3 Gy IR. Mitotic cells were not collected during the first 2 h post-IR to prevent analysis of cells that “escape” checkpoint arrest. Chromatid breaks were scored. (C) Mitotic chromosomal break analysis of 2 WT, *MDC1*^{-/-}, *53BP1*^{-/-}, *Artemis*^{-/-}, and *ATM*^{-/-} MEFs. Chromatid break numbers were normalized to 100 chromosomes to account for differences in chromosome numbers for different lines. (D) Model showing the mechanisms contributing to checkpoint maintenance and the contribution of 53BP1 and MDC1.

PCC analysis, we show here that *53BP1*^{-/-}, *MDC1*^{-/-}, *Artemis*^{-/-}, and *ATM*^{-/-} MEFs have similar DSB repair defects in G₂ (Fig. 7A). We next examined whether the combined checkpoint and repair defects of *53BP1*^{-/-} and *MDC1*^{-/-} cells enhanced mitotic chromosome breakage by comparing breakage in mediator-defective cells (repair and checkpoint mainte-

nance defective) with that in *Artemis*-defective cells, which show prolonged checkpoint arrest (i.e., checkpoint proficient and repair deficient) (6). Previous studies have shown that *53BP1*^{-/-} and *MDC1*^{-/-} MEFs display elevated chromosome breakage, but chromosome aberrations per cell were measured at a single, early time point post-IR (16, 19). This procedure

monitors chromosome breakage in cells that enter mitosis through checkpoint escape and may not reflect the response of the total G₂ population and the contribution of checkpoint arrest in preventing chromosome breakage (12). In short, although chromosome breakage per cell may be high at early times post-IR, it may not significantly contribute to the breakage observed with the population if most cells remain arrested. We, therefore, developed an approach to assess the contribution of the entire pool of G₂-phase cells to mitotic breakage. We utilized standard chromosome analysis to score chromatid breaks, but added APH to preclude the mitotic entry of irradiated S-phase cells. To examine the entire G₂ population, colcemid was added from 2 to 12 h post-IR, by which time all G₂ cells had entered mitosis (Fig. 7B) (6). To avoid analysis of irradiated mitotic cells as well as cells that avoid checkpoint arrest at very early times post-IR, we did not collect cells in the first 2 h posttreatment.

We observed only modestly increased breakage in *Artemis*^{-/-} MEFs compared to control cells, consistent with their prolonged checkpoint arrest limiting (but not ablating) cells with DSBs entering mitosis (Fig. 7C). *MDC1*^{-/-} and *53BP1*^{-/-} MEFs, in contrast, display elevated mitotic breakage that is intermediate between those of *ATM*^{-/-} and WT MEFs. Since we excluded analysis of cells entering mitosis within 0 to 2 h post-IR, we likely underestimated chromosome breakage in checkpoint-defective *ATM*^{-/-} MEFs. This will have little impact on *53BP1*^{-/-} MEFs since they initiate arrest normally. Taken together, the data suggest that although 53BP1 and MDC1 function in a subcomponent of DSB repair that likely contributes to their radiosensitivity, their defect in maintaining checkpoint arrest contributes to their elevated chromosome breakage.

DISCUSSION

Although the molecular steps activating G₂/M arrest have been well characterized, the process by which ATM signaling maintains arrest has not been detailed. We evaluate this in the light of recent findings that (i) ATM-dependent resection can lead to ATR activation in G₂ phase, conferring a switch from ATM to ATR signaling (11), and (ii) a subset (~15%) of DSBs representing the slow component of DSB repair undergoes resection and repair by HR in G₂ phase (3). We define two ATM-dependent processes that contribute to maintaining the G₂/M checkpoint in irradiated G₂ cells: (i) ATR-dependent Chk1 activation at resected DSBs and (ii) sustained ATM-to-Chk2 signaling at unrepaired DSBs (Fig. 7D). Further, although 53BP1 and MDC1 are dispensable for the initiation of checkpoint arrest at all but low doses, they are required for maintaining arrest, a role that contributes to their function in maintaining genomic stability. We provide insight into the role of 53BP1 by showing that 53BP1-deficient cells fail to activate Chk1 normally after IR and have a diminished ability to affect sustained ATM-Chk2 signaling.

The checkpoint maintenance mechanisms. A subcomponent of DSBs in G₂ undergoes ATM-dependent resection, generating RPA-coated ssDNA that signals via ATR recruitment to Chk1. We uniquely examine Chk1's role following resection in G₂ phase by adding APH to prevent analysis of Chk1 activation at stalled replication forks. Chk1's role in maintaining ATM-

dependent checkpoint arrest is demonstrated by the premature release of Chk1 siRNA and ATR-SS hTERT cells. These findings provide the first evidence in mammalian cells that ATM-dependent Chk1 activation at resected DSBs (as opposed to stalled replication forks) contributes to checkpoint maintenance. The modest impact of Chk1 is consistent with our findings that only 15 to 20% of IR-induced DSBs undergo resection and repair by HR in G₂ phase. However, the DSBs that undergo resection represent the slow DSB repair component (3). Thus, resected DSBs make a greater contribution to unrepaired DSBs at later times post-IR, when the majority of NHEJ (and hence signaling to Chk2) is completed.

We also provide evidence for a mechanism involving sustained ATM-Chk2 signaling. Sustained ATM activation could occur by prolongation of initially activated ATM, by ongoing activation of ATM retained at the DSB site or by continuous recruitment of ATM to DSBs. Although further work is required to distinguish the precise mechanism, the concept of sustained ATM activation has received little consideration hitherto. Sustained ATM signaling is observed most strikingly with cells with impaired NHEJ (e.g., *XLF*^{-/-} or DNA-PK inhibitor-treated cells). To dissect the process, we added ATM inhibitor at 30 min post-IR, when maximal levels of p-Chk1/Chk2 had been attained. ATM inhibitor addition to WT, *XLF*^{-/-}, or DNA-PK inhibitor-treated cells showed premature checkpoint release and diminished p-Chk2 levels, demonstrating a requirement for sustained ATM-Chk2 signaling. Direct evidence for this process was also observed with the premature mitotic entry following Chk2 siRNA treatment, consistent with a previous report that *Chk2*^{-/-} MEFs arrest but are released prematurely (10). Further, we show that slow decay of the initially generated p-Chk2 signal cannot account for the prolonged arrest, although it may provide a period of arrest without the need for further ATM signaling. This process may underlie the slightly longer arrest observed with ATM inhibitor-treated ATR-SS hTERT cells than with cells ablated for Chk1/Chk2 activity (i.e., WT plus Chk1/Chk2 inhibitor-treated cells), since ATR-SS hTERT cells show impaired Chk1 activation and the ATM inhibitor diminishes sustained Chk2 activation.

Overlap between Chk1 and Chk2 in checkpoint arrest. Checkpoint maintenance differs from initiation in two ways. First, above a certain dose, sufficiently activated Chk1 or Chk2 may initiate arrest. In contrast, checkpoint release is determined by a threshold signal as DSB repair ensues. Further, if HR and NHEJ do not proceed at equal rates, the ratio of resected to nonresected DSBs will change over time (3).

Our results suggest that after 3 Gy IR, either Chk1 or Chk2 alone is sufficient to initiate arrest, while loss of either kinase impairs checkpoint maintenance. The lack of requirement for Chk2 for checkpoint initiation after 3 Gy is consistent with published findings using *Chk2*^{-/-} MEFs (10). Although it is difficult to fully ablate Chk1 since it is essential, we show that following siRNA Chk1 treatment and in ATR-SS cells, checkpoint arrest is initiated normally after IR, but its maintenance is impaired. Further, both treatments abolish 53BP1 focus formation after hydroxyurea (HU) treatment, a known Chk1-dependent process. Thus, there is either no or a less stringent requirement for Chk1 and Chk2 for initiation versus checkpoint maintenance. It is possible and indeed likely that check-

point arrest could have different requirements after exposure to lower doses, however. Checkpoint arrest in DT40 chicken cells has been reported to be Chk1 dependent after 4 Gy, consistent with the fact that most DSBs undergo resection and repair by HR in G₂ in DT40 cells (23, 27). Our finding that in mammalian cells both Chk1 and Chk2 are activated and able to initiate checkpoint arrest is, thus, consistent with the notion that both HR and NHEJ contribute to DSB repair in G₂ and that some but not all DSBs undergo resection. The fact that loss of either checkpoint kinase impairs the maintenance of arrest suggests that both kinases contribute to the checkpoint signal as it approaches a threshold level as DSB repair ensues, consistent with the suggestion that both HR and NHEJ contribute to DSB repair in G₂. It is noteworthy that there appears to be a greater contribution of Chk1 in control cells, in agreement with the notion that resected DSBs and HR represent the slow DSB repair component (3).

The role of the mediator proteins 53BP1 and MDC1. We demonstrate that 53BP1 and MDC1 have roles in maintaining checkpoint arrest and hence have checkpoint defects following exposure to high IR doses. In contrast, 53BP1 and MDC1 are dispensable for checkpoint initiation, except after low IR doses (7, 16, 28, 31). We show that 53BP1 impacts ATR-Chk1 activation and sustained ATM-Chk2 signaling. Interestingly, at 30 min post-IR, loss of 53BP1 had a greater impact on p-Chk1 than on p-Chk2 levels and a greater impact on maintaining p-Chk2 levels than on the initial signal. Perhaps surprisingly, we observed that 53BP1 is not essential for sustained ATM-Chk2 signaling but enhances the efficacy of the process. We have recently shown that the mediator proteins help to maintain ATM at the DSB (14, 20). We suggest that this promotes the maintenance of active ATM at the DSB, enhancing its ability to phosphorylate Chk2 and to promote resection and Chk1 activation. Importantly, we demonstrate that the mediator proteins have a significant function in maintaining ATM-Chk2 signaling, a notion that has not fully been considered hitherto.

Our study also provides insight into how the mediator proteins function to limit genomic instability. Despite their subtle DSB repair defect and the previously described subtle checkpoint defect, 53BP1^{-/-} and MDC1^{-/-} MEFs show marked genomic instability. Chromosome breakage represents a reliable monitor of genomic instability. We present evidence that defective checkpoint maintenance when coupled with a DSB repair defect significantly contributes to IR-induced chromosome breakage. Thus, while being apparently minor defects, they are of major significance when considering genomic instability.

Conclusion. We have dissected mechanisms regulating the checkpoint maintenance in irradiated mammalian G₂ cells. We show that (i) Chk1 activation at resected DSBs contributes to maintaining checkpoint arrest and (ii) sustained signaling from ATM to Chk2 at unrepaired DSBs can prolong arrest, most strikingly in NHEJ-defective cells. We demonstrate that the mediator proteins 53BP1 and MDC1, while impacting the checkpoint initiation only at low doses, are required to maintain arrest at all doses. They achieve this by enhancing Chk1 activation in G₂ and by facilitating sustained ATM-Chk2 signaling. Thus, 53BP1^{-/-} and MDC1^{-/-} cells have checkpoint

defects after high and low IR doses, significantly contributing to their elevated chromosome breakage.

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