The Limitations of the G1-S Checkpoint

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Abstract

It has been proposed that the G1-S checkpoint is the critical regulator of genomic stability, preventing the cell cycle progression of cells with a single DNA double-strand break. Using fluorescence-activated cell sorting analysis of asynchronous cells and microscopic analysis of asynchronous and synchronized cells, we show that full blockage of S-phase entry is only observed >4 hours after irradiation. The process is ataxia-telangiectasia mutated (ATM) dependent and Chk1/2 independent and can be activated throughout G1 phase. By monitoring S-phase entry of irradiated synchronized cells, we show that the duration of arrest is dose dependent, with S-phase entry recommencing after arrest with kinetics similar to that observed in unirradiated cells. Thus, G1-S checkpoint arrest is not always permanent. Following exposure to higher doses (>2 Gy), G1-S arrest is inefficiently maintained, allowing progression of G1-phase cells into G2 with elevated γH2AX foci and chromosome breaks. At early times after irradiation (<4 h), G1-S checkpoint arrest is not established but cells enter S phase at a reduced rate. This early slowing in S-phase entry is ATM and Chk2 dependent and detectable after 100 mGy, showing a novel and sensitive damage response. However, the time needed to establish G1-S checkpoint arrest provides a window when cells can progress to G2 and form chromosome breaks. Our findings detail the efficacy of the G1-S checkpoint and define two significant limitations: At early times after IR, the activated checkpoint fails to efficiently prevent S-phase entry, and at later times, the checkpoint is inefficiently maintained. Cancer Res; 70(11): OF1–10. ©2010 AACR.

Introduction

Cell cycle checkpoints function to limit genomic instability arising from DNA damage. The significance of the G1-S checkpoint regulated by p53 is shown by its downregulation in tumors and established cell lines. p53 and its regulatory protein, MDM2, are phosphorylated directly or indirectly by the damage response kinase ataxia-telangiectasia mutated (ATM), enhancing p53 stability and DNA binding activity (1, 2). To achieve G1-S checkpoint arrest, p53 regulates transcription of the cyclin-dependent kinase (Cdk) inhibitor p21Waf1/Cip1 (3, 4). It has been argued that p53-dependent G1-S arrest is activated by a single DNA double-strand break (DSB) and that it primarily regulates permanent checkpoint arrest to prevent proliferation of irreparably damaged cells rather than enhancing time for DNA repair (5–7). It is likely, however, that activation of p53-dependent G1-S arrest is established slowly because the process is transcription dependent (3).

The damage response signaling kinases ATM and ATR (ATM and Rad3-related) phosphorylate and activate the transducer kinases Chk2 and Chk1, respectively (3, 8). The phosphatase Cdc25A, a critical Chk1/2 substrate, regulates inhibitory phosphorylation of Cdk1/Cdk2. Following exposure to ionizing radiation (IR), Cdc25A is rapidly ubiquitylated and degraded, inhibiting Cdk1/Cdk2 activation (9). Thus, a more rapidly activated pathway for G1-S arrest involving the inhibition of Cdc25A-mediated activation of cyclin E–Cdk2 has been proposed (3, 9, 10). Collectively, a dual wave of checkpoint responses at the G1-S boundary has been proposed: an initial transient response causing Cdk2 inhibition within 20 to 30 minutes and lasting several hours and a slowly activated but sustained process involving p53/p21 (3).

Previously, we and others presented evidence that radiation-induced G2-M checkpoint arrest is rapidly activated and that its duration is dose and DSB repair capacity dependent (11–14). However, release from arrest occurs before the completion of DSB repair, resulting in chromosome breakage in cells released from checkpoint arrest (12). Further, in primary fibroblasts, G2-M checkpoint activation requires doses >200 mGy, a phenomenon underlying low-dose radiation hypersensitivity (15). These findings, coupled with the significant effect of G1-S checkpoint loss on tumor predisposition, have led to the suggestion that a sensitive G1-S checkpoint may be the master regulator of genomic stability in mammalian cells (16, 17).
Our aim here was to examine the proficiency of the G1-S checkpoint in preventing chromosome breakage. Current evidence suggests a paradox; the p53-dependent G1-S checkpoint has been reported to be sensitive to a single DSB, yet cytologic studies have shown that mitotic chromosome breaks arise in irradiated G0-G1-phase cells (5–7, 18). Our study provides an explanation consistent with both findings. The G1-S checkpoint is highly sensitive and efficiently limits genomic instability. However, two aspects compromise its efficacy; at early times after IR, S-phase entry is slowed but not abolished. Additionally, G1-S arrest once fully activated is inefficiently maintained, allowing cells with γH2AX foci, a marker for DSBs (19), to escape from arrest and enter S phase. We show that both routes can lead to chromosome breakage in the subsequent G2 phase.

Materials and Methods

Cell culture
Primary human wild-type (WT) fibroblasts (HSF1 and 48BR), Artemis−/− (CJ79) fibroblasts, and hTert derivatives (WT: 82-6hTert and 1BRhTert; Artemis−/−: CJ79hTert; ATM−/−: AT1BRhTert) were grown in MEM with 15% FCS/1% antibiotics. Primary AT1BR fibroblasts were grown in HAM’s F10 supplemented with 15% FCS, 1% nonessential amino acids, and 1% antibiotics. Primary AT1BR fibroblasts were grown in HAM’s F10 supplemented with 15% FCS/1% antibiotics. For synchronization, fibroblasts were serum starved for 3 to 4 days in MEM containing 0.5% (primary) or 0.1% (immortalized) FCS to promote G0 entry. Cells were released by addition of FCS-containing MEM. S-phase cells were labeled with 10 μmol/L bromodeoxyuridine (BrdUrd; Becton Dickinson), 10 μmol/L 5-ethyl-2′-deoxyuridine (EdU; Molecular Probes), or 20 μmol/L chlorodeoxyuridine (CldU; MP Biomedicals). Nocodazole was used at 80 ng/mL. UCN01 (600 nmol/L; Sigma) was added 30 minutes before IR for Chk1/2 inhibition. p53, Chk1, Chk2, and control siRNA were transfected with Metafectene Transfection Reagent (Biontex). X-irradiation was carried out using the Dharmacon SMARTpool and Artemis

Results

G1-S entry is only abolished >4 hours after IR
As one approach to monitor the G1-S checkpoint, we labeled WT (82-6hTert) cells with BrdUrd 1 hour before IR to identify preexisting S-phase cells and then assessed the percentage of BrdUrd− S-phase cells by FACS following IR (21). Nocodazole was added to prevent progression of irradiated G2-phase cells. Hence, we monitor S-phase entry of irradiated G1 cells. Without IR, BrdUrd− S-phase cells increase for 6 hours, reaching a plateau as cells enter and exit S phase. S-phase entry of irradiated WT cells is similar to, although slightly less than, unirradiated cells for the first 6 hours (Fig. 1A; Supplementary Fig. S1A). After 6 hours, the BrdUrd− S-phase population decreases consistent with G1-S checkpoint activation, preventing S-phase entry but not exit. The time of initiation of checkpoint arrest (~6 h) seems to be dose independent and detectable after 500 mGy. XLF−/− (2BNhTert) cells, which are defective in a core NHEJ protein and exhibit an extensive DSB repair defect evident at early times after IR (22), show reduced but nonetheless substantial S-phase entry during the first 6 hours. Abolition of S-phase entry is only observed after 6 hours and detectable after 200 mGy (Supplementary Fig. S1B). Interestingly, slow S-phase entry during the first 6 hours seemed more marked for XLF−/− compared with WT cells, suggesting a potential consequence of un repaired DSBs.

We also used a microscopy-based approach to quantify early, middle, and late S-phase cells, exploiting their distinct patterns of replication fork-associated PCNA (Supplementary Fig. S1C; ref. 23). Although the level of these S-phase populations remained similar throughout 16-hour analysis of untreated cells, the pattern changed following irradiation (Fig. 1B). Consistent with the FACS analysis, total S-phase cells only decreased at 6 to 8 hours after IR, although a diminution of early S-phase cells was already evident by 4 hours, suggesting an effect on S-phase entry at early times after IR.

Finally, we applied an immunofluorescence-based procedure to monitor S-phase entry using DNA synthesis. We pulse-labeled (30 min) exponentially growing human fibroblasts with the thymidine analogue EdU to identify preexisting S-phase cells. Following IR, we added BrdUrd to detect cells entering S-phase after IR and nocodazole to prevent mitotic progression. S-phase entry after IR was monitored by enumerating EdU−/BrdUrd− cells (Fig. 1C). Following exposure to 0.5,
1, or 2 Gy, cells enter S phase for 4 hours with slower kinetics compared with unirradiated cells. Full checkpoint arrest, evident by a plateau of EdU−/BrdUrd+ cells, is only observed >4 hours after IR. Only a small plateau was observed in cells exposed to 500 mGy, suggesting a transient block in S-phase entry consistent with the data in Fig. 1A and B (NB cells remain labeled following S-phase exit and hence S-phase entry continuously increases in contrast to the FACS approach).

These experiments show that abolition of S-phase entry is only observed several hours (>4) after IR. Thus, full G1-S arrest is activated slowly. However, at early times after IR, S-phase entry slows in a dose-dependent and DSB repair
capacity–dependent manner. Below, we investigate if this represents a distinct process or partial activation of the G₁-S checkpoint observed at later times.

**Early slowing of S-phase entry is an ATM- and Chk2-dependent response**

The experiments above used hTert fibroblasts. To validate our results and dissect events at early versus late times after IR, we serum-starved primary fibroblasts to promote entry into G₀ phase. Three to 4 days later, serum was readded together with CldU, and S-phase entry was monitored by enumerating CldU⁺ cells. Cells commenced S-phase entry ~10 hours following serum addition, reflecting the time required to exit G₀ and traverse G₁. To examine S-phase entry at early times after IR, cells were irradiated at 8.5 hours after serum readdition (i.e., 1.5 h before their predicted S-phase entry). A dose-dependent decrease in CldU⁺ cells was observed at early times (Fig. 2A). Following higher doses (≥ 1 Gy), a plateau in CldU⁺ cells suggesting full checkpoint arrest was only observed ~10 hours after IR (i.e., at ~18 h after release from G₀, Fig. 2A). At lower doses (≤ 500 mGy), the defined plateau is replaced by a “kink” in the S-phase entry rate, suggesting transient G₁-S checkpoint arrest. We also studied Artemis⁻/⁻ (CJ179) cells, which rejoin most DSBs normally but are defective in the slow component of DSB repair, providing an ideal model system to study the effect of persisting DSBs on cell cycle progression (Supplementary Fig. S2A and B; ref. 24). Artemis⁻/⁻ cells showed a similar response to WT cells at early times after IR, but at later times, they remained arrested and failed to reenter S phase (Fig. 2B). 53BP1 foci analysis, used to monitor DSBs, revealed that G₁ cells harbor foci when S-phase entry occurs at early times after IR, suggesting that entry occurs despite the presence of unrepaired DSBs (Supplementary Fig. S2A and B).

To examine whether the slowing of S-phase entry represents a regulated damage response, we examined primary ataxia-telangiectasia (A-T) cell lines and observed no perturbation in S-phase entry even after high doses (4 Gy; Fig. 2C). Thus, slow S-phase entry after IR represents an ATM-regulated process. Similar findings were obtained using exponentially
growing A-T fibroblasts and the EdU/BrdUrd approach (Supplementary Fig. S2C). Previous studies have described two G1-S checkpoints: one p53 and one Chk1/2 dependent. To determine whether the early slowing is Chk1/2 and/or p53 dependent, we used RNAi. Chk2-downregulated, but not Chk1-downregulated, cells show early S-phase entry unperturbed even after high IR doses (4 and 10 Gy), suggesting that the process is Chk2 dependent (Fig. 2C; Supplementary Fig. S2D). UCN01, a Chk1/2 inhibitor at 600 nmol/L, provided the same result as Chk2 inactivation (Supplementary Fig. S2D; refs. 25, 26). Without IR, p53 siRNA–treated cells enter S phase earlier (at 6.5 h) than control siRNA–treated cells. IR exposure failed to cause a significant delay in S-phase entry (Fig. 2C). Although this could suggest a role of p53 in early S-phase slowing, this conclusion is limited by the fact that p53 loss accelerates S-phase entry in undamaged cells.

Collectively, these experiments show that slowing of S-phase entry after IR is a sensitive ATM- and Chk2-dependent response detectable after 200 mGy.

Late G1-S checkpoint arrest is Chk1/2 independent
Abolition of S-phase entry is only observed several hours (>4) after IR, suggesting that full G1-S arrest is activated slowly. To examine this further, we irradiated cells 0.5 hour after release from serum starvation, added CldU after IR, and monitored S-phase entry by enumerating CldU+ cells. CldU+ unirradiated cells increased from ∼9 hours after release. Strikingly, a dose-dependent delay in S-phase entry was observed with a minor delay evident even following exposure to 100 mGy (Fig. 3A), a dose that induces at least one focus in 90% of cells (Supplementary Fig. S3A). For doses up to 4 Gy, S-phase entry after arrest, although delayed, commenced at a similar rate to that observed in untreated cells, although a diminished fraction of cells entered S phase. Similar analysis with Artemis−/− cells revealed a prolonged arrest and reduced S-phase entry, showing that checkpoint release requires DSB repair (Fig. 3B; Supplementary Fig. S3B).

A-T cells showed no significant delay in S-phase entry even following exposure to 10 Gy (Fig. 3C). Chk1 and Chk2 down-regulation did not affect the delay in S-phase entry (Fig. 3C;
Further, although UCN01 treatment affected the timing of S-phase entry, it did not abolish the IR-induced delay in S-phase entry (Supplementary Fig. S3C), confirming that the process is Chk1/Chk2 independent. p53 siRNA caused earlier S-phase entry of unirradiated cells compared with mock siRNA–treated cells, as observed previously (Fig. 3C). Irradiation failed to delay entry in p53 siRNA–treated cells in contrast to control siRNA cells. Although this is consistent with a p53-dependent process, this conclusion is limited by the effect of p53 depletion on S-phase entry in unirradiated cells.

Collectively, these experiments show that full G1-S arrest takes >4 hours and is distinct to the early slowing of S-phase entry, the latter but not the former being Chk2 dependent. Both processes are ATM dependent and abolished by loss of p53.

**Early S-phase entry leads to γH2AX foci in S phase and chromosome breaks in G2**

We applied a procedure to examine γH2AX foci formation in cells that enter S phase at early times after IR and to minimize analysis of replication-associated breakage. EdU was added 30 minutes before IR and maintained until 2 hours before sampling to mark preexisting S-G2 cells. Two hours before analysis, EdU was replaced with BrdUrd and γH2AX foci were scored in EdU−/BrdUrd− cells, representing cells that enter S phase within 2 hours before sampling (Fig. 4A). In

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**Figure 4.** Early S-phase entry of irradiated asynchronous WT fibroblasts (82-6hTert) causes γH2AX foci formation and chromosome breakage in S and G2, respectively. A, immunofluorescence microscopic analysis of WT cells after IR in G1. Cells were labeled with EdU 30 min before IR and until 2 h before fixation (1 h for the 1-h time point). At this time, EdU (TR) was replaced with BrdUrd (Cy5), and γH2AX foci (FITC) were counted in EdU−/BrdUrd− cells (NB γH2AX foci in S-phase cells are visible above a slight background lawn of γH2AX staining). B, γH2AX foci analysis of WT cells. Cells were treated as in A and γH2AX foci were assessed in EdU−/BrdUrd− cells (representing G1 cells; left) and EdU+/BrdUrd− cells (representing cells that enter S phase within 2 h before fixation; right). At early times, G1- and G2-phase cells were distinguished by their DAPI signal intensity (22). C, G2-PCC analysis in WT cells after IR in G1. Cells were labeled with EdU 6 h before IR to mark cells in S and G2 at the time of IR. Ten hours after IR, chromosomes were prematurely condensed by calyculin A treatment and chromosome spreads were stained for EdU. Chromosome breaks were quantified in EdU−G2 cells. Points and columns, mean of two to five experiments; bars, SE.
parallel, foci were enumerated in G₁-phase cells. γH2AX foci numbers were similar in early S- and G₁-phase cells, showing that cells entering S phase are representative of the G₁ population in their DSB repair status (Fig. 4B). The number of EdU+/BrdUrd⁻ cells decreased significantly from 6 to 12 hours after IR, particularly at higher doses, as full G₁-S checkpoint arrest became established (data not shown).

We examined chromosomal breakage in the subsequent G₂-M phase using premature chromosome condensation (PCC). Exponentially growing cells were labeled with EdU for 6 hours before IR to mark S and G₂ cells (Supplementary Fig. S4A). Ten hours after IR, some EdU⁻ cells (representing cells that enter S phase after IR) have progressed into G₂ (Supplementary Fig. S4A). PCC was induced in G₂ cells using

**Figure 5.** A to C, WT hTert fibroblasts (1BRhTert) released from G₀-S arrest have γH2AX foci and chromosome breaks in G₂. Following serum starvation of hTert fibroblasts, fresh medium was added. Irradiation was at 0.5 h after serum addition. A, γH2AX foci analysis in G₀ (CENP-F⁻) and G₂ (CENP-F⁺) cells after IR. B, the percentage of CENP-F⁺ G₂ cells was quantified. C, G₂-PCC analysis of WT cells after IR in G₁. Cells treated as in A were processed for chromosome analysis (using calyculin A) 24 h after IR. D, cells released from G₂-M checkpoint stop at the subsequent G₁-S checkpoint. Left, after IR in G₂, the mitotic index (lines) and γH2AX foci numbers per G₂-phase cell (columns) were estimated according to Deckbar and colleagues (12); right, cells were untreated or irradiated 4 h after BrdUrd pulse labeling (when BrdUrd⁻ cells are in G₂; time 0). Twelve hours after IR, when 90% of BrdUrd⁺ cells have entered G₁, nocodazole was added to arrest cells in the subsequent G₂-M phase. The percentage BrdUrd⁺ G₂ cells of all BrdUrd⁺ cells was estimated by FACS analysis (lines). Right, in parallel experiments, EdU was added at 12 h after IR, and the γH2AX foci number in BrdUrd⁺ and EdU⁺ G₂ cells (columns) was estimated. Points and columns, mean of two to three experiments; bars, SE.
cylcalin A, and excess chromosome fragments were quantified in EdU+ cells (Supplementary Fig. S4B). Increased chromosome breakage was observed after 1, 2, and 4 Gy (Fig. 4C). Thus, EdU+ cells irradiated in G1 progress through S and exhibit chromosome breaks in G2. To validate that the cells originated from G1, we examined chromosome versus chromatid breakage in mitotic cells because they arise in cells irradiated in G1 or G2, respectively (18, 26). Mitotic cells were used because they allow the breakage class to be distinguished. EdU+ cells exhibited more chromatid than chromosome breaks (Supplementary Fig. S4C), whereas EdU− cells had primarily chromosome breaks, confirming their G1 origin.

In summary, these data suggest that slow G1-S checkpoint activation enables cells to enter S phase with unrepaired DSBs (and possibly unrepaired single-strand breaks), which causes chromosome breaks in the subsequent G2-M phase.

The G1-S checkpoint is inefficiently maintained

We addressed whether cells released from full G1-S arrest have DNA damage and chromosome breakage. In our synchronization experiments, cells irradiated with 100 mGy to 1 Gy at 30 minutes after release from serum starvation have completed DSB repair when they enter S phase 12 to 16 hours after IR (Supplementary Fig. S3B). In contrast, following >2 Gy, S-phase entry occurred in a low frequency of cells at ∼20 hours, a time when foci remain detectable in G1 cells (Fig. 5A). Thus, we monitored foci numbers and chromosome breakage in G2 cells that have undergone G1-S arrest following irradiation at 30 minutes following serum starvation.

G2 cell numbers were low following exposure to higher doses but increased from 16 to 24 hours (Fig. 5B). γH2AX foci numbers were dose dependent and decreased from 16 to 24 hours. γH2AX foci numbers in G2 were >2-fold higher than in parallel G1 cells (Fig. 5A), suggesting that additional DSBs arise during progression through S phase. Strikingly, the small fraction of cells (∼5%) escaping G1-S arrest after high doses (>2 Gy) show multiple γH2AX foci in G2 (Fig. 5A).

We also examined chromosome breakage in the same cells at 24 hours using PCC analysis. We observed increased chromosome breakage, which was dose dependent and evident even after 1 Gy (Fig. 5C; Supplementary Fig. S5A). Collectively, these data show that the G1-S checkpoint is inefficiently maintained and that cells are released or escape from G1 arrest with unrepaired DSBs, causing chromosome breakage in the subsequent G2-M phase.

Cells released from G2-M arrest accumulate at the subsequent G1-S checkpoint

Previous findings showed that at the time of G2-M checkpoint release (4–6 h after 1 Gy), G2 cells harbor ∼10 to 15 γH2AX foci, suggesting that the G2-M checkpoint is insensitive (Fig. 5D; ref. 12). This is further supported by the finding that cells released from G2-M checkpoint arrest have one to two chromosome breaks in mitosis (12). We therefore examined whether the G1-S checkpoint might be activated in such cells. To examine G1-S checkpoint arrest in G2-irradiated cells, WT and Artemis−/− fibroblasts were BrdUrd pulse labeled, incubated for 4 hours to allow BrdUrd+ cells to reach G2, and exposed to 1 Gy. Twelve hours after IR, when cells had undergone division, nocodazole was added to accumulate cells at the next G2-M phase, and the fraction of BrdUrd+ G2-M phase cells was assessed by FACS (Fig. 5D; Supplementary Fig. S5B). At 24 and 36 hours after IR, when BrdUrd+ G1-phase cells harbor approximately one focus, the number of BrdUrd+ G2-M phase cells was lower than in unirradiated cells (Fig. 5D), a feature marked in Artemis−/− cells (Supplementary Fig. S5B). This suggests that cells released from G2-M checkpoint arrest might undergo prolonged G1-S arrest, raising the possibility that one function of the G1-S checkpoint is to prevent progression of G2 irradiated cells.

Discussion

Our aim here was to evaluate the proficiency of processes underlying G1-S checkpoint arrest in preventing chromosome breakage. This is important because as the G2-M checkpoint has limitations, the G1-S checkpoint has been proposed to be a master regulator that precludes cell cycle progression of damaged cells (12, 17).

Two processes affect S-phase entry following IR

Previous studies have defined two molecularly distinct pathways regulating G1-S entry following DNA damage: the well-characterized, p53-dependent pathway that regulates the Cdk inhibitor p21 and a rapid response that involves Cdc25A degradation (3, 27). Our findings are consistent with these studies but evaluate them from the perspective of their limitations. At early times after IR, we observed a slowing but incomplete block of S-phase entry, which is ATM and Chk2 dependent but Chk1 independent. This finding is consistent with ATM activation of Chk2, whereas ATR and Chk1 are not activated after IR in G1. Although abolished in the absence of p53, the process may not necessarily be p53 regulated because p53 siRNA accelerates S-phase entry in undamaged cells. Nonetheless, loss of p53 overrides the process. The Chk2 dependency of early slow S-phase entry in contrast to full G1-S arrest at later times, which is Chk2 independent, strongly argues that they represent distinct processes. Collectively, our findings suggest that this represents the previously described rapid response involving Cdc25A phosphorylation by Chk1/2 and subsequent degradation. The previous studies, however, did not examine G1-S entry. Surprisingly, this process does not abolish S-phase entry, a feature observed by all approaches. Redundancy between the Cdc25 phosphatases and failure of ATM to inactivate all three phosphatases may provide an explanation. The process is dose dependent and activated after very low IR doses (100 mGy). Our analysis of distinct S-phase stages using the PCNA staining technique that does not depend on measuring DNA synthesis shows that inhibition of early S-phase cells is the major change observed at early times, suggesting inhibition of S-phase entry rather than S-phase progression.

Full S-phase arrest was ATM dependent but Chk1/2 independent and occurred only >4 hours after IR in all our
approaches, consistent with it representing slow p21 transcriptional activation. The time to achieve full G1-S arrest differed between our approaches and was substantially longer following synchronization. Primary cells were used for this procedure, and indeed, a shorter delay was observed with 1RBrTert cells (data not shown). Additionally, the synchronization protocol may delay activation of G1-S arrest in some way. The significant time taken to achieve full S-phase arrest is perhaps surprising but likely represents its transcriptional dependence and the necessity for its function before commitment to S-phase entry (28). Additionally, the process is activated by 100 mGy, a dose introducing on average two to three DSBs per cell (Fig. 3A; Supplementary Fig. S3A). The duration of delay seems to be dose dependent but independent of the point in G1 phase when irradiation occurs. Thus, in Fig. 2A, where irradiation is given 8.5 hours after serum addition, full S-phase arrest commences ~10 hours later and lasts 5 to 6 hours after 1 Gy. In Fig. 3A, where irradiation is given 0.5 hour after serum addition, full arrest occurs on S-phase entry but has a similar duration. In Fig. 1B, the duration of arrest after 1 Gy is also 5 to 6 hours. These findings suggest that G1-S arrest does not occur at the G1-S boundary but rather stops progression through G1 phase, consistent with the notion that it halts assembly of a replication-competent state involving Rb phosphorylation (29, 30). This notion is further supported by the observation that 100-mGy irradiated cells have repaired their breaks by the time they reach the G1-S border and show delayed S-phase entry (Supplementary Fig. S3B).

It has previously been suggested that p53-dependent checkpoint arrest permanently eliminates damaged cells rather than providing a transient arrest to enhance the DSB repair time (5, 7). Our findings, however, show that cells can be released from full G1-S arrest. Thus, we propose that G1-S arrest can operate in a transient manner, increasing the time for DSB repair.

Our study shows two limitations of the G1-S checkpoint that can result in chromosome breakage. First, the failure to fully arrest G1-S entry at early times can cause chromosome breakage in G2 phase. Second, cells that have undergone full G1-S arrest can subsequently "escape" arrest and enter S phase with unrepaired DSBs and form chromosome breaks in G2. The phenomenon of G1-S checkpoint escape is best shown following high-dose exposure (≥2 Gy; Fig. 5B), which provides substantial arrest for 24 hours. However, a few cells escape arrest and can be detected in G2 with γH2AX foci and chromosome breaks. Further, we provide evidence that cells released from checkpoint arrest after ≤1 Gy may also harbor unrepaired DSBs, leading to G2 chromosome breakage.

Despite these limitations, the G1-S checkpoint represents a sensitive process that efficiently limits the progression of cells with DSBs into S phase. However, the checkpoint is not foolproof in preventing chromosome breakage. Indeed, our results are consistent with classic cytogenetic studies, which show that chromosome breaks can arise in mitotic cells derived from irradiated G1/G2 cells in a dose-dependent manner. We describe the basis underlying this breakage. These findings are important in considering the effect of low doses of radiation. They suggest that even following low-dose IR exposure, cells can progress to G2 phase with unrepaired DSBs. Although subsequent DSB repair may ensue, the possibility of misrepair will be enhanced following replication past a DSB, providing a significant window for survival with rearrangements. In conclusions, our findings show that the G1-S checkpoint, like the G2-M checkpoint, has defined limitations, and hence, even low doses have the potential to cause genomic instability.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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