Cell Cycle 9:4, 662-669; February 15, 2010; © 2010 Landes Bioscience

γH2AX foci analysis for monitoring DNA double-strand break repair

Strengths, limitations and optimization

Markus Löbrich,[1,*] Atsushi Shibata,[2] Andrea Beucher,[1] Anna Fisher,[2] Michael Ensminger,[1] Aaron A. Goodarzi,[2] Olivia Barton[1] and Penny A. Jeggo[2,*]

[1]Darmstadt University of Technology; Radiation Biology and DNA Repair; Darmstadt, Germany; [2]Genome Damage and Stability Centre; University of Sussex; East Sussex, UK

DNA double-strand breaks (DSBs) represent an important radiation-induced lesion and impaired DSB repair provides the best available correlation with radiosensitivity. Physical techniques for monitoring DSB repair require high, non-physiological doses and cannot reliably detect subtle defects. One outcome from extensive research into the DNA damage response is the observation that H2AX, a variant form of the histone H2A, undergoes extensive phosphorylation at the DSB, creating γH2AX foci that can be visualized by immunofluorescence. There is a close correlation between γH2AX foci and DSB numbers and between the rate of foci loss and DSB repair, providing a sensitive assay to monitor DSB repair in individual cells using physiological doses. However, γH2AX formation can occur at single-stranded DNA regions which arise during replication or repair and thus does not solely correlate with DSB formation. Here, we present and discuss evidence that following exposure to ionizing radiation, γH2AX foci analysis can provide a sensitive monitor of DSB formation and repair and describe techniques to optimize the analysis. We discuss the limitations and benefits of the technique, enabling the procedure to be optimally exploited but not misused.

Key words: γH2AX foci, double-strand break repair, ionizing radiation, hydrogen peroxide, pulsed-field gel electrophoresis, ataxia telangiectasia, single-stranded DNA

Submitted: 11/20/09

Accepted: 11/30/09

Previously published online: www.landesbioscience.com/journals/cc/article/10764

Correspondence to: Markus Löbrich and Penny A. Jeggo;
Email: lobrich@bio.tu-darmstadt.de and P.A.Jeggo@sussex.ac.uk

Introduction

Recent research has provided a detailed understanding of the damage response mounted by the presence of a DNA double-strand break (DSB).[1] The response involves an exquisitely orchestrated assembly of proteins. This knowledge has recently been exploited to develop DSB detection assays. γH2AX foci analysis is one such assay that exploits an early step in the damage response, namely the phosphorylation of H2AX, a variant form of the histone, H2A.[2,3] Since phosphorylated H2AX, designated γH2AX, expands a large distance (Mbp) from the DSB site and since specific γH2AX antibodies are available, the DSBs can be visualized as discrete foci. The assay is highly sensitive and can potentially detect all induced DSBs. However, sceptics have argued that γH2AX occurs at lesions other than DSBs, that it is an indirect rather than a direct monitor of DSBs, and that the rate of DSB repair does not precisely correlate with the rate assessed by other methods. Notwithstanding some limitations, the assay has such potential utility that it is a shame to preclude its usage without serious considerations. Previous reviews have considered γH2AX foci expansion from the chromatin perspective and its use in the analysis of cancer cells.[4-6] We review and present data arguing that after ionizing radiation (IR) exposure, γH2AX foci can represent a reliable and exquisitely sensitive monitor of DSB formation and repair. We review the procedures that we, and others, have developed to enhance the sensitivity of the technique. We discuss the limitations that have to be carefully evaluated and consider how the analysis can be exploited for specific end points notwithstanding these limitations.

Comparison of γH2AX Foci Analysis and Other Techniques

Traditional methods of DSB analysis rely on physical estimation of DNA size or on procedures that assess chromosome breakage. Of the physical methods, pulsed-field gel electrophoresis (PFGE) is the most widely used.[7] Chromosome analysis is undertaken on mitotic cells but procedures causing premature chromosome condensation (PCC) can allow analysis of G_1- or G_2-phase cells. PCC analysis in G_0/G_1 requires fusion with mitotic cells whereas in G_2-phase cells condensation can be promoted by calyculin A.[8] Below we compare γH2AX foci analysis with PFGE and chromosome analysis and consider questions that can be uniquely addressed by γH2AX analysis.

Dose. The dose necessary for γH2AX analysis primarily depends on endogenous foci levels. The technique has been utilized with mGy doses in primary fibroblasts and lymphocytes where endogenous foci numbers were low.[9] However, transformed cells frequently have high endogenous foci numbers due to their genetic instability. Senescent cells can also harbour γH2AX foci at uncapped telomeres and induced cellular senescence can produce γH2AX foci without DNA damage.[10,11] As well recognized, PFGE necessitates much higher doses (10 to 80 Gy; ~250 to 2,000 γH2AX foci). Chromosome analysis has been utilized with doses from 0.5 Gy and, as for γH2AX analysis, the sensitivity largely depends on the endogenous level of breakage. Current estimations have suggested that ~1 in 10 DSBs can be visualized as a mitotic chromosome break and 1 in 3–6 DSBs can be observed as PCC break (reviewed in ref. 12).

Sensitivity. Cell lines lacking ATM, Artemis and ATM-dependent signalling proteins (e.g., 53BP1) have a subtle DSB repair defect due to a failure to carry out the slow component of DSB repair, which represents ~15% of IR-induced DSBs.[13] Further, it has been suggested that radiosensitivity correlates with DSB numbers remaining at 24 h post IR.[14] Thus, the ability to detect a small number of persisting DSBs is important. One limitation of PFGE analysis for monitoring persisting DSBs is that the high doses required can cause apoptosis at later times after IR, particularly in transformed cells and mouse embryo fibroblasts (MEFs). Indeed, this was likely the explanation for the failure to detect a repair defect in A-T cells using PFGE and, significantly, the study which described such a defect exploited primary A-T cells.[15] Due to its sensitivity γH2AX foci analysis is particularly suitable for detecting subtle and persisting DSB repair defects.

Cell cycle progression during analysis. An important consideration for both techniques is the progression of cells through the cell cycle during analysis. This can create further breakage in S phase and can also potentially lead to non-DSB γH2AX formation. The use of non-cycling (G_0 phase) cells avoids this problem whilst transformed cells can be problematic given their rapid cell cycle progression and their inability to enter G_0 phase. The use of BrdU or other markers to identify cells that progress through the cycle can be exploited to preclude the analysis of replicating cells.

Individual cell and cell cycle phase analysis. γH2AX and chromosome analysis can be exploited to study distinct cell cycle phases and individual cells. Procedures for discriminating cell cycle phases are discussed below. Although we developed a procedure to study DSB formation in G_2 by PFGE, the technique has limited sensitivity and is not readily applicable for assessment of DSB repair kinetics, partly because G_2-phase cells irradiated with non-physiological doses are prone for apoptosis.[12] PFGE requires the analysis of 10^5–10^6 cells which is a further restriction if limited cell numbers are available.

Analysis of distinct genomic regions. Markers for specific genomic regions, such as heterochromatic chromocentres, allow the analysis of DSB repair in discrete chromatin regions that differ in condensation or transcriptional status.[16] DSB repair can also be examined using siRNA knockdown techniques by analyzing specifically those cells of a population for which knock-down was most efficient.

Visualizing tracks of radiation particles. The ability to visualize the spatial organization of DSBs induced along a track of radiation particles will allow further questions of chromatin movement in a temporal manner to be addressed.[17]

Exploitation of live cell imaging. The generation of cells with fluorescent tagged proteins (such as GFP-labelled 53BP1) allows the visualization of damaged sites and their repair in living cells.[18,19]

The DSB Signalling Response

ATM, ATR and DNA-PK, are the major kinases that phosphorylate H2AX following DNA damage. Extensive research using model organisms and mammalian cells has established the distinct parameters underlying the activation of each kinase. We briefly overview this here, since the detailed current understanding of these processes justifies our contention that γH2AX analysis can be exploited for DSB assessment.

ATM and ATR represent the major damage response signalling kinases. ATM is activated by DNA DSBs whilst ATR is activated by single-stranded DNA (ssDNA) regions that arise from replication fork stalling or processing of bulky lesions. Although ATM can bind to DNA ends, current in vivo, in vitro and structural studies suggest that the Mre11/Rad50/NBS1 (MRN) complex is the DSB sensor that recruits ATM to the DSB, which occurs via its interaction with the C-terminus of NBS1.[20-23] Activated ATM phosphorylates H2AX facilitating the recruitment of MDC1, the ubiquitin ligases RNF8 and RNF168, and the mediator protein, 53BP1.[1] A complex web of interactions between these proteins enhances their retention at the DSB. Studies have suggested that ATM can be activated by chromatin changes in the absence of DSBs but defined ATM-dependent foci only arise at DSBs.[24] Further, a defined and orchestrated process acts to prevent ATM activation at intact telomeres.[25]

In contrast, ATR is activated by ssDNA regions via a process that requires the ATR interacting protein (ATRIP), RPA binding to ssDNA and TopBP1.[26] Efficient phosphorylation of ATR substrates also requires Rad17 and the so-called 9-1-1 complex of Rad9, Hus1 and Rad1. ATR is activated, at least to a low level, in normal S-phase cells, since they show a background lawn of ATR-dependent γH2AX. Current evidence suggests that replication fork stalling leading to ssDNA generation

and ATR activation occurs frequently during normal S-phase progression either at endogenous lesions or at regions that are difficult to replicate.[27] Replication fork collapse may result in one-ended DSBs, which can activate ATM, but can also lead to the activation of homologous recombination without the induction of a DSB.[28] These mechanistic studies suggest that ATR activation requires a significant stretch of ssDNA for RPA binding and that ATR is not activated by single-stranded nicks or small gaps. The precise length of ssDNA required for ATR activation is unclear but is likely to be around, or even greater than, 30 nucleotides.

DNA-PK, encompassing the Ku heterodimer and a catalytic subunit, DNA-PKcs, is the end-binding complex that functions during DNA non-homologous end-joining (NHEJ).[29] Although DNA-PK functions in NHEJ, it has an overlapping role with ATM in phosphorylating H2AX at DSBs. However, it cannot substitute for ATM in phosphorylating other substrates such as Chk2 or p53.[30]

Although ATM and ATR are activated by distinct substrates, there is interplay between them. Most importantly, ssDNA generated by DSB resection can cause ATR activation.[31] Since resection at DSBs is ATM dependent, this results in ATM-dependent ATR activation in G_2 phase following IR. Interestingly, a recent study has suggested that ATM ceases to be activated following extensive resection, suggesting that there could be a switch from ATM to ATR activation in G_2 at resected DSBs.[32] It is important to distinguish this process of ATR activation from ATR activation in cells which were irradiated in G_1 or S phase and subsequently progressed into G_2. In the latter case, ATR activation occurs in S phase and is ATM independent.

In summary, ATM and DNA-PK are activated by DSBs whilst ATR is activated by ssDNA regions. Further, single-stranded nicks, small gaps or base damage are not recognized by any of the PIKKs and do not appear to activate γH2AX formation unless subsequent processing occurs.

IR-Induced γH2AX Foci Analysis

G_0/G_1-phase cells. The lesions induced by IR can be broadly classified into DSBs, SSBs and base damage. Cross link damages can also arise but represent less frequent lesions. The ratio of SSBs:DSBs induced by IR is 20:1. As discussed above, mechanistic studies strongly suggest that neither base damage nor SSBs activate damage response signalling and we provide evidence to support this contention below. We also discuss data that argue that γH2AX foci formation after IR in G_0/G_1 cells represents DSBs and that the kinetics of γH2AX foci loss allows researchers to monitor DSB repair.

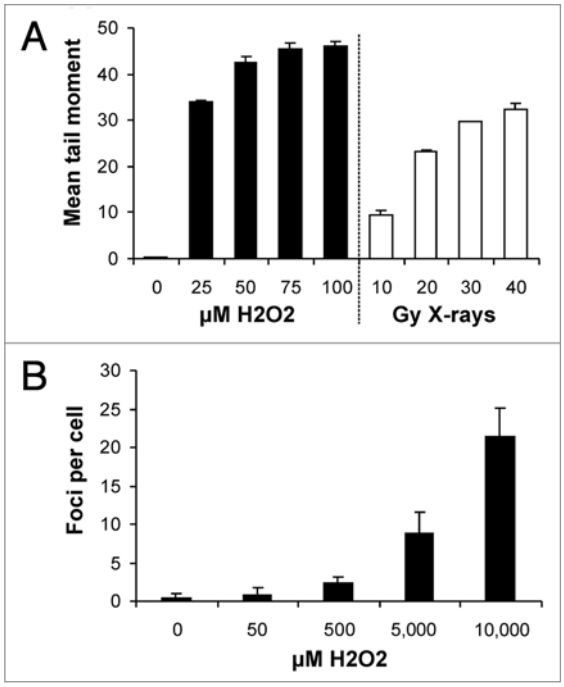

Figure 1. H_2O_2 at a concentration of 25 μM induces a level of SSBs similar to 40 Gy IR, as assessed by the alkaline comet assay at 15 min post treatment (A). In strong contrast, a concentration of 10 mM H_2O_2 is required to induce 20–25 γH2AX foci, a level which is reached after a dose of 1 Gy (B).

The induction of γH2AX foci numbers and estimated DSBs correlate. Although maximum foci numbers are observed by 3 min post IR, their detection is difficult at this time due to their small size and foci are more reliably scored 15–30 min post exposure. Since "simple" DSBs can be repaired rapidly, it is likely that repair occurs during this time and hence that foci numbers at 15–30 min underestimate DSB induction levels. Consistent with this, DSB repair-defective cells frequently harbour enhanced foci numbers at 15–30 min compared to control cells.[33] It has been argued that DSB induction levels estimated by PFGE may be overestimated due to the conversion of labile sites to DSBs. Using human fibroblasts, we routinely observe 15–20 γH2AX foci at 15 min post exposure to 1 Gy. Assuming that some repair occurs in 15 min post IR, this is consistent with the current estimation of 25 DSBs induced per Gy by PFGE.[34] It is worthwhile noting that X-irradiating cells attached to glass cover slides can have a dose doubling impact since photoelectrons arising in the glass material can enhance the damage to cells. This needs to be considered when comparing γH2AX with PFGE data.[35] DNA content will also influence γH2AX foci numbers and we have observed variable induction numbers in transformed cells. Most significantly, the number of γH2AX foci formed is twenty times lower than the estimated SSB induction level.

High SSB induction does not cause γH2AX foci formation. Hydrogen peroxide and other agents inducing oxidative damage produce 2,000 SSBs per DSB.[36] Treatment with 0.1 mM H_2O_2 caused high SSB numbers assessed by comet assay but only few γH2AX foci (**Fig. 1**). Moreover, no foci were detected during repair incubation demonstrating that γH2AX foci are not formed during the processing of SSBs. 1 Gy IR, in contrast,

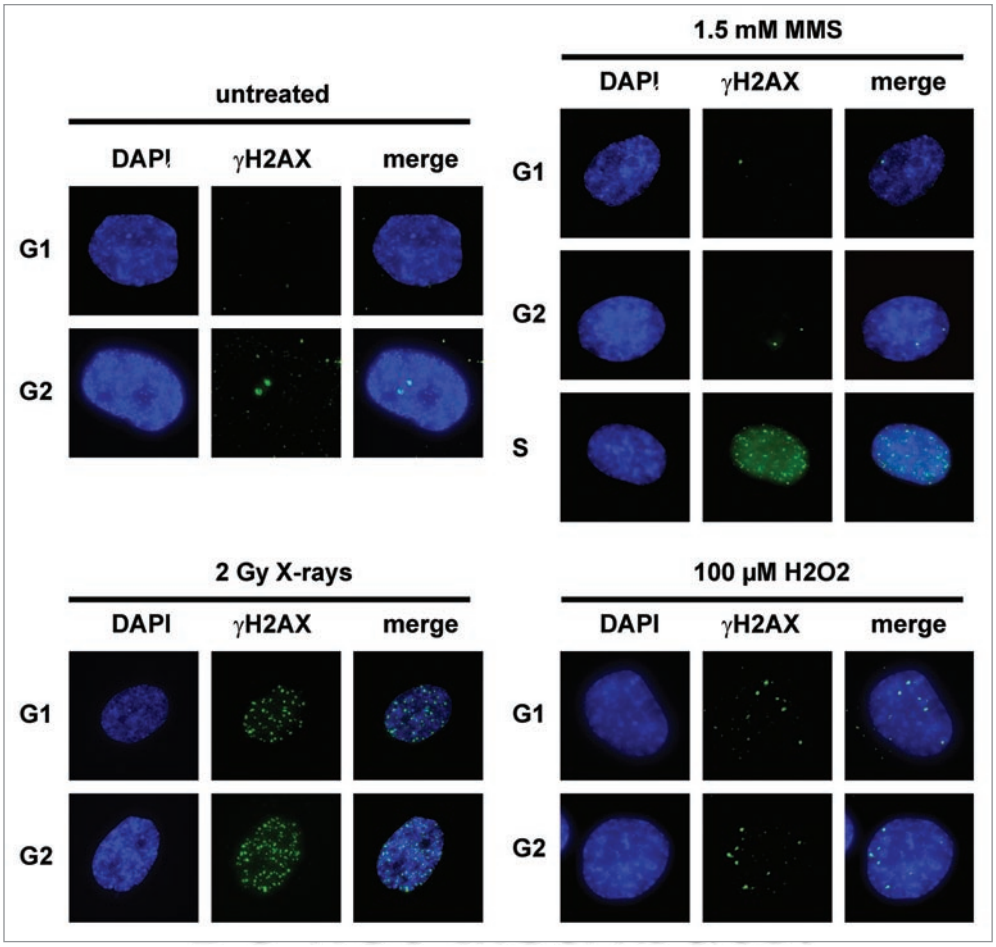

Figure 2. γH2AX foci formation in G_1, S and G_2 human cells 15 min after treatment with various agents. Untreated cells exhibit usually 0 or 1 γH2AX foci in G_1 and a slightly higher level in G_2. After 2 Gy of IR, clear distinct foci are observed which are twice the number in G_2 as in G_1. 1.5 mM of the alkylating agent MMS induces no or very few foci in G_1 or G_2 but significant H2AX phosphorylation in S-phase cells. The S-phase signal shows a pan nuclear lawn likely due to ATR activation at stalled replication forks and distinct foci possibly due to secondary DSBs arising when a replication fork encounters a SSB. 100 μM H_2O_2 induces about 3–6 foci in G_1 and about 6–12 foci in G_2-phase cells. At this concentration of H_2O_2, several thousand SSBs are induced suggesting that overlapping SSBs can cause a few DSBs.

leads to 15–20 γH2AX foci at 15 min but no detectable comet tails, consistent with the higher DSB:SSB induction ratio. At very high H_2O_2 doses, γH2AX foci did form but likely represent DSBs arising from overlapping SSBs since their loss is dependent upon functional DSB repair (data not shown). Further, γH2AX foci do not arise following treatment of G_0/G_1 cells with the alkylating agent, methyl methanesulphonate (MMS), although pan nuclear phosphorylation readily forms in S-phase cells (**Fig. 2**). These findings are consistent with the notion that γH2AX foci in G_0/G_1 cells do not form at SSBs or at alkylation damage but can arise when such lesions are replicated.

IR-induced γH2AX foci are ATM/DNA-PK dependent and ATR independent. We have previously shown that in G_0/G_1 cells γH2AX foci formation after IR is redundantly dependent upon ATM or DNA-PK, that loss of both kinases abolishes foci formation and that ATR deficiency is without impact despite its impact on H2AX formation after HU or UV treatment.[30]

γH2AX foci loss is dependent upon known DSB repair components. NHEJ represents the major process repairing DSBs in G_0/G_1 phase.[37] DNA ligase IV (LigIV) is an essential NHEJ protein and $LigIV^{-/-}$ cells show little diminution of γH2AX foci numbers up to 7 days post IR (**Fig. 3**).

γH2AX foci loss closely correlates with the rate of DSB repair estimated by PFGE. Studies using a range of physical methods have shown that DSBs are repaired more slowly than SSBs and with a fast and slow component. γH2AX foci loss also occurs with two component kinetics with relative ratios that correlate with those estimated by PFGE studies (**Fig. 3**). The kinetics are quite distinct to the rate of SSB repair (half life < 30 min). Further, DSB repair-deficient cell lines show similar DSB repair defects when monitored by either PFGE or γH2AX foci analysis.

S-phase cells. Although S-phase cells are characterized by weak pan-nuclear γH2AX, defined foci can be discerned after IR. Since IR-induced lesions likely cause replication fork stalling or collapse, it is currently difficult and beyond the scope of this paper to evaluate the specificity of the γH2AX foci assay after IR of S-phase cells. Nevertheless, we observed

that IR-induced foci in S (at 15 min post IR) are ATM and DNA-PK dependent and ATR independent whilst foci arising after agents which do not induce direct DSBs (e.g., MMS) are only abolished when all three kinases are inactivated (unpublished findings). This suggests that even in S phase the majority of IR-induced foci represent direct DSBs, at least at short times after IR.

G_2-phase cells. DSB formation and repair in G_2 differs from that in G_1 in several ways. Firstly, homologous recombination (HR) can function in G_2 due to cell cycle-dependent resection. Secondly, S-phase cells can progress into G_2 during repair analysis. As discussed above, ATR activation can occur in S-phase cells at stalled replication forks leading to non-DSB-dependent γH2AX formation. Below we discuss the evidence that under conditions that avoid analysis of S-phase cells, IR-induced γH2AX foci analysis in irradiated G_2-phase cells can be used to monitor DSB formation and repair.

γH2AX foci induction in G_2-phase cells correlates with predicted DSB formation. γH2AX foci number in G_2 are almost exactly double those obtained in G_1-phase cells, consistent with the doubling in DNA content.[12]

High SSB induction by H_2O_2 treatment yields few γH2AX foci. Following exposure to 10 μM H_2O_2, we observed no detectable γH2AX phosphorylation in G_2 as in G_1 strongly suggesting that SSBs do not lead to γH2AX formation in G_2. Exposure to higher doses (>100 μM) produced foci numbers approximately double that observed in G_1. Similarly, MMS does not cause H2AX phosphorylation in G_2 (**Fig. 2**).

ATR contributes to γH2AX formation at later times in G_2-phase cells. At 30 min post IR, γH2AX foci formation was ATM and DNA-PK dependent and ATR independent, identical to the situation in G_1-phase cells. However, by 6 h post IR, foci were observed in ATM/DNA-PK inhibitor treated cells and, conversely, foci number or their size were reduced in ATR-deficient cells (**Fig. 4**). Although γH2AX phosphorylation in G_2 is ATR dependent at 6 h, foci numbers are approximately double in G_2 versus G_1 cells and the rate of repair is similar (although subtly different). These findings are consistent with the notion that γH2AX foci in G_2 as in G_1 form only at DSBs and that ATR signalling can contribute as resection ensues. It is also noteworthy that, although ATR activation is ATM dependent at early times post IR, there appears to be ATR activation at later times in the absence of ATM suggesting that, although resection is largely ATM dependent, slow and inefficient resection can occur in its absence.

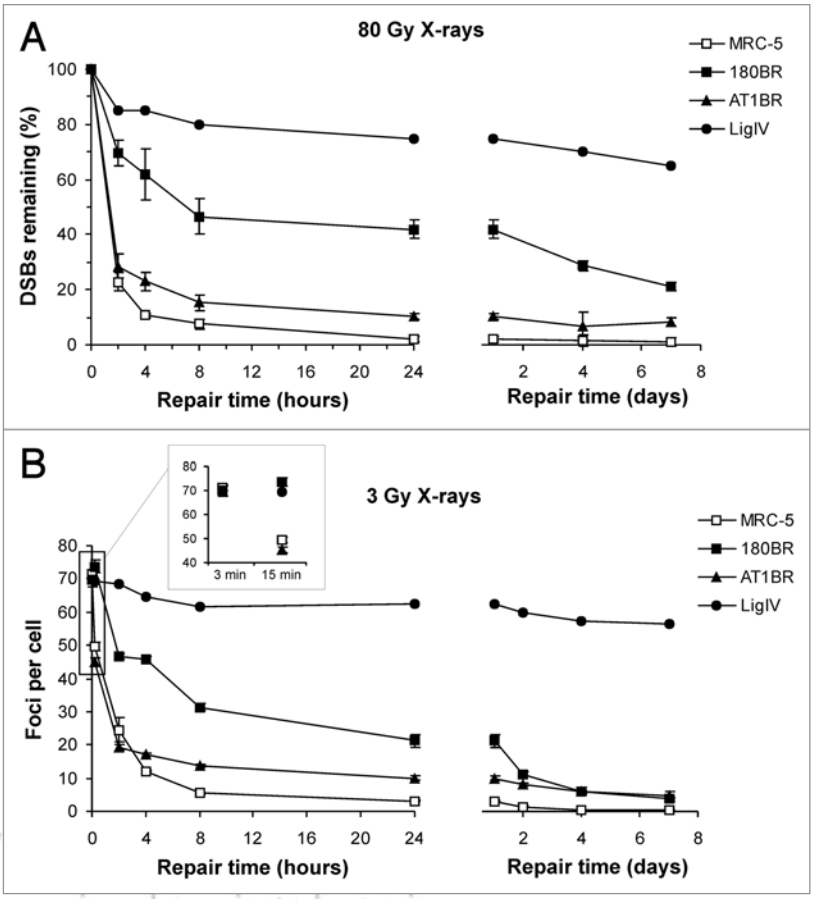

Figure 3. The rate of loss of γH2AX foci correlates with the physical joining of DSBs assessed by PFGE. Cells were grown to confluency, irradiated with 80 or 3 Gy and analysed by PFGE (A) or with the γH2AX foci assay (B). Wild-type human fibroblasts (MRC-5) exhibit a fast repair component during the first 2–4 h and a second much slower component during the following hours. Human A-T cells (AT1BR) fail to repair a subset of approximately 10–15% of the breaks which are repaired with slow kinetics in wt cells. Human cells with a hypomorphic mutation in DNA ligase IV (180BR), which compromises but does not abolish LigIV function, show substantially impaired repair. The residual rejoining is particularly obvious at prolonged repair times and lower doses. In contrast, LigIV knock-out mouse cells are nearly completely deficient in DSB repair. The data were redrawn from previous publications.[13,33]

γH2AX foci loss is substantially dependent upon LigIV in G_2 phase. The rate of γH2AX foci loss in G_2-phase $LigIV^{-/-}$ cells is similar to, although slightly greater than, that observed in G_1.[38] These findings suggest that NHEJ represents the major DSB repair pathway in G_2 phase and substantiate the notion that radiation-induced γH2AX foci in G_2 represent DSBs. The slightly greater rate of foci loss in G_2-phase $LigIV^{-/-}$ cells likely represents repair by HR. Thus, although the analysis with $LigIV^{-/-}$ cells cannot allow the conclusion that all γH2AX foci in G_2 represent DSBs, the results are consistent and, indeed, supportive of that notion.

Persistent γH2AX foci correlate with persistent DSBs and chromosome breaks analysed by PFGE and PCC, respectively. Although PFGE analysis in G_2-phase cells has limitations, it has demonstrated a subtle DSB repair defect in ATM and Artemis-deficient cells consistent with γH2AX foci

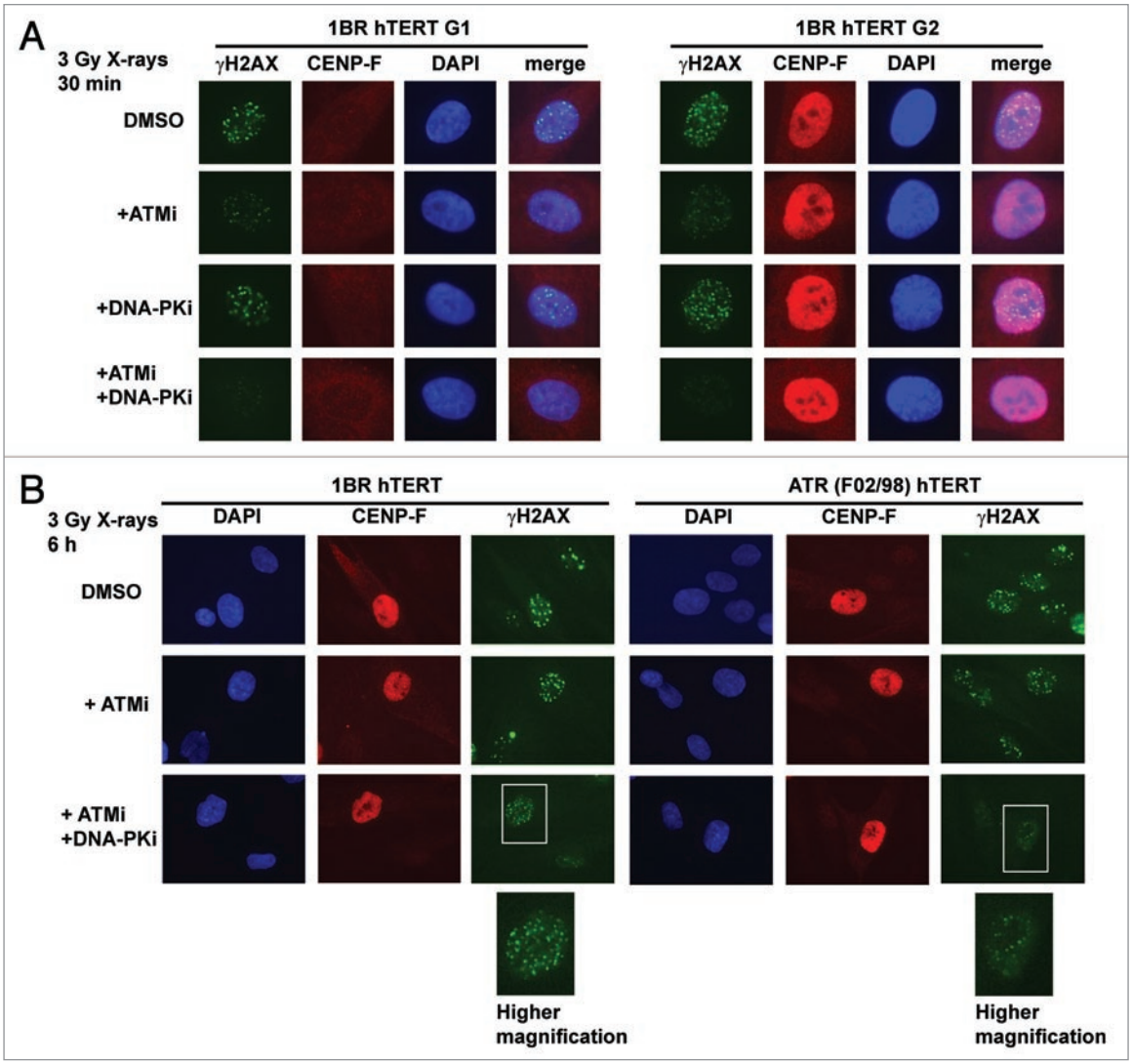

Figure 4. (A) γH2AX foci formation at early times post IR is completely abolished in G_1- and G_2-phase wt cells (1BR hTert) treated with an ATM and DNA-PK inhibitor. (B) At later times post IR (6 h) foci arise in G_2 phase despite ATM and DNA-PK inhibition. These foci are significantly reduced in ATR-deficient cells (F02/98 hTert). ATR mutants show normal γH2AX phosphorylation at early times.

data.[12] Furthermore, calyculin A-induced PCC analysis has also shown that unrepaired DSBs persist in G_2-phase ATM and Artemis-deficient cells. Thus, genetic situations leading to γH2AX foci persistence in G_2 also lead to persistent DSBs assessed by PFGE or PCC breakage.

Optimization of γH2AX Foci Analysis

Below, we consider important parameters that we have found help to enhance the sensitivity of our analysis as well as to limit the analysis of non-DSB lesions.

Consideration of cell cycle progression. Following irradiation, cells can progress from one cell cycle phase to another. This is important at prolonged times post irradiation when cells with γH2AX activated by replication stalling, which may not represent DSBs, progress from S into G_2 phase. One way to avoid difficulties of cell cycle progression is to examine G_0-phase cells. However, for studying G_2-phase repair, cells have to be maintained in G_2 from the time of irradiation to analysis and the entry of S-phase cells into G_2 needs to be prevented. This has been achieved by using aphidicolin, an inhibitor of replication polymerases. Importantly, aphidicolin does not affect DSB repair in G_2 and does not appear to inhibit polymerases involved in homologous recombination.[38] A second consideration for studying repair in G_2 for prolonged times is the duration of G_2 checkpoint arrest, which is dependent on dose and repair capacity with checkpoint release occurring at a threshold level of 10–20 DSBs.[39,40] This allows the analysis of G_2-phase cells irradiated with 1 Gy for up to 8 h. Studies of longer repair times require higher doses.

Identification of cell cycle phases. Since most methods to synchronize cells induce γH2AX foci, we utilize asynchronous populations and cell cycle specific markers. Established G_2 markers are CENP-F or serine 10 phosphorylation of histone H3 (pH3), the latter providing a focal signal in G_2 which is distinct from

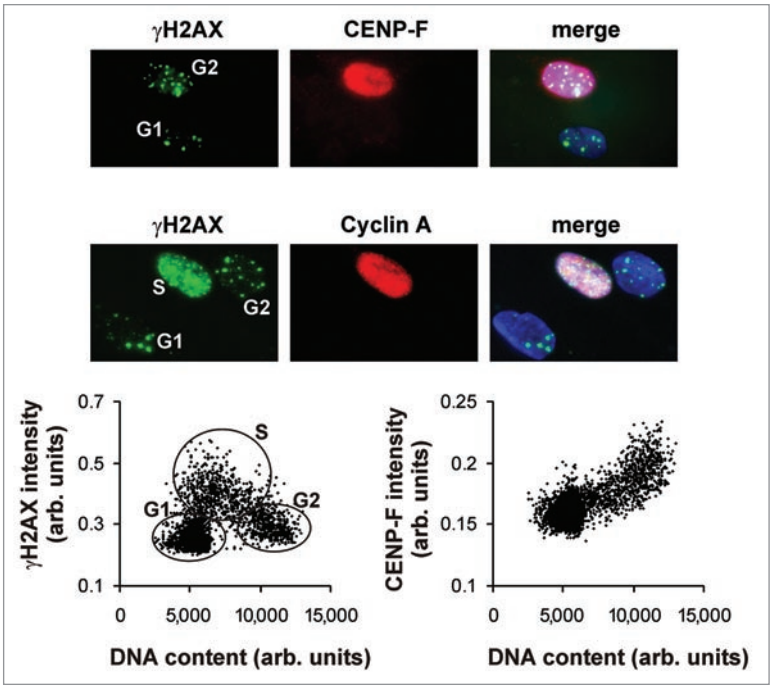

Figure 5. Cell cycle phases are readily distinguished using specific markers or by quantifying the DAPI signal intensity. G_2-phase human cells are positive for CENP-F, G_1 cells are negative. S-phase cells are positive for cyclin A. The images shown represent a cell population treated with aphidicolin, an inhibitor of replicative polymerases. As a consequence of this, S phase cells show pronounced γH2AX phosphorylation. The DAPI signal can also be quantified under the microscope. A typical dot-blot showing γH2AX signal as a function of DAPI is shown in the lower left panel. The G_1-, S- and G_2-phase populations can be readily distinguished which is confirmed by the lower right panel showing that cells with higher DAPI content are high in CENP-F signal. The picture was redrawn from a previous publication.[1,2]

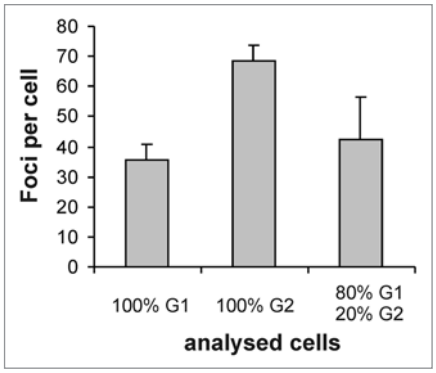

Figure 6. A cell population of 80% G_1- and 20% G_2-phase cells is compared with a population of only G_1-phase or only G_2-phase cells. The mixed population exhibits considerably higher error bars (standard deviation) demonstrating the benefit of a cell cycle specific analysis. The foci values were taken from a typical experiment analyzing 40 human cells at 15 min after 2 Gy IR.

the pronounced pan-nuclear pH3 signal of mitotic cells. Cyclin A is a marker of S-phase cells. However, S-phase cells can usually be readily identified by pan-nuclear γH2AX signal, most particularly when aphidicolin is used. Cell cycle phases can also be identified under the microscope by assessing the magnitude of the DAPI signal, which is particularly useful if limited antibody combinations are available for cell cycle phase identification and foci measurements (**Fig. 5**).

Concomitant analysis of G_1- and G_2-phase cells. The analysis of cells in distinct cell cycle phases (and the usage of appropriate cell lines which can be maintained in specific cell cycle phases) is particularly important to improve the accuracy of measurement as even a low percentage of G_2-phase cells in a population (with twice the number of foci) will significantly enlarge the error bars (**Fig. 6**).

Choice of cell line. Primary cells with low background numbers of γH2AX foci are particularly beneficial for analysis. However, they are less suitable for siRNA experiments. For such approaches, transformed cells are used but they can have significant problems of causing high background foci numbers. We have successfully exploited hTert lines, which normally have lower background foci numbers than transformed cells but are suitable for siRNA analysis. Since γH2AX foci form at uncapped telomeres, care should be taken with senescent cells. A further difficulty of using tumour or transformed cells, particularly if they are p53 deficient, is their rapid cell cycle progression and failure to remain for prolonged times in a single cell cycle phase.

Foci scoring and data evaluation. The statistical error obtained from several independent experiments provides a measure for the reproducibility of the data. However, statistical analysis, such as the student's t-test, should be performed at critical time points to evaluate the significance of differences in foci level. Another important aspect is that samples are scored in a blinded manner to avoid bias. Alternatively, software approaches are now available providing the opportunity to perform automated foci scoring. The microscopic facility used also determines the foci limit up to which scoring can be performed reliably and reproducibly. Pilot experiments measuring foci numbers as a function of dose can be performed to evaluate the dose range yielding linearity between foci number and dose.

Limitations of γH2AX Foci Analysis

γH2AX foci do not always represent DSB formation. This limitation has been discussed in detail above. This review has focused on the use of the technique for monitoring radiation-induced DSBs. For other agents, a careful consideration of the lesions formed and their repair needs to be considered.

γH2AX foci analysis represents an indirect monitor of DSB formation. Whilst this is a valid concern, it need not limit the utility of the procedure. It is important to consider, however, that genetic factors may influence the ability to form γH2AX (e.g., the presence of H2AX) and to remove the phosphorylated residues after repair (e.g., protein phosphatases).

The timing of foci loss does not fully correlate with DSB repair. Currently, it is unclear whether loss of visible foci precisely correlates with the final step in the rejoining process or whether a delay of 1–2 h occurs due to the additional time required to fully reconstitute the chromatin status.[4] However, this does not negate the utility of the analysis to monitor the process of DSB repair as long as the caveats are considered carefully during interpretation.

Summary

γH2AX foci analysis is an important outcome of many years of research into the DNA damage responses. It has enhanced our ability to detect DNA damage in individual cells enormously and has led to novel insight into the DNA damage response. Laboratories are now trying to exploit the technique for bio-monitoring purposes and for further research avenues. The approach, however, can be readily misused. Nonetheless, it is far too valuable a technique to be disregarded. A detailed understanding of the molecular steps leading to γH2AX formation is important to allow a rational exploitation of the technique. We argue that the approach has the capacity to monitor DSB formation and repair after exposure to ionizing radiation and should be exploited provided careful consideration is given to the restraints of the procedure.

References

1. Panier S, Durocher D. Regulatory ubiquitylation in response to DNA double-strand breaks. DNA Repair 2009; 8:436-43.
2. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000; 10:886-95.
3. Rothkamm K, Lobrich M. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Proc Natl Acad Sci USA 2003; 100:5057-62.
4. Kinner A, Wu W, Staudt C, Iliakis G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res 2008; 36:5678-94.
5. Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, Pommier Y. GammaH2AX and cancer. Nat Rev Cancer 2008; 8:957-67.
6. Sedelnikova OA, Bonner WM. GammaH2AX in cancer cells: a potential biomarker for cancer diagnostics, prediction and recurrence. Cell Cycle 2006; 5:2909-13.
7. Iliakis G, Cicilioni O, Metzger L. Measurement of DNA double strand breaks in CHO cells at various stages of the cell cycle using pulse field gel electrophoresis: Calibrations by means of 125I decay. Int J Radiat Biol 1991; 59:343-57.
8. Gotoh E, Durante M. Chromosome condensation outside of mitosis: mechanisms and new tools. J Cell Physiol 2006; 209:297-304.
9. Lobrich M, Rief N, Kuhne M, Heckmann M, Fleckenstein J, Rube C, Uder M. In vivo formation and repair of DNA double-strand breaks after computed tomography examinations. Proc Natl Acad Sci USA 2005; 102:8984-9.
10. Reaper PM, di Fagagna F, Jackson SP. Activation of the DNA damage response by telomere attrition: a passage to cellular senescence. Cell Cycle 2004; 3:543-6.
11. Pospelova TV, Demidenko ZN, Bukreeva EI, Pospelov VA, Gudkov AV, Blagosklonny MV. Pseudo-DNA damage response in senescent cells. Cell Cycle 2009; 8: 4112-8.
12. Deckbar D, Birraux J, Krempler A, Tchouandong L, Beucher A, Walker S, et al. Chromosome breakage after G_2 checkpoint release. J Cell Biol 2007; 176:748-55.
13. Riballo E, Kuhne M, Rief N, Doherty A, Smith GC, Recio MJ, et al. A pathway of double-strand break rejoining dependent upon ATM, Artemis and proteins locating to gamma-H2AX foci. Mol Cell 2004; 16:715-24.
14. Dikomey E, Dahm-Daphi J, Brammer I, Martensen R, Kaina B. Correlation between cellular radiosensitivity and non-repaired double-strand breaks studied in nine mammalian cell lines. Int J Radiat Biol 1998; 73:269-78.
15. Foray N, Priestley A, Alsbeih G, Badie C, Capulas EP, Arlett CF, Malaise EP. Hypersensitivity of ataxia-telangiectasia fibroblasts to ionizing radiation is associated with a repair deficiency of DNA double-strand breaks. Int J Radiat Biol 1997; 72:271-83.
16. Goodarzi AA, Noon AT, Deckbar D, Ziv Y, Shiloh Y, Lobrich M, Jeggo PA. ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Mol Cell 2008; 31:167-77.
17. Jakob B, Scholz M, Taucher-Scholz G. Biological imaging of heavy charged-particle tracks. Radiat Res 2003; 159:676-84.
18. Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, Lukas J. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol 2006; 173:195-206.
19. Mari PO, Florea BI, Persengiev SP, Verkaik NS, Bruggenwirth HT, Modesti M, et al. Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. Proc Natl Acad Sci USA 2006; 103:18597-602.
20. Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. EMBO J 2003; 22:5612-21.
21. Williams RS, Moncalian G, Williams JS, Yamada Y, Limbo O, Shin DS, et al. Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. Cell 2008; 135:97-109.
22. Williams RS, Dodson GE, Limbo O, Yamada Y, Williams JS, Guenther G, et al. Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. Cell 2009; 139:87-99.
23. Falck J, Coates J, Jackson SP. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature 2005; 434:605-11.
24. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003; 421:499-506.
25. Dimitrova N, de Lange T. Cell cycle-dependent role of MRN at dysfunctional telomeres: ATM signaling-dependent induction of nonhomologous end joining (NHEJ) in G_1 and resection-mediated inhibition of NHEJ in G_2. Mol Cell Biol 2009; 29:5552-63.
26. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 2003; 300:1542-8.
27. Meister P, Taddei A, Ponti A, Baldacci G, Gasser SM. Replication foci dynamics: replication patterns are modulated by S-phase checkpoint kinases in fission yeast. EMBO J 2007; 26:1315-26.
28. Mizuno KL S, Baldacci G, Murray JM, Carr AM. Nearby inverted repeats fuse to generate acentric and dicentric palindromic chromosomes by a replication template exchange mechanism. Genes Dev 2009; 23:2876-86.
29. Wyman C, Kanaar R. DNA double-strand break repair: all's well that ends well. Annu Rev Genet 2006; 40:363-83.
30. Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Lobrich M, Jeggo PA. ATM and DNA-PK function redundantly to phosphorylate H2AX following exposure to ioninsing radiation. Cancer Res 2004; 64:2390-6.
31. Jazayeri A, Falck J, Lukas C, Bartek J, Smith GC, Lukas J, Jackson SP. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol 2006; 8:37-45.
32. Shiotani B, Zou L. Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. Mol Cell 2009; 33:547-58.
33. Kuhne M, Riballo E, Rief N, Rothkamm K, Jeggo PA, Lobrich M. A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. Cancer Res 2004; 64:500-8.
34. Rydberg B. Radiation-induced heat-labile sites that convert into DNA double-strand breaks. Radiat Res 2000; 153:805-12.
35. Kegel P, Riballo E, Kuhne M, Jeggo PA, Lobrich M. X-irradiation of cells on glass slides has a dose doubling impact. DNA Repair 2007; 6:1692-7.
36. Bradley MO, Kohn KW. X-ray induced DNA double-strand break production and repair in mammalian cells as measured by neutral filter elution. Nucleic Acids Res 1979; 7:793-804.
37. Rothkamm K, Kruger I, Thompson LH, Löbrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 2003; 23:5706-15.
38. Beucher A, Birraux J, Tchouandong L, Barton O, Shibata A, Conrad S, et al. ATM and Artemis promote homologous recombination of radiation-induced DNA double-strand breaks in G_2. EMBO J 2009; 28:3413-27.
39. Lobrich M, Jeggo PA. The impact of a negligent G_2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer 2007; 7:861-9.
40. Krempler A, Deckbar D, Jeggo PA, Lobrich M. An imperfect G_2M checkpoint contributes to chromosome instability following irradiation of S and G_2 phase cells. Cell Cycle 2007; 6:1682-6.