RADIATION RESEARCH **161**, 247–255 (2004)
0033-7587/04 $15.00

REVIEW

Low-Dose Hyper-radiosensitivity: A Consequence of Ineffective Cell Cycle Arrest of Radiation-Damaged G_2-Phase Cells

B. Marples,[a,1] B. G. Wouters,[b] S. J. Collis,[c] A. J. Chalmers[d] and M. C. Joiner[a]

[a] *Karmanos Cancer Institute, Wayne State University, Detroit, Michigan;* [b] *MAASTRO Laboratory, University of Maastricht, Maastricht, The Netherlands;* [c] *The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Department of Radiation Oncology, Baltimore, Maryland; and* [d] *Radiotherapy Department, The Royal Marsden NHS Trust, Fulham Road, London, United Kingdom*

Marples, B., Wouters, B. G., Collis, S. J., Chalmers, A. J. and Joiner, M. C. Low-Dose Hyper-radiosensitivity: A Consequence of Ineffective Cell Cycle Arrest of Radiation-Damaged G_2-Phase Cells. *Radiat. Res.* 161, 247–255 (2004).

This review highlights the phenomenon of low-dose hyper-radiosensitivity (HRS), an effect in which cells die from excessive sensitivity to small single doses of ionizing radiation but become more resistant (per unit dose) to larger single doses. Established and new data pertaining to HRS are discussed with respect to its possible underlying molecular mechanisms. To explain HRS, a three-component model is proposed that consists of damage recognition, signal transduction and damage repair. The foundation of the model is a rapidly occurring dose-dependent pre-mitotic cell cycle checkpoint that is specific to cells irradiated in the G_2 phase. This checkpoint exhibits a dose expression profile that is identical to the cell survival pattern that characterizes HRS and is probably the key control element of low-dose radiosensitivity. This premise is strengthened by the recent observation coupling low-dose radiosensitivity of G_2-phase cells directly to HRS. The putative role of known damage response factors such as ATM, PARP, H2AX, 53BP1 and HDAC4 is also included within the framework of the HRS model.

INTRODUCTION

The response of a population of cells to radiation exposure can be affected by passive biological factors (e.g. disparate cell cycle phase radiosensitivities) and/or physical environmental conditions (e.g. redox status). However, intrinsic cellular radiation sensitivity is largely determined by the efficiency and fidelity of dynamic processes that control and govern cell cycle arrest, DNA repair and apoptosis. Many radiation-induced lesions in DNA (e.g. base damage, single-strand breaks) are repaired readily by error-free mechanisms and therefore have little consequence for survival, mutagenesis or genomic instability in repair-competent cells. In contrast, radiation-induced DNA double-strand breaks (DSBs) are prime lesions in radiation-induced inactivation, and failure to repair (or the misrepair of) DSBs is a prime cause of cell death even in the context of repair-proficient cells (*1*). Consequently, conserved cellular processes have evolved to facilitate sensitive detection and activation of DSB repair pathways to preserve genomic integrity. These pathways consist of integrated responses that associate DNA damage recognition with signal transduction pathways that, particularly for ionizing radiation, activate cell cycle arrest, apoptosis and DNA repair machinery. The coordination of these processes ensures that cell cycle progression is halted or slowed after the detection of DNA damage, to allow time for genome repair prior to critical events such as cell division or DNA replication. Recently, considerable progress has been made establishing the molecular associations between damage recognition, checkpoint arrest and damage repair that occur in the response of cells to high-dose radiation injury. However, the way in which these processes dictate and control the response of cells at low radiation doses remains poorly understood.

RADIATION-ACTIVATABLE PROTECTIVE SYSTEMS

It has become clear that the restoration of genomic integrity after DNA damage does not occur through constitutively active cellular processes. Rather, cells have developed highly specialized pathways to recognize DNA damage and ''activate'' downstream molecular pathways. The induction of these ''protective'' responses after radiation exposure has been described at both the molecular and whole cell levels (see refs. *2–4* for reviews). After irradiation, an ordered sequence of biological events is rapidly induced that includes the molecular monitoring of lesions, the initiation of cell cycle arrest and damage repair processes, each of which is brought into play in a measured

[1] Address for correspondence: Radiation Biology Group, Karmanos Cancer Institute, Hudson Webber Building, Room 824, 4100 John R., Detroit, MI 48201-2013; e-mail: marplesb@kci.wayne.edu.

manner as needed (for a review see ref. *5*). Such an ''*active*'' response from irradiated cells is thought to contribute to the highly non-linear relationship that has been observed between measurements of (log) cell survival and radiation dose. This is particularly true for radiation doses less than 1 Gy, for which cells are very often hypersensitive to cell killing. Low-dose hyper-radiosensitivity (HRS) and the ''adaptive response'' are likely two examples of the distinct manifestations of such ''*active*'' responses to low-dose radiation exposure (see reviews in refs. *2, 4, 6*).

LOW-DOSE HYPER-RADIOSENSITIVITY (HRS)

In vitro radiation sensitivity is typically measured by clonogenic assay in a manner similar to that first described by Puck and Marcus (*7*). This well-established method determines the ability of irradiated cells to form colonies of 50 cells or more. However, the traditional implementation of this technique lacks the resolution to measure radiosensitivity at doses below 1 Gy with high precision due to statistical uncertainties associated with cell plating (*8*). This ''low-dose'' limitation can be overcome using cell plating approaches that use a microscope relocation technique (*9*) or flow cytometry-based methods (*10, 11*) to assess the exact number of cells at risk. Using the former technique, V79 Chinese hamster cells were initially discovered to be hypersensitive to radiation doses below 25 cGy, a phenomenon that has since become known as low-dose hyper-radiosensitivity (HRS) (*9*). As the radiation dose is increased to 1 Gy, the cell population becomes increasingly resistant per unit dose (Fig. 1), a phenomenon that was named increased radioresistance (IRR). Although the observation of these two phenomena was novel for mammalian cells (*9*), atypical survival responses akin to HRS/IRR had been reported previously in lower cell systems (e.g. *12–14*; reviewed in ref. *15*).

HRS has been reported in cells of more than 40 X-irradiated human cell lines (see reviews in refs. *4, 6*) and for different radiation qualities and biological end points from several independent laboratories (*16–24*). The requirement for nuclear-located radiation injury, and by implication DNA damage (see also *The Dependence of HRS on LET Implicates DNA Repair* below), was eloquently demonstrated in recent charged-particle microbeam studies by Schettino and colleagues (*25*). The prevalence of HRS (*4*) indicates that this phenomenon is, in all probability, the default response of cells to small doses of both high- and low-LET ionizing radiations. The minority of cell lines that do not exhibit HRS have a survival response usually described by a pure linear-quadratic equation; for example, we (*26*) and others (*27*) have consistently failed to detect an HRS response in Chinese hamster ovary or human U373 glioma cells. The explanation why some cell lines do not show HRS undoubtedly reflects either deregulation of the underlying molecular pathway or alternatively the dominance of a subpopulation of cells which lack such a response due to their position in the cell cycle (see *Transducer Signal: Role of Checkpoints* below and ref. *28*).

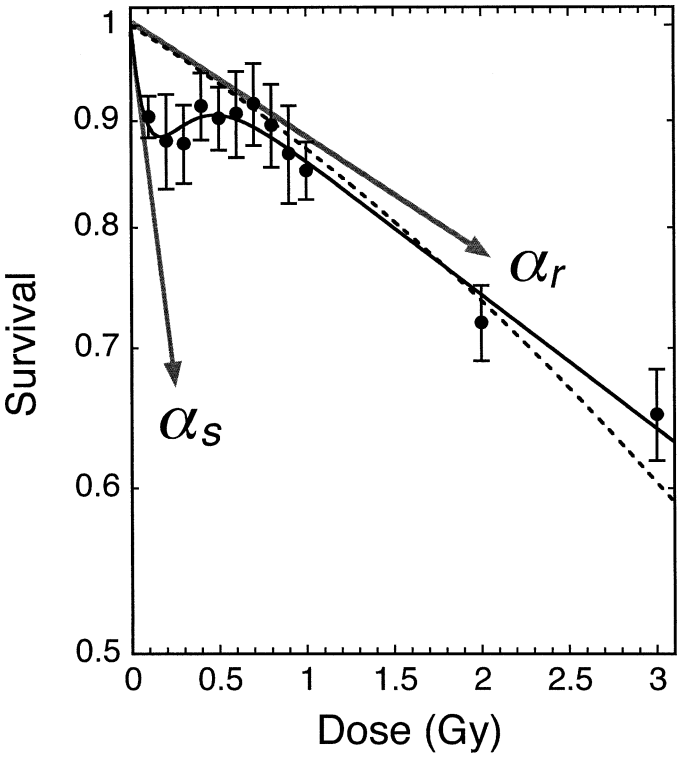

FIG. 1. Low-dose clonogenic cell survival of V79 hamster fibroblasts irradiated with 240 kVp X rays. Each point represents the mean of six individual experiments (±SD). The solid line shows the fit to the data set using the Induced Repair model (*9*). The dashed line represents the fit using the linear-quadratic model over the dose range of 0.5–10 Gy (data for 4–10 Gy not shown). The α_s represents the low-dose value of α (derived from the response at very low doses) while α_r is the value extrapolated from the conventional high-dose response. The ratio of α_s/α_s is used as a measure of the HRS response (*4*).

The extent of the HRS response varies considerably between different cell lines, being more prominent in malignant cells than normal tissues (*4*). The α_s/α_r ratio (see Fig. 1), which compares the initial slopes of the fits for low- and high-dose radiation to the cell survival curve, has been adopted as quantitative measure of HRS, typically varying within the range of 7–10. However, no clear association has been found between the α_s/α_r ratio and other commonly investigated measures of radiosensitivity such as cell pedigree, apoptosis, radiation-induced G_1 cell cycle arrest, or TP53 status (*4*). Interestingly, the α_s/α_r ratio shows a significant but weak correlation with surviving fraction at 2 Gy ($P = 0.023$, $r^2 = 0.36$). This suggests that the radioresistant phenotype that is induced after the inactivation of HRS may relate to overall high-dose intrinsic radiosensitivity.

Two independent groups have reported hyper-radiosensitivity after fractionated X irradiation in a tumor model system (*24, 29*), and to a lesser extent in normal epithelial tissue during radiotherapy (*30, 31*), indicating recoverability of HRS between fractions. This offers the intriguing

possibility that radiotherapy treatment regimens could be devised that consist of multiple small dose fractions (<0.5 Gy) to exploit low-dose hyper-radiosensitivity. However, for this to be realized clinically, a better understanding of the mechanisms underlying HRS (and IRR) is needed. A number of hypotheses have been proposed, but the precise explanation remains unclear (*4, 6, 32, 33*). The balance of evidence suggests that DNA repair itself or process(es) associated with the repair of radiation-induced DNA damage are important (*4, 6, 34*). For example, the development of IRR requires a process(es) that involves poly(ADP-ribose) polymerase 1 (PARP) (*26, 33*) and functional DNA-PK activity (*34, 35*), implying a role for the recognition and repair of DNA damage.

HRS: THE UNDERLYING MECHANISM

This review brings together historical data and our recent observations, and those of others, which now form the framework of an improved understanding of the putative underlying mechanism of HRS. Adopting the nomenclature of others (*5*), the mechanistic process of HRS/IRR can be operationally divided into three response phases after the production of radiation-induced damage in DNA: (1) sensor stage, (2) transducer stage and (3) effector stage. The recognition of DNA damage is mediated by the *sensor stage*; this activates one or more downstream signaling pathways (the *transducer stage*), leading to a measurable biological change in behavior such as apoptosis, cell cycle arrest, or DNA repair (*effector stage).*

The Dependence of HRS on LET Implicates DNA Repair

HRS was originally observed in V79 cells irradiated with single acute doses of X rays (*9*). The X-ray survival response over 0–20 cGy was almost indistinguishable from that of high-LET d(4)-Be neutrons, producing a relative biological effectiveness (RBE) of close to 1. However, as the X-ray dose was increased up to 1 Gy, the survival curve became less steep (the IRR response), while the survival curve for neutrons continued with the same slope, giving rise to a maximal RBE of approximately 3 at 1 Gy. Since the very low-dose survival response of cells (0–10 cGy) appeared to be independent of LET, the IRR component was considered a phenomenon specific to low-LET radiation injury, perhaps accounting for the curvature on survival curves for low-LET radiation. This hypothesis gained some support when a correspondence was observed between the extent of IRR and LET (*16, 36*). However, a likely HRS/IRR response was evident after low-dose-*rate* high-LET neutrons, indicating that IRR was a general response to radiation injury (*21*). The apparent contradiction between the original data for acute-dose-rate neutrons (*28*) and those of Dionet *et al.* (*21*) using low-dose-rate neutrons can be resolved by considering the potential involvement of DNA repair processes in the outcome of irradiated cells in these contexts. The severe nature of the complex DNA lesions produced by acute-dose-rate neutrons renders the activation of DNA repair pathways irrelevant since these processes are unable to repair the damage induced by this type of radiation. We verified this hypothesis in dual dosing experiments; cells that survived a small acute dose of neutrons were adapted to subsequent doses of lower-LET radiation, suggesting that damage produced by high-LET radiation can activate DNA repair processes that subsequently protect cells against the repairable damage produced by the low-LET radiation (*16*).

Sensitivity of Damage Recognition: The Requirement for a Threshold Level of DNA Damage

The phenomenon of increased radioresistance (IRR) is not unexpected in mammalian cells in light of historical reports in lower organisms (see review in ref. *15*) and from observations assessing the radiosensitivity of insect cells (*14, 37*). Indeed, the concept of needing to exceed a threshold level of radiation injury for the full induction of repair processes was established by the work on the adaptive response (see reviews refs. *2, 38*). In an experimental design akin to that used in the ''adaptive'' studies, pre-exposing cells with small priming doses of X rays was shown to eliminate the HRS response in subsequently X-irradiated cell populations (*39*). Priming V79 cells with 20 cGy, a dose above the putative threshold for induction of IRR (d_c) of ~10 cGy, induced resistance to a second radiation dose given within a few hours, thereby eliminating HRS to the second exposure. In contrast, a priming dose of 5 cGy (below the d_c induction threshold) produced limited protection against a second exposure. The dose dependence of the ''protective'' effect induced by the priming treatments in V79 cells supported the concept of a dose threshold for induction of IRR in the single-dose studies. The d_c value estimated for any given cell type is likely to vary slightly between repeat measurements since the transition between the HRS and IRR survival responses is thought to be exquisitely dependent on several ''*active*'' biological factors (e.g. cycle cell phase distribution, cell cycle progression). The distribution of cells within the different phases of the cell cycle is likely to be the largest source of variability in estimating the value of d_c, with the proportion of G_2-phase cells in the population being the most important (see below). In our laboratory, we have observed that the d_c parameter can vary a few centigrays between different exponentially growing populations of V79 cells (unpublished results).

The ''threshold'' hypothesis was consolidated when pretreatment with a low concentration of hydrogen peroxide did not eliminate HRS, in contrast to the effect seen using a 100-fold higher concentration (*39*). Moreover, these data for H_2O_2 suggested that the formation of DNA (double) strand breaks may be an important event for activation of IRR. Finally, cells that expressed HRS after small single

exposures at acute dose rates were also reported to be sensitive to continuous radiation exposures given to much larger doses at very low dose rates (*40, 41*). Lowering the dose rate from 1 Gy/h down to 2–5 cGy/h enhanced net radiosensitivity by about a factor of 4, an observation that supports the idea that DNA damage must exceed some threshold to induce a protective pathway. The concept that small amounts of DNA damage remain unrecognized and unrepaired gained support recently from the observation that DNA DSBs in *nondividing* human cells persist for many days after radiation doses less than 2 cGy (*42*). Moreover, the time course of repair of DSBs after 2 cGy, 20 cGy and 2 Gy was similar but was considerably longer at doses <0.5 cGy, with no repair detected 24 h after a dose of 0.12 cGy. These data are consistent with the concept that repair factors are activated only after the number of DSBs exceeds a putative threshold, whereas repair is strongly compromised at or below the threshold number of events.

The Sensor: Detection of Radiation-Induced Damage (Sensing the Signal)

Recognition of DNA damage after radiation exposure activates several different downstream pathways, often dependent on the position of the cell in the cell cycle. As described above, the activation of these biological responses and correspondingly the activation of IRR has been linked with the production of strand breaks in DNA (*39*). Several ''damage-sensing'' molecules have been described in mammalian cells, including PARP, which is efficiently activated by DNA strand breaks (*43–45*). Interestingly, a deficiency in functional PARP, or depletion or inhibition of functional PARP activity (*46–48*), negatively affects the ability of cells to repair radiation injury. The importance of PARP-mediated DNA damage-sensing pathways for inactivating HRS was initially implied using the PARP inhibitor 3-aminobenazide (*33*). Chemical inhibition of PARP activity prevented the development of increased radioresistance in V79 Chinese hamster cells, implicating a role for PARP in damage signaling. The importance of PARP functionality for activation of IRR was supported using the potent and more specific PARP inhibitor PJ34 in T98G human cells (*26*). PJ34 prolonged the HRS response to higher doses, suggesting that more damage was required to exceed the threshold and trigger increased radioresistance. The status of PARP as a credible candidate for an IRR damage sensor was reinforced by the failure of PJ34 to modify low-dose radiosensitivity in *confluent* cultures of T98G predominantly enriched in G_1-phase cells that do not show an HRS/IRR survival response (*26*).

During the past several years, the protein kinase ataxia telangiectasia mutated (ATM) has emerged as a central protein in the recognition of DNA DSBs and the activation of several downstream biological pathways that control cell cycle progression and DNA repair. To date, no role for ATM has been established in the HRS/IRR response. However, recent discoveries regarding the activation and function of this protein provide evidence that this protein may in fact be involved. Although changes in the abundance of ATM protein are not observed in response to radiation exposure (*49*), the kinase activity of ATM is rapidly induced in response of radiation-induced damage (*50–53*). Bakkenist and Kastan (*54*) have recently provided a model for activation of ATM after DNA damage, involving the rapid dual autophosphorylation of the inactive 1981 serine of ATM dimers. This process was shown to be exquisitely responsive to modifications in DNA structure or strand damage. Phosphorylation of 1981S and activation of ATM occurred over the dose range of 0–1 Gy, with maximal phosphorylation occurring at only 40 cGy. Moreover, 50% of the protein is activated within 15 min of radiation exposure, suggesting an early involvement of ATM in the damage signal transduction pathway. The overlapping radiation dose–response profiles for activation of ATM and HRS encourages the hypothesis that ATM may be a major controlling factor responsible for the activation of radiation protective responses that are reflected in cell lines that exhibit HRS and IRR (see *HRS: The Current Understanding*). The role of other DNA damage response proteins in HRS that associate with ATM after DNA damage is also unclear [e.g. H2AX (*55, 56*), 53BP1 (*57, 58, 59*), HDAC4 (*60*)]. Phosphorylation of H2AX has been identified as an early marker for DNA strand breaks (*55*), and its expression can serve as a surrogate of cell killing by drugs that produce DNA DSBs (*56*). The persistence of γH2AX foci in cells exposed to very low radiation doses demonstrates that DNA DSBs can remain unrepaired and therefore presumably undetected in the genome (*42*). Ward *et al.* (*61*) recently suggested that phosphorylation of H2AX is needed for the accumulation of 53BP1 at DNA break sites. 53BP1 has been shown to localize rapidly at sites of DNA DSBs and has been implicated in the ATM-dependent G_2/M-phase cell cycle arrest process after irradiation (*57, 58*). Moreover, Fernandez-Capetillo *et al.* have proposed that the activation of 53BP1 may also be dependent on DNA damage exceeding a threshold (*59*).

The Transducer Signal (Processing Damage)

HRS is strongly dependent on cell cycle phase (*23, 28*), but it does *not* reflect the ''*passive*'' response of a subpopulation of radiosensitive cells in one phase of the cell cycle (*9*). Rather, HRS and the mechanism of the transition between the HRS and IRR responses are likely to be due to an ''*active*'' process occurring in a small fraction of G_2-phase cells. A population of V79 cells enriched in G_1 ($\sim$92%) or S ($\sim$60%)-phase cells did not show HRS. In contrast, G_2-phase-enriched populations exhibited a pronounced HRS response compared with an asynchronous population. Indeed, the HRS response expressed in the asynchronous cell population could be fully accounted for by the sum of HRS responses in the G_1-, S- and G_2-phase

cell populations. Moreover, enhanced HRS responses for G_2-phase enriched populations have also been reported for human T98 and U373 cells (*23*). Interestingly, *asynchronous* cultures of U373 cells do not show an HRS/IRR response, an observation that may be rationalized by speculating that the small HRS response seen in the enriched G_2-phase cell population is masked by the lack of an HRS response of G_1- and S-phase cells that comprise the bulk of an asynchronous population. Thus the hypersensitivity at low doses and the unusual increase in radiosensitivity between 0.5 and 1 Gy appear to be limited to cells in the G_2 phase of the cell cycle.

Transducer signal: role of checkpoints. The significance of G_2-phase cells in HRS focused the search for the underlying mechanism to processes that are specific to, or dominant in, G_2-phase cells. DNA damage triggers a series of checkpoints that arrest the cell cycle in either the G_1, S or G_2 phase to allow repair of radiation injury (*62*). The biochemical events controlling the S-phase checkpoint are dependent upon functional ATM and seem to be critical for avoiding mutations, but they do not have a significant impact on cell survival after irradiation (*63, 64*). The checkpoints at the G_1/S and G_2/M boundaries prevent cells from initiating DNA replication or cell division with damaged DNA. The G_1 checkpoint, which is largely controlled by activation of TP53, also does not seem to significantly influence cell survival and does not influence HRS/IRR.

Two distinct checkpoints in G_2/M phase have been described. The first, which has been known for many decades (*65*), is due to the accumulation of cells in G_2 that were irradiated in the G_1 or S phase of the cell cycle. The second recently described checkpoint is believed to protect radiation-damaged G_2-phase cells from prematurely entering mitosis (*66*). While the classic G_2/M checkpoint exhibits a dose dependence of activation, the second checkpoint is independent of dose over the range 1–10 Gy with a distinct threshold for activation at ~40 cGy and is transient over a short time frame. This checkpoint has been shown to be dependent upon ATM, and this dependence is reflected by the fact that the dose response for activation of ATM parallels the dose response for activation of the checkpoint. Of interest to the field of low-dose radiation is that the ''*threshold*'' dose required to activate the ''second'' G_2/M checkpoint is similar to that required to cause IRR. Thus a plausible hypothesis is that a failure to activate this checkpoint at doses less than 40 cGy would manifest as a radiosensitive phenotype since radiation-damaged G_2-phase cells would proceed into mitosis carrying unrepaired breaks, a situation that would deliver a survival response analogous to HRS. Moreover, the G_2-phase specificity of this highly thresholded checkpoint would imply an enhanced HRS response for G_2-phase-enriched cell populations, as has been demonstrated (*23, 28*).

A relationship between this newly described G_2/M checkpoint (*66*) and HRS/IRR has already been established (*28*), albeit from a limited data set. Activation of the G_2/M checkpoint occurred in the same dose range as induction of IRR. Indeed, a pattern emerged in which the dose required to change from the HRS to the IRR response in the survival experiments corresponded with the activation point of the G_2/M checkpoint. Although this does not imply a cause and effect relationship, it does strongly suggest that the same underlying pathway is responsible for both effects. Moreover, cells that failed to exhibit HRS in asynchronous culture did not have a threshold-activated second G_2/M checkpoint; rather it was active even at the lowest dose examined, predicating HRS. These data demonstrated for the first time a mechanism that could link the detection and repair of DNA damage to the phenomenon of HRS.

The Effector Step (Repair of Damage)

Unrepaired DNA double-strand breaks are considered the most relevant lesion in radiation-induced cell killing (*1*). Failure to repair a single DNA DSB can lead to cell death, and cells deficient in essential elements of critical DNA repair pathways exhibit extreme radiosensitivity. Radiation-induced DNA DSBs are repaired efficiently by the process of non-homologous end joining (NHEJ), with homologous recombination contributing to repair of breaks during the S and G_2 phase of the cell cycle. The importance of NHEJ in the phenomenon of HRS/IRR was first suggested by the failure to detect a defined HRS-like substructure in the low-dose survival curves of Ku80-deficient XR-V15B cells (*67*). More recently, an association was established between functionality of the NHEJ repair pathway and HRS/IRR in a panel of eight cell lines (*35*). The importance of the NHEJ pathway in HRS/IRR was later confirmed by the disparate survival responses of three genetically related cell lines with disparate PRKDC status, a critical component of NHEJ repair complex. Clonogenic survival for the cell lines was indistinguishable over the 0–30-cGy dose range of HRS, but differences in radiosensitivity became evident at doses >40 cGy. M059K and M059J/Fus1 cells (both PRKDC competent) had discrete HRS and IRR responses, albeit to somewhat different extents. However, M059J cells (PRKDC mutant) were extremely radiosensitive, exhibiting an exponential survival curve with an extended and prolonged HRS response, with no evidence of IRR. Therefore, the inactivation of HRS (and associated activation of the increased radioresistant response) was coincident with the presence of PRKDC protein and functional DNA-PK activity. Therefore, a plausible conclusion from these data, when considered with the reports of Skov (*67*) and Vagancy-Juery (*35*) is that the initial response (HRS) is *independent* of DNA-PK activity, whereas the activation of IRR showed a *dependence* on the presence of PRKDC protein and/or functional DNA-PK activity.

HRS: THE CURRENT UNDERSTANDING

HRS appears to be the default survival response of cells to radiation injury at doses below ~10–20 cGy (*4*) and to

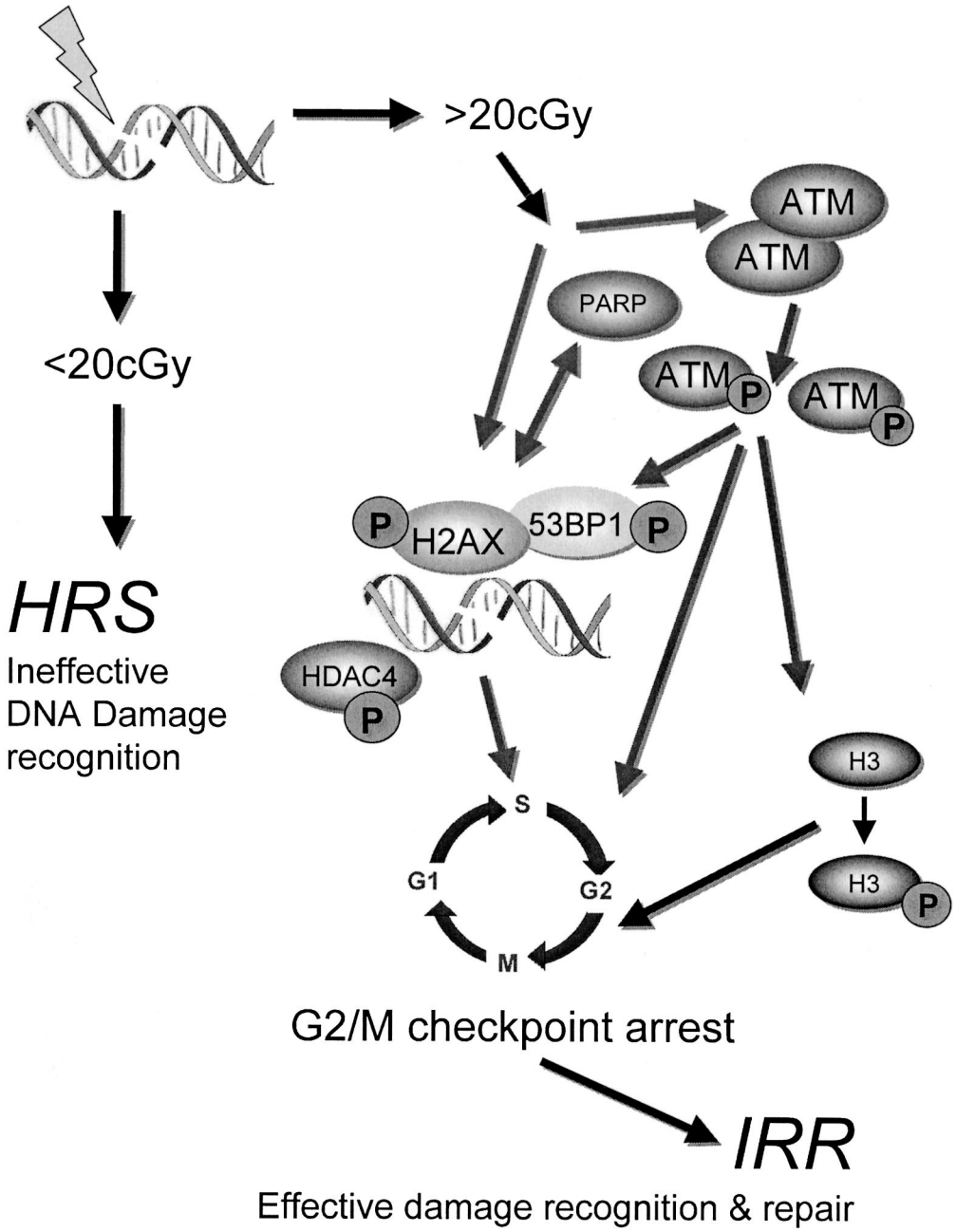

FIG. 2. A schematic representation of the current understanding of the mechanism of HRS/IRR. For the purposes of this illustrative cartoon, a threshold value of 20 cGy has been selected for the induction of IRR. DNA DSBs themselves, or the resultant DNA conformation change that arises from the damage, cause the activation of ATM. This results in the recognition of the strand breaks by damage recognition proteins. Ultimately, a G_2-phase-specific checkpoint is activated which promotes repair and cell survival. HRS: low-dose hyper-radiosensitivity; IRR: increased radioresistance; ATM: ataxia telangiectasia mutated; H2AX-P: phosphorylated form of histone H2AX; 53BP1-P; phosphorylated 53BP1; HDAC4; histone deactylase 4; H3-P: phosphorylated histone H3; PARP: poly(ADP-ribose) polymerase.

all doses in NHEJ repair-deficient mutants. In repair-competent cells, increasing levels of radiation-induced damage activate a protective response resulting in IRR. One question that arises is why a DNA repair system has evolved that needs to be activated by a threshold of damage? One possibility is that it is beneficial for the organism to allow small numbers of cells with low levels of damage to die rather than risk mutation through repair and survival. This explanation predicts that the persistence of some unrepaired DSBs does not increase the risk of mutation, but rather serves to identify damaged cells for elimination from the population. Presumably, these breaks would be present in G_2-phase cells committed to enter mitosis or that have passed other critical points in the cell cycle. To verify such

a hypothesis, a detailed evaluation of mutagenesis in this dose range is needed. The second possible explanation is that activation of DNA repair systems is inherently dangerous to the genome. In particular, NHEJ is an error-prone DNA repair system that results in the deletion or gain of genetic material at the sites of DNA damage. One can thus imagine a beneficial effect in keeping this pathway ''off'' until it is clearly required.

Despite the fact that the ''phenomenon'' of HRS/IRR is well established, the underlying molecular mechanism(s) has not been conclusively identified. Perhaps this reflects a reliance on evaluating the presence of HRS/IRR with a survival assay that provides no direct information on important molecular events. Pathways or processes essential in the HRS/IRR responses have been identified previously in survival experiments using mutant cells (e.g. *4, 33, 34*), but new evidence indicates the potential involvement of a cell cycle checkpoint (*28*). Moreover, key molecular events have been described that fundamentally support the concept of cellular checkpoint and repair processes induced by radiation doses within the HRS/IRR dose range. For example, the discovery of a cell cycle arrest checkpoint specific to cells irradiated in G_2 phase (*66*), the characterization of the ATM activation (*54*), and the observation that DSBs can persist unrepaired in cells (*42*) can all be rationalized within the framework of HRS/IRR.

Evidence from survival experiments implies that DNA DSBs may function as the trigger for activating the IRR survival response. However, it is unknown if the presence of strand breaks or the consequence of alterations in chromatin structure produced by the breaks is critical. The involvement of DNA DSBs circumstantially implies that ATM is the most probable candidate as the controlling initial sensor of damage, inducing checkpoint arrest through CHK2 (*68–70*). However, we found no difference in low-dose survival between HCT116 cells and an isogenic CHK2 mutant (unpublished results), possibly suggesting the involvement of other damage-sensing pathways or some redundancy in CHK2 function. Alternatively, ATM may function in conjunction with other stress response proteins, independent of CHK2, to reinforce the activation signal. Again, a credible candidate is PARP since this molecule has been associated with HRS/IRR (*26, 33*). However, the significance of ATM is indicated by the comparable dose response patterns of ATM expression through Ser-1981 phosphorylation (*54*) and transition between HRS and IRR. Both effects become established over the dose range of 10–50 cGy, and the triggering of ATM function would conceivably predicate an enhanced survival response through the activation of cell cycle arrest and DNA repair processes (*68, 70, 71*).

A relationship between DNA repair through NHEJ and the presence of HRS/IRR has been demonstrated (*34, 35*). We now propose that these events, i.e. the activation of ATM and the transition from an HRS to the IRR survival response due to increase NHEJ activity, are linked through the recently discovered cell cycle checkpoint mechanism (*66*) that is specific to G_2-phase cells (see model in Fig. 2). Indeed, an association has already been established between this novel cell cycle checkpoint and the prevalence of low-dose hypersensitivity (*28*). Moreover, HRS/IRR is known to be a phenomenon predominant in G_2-phase cells (*23, 28*), and it may be a response exclusive to G_2-phase cells. This is consistent with the concept of a cell cycle arrest process specific for G_2-phase cells being the mechanism that links DNA damage detection (sensor step) with repair (effector step) by allowing time for the latter to occur. Nonetheless, the data at this point only demonstrate a correlation between the early G_2 checkpoint and HRS/IRR. The data strongly support the idea that both of these responses are linked through a common biological pathway. It remains to be established whether the G_2 checkpoint itself is responsible for the HRS/IRR phenomenon. This provides ample opportunity for future research.

The consolidation of the low-dose expression patterns of (1) ATM activation, (2) G_2-phase-specific arrest, (3) NHEJ function, and (4) changes in radiosensitivity over the dose range of 10–50 cGy into a unifying hypothesis to explain HRS/IRR is provocative. Clearly, a considerable amount of work needs to be done to address the many unanswered questions of HRS/IRR, most notably concerning why certain cells fail to exhibit HRS, and what the cause and effect relationship is between the G_2 checkpoint and HRS. However, the recent molecular data provide avenues to continue the search for the control of low-dose hyper-radiosensitivity and increased radioresistance.

Received: September 11, 2003; accepted: October 31, 2003

REFERENCES

1. P. A. Jeggo, DNA breakage and repair. *Adv. Genet.* **38**, 185–218 (1998).
2. G. P. Raaphorst and S. Boyden, Adaptive response and its variation in human normal and tumour cells. *Int. J. Radiat. Biol.* **75**, 865–873 (1999).
3. K. S. Leskov, T. Criswell, S. Antonio, J. Li, C. R. Yang, T. J. Kinsella and D. A. Boothman, When X-ray-inducible proteins meet DNA double strand break repair. *Semin. Radiat. Oncol.* **11**, 352–372 (2001).
4. M. C. Joiner, B. Marples, P. Lambin, S. C. Short and I. Turesson, Low-dose hypersensitivity: Current status and possible mechanisms. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 379–389 (2001).
5. I. Szumiel, Monitoring and signaling of radiation-induced damage in mammalian cells. *Radiat. Res.* **150** (Suppl.), S92–S101 (1998).
6. B. Marples, P. Lambin, K. A. Skov and M. C. Joiner, Low dose hyper-radiosensitivity and increased radioresistance in mammalian cells. *Int. J. Radiat. Biol.* **71**, 721–735 (1997).
7. T. T. Puck and P. I. Marcus, Action of X rays on mammalian cells. **103**, 653–666 (1956).
8. J. W. Boag, The statistical treatment of cell survival data. In *Cell Survival after Low Doses of Radiation: Theoretical and Clinical Implications* (T. Alper, Ed.), pp. 40–53. Wiley, London, 1975.
9. B. Marples and M. C. Joiner, The response of Chinese hamster V79 cells to low radiation doses: Evidence of enhanced sensitivity of the whole cell population. *Radiat. Res.* **133**, 41–51 (1993).
10. B. G. Wouters and L. D. Skarsgard, Low-dose radiation sensitivity

and induced radioresistance to cell killing in HT-29 cells is distinct from the ''adaptive response'' and cannot be explained by a subpopulation of sensitive cells. *Radiat. Res.* **148**, 435–442 (1997).

11. R. E. Durand, Use of a cell sorter for assays of cell clonogenicity. *Cancer Res.* **46**, 2775–2778 (1986).
12. C. A. Beam, R. K. Mortimer, R. G. Wolfe and C. A. Tobias, The relation of radioresistance to budding in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **49**, 110–122 (1954).
13. J. Calkins, An unusual form of response in X-irradiated protozoa and a hypothesis as to its origin. *Int. J. Radiat. Biol.* **12**, 297–301 (1967).
14. T. M. Koval, Inducible repair of ionizing radiation damage in higher eukaryotic cells. *Mutat. Res.* **173**, 291–293 (1986).
15. M. C. Joiner, P. Lambin, E. P. Malaise, T. Robson, J. E. Arrand, K. A. Skov and B. Marples, Hypersensitivity to very-low single radiation doses: Its relationship to the adaptive response and induced radioresistance. *Mutat. Res.* **358**, 171–183 (1996).
16. B. Marples, H. Adomat, C. J. Koch and K. A. Skov, Response of V79 cells to low doses of X-rays and negative pi-mesons: Clonogenic survival and DNA strand breaks. *Int. J. Radiat. Biol.* **70**, 429–436 (1996).
17. B. G. Wouters, A. M. Sy and L. D. Skarsgard, Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. *Radiat. Res.* **146**, 399–413 (1996).
18. B. G. Wouters and L. D. Skarsgard, The response of a human tumor cell line to low radiation doses: Evidence of enhanced sensitivity. *Radiat. Res.* **138** (Suppl.), S76–S80 (1994).
19. A. Vral, H. Louagie, H. Thierens, J. Philippe, M. Cornelissen and L. de Ridder, Micronucleus frequencies in cytokinesis-blocked human B lymphocytes after low dose gamma-irradiation. *Int. J. Radiat. Biol.* **73**, 549–555 (1998).
20. E. Tsoulou, L. Baggio, R. Cherubini and C. A. Kalfas, Low-dose hypersensitivity of V79 cells under exposure to gamma-rays and ^{4}He ions of different energies: Survival and chromosome aberrations. *Int. J. Radiat. Biol.* **77**, 1133–1139 (2001).
21. C. Dionet, A. Tchirkov, J. P. Alard, J. Arnold, J. Dhermain, M. Rapp, V. Bodez, J. C. Tamain, I. Monbel and P. Verrelle, Effects of low-dose neutrons applied at reduced dose rate on human melanoma cells. *Radiat. Res.* **154**, 406–411 (2000).
22. J. L. Redpath, Q. Lu, X. Lao, S. Molloi and E. Elmore, Low doses of diagnostic energy X-rays protect against neoplastic transformation *in vitro*. *Int. J. Radiat. Biol.* **79**, 235–240 (2003).
23. S. C. Short, M. Woodcock, B. Marples and M. C. Joiner, The effects of cell cycle phase on low dose hyper-radiosensitivity. *Int. J. Radiat. Biol.* **79**, 99–105 (2003).
24. S. Dey, P. M. Spring, S. Arnold, J. Valentino, D. Chendil, W. F. Regine, M. Mohiuddin and M. M. Ahmed, Low-dose fractionated radiation potentiates the effects of Paclitaxel in wild-type and mutant p53 head and neck tumor cell lines. *Clin. Cancer Res.* **9**, 1557–1565 (2003).
25. G. Schettino, M. Folkard, K. M. Prise, B. Vojnovic, A. G. Bowey and B. D. Michael, Low-dose hypersensitivity in Chinese hamster V79 cells targeted with counted protons using a charged-particle microbeam. *Radiat. Res.* **156**, 526–534 (2001).
26. A. Chalmers, P. J. Johnston, M. Woodcock, M. C. Joiner and B. Marples, PARP-1, PARP-2 and the cellular response to low doses of ionizing radiation. *Int. J. Radiat. Oncol. Biol. Phys.*, in press.
27. D. Bartkowiak, S. Hogner, W. Nothdurft and E. M. Rottinger, Cell cycle and growth response of CHO cells to X-irradiation: Threshold-free repair at low doses. *Int. J. Radiat. Oncol. Biol. Phys.* **50**, 221–227 (2001).
28. B. Marples, B. G. Wouters and M. C. Joiner, An association between the radiation-induced arrest of G_2 phase cells and low-dose hyper-radiosensitivity: A plausible underlying mechanism? *Radiat. Res.* **160**, 38–45 (2003).
29. S. C. Short, J. Kelly, C. R. Mayes, M. Woodcock and M. C. Joiner, Low-dose hypersensitivity after fractionated low-dose irradiation *in vitro*. *Int. J. Radiat. Biol.* **77**, 655–664 (2001).
30. I. Turesson and M. C. Joiner, Clinical evidence of hypersensitivity to low doses in radiotherapy. *Radiother. Oncol.* **40**, 1–3 (1996).
31. C. S. Hamilton, J. W. Denham, M. O'Brien, P. Ostwald, T. Kron, S. Wright and W. Dorr, Underprediction of human skin erythema at low doses per fraction by the linear quadratic model. *Radiother. Oncol.* **40**, 23–30 (1996).
32. K. A. Skov, Radioresponsiveness at low doses: Hyper-radiosensitivity and increased radioresistance in mammalian cells. *Mutat. Res.* **430**, 241–253 (1999).
33. B. Marples and M. C. Joiner, Modification of survival by DNA repair modifiers: A probable explanation for the phenomenon of increased radioresistance. *Int. J. Radiat. Biol.* **76**, 305–312 (2000).
34. B. Marples, N. E. Cann, C. R. Mitchell, P. J. Johnston and M. C. Joiner, Evidence for the involvement of DNA-dependent protein kinase in the phenomena of low dose hyper-radiosensitivity and increased radioresistance. *Radiat. Res.* **78**, 1151–1159 (2002).
35. S. Vaganay-Juery, C. Muller, E. Marangoni, B. Abdulkarim, E. Deutsch, P. Lambin, P. Calsou, F. Eschwege and B. Salles, Decreased DNA-PK activity in human cancer cells exhibiting hypersensitivity to low-dose irradiation. *Br. J. Cancer* **83**, 514–518 (2000).
36. B. Marples, G. K. Lam, H. Zhou and K. A. Skov, The response of Chinese hamster V79–379A cells exposed to negative pi-mesons: Evidence that increased radioresistance is dependent on linear energy transfer. *Radiat. Res.* **138** (Suppl.), S81–S84 (1994).
37. T. M. Koval, Enhanced recovery from ionizing radiation damage in a lepidopteran insect cell line. *Radiat. Res.* **115**, 413–420 (1988).
38. J. D. Shadley, V. Afzal and S. Wolff, Characterization of the adaptive response to ionizing radiation induced by low doses of X rays to human lymphocytes. *Radiat. Res.* **111**, 511–517 (1987).
39. B. Marples and M. C. Joiner, The elimination of low-dose hypersensitivity in Chinese hamster V79–379A cells by pretreatment with X rays or hydrogen peroxide. *Radiat. Res.* **141**, 160–169 (1995).
40. C. R. Mitchell, M. Folkard and M. C. Joiner, Effects of exposure to low-dose-rate ^{60}Co gamma rays on human tumor cells *in vitro*. *Radiat. Res.* **158**, 311–318 (2002).
41. C. R. Mitchell and M. C. Joiner, Effect of subsequent acute-dose irradiation on cell survival *in vitro* following low dose-rate exposures. *Int. J. Radiat. Biol.* **78**, 981–990 (2002).
42. K. Rothkamm and M. Lobrich, 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* **100**, 5057–5062 (2003).
43. Y. Le Rhun, J. B. Kirkland and G. M. Shah, Cellular responses to DNA damage in the absence of poly(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.* **245**, 1–10 (1998).
44. J. Menissier-de Murcia, M. Molinete, G. Gradwohl, F. Simonin and G. de Murcia, Zinc-binding domain of poly(ADP-ribose)polymerase participates in the recognition of single strand breaks on DNA. *J. Mol. Biol.* **210**, 229–233 (1989).
45. M. Weinfeld, M. A. Chaudhry, D. D'Amours, J. D. Pelletier, G. G. Poirier, L. F. Povirk and S. P. Lees-Miller, Interaction of DNA-dependent protein kinase and poly(ADP-ribose) polymerase with radiation-induced DNA strand breaks. *Radiat. Res.* **148**, 22–28 (1997).
46. J. H. Kupper, M. Muller, M. K. Jacobson, J. Tatsumi-Miyajima, D. L. Coyle, E. L. Jacobson and A. Burkle, trans-dominant inhibition of poly(ADP-ribosyl)ation sensitizes cells against gamma-irradiation and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine but does not limit DNA replication of a polyomavirus replicon. *Mol. Cell Biol.* **15**, 3154–3163 (1995).
47. S. Shall and G. de Murcia, Poly(ADP-ribose) polymerase-1: What have we learned from the deficient mouse model? *Mutat. Res.* **460**, 1–15 (2000).
48. S. Boulton, S. Kyle and B. W. Durkacz, Interactive effects of inhibitors of poly(ADP-ribose) polymerase and DNA-dependent protein kinase on cellular responses to DNA damage. *Carcinogenesis* **20**, 199–203 (1999).
49. K. D. Brown, Y. Ziv, S. N. Sadanandan, L. Chessa, F. S. Collins, Y. Shiloh and D. A. Tagle, The ataxia-telangiectasia gene product, a constitutively expressed nuclear protein that is not up-regulated fol-

lowing genome damage. *Proc. Natl. Acad. Sci. USA* **94**, 1840–1845 (1997).

50. J. D. Siliciano, C. E. Canman, Y. Taya, K. Sakaguchi, E. Appella and M. B. Kastan, DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.* **11**, 3471–3481 (1997).

51. S. Banin, L. Moyal, S. Shieh, Y. Taya, C. W. Anderson, L. Chessa, N. I. Smorodinsky, C. Prives, Y. Reiss and Y. Zin, Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**, 1674–1677 (1998).

52. C. E. Canman and D. S. Lim, The role of ATM in DNA damage responses and cancer. *Oncogene* **17**, 3301–3308 (1998).

53. C. E. Canman, D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan and J. D. Siliciano, Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281**, 1677–1679 (1998).

54. C. J. Bakkenist and M. B. Kastan, DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**, 499–506 (2003).

55. S. Burma, B. P. Chen, M. Murphy, A. Kurimasa and D. J. Chen, ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J. Biol. Chem.* **276**, 42462–42467 (2001).

56. J. P. Banath and P. L. Olive, Expression of phosphorylated histone H2AX as a surrogate of cell killing by drugs that create DNA double-strand breaks. *Cancer Res.* **63**, 4347–4350 (2003).

57. R. A. DiTullio, Jr., T. A. Mochan, M. Venere, J. Bartkova, M. Sehested, J. Bartek and T. D. Halazonetis, 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat. Cell Biol.* **4**, 998–1002 (2002).

58. B. Wang, S. Matsuoka, P. B. Carpenter and S. J. Elledge, 53BP1, a mediator of the DNA damage checkpoint. *Science* **298**, 1435–1438 (2002).

59. O. Fernandez-Capetillo, H. T. Chen, A. Celeste, I. Ward, P. J. Romanienko, J. C. Morales, K. Naka, Z. Xia, R. D. Camerini-Otero and A. Nussenzweig, DNA damage-induced G_2-M checkpoint activation by histone H2AX and 53BP1. *Nat. Cell Biol.* **4**, 993–997 (2002).

60. G. D. Kao, W. G. McKenna, M. G. Guenther, R. J. Muschel, M. A. Lazar and T. J. Yen, Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. *J. Cell Biol.* **160**, 1017–1027 (2003).

61. I. M. Ward, K. Minn, K. G. Jorda and J. Chen, Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. *J. Biol. Chem.* **278**, 19579–19582 (2003).

62. B. B. Zhou and S. J. Elledge, The DNA damage response: Putting checkpoints in perspective. *Nature* **408**, 433–439 (2000).

63. B. Xu, S. Kim and M. B. Kastan, Involvement of Brca1 in S-phase and G_2-phase checkpoints after ionizing irradiation. *Mol. Cell Biol.* **21**, 3445–3450 (2001).

64. J. Falck, J. H. Petrini, B. R. Williams, J. Lukas and J. Bartek, The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. *Nat. Genet.* **30**, 290–294 (2002).

65. W. K. Sinclair, Cyclic X ray responses in mammalian cells *in vitro*. *Radiat. Res.* **33**, 620–643 (1968).

66. B. Xu, S. T. Kim, D. S. Lim and M. B. Kastan, Two molecularly distinct G_2/M checkpoints are induced by ionizing irradiation. *Mol. Cell Biol.* **22**, 1049–1059 (2002).

67. K. Skov, B. Marples, J. B. Matthews, M. C. Joiner and H. Zhou, A preliminary investigation into the extent of increased radioresistance or hyper-radiosensitivity in cells of hamster cell lines known to be deficient in DNA repair. *Radiat. Res.* **138** (Suppl.), S126–S129 (1994).

68. Y. Shiloh, ATM and related protein kinases: Safeguarding genome integrity. *Nat. Rev. Cancer* **3**, 155–168 (2003).

69. R. T. Abraham, Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* **15**, 2177–2196 (2001).

70. R. T. Abraham, Checkpoint signaling: Epigenetic events sound the DNA strand-breaks alarm to the ATM protein kinase. *Bioessays* **25**, 627–630 (2003).

71. M. B. Kastan, D. S. Lim, S. T. Kim, B. Xu and C. Canman, Multiple signaling pathways involving ATM. *Cold Spring Harbor Symp. Q. Biol.* **65**, 521–526 (2000).