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The Rad9 protein enhances survival and promotes DNA repair following exposure to ionizing radiation

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Abstract

Following DNA damage cells initiate cell cycle checkpoints to allow time to repair sustained lesions. Rad9, Rad1, and Hus1 proteins form a toroidal complex, termed the 9-1-1 complex, that is involved in checkpoint signaling. 9-1-1 shares high structural similarity to the DNA replication protein proliferating cell nuclear antigen (PCNA) and 9-1-1 has been shown *in vitro* to stimulate steps of the repair process known as long patch base excision repair. Using a system that allows conditional repression of the Rad9 protein in human cell culture, we show that Rad9, and by extension, the 9-1-1 complex, enhances cell survival, is required for efficient exit from G2-phase arrest, and stimulates the repair of damaged DNA following ionizing radiation. These data provide *in vivo* evidence that the human 9-1-1 complex participates in DNA repair in addition to its previously described role in DNA damage sensing.

Keywords: Rad9; 9-1-1 complex; DNA damage; Cell cycle checkpoints; Base excision repair

Detection and repair of damaged DNA is necessary to maintain genomic fidelity and prevent aberrant cellular phenotypes including cancer. The 9-1-1 complex, composed of Rad9, Rad1, and Hus1, is a toroidal molecule that associates with DNA early in the response to genotoxic stress [1–4]. Following DNA damage, single-stranded DNA is coated by replication protein A (RPA). RPA-coated stretches of DNA promote recruitment of the Rad17-RFC clamp loader [5], which loads the 9-1-1 ring structure around DNA [6–8]. Association of Rad17-RFC and 9-1-1 with chromatin is a well-defined event following DNA damage [9–11]. Once bound to chromatin, 9-1-1 is poised to participate in downstream processes necessary for eventual remediation of the DNA damage. For example, 9-1-1 facilitates Chk1 phosphorylation in the ATR-dependent cell cycle signaling pathway [12,13].

The 9-1-1 complex bears striking structural similarity to the homotrimeric toroid proliferating cell nuclear antigen (PCNA) that tethers polymerases to DNA and stimulates known DNA repair proteins [14,15]. In addition, the 9-1-1 clamp loader, Rad17-RFC, has 4 of 5 subunits in common with the PCNA clamp loader RFC [3,16,17]. These similarities and the fact that 9-1-1 remains attached to chromatin during the damage response have led to speculation that 9-1-1 fills an accessory role for DNA repair proteins [18]. Indeed, recent findings indicate that the 9-1-1 complex interacts with a variety of enzymes involved in base excision repair (BER).

The BER pathway is responsible for the repair of thousands of damaged bases per cell per day [19]. 9-1-1 stimulates or associates with many enzymes involved in one branch of this pathway called long patch (LP) BER. Briefly, LP-BER involves removal of the damaged base by a DNA glycosylase, phosphodiester backbone cleavage at the abasic site, strand displacement polymerization to create a flap, flap removal to generate a nick, and ligation to complete repair [20]. 9-1-1 was found to co-immunoprecip-

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itate with the DNA glycosylase MutY homolog in yeast [21]. The activity of both the structure-specific flap-endonuclease-1 (FEN1) [22,23] and the DNA repair polymerase Pol β [24] is stimulated by 9-1-1. Finally, nick joining by DNA ligase I is also stimulated by 9-1-1 [25]. These results suggest that 9-1-1 is an important DNA repair component.

Here, we report the establishment of a human cell line that conditionally represses Rad9 expression. Presumably, concomitant with Rad9 depletion is the disruption of 9-1-1 functionality. Using this system, we determined that 9-1-1 moderately enhances cell viability following ionizing radiation (IR). Repression of Rad9 also delays release of IR-exposed cells from G2 arrest following exposure, consistent with impairment of DNA repair. Moreover, IR-exposed cells with depleted Rad9 showed prolonged DNA damage compared to cells expressing normal levels of Rad9. We conclude that Rad9, and by extension the 9-1-1 clamp complex, increases cell viability by stimulating DNA repair in damaged cells.

Materials and methods

Cell culture and generation of cell lines. H1299 cells were cultured at 37 °C (95% room air, 5% CO_2) and maintained in Delbecco's modified Eagle's medium (DMEM, high glucose) with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco-BRL, Grand Island, NY). The human Rad9 antisense-reading-frame was generated by RT-PCR of RNA isolated from human lung adenocarcinoma H1299 cells using forward 5′-gatcttacagcgcagcaggtcc-3′ and reverse 5′-ctggggcagcatgaag tgcctg-3′ primers. The Rad9 antisense sequence was designed to complement the native Rad9 mRNA sequence from −10 to +225. *Nhe*I and *Bam*HI sites were added to the forward and reverse primers, respectively. The full-length p21 sequence was removed from the pBIG2i Flag p21 Full Length vector [26] by digestion with *Nhe*I and *Bam*HI. The Rad9 antisense sequence was then ligated into the pBIG2i Flag p21 Full Length plasmid. Thus the pBig2i Rad9 AS vector was produced. All plasmids were purified by Qiagen preparation and transfected into H1299 cells using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions.

Cells with inducible expression of Rad9 antisense sequence and EGFP were initially selected by treating transfected populations with 2 μg/mL doxycycline for 24 h, then screening for EGFP fluorescence using a B-D FACSVantage SE cell sorter (Becton–Dickinson Immunocytometer Systems, Palo Alto, CA). Further sorting steps were performed as outlined in Roper et al. [27].

Colony survival assays. Colony survival assays were initiated by incubating the cells in 8 μg/mL doxycycline for one week to effectively repress Rad9 protein levels. Cells were then trypsinized and counted using a hemacytometer. Serial dilutions of the cells in the absence or presence of doxycycline were made and exposed to the indicated doses of ionizing radiation using a Shepard 1300 Curie radiation source at a dose rate of approximately 3.0 gray (Gy)/min. Dosed cells were replated in 60 mm culture dishes and allowed to grow undisturbed at 37 °C for 10 days, at which time colonies were stained with crystal violet diluted in methanol (2.5 g crystal violet/L methanol). The total number of colonies per plate was counted to determine the surviving fraction.

Cell cycle analysis. In order to repress Rad9 protein expression, cells were plated at low density and incubated in normal media with doxycycline (Sigma, St. Louis, MO) (8 μg/mL) for one week prior to plating. Initial characterization of the cell populations indicated that a 7-day exposure of doxycycline was optimal for maximum inhibition of Rad9 protein levels. For IR exposures, cells were counted on a hemacytometer, plated on 100 mm dishes (Becton–Dickinson, Franklin Lakes, NJ), and allowed to adhere overnight.

The cells were then exposed to the respective doses of IR using a Shepard 1300 Curie radiation source at a dose rate of 3.0 Gy/min. At the indicated times after the initial IR exposure, cells were trypsinized, centrifuged, and fixed in 75% ethanol for a minimum of 24 h prior to flow cytometric analysis. RNA was degraded by exposure to 1 mg/mL RNase (Sigma, St. Louis, MO) for 45 min. DNA content was determined by staining with 20 μg/mL propidium iodide (Sigma, St. Louis, MO). Measurement of red fluorescence (DNA content) was analyzed on an EPICS Elite flow cytometer (Coulter Electronics, Hialeah, FL). The cell cycle analysis was done in triplicate.

Western blot analysis. Adherent cells were lysed with Tris-buffered saline (TBS) containing protease inhibitors (Complete, Mini-Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN) supplemented with 0.1% Tween-20 and 5 μM EDTA. The protein lysates were then denatured in a heat block at 95 °C for 5 min in Laemmli buffer. Laemmli at 1× contains 50 mM Tris, pH 6.8, 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Protein concentrations were determined by the Bicinchoninic acid (BCA) method (BCA Protein Assay, Pierce, Rockford, IL). The extracted protein was separated by SDS–polyacrylamide gel electrophoresis using the Criterion Precast Gel System (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA). Membranes were blocked with TBST + 5% blocking grade non-fat dried milk (Bio-Rad, Hercules, CA) for 1 h. The membranes were then either washed three times in TBST for 5 min and then placed in Rad9 primary antibody (1:500 in TBST + 5% BSA; EMD Biosciences, San Diego, CA) or immediately exposed to β-actin (1:5000; Sigma, St. Louis, MO) or EGFP (1:1000; Clontech, Mountain View, CA) as per manufacturer's instructions. Membranes were then incubated in the appropriate secondary antibody: goat anti-mouse (1:4000, Southern Biotechnology, Birmingham, AL) or goat anti-rabbit (1:5000, Jackson Labs, West Grove, PA). After washing the membranes with TBST, specific antibody interactions were visualized by chemiluminescence (ECL-Plus Western blotting detection system, Amersham, Arlington Heights, IL). Blots were exposed to Kodak BioMax MR Film (Kodak, Rochester, NY) and developed using a Kodak X-OMAT 2000A Film Developer (Kodak, Rochester, NY).

Comet assays. Stably transfected H1299 cells were plated at low density on 100 mm dishes and allowed to grow in the presence or absence of 8 μg/mL doxycycline for 7 days prior to exposure to 15 Gy IR using a Shepard 1300 Curie radiation source at a dose rate of 3.0 Gy/min. Cells were kept at room temperature and harvested 0, 20, 40, 60, or 120 min after exposure. Comet assays were performed using the Trevigen Comet Assay Kit. Briefly, harvested cells were diluted to a concentration of 5000 cells per mL. The cells were then mixed with low melt agarose and allowed to solidify at 4 °C for 30 min. The slides were then incubated in the manufacturer's lysis solution for 50 min to lyse cell membranes. After a 35-min pre-incubation in alkaline solution, the slides were placed in an electrophoresis tank and exposed to 300 mA of current at ~25 V for 30 min. Slides were briefly washed then fixed in 70% ethanol for 5 min and allowed to air dry overnight. DNA was visualized by incubating the slides in SYBR-Green solution as per manufacturer's instructions and capturing images using a Nikon Eclipse TE2000 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a Photometrix CCD camera (Photometrix, Tucson, AZ). Sixty individual comet images were captured for each comet population. Mean tail moment of the captured images was determined using a macro developed for Scion Image software (Meyer Instruments, Houston, TX) by Helma and Uhl [28]. The comet assay was done in triplicate.

Results and discussion

Rad9 protein abundance can be inhibited by induction of the Rad9-antisense sequence

The tetracycline-inducible promoter system is frequently used to over-express transgenes of interest. In a variation

on the traditional system, we inserted a bicistronic sequence, which, when translated, produces Rad9 antisense mRNA and enhanced green fluorescing protein (EGFP) mRNA separated by an internal ribosomal entry site (IRES) (Fig. 1A). The IRES promotes translation of the EGFP marker following successful cellular integration and exposure to the tetracycline analog, doxycycline. The human lung adenocarcinoma cell line, H1299, which lacks p53, was chosen for stable transfection. Without p53, the G1 checkpoint is disabled and any minor effect of p53 on the G2 checkpoint is also avoided. We were then able to focus on the effects of Rad9 ablation exclusively on the G2 checkpoint and post-S phase repair.

Due to the nature of the insert, any cell that expresses EGFP will also express the Rad9 antisense sequence (Fig. 1A). The Rad9 antisense sequence is complementary to 235 nucleotides of the 5′ end of the native Rad9 mRNA, including the transcription start site. Therefore, the resulting duplex hinders ribosomal entry and translation of native Rad9 mRNA into protein. H1299 cells that were stably transfected with the inducible Rad9 repression system showed significantly decreased levels of Rad9 protein after 3 days of exposure to 8 μg/mL doxycycline (Fig. 1B). We determined that exposure of cells to 8 μg/mL doxycycline for 7 days consistently inhibited Rad9 protein translation in the range 80–95% as determined by Western blotting.

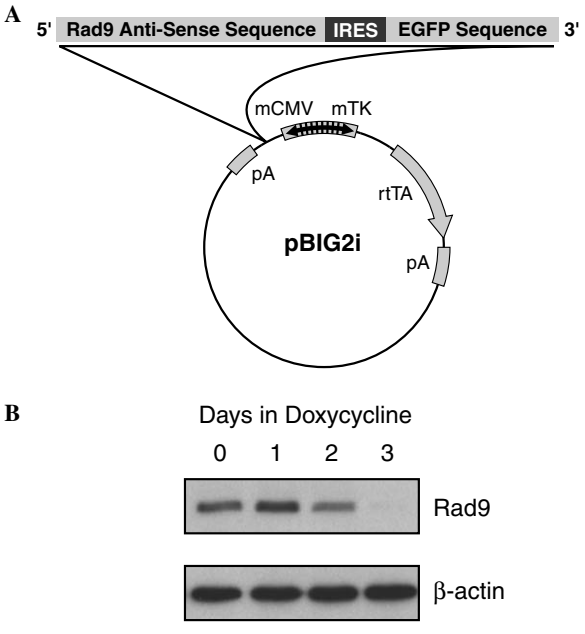

Fig. 1. Schematic of the Rad9 knockdown construct used in this study. (A) Plasmid map of the pBig2i tetracycline-on system used for inducing expression of the Rad9 antisense sequence. Presence of the tetracycline analog, doxycycline, binds to the reverse tetracycline transactivator (rtTA) gene product, which enhances utilization of the cytomegalovirus (mCMV) and thymidine kinase (mTK) minimal promoters to transcribe our engineered Rad9 antisense-EGFP insert and additional rtTA, respectively. PolyA (pA) sites 3′ to each gene serve to stabilize the resulting mRNA. (B) Doxycycline represses Rad9 protein abundance in stably transfected H1299 cells. Cells were exposed to doxycycline (8 μg/mL) for 0, 1, 2, or 3 days; cell lysates (40 μg) were immunoblotted with anti-Rad9 and anti-β-actin antibodies. Immune complexes were visualized by chemiluminescence.

Rad9 protects cells from ionizing radiation

It was previously established that ablation of Rad9 sensitizes cells to various forms of genotoxic stress [29–31]. In order to determine the effect of Rad9 depletion on cell survival in our model system, we exposed cells to doxycycline for 7 days to inhibit Rad9 protein translation, then exposed the cells to 0, 3, 6, or 9 Gy of ionizing radiation. The cells were then replated at low density and incubated at 37 °C for 10 days to allow surviving cells to form colonies. Depletion of Rad9 resulted in a modest increase in sensitivity to IR (Fig. 2). We interpret this result to mean that Rad9 is required in some capacity for efficient recovery from IR-induced damage.

Rad9 protein is required for efficient exit from G2 arrest

Rad9 is a vital protein implicated in the initiation and maintenance of cell cycle checkpoints [32,33]. Therefore, we tested the ability of our Rad9 depleted H1299 cells to arrest in G2-phase following IR. We tracked the progress of irradiated cells through the cell cycle using flow cytometry and saw a clear arrest of both control cells and Rad9 depleted cells in G2-phase 12 h after the initial exposure to 9 Gy of IR (Fig. 3). This observation is in apparent conflict with previous reports that Rad9 is necessary for G2 arrest in *Saccharomyces pombe* [34–36] but is consistent with others' findings that Rad9 is not required for G2 arrest in some mammalian cell lines [30,37]. These findings appear

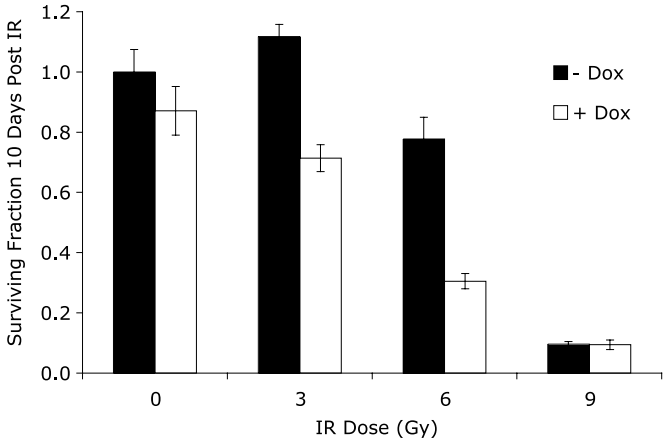

Fig. 2. Rad9 protects cells from ionizing radiation. Rad9 levels were depleted by incubating cells in doxycycline (8 μg/mL) for 1 week. Rad9 depleted cells and control populations were then dosed with 0, 3, 6, and 9 Gy of ionizing radiation. Cell populations were serially diluted, plated, and allowed to grow undisturbed for 10 days. Colonies were counted after 10 days with the aid of crystal violet stain. The surviving fraction of control cells (−Dox, black bars) and Rad9 depleted cells (+Dox, white bars) is depicted graphically with error bars showing standard deviation of the mean. All values were normalized to the unirradiated, no doxycycline control population.

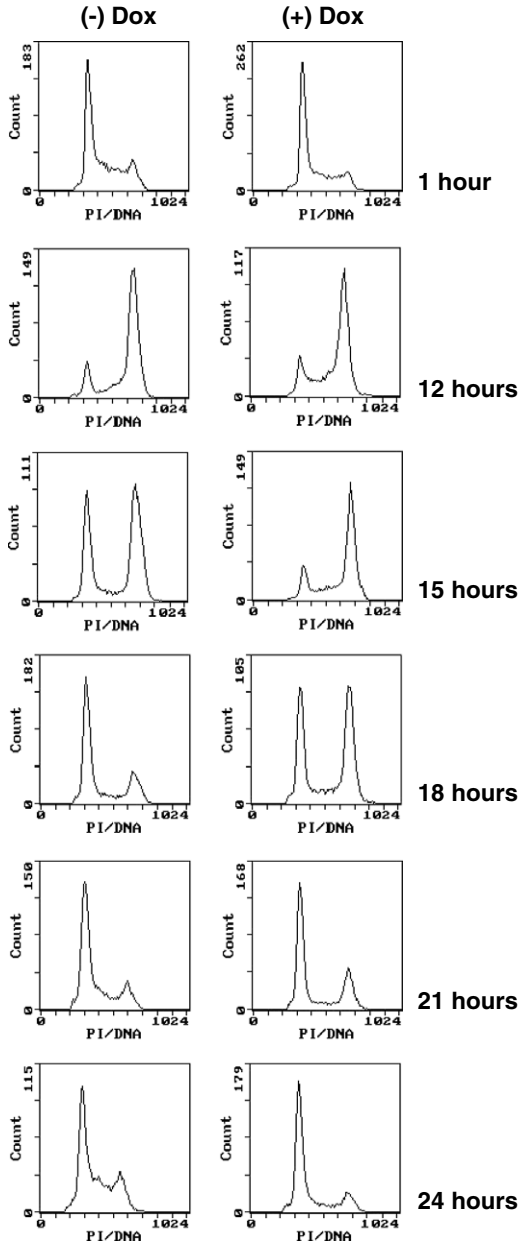

Fig. 3. Inhibition of Rad9 protein expression produces a lag in the G2 checkpoint following exposure to ionizing radiation. Cells were exposed to doxycycline (8 μg/mL) for 1 week to deplete Rad9 protein then dosed with 9 Gy of ionizing radiation. Cells were fixed in 70% ethanol 1, 12, 15, 18, 21, and 24 h after exposure to IR. Propidium iodide (PI) was used to stain genomic DNA for flow cytometric analysis. Two other identically designed experiments provided equivalent results.

to highlight a difference between the checkpoint control activities of the yeast and mammalian Rad9 homologs. Still, we cannot rule out that the residual level of Rad9 present in our cell system aids the initiation of G2 arrest.

Although control cells and Rad9 depleted cells initiated G2 arrest similarly, exit from G2-phase was delayed in the Rad9 depleted cells. There was a 3-h lag in exit from G2-phase following IR in Rad9 depleted cells compared to control cells (Fig. 3). The lengthened G2 arrest is not an artifact of doxycycline exposure since unirradiated cells exposed to doxycycline for 7 days show identical cell cycle activity compared to uninduced cells (data not shown). We interpret our result to mean that hRad9 is not required for the initiation of G2 arrest, rather it aids the cell in repairing damaged DNA thus leading to a shorter total time spent in G2 arrest. Consistent with biochemical data showing that the 9-1-1 complex stimulates DNA repair enzymes, we hypothesize that Rad9, in the form of the 9-1-1 complex, promotes exit from G2 arrest by stimulating DNA repair. To test this hypothesis, we measured DNA repair kinetics in Rad9 depleted cells compared to control cells.

Rad9 promotes DNA repair in H1299 cells after exposure to IR

The comet assay is frequently used to determine the amount of DNA damage sustained by individual cells. DNA damage is commonly quantified as the "tail moment" which is a standardized measurement of the amount of fragmented DNA that has migrated out of the cell's nucleus under the influence of an electric field. We used the comet assay to establish the kinetics of DNA repair following a single dose of 15 Gy of ionizing radiation in Rad9 depleted cells compared to control cells. Rad9 depleted cells and control cells were harvested immediately after IR exposure and again at 20, 40, 60, and 120 min following exposure to IR. Comet assay harvest times are of necessity earlier than those for cell cycle experiments because the comet assay provides an immediate snapshot of DNA damage whereas cell cycle phenomena are evident only after a series of signaling events have occurred. The comet assay harvest times we selected correlate with the half times for single strand break repair and double strand break repair, which are reported to be approximately 20 min and 2–3 h, respectively [38,39]. Cells deficient in Rad9 exhibited a decreased ability to repair IR induced damage compared to control cells as shown by higher tail moment values at 20, 40, and 60 min post-IR (Fig. 4). These data are consistent with the hypothesis that the 9-1-1 complex stimulates one or more DNA repair pathways.

We have established a system *in vivo* to analyze the role of Rad9 and presumably the entire 9-1-1 complex in response to genotoxic stress. Our results show that Rad9 expression can be repressed by the presence of a complementary RNA sequence resulting in a major reduction in Rad9 protein abundance. Rad9 depleted cells exhibited increased sensitivity to IR and exit IR-induced G2-phase growth arrest roughly 3 h after control cells. We believe this result is due to inability of the cells to properly stimulate repair in G2-phase producing a "lag" in the exit time from the G2-compartment. In addition, quantification of DNA repair kinetics revealed that Rad9 depletion decreases the rate at which DNA is repaired.

Several observations can be noted from these data. First, depletion of Rad9 has only a moderate effect on survival after IR exposure. This was observed directly in the

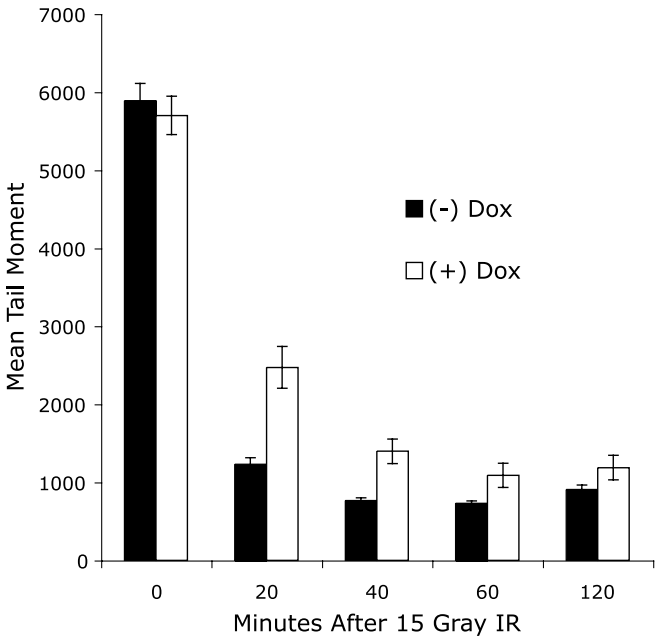

Fig. 4. Rad9 depleted cells repair damaged DNA slower than control cells. Cells were exposed to doxycycline (8 μg/mL) for 7 days to deplete Rad9 protein, then dosed with 15 Gy of IR. Cells were harvested 0, 20, 40, 60, and 120 min after exposure, suspended in molten low melt agarose, and spotted onto Trevigen CometSlides©. Slides were subjected to electrophoresis in alkaline buffer for 30 min. Images of individual cells stained with SyBR-Green 1 were captured using an epifluorescent microscope. Mean tail moment was calculated using a macro developed for Scion Image software from Meyer Instruments. Values are means of 60 individually imaged comet tails. Error bars show standard error calculated by dividing the standard deviation of the mean by the square root of the number of samples in the mean. Two other identically designed experiments provided equivalent results.

colony survival assay and is also consistent with our cell cycle data. Rad9 depletion does not ablate G2 checkpoint initiation; however, it does delay the exit of cells from this cell cycle compartment. When the G2 checkpoint is entirely suppressed, for example by caffeine, cells are overwhelmingly sensitized to genotoxic stress [40,41]. Since our model system produces only a 3-h delay in the exit from G2-phase, it is reasonable that we observe only a modest effect on cell survival when Rad9 is depleted.

The lengthened period of G2 arrest and the slower kinetics of DNA repair in IR damaged cells are consistent with a direct role of Rad9 in repair pathways. This conclusion is compatible with a recent report by Pandita and colleagues [37]. Our results are consistent with biochemical observations showing that 9-1-1 interacts with and stimulates DNA repair proteins. Overall we interpret our results to mean that one or more repair pathways are operating inefficiently in the absence of Rad9. This allows damage-induced breaks in the DNA to endure longer as shown by the persistence of comet tails in Rad9 depleted cells. The cells attempt to compensate for the loss of repair activity by maintaining the G2 checkpoint. Sufficient repair to trigger release from G2 now takes longer, resulting in the observed delay. Ultimately, repair is completed; supporting the conclusion that 9-1-1 is a facilitator, but not an essential factor for DNA repair. These data are consistent with biochemical results showing that 9-1-1 does not have a unique activity, but stimulates the activities of other proteins.

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