Extracellular Signal-Related Kinase Positively Regulates Ataxia Telangiectasia Mutated, Homologous Recombination Repair, and the DNA Damage Response

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Abstract

The accurate joining of DNA double-strand breaks by homologous recombination repair (HRR) is critical to the long-term survival of the cell. The three major mitogen-activated protein (MAP) kinase (MAPK) signaling pathways, extracellular signal-regulated kinase (ERK), p38, and c-Jun-NH_2-kinase (JNK), regulate cell growth, survival, and apoptosis. To determine the role of MAPK signaling in HRR, we used a human *in vivo* I-*Sce*I–based repair system. First, we verified that this repair platform is amenable to pharmacologic manipulation and show that the ataxia telangiectasia mutated (ATM) kinase is critical for HRR. The ATM-specific inhibitor KU-55933 compromised HRR up to 90% in growth-arrested cells, whereas this effect was less pronounced in cycling cells. Then, using well-characterized MAPK small-molecule inhibitors, we show that ERK1/2 and JNK signaling are important positive regulators of HRR in growth-arrested cells. On the other hand, inhibition of the p38 MAPK pathway generated an almost 2-fold stimulation of HRR. When ERK1/2 signaling was stimulated by oncogenic RAF-1, an ~2-fold increase in HRR was observed. KU-55933 partly blocked radiation-induced ERK1/2 phosphorylation, suggesting that ATM regulates ERK1/2 signaling. Furthermore, inhibition of MAP/ERK kinase (MEK)/ERK signaling resulted in severely reduced levels of phosphorylated (S1981) ATM foci but not γ-H2AX foci, and suppressed ATM phosphorylation levels >85% throughout the cell cycle. Collectively, these results show that MAPK signaling positively and negatively regulates HRR in human cells. More specifically, ATM-dependent signaling through the RAF/MEK/ERK pathway is critical for efficient HRR and for radiation-induced ATM activation, suggestive of a regulatory feedback loop between ERK and ATM. [Cancer Res 2007;67(3):1046–53]

Introduction

Mitogen-activated protein (MAP) kinases (MAPK) play an evolutionarily conserved role in mediating and amplifying growth factor–mediated and mitogenic signals from the cytoplasm to the nucleus (1). Three major MAPK pathways have been described; the extracellular signal-related kinase (ERK), the c-Jun NH_2-terminal kinase (JNK), and the p38 MAPK pathways. In addition to their roles in regulating normal cell growth, all three classes are also known to be activated in response to genotoxic stresses such as ionizing radiation (IR) and UV light. The MAPKs seem to work in concert to balance cell death with growth and survival. The JNK and p38 MAPKs are largely associated with apoptosis, whereas signaling via the ERK pathway is primarily linked with cell proliferation and survival, and seems to prevent apoptosis. Deregulation of the MAPK pathways is associated with genomic instability and cancer.

Another highly conserved cellular process is the repair of DNA double-strand breaks. Whether in response to naturally occurring double-strand breaks from stalled replication forks and V(D)J recombination or DNA damage induced by IR, the cell must repair these breaks to maintain genomic integrity (2). Homologous recombination repair (HRR) is one of two major double-strand break repair mechanisms, the other one being nonhomologous end-joining. Using the sister chromatid or DNA repeats as templates, HRR produces a perfect repair of these DNA lesions. HRR is associated with the repair of more toxic DNA lesions occurring in vital areas of the genome (3).

In response to double-strand breaks, the cell triggers checkpoints that halt the cell cycle while a decision is made regarding repair and survival, or death (4). Ataxia telangiectasia mutated (ATM), and other phosphatidylinositol 3-kinase–like kinases (PIKK) such as ATR (ATM- and RAD3-related kinase), and DNA-dependent protein kinase catalytic subunit are the primary DNA damage sensors that, together with the MRE11/RAD50/NBS1 complex, initiate the damage response, trigger cell cycle checkpoints, and coordinate DNA repair. Upon DNA damage or alterations in chromatin structure, ATM dimers are quickly autophosphorylated on serine 1981 (5), thereby activating ATM and leading to the phosphorylation of a number of downstream targets, including H2AX, p53, CHK2, NBS1, hMDM2, SMC1, and BRCA1 (2). ATM is physically associated with the double-strand breaks induced by IR, restriction endonucleases, and V(D)J recombination, and has been proposed to regulate HRR (6–8). ATM colocalizes and phosphorylates H2AX (γ-H2AX), rapidly forming discrete foci at double-strand breaks in response to IR, referred to as IR-induced foci (IRIF). Numerous other proteins involved in cell cycle control, DNA repair, and apoptosis colocalize to IRIF presumably to coordinate these processes. Several members of the MAPK family have been linked to the DNA damage response and ATM-mediated signaling events. For example, low levels of DNA damage can trigger prosurvival signals mediated by ERK1/2 phosphorylation (9); p38γ MAPK triggers G_2-M arrest in response to IR in an ATM-dependent manner (10); and JNK activation has been shown to promote base excision repair of cisplatin DNA

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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lesions (11). However, little is known about the role of MAPKs in the repair of double-strand breaks. This report shows that all three major MAPK pathways regulate HRR in human cancer cells. Most importantly, ERK1/2 signaling is a positive, ATM-dependent regulator of HRR, and phosphorylated (S1981) [phospho-(S1981)] ATM foci formation in response to IR critically depends on MAP/ERK kinase (MEK)/ERK signaling.

Materials and Methods

Reagents. Antibodies used were as follows: phospho-(T202/Y204) ERK1/2, phospho-(S63) c-Jun, ERK2, β-actin, estrogen receptor (ER) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-(S1981) ATM, phospho-(S133) cyclic AMP–responsive element binding protein (CREB), and phospho-(S15) p53 antibodies were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-p53 (Ab-6) antibody came from EMD Biosciences (San Diego, CA), and γ-H2AX antibody was from Trevigen (Gaithersburg, MD). SB203580 and PD98059 were purchased from EMD Biosciences, and SP600125 was from A.G. Scientific, Inc. (San Diego, CA). PD184352 has been described (12). KU-55933 was kindly donated by Graeme Smith (KuDOS Pharmaceuticals Ltd., Cambridge, United Kingdom; ref. 13). All drugs were dissolved in DMSO, except 4-hydroxytamoxifen (EMD Biosciences) that was dissolved in ethanol.

Cell culture and treatments. Human malignant glioma U87/DR-GFP ($p53^+$) cells were cultured as described (6). The Ad-*Sce*I-NG and AdCMV-EGFP adenoviruses (6, 14) were added to the culture medium at a multiplicity of infection (MOI) of 30 and incubated with cells while slowly rocking for 4 h at 37°C. U87/DR-GFP cells were infected with ΔRAF-ER* or empty (pBabe-hygro) retrovirus (15, 16), and cell clones were selected with hygromycin. Clones were screened for increased ERK1/2 phosphorylation in response to 4-hydroxytamoxifen by Western blotting. Cells were irradiated with UV-C (254 nm) and IR (17, 18). Inhibitors were added to the cell culture medium to the indicated final concentrations 1 h before treatment and left in the medium throughout the experiment.

Western blotting. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were exposed to antibodies at 1:500 to 1:2,000 dilutions. Specific protein bands were detected and quantified using infrared-emitting conjugated secondary antibodies—anti-mouse 680 Alexa (Molecular Probes, Eugene, OR) or anti-rabbit IRDYE 800 (Rockland Immunochemicals, Gilbertsville, PA), using the Odyssey Infrared Imaging System and the Application software version 1.2 from Li-Cor Biosciences (Lincoln, NE).

Double-strand break repair and IRIF assays. The green fluorescent protein (GFP) HRR assay has been described previously (6). The system uses a DNA cassette that contains two incomplete copies of the *GFP* gene separated by a puromycin resistance gene (for more detail, see refs. 6, 19). Briefly, the 5′ copy of the *GFP* gene is mutated by an I-*Sce*I restriction site inserted into a *Bcg*I site resulting in two in-frame stop codons and a truncated, nonfunctional GFP protein. The 3′ copy of the *GFP* gene is an 812-bp internal fragment of GFP spanning the *Bcg*I site in the 5′ GFP copy. Thus, upon infection with I-*Sce*I adenovirus, a single double-strand break is created. Repair can occur by a number of mechanisms, including nonhomologous end joining, homologous recombination (gene conversion), and single-strand annealing. However, only gene conversion will result in a functional GFP (19) because the internal GFP fragment does not have an intact 3′ portion of the *GFP* gene single-strand annealing would not result in a functional GFP. In the IRIF assay, U87/DR-GFP cells were grown on glass

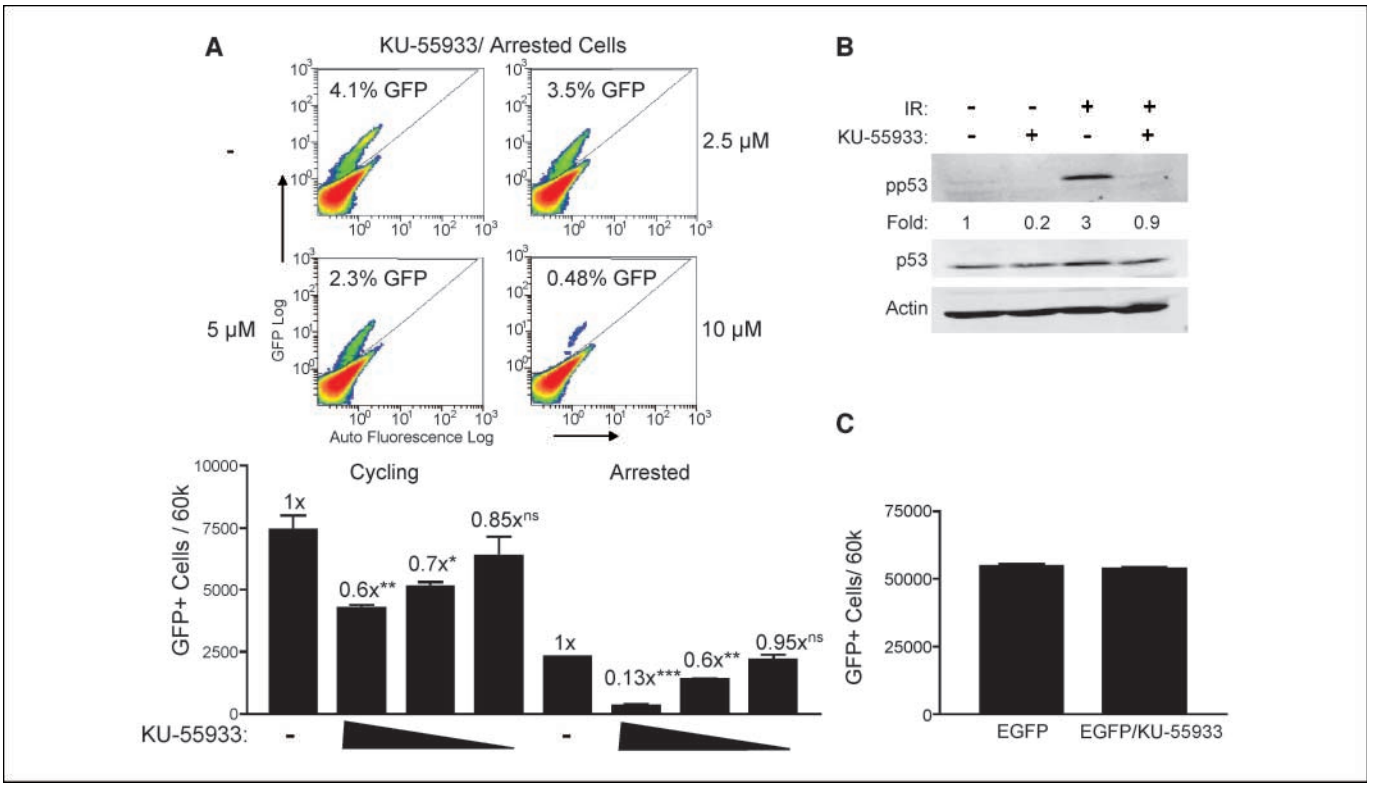

Figure 1. KU-55933 inhibits ATM signaling and HRR. Cycling and growth-arrested U87/DR-GFP cells were infected with Ad-*Sce*I-NG and treated with KU-55933 (2.5, 5, and 10 μmol/L) 4 h after virus infection. GFP+ cells/HRR events were determined by fluorescence-activated cell sorting (FACS) 48 h after virus infection. *A,* FACS images represent data from growth-arrested cells with and without KU-55933 (*top*). HRR events from cycling and growth-arrested cells are depicted in graph form (*bottom*). Fold (*X*) increases shown over each column indicate relative levels compared with untreated (*−KU-55933*) in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *ns,* not significant ($n = 3$). *B,* parallel cultures of U87/DR-GFP cells were treated or not with KU-55933, exposed to 10 Gy, and collected after 1 h for Western blot analysis. Fold depicts phospho-p53(S15) densitometric levels normalized to p53 protein. *C,* growth-arrested U87/DR-GFP cells were infected with AdCMV-EGFP (10 MOI) and exposed to KU-55933 (10 μmol/L) or not. Relative GFP expression was determined by FACS analysis after 48 h. *Columns,* number of GFP+ cells/60k cells ($n = 3$); *bars,* SE. *EGFP,* AdCMV-EGFP; *EGFP/KU-55933,* AdCMV-EGFP + KU-55933.

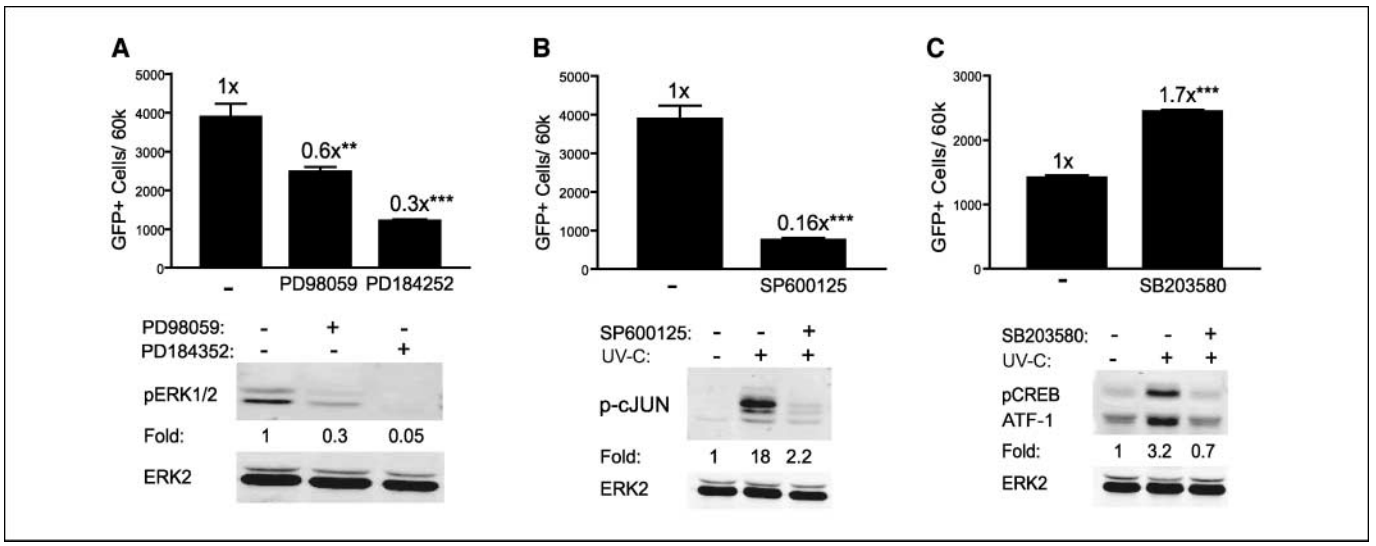

Figure 2. Inhibition of MAPK signaling affects HRR. Arrested U87/DR-GFP cells were infected with Ad-*Sce*I-NG. Four hours after infection, cells were treated with MAPK inhibitors, and 48 h after infection triplicate data sets were analyzed by GFP FACS to determine HRR. Parallel cell cultures were collected for Western blot analysis to determine drug efficacy. Fold depicts phosphoprotein levels normalized to ERK2 protein levels. *A,* PD98059 (10 μmol/L) and PD184352 (3 μmol/L) reduce basal ERK1/2 phosphorylation levels. *B,* SP600125 (30 μmol/L) reduces UV-induced c-Jun phosphorylation. Parallel cell cultures were exposed to UV-C (20 J/m^2) 1 h after drug treatment to activate JNK signaling, and cells were collected after 15 min. *C,* SB203580 (3 μmol/L) reduces CREB(S133) and ATF-1(S63) phosphorylation. Parallel cell cultures were exposed to UV-C (20 J/m^2) 1 h after drug treatment to activate p38 MAPK signaling, and cells were collected after 15 min. *Columns,* number of cells for triplicate data sets; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

chamber slides. After treatment, cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100/PBS, and blocked with 10% nonfat dry milk in PBS before the exposure to primary antibodies followed by secondary antibodies [Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes) at 1:500 dilution, and 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI)]. Primary antibodies used for immunostaining were anti–γ-H2AX (S139) and anti–phospho-(S1981) ATM, both at 1:500 dilution. Cells were imaged and analyzed using a Zeiss LSM 510 Meta imaging system in the Massey Cancer Center Flow Cytometry and Imaging Facility.

Flow cytometry. Cells were fixed in 70% ethanol, resuspended in 1% fetal bovine serum-PBS, and incubated with anti–phospho-(S1981) ATM antibody at 1:500 dilution for 30 min on ice. Cells were washed in PBS and incubated with goat anti-mouse Alexa Fluor 488 at 1:500 dilution for 30 min on ice. Cell cycle distribution was analyzed by propidium iodide staining (5 μg/mL, 0.1% Triton X-100/PBS). Flow cytometry was done on a Beckman Coulter XL-MC flow cytometer at the Massey Cancer Center Flow Cytometry Core Facility. Data was analyzed using EXPO32 ADC software.

Statistics. Unpaired two-tailed *t* tests were done on triplicate data sets using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). *P* values are indicated as follows: *, <0.05; **, <0.01; ***, <0.001. All error bars depict SE for triplicate data sets.

Results

ATM kinase–specific small-molecule inhibitor KU-55933 blocks HRR. The I-*Sce*I–based DNA double-strand break repair assay is now widely used for the study of HRR in mammalian cells (6, 19, 20). Using this system, previous studies by our group and others have shown that ATM is important for HRR (6, 21). Our particular system results in the generation of a GFP-positive cell when HRR occurs as a result of gene conversion, whereas repair by single-strand annealing does not (19). To first determine whether the I-*Sce*I repair system would be suitable for assessing the effects of pharmacologic inhibitors on double-strand break repair, we investigated the effect of a small-molecule inhibitor specific for the ATM kinase, KU-55933, on HRR (13). Many of the proteins involved in DNA double-strand break repair also play important roles in cell cycle checkpoint control (2). Therefore, U87/DR-GFP cells were growth-arrested in G_0-G_1 by serum starvation before and during the course of the experiment (6), thus facilitating a closer investigation of double-strand break repair by minimizing the interference of cell cycle effects. Cycling and growth-arrested U87/DR-GFP cells were infected with an adenovirus expressing I-*Sce*I (Ad-*Sce*I-NG) and treated with KU-55933. In line with our previous findings using caffeine and dominant-negative ATM expressed from adenovirus (6), KU-55933 inhibited HRR ~40% in cycling and ~85% in growth-arrested cells in a dose-dependent manner (Fig. 1*A*). Serine 15 of p53 is a well-documented target for ATM phosphorylation in response to IR (2). In agreement with previous findings, serine 15 phosphorylation was completely inhibited by KU-55933 in response to IR (Fig. 1*B*; ref. 13). The I-*Sce*I–based repair assay relies on the stable expression of the integrated DR-GFP cassette and expression of I-*Sce*I, in this case from an adenovirus (6, 19). Transcription of both genes is under control of a hybrid cytomegalovirus (CMV)/β-actin or CMV promoter, respectively (19). Conceivably, KU-55933 could affect expression from these promoters either at the transcriptional or translational levels. To rule this out, U87 cells were infected with AdCMV-EGFP, treated with KU-55933, and relative GFP expression levels were determined closely following the experimental procedure used for the HRR assay. Figure 1*C* shows that KU-55933 did not affect GFP levels, suggesting that KU-55933 inhibited HRR and not GFP or I-*Sce*I expression, or any other critical step in transcription or translation. These results substantiate our previous finding that ATM is important for efficient HRR throughout the cell cycle. Furthermore, these results suggest that we can use this approach to investigate the effect of pharmacologic manipulation of other protein kinases on HRR.

Interfering with signaling through the major MAPK signaling pathways modulates HRR. ERK1/2 signaling has long been associated with prosurvival responses after cell stress and radiation

(1, 22). To investigate a role for ERK1/2 signaling in HRR, we used the well-characterized MEK1/2 inhibitors PD98059 and PD184352. PD184352 is highly specific for MEK1/2 and is documented to have no effects on other kinases (23). PD98059 likewise inhibits MEK1/2 but has been shown to also exert some inhibitory effects on ERK5 (23). Arrested U87/DR-GFP cells were infected with Ad-*Sce*I-NG, treated with MEK1/2 inhibitors, and the effect on HRR was examined. When ERK1/2 signaling was inhibited, HRR levels were reduced. PD184352 had a more potent inhibitory effect reducing HRR by 70% compared with 40% with PD98059 (Fig. 2*A*). To confirm that these inhibitors were inhibiting MEK1/2, parallel protein samples from treated cells were assessed by Western blotting of phosphorylated (T202/Y204) ERK1/2. We found that PD184352 inhibited basal ERK1/2 phosphorylation almost completely compared with only 70% by PD98059 (Fig. 2*A*). Under our starvation conditions, these drugs did not influence cell cycle distribution when determined by flow cytometry (Supplementary Fig. S1). This result suggests that ERK1/2 signaling regulates HRR in human cancer cells.

JNK signaling is activated by DNA-damaging agents and is believed to enhance DNA repair (11). Also, c-Jun colocalizes with ATM and γ-H2AX in nuclear foci (24). To determine the effect of JNK signaling on HRR, growth-arrested U87/DR-GFP cells were infected with Ad-*Sce*I-NG, and JNK signaling was inhibited with SP600125 (25). As was the case with ERK, inhibiting JNK severely compromised HRR. In several experiments, HRR was inhibited ~80% (Fig. 2*B*). Western blot analysis showed that SP600125 blocked UV-C–induced (S63)-c-Jun phosphorylation by almost 90%, demonstrating that the drug performed as expected (25). These data suggest that JNK signaling is important for efficient HRR.

Signaling via the p38 MAPK pathway is vital to the cellular response to stress and has been linked to the regulation of apoptosis and G_2 arrest in response to UV and IR (1), which, in the case of IR, seems to be controlled by ATM (10). SB203580 is a well-documented p38α/β MAPK inhibitor (23); thus, this drug was used to investigate the importance of p38 signaling in HRR. We found that treatment with SB203580 enhanced HRR 1.7-fold (Fig. 2*C*). Western blot analysis showed that SB203580 inhibited phosphorylation of (S133)-CREB and (S63)–ATF-1 by 80% in response to UV-C in line with the literature (17). This finding suggests that p38 MAPK signaling somehow dampens HRR, perhaps directly or indirectly through downstream signaling events. To further investigate the increase in repair observed when p38 MAPK signaling was blocked, ERK1/2 phosphorylation levels were analyzed by Western blotting. Strikingly, treatment with SB203580 alone was able to enhance ERK1/2 phosphorylation nearly 2-fold (Supplementary Fig. S2). Furthermore, inhibition of p38 MAPK amplified the effect of EGF on ERK phosphorylation. ERK1/2 phosphorylation increased 3-fold in response to EGF alone, an effect that further increased to 5.3-fold upon the addition of SB203580 (Supplementary Fig. S2). A Yin-Yang relationship seems to exist between p38 MAPK and ERK1/2 (26), possibly explaining this stimulatory effect of SB203580 on HRR acting through ERK. As with KU-55933, none of the MAPK inhibitors had any effect on the transcription or translation of CMV-mediated EGFP expression or cell cycle distribution of growth-arrested cells (data not shown). Thus, the changes observed in HRR are directly related to the inhibition of MAPK signaling. Altogether, MAPK signaling affects HRR positively through ERK and JNK, and negatively through p38.

Expression of oncogenic RAF-1 increases HRR repair. MEK1/2 signaling is mostly associated with the RAF/MEK/ERK pathway (1). To confirm that the events we were studying follow this pathway, we expressed an oncogenic RAF-1 to genetically activate this cascade and then determined the effect on HRR. A truncated human oncogenic RAF-1 construct consisting of the activation domain fused to the ligand-binding domain of the ER was stably expressed from a mouse retrovirus (15). The ER domain carries a mutation that makes it unresponsive to its natural ligand,

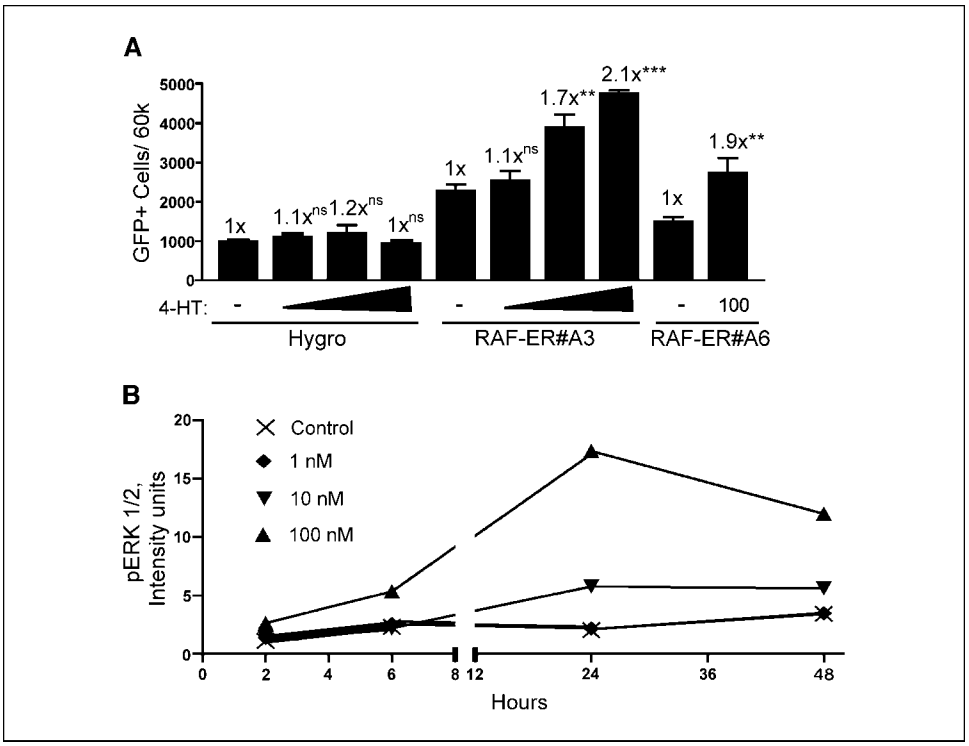

Figure 3. Genetic up-regulation of RAF-1 signaling stimulates HRR. *A,* growth-arrested U87/DR-GFP/ΔRAF-ER* (clones A3 and A6) and U87/DR-GFP/Hygro cells were exposed to 1, 10, and 100 nmol/L 4-hydroxytamoxifen (*4-HT*) 4 h after infection with Ad-*Sce*I-NG and analyzed by FACS. *Columns,* number of GFP+ cells/60k cells ($n = 3$). Fold (*X*) increases shown over each column indicate relative levels compared with untreated (without 4-hydroxytamoxifen) in each group. *B,* parallel U87/DR-GFP/ΔRAF-ER* (clone A3) cell cultures were analyzed for ERK1/2 phosphorylation levels by Western blotting. *Points,* ERK1/2 phosphorylation levels plotted against time (for triplicate data sets); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

β-estradiol. Upon the addition of 4-hydroxytamoxifen, a β-estradiol antagonist, the ER domain undergoes a conformational change exposing the RAF-1 moiety of ΔRAF-ER*, thereby triggering RAF/MEK/ERK signaling (15). Hygromycin-resistant clonal cell lines were established, including a vector control. ΔRAF-ER* expression was evident in response to 4-hydroxytamoxifen (Supplementary Fig. S3*A*). A consequent increase in ERK1/2 phosphorylation was observed in two ΔRAF-ER* clones in response to 4-hydroxytamoxifen (Supplementary Fig. S3*B*). Because 4-hydroxytamoxifen itself is known not to result in DNA damage (27), and no ERK1/2 phosphorylation was detected in the vector control cell line, these data strongly suggest that signaling originated from ΔRAF-ER* and not from 4-hydroxytamoxifen (Supplementary Fig. S3*B*). To determine whether the activation of the RAF/MEK/ERK pathway would exert a positive effect on repair, we again carried out the HRR assay under the conditions described above. Consistent with a positive role of ERK1/2 signaling in HRR, 4-hydroxytamoxifen treatment and activation of ΔRAF-ER* resulted in an ~2-fold increase in HRR (Fig. 3*A*). Both ΔRAF-ER* cell clones tested gave similar increases in HRR with 100 nmol/L of 4-hydroxytamoxifen. In parallel, samples were collected and ERK1/2 phosphorylation levels were determined by Western blotting. Relative ERK phosphorylation was plotted as a function of time (Fig. 3*B*). As expected, 4-hydroxytamoxifen treatment induced significant ERK1/2 phosphorylation as early as 2 h after exposure. Activation peaked at 24 h and begun to diminish by 48 h when HRR was determined. Interestingly, 1 μmol/L 4-hydroxytamoxifen induced much higher levels of ERK1/2 phosphorylation but had a lesser stimulatory effect on HRR (data not shown), suggesting that overstimulation of ERK1/2 signaling is counter-effective at the level of HRR. This result corroborates the results with the MEK inhibitors. Taken together, these data provide genetic evidence that RAF/MEK/ERK signaling promotes efficient HRR in human cancer cells.

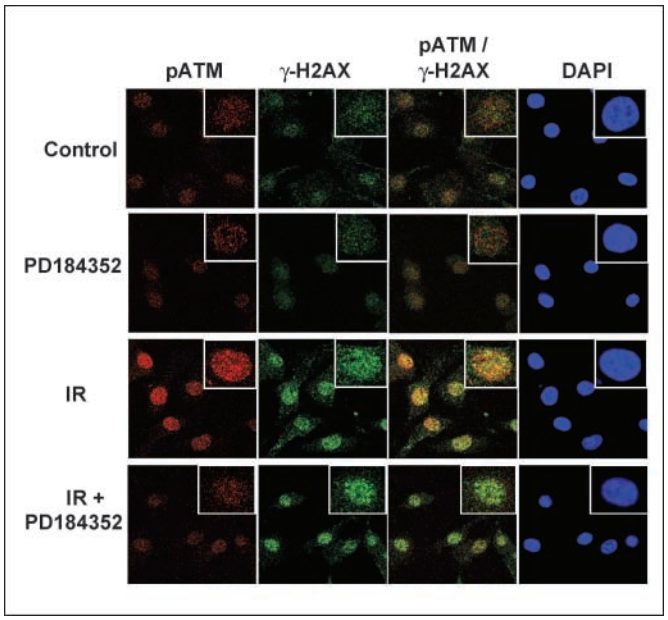

Figure 4. MEK/ERK signaling is important for phospho-(S1981) ATM foci formation. Growth-arrested U87/DR-GFP cells were exposed to 5 Gy IR with or without PD184352 (3 μmol/L) or KU-55933 (10 μmol/L) present and fixed after 15 min. Cells were prepared for confocal microscopy as described in Materials and Methods and exposed to anti–phospho-(S1981) ATM and/or anti–γ-H2AX antibodies. DAPI staining shows nuclei.

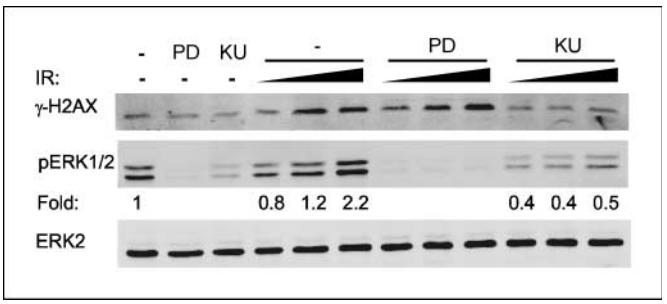

Figure 5. KU-55933 inhibits ERK1/2 signaling. Growth-arrested U87/DR-GFP cells were treated with KU-55933 (10 μmol/L) or PD184352 (3 μmol/L) and exposed to IR at 2.5, 5, or 10 Gy and collected for Western blot analysis after 15 min. Fold depicts phospho-ERK1/2 levels normalized to ERK2 protein levels. *PD,* PD184352; *KU,* KU-55933.

ERK1/2 signaling is important for phospho-(S1981) ATM foci formation. As a single I-*Sce*I–generated double-strand break is not sufficient to trigger a substantial DNA damage response in the U87/DR-GFP cells,[6] we wanted to determine whether MEK/ERK signaling is important in the IR-induced DNA damage response. To address this point, we exposed growth-arrested U87/DR-GFP cells to IR with or without PD184352 present (Fig. 4). As previously reported (28), we observed distinct γ-H2AX foci in response to IR. We also observed significant phospho-(S1981) ATM foci, which clearly colocalized with γ-H2AX as indicated by the shift toward yellow in the overlay (Fig. 4). However, most striking was the near-complete abrogation of phospho-ATM foci in the cells treated with PD184352 before IR. On the other hand, we observed only a slight reduction in the formation of γ-H2AX foci. This finding not only supports a role for ERK1/2 in the DNA damage response but also suggests that its role may be critical for ATM activation and/or foci formation. A Western blot of cell extracts from irradiated cells treated with or without inhibitors confirmed that KU-55933 not only blocked IR-induced γ-H2AX and p53 phosphorylation but also reduced ERK1/2 phosphorylation by 60% to 75% (Fig. 5; Supplementary Fig. S4). PD184352 did not block either p53 or H2AX phosphorylation in response to IR (Fig. 5; Supplementary Fig. S4), suggesting that DNA-dependent protein kinase catalytic subunit and/or ATR are able to phosphorylate H2AX and p53 when the ATM kinase is inhibited. In addition, flow cytometry showed that (S1981) ATM autophosphorylation occurred throughout the cell cycle after irradiation, a response that was compromised >85% in the presence of PD184352 (Fig. 6). KU-55933 blocked ATM autophosphorylation >90% in line with a previous report (13). Collectively, and to reconcile our findings, we propose that MEK/ERK signaling is critical for the phosphorylation of (S1981) ATM and/or the localization of ATM to repair foci, a step that is expected to be important for efficient HRR that would explain our results using the I-*Sce*I repair assay.

Discussion

HRR is considered to be most important in late S and G_2 phases of the cell cycle when sister chromatids are available. However, HRR could potentially also occur between homologous DNA repeats on heterologous chromosomes outside of the S and G_2 phases (6, 29, 30). Approximately half of the human genome

[6] A. Khalil and K. Valerie, unpublished observations.

consists of repetitive DNA sequences, and, thus, opportunities exist for HRR outside of S and G_2 although this would represent a minor component. Double-strand break repair and cell cycle regulation are intimately connected. Therefore, we used growth-arrested G_0-G_1 cells to minimize indirect cell cycle effects on double-strand break repair in our studies. The DR-GFP plasmid we used as a target or template for HRR consists of two defective copies of the *GFP* gene inserted in tandem between a stuffer DNA fragment carrying a puromycin expression cassette (19), thus potentially permitting HRR during all cell cycle phases. We previously showed that in human cells with an integrated copy of DR-GFP, HRR is detected in growth-arrested cells at ~40% of the levels seen with cycling cells (6). Similarly, Saleh-Gohari and Helleday detected HRR events in G_0-G_1 cells albeit at lower relative levels than what we have observed, and several recent studies showed that HRR occur at RAG-induced double-strand breaks during V(D)J recombination in G_0-G_1 (31–33). Furthermore, most if not all proteins necessary for HRR are expressed in G_0-G_1 cells. For example, both ATM and BRCA1 are expressed and activated by IR in growth-arrested human fibroblasts (34). Therefore, HRR seems to function outside of S and G_2, perhaps by using different subsets of proteins (30, 31).

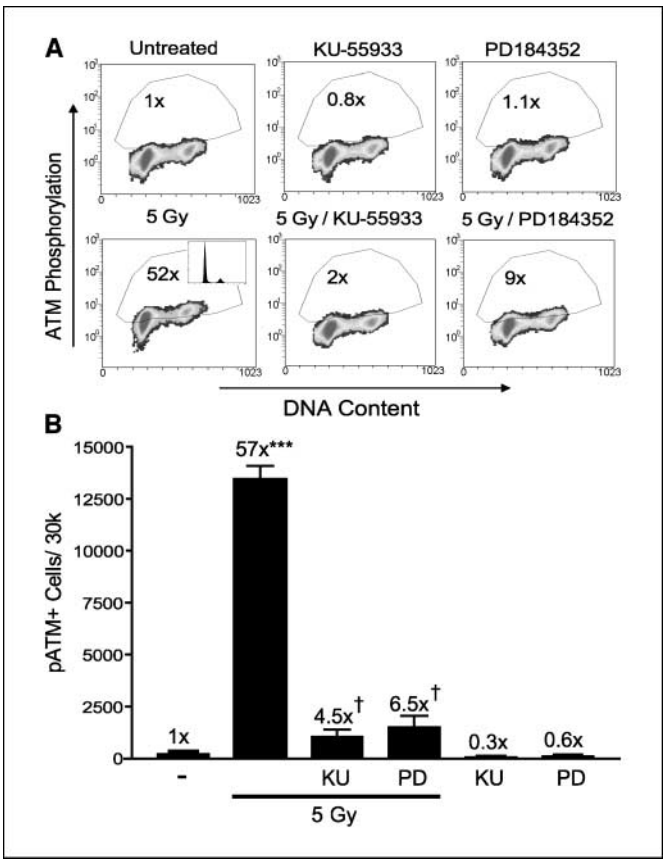

Figure 6. PD184352 compromises IR-induced ATM phosphorylation. Cycling U87/DR-GFP cells were exposed to 5 Gy with or without KU-55933 (10 μmol/L) or PD184352 (3 μmol/L) and fixed after 15 min. Cells were prepared for flow cytometry as described in Materials and Methods. *A,* FACS images represent DNA content (propidium iodine) versus relative phospho-(S1981) ATM levels. The histogram inserted into the 5 Gy density plot depicts the cell cycle profile of the phospho-ATM–positive cells. The area used to determine fold (*X*) phospho-(S1981) ATM levels were obtained from FACS of cells treated with the secondary anti-mouse Alexa Fluor 488 antibody only. *B,* phospho-ATM levels obtained from triplicate samples are depicted in graph form (*pATM+ Cells/30k*). Fold (*X*) increases shown over each column indicate relative levels compared with untreated cells (basal ATM phosphorylation levels). *Bars*, SE. ***, $P < 0.001$ ($n = 3$), when compared with basal ATM phosphorylation levels. †, $P < 0.001$, when compared with ATM phosphorylation levels after 5 Gy IR.

We previously showed that ATM is critical for HRR in human cells using caffeine, a relatively nonspecific PIKK inhibitor, and by the expression of a dominant-negative allele of ATM (6). In the present study, we found that the ATM kinase–specific inhibitor KU-55933 significantly reduced HRR at doses that inhibit the ATM kinase. Interestingly, inhibition of HRR was seen under conditions when ATM was not activated by IR or any other DNA damage except for the single I-*Sce*I–generated double-strand break, suggesting that the ATM kinase is important for coordinating double-strand break repair at the site of double-strand breaks even at very low levels of DNA damage. In support of this idea, ATM was shown to stabilize double-strand break repair complexes during V(D)J recombination in growth-arrested cells (35), and we previously reported that ATM is present at the I-*Sce*I double-strand break by chromatin immunoprecipitation analysis (6). We observed an enhanced inhibitory effect of KU-55933 on HRR in growth-arrested cells compared with cycling cells. This may be because ATR or other PIKKs are available and can serve as a backup for ATM during other phases of the cell cycle, especially during the S phase. ATR is an essential DNA damage sensor and is critical for HRR and the repair of double-strand breaks occurring during DNA replication (36). Thus, we have confirmed in this study our previous finding that the ATM kinase is important for HRR using the ATM-specific pharmacologic inhibitor KU-55933 (6).

Irrespective of the specific mechanisms involved, our results strongly suggest that RAF/MEK/ERK signaling is associated with ATM regulation and HRR, and, clearly, the blockade of MEK/ERK signaling has a major effect on phospho-(S1981) ATM foci formation in response to radiation. Strikingly, in our study, ERK kinase activity was required for ATM phosphorylation. However, inhibition of MEK/ERK signaling did not affect IR-induced phosphorylation of p53 and H2AX, which are both direct ATM phosphorylation targets. This is probably due to redundant phosphorylation of (S15) p53 and (S139) H2AX by other PIKKs, such as DNA-PK and ATR (3, 37–39). In addition, ERK phosphorylation was significantly reduced in the presence of KU-55933. The KU-55933 inhibitor was screened against a panel of 60 protein kinases, including ERK, without observing any significant nonspecific effects (13), suggesting that the inhibition of ERK phosphorylation by KU-55933 is ATM mediated. Our result is in agreement with a previous study suggesting that ATM regulates ERK in a p53-independent manner in response to DNA damage (22). However, the mechanism by which ATM affects ERK signaling is not yet known. Previous studies have shown a role for ATM in epidermal growth factor receptor and insulin-like growth factor-IR signaling and regulation (40–42). Conceivably, signaling could thus occur at the level of an as of yet unidentified growth factor receptor that would stimulate ERK. How ERK affects ATM activation is also not known. Potentially, this could occur by direct phosphorylation of ATM by ERK, but whether this is the case or not remains to be determined. Recent work has shown that ATM activation is tightly regulated by the actions of protein phosphatases PP2A and PP5 (43, 44), and the acetyl transferases Tip60 and hMOF (45, 46). Thus, one possibility that could explain our findings is that ERK may regulate ATM activation, nuclear transport, or protein stability indirectly through a protein phosphatase or acetyl transferase. Recently, one study showed that MEK/ERK signaling was important for the formation of ATR nuclear foci in response to hydroxyurea treatment, which resulted in DNA replication arrest and double-strand

break formation in the S phase (47). A similar mechanism could link ERK and ATM perhaps through a feedback loop that might regulate cellular homeostasis and sense the well being of the cell; if DNA damage is repairable, prosurvival ERK signaling might positively influence HRR, whereas overwhelming DNA damage might inhibit ERK signaling and HRR. If this is the case, it could be a possible mechanism by which ERK signaling intersects with ATM signaling to coordinate cell cycle checkpoints and HRR. It should be pointed out that this effect seems bimodal because it is clear from our studies (data not shown),[7] and those of others, that the extent of ERK signaling is critical for achieving either positive or negative effects on HRR, cell growth, and radiosurvival (1, 48).

Although the emphasis of the present work was on the role of ERK signaling in HRR, we also report on the importance of JNK and p38 signaling in HRR. These results are preliminary and serve as controls for the results generated by interfering with ERK signaling. Nevertheless, similar to the effect of abrogating ERK signaling when HRR was examined, inhibiting JNK signaling also resulted in an inhibition of HRR. Although this finding needs to be confirmed by more detailed experimentation in future studies, it suggests that JNK signaling is also important for efficient HRR in human cancer cells. Keeping with the notion that ATM might be involved in regulating JNK signaling important for HRR, A-T cells have deregulated JNK/c-Jun signaling, c-Jun colocalizes with ATM and γ-H2AX in IRIF, and ATM has been shown to phosphorylate ATF-2, which forms the activator protein-1 transcription factor together with c-Jun (11, 24, 49, 50). Thus, the finding that JNK is important for HRR fits conceptually with earlier work.

As a striking difference to the results obtained using inhibitors aimed at ERK and JNK signaling, SB203580, the p38α/β MAPK-specific drug, stimulated HRR in growth-arrested cells. One possibility for this enhancement could be that ERK signaling is stimulated, as we have shown here. Other possibilities are plausible as well. However, more thorough future studies may reveal what role p38 might play in HRR and whether this is an indirect or direct effect.

In summary, we have shown here that all three major MAPK signaling pathways affect HRR. The role of ERK was clearly defined as a positive regulator of HRR, whereas the roles of JNK and p38 seems to be positive and negative, respectively, and are currently being investigated in more detail. We have also shown that inhibiting the ATM kinase with KU-55933 reduced ERK phosphorylation, suggesting that ATM controls signaling through the ERK pathway. Furthermore, a highly specific inhibitor of MEK/ERK signaling compromised ATM kinase activity and severely attenuated the phosphorylation and localization of ATM to foci, suggesting that ERK signaling affects repair protein complex formation or stability, and/or localization of ATM necessary for efficient HRR. Thus, ATM and ERK signaling could be under the control of a regulatory feedback loop.

[7] S. Golding, A. Khalil, and K. Valerie, unpublished observations.

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References

1. Dent P, Yacoub A, Contessa J, et al. Stress and radiation-induced activation of multiple intracellular signaling pathways. Radiat Res 2003;159:283–300.
2. Valerie K, Povirk LF. Regulation and mechanisms of mammalian double-strand break repair. Oncogene 2003; 22:5792–812.
3. Thompson LH, Schild D. Recombinational DNA repair and human disease. Mutat Res 2002;509:49–78.
4. Bernstein C, Bernstein H, Payne CM, Garewal H. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. Mutat Res 2002;511:145–78.
5. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499–506.
6. Golding SE, Rosenberg E, Khalil A, et al. Double-strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells. J Biol Chem 2004;279:15402–10.
7. Perkins EJ, Nair A, Cowley DO, et al. Sensing of intermediates in V(D)J recombination by ATM. Genes Dev 2002;16:159–64.
8. Andegeko Y, Moyal L, Mittelman L, et al. Nuclear retention of ATM at sites of DNA double strand breaks. J Biol Chem 2001;276:38224–30.
9. Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. MAPK pathways in radiation responses. Oncogene 2003; 22:5885–96.
10. Wang X, McGowan CH, Zhao M, et al. Involvement of the MKK6-38γ cascade in γ-radiation-induced cell cycle arrest. Mol Cell Biol 2000;20:4543–52.
11. Hayakawa J, Depatie C, Ohmichi M, Mercola D. The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. J Biol Chem 2003;278:20582–92.
12. Sebolt-Leopold JS, Dudley DT, Herrera R, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. Nat Med 1999;5:810–6.
13. Hickson I, Zhao Y, Richardson CJ, et al. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Res 2004;64:9152–9.
14. Valerie K, Brust D, Farnsworth J, et al. Improved radiosensitization of rat glioma cells with adenovirus-expressed mutant HSV-TK in combination with acyclovir. Cancer Gene Ther 2000;7:879–84.
15. Samuels ML, Weber MJ, Bishop JM, McMahon M. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. Mol Cell Biol 1993;13:6241–52.
16. Auer KL, Park JS, Seth P, et al. Prolonged activation of the mitogen-activated protein kinase pathway promotes DNA synthesis in primary hepatocytes from p21Cip-1/WAF1-null mice, but not in hepatocytes from p16INK4a-null mice. Biochem J 1998;336:551–60.
17. Taher MM, Baumgardner T, Dent P, Valerie K. Genetic evidence that stress-activated p38 MAP kinase is necessary but not sufficient for UV activation of HIV gene expression. Biochemistry 1999;38:13055–62.
18. Rosenberg E, Hawkins W, Holmes M, et al. Radiosensitization of human glioma cells *in vitro* and *in vivo* with acyclovir and mutant HSV-TK75 expressed from adenovirus. Int J Radiat Oncol Biol Phys 2002;52:831–6.
19. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev 1999;13:2633–8.
20. Taghian DG, Nickoloff JA. Chromosomal double-strand breaks induce gene conversion at high frequency in mammalian cells. Mol Cell Biol 1997;17: 6386–93.
21. Morrison C, Sonoda E, Takao N, et al. The controlling role of ATM in homologous recombinational repair of DNA damage. EMBO J 2000;19:463–71.
22. Tang D, Wu D, Hirao A, et al. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem 2002;277: 12710–7.
23. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95–105.
24. MacLaren A, Black EJ, Clark W, Gillespie DA. c-Jun-deficient cells undergo premature senescence as a result of spontaneous DNA damage accumulation. Mol Cell Biol 2004;24:9006–18.
25. Bennett BL, Sasaki DT, Murray BW, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 2001;98:13681–6.
26. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.
27. Kim SY, Suzuki N, Laxmi YR, et al. Antiestrogens and the formation of DNA damage in rats: a comparison. Chem Res Toxicol 2006;19:852–8.
28. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 1998;273: 5858–68.
29. Richardson C, Moynahan ME, Jasin M. Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. Genes Dev 1998;12:3831–42.

30. Fabre F. Induced intragenic recombination in yeast can occur during the G_1 mitotic phase. Nature 1978;272:795–8.

31. Lee GS, Neiditch MB, Salus SS, Roth DB. RAG proteins shepherd double-strand breaks to a specific pathway, suppressing error-prone repair, but RAG nicking initiates homologous recombination. Cell 2004;117:171–84.

32. Weinstock DM, Jasin M. Alternative pathways for the repair of RAG-induced DNA breaks. Mol Cell Biol 2006;26:131–9.

33. Saleh-Gohari N, Helleday T. Conservative homologous recombination preferentially repairs DNA double-strand breaks in the S phase of the cell cycle in human cells. Nucleic Acids Res 2004;32:3683–8.

34. Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB. Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-1 pathway. Genes Dev 2004;18:1423–38.

35. Bredemeyer AL, Sharma GG, Huang CY, et al. ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. Nature 2006;442:466–70.

36. Wang H, Wang H, Powell SN, Iliakis G, Wang Y. ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of nonhomologous end joining. Cancer Res 2004;64:7139–43.

37. Achanta G, Pelicano H, Feng L, Plunkett W, Huang P. Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. Cancer Res 2001;61:8723–9.

38. Woo RA, Jack MT, Xu Y, et al. DNA damage-induced apoptosis requires the DNA-dependent protein kinase, and is mediated by the latent population of p53. EMBO J 2002;21:3000–8.

39. Stiff T, O'Driscoll M, Rief N, et al. ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. Cancer Res 2004;64:2390–6.

40. Peretz S, Jensen R, Baserga R, Glazer PM. ATM-dependent expression of the insulin-like growth factor-I receptor in a pathway regulating radiation response. Proc Natl Acad Sci U S A 2001;98:1676–81.

41. Keating KE, Gueven N, Watters D, Rodemann HP, Lavin MF. Transcriptional downregulation of ATM by EGF is defective in ataxia-telangiectasia cells expressing mutant protein. Oncogene 2001;20:4281–90.

42. Macaulay VM, Salisbury AJ, Bohula EA, et al. Downregulation of the type 1 insulin-like growth factor receptor in mouse melanoma cells is associated with enhanced radiosensitivity and impaired activation of Atm kinase. Oncogene 2001;20:4029–40.

43. Goodarzi AA, Jonnalagadda JC, Douglas P, et al. Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. EMBO J 2004;23:4451–61.

44. Ali A, Zhang J, Bao S, et al. Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. Genes Dev 2004;18:249–54.

45. Sun Y, Jiang X, Chen S, Fernandes N, Price BD. A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci U S A 2005;102:13182–7.

46. Gupta A, Sharma GG, Young CS, et al. Involvement of human MOF in ATM function. Mol Cell Biol 2005;25:5292–305.

47. Wu D, Chen B, Parihar K, et al. ERK activity facilitates activation of the S-phase DNA damage checkpoint by modulating ATR function. Oncogene 2006;25:1153–64.

48. Kwok TT, Sutherland RM. Cell cycle dependence of epidermal growth factor induced radiosensitization. Int J Radiat Oncol Biol Phys 1992;22:525–7.

49. Weizman N, Shiloh Y, Barzilai A. Contribution of the Atm protein to maintaining cellular homeostasis evidenced by continuous activation of the AP-1 pathway in Atm-deficient brains. J Biol Chem 2003;278:6741–7.

50. Bhoumik A, Takahashi S, Breitweiser W, et al. ATM-dependent phosphorylation of ATF2 is required for the DNA damage response. Mol Cell 2005;18:577–87.

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