

# ATM Acts Downstream of ATR in the DNA Damage Response Signaling of Bystander Cells

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## Abstract

**This study identifies ataxia-telangiectasia mutated (ATM) as a further component of the complex signaling network of radiation-induced DNA damage in nontargeted bystander cells downstream of ataxia-telangiectasia and Rad3-related (ATR) and provides a rationale for molecular targeted modulation of these effects. In directly irradiated cells, ATR, ATM, and DNA-dependent protein kinase (DNA-PK) deficiency resulted in reduced cell survival as predicted by the known important role of these proteins in sensing DNA damage. A decrease in clonogenic survival was also observed in ATR/ATM/DNA-PK-proficient, nonirradiated bystander cells, but this effect was completely abrogated in ATR and ATM but not DNA-PK-deficient bystander cells. ATM activation in bystander cells was found to be dependent on ATR function. Furthermore, the induction and colocalization of ATR, 53BP1, ATM-S1981P, p21, and BRCA1 foci in nontargeted cells was shown, suggesting their involvement in bystander DNA damage signaling and providing additional potential targets for its modulation. 53BP1 bystander foci were induced in an ATR-dependent manner predominantly in S-phase cells, similar to  $\gamma$ H2AX foci induction. In conclusion, these results provide a rationale for the differential modulation of targeted and nontargeted effects of radiation.** [Cancer Res 2008;68(17):7059–65]

## Introduction

There is now an extensive body of evidence for the spatial and temporal transmission of adverse effects from irradiated cells to unirradiated “bystander” cells. Such ionizing radiation-induced nontargeted effects have been reported for a range of end points (1–6), including the induction of  $\gamma$ H2AX foci (7–11), which serve as a marker for DNA double-strand breaks (12). Links between bystander responses in nontargeted cells and DNA repair pathways have been proposed (reviewed in ref. 13).

Most recently, ataxia-telangiectasia and Rad3-related (ATR) has been identified as a central figure within the bystander signaling cascade leading to  $\gamma$ H2AX foci formation, whereas ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) function were not essential for the induction of bystander  $\gamma$ H2AX foci. Bystander foci induction is cell cycle dependent and was observed predominantly in S-phase cells (8).

These observations support the hypothesis of an accumulation of stalled replication forks in bystander cells, which induces  $\gamma$ H2AX foci in an ATR-dependent manner. In support of this hypothesis, H2AX phosphorylation at sites of gemcitabine-induced stalled replication forks has recently been reported (14).

DNA replication fork stalling can be caused by double-stranded DNA or ssDNA breaks produced by reactive oxygen species (ROS), or by adverse secondary structures of the DNA. ATR is involved in the recognition of stalled replication forks, as failure to stabilize them results in their collapse and ultimately in genetic instability (reviewed in ref. 15).

ATR, ATM, and DNA-PK are members of the phosphoinositol 3-kinase-like kinase (PIKK) family, which act as sensors of DNA damage and translate the signal into responses of cell cycle arrest and DNA repair (16). ATR is associated with an activating subunit ATRIP, which responds to ssDNA-RPA complexes (17). ATR recruitment to DNA damage induced by ionizing radiation depends on the ATM-MRN complex and results in Chk1 phosphorylation (18). In contrast, ATR is recruited to sites of stalled replication and UV damage independent of ATM, and the phosphorylation and activation of ATM in response to UV treatment or replication fork stalling is ATR dependent (19). There is also substantial overlap between ATR and ATM downstream signaling, which merges into an extensive signaling network (20).

This study reports a radiation-induced decrease in clonogenic survival in ATR/ATM-proficient bystander cells but a complete abrogation of this effect in ATR/ATM but not DNA-PK-deficient cells. We conclude that both ATR and ATM participate in bystander signaling leading to decreased survival in nontargeted cells. In contrast, in directly targeted cells, survival decreased on ATR, ATM, and DNA-PK inhibition, suggesting the possibility of differential modulation of targeted and nontargeted effects through ATM and ATR inhibitors. Furthermore, we show the induction and colocalization of ATR, 53BP1, ATM-S1981P, p21 (WAF1/CIP1), and BRCA1 foci in nontargeted cells. 53BP1 foci induction occurred, similar to  $\gamma$ H2AX foci, in an ATR-dependent manner in S-phase bystander cells, and ATM activation was also found to be dependent on ATR function.

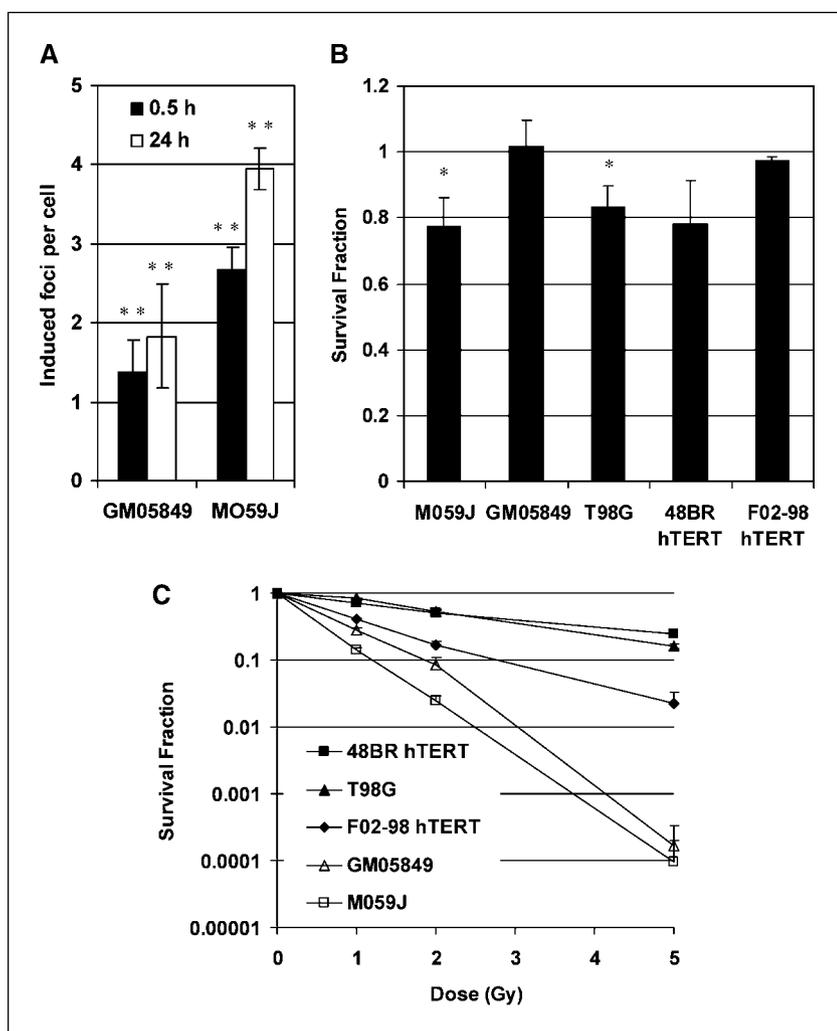
## Materials and Methods

**Cell culture.** T98G glioma cells, GM05849 ATM-deficient fibroblasts, and M059J (DNA-PK mutated) glioma cells were cultured in RPMI 1640 (Cambrex) supplemented with 10% fetal bovine serum (FBS; PAA), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin (all Cambrex). T98G cells were obtained from the European Collection of Animal Cell Cultures, and GM05849 ATM-deficient fibroblasts were obtained from Cancer Research UK. M059J glioma cells were a kind gift from A. Kiltie (Leeds Institute of Molecular Medicine, Leeds, United Kingdom). The DNA-PK mutant cell line M059J, which lacks the p350 DNA-PK subunit (21), has been used in previous studies on the effect of DNA-PK deficiency on a range of biological end points (22–25).

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**Figure 1.** A, bystander  $\gamma$ H2AX foci induction in DNA-PK–mutated M059J cells and ATM–mutated GM05849 fibroblasts. Induced foci numbers per cell in bystander cultures were calculated as average foci number per cell minus average foci number in control cultures. B, clonogenic survival in bystander cells. DNA-PK–mutated M059J cells, ATM–mutated GM05849 fibroblasts, T98G glioma cells, 48BR hTERT fibroblasts, and ATR–mutated F02-98 hTERT fibroblasts were treated with medium derived from irradiated T98G cells. C, clonogenic survival in directly irradiated cells. DNA-PK–mutated M059J cells, ATM–mutated GM05849 fibroblasts, T98G glioma cells, 48BR hTERT fibroblasts, and ATR–mutated F02-98 hTERT fibroblasts were irradiated with 1, 2, or 5 Gy of X-rays. Bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , paired  $t$  test.

ATR–mutated Seckel cells (F02-98 hTERT; ref. 26) and matched ATR–proficient fibroblasts (48BR hTERT), received as gift from P. Jeggo (University of Sussex, Brighton, United Kingdom), were cultivated in MEM supplemented with 15% FBS, 2 mmol/L L–glutamine, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin (all Cambrex), and 0.4  $\mu$ g/mL puromycin (Sigma).

All cells were incubated at 37°C, 5% CO<sub>2</sub>. For all experiments, nonconfluent cell cultures were used.

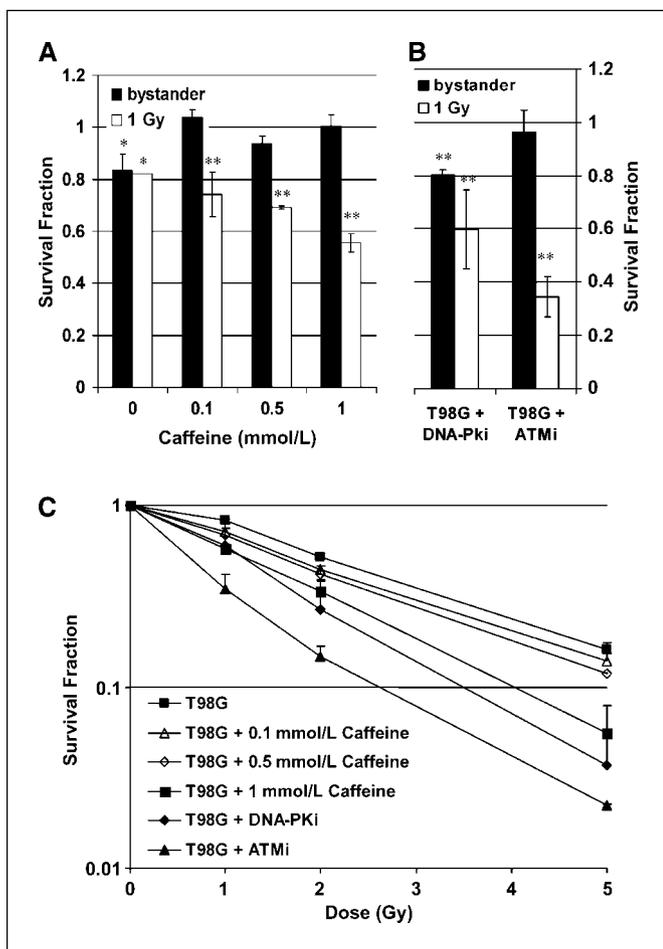
For medium transfer experiments, cells were seeded on 22  $\times$  22 mm coverslips placed in six-well tissue culture dishes and were treated with filtered medium obtained from T98G cells, which had been irradiated with 2 Gy of X-rays (240 kV; Pantak) followed by 30 to 60 min of incubation. The recipient cells were incubated with the conditioned medium at 37°C, 5% CO<sub>2</sub>.

**Clonogenic cell survival.** Cells were seeded in T25 tissue culture flasks containing filtered medium derived from irradiated (2 Gy, X-rays) or sham-irradiated T98G cells and incubated for 10 to 14 d at 37°C, 5% CO<sub>2</sub>. For experiments involving direct irradiation, individual doses were 0, 1, 2, and 5 Gy. Flasks were stained with crystal violet staining solution, and individual colonies were counted. Average survival fractions and SE of two to four independent experiments performed in triplicate were calculated.

The final concentration for the ATM inhibitor KU-55933 (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one, Calbiochem/Merck) and the DNA-PK inhibitor NU7026 (KuDos) was 10  $\mu$ mol/L. Caffeine (Sigma) was used as an ATR/ATM inhibitor at a concentration of 0.1, 0.5, and 1 mmol/L. The

inhibitors were added to the culture medium 15 min before direct irradiation or were added into the conditioned medium before transfer to recipient cells for bystander experiments.

**Immunocytochemistry and microscopy.** For immunocytochemistry, cells were fixed for 15 min with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (both Sigma), and blocked with 3% FBS in PBS for 30 min at room temperature. Incubation with a primary antibody specific for H2AX p139S (Upstate), 53BP1 (Novus Biologicals), BRCA1 (Oncogene), p21/Cip1/WAF1 (PharMingen/BD Biosciences), ATM p1981S (Upstate), and ATR (N-19; Santa Cruz Biotechnology) for 1 h at room temperature was followed by incubation with a matching Alexa Fluor 488– or Alexa Fluor 568–labeled secondary antibody (Molecular Probes) and nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Cells were grown on coverslips and the inverted coverslip was placed on a glass slide with Vectashield mounting medium for fluorescence microscopy (Vector Laboratories) after staining was completed, and the edges were sealed with clear nail varnish. A fluorescence microscope was used for imaging and analysis (Zeiss). For quantification of foci numbers, cells were microscopically analyzed for individual foci. Costaining for 53BP1 and  $\gamma$ H2AX was analyzed in one representative experiment scoring  $\geq 50$  cells per data point. For 53BP1/bromodeoxyuridine (BrdUrd) double staining,  $\geq 99$  cells per slide were scored for 53BP1 foci numbers in BrdUrd<sup>+</sup> and BrdUrd<sup>-</sup> cells in one representative experiment. For single analysis of 53BP1,  $\gamma$ H2AX, and ATM-S1981P foci induction, two to five independent experiments were evaluated; average induced foci and SE were calculated. Average foci per cell and distribution of foci numbers in the analyzed cell population were calculated



**Figure 2.** Modulation of clonogenic survival in directly irradiated T98G cells or T98G bystander cells treated with medium derived from irradiated cells. *A*, caffeine pretreatment. *B*, pretreatment with 10  $\mu$ mol/L of the ATM inhibitor (*ATMi*) KU-55933 or the DNA-PK inhibitor (*DNA-PKi*) NU7026. *C*, dose-response curves for direct irradiation after pretreatment with caffeine, ATM inhibitor, and DNA-PK inhibitor. Bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , paired  $t$  test.

using Microsoft Excel. For the calculation of induced foci per cell, background foci numbers were subtracted.

For the detection of BrdUrd incorporation, cells were incubated with medium containing 20  $\mu$ mol/L BrdUrd for 15 min before fixation. A BrdUrd-specific primary antibody followed by a matching Alexa Fluor 488-labeled secondary antibody (both Molecular Probes) was applied after treatment with 2 mol/L HCl for 15 min at room temperature and neutralization with 0.1 mol/L sodium borate buffer. For colocalization analysis, ImageJ software and Colocalisation Test plugin (T. Collins and W. Rasband)<sup>3</sup> were used to calculate Pearson's correlation coefficients (27) and determine the significance of correlation coefficients for individual cells using Costes' spatial statistics approach (28). DAPI images were used as masks to restrict the analysis to the cell nucleus.

**Statistical analysis.** Average foci numbers per cell, average survival fractions, and SEs were calculated using Microsoft Excel. The significance of reported findings was tested with a  $t$  test comparing treated samples with their sham-treated controls. Significance levels of  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) were indicated in the diagrams.

<sup>3</sup> [http://www.uhnresearch.ca/facilities/wcif/software/Plugins/colocalisation\\_test.html](http://www.uhnresearch.ca/facilities/wcif/software/Plugins/colocalisation_test.html)

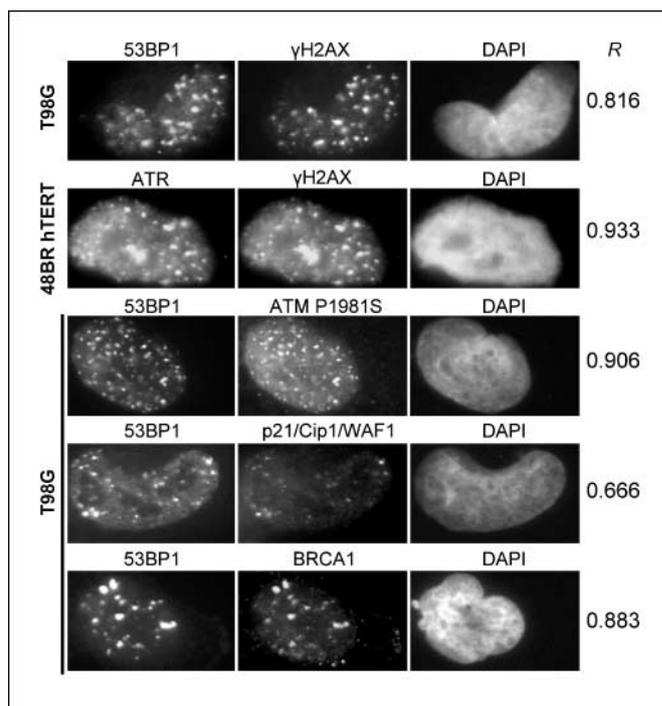
## Results

**Reduced clonogenic survival in bystander cells.** A robust bystander response has previously been shown for T98G glioma cells for various end points, including the induction of  $\gamma$ H2AX foci and micronuclei (1, 8, 29).  $\gamma$ H2AX bystander foci have previously also been shown in 48BR hTERT cells but did not occur in ATR-mutated F02-98 hTERT cells (8). In this study, we confirmed significant  $\gamma$ H2AX bystander foci induction ( $P < 0.01$ ,  $t$  test) in DNA-PK-mutated M059J cells and ATM-mutated GM05849 fibroblasts after 0.5 and 24 h of incubation with conditioned medium derived from irradiated T98G cells (Fig. 1A). Induced foci per cell describe the average increase in foci per cell after subtraction of average background foci, which are 1.7 foci per cell for GM05849 and 3.9 foci per cell for M059J cells.

We also investigated the effect of medium transferred from irradiated T98G cells to nonirradiated bystander cells on clonogenic survival in a colony formation assay. A reduction of clonogenic survival by 20% was shown for T98G bystander cells ( $P < 0.05$ ,  $t$  test), and a similar effect was seen in 48BR hTERT fibroblasts ( $P = 0.059$ ) and DNA-PK-mutated M059J cells ( $P < 0.05$ ). In contrast, ATM-mutated GM05849 fibroblasts as well as ATR-mutated F02-98 hTERT cells did not show a bystander effect for clonogenic survival (Fig. 1B).

As expected, following direct irradiation, M059J, F02-98 hTERT, and GM05849 cells that bear mutations in DNA-PK, ATR, or ATM, respectively, were much more radiosensitive than T98G cells and 48BR hTERT fibroblasts that are DNA-PK, ATR, and ATM proficient (Fig. 1C).

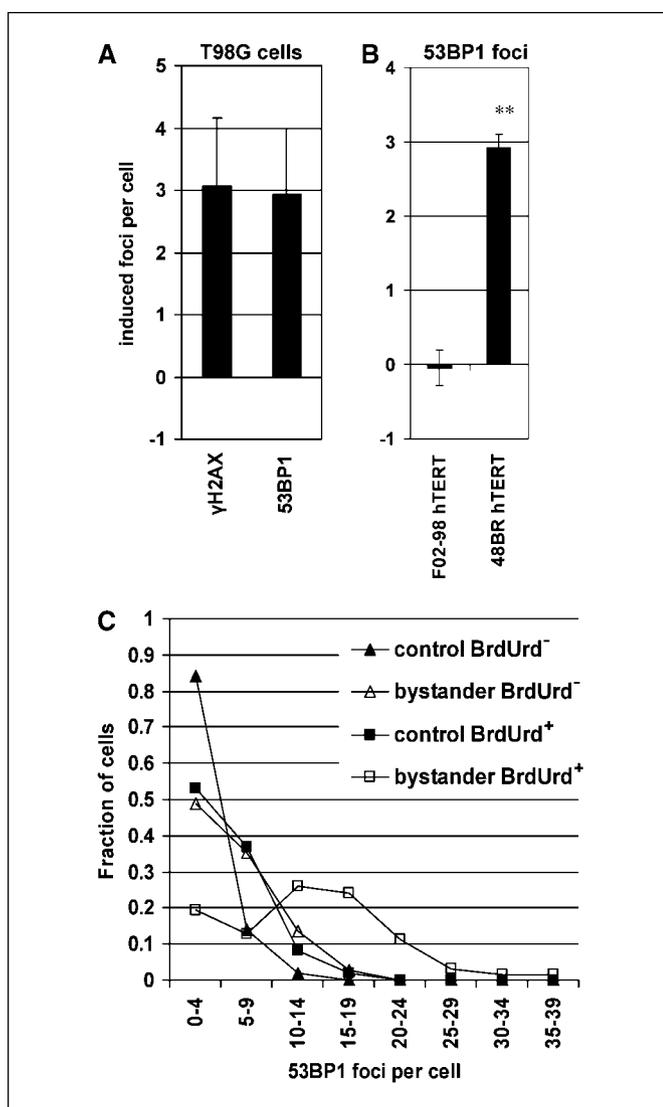
**Modulation of the bystander effect on clonogenic survival.** The above results suggest a dependency of the bystander effect, measured as reduced clonogenic survival, on the presence of functional ATR and ATM. To test this hypothesis, T98G cells were



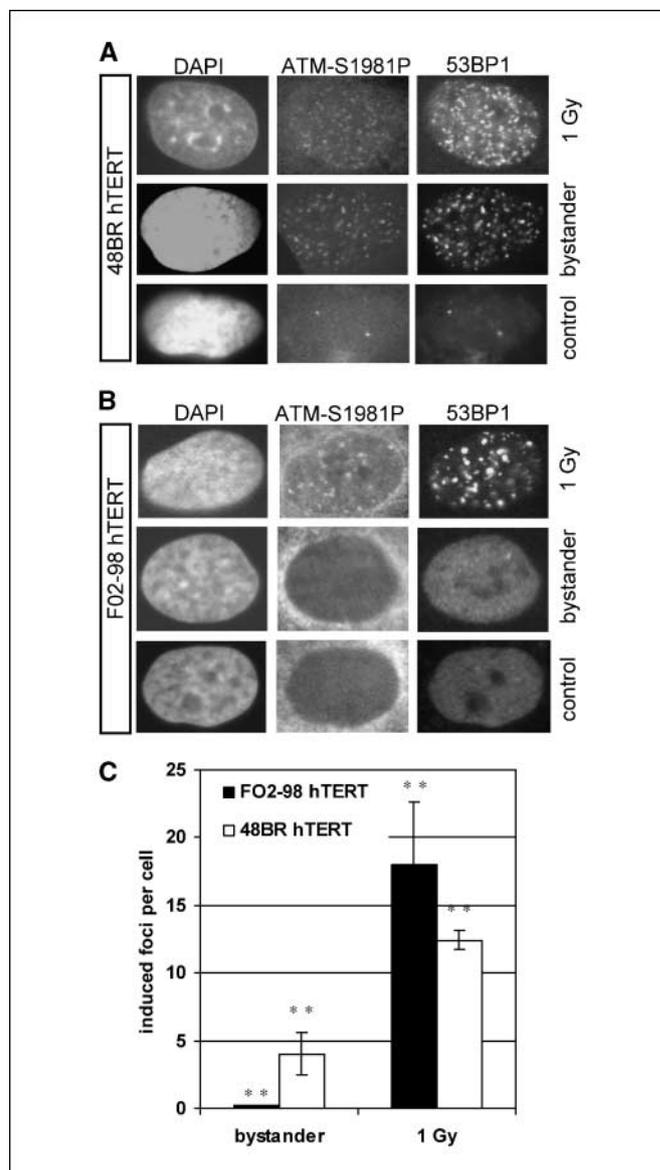
**Figure 3.** Colocalization of proteins involved in DNA damage recognition and repair and in cell cycle control in 48BR hTERT or T98G bystander cells at 30 min after transfer of medium from irradiated T98G cells. *R* values are Pearson's correlation coefficients.

treated with caffeine at 0.1, 0.5, and 1 mmol/L and either incubated with medium derived from irradiated cells (Fig. 2A) or subjected to direct irradiation (Fig. 2A and C). Caffeine has been reported to inhibit ATM and ATR causing G<sub>2</sub> checkpoint abrogation (30), although there is evidence for other pathways being involved (31). For directly irradiated cells, a significant radiosensitization was detected with 1mmol/L caffeine ( $P < 0.05$  at 5 Gy, *t* test), whereas a smaller effect was seen at 0.1 and 0.5 mmol/L. In contrast, an abrogation of the bystander effect on clonogenic survival was shown at all concentrations tested.

In a further experiment, the DNA-PK inhibitor NU7026 and the ATM inhibitor KU-55933 were tested in T98G cells for a modulation of clonogenic survival in directly irradiated and bystander cells. For direct irradiation, both inhibitors caused significant radiosensitization of T98G cells at 10  $\mu$ mol/L ( $P < 0.05$  for DNA-PK inhibitor and  $P < 0.01$  for ATM inhibitor at 5 Gy, *t* test; Fig. 2C). The ATM inhibitor KU-55933 abrogated the bystander effect, whereas in cells treated with the DNA-PK inhibitor NU7026 a significant bystander



**Figure 4.** Induction of 53BP1 bystander foci at 30 min after transfer of medium from irradiated T98G cells. **A**, 53BP1 versus  $\gamma$ H2AX foci in T98G bystander cells. **B**, 53BP1 foci in ATR-proficient 48BR hTERT versus ATR-mutated F02-98 hTERT cells. Bars, SE. \*\*,  $P < 0.01$ , paired *t* test. **C**, distribution of 53BP1 foci in bystander and control cell cultures after BrdUrd pulse labeling.



**Figure 5.** Phosphorylation of ATM (Ser<sup>1981</sup>) and colocalization with 53BP1 foci in directly irradiated bystander and control cells at 30 min after irradiation/medium transfer. **A**, ATR-proficient 48BR hTERT cells. **B**, ATR-mutated F02-98 hTERT cells. **C**, columns, average induced phospho-ATM foci per cell; bars, SE. \*\*,  $P < 0.01$ , paired *t* test.

response was still observed ( $P < 0.01$ , *t* test; Fig. 2B). These results suggest a key role for ATM in the bystander response but do not identify a role for DNA-PK, thus confirming the results observed in the mutant cell lines.

**Colocalization of  $\gamma$ H2AX foci with DNA repair-associated proteins in bystander cells.** The induction of  $\gamma$ H2AX foci, a marker for DNA damage, has been shown previously in bystander cells (7–11), and therefore, colocalization of  $\gamma$ H2AX bystander foci with other proteins associated with the recognition of DNA damage and repair was predicted. Accordingly, 48BR hTERT fibroblasts and T98G cells were analyzed for the induction of  $\gamma$ H2AX, 53BP1, ATR, ATMpS1981, p21/Cip1/WAF1, and BRCA1 foci (Fig. 3) 30 min after the transfer of conditioned medium from irradiated cells.  $\gamma$ H2AX bystander foci were found to colocalize with 53BP1 foci and with ATR foci, and

colocalization was also observed for 53BP1 with p21/Cip1/WAF1 foci, 53BP1 with BRCA1 foci, and 53BP1 with ATMpS1981 foci in bystander cells. Pearson's correlation coefficients were calculated for channel 1 versus channel 2 (Fig. 3, *left* and *middle column*) and Costes' spatial statistics approach showed significant colocalization for all tested combinations (i.e., in each case, Pearson's correlation coefficients were greater than any correlation coefficient calculated between channel 1 and 10 randomly scrambled channel 2s).

**ATR-dependent 53BP1 foci induction in bystander cells.** 53BP1 is a central figure in the DNA damage response (32–36). The induction of 53BP1 foci has previously been investigated in a study of effects induced by targeted cytoplasmic irradiation (37).

Here, a similar induction of 53BP1 and  $\gamma$ H2AX foci in bystander cells could be detected in T98G glioma cells after 30 min of incubation with conditioned medium from irradiated cells (Fig. 4A). The induction of 53BP1 foci depended on functional ATR within the recipient cells, as ATR-mutated F02-98 hTERT fibroblasts did not show 53BP1 foci induction in bystander cells in contrast to 48BR hTERT ATR-proficient fibroblasts (Fig. 4B). Background 53BP1 foci numbers were cell line dependent and highly variable between different cell lines with an average of 1.8 foci in F02-98 hTERT, 5.2 foci in 48BR hTERT, and 8.4 in T98G cells. In T98G cells, high 53BP1 foci numbers ( $\geq 10$  foci per cell) were predominantly observed in BrdUrd<sup>+</sup> bystander cells (Fig. 4C), confirming a link between S-phase/DNA replication and the early bystander response. These results show an ATR-dependent 53BP1 bystander foci induction

in S-phase cells comparable with the previously reported induction of  $\gamma$ H2AX foci.

**ATR-dependent ATM phosphorylation in bystander cells.** To further investigate the mechanism of ATM activation in the bystander signaling cascade, ATR-proficient 48BR hTERT cells and ATR-mutated F02-98 hTERT cells were analyzed for ATM phosphorylation (Ser<sup>1981</sup>) in directly irradiated and bystander cells (Fig. 5). For both 48BR hTERT and F02-98 hTERT cells, ATM phosphorylation was detected in directly irradiated cells (1 Gy, X-rays) as early as 30 min after irradiation and these phospho-ATM foci colocalized with 53BP1 foci. When medium was transferred from irradiated to nonirradiated cells, recipient bystander 48BR hTERT cells developed; on average, 4 additional phospho-ATM foci per cell colocalized with 53BP1 foci, whereas recipient bystander F02-98 hTERT cells showed only 0.3 additional phospho-ATM foci per cell consistent with previously described residual ATR activity in F02-98 hTERT cells (19). These findings support the hypothesis of a dependency of ATM phosphorylation on ATR signaling in bystander cells similar to 53BP1 foci formation. Background ATM-S1981P foci numbers were 0.7 in F02-98 hTERT and 4.0 in 48BR hTERT cells.

## Discussion

This study contributes to our understanding of mechanisms of radiation-induced signaling in nontargeted bystander cells, which differs in several key aspects from the response of targeted cells. We have previously reported the ATR-dependent induction of  $\gamma$ H2AX foci for a prolonged time in bystander cells (8), and in this

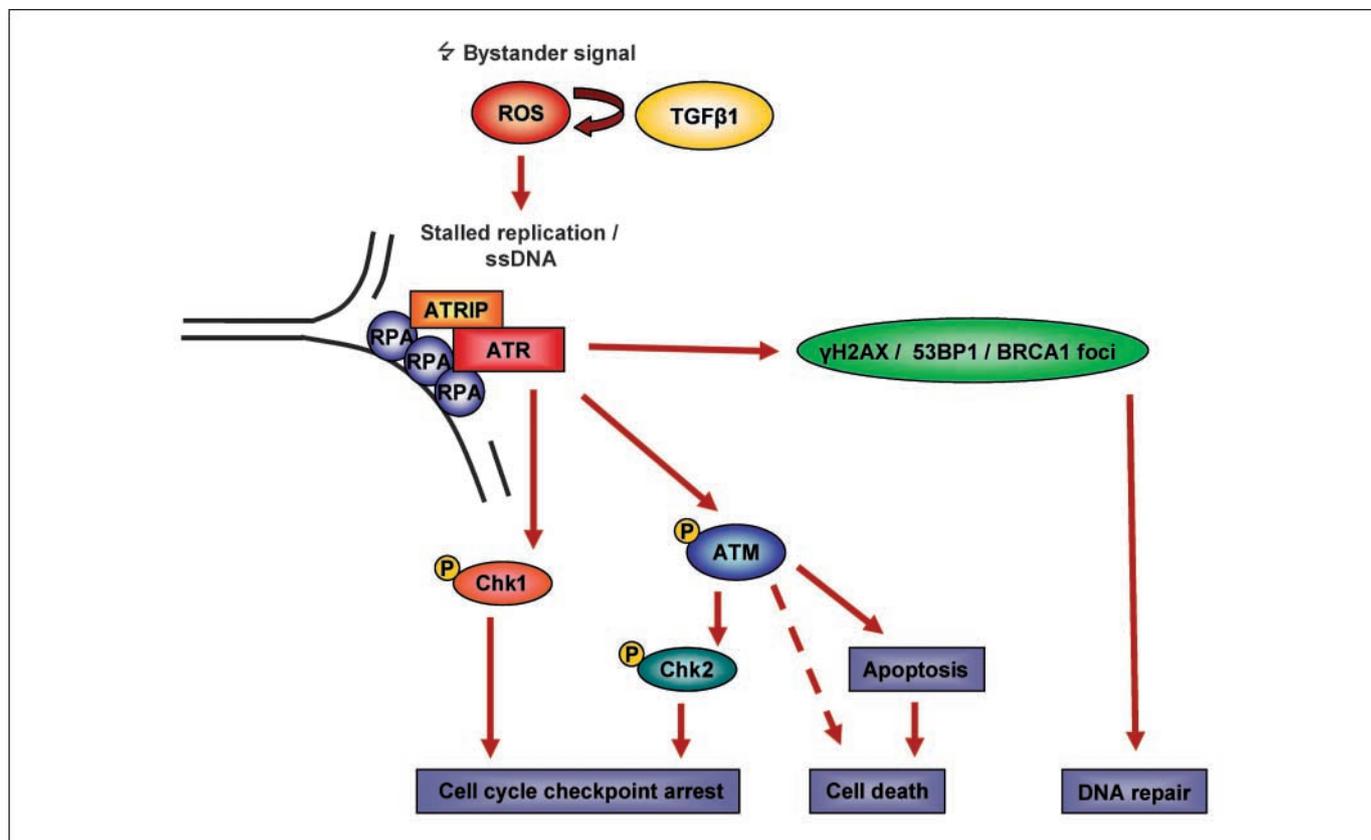


Figure 6. Model of ATR/ATM involvement in bystander signaling. *TGFβ1*, transforming growth factor- $\beta$ 1.

study, we report a similar response for 53BP1 foci induction. This suggests an induction of DNA damage presumably linked to stalled replication forks in S-phase cells and the consecutive activation of proteins involved in DNA damage recognition and repair as well as cell cycle checkpoint control involving ATM, BRCA1, and p21/Cip1/WAF1.

In this study, we have shown a reduction in clonogenic bystander cell survival in T98G, 48BR hTERT, and DNA-PK-mutated M059J cells but not in ATM-deficient GM05849 and ATR-mutated F02-98 hTERT cells. These findings support the hypothesis of a dependency on ATR and ATM, but not DNA-PK, for bystander signaling resulting in reduced clonogenic survival. Further confirmation was obtained by treatment of T98G bystander cells with the ATM/ATR inhibitor caffeine (30) and the specific ATM inhibitor KU-55933 (38), which both abrogated the effect. In contrast, the specific DNA-PK inhibitor NU7026 (39) did not block the bystander effect on clonogenic survival in T98G cells. Following direct irradiation, clonogenic survival was reduced by each of the PIKK inhibitors caffeine, KU-55933, and NU7026 as would have been predicted for these radiosensitizers.

Further potential downstream effects of this emerging bystander signaling network are cell cycle checkpoint and apoptosis induction, the latter having already been reported as part of radiation-induced bystander responses (40, 41). An updated model for a bystander signaling network is proposed, taking into account the novel findings derived from this study (Fig. 6). The initial DNA damage in bystander cells is thought to be caused by ROS, which are amplified by the induction of transforming growth factor- $\beta$ 1. Oxidative DNA damage interferes with the replication fork in S-phase cells and ssDNA is exposed at sites of stalled replication initially attracting RPA and ATR/ATRIP, which initiate a DNA damage response and lead to the recruitment of DNA repair factors, including  $\gamma$ H2AX, 53BP1, and BRCA1, with the aim of replication fork stabilization and DNA repair. ATR can further phosphorylate several target proteins, such as ATM and Chk1, potentially promoting cell cycle arrest and apoptosis induction.

p53 status seems not to be critical for the bystander responses investigated in this study. T98G cells express mutant although probably partly functional p53 (42), whereas M059J, 48BR, F02-98, and GM05849 ATM-deficient fibroblasts to our knowledge carry no p53 mutations. Previous bystander studies involved Chinese

hamster ovary cells (43) that carry a p53 mutation (44) and primary cells [e.g., primary human astrocytes (8)] that most probably are not p53 mutated. More recently, p53 status was not found to play a role in the production or response to a bystander signal leading to mutation induction (45). Therefore, for certain end points, the bystander DNA damage signaling presumably is independent of or can bypass p53, although it is a known ATM target. Nevertheless, it may be of interest to investigate a potential p53 involvement for selected bystander responses (e.g., bystander cell apoptosis).

From a therapeutic aspect, our study suggests ATR, ATM, and their downstream effectors to be suitable therapeutic targets to differentially modulate targeted and nontargeted effects of radiation. Understanding the role of ATR and ATM pathways for radiation-induced effects in nontargeted cells will complement the already established research into potential radiosensitizing effects in directly targeted cells. Further research beyond this study will have to establish the fate of cells that are spared from obvious bystander responses due to impaired ATR or ATM function. In particular, long-term effects, such as increased mutation rates and genomic instability (46, 47), in these surviving cells have to be considered because drugs inhibiting ATR, ATM, and Chk1 are under development or already undergoing clinical trials. In combination with radiotherapy, the abrogation of bystander cell killing in ATR- and ATM-deficient cells could have unwanted late effects, including the potential of secondary cancers arising from surviving bystander cells accumulating DNA damage.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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