

ATM and DNA-PK Function Redundantly to Phosphorylate H2AX after Exposure to Ionizing Radiation

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ABSTRACT

H2AX phosphorylation is an early step in the response to DNA damage. It is widely accepted that ATM (ataxia telangiectasia mutated protein) phosphorylates H2AX in response to DNA double-strand breaks (DSBs). Whether DNA-dependent protein kinase (DNA-PK) plays any role in this response is unclear. Here, we show that H2AX phosphorylation after exposure to ionizing radiation (IR) occurs to similar extents in human fibroblasts and in mouse embryo fibroblasts lacking either DNA-PK or ATM but is ablated in ATM-deficient cells treated with LY294002, a drug that specifically inhibits DNA-PK. Additionally, we show that inactivation of both DNA-PK and ATM is required to ablate IR-induced H2AX phosphorylation in chicken cells. We confirm that H2AX phosphorylation induced by DSBs in nonreplicating cells is ATR (ataxia telangiectasia and Rad3-related protein) independent. Taken together, we conclude that under most normal growth conditions, IR-induced H2AX phosphorylation can be carried out by ATM and DNA-PK in a redundant, overlapping manner. In contrast, DNA-PK cannot phosphorylate other proteins involved in the checkpoint response, including chromatin-associated Rad17. However, by phosphorylating H2AX, DNA-PK can contribute to the presence of the damage response proteins MDC1 and 53BP1 at the site of the DSB.

INTRODUCTION

Three related phosphatidylinositol 3'-kinase-like kinases play important roles in the response to DNA damage (1). ATM [ataxia telangiectasia (A-T) mutated protein], which is defective in the hereditary disorder A-T, is a central component of the signal transduction pathway that responds to DNA double-strand breaks [DSBs (2)]. ATM is activated by DSBs and phosphorylates key proteins that lead to cell cycle checkpoint arrest and/or apoptosis. ATM substrates include Chk1, Chk2, Rad17, NBS1, BRCA1, BLM, SMC1, 53BP1, and MDC1 (3–7). Consistent with a role of ATM in cell cycle checkpoint control, A-T cells show impaired G₁-S, S, and G₂-M checkpoint arrest after exposure to ionizing radiation [IR (8–10)]. ATR (ataxia telangiectasia and Rad3-related protein) phosphorylates many of the same proteins phosphorylated by ATM. Current evidence suggests that ATR, in contrast to ATM, responds to stalled replication forks and bulky lesions (11–14).

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is distinct from the former phosphatidylinositol 3'-kinase-like kinases because it does not appear to function in signal transduction damage response pathways but rather functions in DNA nonhomologous end joining, a pathway for DSB repair (Ref. 15; for a review, see Ref. 16).

Thus, cell cycle checkpoint arrest and p53 phosphorylation occur normally in DNA-PKcs-deficient cells (17).

An early response to DSBs is phosphorylation of a variant form of the histone H2A designated H2AX. Phosphorylated H2AX (called γ -H2AX) can be visualized as foci by immunofluorescence using phospho-specific antibodies (18, 19). H2AX foci colocalize with foci of other proteins, including NBS1, 53BP1, MDC1, and BRCA1 (3, 6, 18, 20). Although the initial recruitment of these proteins appears to be γ -H2AX independent, their retention as foci at longer times post-irradiation does not occur in cells lacking H2AX, leading to the suggestion that γ -H2AX plays a critical role in the retention of repair factors at the sites of DSBs (21, 22). Serine 139, which lies within an ATM consensus sequence, is the major H2AX residue phosphorylated in response to DNA damage (19). One study examining ATM knock-out cell lines concluded that IR-induced γ -H2AX foci formation is ATM dependent (23). We have also observed failure to phosphorylate H2AX in A-T lymphoblastoid cell lines [LCLs (24)]. Together, these data have led to the widely held belief and logical conclusion that γ -H2AX formation in response to DSBs is an early step in the ATM-dependent signal transduction process. However, another study reported defective H2AX phosphorylation in the DNA-dependent protein kinase (DNA-PK)-defective tumor line M059J (18). Here, we have addressed the roles of ATM, ATR, and DNA-PK in H2AX phosphorylation after exposure to IR. We demonstrate that IR-induced H2AX phosphorylation can be carried out by ATM or DNA-PK in a redundant, overlapping manner.

MATERIALS AND METHODS

Cell Culture. 1BR3 are control primary fibroblasts. AT7BI and AT1BR are A-T primary fibroblast cell lines (25). F02-98 is an ATR-defective fibroblast derived from a Seckel Syndrome patient (14). AT5BIVA is a SV40-transformed A-T fibroblast line. M059J and M059K are glioma cell lines lacking and expressing DNA-PKcs, respectively (26). Cells were cultured in MEM supplemented with 20% (primary cells) or 15% (transformed lines) FCS, penicillin, and streptomycin as described previously (27). Sweig is a control EBV transformed LCL; AO and LB541 are A-T LCLs (24). LCLs were grown in RPMI 1640 supplemented with 15% FCS, penicillin, and streptomycin. DNA-PKcs^{-/-} and ATM^{-/-} mouse embryo fibroblasts (MEFs) were derived as described previously (28, 29). Chicken DT40 cells were grown in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 1% antibiotics, and 100 μ M β -mercaptoethanol as described previously (30).

Treatment with DNA-Damaging Agents. Irradiation was performed using a ¹³⁷Cs source (8.5 Gy/min). X-irradiation (95 kV, 25 mA, ~6 Gy/min) was used for the experiments involving DT40 chicken cells. When present, LY294002 (200 μ M) was added to the cells 30 min before treatment with the relevant agent. LY294002 was purchased from Sigma-Aldrich (Poole, United Kingdom).

Antibodies. Anti-p53^{Ser15} and anti-Rad17^{Ser645} were purchased from Cell Signaling Technology (Beverly, MA) and New England Biolabs (Hitchin, United Kingdom), respectively. Anti-H2AX^{Ser139} antibodies were obtained from Upstate Technology (Buckingham, United Kingdom). The anti-53BP1 mouse monoclonal antibodies were as described previously (31). The anti-MDC1 (NH₂-terminal FHA domain) rabbit polyclonal antibodies were kindly provided by Dr. M. Goldberg and Prof. S. Jackson. Antirabbit and antimouse secondary antibodies were purchased from Dako (Glostrup, Denmark).

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Immunofluorescence. Cells were fixed in 3% paraformaldehyde and 2% sucrose PBS for 10 min at room temperature and permeabilized in 20 mM HEPES (pH 7.4), 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, and 0.5% Triton X-100 (Sigma-Aldrich) for 5 min at 4°C. Thereafter, coverslips were washed in PBS before immunostaining. Primary antibody incubations were performed for 40 min at 37°C at 1:100 dilutions (1:800 for anti- γ -H2AX) in PBS supplemented with 2% bovine serum fraction V albumin (Sigma-Aldrich) and followed by washing in PBS. Incubations with antimouse tetramethylrhodamine isothiocyanate and FITC or with antirabbit FITC secondary antibodies (Sigma-Aldrich) were performed at 37°C at 1:100 dilutions in 2% bovine serum fraction V albumin for 20 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich; data not shown) for 10 min at 4°C. Coverslips were mounted in Vectashield (Vector Laboratories, Peterborough, United Kingdom). Foci were counted using a fluorescence microscope. Foci present in 200 cells were counted in each experiment. The error bars represent the SD of the mean. A minimum of three experiments were carried out where error bars are shown. For experiments involving the DT40 cells, cells in suspension were spotted onto glass slides coated with 0.1% gelatin, fixed, and permeabilized with 100% ice-cold methanol for 30 min and ice-cold acetone for 1 min. Further analysis was as described previously (32).

DNA-PK Kinase Assay. Whole cell extracts were prepared by freezing and thawing as described previously (33). Cell extract was mixed with pre-swollen and prewashed DNA-cellulose beads (Sigma; 5 mg/reaction) and incubated for 30 min at 4°C. After two washes in Z'0.05 buffer [25 mM HEPES (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1% NP40, and 20% glycerol], the beads were resuspended in the same buffer and used for the kinase assay. The beads were incubated with 1 μ l of p53-derived substrate (Promega) and 0.52 μ Ci of [γ -³²P]ATP for 15 min at 30°C, and then the reaction was stopped by the addition of 30% acetic acid. Reactions were spotted onto P81 cation exchange paper to bind the peptide substrate, washed three times with 15% acetic acid, and then dried. Radioactivity retained on the filters was quantified by liquid scintillation counting.

RESULTS

IR-Induced H2AX Phosphorylation Occurs Efficiently in Human Cells Lacking ATM or DNA-PK. To examine H2AX phosphorylation, we used phospho-specific anti-H2AX^{Ser139} antibodies for immunofluorescence and counted the number of foci formed per cell. The time course of γ -H2AX formation after exposure to 2 Gy was examined in two A-T primary fibroblast cell lines (AT1BR and AT7BI), an immortalized A-T cell line (AT5BIVA), and DNA-PKcs-deficient M059J cells. Although the foci formed with slightly slower kinetics in the three A-T cell lines, by 30 min postirradiation, a similar number of foci were observed in A-T and control cell lines (Fig. 1A). M059J (DNA-PKcs^{-/-}) and the parental line, M059K (DNA-PKcs^{+/+}), a glioma cell line, gave a more robust response relative to the primary fibroblast cell lines, but a similar peak of foci was observed in both lines (Fig. 1A). The elevated number of foci in these glioma cell lines relative to primary cell lines is most likely explained by the fact that the cells are hyperdiploid (modal chromosome number approximately 110), allowing the formation of more DSBs (34). Foci both form and commence disappearing more rapidly in the M059K (DNA-PKcs^{+/+}) cells relative to the fibroblasts. By 1 h postirradiation, the number of foci in M059K cells had already decreased, whereas foci were lost more slowly in DNA-PKcs^{-/-} M059J cells, indicative of their impaired DSB repair (26). With the fibroblasts, foci remained at a similar level until around 60 min postirradiation, most likely representing a steady state between continued formation and disappearance due to DSB repair. With prolonged incubation, the number of foci decreased, and this occurred to similar extents in A-T and control cells over a 3-h period examined (data not shown).

Examination of foci formation at 30 min after exposure to different doses of IR also demonstrated a similar response in the A-T and DNA-PKcs^{-/-} cell lines relative to their control counterpart (Fig. 1B). At higher doses, the response ceased to be linear, probably due to inaccuracy in scoring >60 foci/cell. Taken together, these results

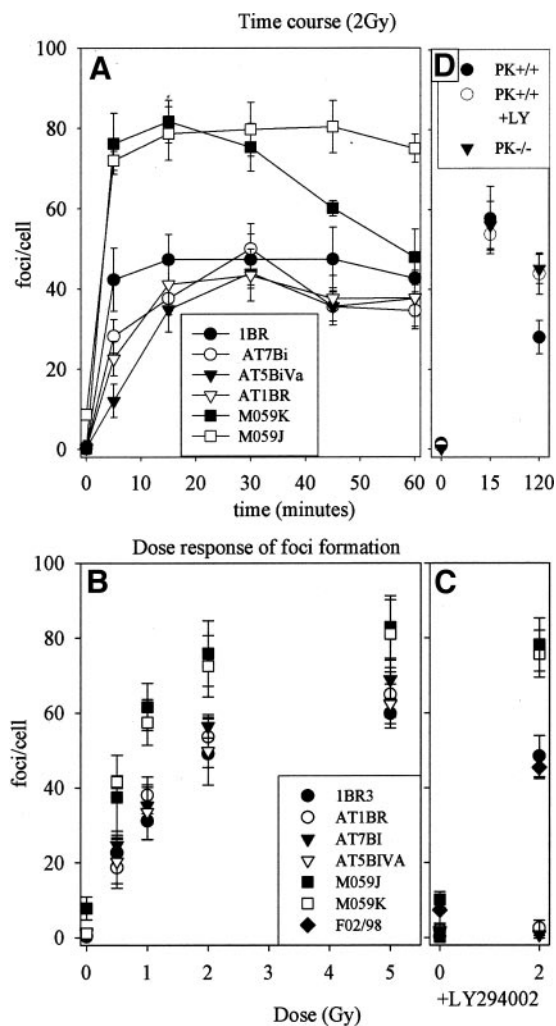


Fig. 1. Ataxia telangiectasia and DNA-dependent protein kinase (DNA-PK) catalytic subunit-deficient cell lines efficiently form H2AX foci after exposure to ionizing radiation. **A**, time course of H2AX foci formation after 2 Gy of γ -rays. Cells were grown to near confluence, irradiated, and sampled at the times indicated. Immunofluorescence was carried out using the phospho-specific anti-H2AX antibody (anti-H2AX^{Ser139}), and the number of γ -H2AX foci/cell was counted. Error bars represent the SD of the mean between among independent experiments. **B**, dose response of γ -H2AX foci sampled at 30 min postirradiation. **C**, the results of experiments in which the DNA-PK inhibitor LY294002 was added 30 min before irradiation. The legend is the same as that for **B**. 1BR3, AT1BR, AT7BI, F02-98, M059J, and M059K were examined. No induction of foci was observed in either AT1BR or AT7BI. Normal induction of foci was observed in 1BR3 and F02-98 cells, and higher induction of foci was observed in M059J and M059K cells. **D**, impact of LY294002 on the rate of loss of γ -H2AX foci in mouse embryo fibroblasts. LY294002 was added to the mouse embryo fibroblasts indicated 30 min before irradiation and maintained in medium until sampling. More foci persisted at 120 min postirradiation in DNA-PK^{-/-} cells and in DNA-PK^{+/+} cells treated with LY294002.

show that foci form efficiently in human cells lacking either ATM or DNA-PK.

H2AX Phosphorylation Is Ablated in Human A-T Fibroblasts Treated with a DNA-PK-Inhibiting Drug. One explanation for these findings is that ATM and DNA-PKcs function redundantly to phosphorylate H2AX. To examine this possibility, we measured γ -H2AX formation in the presence of the drug LY294002, a specific inhibitor of DNA-PKcs, in cells lacking ATM (35–37). Whereas IR-induced foci form normally in the presence of LY294002 in 1BR3, M059K (DNA-PKcs^{+/+}), and M059J (DNA-PKcs^{-/-}) cells, foci were barely detectable in the two A-T fibroblast cell lines (AT1BR and AT7BI) examined (Fig. 1C). To confirm that LY294002 does not affect ATR, we exploited a recent finding that F02-98 cells, which have a mutational change in ATR leading to aberrant splicing and

impaired ATR activity, show dramatically reduced UV-induced γ -H2AX formation, demonstrating that this event is ATR dependent (14). LY294002 did not impair UV-induced H2AX phosphorylation in 1BR3 cells, demonstrating that ATR remains active in the presence of the drug (data not shown). To verify that LY294002 does not impair ATM function, we examined the impact of the drug LY294002 on IR-induced p53 and Rad17 phosphorylation by immunofluorescence using phospho-specific antibodies (anti-p53^{Ser15} and anti-Rad17^{Ser645}) and observed similar levels of phosphorylated p53 and Rad17 in control and drug-treated control cells but impaired phosphorylation in AT1BR (data not shown). Taken together, these findings demonstrate that LY294002 does not impair either ATM or ATR activity. To verify that LY294002 does impair DNA-PK activity, we first carried out a DNA-PK kinase assay in the presence of the drug and observed significant inhibition of DNA-PK activity consistent with previous findings (data not shown; Refs. 36 and 37). To examine the impact on DNA-PK activity *in vivo*, we counted the H2AX foci remaining at 2 h postirradiation in wild-type MEFs in the presence of LY294002 (Fig. 1D). In a manner similar to the results obtained with DNA-PKcs^{-/-} M059J and DNA-PKcs^{+/+} M059K cells, we observed that H2AX foci form normally in DNA-PKcs^{+/+} and DNA-PK^{-/-} MEFs (see also below). However, the H2AX foci are lost more slowly in the DNA-PK^{-/-} MEFs, presumably due to the reduced DSB repair observed in DNA-PK-deficient cells (Fig. 1D; Ref. 26). Treatment of wild-type MEFs with LY294002 caused the same reduced rate of loss of H2AX foci as that observed in DNA-PKcs^{-/-} MEFs (Fig. 1D).

Finally, to examine the contribution of ATR to IR-induced H2AX phosphorylation, we examined foci formation in F02-98 (ATR^{m/m}) cells in the presence and absence of LY294002. In all cases, a similar level of IR-induced H2AX foci relative to control cells was observed (Fig. 1C). Thus, we conclude that ATR does not significantly contribute to IR-induced H2AX foci formation, at least over the time scale examined in these experiments.

Taken together, these findings show that in addition to ATM, another phosphatidylinositol 3'-kinase that is inhibited by LY294002 can phosphorylate H2AX after IR treatment. We show that DNA-PK, but not ATM or ATR, is inhibited by LY294002.

IR-Induced H2AX Phosphorylation Occurs Efficiently in MEFs Lacking ATM and Is Ablated by Drug-Induced Inhibition of DNA-PK. An earlier study carried out on primary and transformed MEFs reported that H2AX phosphorylation is ATM dependent (23). To verify that our findings were not specific to human cells, which have 50-fold higher levels of DNA-PK activity compared with mouse cells, we also examined transformed ATM^{-/-} and DNA-PKcs^{-/-} MEFs for induction of γ -H2AX foci. Similar to our findings with human cells, we observed slightly slower induction of γ -H2AX foci in ATM^{-/-} MEFs compared with control and DNA-PKcs^{-/-} MEFs, but by 15 min postirradiation, a similar level of foci was observed (Fig. 2). Addition of LY294002 ablated H2AX foci formation in ATM^{-/-} MEFs, identical to the results found with human fibroblasts (Fig. 2). Thus, our findings suggest that in rodent cells, as in human cells, DNA-PK has a redundant, overlapping function with ATM in phosphorylating H2AX.

Analysis of IR-Induced γ -H2AX Foci in LCLs. In contrast to these results, we have previously reported the lack of H2AX phosphorylation in an A-T LCL after exposure to IR (24). In initial experiments, we observed, as had been seen previously, the lack of γ -H2AX foci formation in A-T LCLs (24). These experiments were carried out with LCLs that had grown to saturation after several days of growth without medium change. To examine whether this might be a factor influencing the response, we monitored IR-induced γ -H2AX formation in two A-T LCLs replenished by the addition of fresh

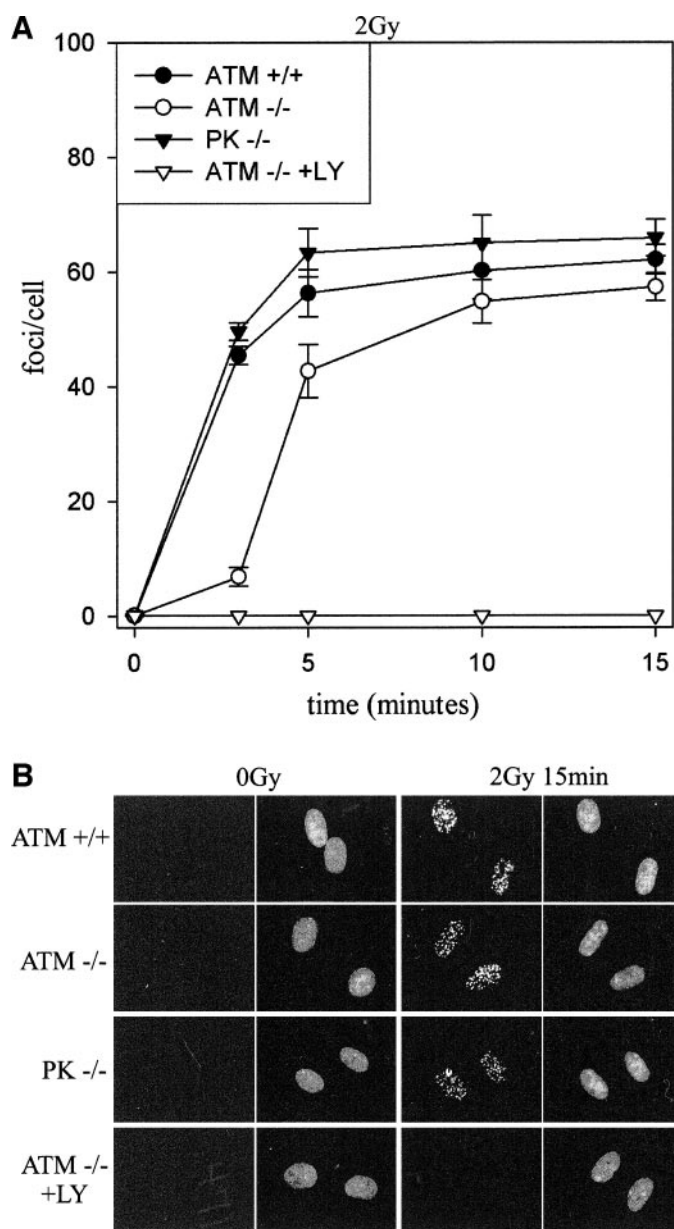
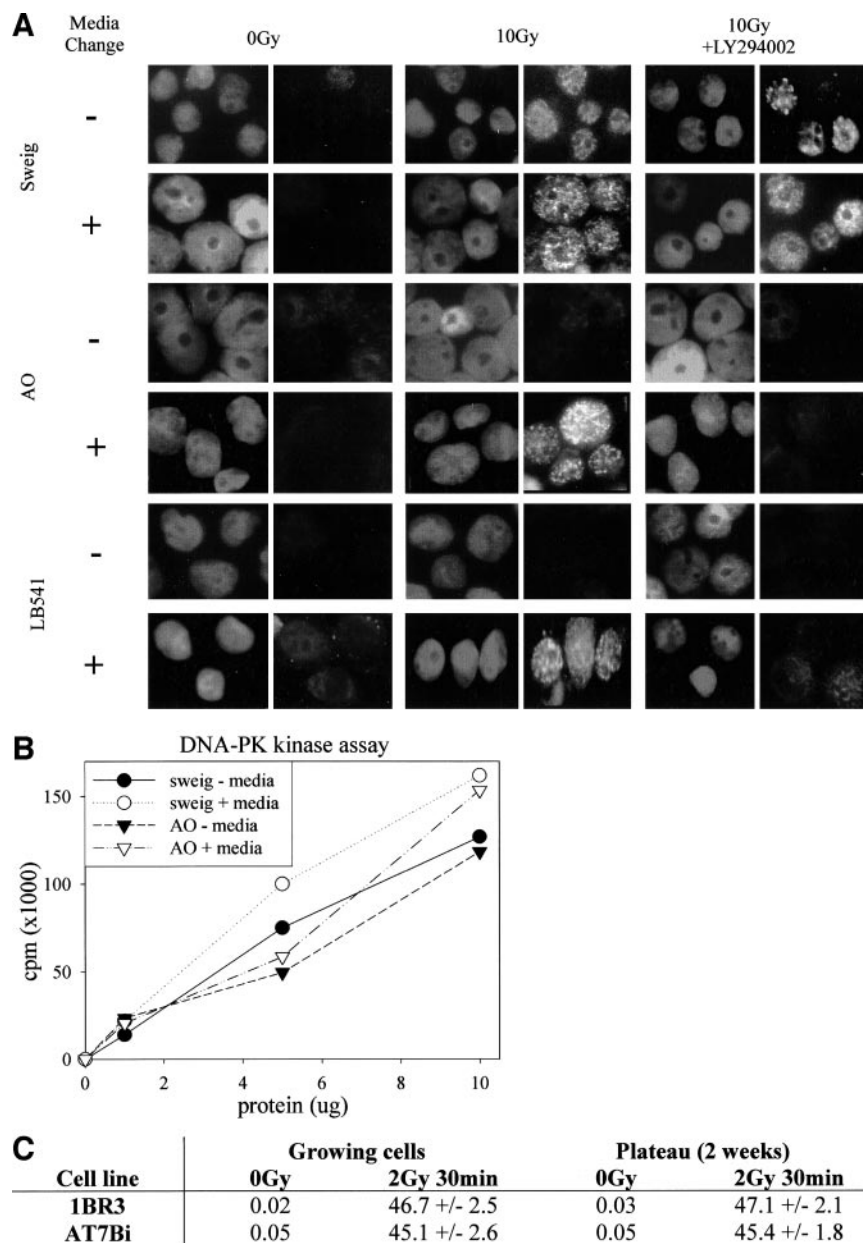


Fig. 2. γ -H2AX foci formation in ATM^{-/-} and DNA-dependent protein kinase catalytic subunit (DNA-PKcs)^{-/-} mouse embryo fibroblasts (MEFs) but not in ATM^{-/-} MEFs treated with LY294002. A, time course of γ -H2AX foci formation in MEFs after exposure to 2 Gy of γ -rays. Error bars represent the SD of the mean of three independent experiments. B, foci formation in ATM^{-/-}, DNA-PKcs^{-/-} MEFs and their ablation in ATM^{-/-} MEFs treated with LY294002. Cells were immunostained for γ -H2AX (left panels) and counterstained with 4',6-diamidino-2-phenylindole (right panels).

medium postirradiation in parallel to cells maintained in growth saturating conditions. No significant IR-induced H2AX phosphorylation was observed in the two A-T LCLs [AO (ATM^{-/-}) and LB541 (ATM^{m/m})] that were maintained in their original medium postirradiation (Fig. 3A). In contrast, efficient γ -H2AX formation similar to that observed in control cells was observed in both A-T LCLs when the medium was changed immediately after irradiation (Fig. 3A). The addition of LY294002 inhibited this H2AX phosphorylation. To examine whether DNA-PK is down-regulated in growth-saturated LCLs, we monitored DNA-PK activity in extracts prepared under both conditions (Fig. 3B). Although the level of DNA-PK activity was slightly lower when held under nongrowing conditions, the difference was small and unlikely to explain the dramatic impact on H2AX phosphorylation. Thus, the inability of DNA-PK to phosphorylate

Fig. 3. Ataxia telangiectasia (A-T) lymphoblastoid cell lines (LCLs) form H2AX foci normally if growth is stimulated after irradiation; they fail to form foci if they are maintained under growth-saturating conditions. **A**, control (Sweig) and two A-T (AO and LB541) LCLs were grown to near saturation and exposed to 10 Gy of γ -rays. LY294002 was added 30 min before irradiation. After irradiation, cells were either maintained in their original medium (with or without LY294002) or centrifuged and placed in fresh medium (with or without LY294002). Cells were immunostained for γ -H2AX (right panels) and counterstained with 4',6-diamidino-2-phenylindole (left panels). Unirradiated cells were also treated with LY294002, and the endogenous level of foci was identical to that observed in untreated cells (data not shown). It is more difficult to quantify foci formation in LCLs as compared with fibroblasts, but the H2AX phosphorylation is readily visible by the clear elevated fluorescent signal. **B**, DNA-dependent protein kinase activity present in extracts obtained from cells used in **A**. DNA-dependent protein kinase was pulled down using DNA cellulose beads, and the kinase activity was measured using a p53-derived peptide as substrate. The results represent the average of two experiments. The counts produced by the control (peptide alone) were subtracted from the results. **C**, examination of H2AX phosphorylation in growing and plateau phase-arrested human fibroblasts. Human control (1BR3) and A-T (AT7BI) fibroblasts were either maintained under actively growing conditions or grown to and maintained in plateau phase for 2 weeks before irradiation and scoring of γ -H2AX foci. Numbers represent the number of foci/cell.



H2AX in growth-saturated LCLs cannot be explained simply by down-regulation of DNA-PK levels or activity.

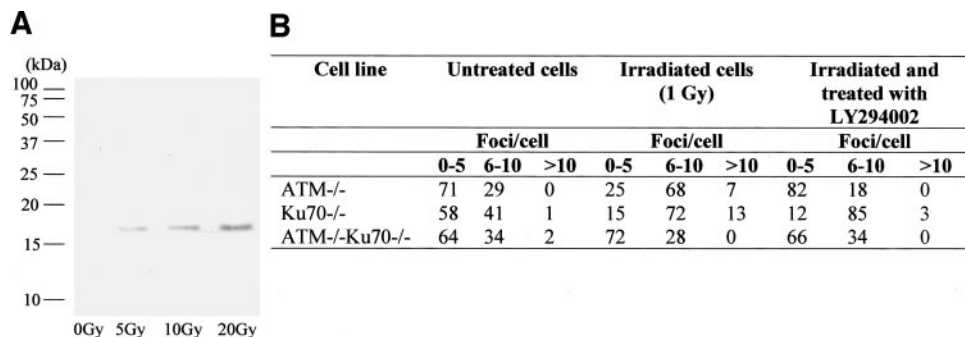
To verify whether proliferation status might influence IR-induced H2AX phosphorylation in primary fibroblasts, we compared γ -H2AX foci formation in primary fibroblasts that were actively growing with fibroblasts maintained in plateau phase for 2 weeks. γ -H2AX foci formation occurred to a similar extent in all samples (Fig. 3C).

Taken together, we conclude that ATM and DNA-PK can phosphorylate H2AX in a redundant, overlapping manner in response to DSBs. However, under certain growth conditions (e.g., growth-inhibited LCLs), DNA-PK does not appear to play a major role.

ATM and DNA-PK Contribute to IR-Induced H2AX Phosphorylation in Chicken Cells. To substantiate that it is DNA-PK rather than another phosphatidylinositol 3'-kinase inhibited by LY294002 that overlaps with ATM in H2AX phosphorylation, we sought to examine cells lacking both DNA-PK and ATM and exploited chicken DT40 cells because double mutant cell lines lacking Ku70 and ATM have been described previously (30). Because DNA-PK activation

requires Ku binding to DNA ends, Ku70 mutants are devoid of DNA-PK activity. We therefore examined foci formation in ATM^{-/-}, Ku70^{-/-}, and ATM^{-/-}Ku70^{-/-} cells at 2 h posttreatment with 1 and 2 Gy of X-rays in the presence and absence of the drug LY294002 using human anti-phospho-H2AX antibody. By Western blotting, we verified that human anti-H2AX antibodies cross-react with chicken H2AX in a manner inducible by IR (Fig. 4A). To allow a quantitative examination of H2AX phosphorylation, we examined foci formation as carried out with human and mouse cells (Fig. 4B). All three chicken cell lines had a high background of γ -H2AX foci relative to that observed with mammalian cells, which we attribute to ATR-dependent events (Fig. 4B). After irradiation, there was an increase in the number of cells with >5 foci/cell in the ATM^{-/-} and Ku70^{-/-} single mutant cells that was not observed in the ATM^{-/-} Ku70^{-/-} double mutants. The slightly greater increase in γ -H2AX foci in the Ku70^{-/-} cells relative to ATM^{-/-} cells possibly reflects the inability of Ku-defective cells to repair their DSBs relative to ATM^{-/-} cells during the 2-h incubation period. Furthermore, LY294002 inhibited the ra-

Fig. 4. H2AX foci formation in chicken cells lacking ATM, Ku, or both ATM and Ku. *A*, Western blot demonstrating that human anti-phospho-H2AX recognizes the chicken H2AX. DT40 cell extracts (200 μ g) made 1 h after exposure to γ -rays were examined by Western blotting using human anti- γ -H2AX antibody. *B*, γ -H2AX foci formation in the indicated chicken cells. Categories represent the percentage of cells with 0–5, 6–10, and >10 foci/cell. Approximately 100 cells were counted for each category. Similar results were obtained after exposure to 2 Gy of X-rays.



diation-induced γ -H2AX foci in ATM^{-/-} cells but not in the cell line lacking Ku, consistent with our conclusion that the impact of LY294002 on γ -H2AX foci formation is due to its inhibition of DNA-PK. Taken together, we conclude that ATM and DNA-PK jointly contribute to H2AX phosphorylation in vertebrate cells.

p53 and Rad17 Phosphorylation Occurs Normally in M059J Cells. Recently, we have shown that BRCA1 is required for a subset of ATM and ATR phosphorylation events but is dispensable for both H2AX and Rad17 phosphorylation (38). Because Rad17 and H2AX are located on the chromatin, we proposed that BRCA1 might be required for ATM-dependent phosphorylation of non-histone-bound downstream substrates. Although previous studies have shown convincingly that DNA-PK is not required for p53 phosphorylation, the role of DNA-PK in phosphorylating chromatin-bound substrates has not been studied (17). The overlapping role of DNA-PK in phosphorylating H2AX, therefore, prompted us to examine its ability to phosphorylate Rad17. We observed normal phosphorylation of Rad17 and p53 in M059J (DNA-PKcs^{-/-}) and M059K (DNA-PKcs^{+/+}) cells (Fig. 5). These findings are consistent with the dependency of Rad17 and p53 phosphorylation on ATM (39–41) and demonstrate that whereas DNA-PK can phosphorylate H2AX, it is not able to phosphorylate these other components of the signaling pathway. It is worth noting that previous studies have reported that M059J cells have low ATM activity (42). The present results show that the level of ATM in M059J cells is sufficient to efficiently phosphorylate H2AX, Rad17, and p53.

DNA-PK Can Impact on the Presence of Damage Response Proteins at DSBs. In addition to H2AX, other proteins, including the MRE11/RAD50/NBS1 complex, MDC1, and 53BP1 accumulate in foci at the sites of DSBs (3–6, 20, 43–45). Recent evidence has demonstrated that phosphorylated H2AX is not required for the initial recruitment of these proteins but is required for their retention as foci (22). By 60 min post-IR, foci formation of these proteins appears to be

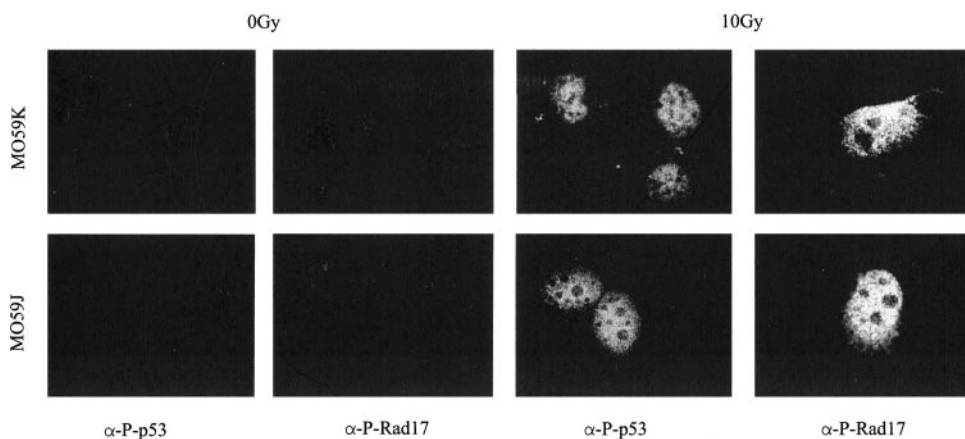
ATM independent but γ -H2AX dependent (22). To examine the contribution of DNA-PK to this process, we examined the formation of MDC1 and 53BP1 foci at 1 h post-IR in 1BR3, AT1BR, M059J, and M059K cells. In control cells, both 53BP1 and MDC1 changed from diffuse nuclear staining with the occasional (1–3) large foci in the absence of irradiation to abundant small nuclear foci after radiation exposure. 53BP1 and MDC1 foci formed normally in M059J and AT1BR but were abolished in drug-treated AT1BR (Fig. 6). These findings consolidate previous reports that retention of these two proteins as foci post-irradiation is ATM independent (22). Our results suggest that this is because there is DNA-PK-dependent H2AX phosphorylation, thereby demonstrating that DNA-PK can contribute to the retention of these complexes at the sites of DSBs in the absence of ATM.

DISCUSSION

To gain insight into the early events initiated by DNA damage, we examined which phosphatidylinositol 3'-kinase contributes to H2AX phosphorylation after exposure to IR. Because γ -H2AX foci are required for the stable formation of NBS1, 53BP1, MDC1, and BRCA1 foci at the damage sites, this represents an important step that determines subsequent events in the signal transduction pathway (3, 6, 18, 20–22).

Firstly, we show that inactivation of ATM and a second phosphatidylinositol 3'-kinase that is inhibited by the drug LY294002 is required to ablate IR-induced H2AX phosphorylation. Consistent with reported data that LY294002 is a specific DNA-PK inhibitor, we show that *in vivo* DNA-PK is inhibited by LY294002, whereas both ATM and ATR remain active. We substantiate the notion that DNA-PK is the inhibited kinase by using the DT40 model system to examine chicken cells lacking both ATM and DNA-PK activity. Thus, we conclude that ATM and DNA-PK function redundantly to phosphor-

Fig. 5. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is dispensable for p53 and Rad17 phosphorylation. Control (M059K) and DNA-PKcs-deficient (M059J) cells were exposed to 10 Gy of γ -rays and examined for immunofluorescence using phospho-specific anti-p53^{Ser15} and anti-Rad17^{Ser645} antibodies 2 h postirradiation. 4',6-Diamidino-2-phenylindole staining showed the presence of nuclei in all fields (data not shown). Phosphorylation of both proteins occurred normally in M059J cells, demonstrating that DNA-PKcs does not contribute to these phosphorylation events and that ATM is active in M059J cells.



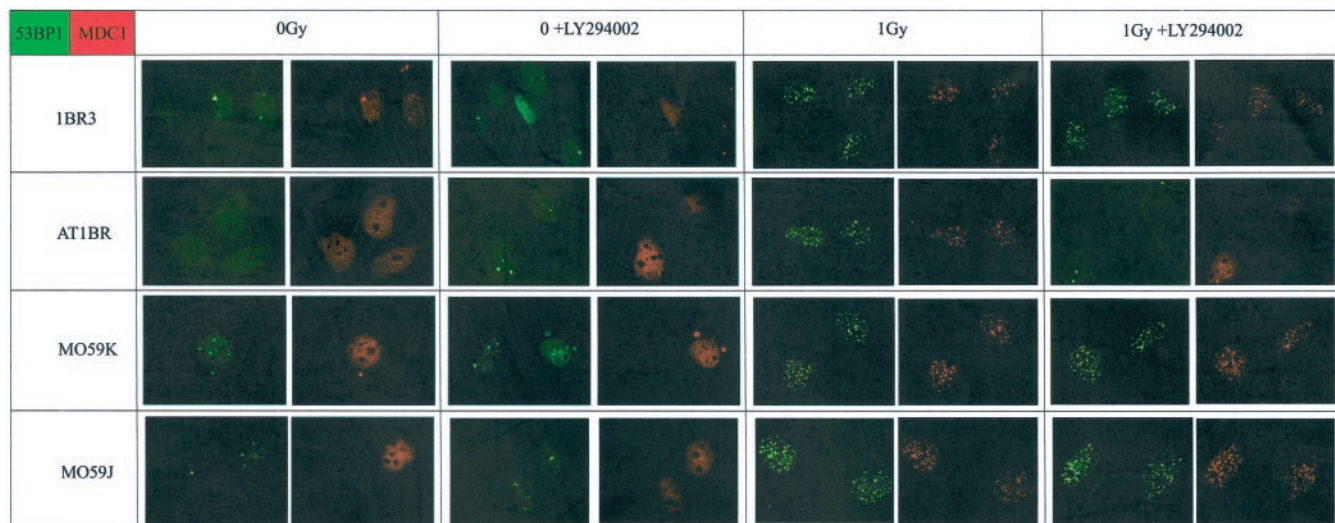


Fig. 6. Both ATM and DNA-dependent protein kinase catalytic subunit can contribute to 53BP1 and MDC1 foci formation after exposure to ionizing radiation. Cells were untreated or irradiated with 10 Gy in the presence or absence of LY294002. LY294002 was added 30 min before irradiation and maintained in the medium until sampling. One h after irradiation, cells were analyzed for 53BP1 or MDC1 foci formation using anti-53BP1 or anti-MDC1 antibodies. The 53BP1 and MDC1 foci are visible as small discrete foci. MDC1 foci were analyzed after two-dimensional blind deconvolution using Simple PCI software. Merged images showed that the 53BP1 foci overlap with MDC1 foci as shown previously (Ref. 4; data not shown). Radiation-induced foci develop efficiently in AT1BR and M059J cells but not in AT1BR cells treated with LY294002.

ylate H2AX in response to DSBs. This overlapping function is observed in human, mouse, and vertebrate cells. Whereas our findings show that each kinase can function in the absence of the other, they do not demonstrate which protein phosphorylates H2AX when both proteins are present. Interestingly, DSBs undergo repair and initiate ATM-dependent phosphorylation events concomitantly, indicating that the DNA-PK complex and ATM are both localized to or activated by the double-stranded DNA ends noncompetitively. The slightly slower kinetics of induction of γ -H2AX foci in ATM-deficient human and mouse cells indicates that ATM may play the dominant role, at least at early times postirradiation. Additionally, we observed ATM-dependent H2AX phosphorylation in growth-saturated A-T LCLs. However, nonproliferating (plateau phase) A-T human fibroblasts phosphorylate H2AX efficiently, suggesting that this observation is not linked to proliferation *per se*. DNA-PK was not markedly downregulated under these conditions, suggesting that there may be factors influencing its access to H2AX that remain to be elucidated. Nonetheless, this was only one of many conditions examined where H2AX phosphorylation was found to be specifically ATM dependent. Thus, we conclude that under most normal conditions (actively growing and plateau phase human fibroblasts, growing MEFs and LCLs, and in chicken cells), the two kinases function redundantly, although there are kinetic and growth conditions where ATM predominates.

Two previous studies have addressed which phosphatidylinositol 3'-kinase-like kinase is responsible for IR-induced H2AX phosphorylation (18, 23). Our findings disagree with the study of Burma *et al.* (23), which concluded that ATM is the responsible kinase. It is possible that the growth status of the cells could explain this result. Nonetheless, our findings show clearly that DNA-PK can contribute to H2AX phosphorylation after IR. The other study reported a very high background of γ -H2AX foci in M059J cells that increased only slightly after IR (18). In our experiments using 2 Gy, although the background level of foci was elevated in M059J cells, we observed a substantial increase after IR treatment, and the induced level of phosphorylation was similar to that observed in DNA-PKcs^{+/+} M059K cells. It has been shown previously that M059J cells carry a mutational change in *ATM* and have reduced ATM as well as DNA-PKcs activity (42). However, here we observed efficient Rad17 and p53 phosphorylation (Fig. 5), indicating that ATM is functional and

capable of efficiently phosphorylating its substrates. Nonetheless, certain conditions might facilitate exposure of the dual phosphatidylinositol 3'-kinase-like kinase requirement in M059J. Paull *et al.* (18) used two-dimensional gel analysis to examine H2AX phosphorylation and observed decreased phosphorylation in M059J cells (18). This procedure required high doses of IR, and it is possible that the reduced ATM activity in M059J cells could not fully achieve the extensive phosphorylation required, thereby exposing a role for DNA-PK. Although our results are substantially different, our conclusion is similar: namely that ATM and DNA-PK both contribute to H2AX phosphorylation in response to DSBs. Furthermore, the fact that H2AX phosphorylation is reduced after high radiation doses in M059J cells supports our contention that DNA-PK can contribute to H2AX phosphorylation. We also show that ATR does not contribute to IR-induced H2AX phosphorylation in primary fibroblasts, at least over the time scale examined. It is likely, however, that at longer times postirradiation after replication of IR-induced lesions, a contribution of ATR may occur. Our findings do not eliminate this possibility but do show, importantly, that ATR is not activated by DSBs in G₁-phase cells.

Activation of ATM by DSBs results in phosphorylation of substrates further downstream that include Chk2, NBS1, CtIP, BLM, and p53. None of these events are observed in A-T cell lines, demonstrating that DNA-PK does not contribute significantly to this aspect of the checkpoint response. Consistent with this, it has been shown that DNA-PKcs-defective cells show checkpoint arrest and p53 phosphorylation (17). We show that Rad17, which, like H2AX, is located on the chromatin, is also efficiently phosphorylated in DNA-PK-defective cells but not in A-T cells. The fact that DNA-PK phosphorylates H2AX but not other substrates demonstrates the constraints of phosphorylation events *in vivo*. More recently, another aspect of the DNA damage response has been described in which the role of ATM appears to be redundant, namely, the recruitment of proteins that form as foci that overlap with γ -H2AX foci (4, 6, 21, 22). The proteins that fall into this class include MCD1, 53BP1, MRE11/RAD50/NBS1, and BRCA1. We show that at 1 h post-IR treatment, MDC1 and 53BP1 foci are not visible when both DNA-PK and ATM activities are inhibited but are visible in cells lacking either DNA-PK or ATM alone. Because this correlates with the abolition of γ -H2AX foci

formation, this is consistent with the model that retention of these proteins as foci is dependent on H2AX phosphorylation but not ATM activity. Interestingly, a recent study has suggested that H2AX is not required for the recruitment of these proteins to the DSB but is required for their retention (22). Our findings, therefore, provide evidence that DNA-PK plays a redundant, overlapping role with ATM in the retention of these proteins as foci at the damage site by contributing to H2AX phosphorylation.

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REFERENCES

- Jackson S. Sensing and repairing DNA double strand breaks. *Carcinogenesis (Lond.)* 2002;23:687–96.
- Savitsky K, Bar-Shira A, Gilad S, et al. A single ataxia telangiectasia gene with a product similar to PI 3-kinase. *Science (Wash. DC)* 1995;268:1749–53.
- Wang B, Matsuoka S, Carpenter PB, Elledge SJ. 53BP1, a mediator of the DNA damage checkpoint. *Science (Wash. DC)* 2002;298:1435–8.
- Goldberg M, Stucki M, Falck J, et al. MDC1 is required for the intra-S-phase DNA damage checkpoint. *Nature (Lond.)* 2003;421:952–6.
- Lou Z, Chini CC, Minter-Dykhouse K, Chen J. MDC1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control. *J Biol Chem* 2003;278:13599–602.
- Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ. MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature (Lond.)* 2003;421:961–6.
- Yazdi PT, Wang Y, Zhao S, et al. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev* 2002;16:571–82.
- Lavin MF, Khanna KK. ATM: the protein encoded by the gene mutated in the radiosensitive syndrome ataxia-telangiectasia. *Int J Radiat Biol* 1999;75:1201–14.
- Rotman G, Shiloh Y. ATM: from gene to function. *Hum Mol Genet* 1998;7:1555–63.
- Rotman G, Shiloh Y. The ATM gene and protein: possible roles in genome surveillance, checkpoint controls and cellular defense against oxidative stress. *Cancer Surv* 1997;29:285–304.
- Bentley NJ, Holtzman DA, Flagg G, et al. The *Schizosaccharomyces pombe* rad3 checkpoint gene. *EMBO J* 1996;15:6641–51.
- Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. *Nature (Lond.)* 2000;408:433–9.
- Caspari T, Carr AM. Checkpoints: how to flag up double-strand breaks. *Curr Biol* 2002;12:R105–7.
- O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nat Genet* 2003;33:497–501.
- Hartley KO, Gell D, Smith GCM, et al. DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* 1995;82:849–56.
- Jeggo PA. DNA breakage and repair. *Adv Genet* 1998;38:185–211.
- Jimenez GS, Bryntesson F, Torres-Arzayus MI, et al. DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage. *Nature (Lond.)* 1999;400:81–3.
- Paull TT, Rogakou EP, Yamazaki V, et al. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000;10:886–95.
- 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.
- Fernandez-Capetillo O, Chen HT, Celeste A, et al. DNA damage-induced G₂-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol* 2002;4:993–7.
- Celeste A, Petersen S, Romanienko PJ, et al. Genomic instability in mice lacking histone H2AX. *Science (Wash. DC)* 2002;296:922–7.
- Celeste A, Fernandez-Capetillo O, Kruhlak MJ, et al. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol* 2003;5:675–9.
- Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* 2001;276:42462–7.
- Girard P-M, Riballo E, Begg A, Waugh A, Jeggo PA. Nbs1 promotes ATM dependent phosphorylation events including those required for G₁/S arrest. *Oncogene* 2002;21:4191–9.
- Taylor AMR, Harnden DG, Arlett CF, et al. Ataxia-telangiectasia, a human mutation with abnormal radiation sensitivity. *Nature (Lond.)* 1975;258:427–9.
- Allalunis-Turner MJ, Lintott LG, Barron GM, Day RS, Lees-Miller SP. Lack of correlation between DNA-dependent protein kinase activity and tumor-cell radiosensitivity. *Cancer Res* 1995;55:5200–2.
- Arlett CF, Green MHL, Priestley A, Harcourt SA, Mayne LV. Comparative human cellular radiosensitivity. I. The effect of SV40 immortalisation on the γ -irradiation survival of skin derived fibroblasts from normal individuals and from ataxia-telangiectasia patients and heterozygotes. *Int J Radiat Biol* 1988;54:911–28.
- Taccioli GE, Amatucci AG, Beamish HJ, et al. Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity. *Immunity* 1998;9:355–66.
- Hirao A, Cheung A, Duncan G, et al. Chk2 is a tumour suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol Cell Biol* 2002;22:6521–32.
- 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.
- Iwabuchi K, Basu BP, Kysela B, et al. Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. *J Biol Chem* 2003;278:36487–95.
- Rothkamm K, Lobrich M. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci USA* 2003;100:5057–62.
- Blunt T, Finnie NJ, Taccioli GE, et al. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell* 1995;80:813–23.
- Anderson CW, Dunn JJ, Freimuth PI, Galloway AM, Allalunis-Turner MJ. Frameshift mutation in PRKDC, the gene for DNA-PKcs, in the DNA repair-defective, human, glioma-derived cell line M059J. *Radiat Res* 2001;156:2–9.
- Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 1994;269:5241–8.
- Izzard RA, Jackson SP, Smith GC. Competitive and noncompetitive inhibition of the DNA-dependent protein kinase. *Cancer Res* 1999;59:2581–6.
- Rosenzweig KE, Youmell MB, Palayoor ST, Price BD. Radiosensitization of human tumor cells by the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 correlates with inhibition of DNA-dependent protein kinase and prolonged G₂-M delay. *Clin Cancer Res* 1997;3:1149–56.
- Foray N, Marot D, Gabriel A, et al. A subset of ATM and ATR-dependent phosphorylation events requires the BRCA1 protein. *EMBO J* 2003;22:2860–71.
- Canman CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science (Wash. DC)* 1998;281:1677–9.
- Banin S, Moyal L, Shieh S, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science (Wash. DC)* 1998;281:1674–7.
- Bao S, Tibbetts RS, Brumbaugh KM, et al. ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. *Nature (Lond.)* 2001;411:969–74.
- Tsuchida R, Yamada T, Takagi M, et al. Detection of ATM gene mutation in human glioma cell line M059J by a rapid frameshift/stop codon assay in yeast. *Radiat Res* 2002;158:195–201.
- Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol* 2001;21:1719–29.
- Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol* 2001;153:613–20.
- Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol* 2000;151:1381–90.

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