ATM Phosphorylates Histone H2AX in Response to DNA Double-Strand Breaks

Sandeep Burma[1], Benjamin P. Chen[1], Michael Murphy[1], Akihiro Kurimasa[1, 2], and David J. Chen[1, *]

Lawrence Berkeley National Laboratory
Life Sciences Division
1 Cyclotron Road, Berkeley, CA 94720

[1]Lawrence Berkeley National Laboratory, Berkeley, CA
[2]Current Address: Life Sciences Division, Tottori University, Tottori, JAPAN

[*] Corresponding author
Tel: 510-495-2861
Fax: 510-486-6816
E.mail: djchen@lbl.gov

Running title: *Phosphorylation of H2AX by ATM*

SUMMARY

A very early step in the response of mammalian cells to DNA double-strand breaks (DSBs) is the phosphorylation of histone H2AX at serine 139 at the sites of DNA damage. While the PI-3 kinases, DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated), and ATR (ATM and Rad3-related), have all been implicated in H2AX phosphorylation, the specific kinase involved has not yet been identified. In order to definitively identify the specific kinase(s) that phosphorylates H2AX *in vivo*, we have utilized DNA-PKcs-/- and Atm-/- cell lines and mouse embryonic fibroblasts. We find that H2AX phosphorylation and nuclear focus formation are normal in DNA-PKcs-/- cells and severely compromised in Atm-/- cells. We also find that ATM can phosphorylate H2AX *in vitro* and that ectopic expression of ATM in Atm-/- fibroblasts restores H2AX phosphorylation *in vivo*. The minimal H2AX phosphorylation in Atm-/- fibroblasts can be abolished by low concentrations of wortmannin suggesting that DNA-PK, rather than ATR, is responsible for low levels of H2AX phosphorylation in the absence of ATM. Our results clearly establish ATM as the major kinase involved in the phosphorylation of H2AX and suggest that ATM is one of the earliest kinases to be activated in the cellular response to DSBs.

INTRODUCTION

DNA double-strand breaks (DSBs) are probably the most dangerous of the many different types of DNA damage that occur within the cell. DSBs are generated by exogenous agents such as ionizing radiation (IR) or by endogenously generated reactive oxygen species and occur as intermediates during meiotic and V(D)J recombination (1). A very early step in the cellular response to DSBs is the phosphorylation of a histone H2A variant, H2AX, at the sites of DNA damage (2). H2AX is rapidly phosphorylated (within seconds) at serine 139 when DSBs are introduced into mammalian cells (3) resulting in discrete γ-H2AX (phosphorylated-H2AX) foci at the DNA damage sites (4). In experiments involving the use of 'laser scissors' to introduce breaks into living cells, γ-H2AX foci localized specifically with the laser path through the cell nuclei clearly demonstrating that H2AX phosphorylation is specific to the sites of DNA damage (4,5). H2AX phosphorylation also appears to be a general cellular response to processes involving DSB intermediates including V(D)J recombination in lymphoid cells (6) and meiotic recombination in mice (7). Phosphorylation of yeast H2A at serine 129 (homologous to serine 139 of mammalian H2AX) causes chromatin decondensation and is required for efficient DNA double-strand break repair (8). In mammals, phosphorylation of H2AX appears to play a critical role in the recruitment of repair or damage-signaling factors to the sites of DNA damage (5,9).

As H2AX phosphorylation plays a very early and important role in the cellular response to DNA double-strand breaks, it is important to specifically identify the kinase(s) involved in this event. Members of the PI-3 kinase family, including DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated), and ATR (ATM and Rad3-related), are involved in the responses of mammalian cells to DSBs (10). γ-H2AX focus formation is inhibited by the PI-3 kinase inhibitor wortmannin and H2AX phosphorylation is reduced in the

DNA-PK-deficient human cell line M059J (5). This led to the conclusion that DNA-PK, and at least one other kinase, possibly ATM and/or ATR, can phosphorylate H2AX upon DNA damage (2,5,10,11).

In order to unambiguously define the roles of ATM and DNA-PK in H2AX phosphorylation, we utilized cells derived from knockout mice for ATM or DNA-PKcs (the catalytic subunit of DNA-PK). We observed normal H2AX phosphorylation and γ-H2AX focus formation in irradiated fibroblasts derived from wild type or DNA-PKcs-/- mice. In contrast, H2AX phosphorylation and γ-H2AX focus formation were strikingly reduced to near background levels in fibroblasts from Atm-/- mice. Ectopic expression of ATM in Atm-/- cells restored H2AX phosphorylation. Moreover, we show that immunoprecipitated ATM can phosphorylate recombinant H2AX *in vitro*. These results indicate that ATM, not DNA-PK, is the major kinase responsible for modifying H2AX upon irradiation. The minimal H2AX phosphorylation in Atm-/- cells could be abolished by low concentrations of wortmannin suggesting that DNA-PK, rather than ATR, is responsible for low levels of γ-H2AX formation in the absence of ATM.

EXPERIMENTAL PROCEDURES

Cell Culture and Induction of DNA Damage - Spontaneously immortalized mouse fibroblasts, derived from wild type, DNA-PKcs-/- (12), or Atm-/- mice (13), were maintained in a humidified atmosphere with 5% CO_2 in α-MEM medium supplemented with 10% fetal calf serum, 100 units / ml penicillin and 100 μg / ml streptomycin. Mouse embryonic fibroblasts (MEFs) were isolated from 13.5-day old embryos and maintained in α-MEM medium supplemented with 15% fetal calf serum. Cells were grown to about 70% confluence and irradiated with X-rays (300 kV, 12 mA, 0.5 mm Cu) at the rate of 5.5 Gy / min to achieve a cumulative dose of 10 Gy for all experiments unless otherwise mentioned. Cells were UV irradiated at the rate of 0.15 J / m^2 / sec to achieve a cumulative dose of 10 J / m^2. Cells were harvested after 30 min, except in case of time courses where they were harvested at time points ranging from 5 min to 8 h. Drug treatment of cells was carried out by the addition of the following DNA-damaging agents to the culture media for 1 h at the indicated concentrations: neocarzinostatin (0.2 μg / ml), bleomycin (50 μg / ml), etoposide (30 μg / ml), methyl methanesulfonate (50 μg / ml), hydroxyurea (1 mM).

Antibody Production and Western Blotting - Anti-γ-H2AX antibody was generated against a synthetic peptide consisting of the last nine amino acids of H2AX with phospho-Ser–139 as described before (3). SDS extracts for western blotting were prepared from mock-irradiated or irradiated cells as described previously (14). The antibodies used for western blotting are anti-γ-H2AX, anti-H2A (H-124; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-ATM monoclonal antibody MAT3-4G10/8 (15).

Transient Transfection of Atm-/- Cells – Transient transfection of exponentially growing Atm-/- spontaneously immortalized fibroblasts with the ATM cDNA expression vector pMAT1 (16) was carried out using Superfect transfection reagent (Qiagen Inc., Valencia, CA) as per manufacturer's protocols. Immediately after transfection, cells were induced for ATM expression with 5 μM $CdCl_2$ for 16 h and then mock-irradiated or irradiated as described above.

ATM Kinase Assay – ATM immunoprecipitations were carried out as described in (17). Approximately $1X10^7$ spontaneously immortalized mouse fibroblasts were grown to 70 % confluence, mock-irradiated or irradiated, harvested after 30 min, and lysed in fresh cold lysis buffer containing protease and phosphatase inhibitors. The lysate was cleared by centrifugation and the supernatant incubated with 10 μg anti-ATM monoclonal antibody MAT3-4G10/8 (15) for 2 h at 4^oC followed by incubation with protein A/G sepharose beads for an additional 2 h. The beads were washed repeatedly with lysis buffer, once with high salt buffer, and twice with kinase buffer. The beads were then incubated in a kinase mix (20 μl kinase buffer, 500 ng recombinant H2AX (purified from bacteria), 2 μl 100 μM ATP, and 10 μCi $\gamma[^{32}P]$-ATP) at 30^oC for 10 min. After SDS-PAGE, the reaction products were visualized by autoradiography.

Immunofluorescence – Spontaneously immortalized fibroblasts were grown on chamber slides to about 70% confluence, then mock-irradiated or irradiated and incubated for 30 min. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized for 10 min in 0.2% Triton X-100, and blocked in 10% normal goat serum for 1 h at room temperature. The slides were incubated with anti-γ-H2AX antibody for 1 h, washed in PBS, and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 h at

room temperature. Cells were washed in PBS and mounted using Vectashield mounting medium with DAPI (Vector Laboratory, Burlingame, CA). Fluorescence images were captured using an Olympus BH2 epifluorescent microscope equipped with a CCD camera and Cytovision software (Applied Imaging, Santa Clara, CA). To allow direct comparisons, all the cells were irradiated and processed simultaneously and all the images were obtained using the same parameters (brightness, contrast, etc.).

RESULTS

Histone H2AX is Phosphorylated Specifically in Response to DNA Double-Strand Breaks – To examine H2AX phosphorylation in mouse cells, a rabbit polyclonal antibody (anti-γ-H2AX) was generated against a synthetic phosphorylated polypeptide consisting of the last nine amino acids of H2AX with phospho-Ser-139 [CKATQAS(PO_4)QEY]. The purified anti-γ-H2AX antibody reacted specifically with the immunizing polypeptide (phosphorylated at serine 139) but not with the unphosphorylated peptide (CKATQASQEY) (Fig. 1a). Thus, the anti-γ-H2AX antibody is immunoreactive only with H2AX specifically phosphorylated at serine 139.

Spontaneously immortalized wild type mouse fibroblasts were mock-irradiated or irradiated with X-rays, harvested after 30 min, and H2AX phosphorylation was analyzed by western blotting of SDS extracts with anti-γ-H2AX antibody. We observed significant phosphorylation of histone H2AX in response to ionizing radiation (Fig. 1b). The observed phosphorylation was specific to serine 139 as no signal was detected in irradiated samples when the immunizing polypeptide (phosphorylated at serine 139) was used as competitor in western blotting (data not shown). Significant phosphorylation of H2AX was also observed after treatment of cells with the DSB-inducing agents neocarzinostatin (NCS), bleomycin (BLM), and etoposide. In contrast, there was no increase in γ-H2AX formation when these cells were irradiated with UV rays or treated with the DNA alkylating agent methyl methanesulfonate (MMS) confirming that H2AX is phosphorylated at serine 139 specifically in response to DNA double-strand breaks. Low levels of H2AX phosphorylation were also observed in cells treated with the DNA replication inhibitor hydroxyurea (HU). This is probably because cells treated with HU accumulate DSBs due to replication fork collapse (18,19).

Ionizing Radiation-Induced H2AX Phosphorylation Can be Inhibited by Low Concentrations of Wortmannin - As the PI-3 kinases, DNA-PK, ATM, and ATR, have all been implicated in H2AX phosphorylation (2,5,10,11) we wanted to determine which of these three kinases played a major role in the process. The fungal PI-3 kinase inhibitor wortmannin inhibits the kinase activities of ATM and DNA-PK in intact cells with half-maximal inhibition at concentrations of about 5 μM (20). The kinase activity of ATR is significantly more resistant to this drug with half-maximal inhibition at concentrations higher than 100 μM. Spontaneously immortalized wild type mouse fibroblasts were treated with increasing concentrations of wortmannin for 30 min, irradiated with X-rays, harvested after 30 min, and analyzed by western blotting. We found that H2AX phosphorylation was inhibited by low concentrations of wortmannin (1-10 μM) indicating that ATM and/or DNA-PK, but not ATR, is involved in this process (Fig. 1c).

H2AX Phosphorylation is Abrogated in Atm-/-, but Not in DNA-PKcs-/-, Cells - Spontaneously immortalized fibroblasts from wild type, DNA-PKcs-/-, or Atm-/- mice were mock-irradiated or irradiated, harvested at time points ranging from 5 min to 8 h, and assayed for H2AX phosphorylation by western blotting. H2AX phosphorylation in both wild type and DNA-PKcs-/- cells occurred very rapidly (within 5 min) and lasted for about 2 h, with maximum levels of phosphorylation observed at 30 min (Fig. 2a). In striking contrast, we observed minimal H2AX phosphorylation in irradiated Atm-/- cells. While we observed robust H2AX phosphorylation in DNA-PKcs-/- cells at 30 min post-irradiation, γ-H2AX formation in Atm-/- cells was reproducibly reduced to about 5% of that in wild type cells (Fig. 2b) indicating that ATM is the major kinase responsible for H2AX phosphorylation upon DNA damage.

Atm-/- fibroblasts were treated with increasing concentrations of wortmannin for 30 min, irradiated with X-rays, harvested after 30 min, and analyzed by western blotting. We found that the minimal H2AX phosphorylation in Atm-/- cells was completely abolished by low concentrations of wortmannin (1-10 μM) (Fig. 2c). As ATR is inhibited by high concentrations of wortmannin (> 100 μM) (20), our results suggest that DNA-PK, rather than ATR, is responsible for low levels of γ-H2AX formation in the absence of ATM.

Lack of H2AX Phosphorylation in Atm-/- Mouse Embryonic Fibroblasts (MEFs) – It is possible that other mutations in the Atm-/- cell line used could also be responsible for the lack of H2AX phosphorylation in these cells. We therefore examined H2AX phosphorylation in a panel of isogenic, early passage (p2 or p3) ATM +/+ or -/- mouse embryonic fibroblasts (MEFs). Normal H2AX phosphorylation was observed in irradiated Atm+/+ MEFs (Fig. 3a; upper panel). In contrast, very low levels of γ-H2AX formation was observed in two independent Atm-/- MEFs confirming that ATM is required for H2AX phosphorylation in response to IR. No significant difference in H2AX phosphorylation was observed between irradiated DNA-PKcs+/- and -/- MEFs (Fig. 3a; lower panel).

Ectopic Expression of ATM Restores H2AX Phosphorylation in Atm-/- Cells – In order to confirm that ATM is required *in vivo* for H2AX phosphorylation, the ATM cDNA expression vector pMAT1 (16) was transiently transfected into Atm-/- spontaneously immortalized fibroblasts. The ectopic expression of ATM in the transfected cells resulted in restoration of H2AX phosphorylation upon irradiation (Fig. 3b; compare lanes 2 and 4). On the other hand, cells transfected with the vector alone showed no increase in γ-H2AX formation

(Fig. 3b; compare lanes 2 and 6). The correlation between ATM expression and H2AX phosphorylation establishes that ATM is required *in vivo* for γ-H2AX formation in response to ionizing radiation.

ATM Can Phosphorylate Recombinant H2AX *In Vitro* – In order to determine whether ATM can directly phosphorylate H2AX *in vitro*, ATM was immunoprecipitated from spontaneously immortalized wild type fibroblasts using an anti-ATM monoclonal antibody raised against a peptide representing positions 1967-1988 of murine ATM (15). The immunoprecipitated ATM efficiently phosphorylated recombinant H2AX *in vitro* (Fig. 3c; lane 1). Furthermore, irradiation of cells resulted in a significant increase in H2AX phosphorylation (Fig. 3c; lane 2). Essentially no ATM protein or kinase activity was detected when immunoprecipitation was performed with normal mouse IgG or from Atm-/- fibroblasts (Fig. 3c; lanes 3-6). The *in vitro* phosphorylation of H2AX by ATM suggests that ATM could directly phosphorylate H2AX within the cell in response to DNA damage.

Poor γ-H2AX Focus Formation in Atm-/- Cells – H2AX phosphorylation in response to DNA damage results in the formation of discrete γ-H2AX foci at the sites of DNA double-strand breaks (4). To determine the status of γ-H2AX focus formation in wild type, DNA-PKcs-/-, and Atm-/- spontaneously immortalized fibroblasts, these cells were irradiated and allowed to recover for 30 min before fixation and immunostaining with anti-γ-H2AX antibody. We observed robust γ-H2AX focus formation upon irradiation of both wild type and DNA-PKcs-/- cells (Fig. 4). In striking contrast, focus formation was very poor in Atm-/- cells confirming that ATM is required for γ-H2AX focus formation at the sites of DSBs.

DISCUSSION

Histone H2AX is rapidly phosphorylated at serine 139 in response to DNA double-strand breaks (3). The PI-3 kinases - DNA-PK, ATM, and ATR - have all been implicated in this process (2,5,10,11). While the substrate specificities of these kinases are overlapping *in vitro*, they have clearly distinct functions *in vivo* (10). For example, while ATM phosphorylates p53, Chk2, and Nbs1 leading to cell cycle arrest, DNA-PK is not required for any of these processes (21,22). On the other hand, DNA-PK, unlike ATM, may be involved in the recruitment of XRCC4 and DNA ligase IV to the sites of DSBs (23). It is therefore important to definitively delineate the roles of these kinases in the phosphorylation of H2AX.

We demonstrate that ATM can phosphorylate H2AX *in vitro* and that H2AX phosphorylation and γ-H2AX focus formation are severely compromised in Atm-/- cells. Ectopic expression of ATM corrects this defect. In contrast, these functions are normal in DNA-PKcs-/- cells. Interestingly, DNA-PK, but not ATR, may be responsible for the minimal levels of H2AX phosphorylation in Atm-/- cells as this can be abolished by low concentrations of wortmannin. We also find that immunoprecipitated ATM can directly interact with recombinant H2AX *in vitro* (Sandeep Burma and David J. Chen; unpublished results) and experiments will be performed to examine complex formation between H2AX and ATM *in vivo*.

Our results establish that ATM is the major kinase responsible for histone H2AX phosphorylation in response to DNA double-strand breaks in murine fibroblasts. The reduced H2AX phosphorylation reported in M059J cells (5) could, therefore, be due to the low levels of ATM in these cells (24-26) rather than due to the absence of DNA-PK. ATM plays a crucial role in the rapid induction of multiple signaling pathways in response to DSBs, leading to repair of

DNA damage, activation of cell cycle checkpoints, and cellular stress responses (27). As γ-H2AX focus formation is a very early event occurring within seconds of DNA damage infliction (3), our results indicate that ATM is one of the earliest kinases to be activated in the cellular response to DNA double-strand breaks. Supporting ATM's important role in damage-induced chromatin modification is a report indicating that ATM is also required for the IR-induced transient dephosphorylation of histone H1 which is thought to contribute to chromatin decondensation (28).

Our results are consistent with a recent report indicating that a fraction of nuclear ATM co-localizes with γ-H2AX at the sites of DSBs in response to DNA damage (15). A very striking correlation was reported between the kinetics of appearance and dissolution of γ-H2AX foci and DNA-bound ATM aggregates. This suggests that DNA localization by ATM and H2AX phosphorylation occur concomitantly, very rapidly after DNA damage, and decrease at the same rate thereafter. In the light of this report, our findings suggest that once ATM is activated at a DSB, it could immediately phosphorylate histone H2AX at the site of the break thereby signaling to the cell that a DSB has occurred. This very early event could then initiate the recruitment of DNA repair or damage-signaling factors to the break mediated by chromatin modification and/or direct interactions of these factors with phospho-H2AX.

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LEGENDS TO FIGURES

FIG. 1. **Inhibition of H2AX phosphorylation by wortmannin.** *(A)* Anti-γ H2AX antibody (directed against H2AX phosphorylated at serine 139) were used to immunoblot synthetic peptides comprising of the last 9 amino acids of H2AX with (134-142^{ser139}) or without (134-142) phosphorylation at serine 139. *(B)* Spontaneously immortalized wild type mouse fibroblasts were mock-irradiated or irradiated with X-rays or UV rays as indicated and harvested after 30 min. Cells were mock-treated or treated for 1h with the DSB-inducing agents neocarzinostatin (NCS), bleomycin (BLM), or etoposide or with the DNA alkylating agent methyl methanesulfonate (MMS) as indicated and then harvested. SDS extracts were analyzed for H2AX phosphorylation by western blotting with anti-γ-H2AX antibody. The blots were stripped and re-probed with anti-H2A antibody as a normalizing control. *(C)* Wild type fibroblasts were mock-irradiated (C) or irradiated with X-rays after a 30 min incubation with increasing concentrations of wortmannin (0-100 μM). Cells were harvested 30 min after irradiation and SDS extracts analyzed by western blotting with anti-γ-H2AX or anti-H2A antibodies.

FIG. 2. **Lack of H2AX phosphorylation in Atm-/- cells.** *(A)* Wild type, DNA-PKcs-/-, or Atm-/- spontaneously immortalized fibroblasts were mock-irradiated (C) or irradiated with X-rays and harvested at time points ranging from 5 min to 8 h as indicated. SDS extracts were analyzed for H2AX phosphorylation by western blotting with anti-γ-H2AX antibody or with anti-H2A antibody as a normalizing control. *(B)* Wild type, DNA-PKcs-/-, or Atm-/- fibroblasts were mock-irradiated (C) or irradiated (IR), harvested after 30 min, and analyzed by western

blotting. The autoradiograph in the figure was scanned, the bands quantified using NIH Image software, and the relative levels of γ-H2AX plotted on the Y-axis. After IR the level of γ-H2AX in Atm-/- cells is about 5% of that in wild type cells. A similar pattern of H2AX phosphorylation was observed in three independent experiments. *(C)* Atm-/- fibroblasts were incubated with increasing concentrations of wortmannin for 30 min and then irradiated. Cells were harvested 30 min after irradiation and SDS extracts analyzed by western blotting.

FIG. 3. **Phosphorylation of H2AX by ATM *in vitro* and *in vivo*.** *(A)* Abrogated H2AX phosphorylation in Atm-/- mouse embryonic fibroblasts (MEFs). Early passage isogenic Atm +/+ or -/- MEFs were mock-irradiated (C) or irradiated with X-rays (IR), harvested after 30 min, and analyzed for H2AX phosphorylation by western blotting with anti-γ-H2AX antibody or with anti-H2A antibody as a normalizing control. DNA-PKcs+/- or -/- MEFs were similarly analyzed for H2AX phosphorylation. *(B)* Restoration of H2AX phosphorylation in Atm-/- cells by ectopic expression of ATM. Spontaneously immortalized Atm-/- fibroblasts were either untransfected (lanes 1 and 2), transiently transfected with the ATM cDNA expression vector pMat1 (lanes 3 and 4), or transiently transfected with the vector alone (lanes 5 and 6). Cells were either mock-irradiated (C) or irradiated (IR), harvested after 30 min, and SDS extracts analyzed for H2AX phosphorylation by western blotting with anti-γ-H2AX antibody. The expression of ATM in transfected cells was monitored by western blotting with anti-ATM antibody. *(C) In vitro* phosphorylation of recombinant H2AX by ATM. ATM was immunoprecipitated from mock-irradiated (C) or irradiated (IR) spontaneously immortalized fibroblasts with anti-ATM monoclonal antibody or normal mouse IgG as control and kinase activity assayed using

recombinant H2AX as substrate. Top panel: ATM immunoprecipitates immunoblotted with anti-ATM monoclonal antibody. Bottom panel: autoradiograph of phosphorylated H2AX.

FIG. 4. **Poor γ-H2AX focus formation in Atm-/- cells.** Wild type, DNA-PKcs-/-, or Atm-/- spontaneously immortalized fibroblasts were mock-irradiated or irradiated with X-rays and incubated for 30 min before fixation, permeabilization, and staining with anti-γ-H2AX antibody (green). Nuclei were stained with DAPI (blue). To allow direct comparisons, all the slides were processed together and all the images were captured using the same parameters.

FIG. 1.

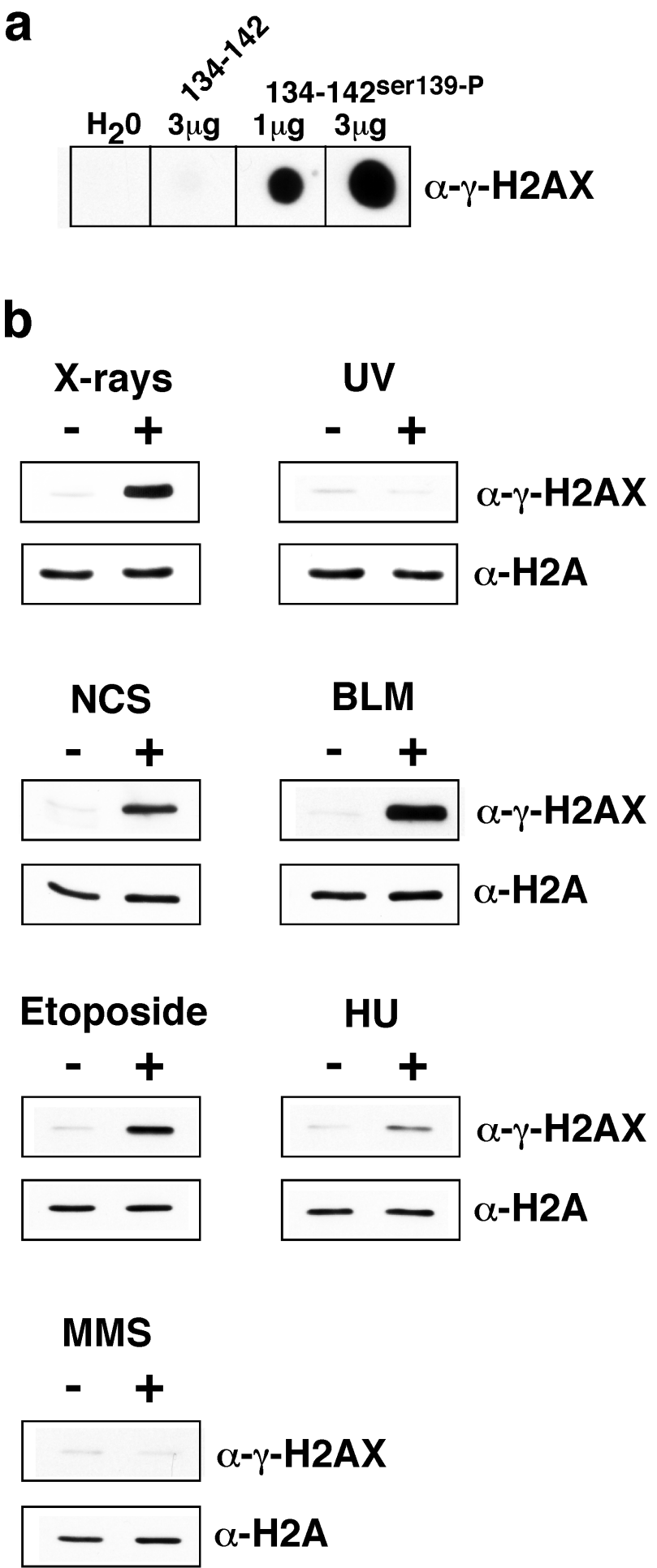
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FIG. 1.

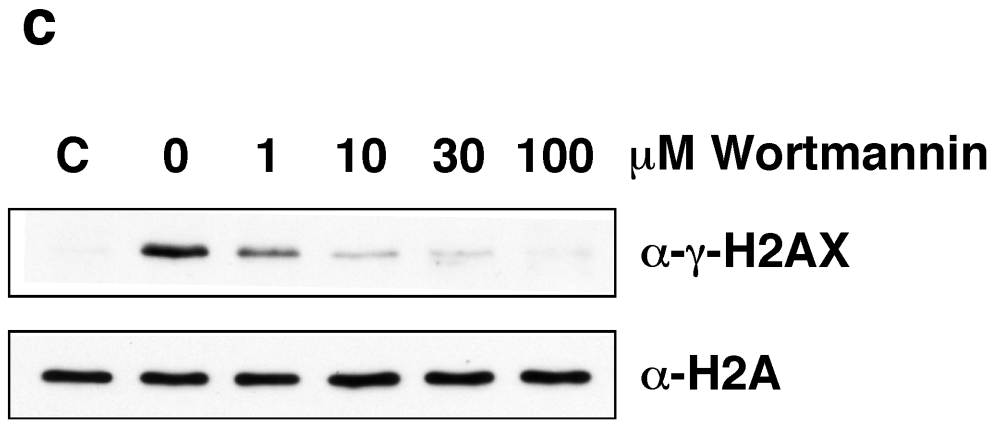
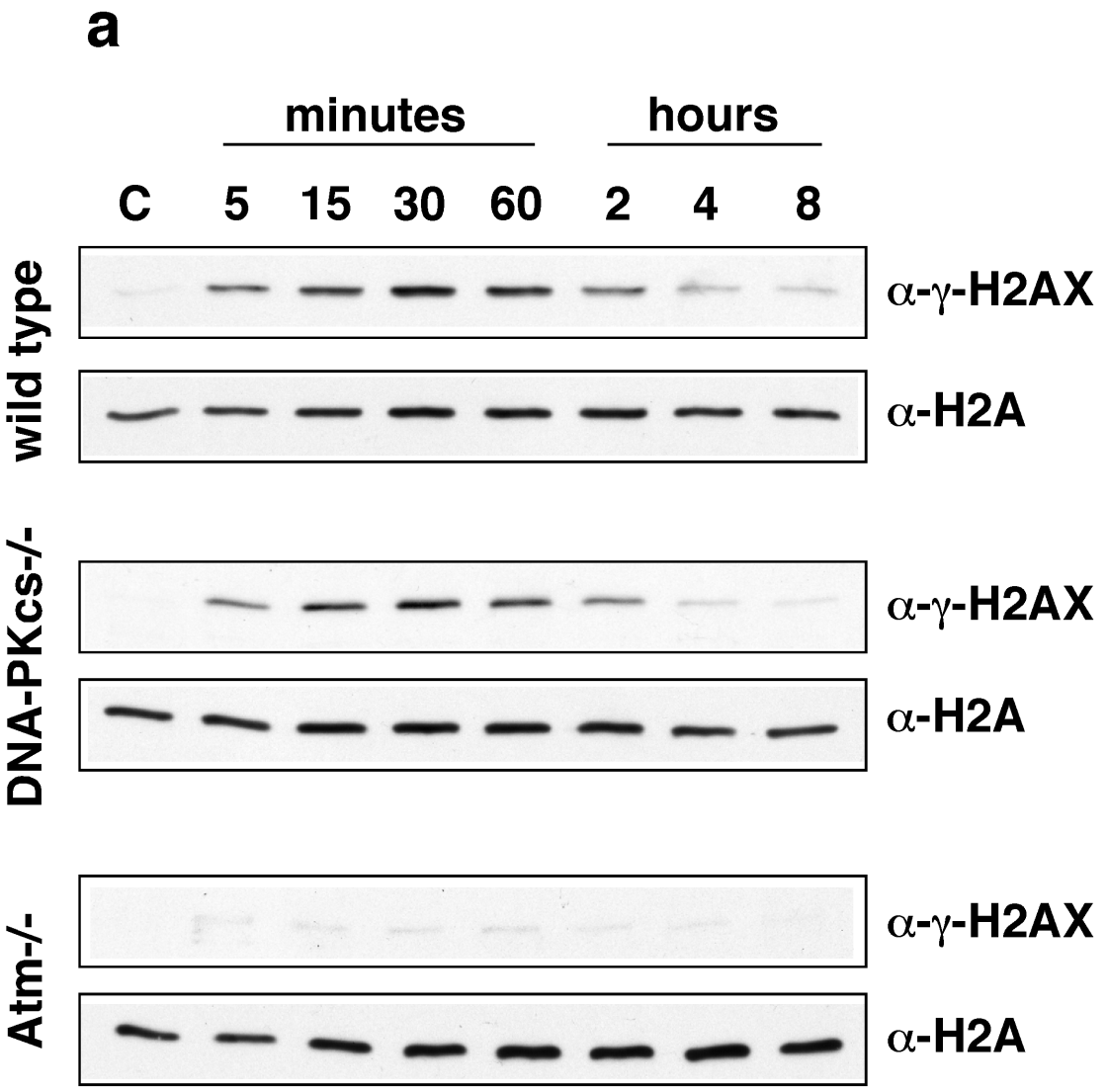


FIG. 2.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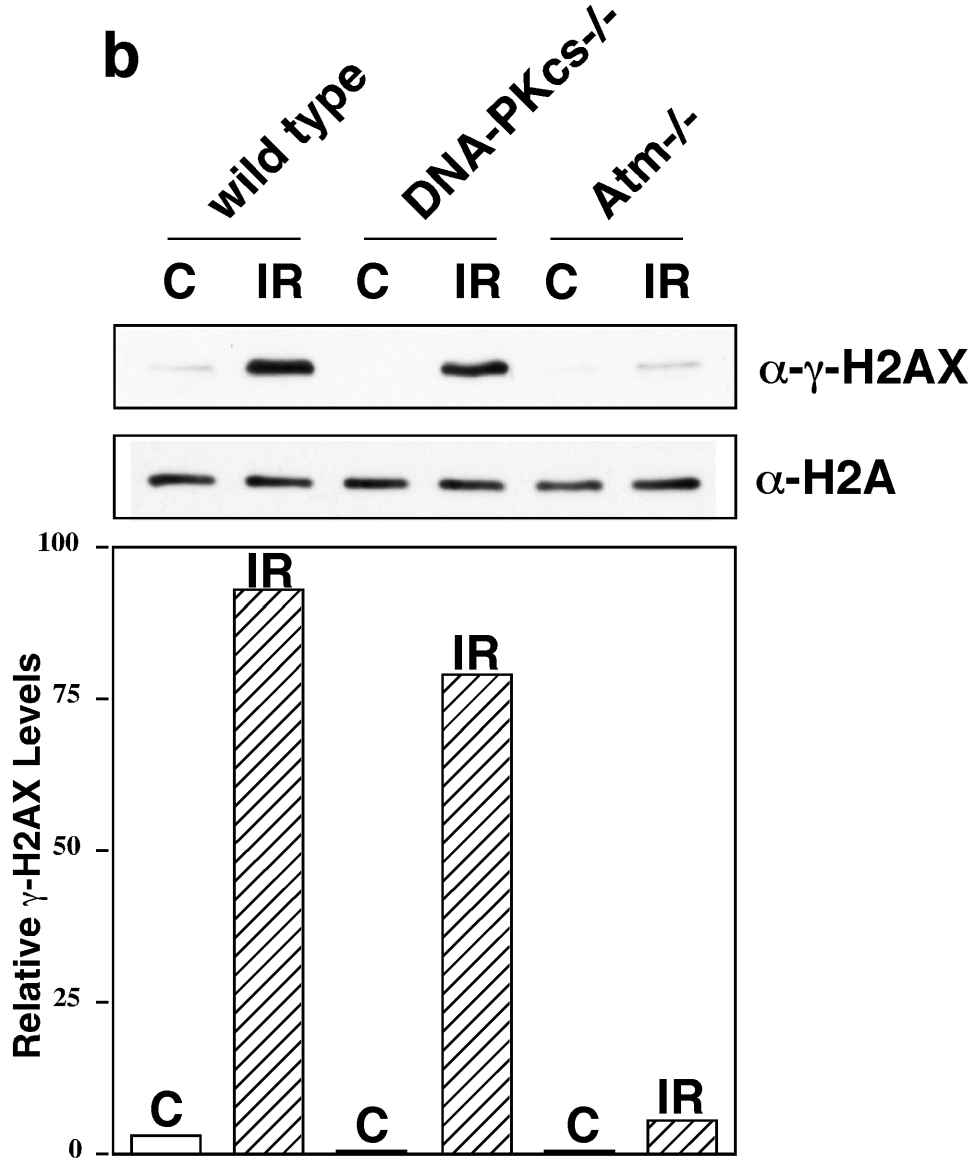
α-H2A

FIG. 2.


b
wild type
DNA-PKcs-/-
Atm-/-
C
IR
C
IR
C
IR
α-γ-H2AX
α-H2A
Relative γ-H2AX Levels
100
75
50
25
0
IR
IR
C
C
C
IR

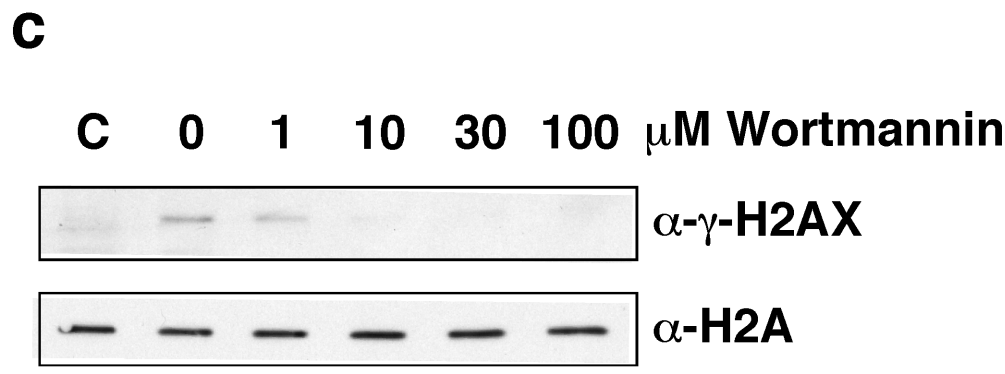
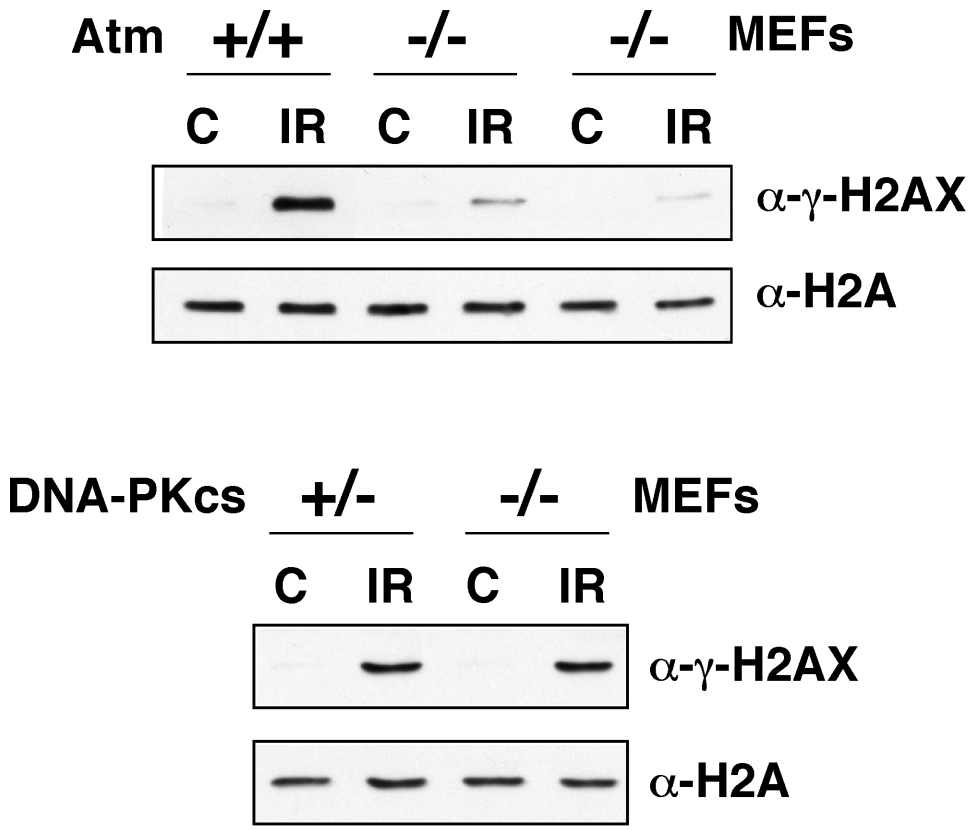

c
C
0
1
10
30
100
μM Wortmannin
α-γ-H2AX
α-H2A

FIG. 3.

a


Atm +/+ -/- -/- MEFs
C IR C IR C IR
α-γ-H2AX
α-H2A
DNA-PKcs +/- -/- MEFs
C IR C IR
α-γ-H2AX
α-H2A

b

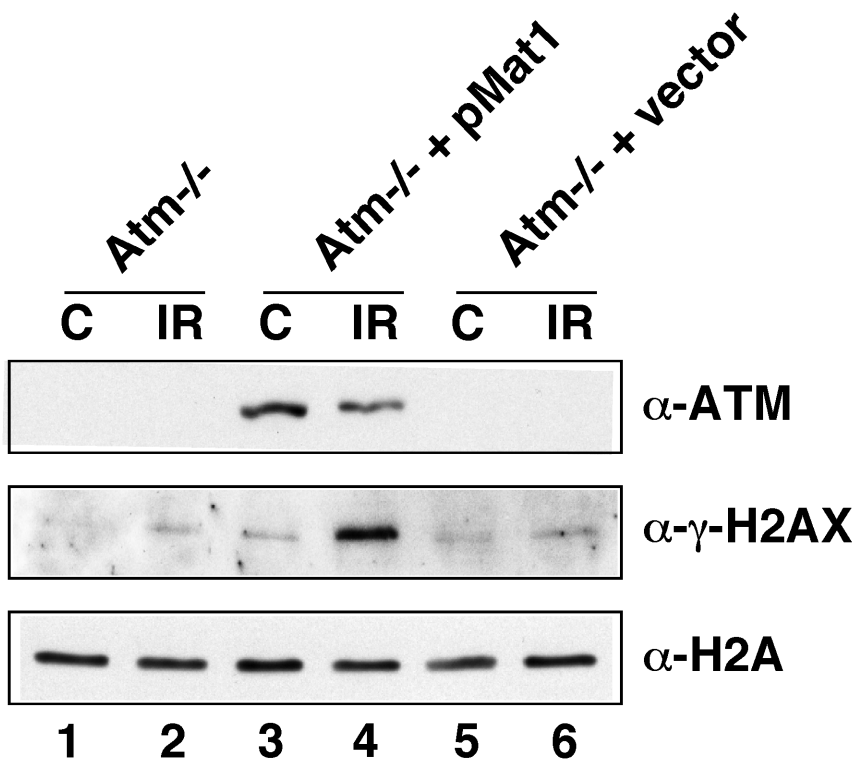

Atm-/-
Atm-/- + pMat1
Atm-/- + vector
C IR C IR C IR
α-ATM
α-γ-H2AX
α-H2A
1 2 3 4 5 6

FIG. 3.

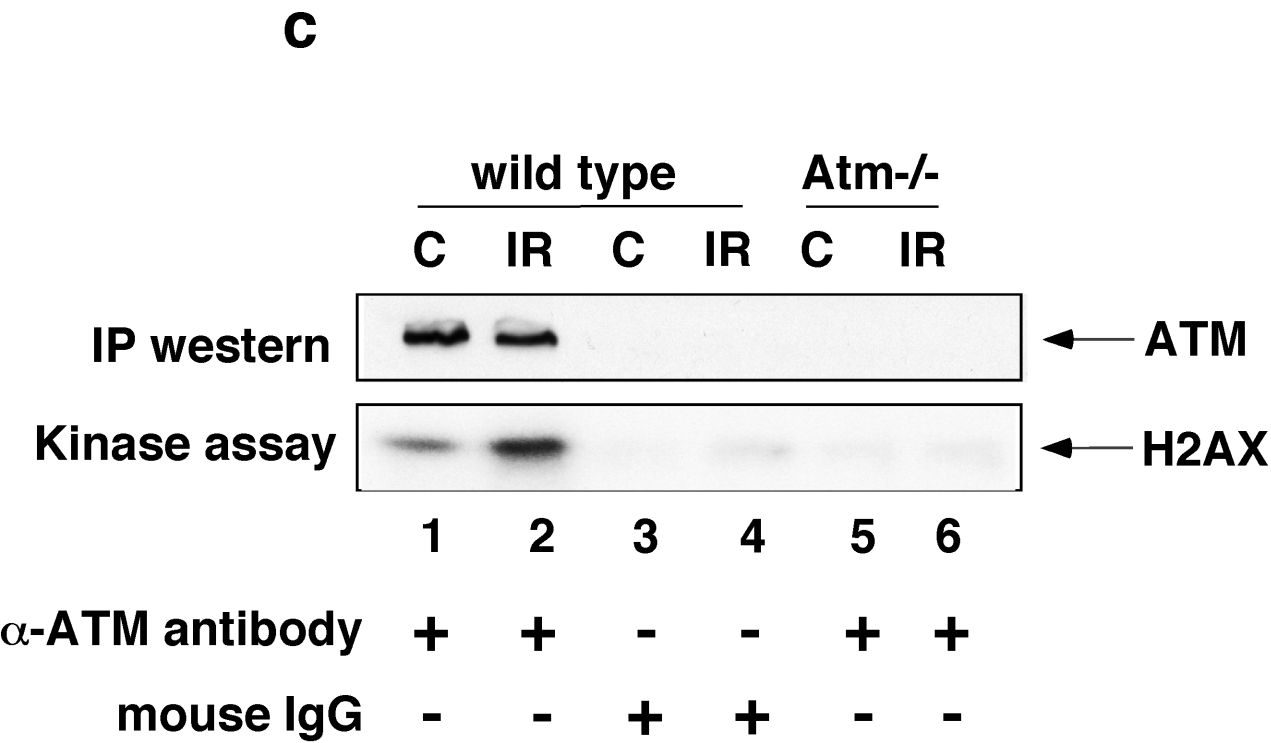
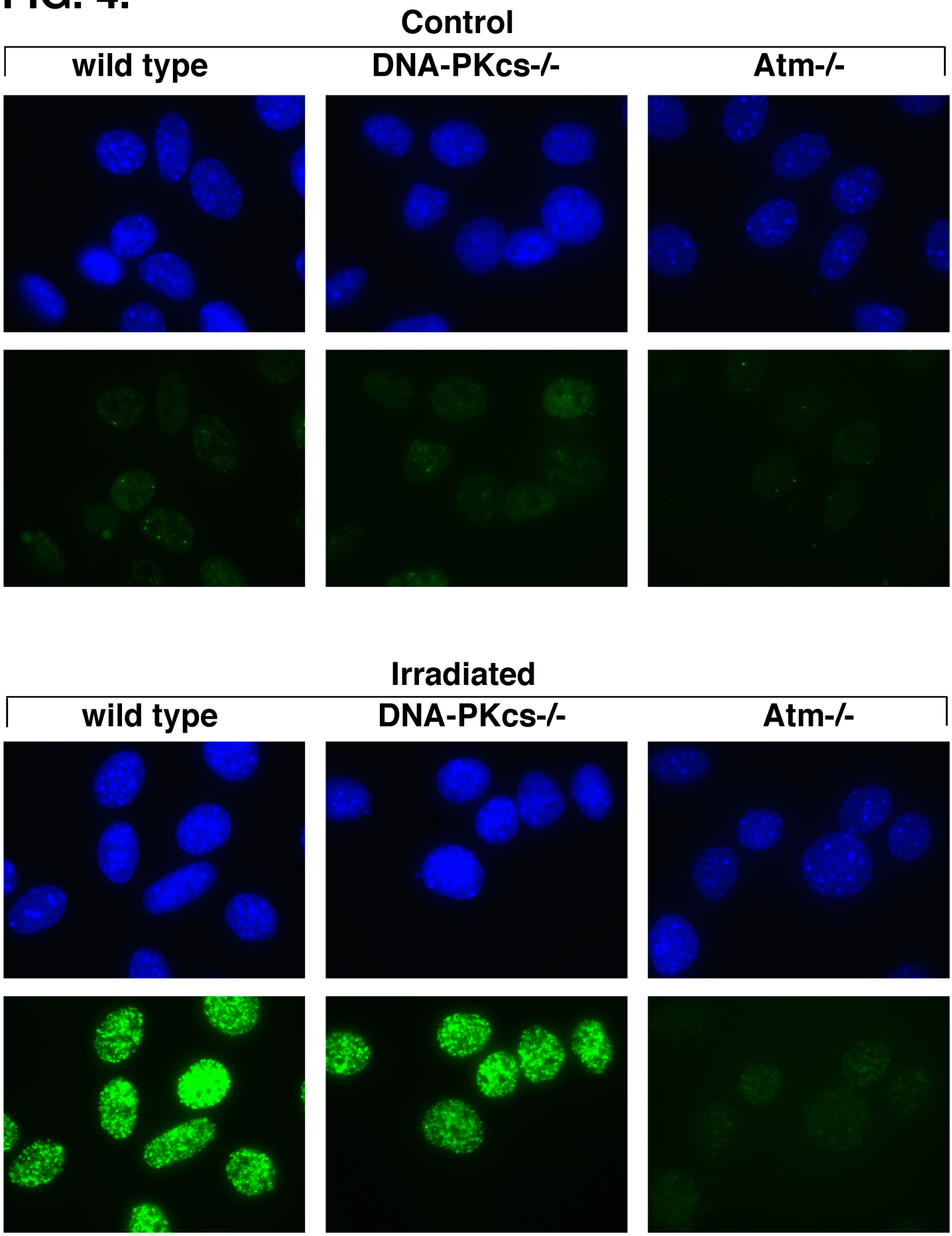

c
wild type
Atm-/-
C
IR
C
IR
C
IR
IP western
ATM
Kinase assay
H2AX
1
2
3
4
5
6
α-ATM antibody
+
+
-
-
+
+
mouse IgG
-
-
+
+
-
-

FIG. 4.


Control
wild type
DNA-PKcs-/-
Atm-/-
Irradiated
wild type
DNA-PKcs-/-
Atm-/-