

Phosphorylation of H2AX at Ser139 and a New Phosphorylation Site Ser16 by RSK2 Decreases H2AX Ubiquitination and Inhibits Cell Transformation

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Abstract

Histone H2AX is a histone H2A variant that is ubiquitously expressed throughout the genome. It plays a key role in the cellular response to DNA damage and has been designated as the histone guardian of the genome. Histone H2AX deficiency decreases genomic stability and increases tumor susceptibility of normal cells and tissues. However, the role of histone H2AX phosphorylation in malignant transformation and cancer development is not totally clear. Herein, we found that ribosomal S6 kinase 2 (RSK2) directly phosphorylates histone H2AX at Ser139 and also at a newly discovered site, Ser16. Epidermal growth factor (EGF)-induced phosphorylation of histone H2AX at both sites was decreased in RSK2 knockout cells. Phosphorylated RSK2 and histone H2AX colocalized in the nucleus following EGF treatment, and the phosphorylation of histone H2AX by RSK2 enhanced the stability of histone H2AX and prevented cell transformation induced by EGF. RSK2 and DNA-PK, but not ATM or ATR, are required for EGF-induced phosphorylation of H2AX at Ser139; however, only RSK2 is required for phosphorylation of H2AX at Ser16. Phosphorylation of histone H3 was suppressed in cells expressing wild-type H2AX compared with H2AX knockout (H2AX^{-/-}) cells. EGF-associated AP-1 transactivation activity was dramatically lower in H2AX^{-/-} cells overexpressing wild-type H2AX than H2AX^{-/-} cells expressing mutant H2AX-AA. Thus, the RSK2/H2AX signaling pathway negatively regulates the RSK2/histone H3 pathway and therefore maintains normal cell proliferation. *Cancer Res*; 71(2); 393–403. ©2011 AACR.

Introduction

Histone H2AX, a variant of histone H2A that is ubiquitously expressed throughout the genome, has been designated as the histone guardian of the genome (1). Deficiency of histone H2AX decreases genomic stability and increases tumor susceptibility of normal cells and tissues (2, 3), suggesting that it might act as a tumor suppressor. Histone H2AX-deficient mice are small in size and H2AX knockout (H2AX^{-/-}) mouse embryonic fibroblasts (MEF) exhibit impaired growth and senesce after only 3 to 4 passages in culture (4). Compared with other 2AX variants, histone H2AX contains a unique C-terminal region, and phosphorylation of histone H2AX at Ser139 (termed γ -H2AX) is located in this region. H2AX is phosphorylated at Ser139 by

various kinases, including the ataxia telangiectasia-mutated (ATM) protein (5–8), the AT and Rad3-related protein (ATR; ref. 9), and DNA-dependent protein kinase (DNA-PK; ref. 10), all of which were reported to be involved in the responses of mammalian cells to DNA double-strand breaks (DSB). An immunocytochemical assay capable of specifically recognizing γ -H2AX has become the gold standard for the detection of DSBs (1, 11). We reported previously that ultraviolet A (UVA) irradiation induces histone H2AX phosphorylation that is mediated by c-Jun N-terminal kinases (JNK), and the JNKs/H2AX pathway is associated with the induction of apoptosis (12). We also found that T-cell-originated protein kinase (TOPK) directly phosphorylates histone H2AX and is involved in arsenic-induced apoptosis of melanoma cells (13). Histone H2AX phosphorylation is induced by various stresses, such as replication stress (9, 12, 14, 15), endogenous stress (16), ionizing radiation (17), and other agents that cause DNA damage (18), including DNA DSBs (19, 20). Histone H2AX phosphorylation is also stimulated by growth factors (21), which affect immune function as well as proliferation (22). The epidermal growth factor (EGF) is a well-known tumor promotion agent used to study malignant transformation in cell and animal models of cancer (23). The mitogen-activated protein kinases (MAPK) are a family of protein kinases that mediate distinct signaling cascades, which are targets for diverse extracellular stimuli including growth factors (24, 25).

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Herein, we report that EGF strongly induces histone H2AX phosphorylation at Ser139 and at a newly identified phosphorylation site, Ser16, *in vitro* and *ex vivo*, and both phosphorylations are mediated by RSK2. Phosphorylation of histone H2AX by RSK2 prevented histone H2AX degradation, and histone H2AX could block cell transformation by inhibiting the ability of RSK2 to phosphorylate histone H3 (Ser10). The RSK2/H2AX signaling pathway, thus, negatively regulates the RSK2/H3 pathway and therefore maintains normal cell proliferation.

Materials and Methods

General Materials and Methods are included as Supplementary Materials and Methods.

Phosphorylated H2AX (Ser 16) antibody preparation

The phospho-H2AX (Ser16) antiserum was prepared by synthesizing an 18-mer phosphopeptide consisting of an N-terminal cysteine (for coupling) and the sequence CGKTGGKARAKAKpSRSSR, then coupling it to keyhole limpet hemocyanin (KLH), and immunizing rabbits by standard methods (Cocalico Biologicals, Inc.). The resulting antiserum was subsequently depleted of reactivity against nonphosphorylated H2AX by passage over an affinity column containing the unmodified peptide. The CGKTGGKARAKAKpSRSSR peptide was suspended in Sulfolink sample preparation buffer (Pierce Sulfolink kit). The IgG fraction was applied 3 times serially to the unmodified peptide column, and the flow-through fraction was collected and used as the phospho-H2AX (Ser16) antiserum.

In vitro kinase assay

Purified fusion GST-H2AX-Wt or GST-H2AX-mutants, His-H2AX-Wt or His-H2AX-mutants constructed as described (Supplementary Materials and Methods), recombinant histone proteins H2AX, H3, and H2A (Upstate), or synthesized peptides (Invitrogen) were used for *in vitro* kinase assays as substrates for active RSK2/MAPKAP kinase 1b, MAP kinase2/ERK2, JNK2 α 2/SAPK1a, p38 α /SAPK2a, mitogen- and stress-activated protein kinase 1 (MSK1; Upstate Biotechnology, Inc.), or PBK/TOPK (Cell Signaling) as appropriate. The inactive substrate and the active kinase (0.1 μ g in 50 μ L reaction) were incubated at 30°C for 30 minutes in 1 \times kinase buffer containing 10 μ mol/L of unlabeled ATP or 10 μ Ci[γ -³²P]ATP. Samples were boiled and then resolved by SDS-PAGE and visualized by autoradiography, Western blotting, or silver staining.

Ubiquitination assay and cycloheximide treatment

The ubiquitin assay system comprised *in vitro* ubiquitination reactions (50 μ L) containing 100 ng of active recombinant His-BRCA1 (Calbiochem), 100 ng of purified His-BARD1 (Supplementary Materials and Methods), 300 ng of recombinant ubiquitin activating enzyme, 300 ng of recombinant UbcH5a, 2 μ g of HA-ubiquitin (Boston Biochem), 1- μ g substrate in 50 mmol/L of HEPES (pH 7.6), and 5 μ L of energy regeneration solution (ERS) buffer (Boston Biochem). Each reaction with or

without RSK2 plus wild-type or mutant H2AX was incubated at 37°C for 3 hours, and then samples were resolved by SDS-PAGE and visualized by Western blot.

For the Fraction II (FII)-HeLa (Boston Biochem)-mediated degradation assay, 5 μ L HA-ubiquitin, 25 μ L HeLa (2 μ g/ μ L) fraction, and 5 μ L ERS buffer were incubated with GST-H2AX-Wt or GST-H2AX-AA at 37°C. For some experiments, H2AX^{-/-}/H2AX-Wt cells and H2AX^{-/-}/H2AX-AA, or RSK2^{+/+} or RSK2^{-/-} MEFs were treated for 15 minutes with EGF (20 ng/mL), and then the medium was supplemented with 50 ng/mL of cycloheximide (CHX; Sigma) to block protein synthesis. Cells were collected at various time points and expression of H2AX was visualized by Western blot.

Results

RSK2 phosphorylates histone H2AX *in vitro*

Our goal was first to identify kinase(s) responsible for phosphorylating histone H2AX. Results of an *in vitro* kinase assay indicated that MSK1 and ribosomal S6 kinase 2 (RSK2) each could phosphorylate histone H2AX (Fig. 1A, left). We compared the ability of RSK2 to phosphorylate histones H2AX and H3. Results indicated that RSK2 phosphorylated histone H2AX more strongly than its phosphorylation of histone H3 (Fig. 1A, right). We next searched for potential H2AX phosphorylation sites using NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>; ref. (26)), and then synthesized 8 peptides (P1–P8) that corresponded to these possible phosphorylation sites (Fig. 1B). The peptides were each incubated with active RSK2 and [γ -³²P]ATP in an *in vitro* kinase assay. The results showed that Ser16 was consistently and strongly phosphorylated by RSK2 (Fig. 1B: P2, P4, P5). Importantly, the phosphorylation was almost completely absent in a peptide containing a mutant Ser16 (S16A; Fig. 1B, P3). To confirm the results of the peptide mapping, we prepared glutathione S-transferase (GST)-wild-type (Wt) and GST-mutant H2AX proteins for an *in vitro* kinase assay in the presence of active RSK2 or inactive RSK2 (as a control) and [γ -³²P]ATP. The results indicated that mutation of Ser16 to alanine (16A) or Ser139 to alanine (139A) resulted in a decreased phosphorylation of H2AX and the total absence of phosphorylation of the double mutant AA (Ser16 and Ser139 to alanine; Fig. 1C, top). The phospho-H2AX (Ser16) antibody was prepared as described in the Materials and Methods section. Western blot analysis confirmed that each respective mutant showed the absence of the targeted phosphorylation site(s); (Fig. 1C, bottom). These results indicated that RSK2 does indeed phosphorylate both Ser139 and the newly identified Ser16 *in vitro*. Results of an additional *in vitro* kinase assay to compare the ability of RSK2 to phosphorylate histones H2AX and H2A, using the phospho-H2AX (Ser16) antibody (Supplementary Fig. 1A), showed that RSK2 does not phosphorylate histone H2A. The human genome contains 16 genes that encode for histone H2A variants (27). Therefore, we examined the specificity of the phospho-H2AX (Ser16) antibody in H2AX^{+/+} and H2AX^{-/-} cells after treatment with EGF. The results also indicated that the phospho-H2AX (Ser16) antibody did not recognize any other histones in H2AX^{-/-}

cells (Supplementary Fig. 1B), confirming that the phospho-H2AX (Ser16) antibody is specific only for H2AX. Next, confocal microscopy was used to show that phosphorylated RSK2 colocalized with phosphorylated histone H2AX in the nucleus following EGF treatment (Fig. 1D). γ -H2AX foci or pan-nuclear γ -H2AX staining was reported to be observed after UV treatment (28). We observed both focus-like and pan-nuclear staining of phospho-H2AX at Ser 16 and Ser139. For the foci staining, both phospho-H2AX signals colocalized with RSK2 staining, indicating that RSK2 and phosphorylated H2AX colocalize in the nucleus (Fig. 1D).

EGF induces phosphorylation of histone H2AX

JB6 Cl41 cells were treated with different doses of EGF for 15 minutes or with 20 ng/mL of EGF for 15 or 30 minutes and harvested to create a dose and time course. Histones were isolated as described previously (13), and phosphorylation of H2AX at Ser16 and Ser139 was assessed by Western blot. Results showed that EGF treatment stimulated histone H2AX phosphorylation at both serine sites in a dose- (Fig. 2A, left) and time-dependent (Fig. 2A, right) manner. JB6 cells stably overexpressing RSK2 were treated for 15 minutes with EGF (20 ng/mL), and the results indicated that

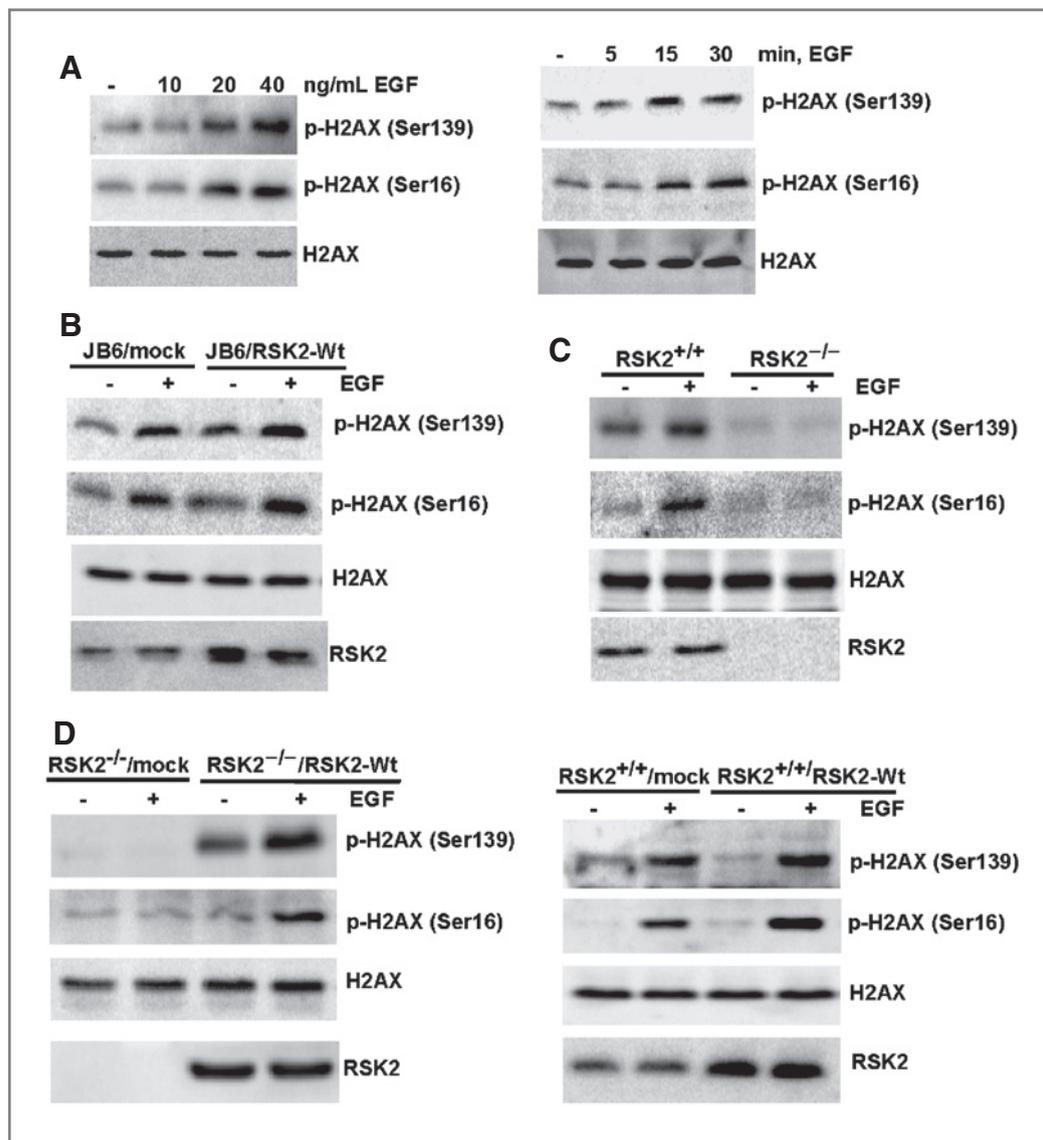


Figure 2. RSK2 phosphorylates histone H2AX at Ser16 and Ser139 *ex vivo*. **A**, left, phosphorylation of histone H2AX at Ser139 or Ser16 is induced dose dependently by EGF; right, EGF induces a time-dependent phosphorylation of histone H2AX at Ser139 or Ser16. Untreated samples served as negative controls. Total histone H2AX was used to verify protein loading. **B** to **D**, lysates from JB6 Cl41 cells stably expressing pcDNA4-mock (JB6/mock) or pHisG-RSK2 (JB6/RSK2-Wt; **B**); or RSK2^{+/+} and RSK2^{-/-} MEFs (**C**); or stable transfectants of RSK2^{-/-} cells expressing pcDNA4-mock (RSK2^{-/-}/mock) or pcDNA4-RSK2 (RSK2^{-/-}/RSK2-Wt; **D**, left); or stable transfectants of RSK2^{+/+} cells expressing pcDNA4-mock (RSK2^{+/+}/mock) or pcDNA4-RSK2 (RSK2^{+/+}/RSK2-Wt; **D**, right) were treated with EGF (20 ng; 15 minutes), and analyzed by Western blot as for (**A**). Total histone H2AX or RSK2 was used to verify equal protein loading or as an internal control for confirmation of RSK2 deficiency (RSK2^{-/-}) or overexpression of RSK2 (JB6/RSK2-Wt, RSK2^{-/-}/RSK2-Wt or RSK2^{+/+}/RSK2-Wt).

overexpression of RSK2 resulted in increased phosphorylation of H2AX at Ser139 and Ser16 (Fig. 2B). RSK2 wild-type (RSK2^{+/+}) and knockout (RSK2^{-/-}) cells were treated similarly, and results showed that phosphorylation of H2AX at both Ser139 and Ser16 was dramatically decreased in RSK2^{-/-} cells compared with RSK2^{+/+} cells (Fig. 2C). To further show that RSK2 phosphorylates H2AX, RSK2 was overexpressed in both RSK2^{-/-} (RSK2^{-/-}/RSK2-Wt) and RSK2^{+/+} (RSK2^{+/+}/RSK2-Wt) cells, and then cells were treated with EGF. The results indicated that RSK2^{-/-}/RSK2-Wt (Fig. 2D, left) and RSK2^{+/+}/RSK2-Wt (Fig. 2D, right) cells exhibited dramatically increased EGF-induced phosphorylation of H2AX at Ser139 and Ser16 compared with mock-transfected cells (RSK2^{-/-}/mock; RSK2^{+/+}/mock). All these data support the idea that RSK2 mediates EGF-induced phosphorylation of histone H2AX at Ser16 and Ser139, which suggests a new function for histone H2AX in addition to its role in DNA damage repair.

H2AX blocks anchorage-independent cell transformation

We generated JB6 Cl41 cells stably overexpressing wild-type H2AX (JB6/H2AX-Wt) or double mutant JB6/H2AX-AA (designated JB6/H2AX-AA representing mutations of Ser16 and Ser139 to alanine) and the growth curves of both cells were compared with JB6/mock cells after EGF treatment (Supplementary Fig. 2). Results demonstrated a slower growth for JB6/H2AX-Wt cells compared with JB6/mock cells, in contrast to faster growth for mutant JB6/H2AX-AA cells compared with JB6/H2AX-Wt cells. These results were surprising because they suggested that histone H2AX, as a substrate of RSK2, does not promote cell growth.

RSK2 is a kinase that has been shown to mediate cell transformation. Therefore, we hypothesized that H2AX might be involved in RSK2-mediated anchorage-independent cell transformation stimulated by tumor promoters such as EGF. Because RSK2 can phosphorylate histone H2AX at both Ser16 and Ser139, we generated JB6 Cl41 cells expressing single mutations of histone H2AX (designated JB6/H2AX-16A and JB6/H2AX-139A representing mutations at Ser16 and Ser139 to alanine, respectively) or double-mutant JB6/H2AX-AA cells for further experiments. JB6/H2AX-Wt, JB6/H2AX-16A, JB6/H2AX-139A, or double-mutant JB6/H2AX-AA cells (Fig. 3A) were used to study anchorage-independent cell transformation induced by EGF. The resulting data indicated that the number of colonies formed by wild-type H2AX cells was significantly less than the number formed by mock-transfected control cells (Fig. 3B, 2nd vs. 3rd panel). In addition, the number of colonies formed by single-mutant H2AX-16A or H2AX-139A or double-mutant H2AX-AA cells was also significantly greater than H2AX-Wt (Fig. 3B, 3rd vs. 4th, 5th, 6th panels). To further confirm this result, siRNA against H2AX was used to create stable JB6 knockdown cells (JB6/siH2AX; Fig. 3C, inset left). Results indicated that after treatment with EGF, the number of colonies was significantly increased in the siH2AX group compared with mock-transfected control cells (Fig. 3C, 3rd vs. 4th panel).

Overexpression of histone H2AX blocks foci formation

H2AX-Wt, H2AX-16A, H2AX-139A, or H2AX-AA expression vectors were then transfected with constitutively active Ras (H-Ras^{G12V}) into NIH3T3 cells to study cell transformation using a foci formation assay. As expected, H-Ras^{G12V} induced cell transformation in NIH3T3 cells that was blocked by cotransfection with H2AX-Wt (Fig. 3D). In contrast, the H2AX-16A, H2AX-139A, or H2AX-AA mutants showed increased foci formation in NIH3T3 cells compared with H2AX-Wt (Fig. 3D). Overall, these results again indicated that histone H2AX plays an important role in preventing cell transformation induced by tumor promoters such as EGF.

Phosphorylation of histone H2AX by RSK2 prevents H2AX protein ubiquitination

In our earlier experiments, we found that histone H2AX is important for suppressing EGF-induced cell transformation, and phosphorylation of H2AX by RSK2 could enhance this function. We thus hypothesized that phosphorylation of histone H2AX might increase H2AX stability thereby allowing it to accumulate, which would enhance its ability to block EGF-induced transformation. We studied histone H2AX ubiquitination using an established method (29) with some modification. First, a kinase assay with active RSK2 and a recombinant histone H2AX protein was performed and fractions were collected to verify RSK2's phosphorylation of histone H2AX [Fig. 4A(i)] and confirm equal protein loading [Fig. 4A(ii)]. Then, an ubiquitin assay system was added to study ubiquitination of histone H2AX. The results indicated that phosphorylation of histone H2AX resulted in less ubiquitination than nonphosphorylated H2AX [Fig. 4A(iii)]. We prepared His-H2AX (Wt), His-H2AX-S16/139AA (AA), and His-H2AX-S16/139EE (EE; designed as a phosphorylation mimic) and used these proteins to further study histone H2AX ubiquitination. The results showed that phosphorylation of H2AX-Wt and the H2AX-EE mutant resulted in less ubiquitination than the ubiquitination level of the nonphosphorylated H2AX-AA mutant, which was dramatically higher than any of the other histone H2AX proteins (Fig. 4B). These results suggested that phosphorylation of histone H2AX by RSK2 prevents the ubiquitination of H2AX.

Phosphorylation of histone H2AX by RSK2 results in stabilization of H2AX

We examined the stability of H2AX using a HeLa fraction and H2AX-Wt or mutant H2AX-AA proteins. Results showed that the degradation of H2AX-Wt was substantially lower than the mutant H2AX-AA (Fig. 5A). To study the stability of H2AX *ex vivo*, we used H2AX knockout (H2AX^{-/-}) cells stably expressing wild-type (H2AX^{-/-}/H2AX-Wt) or mutant H2AX (H2AX^{-/-}/H2AX-AA), or RSK2^{+/+} and RSK2^{-/-} cells. Cells, including controls, were treated with EGF (20 ng) for 15 minutes followed by treatment with cycloheximide (CHX) to assess the stability of histone H2AX at various times. Results showed that the stability of histone H2AX was substantially greater in

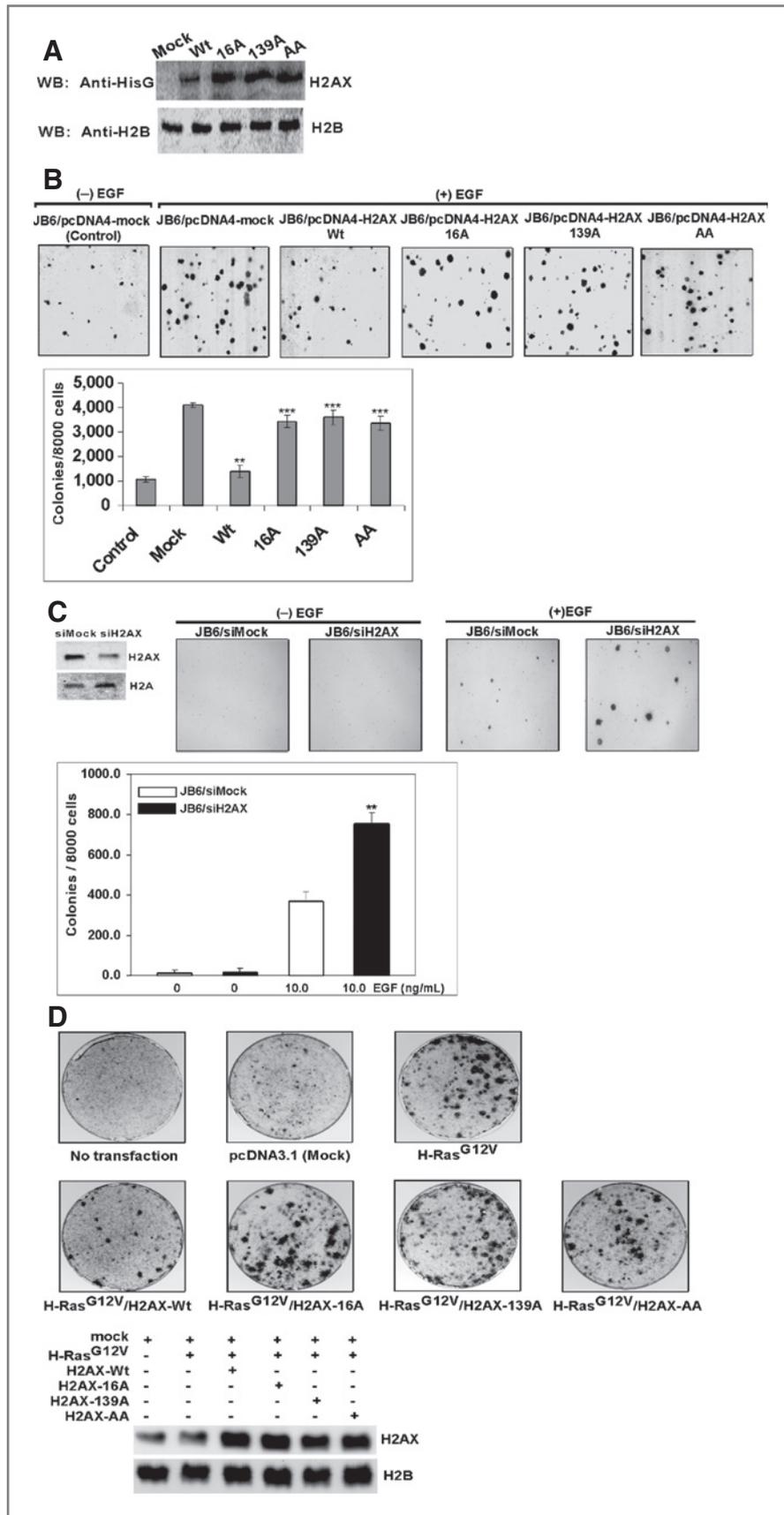


Figure 3. Histone H2AX blocks anchorage-independent cell transformation and foci formation. **A**, JB6 Cl41 cells were transfected with pcDNA4/HisMax-mock (Mock), pcDNA4/HisMax-H2AX (Wt), pcDNA4/HisMax-H2AX-S16A (16A), pcDNA4/HisMax-H2AX-S139A (139A), or double-mutant pcDNA4/HisMax-H2AX-S16/139AA (AA). Cells were selected with zeocin for 14 days and pooled. Mock, Wt-, 16A-, 139A-, or AA-H2AX expression was detected with an antibody against the HisG tag. For visualizing protein loading, total histone H2B was detected by Western blotting. **B**, transfectants of JB6 Cl41 mouse cells stably expressing pcDNA4-mock, pcDNA4-H2AX-Wt, pcDNA4-H2AX-S16A, pcDNA4-H2AX-S139A, or double-mutant pcDNA4-H2AX-AA were compared for EGF-induced colony formation in soft agar. Bottom shows means \pm SD values obtained from triplicate experiments. **, significantly ($P < 0.005$) fewer colonies in H2AX-Wt cells compared with mock-transfected cells; ***, significantly ($P < 0.002$) more colonies in H2AX-S16A, H2AX-S139A, or H2AX-AA cells compared with H2AX-Wt cells. **C**, inset (far left) shows verification of cell types by Western blot. JB6/siMock cells and knockdown H2AX JB6 Cl41 (JB6/siH2AX) cells were compared for EGF-induced colony formation in soft agar. Bottom shows means of values \pm SD obtained from triplicate experiments. **, significantly ($P < 0.005$) greater number of colonies in JB6/siH2AX cells compared with JB6/siMock cells. **D**, Ras-induced foci formation was examined. Various combinations of expression vectors were transfected into NIH3T3 cells as indicated and a foci formation assay was performed using standard protocols. Foci were stained with 0.5% crystal violet. The protein level of transfected H2AX was assessed by Western blot (bottom). Total histone H2B was used for visualizing equal loading of protein.

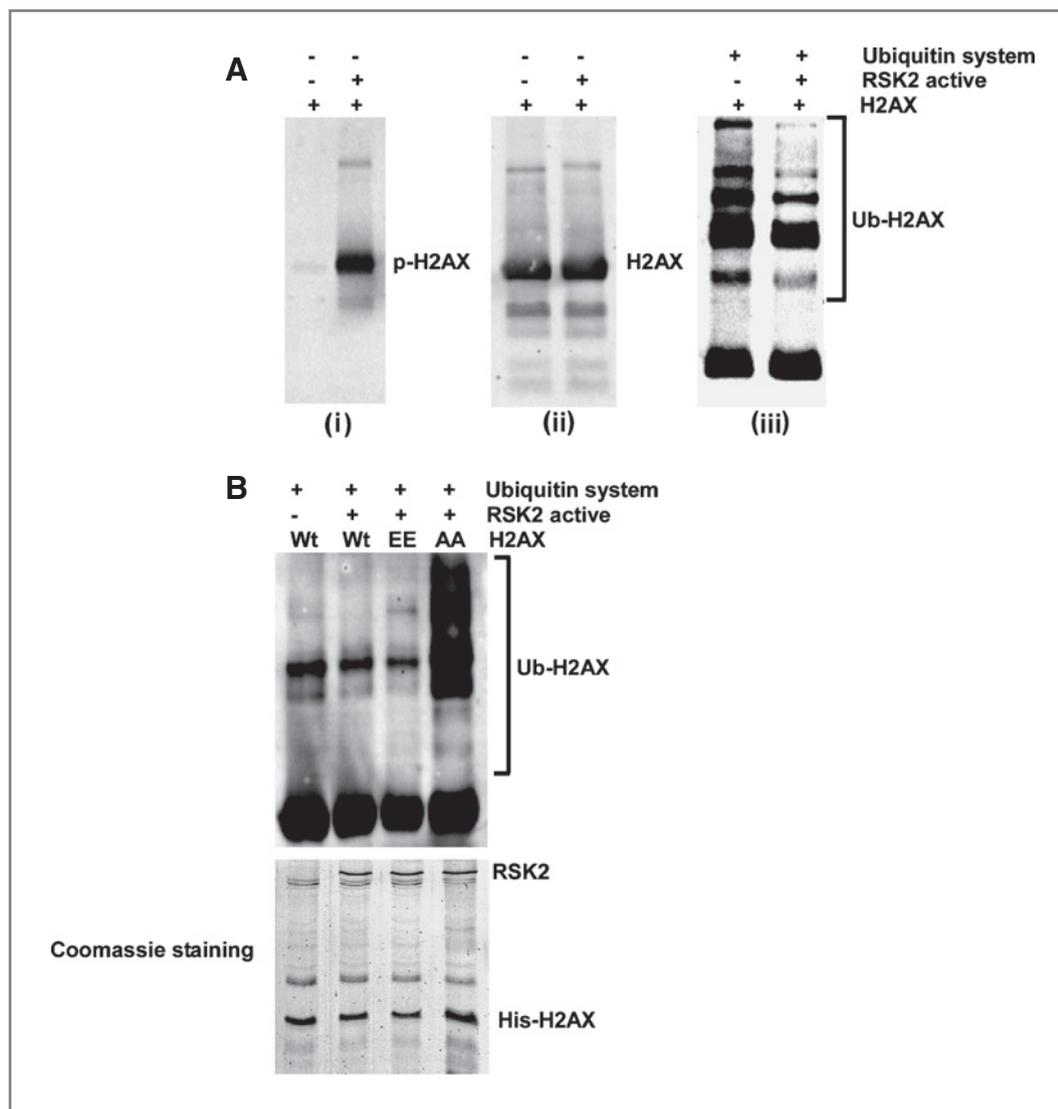


Figure 4. Phosphorylation of H2AX by RSK2 prevents H2AX ubiquitination. **A**, the effect of phosphorylation of H2AX on its ubiquitination was assessed by kinase assay using active RSK2 and recombinant H2AX (H2AX): (i) confirmation of *in vitro* phosphorylation of H2AX by active RSK2; (ii) nonphosphorylated H2AX was used to confirm protein loading; (iii) the ubiquitin assay system (see the Materials and Methods section) was set up in conjunction with a kinase reaction with or without active RSK2, and ubiquitination of H2AX (Ub-H2AX) was detected with anti-H2AX to compare H2AX ubiquitination before and after phosphorylation by RSK2. **B**, ubiquitination of histone wild-type His-H2AX and His-H2AX mutants. The ubiquitin assay was set up in conjunction with a kinase reaction with or without active RSK2, and ubiquitination of H2AX-Wt without phosphorylation was compared with H2AX-Wt, an H2AX (AA) mutant, or a phosphorylation mimic H2AX (EE) after detection with anti-H2AX. Bottom, total H2AX was used to verify equal protein loading by Coomassie staining.

H2AX^{-/-}/H2AX-Wt (Fig. 5B) and in RSK2^{+/+} cells (Fig. 5C) than H2AX^{-/-}/H2AX-AA or RSK2^{-/-} cells, respectively. Taken together, these results suggested that phosphorylation of histone H2AX by RSK2 enhances the stability of H2AX.

H2AX blocks RSK2 phosphorylation of histone H3 (Ser10) and AP-1 luciferase activity

Earlier, we showed that RSK2 phosphorylates histone H3 at Ser10 (30). Because histone H2AX can be phosphorylated by RSK2 to suppress cell transformation, we hypothesized

that H2AX might interfere with the phosphorylation of histone H3 mediated by RSK2. The phosphorylation of histone H3 at Ser10 was examined in H2AX^{+/+} and H2AX^{-/-} cells, (Fig. 6A) and in H2AX^{-/-} cells overexpressing wild-type H2AX (H2AX^{-/-}/H2AX-Wt) or mutant H2AX (H2AX^{-/-}/H2AX-AA; Fig. 6B). Phosphorylation of histone H3 (Ser10) was increased in H2AX^{-/-} cells than H2AX^{+/+} cells (Fig. 6A), and also in H2AX^{-/-}/H2AX-AA cells compared with H2AX^{-/-}/H2AX-Wt cells (Fig. 6B). These results indicated that histone H2AX suppressed phosphorylation of histone H3 at Ser10.

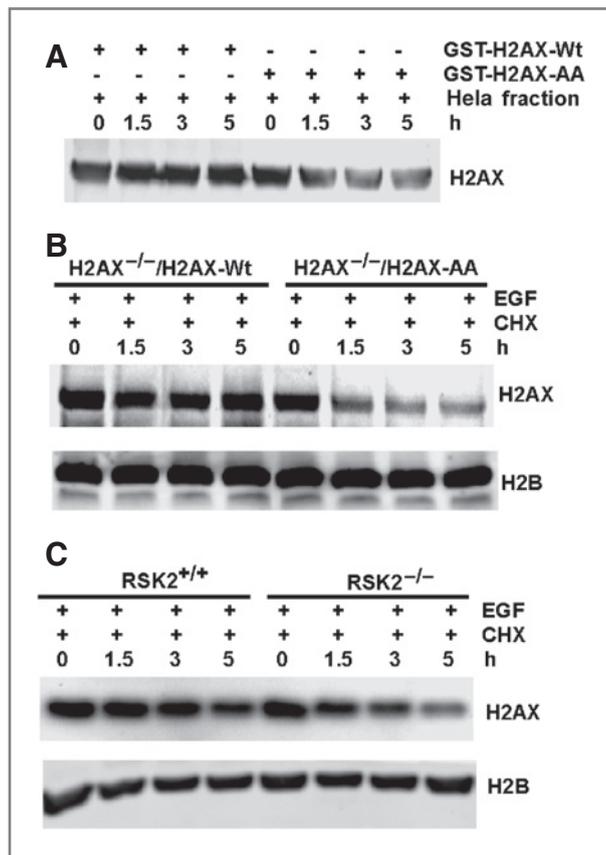


Figure 5. Phosphorylation of H2AX by RSK2 stabilizes H2AX. **A**, *in vitro* degradation of histone H2AX assessed using a HeLa cell fraction. GST-H2AX-Wt or the mutant GST-H2AX-AA was incubated with HeLa fraction II (see the Materials and Methods section), and time-dependent degradation of H2AX was detected with anti-H2AX. **B** and **C**, half-life of histone H2AX. H2AX^{-/-} MEFs overexpressing H2AX-Wt or mutant H2AX-AA (**B**) or RSK2^{+/+} or RSK2^{-/-} (**C**) MEFs were treated with EGF (20 ng, 15 minutes) followed by addition of CHX to prevent new protein synthesis. Proteins in cell lysates were separated by 15% SDS-PAGE followed by Western blot. Time-dependent stability of H2AX was detected with anti-H2AX. Total histone H2B was used to verify protein loading.

EGF stimulates activator protein-1 (AP-1) and induces transformation in many different cell types and animal models (23, 31). To determine whether the overexpression of histone H2AX is responsible for EGF-stimulated AP-1 activation, we next cotransfected the *AP-1 luciferase* reporter plasmid and the *pRL-SV40* gene into H2AX^{-/-} cells overexpressing wild-type H2AX (H2AX^{-/-}/H2AX-Wt) or mutant H2AX (H2AX^{-/-}/H2AX-AA). AP-1 luciferase activity was dramatically increased in H2AX^{-/-}/H2AX-AA mutant cells than H2AX^{-/-} cells overexpressing wild-type H2AX following EGF treatment (Fig. 6C). These results indicated that histone H2AX suppresses AP-1 luciferase activity and phosphorylation of histone H3 at Ser10.

DNA-PK, but not ATM or ATR, is involved in EGF-induced phosphorylation of H2AX at Ser139

Although promoting cell growth and transformation signaling pathway is a major function of EGF, whether EGF can

induce replication stress or oxidative stress indirectly and then cause phosphorylation of H2AX is unclear. Members of the phosphatidylinositol-3 (PI-3) kinase family, including ATM, ATR, and DNA-PK, also regulate histone H2AX phosphorylation and are known to be involved in the DNA damage response induced by replication stress (9, 12, 14, 15). DNA-PK responds to oxidative stress as well in human tumor cell lines (32), and phosphorylation of H2AX at Ser139 is induced in lymphocytes after oxidative stress (33). To determine the role of these kinases in EGF-induced H2AX phosphorylation, we treated cells with the PI-3 kinase family inhibitor, wortmannin (5, 34), for 1 hour before EGF treatment. Wortmannin suppressed EGF-induced phosphorylation of H2AX at Ser139 but had no effect on phosphorylation of Ser16 (Fig. 7A). We also treated DNA-PK-Wt or DNA-PK^{-/-} MEFs (Fig. 7B), ATM-Wt or ATM^{-/-} MEFs (Fig. 7C), and ATR-Wt or ATR-kinase dead (kd) MEFs (Fig. 7D) with EGF. Results indicated that EGF induced H2AX phosphorylation at Ser16 and Ser139 independently of ATM (Fig. 7C) or ATR (Fig. 7D). In contrast, phosphorylation of histone H2AX at Ser139 was dependent on DNA-PK, whereas, phosphorylation of H2AX at Ser16 occurred independently of DNA-PK (Fig. 7B). In our case, the dependence of phosphorylation of H2AX at Ser139 on DNA-PK might be caused by both replication stress and oxidative stress. On the other hand, phosphorylation of H2AX at Ser16 is independent of the DNA damage response that might be caused indirectly by replication or oxidative stress following EGF treatment.

Discussion

MAPKs are important regulators of proliferation and oncogenesis (35). These proteins phosphorylate numerous and varied substrates, including histone H2AX (12, 13). Others (36, 37) and we (30) reported that RSK2 phosphorylates histone H3. In the present study, we found that RSK2 phosphorylates histone H2AX more strongly than it phosphorylates histone H3. We also found that both Ser139 and the newly discovered Ser16 of histone H2AX are important sites for histone H2AX function. RSK2 kinase activity can be stimulated by EGF (30, 38), and we, therefore, determined whether EGF could induce phosphorylation of histone H2AX mediated by RSK2. ATM was reported to be inhibited following EGF treatment (39, 40), but DNA-PK can be activated by the EGF receptor (41). Our data indicated that DNA-PK is required for EGF-induced phosphorylation of H2AX at Ser139 but is not required for phosphorylation at Ser16 (Fig. 7B). RSK2 is required for phosphorylation of both sites. Neither ATM nor ATR is required for the phosphorylation of H2AX induced by EGF. Activation of DNA-PK by EGF might be due to replication stress or oxidative stress. Overall, these data indicate that EGF-induced phosphorylation of H2AX was induced both through proliferation signaling and DNA damage response signaling, which suggests a novel function for phosphorylation of histone H2AX in addition to DNA damage repair.

The colocalization of histone H2AX and ubiquitin has been reported (42). The BRCA1/BRCA-associated ring

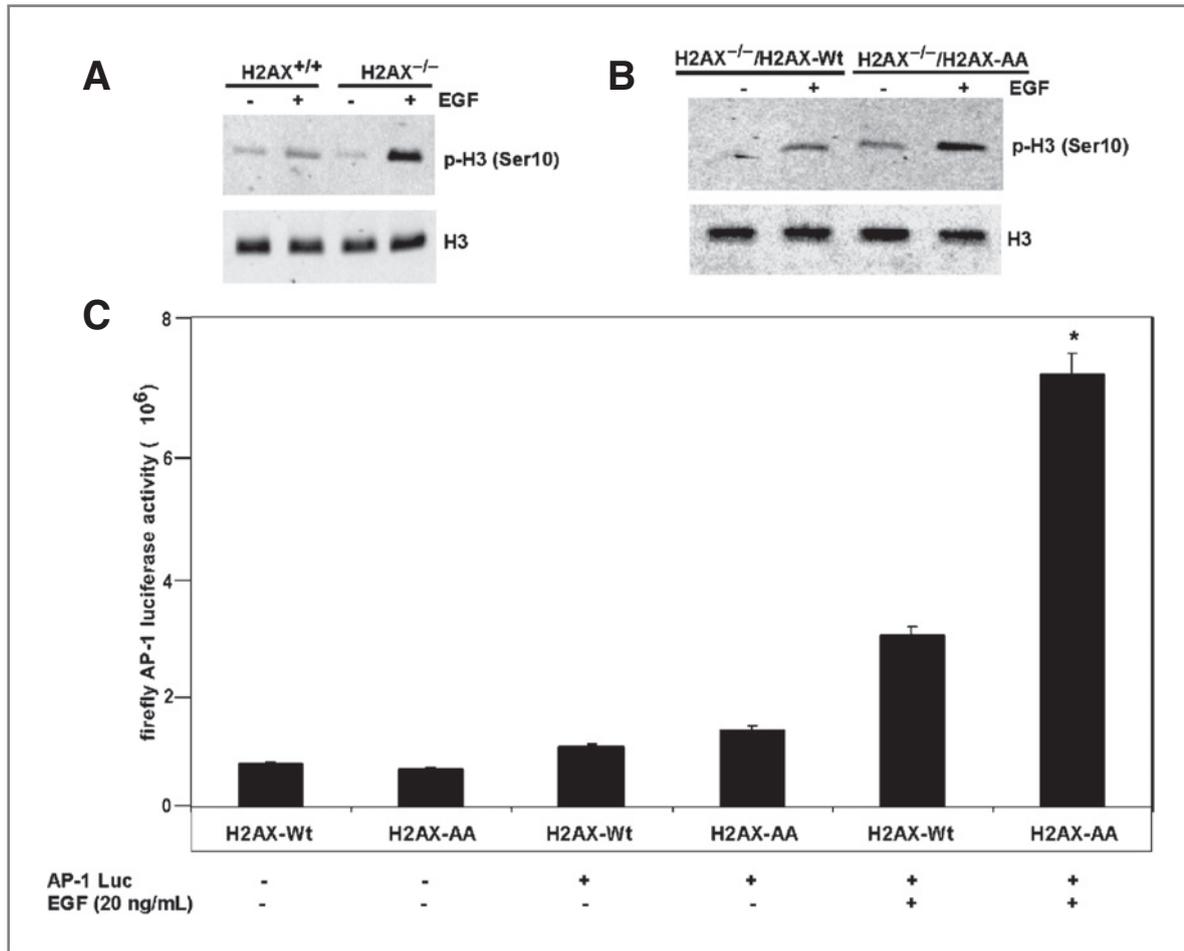


Figure 6. Histone H2AX blocks phosphorylation of histone H3 (Ser10) and AP-1 luciferase activity. A and B, phosphorylation of histone H3 (Ser10) was detected in H2AX^{+/+} or H2AX^{-/-} cells (A) and in H2AX^{-/-} MEFs overexpressing H2AX-Wt or mutant H2AX-AA (B) with or without EGF. Cell lysates were subjected to SDS-PAGE followed by Western blot to detect phosphorylation of histone H3 (Ser10). Total histone H3 was used to verify equal protein loading. C, H2AX blocks AP-1 activity. The AP-1 luciferase plasmid was transfected along with H2AX-Wt or mutant H2AX-AA into H2AX^{-/-} cells. Cells were or were not treated with EGF and lysates assessed for firefly luciferase activity normalized against *Renilla* luciferase activity. Data are expressed as means \pm SD of values from triplicate experiments. The asterisk (*) indicates significantly ($P < 0.02$) increased AP-1 activity in H2AX-AA mutant cells compared with H2AX-Wt cells following treatment with EGF.

domain protein complex (BRCA1/BARD1) promotes ubiquitination of histones H2A and H2AX (29). Moreover, RING-finger-containing nuclear factor (RNF8) was reported to respond to DNA damage and promote ubiquitination of histone H2AX (43, 44). Phosphorylation is known to increase the half-life of some proteins such as c-Jun (45). H2AX-Wt, H2AX-S16/139AA, and H2AX-S16/139EE proteins were used to study histone H2AX ubiquitination *in vitro*, and results indicated that phosphorylation of both Ser16 and Ser139 is important for preventing ubiquitination of histone H2AX.

Overall, our results indicated that EGF induces the phosphorylation of RSK2, which results in the phosphorylation of histone H2AX at a newly discovered Ser16 site and Ser139, both *in vitro* and *ex vivo*. Phosphorylation of histone H2AX increases H2AX stability, which enhances H2AX's ability to block cell transformation that is mediated through

the inhibition of phosphorylation of histone H3 by RSK2. The RSK2-H2AX signaling pathway thus appears to negatively regulate the RSK2-H3 signaling pathway, thereby suppressing cell transformation. EGF can induce DNA-PK activity as well and DNK-PK is required for phosphorylation of H2AX at Ser139, but not Ser16. This supports the idea that DNA-PK plays a key role in regulating γ -H2AX in response to cell-cycle progression. However, DNA damage also induces cell-cycle checkpoints and γ -H2AX is involved in this process. Thus, this response might also account for some of the antiproliferative effects of γ -H2AX as well (46, 47). Our data, therefore, suggest that both a proliferation signaling pathway (RSK2) and a DNA damage response signaling pathway (DNA-PK) are activated by EGF treatment. These results provide new insight into the role of histone H2AX phosphorylation in cell transformation and oncogenesis.

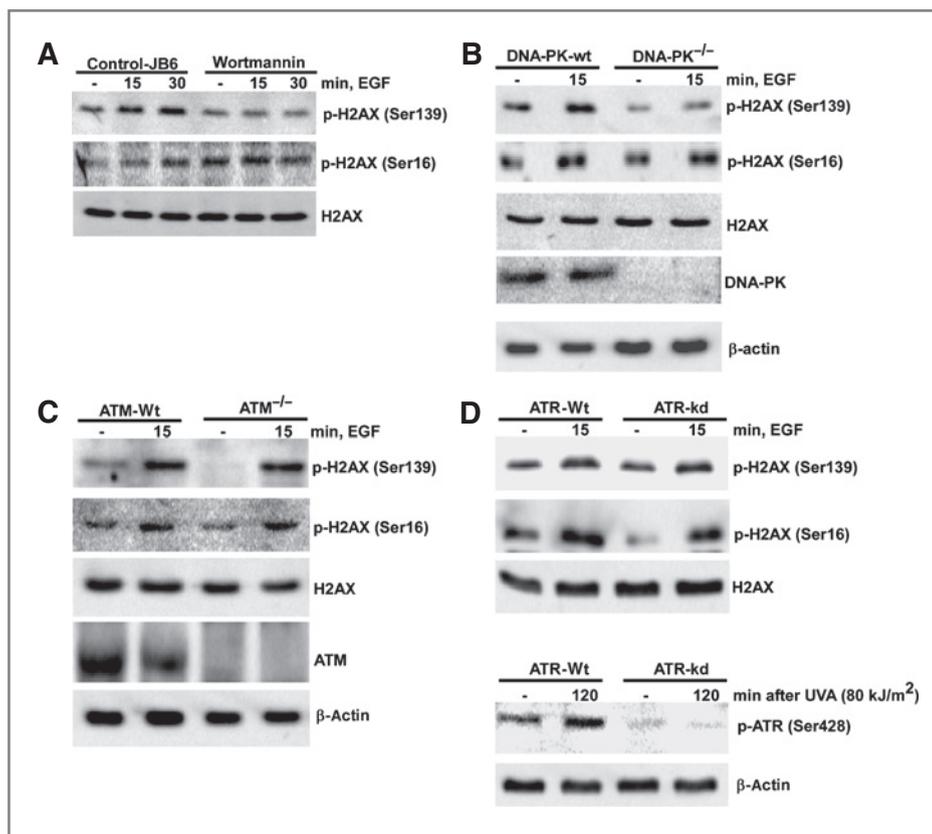


Figure 7. DNA-PK, but not ATM or ATR, is involved in EGF-induced phosphorylation of H2AX at Ser139. **A**, JB6 cells were treated with wortmannin (200 μmol/L) 1 hour before EGF treatment, and harvested at the indicated time. Phosphorylation of H2AX (Ser16) and H2AX (Ser139) was determined by Western blot using specific antibodies. **B**, DNA-PK-Wt and DNA-PK^{-/-} MEFs were treated with 20 ng of EGF and harvested after 15 minutes. Phosphorylation of H2AX at Ser16 or Ser139 or total DNA-PK protein level was determined by Western blot using specific antibodies. **C**, ATM-Wt or ATM^{-/-} MEFs were treated with 20 ng of EGF and harvested after 15 minutes. Phosphorylation of H2AX at Ser16 or Ser139 or total ATM protein level was determined by Western blot using specific antibodies. **D**, ATR-Wt or ATR-kd MEFs were treated with 20 ng of EGF and harvested after 15 minutes. Phosphorylation of H2AX at Ser16 or Ser139 was determined by Western blot using specific antibodies. For verification of cell type, ATR-Wt and ATR-kd MEFs were treated with UVA, and phosphorylation of ATR was determined by Western blot using specific antibodies. For all Western Blots, total H2AX and β-actin were used to verify equal protein loading.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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