HHS Public Access

Author manuscript

Am J Physiol Lung Cell Mol Physiol. Author manuscript; available in PMC 2022 January 24.

Published in final edited form as:

Am J Physiol Lung Cell Mol Physiol. 2008 May ; 294(5): L998–L1006. doi:10.1152/ajplung.00004.2008.

Differential roles of ATR and ATM in p53, Chk1, and histone H2AX phosphorylation in response to hyperoxia: ATR-dependent ATM activation

Amit Kulkarni, **Kumuda C. Das**

Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Abstract

Elevated level of oxygen (hyperoxia) is widely used in critical care units and in respiratory insufficiencies. In addition, hyperoxia has been implicated in many diseases such as bronchopulmonary dysplasia or acute respiratory distress syndrome. Although hyperoxia is known to cause DNA base modifications and strand breaks, the DNA damage response has not been adequately investigated. We have investigated the effect of hyperoxia on DNA damage signaling and show that hyperoxia is a unique stress that activates the ataxia telangiectasia mutant (ATM)- and Rad3-related protein kinase (ATR)-dependent p53 phosphorylations (Ser6, −15, −37, and −392), phosphorylation of histone H2AX (Ser139), and phosphorylation of checkpoint kinase 1 (Chk1). In addition, we show that phosphorylation of p53 (Ser6) and histone H2AX (Ser139) depend on both ATM and ATR. We demonstrate that ATR activation precedes ATM activation in hyperoxia. Finally, we show that ATR is required for ATM activation in hyperoxia. Taken together, we report that ATR is the major DNA damage signal transducer in hyperoxia that activates ATM.

Keywords

ataxia telangiectasia mutant; checkpoint

Oxygen, although irrefutably required for sustenance of aerobic life forms, can also participate in the destruction of the very living tissue it sustains. Higher concentration of oxygen (hyperoxia) often administered to patients in critical care units and in respiratory insufficiencies such as bronchopulmonary dysplasia or acute respiratory distress syndrome (8, 10, 19, 23). Reactive oxygen species (ROS) are products of normal cellular metabolism and are generated in an elevated level in hyperoxia (12, 25, 54). All ROS have the potential to interact with cellular components including DNA to produce damaged bases or strand breaks (9, 13, 24, 26). Overwhelming antioxidant defense system of the cells to counteract oxidative damage, ROS can cause damage to DNA and other macromolecules, which has been proposed to play a key role in the development of diseases such as cancer, heart diseases, neurodegenerative diseases, and diseases of the pulmonary system (2, 14, 47).

Address for reprint requests and other correspondence: K. C. Das, 4301 West Markham St., Slot no. 845, Little Rock, AR 72205 (kdas@uams.edu).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In addition to the antioxidant system, elaborate genomic surveillance and checkpoint systems have been evolved to guard against duplication of genetically altered cells, failure of which could introduce deleterious mutagenesis resulting in cancer and other diseases (1). The cellular response to DNA damage is complex and involves gene products that recognize DNA damage and transduce the signal to various sensors that inhibit proliferation, stimulate repair, or induce apoptosis (16, 17). An important checkpoint system is the p53 tumor suppressor gene. The product of the p53 tumor suppressor gene consists of 393 amino acid polypeptide that function as a homotetrameric transcription factor (17, 48). P53 is normally short lived and is present in low levels in the unstressed cells. Various forms of stress including oxidative stress activate p53 by posttranslational modifications that allow for its stability resulting in its activation as a transcription factor (5 and 6). Genomic approaches have shown that p53 induces or inhibits the expression of more than 150 genes (5 and 6) that mediate arrest of cell cycle, induce apoptosis, or stimulate DNA repair. The biochemical links between p53, cell cycle arrest, senescence, and apoptosis are cell- and stress-type-dependent. Seven serines and two threonines in the NH_2-terminal domain of human p53, specifically Ser6, −9, −15, −20, −33, −37, and −46 and Thr18 and −81, are phosphorylated in response to exposure of cells to ionizing radiation (IR; Refs. 5, 6, 40, 41) or UV light (reviewed in Ref. 5). In the COOH-terminal regulatory domain, Ser315 and Ser392 are phosphorylated, Lys320, −373, and −382 are acetylated, and Lys386 is sumoylated in response to DNA damage (5). Ser392 is known to be phosphorylated by UV light but not by IR (5).

Ataxia telangiectasia mutant (ATM) and the ATM- and Rad3-related protein kinase, ATR, are members of the phosphoinositide 3-kinase-related kinases (PIKK) that play key roles in fundamental cellular processes including proliferation and genomic surveillance (45). ATM is a 370-kDa protein kinase mutated in the human genetic disorder ataxia telangiectasia (45). Cell lines derived from patients lacking ATM are radiosensitive and exhibit defects in checkpoint responses to IR (1) including p53-dependent G_1 cell cycle arrest and p53-independent S and G_2 cell cycle arrest (14, 15, 42). The kinase activity of ATM is activated in response to double-stranded DNA breaks (45), and ATM targets several effectors of checkpoint control, including Cds1 (also known as Chk2), BRCA1 (33), p53 (32), and Mdm2 (35, 37). Unlike ATM, deletion of ATR in mice results in embryonic lethality indicating that ATR is an essential gene (1, 11). Cells lacking ATM are hypersensitive to IR but not to UV or hydroxyurea (HU) (18), whereas cells overexpressing a kinase-inactive form of ATR are sensitive to UV and HU (14) as well as to IR (37). This suggests that ATR plays a more prominent role than ATM during the cellular response to unreplicated DNA or to certain DNA damaging agents. Recent studies have indicated that ATR-interacting protein (ATRIP) is required for the activation of ATR (20). Additionally, ATRIP has been shown to be only associated with ATR and not ATM (20).

Checkpoint kinase 1 (Chk1) is a serine/threonine kinase that is activated by phosphorylation on Ser345 or Ser317 by ATR or ATM. Additionally, Chk2 is a checkpoint kinase that is activated by phosphorylation at Thr68. Whereas Chk2 is known to be phosphorylated by ATM in response to IR, Chk1 is phosphorylated by ATR in response to UV (34, 53) or by ATM in response to IR (46). Activated Chk1 or Chk2 is known to phosphorylate p53 on Ser20 (43). Although Chk1 or Chk2 are known to mediate G_2/M phase cell cycle arrest by

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

phosphorylating and activating Cdc25C phosphatase, these kinases are also known to control G_1 arrest due to activating transcription factor function of p53 by phosphorylation.

Hyperoxia is clinically administered in critical care units and is known to cause DNA damage. Although hyperoxia is a form of oxidative stress, its effect on DNA damage response pathway is relatively under-explored. Hyperoxia has been shown to cause extensive base damage but is relatively less potent in causing strand breaks (9). In addition, hyperoxia does not induce apoptosis in widely studied cell line A549 (30, 49). We show here that hyperoxia induces phosphorylation of p53 on Ser6, Ser15, Ser37, and Ser392 residues that require ATR and ATRIP. In addition, phosphorylation of Ser6 requires ATM. Furthermore, we show that ATR is required for activation of ATM in hyperoxia. Consistent with this finding, we show that activation of ATR precedes that of ATM in response to hyperoxia.

EXPERIMENTAL PROCEDURES

Cell culture and exposure to hyperoxia.

A lung adenocarcinoma cell line derived from human alveolar type II cells (A549) was obtained from American Type Culture Collection (Manassas, VA) and grown in F-12K media supplemented with 10% FBS and 100 units each of penicillin and streptomycin. GM03349 ($ATM^{+/+}$) and GM02052 ($ATM^{-/-}$) cells were obtained from Coriell Institute for Medical Research and cultured in MEM containing 15% FBS and 100 μg each of penicillin and streptomycin as described previously (22). Cells in tissue culture dishes containing 10–12 ml of media were exposed to hyperoxia (95% O_2-5% CO_2) at a flow rate of 10 l/min for 10 min in humidified modular exposure chambers (Billups-Rothenberg, Del Mar, CA), following which the chamber was sealed and incubated in a 37°C incubator. Control cells were maintained in room air containing 5% CO_2 (normoxia, 21% oxygen) in a CO_2 incubator (Steri-Cult, Forma Scientific) for 24 h. Exposure was limited to this duration to prevent glucose depletion in the media (3), which could confound the results. At the end of the incubation, cells were either processed for total cell lysates preparation for Western blotting or for immunoprecipitation. Exposure of cells to hyperoxia was performed in the log phase of cell growth, and cells were seeded at low density to prevent any contact inhibition at the end of the exposure period.

RNA interference.

ATR, ATM, ATRIP, Chk1 or Chk2, and nontargeting small interfering RNAs (siRNAs) were purchased from Dharmacon RNA Technologies (Lafayette, CO). A549 cells were seeded in 100-mm^2 dishes to obtain 30% confluence at the time of transfection. X-tremeGENE siRNA Transfection Reagent (Roche Applied Science) was used to transfect siRNAs to a final concentration of 100 nM. Inhibition of gene expression by siRNA was determined after 48 h by Western blot analysis. The sequences of the siRNAs used in this study were as follows:

ATR: 5′-GCC AAG ACA AAU UCU GUG U dTdT-3′

ATRIP: 5′-GGU CCA CAG AUU AGA U dTdT-3′

Chk1: 5′-GCG UGC CGU AGA CUG UCC A dTdT-3′

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

ATM: 5′-AAU UCA GAA AGC AAC AUU CUU dTdT-3′

Chk2: 5′-GAA CCU GAG GAG CCU ACC C dTdT-3′.

A nontargeting control siRNA (siControl 1, cat. no. D-001210-01-20) was used for nontargeting control transfection.

Immunoprecipitation and Western blotting.

Total cellular lysate was prepared in lysis buffer [50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM PMSF, 30 μl/ml aprotinin (Sigma Chemical, St. Louis, MO), and 1 mM Na_3VO_4]. A549 cell lysate was immunoprecipitated with p53-agarose conjugate antibody (Santa Cruz Biotechnology, Santa Cruz, CA) by incubating at 4°C in a rotating mixer for 2 h. The mixture was then centrifuged at 1,000 *g* for 30 s, the supernatant was carefully aspirated, and the pellet containing the agarose beads was washed three times with lysis buffer. The agarose beads were then suspended in SDS sample buffer without reducing agents to prevent the interference of IgG heavy chain with p53 bands and boiled for 5 min (40).

Aliquots of immunoprecipitate were analyzed by Western blotting. For Chk1 or phospho-Chk1 Western blotting, 30 μg of protein was resolved by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (Bio-Rad, Hercules, CA). After washes, the membrane was incubated with gentle shaking overnight with primary antibody or phosphospecific antibodies (Cell Signaling Technology, Beverly, MA) in TBS containing 0.1% Tween 20 and 5% BSA.

Anti-FLAG polyclonal antibody (Sigma Chemical) was used for detection of FLAG protein. After incubation, the membrane was washed and incubated for 1 h with respective horseradish peroxidase-tagged secondary antibodies. Specific bands were detected by ECL (GE Healthcare Bio-Sciences, Piscataway, NJ 08855–1327), Lumi-GLO reagent (Cell Signaling Technology), or SuperSignal West Femto reagent (Pierce Chemical, Rockford, IL). Western blots were scanned, and image analysis was performed with NIH Image 1.61 software (National Institutes of Health). Statistical analysis was performed by ANOVA and Tukey's test for multiple comparisons on the relative densities using Instat3 statistical software. Means with a $P < 0.05$ were considered significant.

ATM and ATR kinase assay.

A549 cells were grown for 48 h after initial low density seeding and then exposed to normoxia (21% O_2-5% CO_2) or hyperoxia (95% O_2-5% CO_2) for 24 h. Cell lysates were prepared in lysis buffer [50 mM Tris·HCl buffer (pH 7.5) containing 50 mM β-glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 2 mg/ml antipain, and 1 mM DTT]. ATM or ATR protein was immunoprecipitated from total cell lysates using respective antibodies (ATM, Rockland, Rockford, IL; ATR, Genetex, San Antonio, TX) and protein G agarose Plus (Santa Cruz Biotechnology). Immunoprecipitates were washed

Author Manuscript

four times with the lysis buffer, twice with 50 mM Tris·HCl buffer (pH 7.5) containing 0.5 M LiCl, and three times with 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 10 mM $MnCl_2$. Kinase reaction was carried out by incubating immunoprecipitates with purified glutathione S-transferase (GST) fusion protein Chk2 (amino acids 1–96), used as substrate, in the presence of kinase buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM ATP, and 10 mM DTT] for 30 min at 30°C. Proteins were separated by SDS-PAGE. Phosphorylation of Chk2 on Thr68 residues was detected by immunoblotting. Blot was stripped and reprobed with GST antibody to confirm that equal amount of substrate protein was added per kinase reaction. For ATR kinase assay, full-length Chk1 protein (Upstate Biotechnology) was used as substrate, and [γ–^{32}P]ATP was included in the reaction cocktail as a tracer. Radioactive gels were dried in a vacuum drier and were autoradio-graphed after several hours of exposure to film.

RESULTS

Hyperoxia induces phosphorylation of p53 on Ser6, –15, –37, and –392 in an ATR-dependent manner.

We (22) have previously reported phosphorylation of multiple serine residues on p53 in response to hyperoxia. However, the upstream kinases that mediated these phosphorylations were not conclusively established. In addition, conflicting reports were published on the upstream kinases that mediate p53 phosphorylation. For example, phosphorylation of p53 (Ser15) was shown to be dependent on ATM (27) and also on ATR (22). Therefore, we sought to use siRNA approach in A549 cells to evaluate the response of hyperoxia and the role of ATM or ATR on multiple serine phosphorylations on p53. This approach eliminates the use of human embryonic kidney HEK293T cells to overexpress kinase-inactive ATR to delineate the specific role of ATR that has been extensively used in the experiments related to ATR. We downregulated ATR expression by RNA interference and determined the role of ATR in p53 phosphorylations in hyperoxia. Transfection of A549 cells with ATR siRNA downregulated ATR protein (Fig. 1A). Since ATR and ATM have a high degree of homology, we also evaluated the effect of ATR siRNA on ATM protein expression, and, as demonstrated in Fig. 1A, *bottom*, there was no decrease in the ATM protein in response to ATR siRNA suggesting that the effect of ATR siRNA is specific to ATR. ATR-downregulated cells were exposed to 24-h hyperoxia (95% oxygen), and phosphorylations on p53 were detected as described in experimental procedures. As demonstrated in Fig. 1, B, and C, Ser6, –15, –37, and –392 residues were strongly phosphorylated in hyperoxia in cells transfected with nontargeting siRNA and exposed to 95% oxygen. Cells transfected with ATR siRNA did not show phosphorylations on Ser6, –15, –37, and –392 residues, suggesting that ATR is required for phosphorylation of these residues. Our data show that Ser6 is phosphorylated by hyperoxia, and this phosphorylation is dependent on ATR. In addition, hyperoxia did not phosphorylate Ser9 or Thr18 residues on p53 (data not shown).

ATRIP is required for phosphorylations on Ser6, –15, –37, and –392 residues by ATR.

ATRIP is required for ATR functions, and ATRIP and ATR together are essential for many ATR-dependent functions (20). Therefore, if ATR is the kinase that brings about the phosphorylations on p53 in response to hyperoxia, ATRIP should also play a role in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

this process by activating ATR. Therefore, we downregulated ATRIP protein by siRNA and evaluated its effect on p53 phosphorylations. As shown in Fig. 2A, transfection of A549 cells with ATRIP siRNA effectively downregulated ATRIP protein. We exposed A549 cells that were transfected with either nontargeting siRNA or ATRIP siRNA to 24-h hyperoxia or normoxia. As shown in Fig. 2, B and C, cells transfected with nontargeting siRNA and exposed to hyperoxia for 24 h demonstrate significantly increased level of p53 phosphorylations on Ser6, −15, −37, and −392 residues. In contrast, when ATRIP siRNA-transfected cells were exposed to 95% oxygen, we observed significantly lower levels of phosphorylations on Ser6, Ser15, Ser37, and Ser392. Surprisingly, ATRIP downregulation also decreased the level of total p53 in response to hyperoxia (Fig. 2, B and C). There was a 40% increase in p53 expression in hyperoxia, which was decreased to the level of control nontargeting siRNA-transfected cells in ATRIP siRNA. The data show that, in addition to ATR, ATRIP is also required for p53 phosphorylations in hyperoxia, further supporting a role for ATR in phosphorylation of these residues in response to hyperoxia.

ATM is not required for phosphorylation of Ser15, −37, and −392 residues on p53 but is required for Ser6 phosphorylation in response to 24-h hyperoxia.

Using $ATM^{+/+}$ and $ATM^{-/-}$ cells, we (22) had previously demonstrated that phosphorylations on Ser15, −37, and −392 on p53 were dependent on ATR but not ATM. However, another study has shown that p53 (Ser15) phosphorylation is dependent on ATM in hyperoxia (27). Therefore, we used siRNA approach using nontargeting or ATM siRNA to delineate the role of ATM in A549 cells in p53 phosphorylations in response to hyperoxia. As demonstrated in Fig. 3A, transfection of cells with ATM siRNA did not affect the level of ATR protein in A549 cells, whereas transfection of ATM siRNA downregulated ATM protein in A549 cells. These cells were exposed to hyperoxia, and we determined the phosphorylations on Ser6, −15, −37, and −392 residues as described in experimental procedures. Exposure of nontargeting siRNA-transfected cells to hyperoxia phosphorylated Ser6, −15, −37, and −392 residues on p53. Cells transfected with ATM siRNA and exposed to hyperoxia showed a significantly higher level of phosphorylated p53 on Ser15, −37, and −392. However, phosphorylation of Ser6 was significantly reduced in ATM downregulated cells (Fig. 3, B and C). These data suggest that, whereas phosphorylation of Ser6 was dependent on ATM, phosphorylation of Ser15, Ser37, and Ser392 were independent of ATM. In an additional experiment, we used $ATM^{+/+}$ and $ATM^{-/-}$ cells to confirm the role of ATM in response to hyperoxia on Ser6 phosphorylation. As demonstrated in Fig. 4, A and B, $ATM^{-/-}$ cells failed to phosphorylate Ser6 in response to hyperoxia, but $ATM^{+/+}$ cells demonstrated significant phosphorylation of Ser6 in response to hyperoxia. These studies demonstrate that, whereas Ser15, −37, and −392 are phosphorylated in an ATR-dependent manner, Ser6 is phosphorylated involving ATM in hyperoxia.

Phosphorylation of p53 (Ser392) induced by hyperoxia does not involve casein kinase II.

Phosphorylation of Ser392 has been shown to occur involving casein kinase II (CKII; Ref. 31) in response to UV. Therefore, we sought to determine whether hyperoxia-mediated phosphorylation of Ser392 would depend on CKII. We pretreated cells with CKII inhibitor, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), and evaluated Ser392

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

phosphorylation in response to hyperoxia. As shown in Fig. 5, A and B, pretreatment of cells with DMAT did not decrease Ser392 phosphorylation in response to hyperoxia. These data show that in hyperoxia ATR may directly or via an unknown kinase phosphorylate Ser392 on p53.

Time course of phosphorylation of various residues on p53 in response to hyperoxia.

All of our experiments were performed using 24-h time point. Therefore, we sought to determine the time course of phosphorylation of p53 residues to identify the onset of DNA damage response in hyperoxia. As demonstrated in Fig. 6A, phosphorylation of Ser15 occurred as early as 4 h (Fig. 6A). Additionally, phosphorylations of Ser6, −15, −37, and −392 were well pronounced after 8 h of exposure. Phosphorylations on various residues continue to increase at 12 or 16 h. However, most phosphorylations had peaked between 16–24 h (Fig. 6B). These data show that 8 h of hyperoxia is sufficient to evoke a DNA damage response.

Hyperoxia-induced phosphorylation of histone H2AX is dependent on ATR and ATM.

Phosphorylation of histone H2AX on Ser139 is a specific response to genotoxic stress due to double-strand break (DSB; Ref. 1). Since ATM is activated in response to DSB, and Ser6 is phosphorylated in hyperoxia in an ATM- and ATR-dependent manner, we sought to determine the role of hyperoxia in histone H2AX phosphorylation. As shown in Fig. 7A, phosphorylation of H2AX was observed as early as 4 h that remains elevated until 16 h. To further determine the role of ATM or ATR in this phosphorylation, we evaluated H2AX phosphorylation in ATR- or ATM-siRNA transfected cells (Fig. 7B). As shown in Fig. 7B, H2AX phosphorylation was partially downregulated in ATM as well as in ATR siRNA-transfected cells. These data indicate that both ATM and ATR contribute to histone phosphorylation in response to hyperoxia.

Hyperoxia phosphorylates Chk1 in an ATR-dependent pathway without involving ATRIP.

Activated ATR phosphorylates Chk1 on Ser345, its preferred substrate (53). However, recent studies indicate that ATM could also phosphorylate Chk1 in addition to its preferred substrate, Chk2 (28). Therefore, we evaluated whether ATR specifically phosphorylates Chk1 in hyperoxia. Using siRNA approach, we observed that cells transfected with ATR siRNA did not phosphorylate Chk1 in response to hyperoxia (Fig. 8A). Since ATR-ATRIP together is necessary for many phosphorylation events catalyzed by ATR (7), we analyzed the role of ATRIP in Chk1 phosphorylation by ATR. ATRIP downregulation did not inhibit Chk1 phosphorylation by ATR (Fig. 8C). These data suggest that Chk1 phosphorylation by ATR in hyperoxia is independent of ATRIP. Cells transfected with ATM siRNA demonstrated no downregulation in Chk1 phosphorylation, but rather a minor increase was noted, suggesting that ATM does not play a major role in increasing the phosphorylation of Chk1 in hyperoxia.

Hyperoxia induces ATR-ATRIP association.

ATRIP has been shown to associate with ATR during its response to genotoxic stress (7). We observed ATR-ATRIP-dependent phosphorylation of p53 on Ser6, −15, −37, and −392

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

residues but did not observe any effect of ATRIP downregulation on Chk1 phosphorylation. Therefore, we sought to determine whether ATR-ATRIP associate together during exposure to hyperoxia. We transfected FLAG-ATR and hemagglutinin (HA)-ATRIP into HEK293T cells and exposed these cells to hyperoxia. ATRIP was immunoprecipitated using anti-HA antibody. We looked for ATR in the HA immunoprecipitates. As shown in Fig. 9A, we did not detect ATR in cells in normoxia. However, cells that were exposed to hyperoxia showed the presence of ATR in HA-ATRIP immunoprecipitates. These studies show that, under hyperoxia, ATR-ATRIP associated, resulting in the activation of ATR.

ATR is activated at an earlier time point and ATM is activated at a later time point in response to hyperoxia.

We show that p53 (Ser6) is phosphorylated in an ATR- as well as an ATM-dependent manner in response to hyperoxia. However, whereas downregulation of ATR completely inhibited p53 (Ser6) phosphorylation in hyperoxia, ATM downregulation resulted in ~70–80% inhibition of phosphorylation. Since the ATM or ATR siRNAs used in these studies are very specific, we were able to show that downregulation of ATR is sufficient to inhibit p53 (Ser6) phosphorylation in hyperoxia even in the presence of ATM. However, downregulation of ATM does not completely inhibit p53 (Ser6) phosphorylation in the presence of ATR. Additionally, hyperoxia-mediated histone H2AX phosphorylation on Ser139 was dependent on both ATM and ATR (Fig. 7B). These results prompted us to examine the time course of activation of ATR and ATM in response to hyperoxia. ATR or ATM was immunoprecipitated from A549 cells exposed to hyperoxia for 8, 16, or 24 h, and ATR or ATM kinase assay was performed as described in experimental procedures. As demonstrated in Fig. 9B, ATR was activated within 8 h; in contrast, ATM was not activated at this time point in response to hyperoxia. However, by 16 h, ATM was activated, but ATR activation was terminated at this time point. These results show that ATR activation occurs first followed by ATM in response to hyperoxia.

ATR is required for ATM kinase activity in response to hyperoxia.

Because ATR activation occurs first, we sought to determine whether ATR activates ATM in response to hyperoxia. Cells were transfected with ATR siRNA followed by exposure to hyperoxia for 16 h. As demonstrated in Fig. 9C, ATM activation was abolished in ATR siRNA-transfected cells. These data demonstrate that endogenous ATM is indeed activated in an ATR-dependent manner in response to hyperoxia.

DISCUSSION

Hyperoxia is extensively used in critical care units and in conditions of respiratory insufficiencies in adults and children. The concentration of oxygen administered in these circumstances ranges from as low as 40% to as high as 100%, lung being the major organ to receive this oxidative insult. It is well documented that hyperoxia damages the DNA and other components of the cell (13). However, the signaling cascade induced by DNA damage in hyperoxia is less understood than IR- or UV-dependent DNA damage response. Because of its clinical relevance and physiological nature, a clear understanding of hyperoxia-mediated DNA damage response is necessary to evaluate postexposure complications that

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

often arise in clinical situations. However, as we have shown, hyperoxia evokes a distinct DNA damage signaling that is different than IR, UV, or other genotoxic stresses.

In this study, we have extensively used siRNA approach to downregulate ATM, ATR, or ATRIP in the same cell line (A549), thereby eliminating cell type variations and compensatory mechanisms that might have existed in $ATM^{-/-}$ or $ATM^{+/+}$ cells that are extensively used in understanding the role of ATM. Here, we provided evidence that hyperoxia, although an oxidant, activates ATR-ATRIP signaling to phosphorylate specific serine residues on p53. In addition, we also show that ATR is required for the activation of ATM in hyperoxia. Our study shows for the first time that hyperoxia is a unique stress because its DNA damage response signaling differs from that of IR or UV. For example, IR does not phosphorylate p53 on Ser392 but phosphorylates p53 on Ser15. In contrast, hyperoxia phosphorylates both of these residues. IR activates ATM-dependent phosphorylation of histone (Ser139) and Chk1 phosphorylation in an ATM-dependent pathway. In contrast, hyperoxia phosphorylates both of these residues involving ATR. Whereas hyperoxia activates ATR-dependent ATM activation, IR activates ATM-dependent ATR activation (21, 28, 36, 52).

P53 is continuously synthesized and degraded by Mdm2-p53 feedback loop. When cells are exposed to genotoxic agents, ATR/ATM is activated and phosphorylates p53 on several different residues that depend on the type of genotoxic agent. This phosphorylation stabilizes p53, resulting in its activation as a transcription factor (38). Although posttranslational modifications at most sites on p53 occur in response to stress, clear difference in responses at individual sites to different agents have been observed. In this regard, the pattern of hyperoxia p53 activation of phosphorylation differs from that of IR or UV. For example, in response to IR, increased phosphorylation of Ser6 or Ser15 was observed as early as 30 min after treatment (44), whereas hyperoxia takes 4–8 h to induce phosphorylation of these residues. Phosphorylation of Ser392 is the first p53 phosphorylation site to be identified, and this phosphorylation specifically occurs in response to UV (5) but not IR. We have shown here that hyperoxia induces strong phosphorylation of Ser392. Phosphorylation of this residue has been shown to increase p53-dependent transcription (5, 6). ATR depletion abrogated Ser392 phosphorylation in hyperoxia, suggesting a crucial role of ATR in Ser392 phosphorylation. CKII has been shown to phosphorylate Ser392 in response to UV (5, 6). However, we did not observe a role for CKII in phosphorylation of Ser392 in response to hyperoxia (Fig. 5A). Thus it appears that, in hyperoxia, Ser392 is phosphorylated directly by ATR or by another ATR-dependent kinase. NH_2-terminal phosphorylations on Ser15, Ser20, and Ser37 are known to stabilize p53 and to increases its ability to bind to DNA in a sequence-specific manner. Phosphorylation of p53 at NH_2-terminal serines also may enhance interactions with the transcriptional coactivators p300/CBP and PACF. Hyperoxia induced the phosphorylation of Ser15, Ser37, and Ser392. All of these phosphorylations were dependent on ATR and ATRIP. However, phosphorylation of p53 on Ser6 was dependent on ATR as well as ATM. The exact functional role of Ser6 phosphorylation is unknown in hyperoxia.

The activation of the DNA damage response pathway reflects the magnitude and extent of DNA damage due to a specific genotoxic agent. Since ATM is a well-known kinase that

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

is activated in response to DSB, and ATR is known to be activated due to single-strand DNA breaks and in response to nucleotide depletion or stalling of replication fork (1), the phosphorylation program in response to a stress that either activates ATM or ATR could differ. Therefore, activation of ATR in hyperoxia in the first 8 h suggests that no DSB or undetectable level of DSB may occur in hyperoxia. Indeed, supporting this notion, a recent report has shown that damaged bases are the predominant form of DNA damage in response to hyperoxia (9). However, prolonged hyperoxia (>48 h) results in strand breaks. Additionally, base damage in hyperoxia did not correlate with lung injury, but strand breaks did correlate with lung injury in hyperoxia (9). These studies suggest that base damage in hyperoxia could be efficiently repaired during growth arrest in hyperoxia, because hyperoxia does not induce apoptosis in A549 cells (30, 39). Therefore, the activation of ATR and phosphorylation of p53 may contribute to the arrest of cell cycle during hyperoxia. This certainly would have several advantages for the cells, fixing damaged bases by base excision repair mechanism in the growth arrest period during hyperoxia. To this effect, a recent study has shown that overexpression of Ogg1 and APE/Ref-1 could protect hyperoxia-mediated injury to lung cells (51).

Our data indicate that ATR activation precedes ATM activation in hyperoxia. Additionally, ATR is required for ATM kinase activity in hyperoxia. These data are in contrast to several recent reports suggesting ATM-dependent ATR activation and mobilization to DNA damage sites in response to IR. Thus it appears that the type of stress and initial form of DNA damage is probably crucial in determination of whether ATM activates ATR or ATR activates ATM. In hyperoxic exposure, initial activation could be important for DNA repair. During the process of DNA repair, DSB are known to be formed. Therefore, if ATR is indeed required for DNA repair, a process that could generate DSB resulting in the activation of ATM, then inhibition of DNA repair should inhibit ATM activation.

Phosphorylation of histone H2AX by ATM has been shown to be a specific response to DSB; however, a recent study has demonstrated ATR-dependent phosphorylation of histone-H2AX using HU or UV (50). We did observe enhanced phosphorylation of histone H2AX as early as at 4 h that peaked at 16 h. Because we did not find ATM kinase activation in 8 h of hyperoxia, we attribute the early phosphorylation of histone to activation of ATR and delayed histone phosphorylation to ATM. Indeed, our data (Fig. 7B) show that in hyperoxia both ATR and ATM phosphorylate histone. However, since ATR is required for ATM activation in hyperoxia, ATR could be regarded as the principal DNA damage signal transducer in hyperoxia.

Phosphorylation of p53, histone H2AX, and Chk1 suggest that DNA is damaged in response to hyperoxia in A549 cells. However, A549 cells do not undergo apoptotic cell death in hyperoxia (30), but rather necrotic cell death is preferred in prolonged hyperoxia. Using flow cytometry, we did not find significant difference in sub-G_0 peak that suggests no significant apoptosis in hyperoxia in A549 cells (data not shown). This suggests that these cells could either be more resistant to hyperoxia-mediated cell death or more efficiently repair the damaged DNA so that the cells survive and enter cell cycle. The phosphorylation of Ser6, −15, −37, or −392 in hyperoxia suggest a major role of these residues in p53-dependent

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

gene expression or repression program that would favor DNA repair rather than apoptosis, as A549 cells do not undergo apoptosis in hyperoxia.

ACKNOWLEDGMENTS

We acknowledge the generous contribution of ATR plasmid by Dr. Karlene Cimprich, Stanford University, and ATRIP plasmid by Dr. David Cortez, Vanderbilt University.

GRANTS

The study was supported by National Heart, Lung, and Blood Institute Grant HL-55807 to K. C. Das.

REFERENCES

1. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 15: 2177–2196, 2001. [PubMed: 11544175]
2. Albertine KH, Plopper CG. DNA oxidation or apoptosis: will the real culprit of DNA damage in hyperoxic lung injury please stand up? Am J Respir Cell Mol Biol 26: 381–383, 2002. [PubMed: 11919071]
3. Allen CB, White CW. Glucose modulates cell death due to normobaric hyperoxia by maintaining cellular ATP. Am J Physiol Lung Cell Mol Physiol 274: L159–L164, 1998.
5. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 268: 2764–2772, 2001. [PubMed: 11358490]
6. Appella E, Anderson CW. Signaling to p53: breaking the posttranslational modification code. Pathol Biol (Paris) 48: 227–245, 2000. [PubMed: 10858956]
7. Ball HL, Myers JS, Cortez D. ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation. Mol Biol Cell 16: 2372–2381, 2005. [PubMed: 15743907]
8. Barazzone C, Horowitz S, Donati YR, Rodriguez I, Piguet PF. Oxygen toxicity in mouse lung: pathways to cell death. Am J Respir Cell Mol Biol 19: 573–581, 1998. [PubMed: 9761753]
9. Barker GF, Manzo ND, Cotich KL, Shone RK, Waxman AB. DNA damage induced by hyperoxia: quantitation and correlation with lung injury. Am J Respir Cell Mol Biol 35: 277–288, 2006. [PubMed: 16574945]
10. Barnes PJ. Reactive oxygen species and airway inflammation. Free Radic Biol Med 9: 235–243, 1990. [PubMed: 2272532]
11. Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 14: 397–402, 2000. [PubMed: 10691732]
12. Buccellato LJ, Tso M, Akinci OI, Chandel NS, Budinger GR. Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. J Biol Chem 279: 6753–6760, 2004. [PubMed: 14625274]
13. Cacciuttolo MA, Trinh L, Lumpkin JA, Rao G. Hyperoxia induces DNA damage in mammalian cells. Free Radic Biol Med 14: 267–276, 1993. [PubMed: 8458584]
14. Canman CE, Lim DS. The role of ATM in DNA damage responses and cancer. Oncogene 17: 3301–3308, 1998. [PubMed: 9916992]
15. Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281: 1677–1679, 1998. [PubMed: 9733515]
16. Chao C, Saito S, Anderson CW, Appella E, Xu Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. Proc Natl Acad Sci USA 97: 11936–11941, 2000. [PubMed: 11035798]
17. Chao C, Saito S, Kang J, Anderson CW, Appella E, Xu Y. p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. EMBO J 19: 4967–4975, 2000. [PubMed: 10990460]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

18. Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, Friend SH. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 17: 159–169, 1998. [PubMed: 9427750]
19. Coalson JJ, Kuehl TJ, Escobedo MB, Hilliard JL, Smith F, Meredith K, Null DM Jr, Walsh W, Johnson D, Robotham JL. A baboon model of bronchopulmonary dysplasia. II. Pathologic features. Exp Mol Pathol 37: 335–350, 1982. [PubMed: 6924896]
20. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: partners in checkpoint signaling. Science 294: 1713–1716, 2001. [PubMed: 11721054]
21. Cuadrado M, Martinez-Pastor B, Murga M, Toledo LI, Gutierrez-Martinez P, Lopez E, Fernandez-Capetillo O. ATM regulates ATR chromatin loading in response to DNA double-strand breaks. J Exp Med 203: 297–303, 2006. [PubMed: 16461339]
22. Das KC, Dashnamoorthy R. Hyperoxia activates the ATR-Chk1 pathway and phosphorylates p53 at multiple sites. Am J Physiol Lung Cell Mol Physiol 286: L87–L97, 2004. [PubMed: 12959929]
23. Dennery PA, Spitz DR, Yang G, Tatarov A, Lee CS, Shegog ML, Poss KD. Oxygen toxicity and iron accumulation in the lungs of mice lacking heme oxygenase-2. J Clin Invest 101: 1001–1011, 1998. [PubMed: 9486970]
24. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 32: 1102–1115, 2002. [PubMed: 12031895]
25. Freeman BA, Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J Biol Chem 256: 10986–10992, 1981. [PubMed: 7287745]
26. Hagar H, Ueda N, Shah SV. Role of reactive oxygen metabolites in DNA damage and cell death in chemical hypoxic injury to LLC-PK1 cells. Am J Physiol Renal Fluid Electrolyte Physiol 271: F209–F215, 1996.
27. Helt CE, Cliby WA, Keng PC, Bambara RA, O'Reilly MA. Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. J Biol Chem 280: 1186–1192, 2005. [PubMed: 15533933]
28. Jazayeri A, Falck J, Lukas C, Bartek J, Smith GC, Lukas J, Jackson SP. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol 8: 37–45, 2006. [PubMed: 16327781]
30. Kazzaz JA, Xu J, Palaia TA, Mantell L, Fein AM, Horowitz S. Cellular oxygen toxicity. Oxidant injury without apoptosis. J Biol Chem 271: 15182–15186, 1996. [PubMed: 8662947]
31. Keller DM, Zeng X, Wang Y, Zhang QH, Kapoor M, Shu H, Goodman R, Lozano G, Zhao Y, Lu H. A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1. Mol Cell 7: 283–292, 2001. [PubMed: 11239457]
32. Lavin MF, Kozlov S. ATM activation and DNA damage response. Cell Cycle 6: 931–942, 2007. [PubMed: 17457059]
33. Li S, Ting NS, Zheng L, Chen PL, Ziv Y, Shiloh Y, Lee EY, Lee WH. Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. Nature 406: 210–215, 2000. [PubMed: 10910365]
34. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev 14: 1448–1459, 2000. [PubMed: 10859164]
35. Maya R, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, Moas M, Buschmann T, Ronai Z, Shiloh Y, Kastan MB, Katzir E, Oren M. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev 15: 1067–1077, 2001. [PubMed: 11331603]
36. Myers JS, Cortez D. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. J Biol Chem 281: 9346–9350, 2006. [PubMed: 16431910]
37. O'Neill T, Dwyer AJ, Ziv Y, Chan DW, Lees-Miller SP, Abraham RH, Lai JH, Hill D, Shiloh Y, Cantley LC, Rathbun GA. Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. J Biol Chem 275: 22719–22727, 2000. [PubMed: 10801797]
38. Poyurovsky MV, Prives C. Unleashing the power of p53: lessons from mice and men. Genes Dev 20: 125–131, 2006. [PubMed: 16418478]

Author Manuscript

39. Roper JM, Mazzatti DJ, Watkins RH, Maniscalco WM, Keng PC, O'Reilly MA. In vivo exposure to hyperoxia induces DNA damage in a population of alveolar type II epithelial cells. Am J Physiol Lung Cell Mol Physiol 286: L1045–L1054, 2004. [PubMed: 14729512]
40. Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E, Anderson CW. ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. J Biol Chem 277: 12491–12494, 2002. [PubMed: 11875057]
41. Saito S, Yamaguchi H, Higashimoto Y, Chao C, Xu Y, Fornace AJ Jr, Appella E, Anderson CW. Phosphorylation site interdependence of human p53 post-translational modifications in response to stress. J Biol Chem 278: 37536–37544, 2003. [PubMed: 12860987]
42. Shackelford RE, Innes CL, Sieber SO, Heinloth AN, Leadon SA, Paules RS. The *Ataxia telangiectasia* gene product is required for oxidative stress-induced G1 and G2 checkpoint function in human fibroblasts. J Biol Chem 276: 21951–21959, 2001. [PubMed: 11290740]
43. Shieh SY, Ahn J, Tamai K, Taya Y, Prives C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev 14: 289–300, 2000. [PubMed: 10673501]
44. Shieh SY, Taya Y, Prives C. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. EMBO J 18: 1815–1823, 1999. [PubMed: 10202145]
45. Shiloh Y The ATM-mediated DNA-damage response: taking shape. Trends Biochem Sci 31: 402–410, 2006. [PubMed: 16774833]
46. Takai H, Naka K, Okada Y, Watanabe M, Harada N, Saito S, Anderson CW, Appella E, Nakanishi M, Suzuki H, Nagashima K, Sawa H, Ikeda K, Motoyama N. Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. EMBO J 21: 5195–5205, 2002. [PubMed: 12356735]
47. Taylor AM, Byrd PJ. Molecular pathology of ataxia telangiectasia. J Clin Pathol 58: 1009–1015, 2005. [PubMed: 16189143]
48. Vogelstein B, Kinzler KW. p53 function and dysfunction. Cell 70: 523–526, 1992. [PubMed: 1505019]
49. Wang X, Ryter SW, Dai C, Tang ZL, Watkins SC, Yin XM, Song R, Choi AM. Necrotic cell death in response to oxidant stress involves the activation of the apoptogenic caspase-8/bid pathway. J Biol Chem 278: 29184–29191, 2003. [PubMed: 12754217]
50. Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem 276: 47759–47762, 2001. [PubMed: 11673449]
51. Wu M, He YH, Kobune M, Xu Y, Kelley MR, Martin WJ 2nd. Protection of human lung cells against hyperoxia using the DNA base excision repair genes hOgg1 and Fpg. Am J Respir Crit Care Med 166: 192–199, 2002. [PubMed: 12119232]
52. Yoo HY, Kumagai A, Shevchenko A, Shevchenko A, Dunphy WG. Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM. J Biol Chem 282: 17501–17506, 2007. [PubMed: 17446169]
53. Zhao H, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol 21: 4129–4139, 2001. [PubMed: 11390642]
54. Zweier JL, Duke SS, Kuppusamy P, Sylvester JT, Gabrielson EW. Electron paramagnetic resonance evidence that cellular oxygen toxicity is caused by the generation of superoxide and hydroxyl free radicals. FEBS Lett 252: 12–16, 1989. [PubMed: 2547649]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

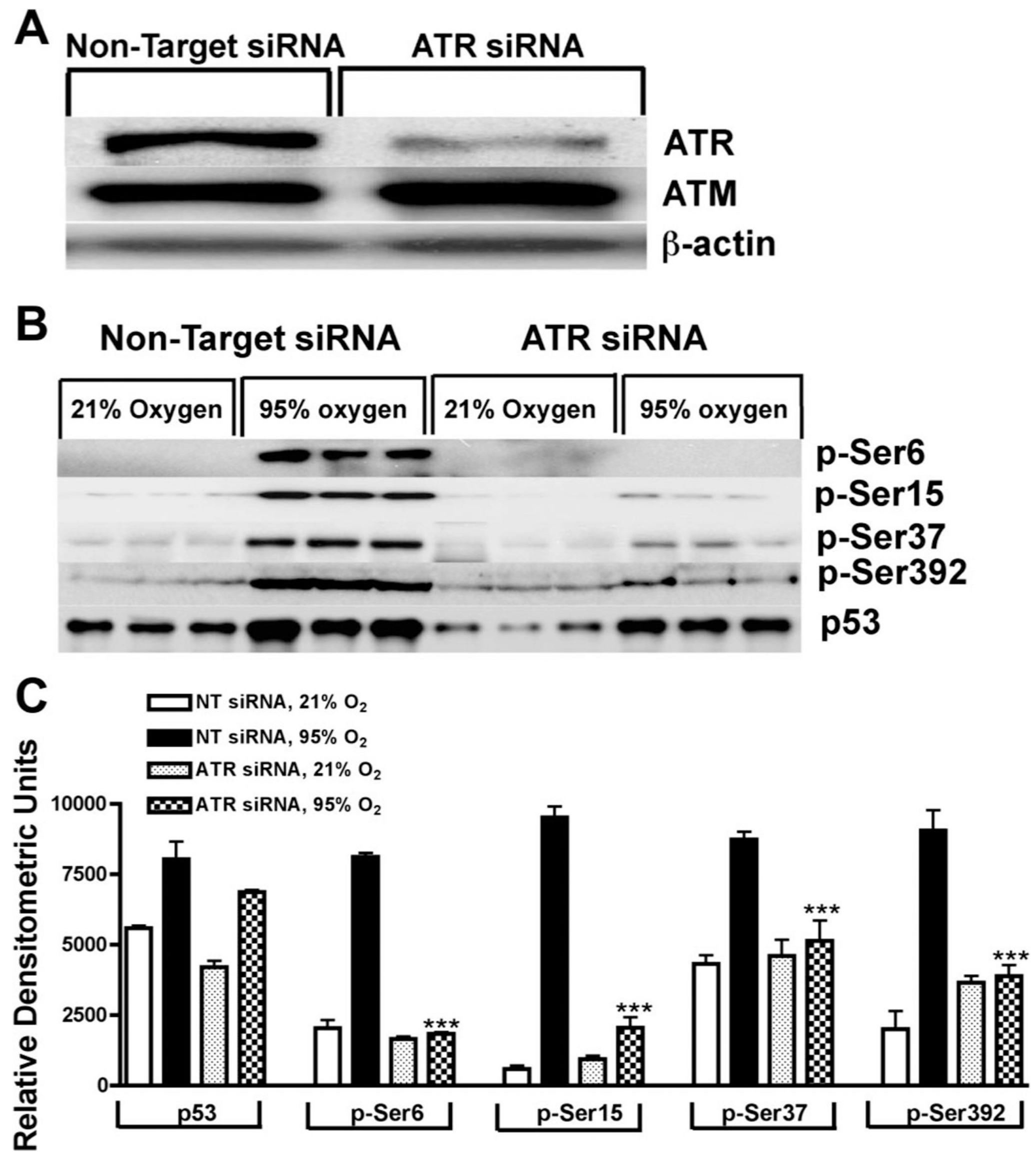

Fig. 1.

Hyperoxia-mediated p53 phosphorylation is dependent on the ataxia telangiectasia mutant (ATM)- and Rad3-related protein kinase (ATR) in A549 cells. *A*: A549 cells were transfected with either nontargeting small interfering RNA (siRNA) (NT) or with ATR siRNA as described in experimental procedures. After 48 h, cells were harvested using lysis buffer, and equal amounts of protein were resolved by 10% SDS-PAGE. ATR, ATM, and β-actin expression were analyzed using Western blot analysis. *B*: A549 cells were seeded and RNA interference of ATR was performed as described for *A*. After 48 h, cells were exposed to either 21% oxygen or 95% oxygen for 24 h. Cells were harvested, total p53 was immunoprecipitated, and samples were resolved by 10% SDS-PAGE and blotted to polyvinylidene difluoride (PVDF) membranes for detection of phosphorylation (p-) of p53 on Ser6, –15, –37, and –392 by Western analysis. *C*: densitometry of Western analysis

presented in *B*. ***Significantly lower than that of NT-transfected cells exposed to 95% oxygen.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

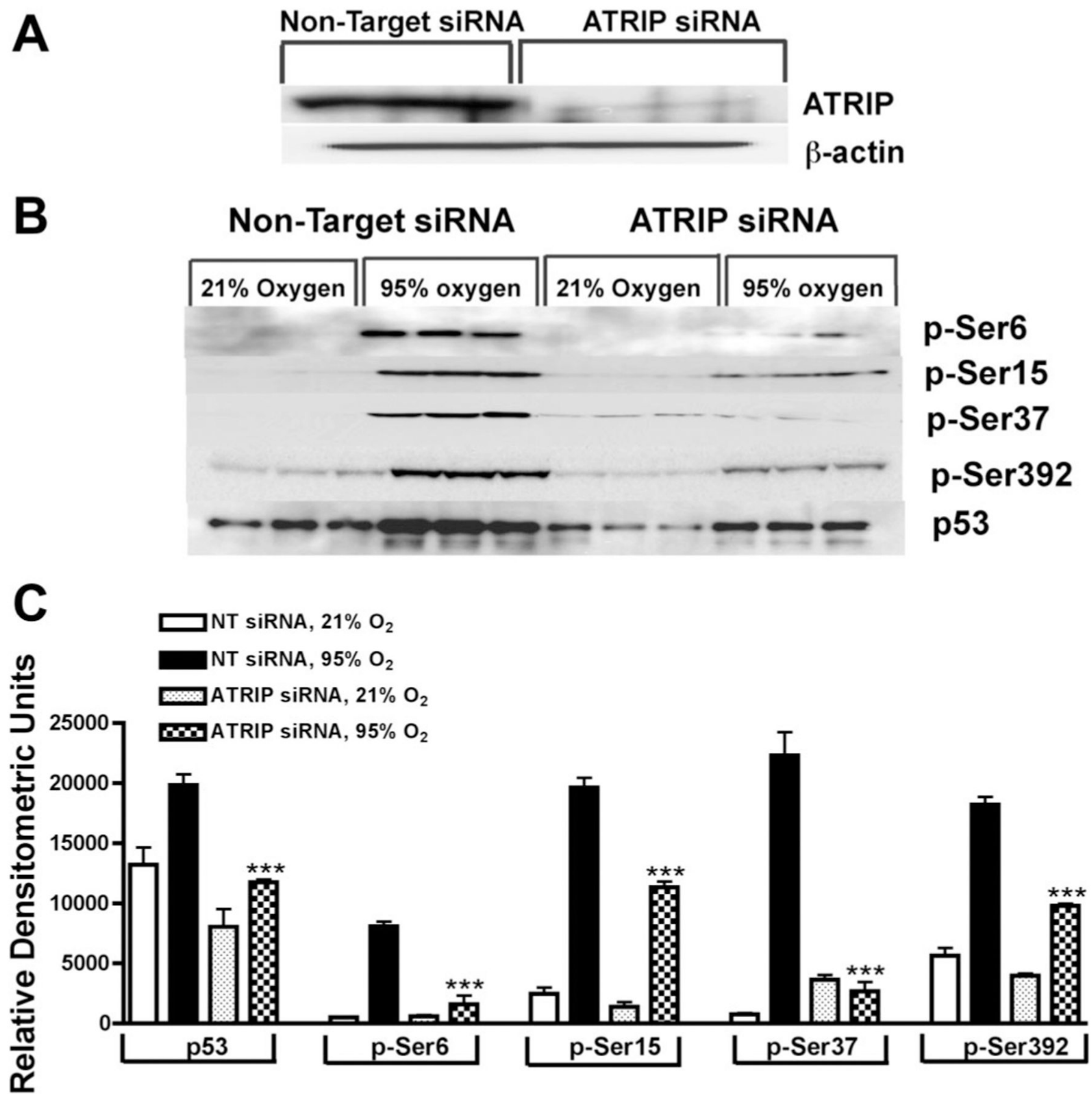

Fig. 2.

ATR-interacting protein (ATRIP) downregulation by RNA interference decreases hyperoxia-mediated phosphorylation of p53 on Ser6, –15, –37, and –392. *A*: A549 cells were transfected with either NT or ATRIP siRNA as described in experimental procedures. After 48 h, cells were harvested using lysis buffer, and equal amounts of protein were resolved by 10% SDS-PAGE. ATRIP and β-actin expression were analyzed using Western blot analysis. *B*: A549 cells were seeded and RNA interference of ATR was performed as described for Fig. 1A. After 48 h, cells were exposed to either 21% oxygen or 95% oxygen for 24 h. Cells were harvested, total p53 was immunoprecipitated, and samples were resolved by 10% SDS-PAGE and blotted to PVDF membranes for detection of phosphorylation of p53 on Ser6, –15, –37, and –392 by Western analysis. *C*: densitometry of Western analysis presented in *B*.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

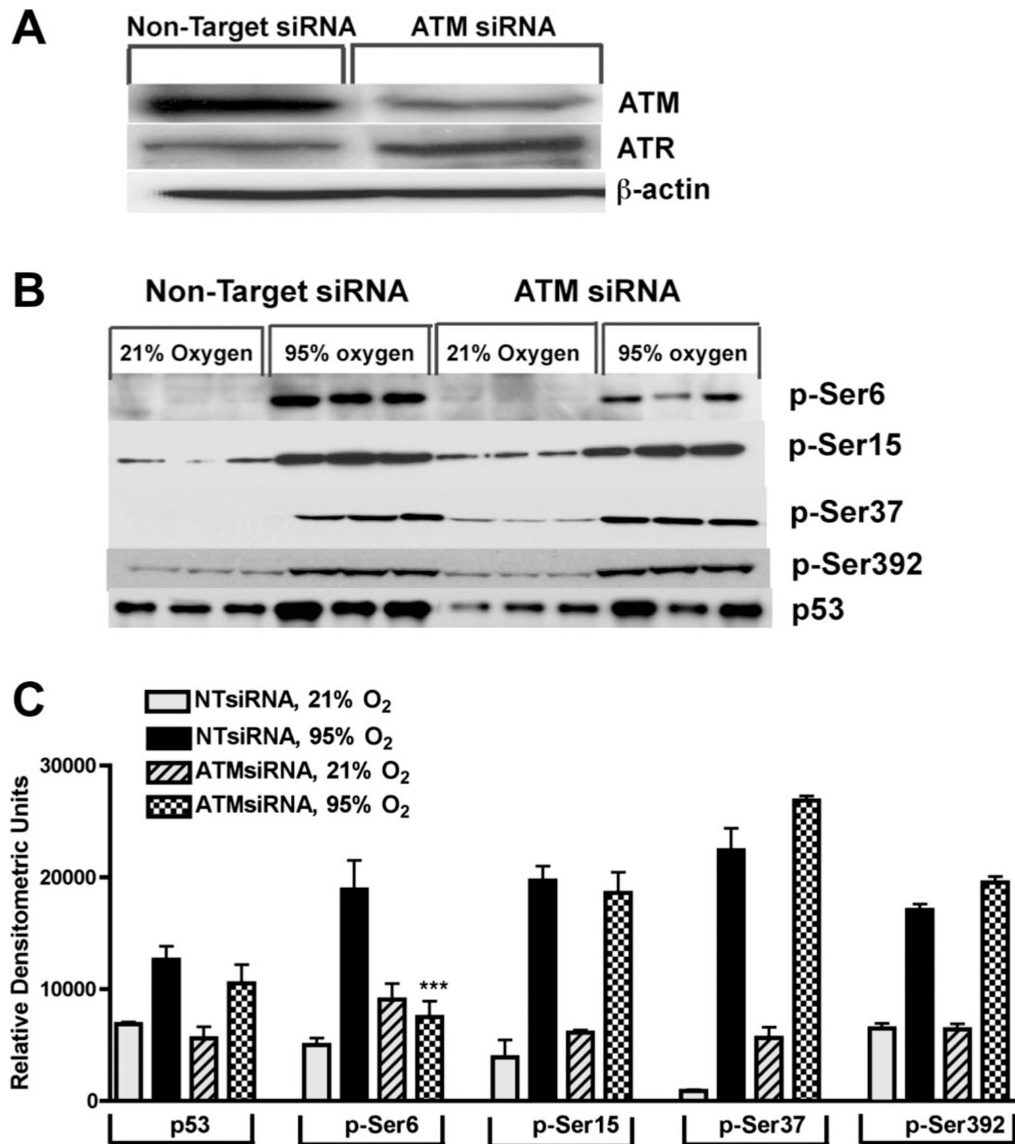

Fig. 3.

Hyperoxia-mediated p53 Ser6 phosphorylation is dependent on ATM, but phosphorylations of p53 on Ser15, −37, and −392 are independent of ATM in A549 cells. *A*: A549 cells were transfected with either NT or ATM siRNA. After 48 h, cells were harvested using lysis buffer, and equal amounts of protein were resolved by 10% SDS-PAGE. ATM, ATR, and β-actin expression were analyzed using Western blot analysis. *B*: A549 cells were seeded and RNA interference of ATM was performed as described for Fig. 1A. After 48 h, cells were exposed to either 21% oxygen or 95% oxygen for 24 h. Cells were harvested in lysis buffer, total p53 was immunoprecipitated, and the samples were resolved by 10% SDS-PAGE and blotted to PVDF membrane for detection of phosphorylation of p53 on Ser6, −15, −37, and −392 as described in experimental procedures. *C*: densitometry of *B*.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

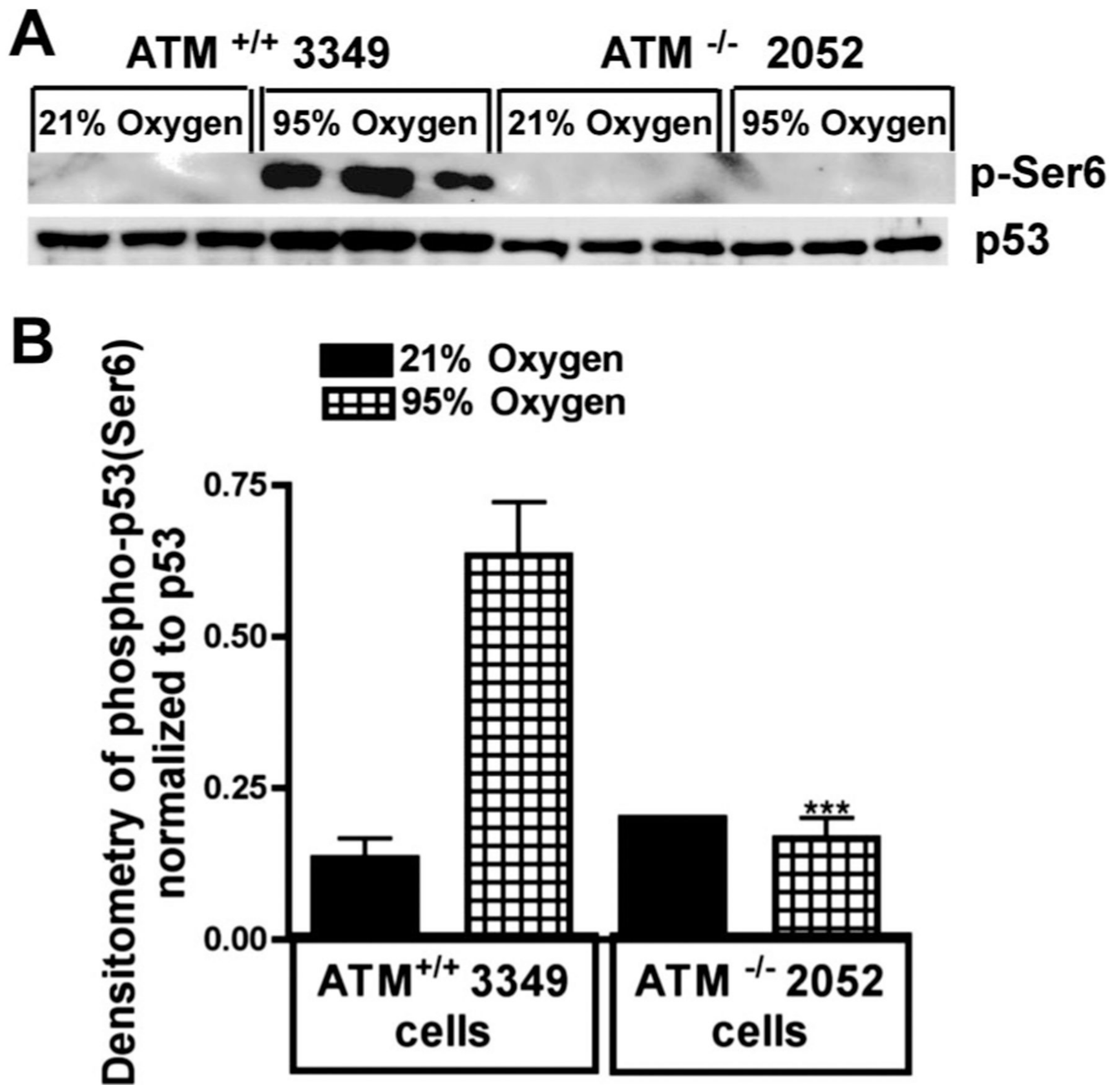

Fig. 4.

Hyperoxia phosphorylates p53 (Ser6) in ATM$^{+/+}$ cells but not in ATM$^{-/-}$ cells. *A*: ATM$^{+/+}$ (GM03349) and ATM$^{-/-}$ (GM02052) fibroblasts were exposed to normoxia or hyperoxia and p53 immunoprecipitates were subjected to Western analysis as described in experimental procedures. *Lanes 1–3*, ATM$^{+/+}$ cells exposed to normoxia; *lanes 4–6*, ATM$^{+/+}$ cells exposed to hyperoxia; *lanes 7–9*, ATM$^{-/-}$ cells exposed to normoxia; *lanes 10–12* ATM$^{-/-}$ cells exposed to hyperoxia. *B*: ratio of relative density of p53 (Ser6) to that of total p53.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

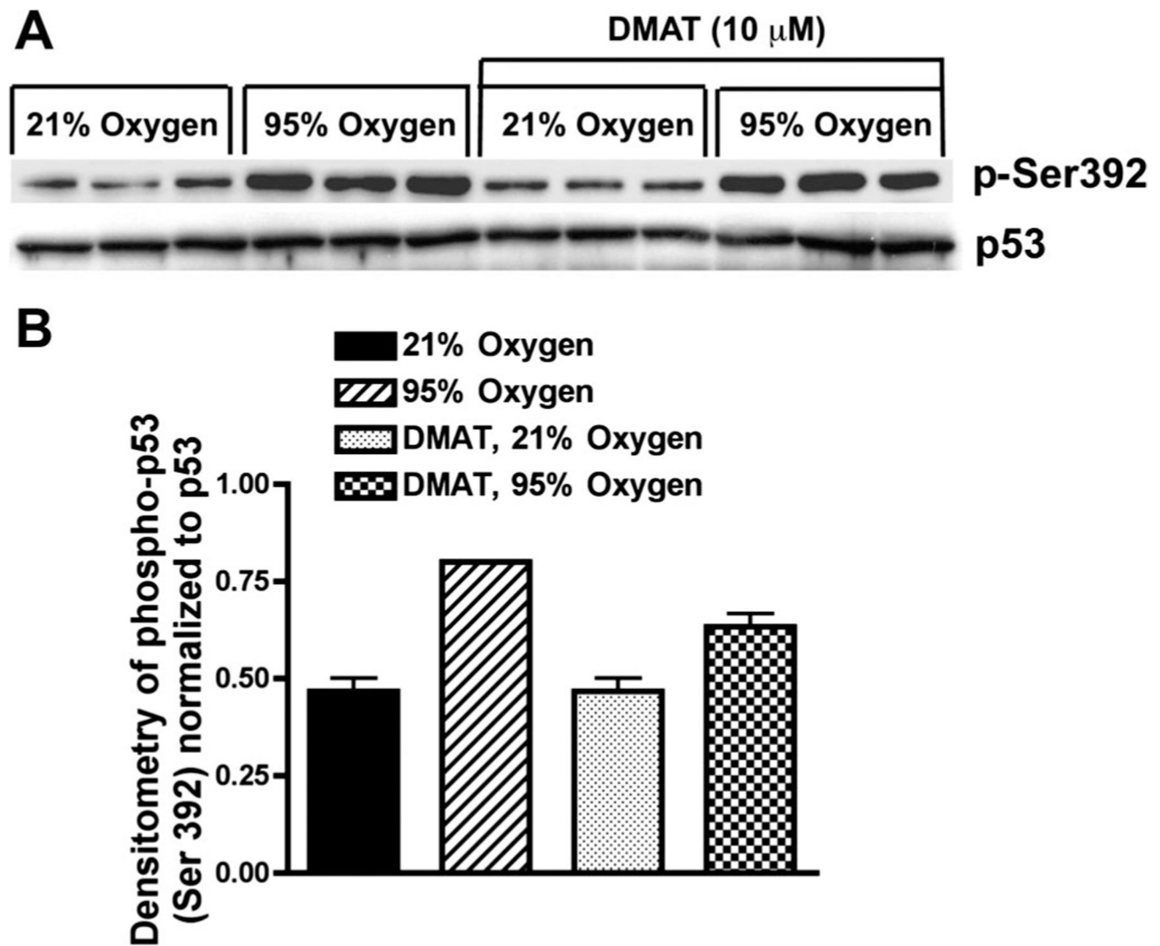

Fig. 5.

Casein kinase inhibitor does not inhibit phosphorylation of p53 (Ser392) in hyperoxia. *A*: A549 cells either untreated or treated with 10 μM 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) for 2 h. Following incubation, cells were either exposed to room air or hyperoxia for 24 h. Total p53 protein was immunoprecipitated and Western analysis of phospho-p53 (Ser392) was performed as described in experimental procedures. *B*: densitometry of p53 (Ser392) band normalized to total p53.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

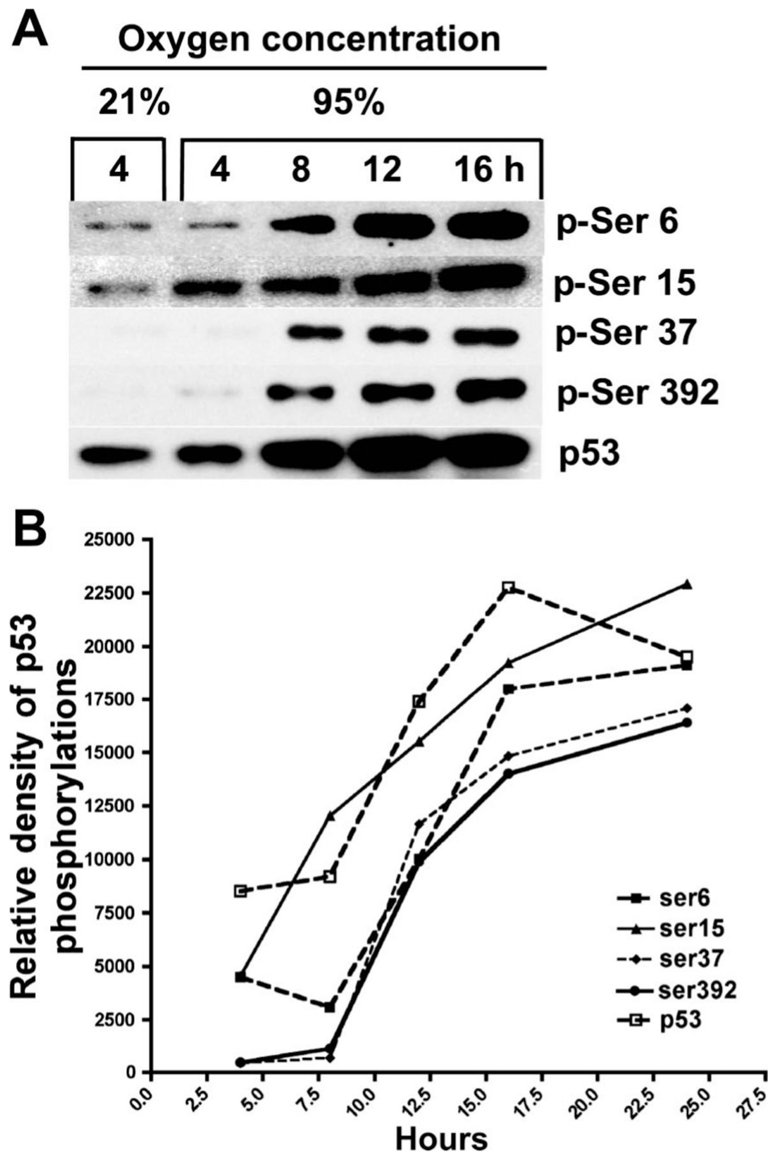

Fig. 6.

A: time course of phosphorylation of p53 Ser6, −15, 37, and −392 in hyperoxia. A549 cells were exposed to normoxia (21% oxygen) or 95% oxygen for 4, 8, 12, or 16 h, followed by immunoprecipitation and detection of phospho-p53 as described in experimental procedures. *B*: relative densitometry of p53 phosphorylations presented in *A*.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

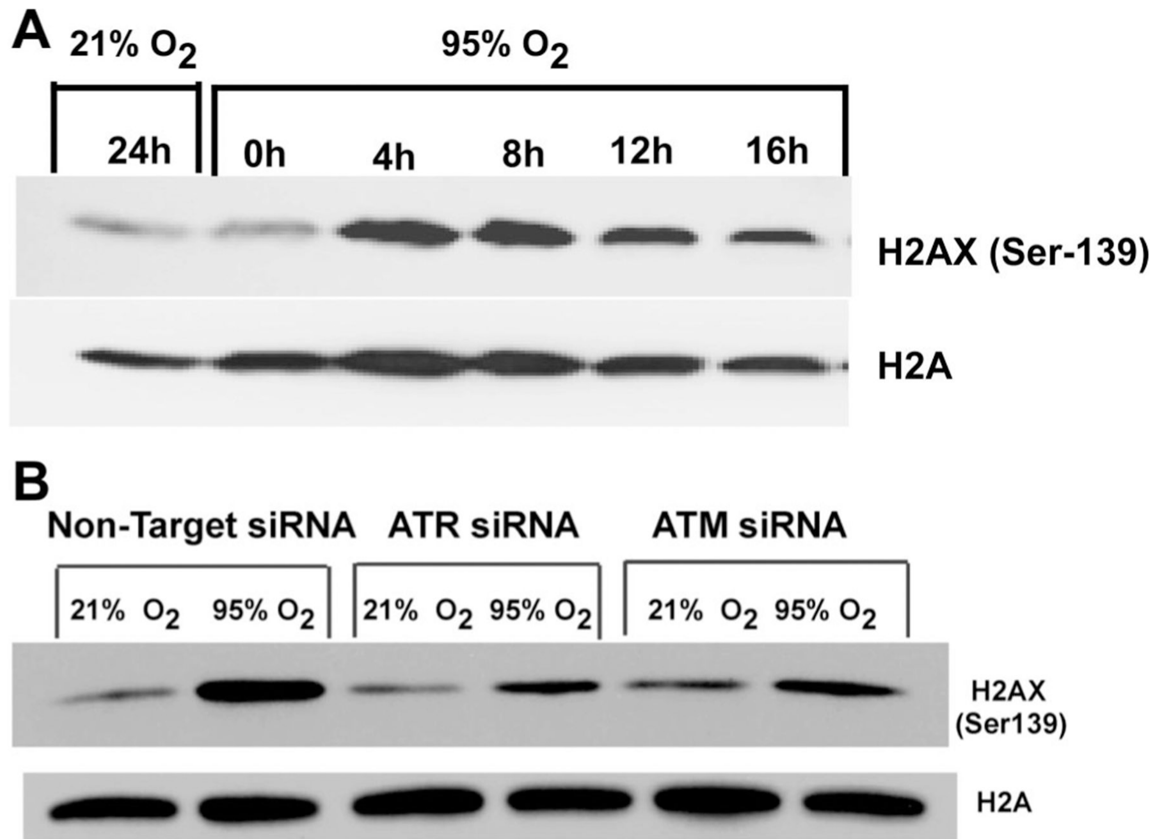

Fig. 7.

Phosphorylation of histone H2AX on Ser139 in hyperoxia is dependent on both ATR and ATM. *A*: A549 cells were exposed to normoxia (21% oxygen) or hyperoxia for 0, 4, 8, 12, or 16 h. Cell lysates were prepared and histone H2AX (Ser139) was detected by Western analysis as described in experimental procedures *B*: A549 cells were either treated with NT, ATM, or ATR siRNA as described in experimental procedures. After 48 h, transfected cells were exposed either to normoxia or hyperoxia for 24 h. Phosphorylation of histone H2AX on Ser139 was detected by Western analysis.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

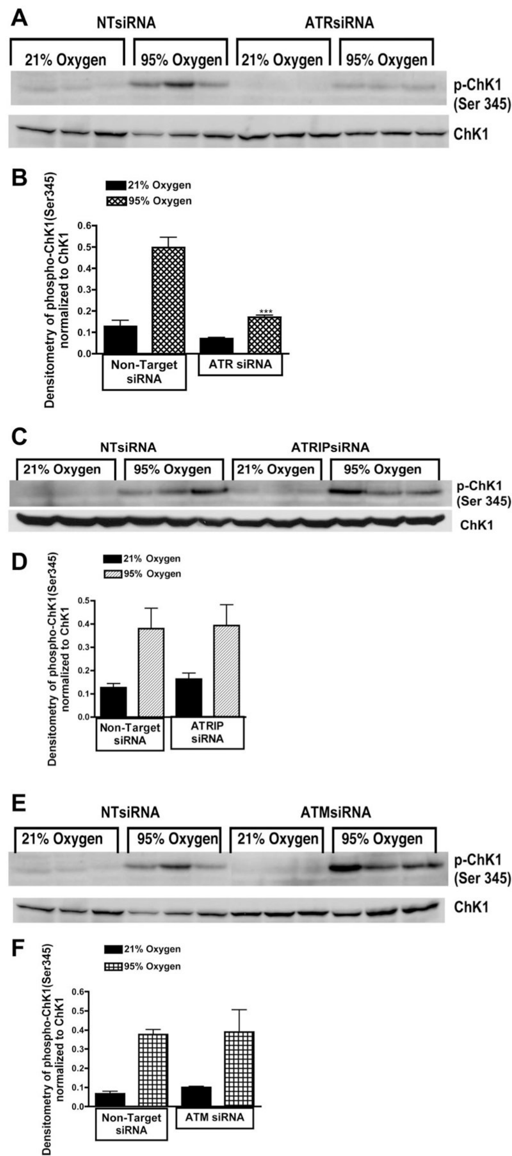

Fig. 8.

Checkpoint kinase 1 (Chk1; Ser345) is phosphorylated in hyperoxia in an ATR-dependent manner but independent of ATRIP. A549 cells were either treated with NT, ATM, or ATR siRNA as described in experimental procedures. After 48 h, transfected cells were exposed either to normoxia or hyperoxia for 24 h. Phosphorylation of Chk1 on Ser345 was detected by Western analysis. *A*: effect of ATR siRNA. *B*: densitometry of *A*. *C*: effect of ATRIP siRNA. *D*: densitometry of *C*. *E*: effect of ATM siRNA. *F*: densitometry of *E*.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

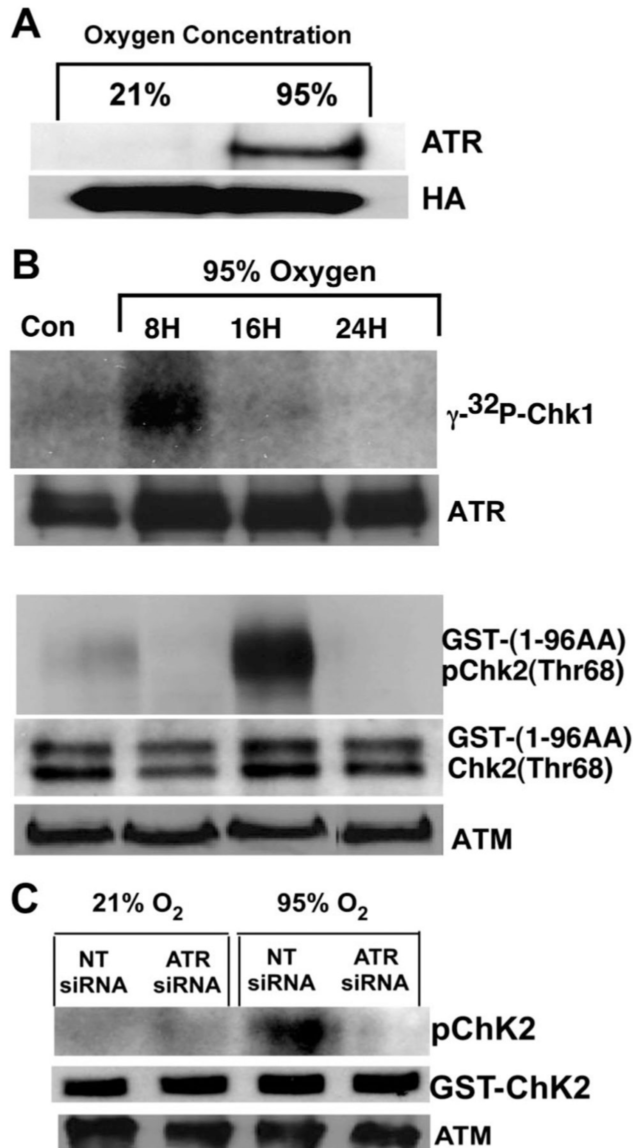

Fig. 9.

A: ATRIP associates with ATR in response to hyperoxia. Human embryonic kidney HEK293T cells were transfected with pcDNA3-hemagglutinin (HA)-ATRIP overexpression vector, and, after 48 h, cells were exposed to normoxia or hyperoxia for 24 h. ATRIP was immunoprecipitated using anti-HA antibody. ATR Western analysis was performed on ATRIP immunoprecipitates. *Top*, ATR; *bottom*, HA. *B*: hyperoxia activates ATR followed by activation of ATM. A549 cells were either exposed to normoxia or hyperoxia for 8, 16, or 24 h, and activation of ATR or ATM activation was determined by ATR or ATM kinase assay as described in experimental procedures. *Top*, phospho-Chk1; *bottom*, ATR as detected in each kinase reaction. Con, control; 1–96AA, amino acids 1–96. *C*: ATR is required for ATM activation in hyperoxia. A549 cells were transfected with ATR siRNA followed by exposure to hyperoxia (16 h). Following incubation, ATM kinase assay

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

was performed using glutathione S-transferase (GST)-Chk2 as substrate as described in experimental procedures.