Hyperoxia activates ATR-Chk1 pathway and phosphorylates p53 at multiple sites.

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Abbreviations

BRCA1- Breast cancer 1, early onset

C-abl- v-abl Abelson murine leukemia viral oncogene

Hus1- HUS1 (S. pombe) checkpoint homologue

MAD2L1- MAD2 (mitotic arrest deficient, yeast homologue).

MCM4 (cdc21)- Minichromosome maintenance deficient (S. cerevisiae) 4.

MCM7 (cdc47)- Minichromosome maintenance deficient (S. cerevisiae) 7.

MCM6 (Mis5)- Minichromosome maintenance deficient (Mis5 S. pombe ) 6.

MRE11A- Meiotic recombination (S. cervisiae) 11 homologue 11.

MRE11B- Meiotic recombination (S. cervisiae) 11 homologue 11.

NBS1 (nibrin)- Nijmegen breakage syndrome 1 (nibrin)

P21 Waf1- Cyclin Kinase Inhibitor 1A (p21, Cip1)

P53- Tumor protein p53 (Li-Fraumeni syndrome)

RAD17- RAD17 (S. pombe) homolog

RAD 50- RAD50 (S. cerevisiae) homolog.

RAD 51- RAD 51 (S. cerevisiae) homolog (E. coli RecA homolog)

RAD9- RAD9 (S. pombe homolog)

Rpa- Replication protein A3 (14kD)

TIMP3- Tissue inhibitor of metalloproteinase 3 (Sorsby fundud dystrophy, pseudo inflammatory)

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase.
Abstract

Hyperoxia has been shown to cause DNA damage resulting in growth arrest of cells in p53-dependent, as well as p53-independent pathway. Although H₂O₂ and other peroxides have been shown to induce ATM-dependent p53 phosphorylation in response to DNA damage, the signal transduction mechanisms in response to hyperoxia are currently unknown. In this report we demonstrate that hyperoxia phosphorylates ser-15 residue of p53 independent of ATM. ATM−/− cells induced p53 expression and ser-15 phosphorylation suggesting that ATM may not be required for hyperoxia-dependent p53 ser-15 phosphorylation. Hyperoxia phosphorylated p53(ser-15) in DNA-PK−/− cells indicating that it may not depend on DNA-PK for phosphorylation of p53 (ser-15). Additionally, we have shown that ser-37 and ser-392 residues of p53 are also phosphorylated in an ATM-independent manner in hyperoxia. In contrast, H₂O₂ did not phosphorylate ser-37 in either ATM+/+ or ATM−/− cells. Furthermore, H₂O₂ failed to phosphorylate ser-15 in ATM+/+ cells. Hyperoxia-mediated p53 phosphorylation was inhibited by wortmannin, a potent inhibitor of PIKKs. Additionally, overexpression of kinase-inactive ATR in HEK293T cells diminished ser-15, ser-37 and ser-392 phosphorylation compared to vector only transfected cells. In contrast, wildtype ATR overexpression did not diminish ser-15, ser-37 or ser-392 phosphorylation. We have also shown that Checkpoint kinase 1 (Chk1) is phosphorylated on ser-345 in response to hyperoxia, which could be inhibited by caffeine or wortmannin, potent inhibitors of PIKKs. In addition, hyperoxia also phosphorylated Chk1 in ATM+/+ as well as in ATM−/− cells demonstrating an ATM-independent mechanism in Chk1 phosphorylation. In contrast, UV light or H₂O₂ did not phosphorylate Chk1 in ATM−/− cells demonstrating that ATM is required for UV light or H₂O₂ –mediated Chk1 phosphorylation. Taken together, our data suggest that hyperoxia activates ATR-Chk1 pathway, and
phosphorylates p53 at multiple sites in an ATM-independent manner, which is different than other forms of oxidative stress such as H₂O₂ or UV light.
Introduction

Hyperoxia-mediated growth inhibition of lung cells is a hallmark of oxygen toxicity. Growth arrest in response to hyperoxia may be mediated by its inhibitory effect on cell cycle regulatory proteins, and/or activation of cell cycle checkpoint proteins in response to DNA damage (23). DNA damage activates signaling pathways that can halt the cell cycle for subsequent repair, or can induce cells to undergo programmed cell death. Therefore, understanding how cells respond to DNA damage is of paramount importance in understanding oxygen toxicity of lung cells. In addition to hyperoxia, lung oxidant burden in various disease states also can induce DNA damage response pathway resulting in delay or inhibition of repair process. Because hyperoxia produces reactive oxygen species (39,9), many investigators use hydrogen peroxide as a surrogate for hyperoxia in their studies. Even though hydrogen peroxide oxidizes DNA there is reason to believe that both, hyperoxia and H₂O₂ are not equivalent stimulus (23). For example, the genotoxic effect of hydrogen peroxide include mutagenic single strand breaks (10-11), whereas hyperoxia induces chromosome aberration and sister chromatid exchange (13). Second, iron chelators such as desferrioxamine have no effect on hyperoxia-induced clonogenic survival, whereas they are protective to cells damaged by hydrogen peroxide (12). It also takes several days for hyperoxia to injure and kill the cell, whereas hydrogen peroxide takes immediate effect. Recent studies have demonstrated that H₂O₂ (37) or other peroxide (34) activates ATM-dependent DNA damage signal transduction pathway. In this study, we investigated whether hyperoxia being an oxidant also uses ATM for transmission of DNA damage signal.

ATM (Ataxia telangiectasia-mutated) and ATR (the ATM-and Rad3-related) are members of PIKK (phosphoinositide 3-kinase-related kinases) that play key roles in fundamental cellular...
processes including proliferation and genomic surveillance (1). ATM is a 370-kDa-protein kinase mutated in the human genetic disorder ataxia telangiectasia (17). Cell lines derived from patients lacking ATM are radiosensitive and exhibit defects in checkpoint responses to ionizing radiation (8) including p53-dependent G1 cell cycle arrest and p53-independent S and G2 cell cycle arrest (8). The kinase activity of ATM is induced in response to double-stranded DNA breaks (1), and it targets several effectors of checkpoint control, including Cds1 (also known as Chk2), BRCA1 (20), p53 (2) and Mdm2 (21).

DNA-dependent protein kinase (DNA-PK) is another member of PIKK family that has been shown to be activated in response to DNA double stranded breaks (18). DNA-PK exhibits strong preference for phosphorylating serine or threonine residues (19). DNA-PK phosphorylates p53 on ser-15 as well as on ser-37 residues. However, unlike ATM null cells, radiation-induced p53 protein induction and p53-dependent G1 checkpoint function appear normal in DNA-PK deficient cells (19). Thus, if DNA-PK plays any role in the regulation of p53 function in vivo, it seems that other kinases such as ATM or ATR fully compensate for this activity in cells that lack DNA-PK.

Unlike ATM, deletion of ATR in mice results in embryonic lethality indicating that ATR is an essential gene (3). Cells lacking ATM are hypersensitive to ionizing radiation (IR), but not to UV or hydroxyurea (HU, 7), whereas cells overexpressing a kinase-inactive form of ATR are sensitive to UV and HU (7), as well as to IR. This suggests that ATR plays a more prominent role than ATM during the cellular response to unreplicated DNA or to certain DNA damaging
agents. ATM and ATR have overlapping substrate specificity. However, both prefer phosphorylating serine or threonine residues that are followed by glutamine (1).

The DNA damage transducer ATM kinase phosphorylates the aminoterminus of p53 at ser-15 that blocks its association with Mdm2 (21). Additionally, ATR phosphorylates ser-15, ser-37, and ser-392 in response to UV radiation (16, 36). Recent studies have reported that checkpoint kinase 1(Chk1), which is activated by ATR in response to DNA damage can phosphorylate p53 on ser-20 (38). Chk1 and Chk2 are serine-threonine kinases that are activated by phosphorylation on ser-345 or on thr-68 by ATR or ATM kinases, respectively (1). Activated Chk1 or Chk2 phosphorylates ser-216 on cdc25C resulting in the inactivation of cdc25C phosphatase that leads to the arrest of cells at G2 transition (26).

Oxidative stress in the form of H2O2 or other peroxide has been demonstrated to activate ATM-mediated DNA damage signaling cascade (34, 37). Additionally, ATM has been shown to be an oxidative stress sensor (30). Although hyperoxia increases p53 expression in vivo and in vitro (22,4), little is known about how this occurs. Furthermore, hyperoxia-mediated DNA damage signal transducers have not yet been identified. In this report we demonstrate that hyperoxia activates ATR-Chk1 pathway and phosphorylates multiple serine residues on p53 involving an ATM-independent mechanism. Additionally, hyperoxia-mediated Chk1 phosphorylation was found to be independent of ATM, but was dependent on ATR.
Experimental Procedure

Cell culture, transfection and exposure to hyperoxia:
Lung adenocarcinoma cell line derived from human alveolar type II cells (A549) was obtained from ATCC and grown in F-12K media supplemented with 10% FBS and 100 units each of penicillin and streptomycin. GM03349 (ATM⁺⁺) and GM02052 (ATM⁻⁻) fibroblasts were obtained from Corell repository (New York), and were grown in minimal essential medium with 15% FBS and antibiotics. DNA-PK⁺⁺ (M059K) and DNA-PK⁻⁻ (M059J) cells were obtained from ATCC and propagated in F-12 and Ham’s media. Cells were either exposed to hyperoxia (95% oxygen + 5% CO₂) at a flow rate of 10L/min for 10 minutes in humidified modular exposure chambers (Billups-Rothenburg, CA) or to room air containing 5% CO₂ (normoxia) in a CO₂ incubator (Stericult, Forma Scientific) in 60 or 100 mm² tissue culture dishes containing 5 or 10 ml of media for 24 hours. At the end of the incubation, cells were either processed for total cell lysate preparation for western blotting or for isolation of total RNA for microarray analysis. For UV exposure dishes were placed in a UV stratalinker (Stratagene, CA) and exposed to 100 mJ/m² energy setting.

HEK293T cells were obtained from ATCC and were propagated in DMEM containing 10% fetal bovine serum (Biowhittakar, MD) and 100 units each of penicillin and streptomycin. For transfection, the cells were grown to 50% confluency in poly-D-lysine coated 60 mm² dishes (BD Biosciences). Twenty micrograms of plasmid DNA was transfected into HEK293T cells using geneporter reagent (GTS Systems, CA) or lipofectamine 2000 reagent (Invitrogen, CA) as
suggested by the manufacturer. Cells were allowed to express the transgene for 48 hours, and the media was changed, and cells were exposed to hyperoxia (95%O₂ + 5% CO₂) or normoxia.

Phospho-specific p53 antibodies and phospho-Chk1 antibodies were obtained from Cell Signaling Technologies, Beverly, MA. Anti-Checkpoint kinase 1 antibody was obtained from Santa Cruz Biotech, CA. Western blotting detection reagent was obtained from Cell Signaling Technologies (Beverly, MA). Super signal femto reagent was obtained from Pierce Chemicals.

**Microarray Analysis of cell cycle checkpoint genes in hyperoxia:**

We used GEArray human cell cycle checkpoint assay kit (SuperArray Inc, Bethesda, MD 20827) for analysis of cell cycle checkpoint genes. This kit is optimized to assay the expression profile of the following cell cycle checkpoint associated genes: ATM, BRCA1, c-Abl, Chk1, Chk2, Gadd45, Hus1, IκBα, MAD2l1, MCM4, MCM6, MCM7, MRE11A, MRE11B, NBS1, p21/WAF1, p53, RAD17, RAD50, RAD51, RAD9, Rpa, TIMP 3. Briefly, 10 µg of total RNA was reverse transcribed with GEArray primer mix (Super array Kit) using 5µl (10 mCi/ml) [α-³²P]dCTP (Perkin Elmer) and 2µl (50U/µl) MMLV RT (Promega, WI) at 42°C for 20 minutes. The reaction was stopped using stop buffer and the probe was denatured at 68°C using denaturation buffer. The labeled cDNA probe was then added to GEArray hybridization solution and incubated for 16-18h at 68°C with continuous agitation. The hybridized membrane was washed with wash buffer (2X SSC, 1% SDS and 0.1X SSC, 0.5% SDS) twice for 20 minutes each at 68°C with agitation. The wet membrane was sealed in hybridization bag and exposed to x-ray film (Kodak Biomax MS film) with intensifying screen at -70°C until sufficient exposure was achieved. The developed spots were identified using grid card provided in the kit. The relative
abundance of a particular transcript was estimated by comparing its signal intensity to the
signal derived from β-actin or GAPDH. This experiment was repeated three independent times
and densitometry was performed using NIH 1.61 image program and statistical analysis was
performed on relative densities using analysis of variance followed by multiple comparison using
Tukey’s test utilizing a statistical software package (In stat).

**Immunoprecipitations and Western blotting:**

Total cellular lysate was prepared in lysis buffer (50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 150
mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM Sodium pyrophosphate, 25 mM B-
glycerophosphate, 1 mM PMSF, 30 µl/ml aprotinin (Sigma) and 1 mM Na₃VO₄). One milligram
of ATM cell lysate, 800 µg of A549 cell lysate or 1500 µg of HEK 293T cell lysate was
immunoprecipitated using p53-agarose conjugate antibody (Santa Cruz Biotechnology Inc., CA).
Briefly, cell lysates were incubated with 10 µl of anti-p53-agarose conjugate, and incubated in
cold in a rotating mixer for two hours. Following incubation, lysates were centrifuged at 1000
Xg for 30 seconds. The supernatant was carefully aspirated and the pellet containing the agarose
beads was washed 4 times with the lysis buffer. Finally, the agarose beads were suspended in 40-
50 µl SDS-sample buffer without reducing agents to prevent the interference of IgG heavy chain
with p53 bands and boiled for 5 minutes. Aliquots of 5 or 10 µl were analyzed in western
blotting. For Chk1 or phospho-Chk1 western blotting, ten or 20 µg of protein was resolved in
10% SDS-PAGE, and electro-blotted onto nitrocellulose membrane. The membrane was blocked
in 5% non-fat dry milk in TBS containing 0.1% Tween-20 (Bio-Rad). Following washes the
membrane was incubated with primary antibody or phospho-specific antibodies (Cell Signaling
Technologies, Beverly, MA) in TBS containing 0.1% Tween-20 and 5% BSA overnight with
gentle shaking. For detection of Flag protein, anti-Flag polyclonal antibody was obtained from Sigma Chemical Co (St. Louis, MO). Following incubation, the membrane was washed and incubated for one hour with respective HRP-tagged secondary antibodies, and specific-bands were detected by ECL (Amersham), LumiGlo reagent (Cell Signaling Technologies, Beverly, MA), or using super signal west femto reagent (Pierce Chemical Co).

Western blots were scanned and image analysis was performed on NIH 1.61 image software. Statistical analysis was performed using ANOVA and Tukey’s test for multiple comparison on the relative densities using In Stat statistical software (In Stat). Means with a significance level of less than 0.05 was considered significant.
Results

**Microarray Analysis of the cell cycle checkpoint gene expression in hyperoxia:**

Although hyperoxia has been shown to cause DNA damage (5), and to induce p53 expression (22,4), the DNA damage response pathway is currently unknown. Therefore, to determine the status of the cell cycle checkpoint genes in response to hyperoxia, we examined 24 genes that are known to mediate cell cycle checkpoint control. We observed that p21, IκBα and Gadd45 were strongly induced in hyperoxia (Fig. 1A). Although p53 was not transcriptionally induced in hyperoxia, p21 was strongly induced (p<0.001), suggesting that post-translational modification of p53 might cause increased p21 expression. In addition, there was significant (p<0.001) induction of the IκBα (6) and Gadd45 genes (Fig. 1B). However, DNA damage transducers such as ATM, Chk1 or Chk2 were not transcriptionally upregulated in hyperoxia, suggesting that post-translational modifications such as phosphorylation of these kinases may play a role in hyperoxia-mediated DNA damage signaling. Additionally, Rad family genes such as Rad9, Rad17 or RaD51 were not transcriptionally upregulated in hyperoxia. There was no significant decrease in any of the 24 cell cycle regulatory genes. These data demonstrate that the sensors of DNA damage such as Rad family genes, or the transducers of DNA damage such as ATM, ATR, Chk1 or Chk2 are not induced or repressed in hyperoxia, whereas the effectors of DNA damage response such as Gadd45 or p21 are induced in hyperoxia.

**Wortmannin a PIKK inhibitor, decreases p53 ser-15, -20, -37, and -392 phosphorylation in hyperoxia in lung adenocarcinoma (A549) cells:**

Members of the PIKK such as ATM or ATR have been shown to be activated in response to DNA damage (1). To determine whether members of PIKKs are involved in hyperoxia-mediated
DNA damage response, we used wortmannin, a potent inhibitor of PIKKs (27). We treated A549 cells with 15 µM wortmannin for 2 hours followed by exposure of cells to hyperoxia containing wortmannin. ATM and ATR have been shown to phosphorylate ser-15 on p53 in response to DNA damage (1). Therefore, we measured the p53 ser-15 phosphorylation in response to hyperoxia in wortmannin treated or wortmannin untreated cells. As demonstrated in Fig.2A, panel B, wortmannin treatment decreased p53 ser-15 phosphorylation, demonstrating that phosphorylation occurs by a wortmannin sensitive pathway. Additionally, the level of p53 protein was also increased in response to hyperoxia (Fig. 2A, panel A), which is consistent with earlier studies in whole mouse lungs (22).

To evaluate whether other p53 residues are also phosphorylated in hyperoxia, we determined phosphorylation of p53 on ser-392 and ser-37 residue in hyperoxia. Hyperoxia phosphorylated ser-392 (Fig. 2A, panel E) and ser-37 (Fig. 2A, panel D) on p53. Additionally, the levels of ser-37 and ser-392 phosphorylations in response to hyperoxia was decreased in response to wortmannin treatment indicating a wortmannin sensitive pathway.

ATM or ATR phosphorylates Ser-20 on p53 in response to DNA damage (35). Additionally, recent studies have shown that Chk1 or Chk2 can also directly phosphorylate ser-20. Thus, ser-20 phosphorylation is a target for multiple kinases (35). To gain further insight into p53 phosphorylation in hyperoxia, we determined ser-20 phosphorylation by western analysis using phospho-specific antibodies. As demonstrated in Fig 2A, panel C, ser-20 was phosphorylated in response to hyperoxia. Additionally, treatment of cells with wortmannin inhibited ser-20 phosphorylation.
phosphorylation in hyperoxia. Thus, these results indicate that ser-20 phosphorylation occurs by a wortmannin-sensitive pathway in hyperoxia.

In additional control experiments we sought to determine whether UV or H_2O_2 phosphorylate similar residues on p53. We treated A549 cells with 100J/m^2 UV light and incubate cells for 5 hours, following which cell lysates were prepared as described in the methods. Additionally, cells were treated with 50 or 100 µM H_2O_2 for 30 minutes following which lysates were made. As shown in Fig. 2B, UV or H_2O_2 also phosphorylated ser-15, -20, -37 or -392 in A549 cells similar to hyperoxia. However, the response of H_2O_2 was stronger with respect to –ser15, -37, and –20 compared to UV light. Although H_2O_2 has earlier been shown to induce ser-15 or ser-20 phosphorylation (34), the phosphorylation of ser-37 and ser-392 has not been previously reported. Since A549 cells have both ATM and ATR proteins, the response could be mediated either by ATM or ATR or both. Therefore, we used ATM^{+/+} or ATM^{-/-} fibroblasts (Corell Repository, NY) to determine whether ATM-mediates any or all of these p53 phosphorylations in hyperoxia.

**Hyperoxia induces p53 ser-15, -20, -37 and -392 phosphorylation in ATM^{+/+} and in ATM^{-/-} cells, whereas H_2O_2 does not phosphorylate ser-15, ser-20 or ser-37 phosphorylation in ATM^{-/-} cells:**

Although hyperoxia increases p53 in vivo and in vitro, little is known about how this occurs. If hyperoxia phosphorylates p53 via an ATM-dependent pathway, we would expect inhibition of p53 phosphorylation in cells that lack a functional ATM or in cells where the ATM gene is functionally inactive due to mutation. Thus, to determine the role of ATM in hyperoxia-mediated
DNA damage response, we exposed ATM<sup>+/+</sup> or ATM<sup>-/-</sup> fibroblasts to 24 hours in hyperoxia, followed by detection of p53, phospho-p53 ser-15, -20, -37 or -392 using anti-p53 or anti-phospho-specific antibodies (Cell Signaling Lab, Beverly, MA). As demonstrated in Fig. 3A, hyperoxia increased p53 protein levels in ATM<sup>+/+</sup> cells as well as in ATM<sup>-/-</sup> cells (Fig. 3A, panel A). In addition, hyperoxia induced ser-15 phosphorylation in ATM<sup>+/+</sup> cells, as well as in ATM<sup>-/-</sup> cells (Fig. 3A, panel B). In fact, there was higher level of ser-15 phosphorylation in ATM<sup>-/-</sup> cells. To determine whether hyperoxia is similar to H<sub>2</sub>O<sub>2</sub> or UV light in inducing p53 ser-15 phosphorylation, we treated ATM<sup>+/+</sup> or ATM<sup>-/-</sup> cells with H<sub>2</sub>O<sub>2</sub> or UV light as described in the method and determined the p53 ser-15 phosphorylation. As demonstrated in Fig 3B, panel B, H<sub>2</sub>O<sub>2</sub> did not induce ser-15 phosphorylation in ATM<sup>-/-</sup> cells. In contrast, UV light phosphorylated ser-15 in ATM<sup>-/-</sup> cells. These data indicate that hyperoxia-mediated ser-15 phosphorylation is independent of ATM, but different from that of H<sub>2</sub>O<sub>2</sub>, which depends on ATM for ser-15 phosphorylation.

To further determine the role of ATM in hyperoxia –mediated p53 phosphorylation we analyzed ser-20, ser-37, and ser-392 phosphorylations in hyperoxia. As shown in Fig 3A (Panels C-E), hyperoxia caused ser-20, ser-37, and ser-392 phosphorylation in ATM<sup>+/+</sup> as well as in ATM<sup>-/-</sup> cells. However, the phosphorylation of ser-20 was significantly less in ATM<sup>-/-</sup> cells compared to ATM<sup>+/+</sup> cells. These results indicate that while hyperoxia-induced phosphorylations of ser-15, -37 and –392 is independent of ATM, phosphorylation of ser-20 in hyperoxia may depend on ATM. In contrast, H<sub>2</sub>O<sub>2</sub> failed to induce ser-37 phosphorylation in either ATM<sup>-/-</sup> or ATM<sup>+/+</sup> cells that suggests that ser-37 phosphorylation is not a target for H<sub>2</sub>O<sub>2</sub> (Fig. 3B, panel D). The effect of H<sub>2</sub>O<sub>2</sub> on ser-37 phosphorylation has not been previously reported. Hyperoxia strongly
phosphorylated ser-392 in ATM\(^{+/+}\) cells compared to ATM \(^{+/+}\) cells (Fig. 3A, panel E), a response similar to UV (Fig. 3B, panel E). However, H\(_2\)O\(_2\) also phosphorylated ser-392 in ATM\(^{+/+}\) or ATM\(^{-/-}\) cells, which demonstrates that H\(_2\)O\(_2\) can phosphorylate ser-392 in an ATM-independent manner.

**Hyperoxia induces p53 (ser-15) phosphorylation in DNA-PK\(^{+/+}\) cells as well as in DNA-PK\(^{-/-}\) cells:**

DNA-PK has been demonstrated to phosphorylate p53(ser-15) (19). Therefore, to determine whether DNA-PK may mediate hyperoxia induced ser-15 phosphorylation, we exposed DNA-PK\(^{+/+}\) or DNA-PK\(^{-/-}\) cells to hyperoxia, and detected p53 or p53 (ser-15) by western analysis. As demonstrated in Fig. 4A hyperoxia phosphorylated p53 (ser-15) in DNA-PK\(^{+/+}\) as well as in DNA-PK\(^{-/-}\) cells indicating that DNA-PK is not required for hyperoxia-mediated p53 phosphorylation. Additionally, UV light also induced p53(ser-15) phosphorylation in DNA-PK\(^{+/+}\) as well DNA-PK\(^{-/-}\) cells (Fig. 4B). In contrast, H\(_2\)O\(_2\) did not induce p53(ser-15) phosphorylation either in DNA-PK\(^{+/+}\) or DNA-PK\(^{-/-}\) cells at a concentration of 50 \(\mu\)M (treated for 30 min (Fig. 4B). To determine whether DNA-PK cells are responsive to H\(_2\)O\(_2\) treatment, we treated DNA-PK\(^{+/+}\) or DNA-PK\(^{-/-}\) cells to 100, 200 or 300 \(\mu\)M H\(_2\)O\(_2\) for 1 hour, and assessed p53 (ser-15) phosphorylation by western analysis. As demonstrated in figure 4C, DNA-PK\(^{+/+}\), as well as DNA-PK\(^{-/-}\) cells induced ser-15 phosphorylation in a dose-dependent manner.

**Kinase-inactive ATR expression decreased ser-15, -20, -37, and -392 phosphorylation in response to hyperoxia:**
Studies to clarify the role of ATR have been hampered by the lack of a viable ATR-deficient animal or a specific chemical inhibitor. Therefore, kinase-inactive mutant ATR protein expression has been used as an effective tool to determine ATR-responsive signal transduction, and has been utilized in several studies (7). By using a kinase-dead ATR overexpression construct, the kinase-dead protein is produced in excess of native wildtype protein in a dominant-negative manner. Because kinase-inactive protein would not phosphorylate downstream targets, we would expect to observe a decreased response in cells expressing kinase inactive protein compared to wildtype protein expressing cells, if the response is mediated by any of these proteins. Thus, by overexpressing kinase-dead or wildtype proteins, we can determine whether ATR plays a role in hyperoxia-induced DNA damage signaling. Therefore, to delineate the role of ATR in DNA damage signaling in hyperoxia, we transiently transfected HEK293T cells with a kinase-dead construct of ATR, wildtype ATR (a generous gift of Dr. Karlene Cimprich, Stanford University) or with the vector alone construct (7). Following 48 hours of transfection, we exposed cells to hyperoxia (95%O₂ + 5% CO₂) for 24 hours as described in the method. Expression of ATRkd was confirmed by western blotting of Flag protein using anti-Flag antibody (Fig.4A, lower panel). As demonstrated in Fig. 5A, panel B hyperoxia potently induced ser-15 phosphorylation in HEK293T cells transfected with vector alone. In contrast, cells transfected with a kinase-inactive ATR construct demonstrated significantly less (p<0.05) ser-15 phosphorylation (Fig. 5A, panel B and Fig. 5B). However, HEK293T cells overexpressing wildtype ATR did not show diminished ser-15 phosphorylation, rather there was an increase in ser-15 phosphorylation in these cells (Fig. 4A, panel B and Fig. 4B). Overexpression of ATRkd also decreased the level of ser-37 and ser-392 (Fig. 5A, panel D and panel E; Fig. 5C and D). In
contrast, overexpression of wildtype ATR did not diminish the expression of these phosphorylations in hyperoxia. Additionally, the level of ser-20 phosphorylation was increased in vector only transfected HEK293T cells exposed to hyperoxia. Cells treated with wildtype ATR showed significantly higher (p<0.01) ser-20 phosphorylation compared to cells transfected with kinase-inactive ATR expression vector in hyperoxia. Additionally, although ATRkd transfected cells demonstrated a lower level (25%) of ser-20 phosphorylation compared to vector only transfected cells in hyperoxia, this difference was not statistically significant. Taken together, these data indicate that while ser-15, ser-37 or ser-392 are increased in response to hyperoxia in an ATR-dependent manner, ser-20 phosphorylation in hyperoxia may not fully depend on ATR activation. As demonstrated in Fig 5F, the expression of Flag protein was significantly decreased in hyperoxia. Thus, the wildtype ATR expression was very high in 21% oxygen exposed HEK293T cells, but there was a significant decrease in the expression of ATRwt in 95% oxygen exposed cells (p<0.0001). Similarly, the expression of ATRkd was also decreased significantly (p<0.0001) in 95% oxygen exposure as compared to 21% oxygen exposure.

**Hyperoxia causes phosphorylation of Chk1 (ser-345), which is sensitive to caffeine or wortmannin:**

ATR has been shown to phosphorylate its downstream target Chk1 on ser-345 in response to DNA damage (38). This phosphorylation activates Chk1, and the activated Chk1 phosphorylates cdc25C on ser-216 causing its sequestration in the cytoplasm, and hence, inhibition of cdc2 kinase activity (26). If hyperoxia activates ATR signaling cascade then Chk1 is a likely target of ATR. Therefore, we determined the phosphorylation of ser-345 on Chk1 in hyperoxia. As
demonstrated in Fig. 6A hyperoxia phosphorylated Chk1 on ser-345. There was no increase in the Chk1 protein in hyperoxia indicating that Chk1 phosphorylation is a specific response of hyperoxia (Fig. 6A, lower panel). These results also agree with our microarray analysis that there was no increase in the Chk1 mRNA in response to hyperoxia (Fig. 1A & B). To define the role of PIKKs in Chk1 phosphorylation, we treated cells with caffeine or wortmannin, inhibitors of PIKKs followed by exposure of cells to hyperoxia. As demonstrated in the Fig. 6A (lanes 7-9) caffeine abolished the phosphorylations of Chk1 in response to hyperoxia. Additionally, wortmannin also inhibited phosphorylation of Chk1 in hyperoxia (Fig. 6B). These results indicate that hyperoxia activates Chk1 involving a wortmannin or caffeine sensitive pathway. Furthermore, these data also indicate that hyperoxia does not directly phosphorylate Chk1. In additional control experiments we treated A549 cells with UV light or H2O2 as described in the legend of figure 5C and determined Chk1 phosphorylation. As demonstrated in Fig. 6C UV light or H2O2 phosphorylated Chk1 in a similar manner as hyperoxia in A549 cells. However, since A549 cells express ATM or ATR proteins, Chk1 phosphorylation in hyperoxia could be mediated by any or both of these signal transducers. Therefore, to determine the role of ATM in Chk1 phosphorylation in hyperoxia we determined the effect of hyperoxia on Chk1 phosphorylation in ATM+/+ or ATM−/− cells.

**Hyperoxia phosphorylates Chk1 (ser-345) in ATM+/+ as well as in ATM−/− cells:**
Next, we determined whether ATM plays a role in hyperoxia-mediated Chk1 phosphorylation. As demonstrate in Fig. 7A, hyperoxia activated Chk1 (Ser-345) phosphorylation in ATM+/+ as well as in ATM−/− cells indicating that Chk1 phosphorylation in hyperoxia is independent of ATM. Additionally, UV light or H2O2 also phosphorylated Chk1 in ATM−/− cells albeit to a lesser
extent indicating that ATM may be required for UV light or \( \text{H}_2\text{O}_2 \)-dependent Chk1 phosphorylation (Fig. 7B).

**Kinase-inactive ATR expression decreases Chk1 phosphorylation in hyperoxia:**

To further determine whether hyperoxia-mediated Chk1 phosphorylation is dependent on ATR, we transfected HEK293T cells with wildtype or kinase-inactive ATR constructs as described in the method, and expression of ATR was confirmed by western blotting of Flag protein. We exposed these cells to normoxia or hyperoxia as described in the method. Hyperoxia significantly (\( p<0.001 \)) increased Chk1 phosphorylation compared to normoxia in vector-only transfected HEK293T cells (Fig. 8A and B). Cells expressing kinase-inactive ATR demonstrated significantly less (\( p<0.05 \)) phospho-Chk1 compared to vector-only transfected cells. In addition, cells transfected with wildtype ATR expression plasmid demonstrated significantly (\( p<0.001 \)) higher level of Chk1 phosphorylation in normoxic cells compared with vector only transfected normoxic cells (Fig. 8A and B). Additionally, ATRwt transfected cells demonstrated significantly higher (\( p<0.01 \)) level of Chk1 phosphorylation compared to ATRkd transfected cells in hyperoxia. Furthermore, there was no change in Chk1 protein expression either in hyperoxia or normoxia. These results demonstrate that hyperoxia-mediated Chk1 phosphorylation is dependent on ATR.
Discussion

In the present study we have demonstrated that hyperoxia phosphorylates ser-15, ser-20, ser-37 and ser-392 residues of p53 in an ATM-independent manner. Hyperoxia phosphorylated p53(ser-15) in DNA-PK\(^{+/+}\) as well as DNA-PK\(^{-/-}\) cells indicating that DNA-PK is not required in hyperoxia-mediated p53 ser15 phosphorylation. In addition, expression of kinase-inactive ATR decreased ser-15, ser-20, ser-37 or ser-392 phosphorylation in response to hyperoxia. Furthermore, ser-15, -20, -37 and –392 phosphorylations could be inhibited by wortmannin. We have also shown that H\(_2\)O\(_2\) phosphorylates ser-15 and ser-20 in an ATM-dependent manner, but phosphorylate ser-392 in an ATM-independent manner. Additionally, H\(_2\)O\(_2\) did not phosphorylate ser-37 residue either in ATM\(^{+/+}\) or in ATM\(^{-/-}\) cells. In contrast to H\(_2\)O\(_2\), UV light phosphorylated ser-15, ser-37, and ser-392 in an ATM-independent manner, but phosphorylated ser-20 in an ATM-dependent manner. We have also shown that Chk1 is phosphorylated in hyperoxia, and this phosphorylation is independent of ATM, but is dependent on ATR. In contrast, UV light or H\(_2\)O\(_2\) phosphorylated Chk1 in an ATM-dependent manner. Our microarray analysis revealed that p53 mRNA does not increase in hyperoxia. A recent study has demonstrated that mouse lung p53 mRNA level does not change in response to hyperoxia (24) which is in agreement with our data. Thus, p53 is controlled in a post-translational manner by phosphorylation in hyperoxia. This has several advantages for immediate “turning on” or “turning off” of p53-dependent genes. We have observed that p53 (ser-15) phosphorylation can occur as early as 4 hours in response to hyperoxia (data not shown). However, p53 level increases in hyperoxia with extensive phosphorylation on various serine residues.
Recent studies have demonstrated that H$_2$O$_2$ (37) or other peroxides (34) transduce DNA damage signaling in an ATM-dependent pathway. Additionally, ATM has been shown to be sensitive to oxidative stress (26). However, ATM or ATR has overlapping substrate specificity, but are activated in response to distinct stimulus. Our present report demonstrate that hyperoxia transduces DNA damage response independent of ATM, which suggests that hyperoxia is a distinct form of oxidative stress, different from that of H$_2$O$_2$ or other peroxides.

The p53 tumor suppressor protein plays an important role in cellular responses to DNA damage and genomic surveillance (2). Activation of p53 can lead either to cell cycle arrest and DNA repair or apoptosis. In addition, p53 is phosphorylated at multiple sites by several different protein kinases (2). Phosphorylation of ser-15 on p53 impairs the ability of Mdm2 to bind p53, prompting both accumulation and functional activation of p53 in response to DNA damage (21). Recent studies have demonstrated that ser-20 on p53 is phosphorylated by Chk1 or Chk2 enhancing its tetramerization, stability and activity (35) in response to DNA damage. Although hyperoxia has been shown to induce ser-15 phosphorylation on p53 (28), the signal transducer has not been identified previously. Our present study provided evidence that ATR may be a signal transducer in hyperoxia-mediated DNA damage signaling.

We have shown that ATM$^{-/-}$ cells can potently induce ser-15 phosphorylation in response to hyperoxia, suggesting that hyperoxia-mediated ser-15 phosphorylation is independent of ATM. Additionally, wortmannin inhibited ser-15 phosphorylation induced by hyperoxia suggesting that PIKKs mediate this phosphorylation. Recent studies have demonstrated that while IC$_{50}$ of wortmannin for ATM is about 250 nM, for ATR it is about 1.8µM (32-33), suggesting that
wortmannin inhibits ATR at a higher concentration. Sarkaria et al. (33) observed that ATR activity was not inhibited by exposure of cells to 100 µM wortmannin for 1 hour in A549 cells. However, in our experiment we pretreated A549 cells for 2 hours followed by 24 hour exposure to hyperoxia. Hence, inhibition of ATR by wortmannin may require higher concentration as well as longer time for interaction of the drug with ATR.

UV-mediated phosphorylation of ser-37 has been shown to be dependent on the activation of ATR (26). In contrast, ATM does not phosphorylate these residues in response to IR. Therefore, phosphorylation of ser-37 in hyperoxia suggests an ATR-mediated DNA damage signaling. As demonstrated in Fig. 2A, we observed ser-37 and ser-392 phosphorylation in response to hyperoxia, which could be inhibited by wortmannin in a dose-dependent manner. These data strongly suggests that ATR, but not ATM may be the DNA damage signal transducer in response to hyperoxia.

We have observed that ser-20 phosphorylation in response to hyperoxia could be inhibited by wortmannin. Ser-20 has been shown to be phosphorylated by ATM, ATR, Chk1 or Chk2 (1, 17, 36). The inhibition of ser-20 phosphorylation by wortmannin suggests that PIKKs are involved in ser-20 phosphorylation in hyperoxia. However, phosphorylation of ser-20 was significantly lower in ATM−/− cells in hyperoxia compared to ATM+/+ cells, which suggests that ATM may be involved in ser-20 phosphorylation in hyperoxia. Additionally, ser-20 phosphorylation in hyperoxia was increased in ATRwt transfected cells, but decreased in ATRkd transfected cells. This decrease was not statistically significant. Taken together, these studies suggest that hyperoxia-mediated phosphorylation of ser-20 may involve both ATM and ATR, predominant
role played by ATM. Recent studies have demonstrated polo-like kinase-3 (Plk3) phosphorylates ser-20 residue of p53 in response to H$_2$O$_2$ treatment (37). In addition, this phosphorylation was caffeine sensitive and ATM dependent (37). The study also demonstrated that UV light, H$_2$O$_2$ or adriamycin can also increase the activity of Plk3 (37). We observed that ATM$^{-/-}$ cells failed to phosphorylate ser-20 in response to UV light or H$_2$O$_2$ suggesting that these stimuli depend on ATM for ser-20 phosphorylation (Fig. 3B, panel C). Additionally, these studies also demonstrate that Plk3 may phosphorylate ser-20 in response to UV or H$_2$O$_2$ in an ATM-dependent manner. However, our data indicate that ATR may, in part, phosphorylate ser-20. Interestingly, H$_2$O$_2$ did not phosphorylate ser-37 either in ATM$^{+/+}$ or in ATM$^{-/-}$ cells, which is consistent with IR, but in contrast with UV response. In addition, ser-392 phosphorylation by H$_2$O$_2$ was independent of ATM, which has not been previously reported (Fig. 3B, panel E).

Chk1 or Chk2 are serine threonine kinases that has been shown to act downstream of ATM or ATR as a signal transducer in DNA damage signaling. Chk1 has been shown to be phosphorylated by ATR (16), whereas ATM phosphorylates Chk2 (1). Therefore, activation of either Chk1 or Chk2 in hyperoxia would shed light on the activation of its upstream signaling molecule. To evaluate this possibility, we determined Chk1 phosphorylation in hyperoxia. As demonstrated in the Fig. 6A, hyperoxia caused Chk1 phosphorylation, and this phosphorylation was inhibited by caffeine (Fig. 6A) or wortmannin (Fig. 6B). Caffeine has been shown to be a potent inhibitor of ATR (32-33). Therefore, our data demonstrate that Chk1 phosphorylation in hyperoxia is dependent on a caffeine-sensitive pathway. Chk1 phosphorylation in hyperoxia was independent of ATM as demonstrated in Fig. 6A. Additionally, overexpression of kinase inactive ATR expression decreased Chk1 phosphorylation in hyperoxia. Taken together our data
demonstrates that hyperoxia induces ATR-Chk1 signaling, which is different than that of H$_2$O$_2$ or UV light. Interestingly, we have observed that UV light-induced Chk1 phosphorylation does not occur in ATM$^+$ cells, whereas ATM$^{++}$ cells phosphorylate Chk1. UV light has been shown to phosphorylate Chk1 in an ATR-dependent manner (38). Therefore, our observation that UV light failed to induce Chk1 phosphorylation in ATM$^+$ cells is rather surprising.

It is important to mention here that our experiments demonstrating the effect of ATRkd expression on p53 phosphorylation in HEK293T cells in hyperoxia may underestimate the level of inhibition. It appears that while the expression of ATRwt is very strong in HEK393T cells exposed to 21% oxygen as demonstrated by immunoblotting of Flag, the expression of ATRwt significantly (p<0.001) decreased in 95% oxygen (Fig 6A, lowest panel). In addition, expression of ATRkd was also significantly (p<0.001) less in 95% oxygen exposed cells compared to 21% oxygen exposed ATRkd transfected cells (Fig. 5F). Therefore, the inhibition of p53 phosphorylation in presence of less expression of ATRkd in 95% oxygen may actually underestimate the level of inhibition. Similarly, less expression of ATRwt in hyperoxia may also underestimate the effect of overexpression of ATRwt on p53 phosphorylations. We do not know the reason for decrease expression of ectopically expressed ATR in hyperoxia. We also do not know whether endogenous ATR expression is decreased in hyperoxia. However, experiments are underway to determine the effect of hyperoxia on ATR expression.

Although hyperoxia has been shown to cause growth arrest of a variety of cells (22, 25), the mechanism of growth arrest has not been elucidated. Additionally, although hyperoxia is known
to cause DNA damage (4), the signaling pathway has not been delineated. Our present study demonstrates that hyperoxia can activate a p53-dependent and/or p53-independent pathway in response to DNA damage. p53-dependent DNA damage response may be transmitted through a p21 or Gadd45-mediated pathway resulting in G1/S phase arrest of cells. In contrast, p53-independent pathway may involve ATR-Chk1 activation resulting in the inactivation of maturation promoting factor and arrest of cells at G2/M boundary. Recent studies have shown that hyperoxia causes growth arrest of A549 cells at G2/M phase boundary (20), supporting our present finding that Chk1 activation may mediate G2 arrest of cells in hyperoxia. In addition, a recent study has demonstrated that DNA strand breaks that occur in exposure of cells to hyperoxia may be due to a replication blockade (29). Since ATR is induced in response to stalled replication, our present study suggests that hyperoxia may activate ATR-mediated signaling in response to a replication block. Additionally, our study demonstrates that hyperoxia is a unique stress that is different from H$_2$O$_2$ or other peroxides in its response to DNA damage.
Acknowledgement:

This study was supported by Scientist Development Grant from American Heart Association, and research project grant from the American Cancer Society (KCD). The authors acknowledge the excellent technical assistance of William Holland. The authors also acknowledge the generous contribution of ATR plasmids by Dr. Karlene Cimprich at Stanford University. The authors sincerely thank Dr. Carl Anderson (Brookhaven National Laboratory) for critical reading of the manuscript and providing helpful suggestions.
References


Figure Legends

Figure 1. (A) Microarray analysis of activation of cell cycle checkpoint genes in hyperoxia: A549 cells were exposed to normal air or 95% O₂ +5% CO₂ for 24 hours. Following exposure, total RNA was prepared (Qiagen RNA isolation kit). 10 µg of total RNA was reverse transcribed with GEAtimer mix (Superarray Kit). The hybridized membrane was washed and exposed to x-ray film (Kodak Biomax MS film) with an intensifying screen. The developed spots were identified using a grid card provided in the kit as described in the methods section. (B) Densitometry was performed on three separate microarrays processed as described in Fig. 1A. Relative densities of genes were analyzed using analysis of variance followed by multiple comparisons and Tukey’s test using in stat Statistical software.

Figure 2. (A) Hyperoxia phosphorylates p53 ser-15, ser-20, ser-37, and ser-392, which is inhibited by wortmannin: A549 cells were exposed to hyperoxia or normoxia and p53 was immunoprecipitated, and subjected to western blotting as described in the methods section. Lanes 1-3, cells exposed to normoxia; lanes 4-6, cells exposed to 95% O₂ + 5% CO₂; lanes 7-9 cells treated with 15 µM wortmannin, and exposed to normoxia for 24 hours; lanes 10-12, cells treated with 15 µM wortmannin and exposed hyperoxia (B) Effect of UV light and H₂O₂ on p53 ser-15, ser-20, ser-37, and ser-392 phosphorylations in A549 cells: Confluent A549 cells were treated with 100 mJ/cm² (5-hour post exposure) or to H₂O₂ (50 or 100 µM, 30 min.) and p53 immunoprecipitates were subjected to western blotting as described in the methods. Lanes 1-3, UV exposure in triplicate; lane 4-5, cells treated with 50 or 100 µM H₂O₂.
Figure 3. (A) Effect of hyperoxia on p53 ser-15, ser-20, ser-37, and ser-392 phosphorylation in ATM^{+/+} and ATM^{+/−} cells:
ATM^{+/+} (GM03349) and ATM^{+/−} (GM 02052) fibroblasts were exposed to normoxia or hyperoxia, and p53 immunoprecipitates were subjected to western analysis as described in the methods. Lanes 1-3, ATM^{+/+} cells exposed to normoxia; lanes 4-6, ATM^{+/−} cells exposed to normoxia; lanes7-9, ATM^{+/+} cells exposed to hyperoxia; lanes 10-12 ATM^{+/−} cells exposed to hyperoxia. Panels A-E, p53, p53-phospho-ser-15, p53-phospho-ser-20, p53-phospho-ser-37, and p53-phospho-ser-392.

(B) Effect of UV light and H_{2}O_{2} on p53 ser-15, ser-20, ser-37, and ser-392 phosphorylation in ATM^{+/+} and ATM^{+/−} cells: ATM^{+/+} (GM03349) and ATM^{+/−} (GM 02052) fibroblasts were exposed to UV light (100 J/cm², 5h post exposure) or H_{2}O_{2} (50 µM, 30 min) and p53 immunoprecipitates were subjected to western analysis as described in the method. Lanes 1-3, ATM^{+/+} cells exposed to UV light; lanes 4-6, ATM^{+/−} cells exposed to UV light; lanes7-9, ATM^{+/+} cells exposed to H_{2}O_{2}; lanes 10-12 ATM^{+/−} cells exposed to H_{2}O_{2}. Panels A-E, p53, p53-phospho-ser-15, p53-phospho-ser-20, p53-phospho-ser-37, and p53-phospho-ser-392.

Figure 4. Hyperoxia phosphorylates p53 ser-15 in DNA-PK^{+/+} as well as DNA-PK^{+/−} cells:
(A) DNA-PK^{+/+} and DNA-PK^{+/−} cells were exposed to hyperoxia as described in the method. Cell lysates were made, and p53 ser-15 phosphorylation was analyzed as described in the methods. Lanes 1-3, DNA-PK^{+/+} cells exposed to normoxia; lanes 4-6, DNA-PK^{+/−} cells exposed to normoxia; lanes7-9, DNA-PK^{+/+} cells exposed to hyperoxia; lanes 10-12 DNA-PK^{+/−} cells exposed to hyperoxia. (B). Effect of UV and H_{2}O_{2} on p53(ser-15) phosphorylation in DNA-PK^{+/+} and DNA-PK^{+/−} cells: DNA-PK cells were exposed to UV light (100 mJ/cm²) or treated with 50 µM H_{2}O_{2} for 30 minutes. Cell lysate was prepared and p53(ser-15) western analysis was
performed as described in the Methods. Lanes 1-3, untreated DNA-PK\textsuperscript{+/+} cells, lanes 4-6. untreated DNA-PK\textsuperscript{-/-} cells; lanes 7-9, DNA-PK\textsuperscript{+/+} cells exposed to UV light; lanes 10-12, DNA- PK\textsuperscript{-/-} cells exposed to UV light; lanes 13-15, DNA-PK\textsuperscript{+/+} cells treated with H\textsubscript{2}O\textsubscript{2}; DNA-PK\textsuperscript{-/-} cells treated with H\textsubscript{2}O\textsubscript{2}. (C) Hyperoxia phosphorylate p53(ser-15) in DNA-PK\textsuperscript{+/+} as well as DNA-PK\textsuperscript{-/-} cells: Lane 1, untreated DNA-PK\textsuperscript{+/+} cells; lanes 2-4, DNA-PK\textsuperscript{+/+} cells treated with H\textsubscript{2}O\textsubscript{2}(100, 200 and 300 µM); Lane 5, untreated DNA-PK\textsuperscript{-/-} cells; lanes 6-8, DNA-PK\textsuperscript{-/-} cells treated with H\textsubscript{2}O\textsubscript{2}(100, 200 and 300 µM). Lower Panel, p53 western analysis.

Figure 5. (A) Effect of ATR\textsubscript{wt} and ATR\textsubscript{kd} overexpression on ser-15, ser-20, ser-37, and ser-392 phosphorylations in HEK293T cells:
HEK293T cells were transfected with 20 µg of pBJ-vector, pBJ-ATR\textsubscript{wt}, or pBJ-ATR\textsubscript{kd} plasmids using geneporter transfection reagent as described in the method. Transfected cells were exposed to normoxia or hyperoxia as described in the method. Western analysis of p53 or phosphorylated forms of p53 was performed in the p53 immunoprecipitates of HEK293T cells. Lanes 1-3, pBJ-vector transfected cells exposed to normoxia; lanes 4-6, pBJ-vector transfected cells exposed to hyperoxia; lanes 7-9, pBJ-ATR\textsubscript{wt} transfected cells exposed to normoxia; lanes 10-12, pBJ-ATR\textsubscript{wt} transfected cells exposed to hyperoxia; lanes 13-16, pBJ-ATR\textsubscript{kd} transfected cells exposed to normoxia; lanes 16-18, pBJ-ATR\textsubscript{kd} transfected cells exposed to hyperoxia. Panels A-E, p53, p53-phospho-ser-15, p53-phospho-ser-20, p53-phospho-ser-37, and p53-phospho-ser-392. (B) Densitometric analysis of ser-15 (panel B). (C), Densitometric analysis of ser-20 (panel C). (D), Densitometric analysis of ser-37, (panel D). (E), Densitometric analysis of ser-392, (panel E). (F) Graph showing statistical comparision of Flag expression in 21% or 95% oxygen.
Figure 6. (A) Hyperoxia-mediated phosphorylation of ser-345 of Chk1 is inhibited by caffeine in A549 cells: A549 cells were exposed to normoxia or hyperoxia (95%O₂+5%CO₂) for 24 hours, followed by preparation of cell lysates with RIPA buffer as described in the method. In some of the treatments cells were treated with 10 mM caffeine and exposed to hyperoxia. Phospho-Chk1 was detected using anti-phospho-Chk1 antibody (Cell Signaling Technology, Beverly, MA), and Chk1 was detected with anti-Chk1 antibody (Santa Cruz Biotech, CA). Lanes 1-3, A549 cells exposed to normoxia; lanes 4-6, cells exposed to 95%O₂+5% CO₂; lanes 7-9, cells treated with 10 mM Caffeine followed by exposure to hyperoxia. (B) Wortmannin inhibits hyperoxia-mediated Chk1 (ser-345) phosphorylation: A549 cells were treated in a similar manner as described for legend of Fig. 5A, except that some cells were treated with wortmannin (15 µM) instead of caffeine. Lanes 1-3, A549 cells exposed to normoxia; lanes 4-6, cells exposed to 95%O₂+5% CO₂; lanes 7-9, cells treated with 15 µM wortmannin followed by exposure to normoxia; lanes, 10-12, cell treated with wortmannin and exposed to hyperoxia. (C) Effect of UV light exposure and H₂O₂ treatment on Chk1 (ser-345) phosphorylation in A549 cells: A549 cells were exposed to UV light (100 mJ/cm², 5-h post exposure) or H₂O₂ (50, 100 or 150 µM, 30 min) and pChk1(ser-345) was determined as described in the methods: Lanes 1-3, cells exposed to UV light; lanes 4-6, cells exposed to 50, 100 or 150 µM H₂O₂ for 30 minutes.

Figure 7. (A) Hyperoxia-mediated phosphorylation of Chk1 (ser-345) is independent of ATM: ATM ++ (GM03349) and ATM +/− (GM 02052) fibroblasts were exposed to normoxia or hyperoxia and cell lysates were subjected to phospho-Chk1 (ser-345) or Chk1 western analysis as
described in the method. Lanes 1-3, ATM+/+ cells exposed to normoxia; lanes 4-6, ATM+/− cells exposed to normoxia; lanes 7-9, ATM+/+ cells exposed to hyperoxia; lanes 10-12 ATM+/− cells exposed to hyperoxia. 

(B) Effect of UV light and H2O2 on Chk1 (ser-345) phosphorylation in ATM+/+ and ATM−/− cells: ATM+/+ (GM03349) and ATM−/− (GM 02052) fibroblasts were exposed to UV light (100 mJ/cm², 5h post exposure) or H2O2 (100 µM, 30 min) and cell lysates were subjected to western analysis as described in the method. Lanes 1-3, untreated ATM+/+ cells; lanes 4-6, untreated ATM−/− cells; lanes 4-6, ATM+/+ cells exposed to UV light; lanes 7-9, ATM−/− cells exposed to UV light; lanes 10-12, ATM+/+ cells exposed to H2O2; lanes 13-16 ATM−/− cells exposed to H2O2.

Figure 8. (A) Effect of ATRwt and ATRkd overexpression on Chk1 (ser-345) phosphorylation in HEK293T cells: HEK293T cells were transfected with 20 µg of pBJ-vector, pBJ-ATRwt or pBJ-ATRkd plasmid using lipofectamine 2000 transfection reagent (Invitrogen Inc., CA) as described in the method. Transfected cells were exposed to normoxia or hyperoxia as described in the method. Western analysis of Chk1 or phosphorylated forms of pChk1 (ser-345) was performed in the lysates of HEK293T cells. Lanes 1-3, pBJ-vector transfected cells exposed to normoxia; lanes 4-6, pBJ-vector transfected cells exposed to hyperoxia; lanes 7-9, pBJ-ATRwt transfected cells exposed to normoxia; lanes 10-12, pBJ-ATRwt transfected cells exposed to hyperoxia; lanes 13-16, pBJ-ATRkd transfected cells exposed to normoxia; lanes 16-18, pBJ-ATRkd transfected cells exposed to hyperoxia. Panels A, pChk1 (ser-345); panel B, Chk1, panel C, Flag. (B) Densitometric analysis of phospho-Chk1 (ser-345) of Fig. 7A.
Figure 1A
**Figure 2A**

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Figure 2B

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Figure 4A

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- **p-Ser-15**
- **p53**

Figure 4B

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- p53 (Ser-15)
- p53

Figure 4C

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<th>DNA-PK&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>DNA-PK&lt;sup&gt;-/-&lt;/sup&gt;</th>
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- p53(ser-15)
- p53
Figure 5A

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<th>pBJ-vec 1</th>
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<th>95% O₂</th>
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<th>21% O₂</th>
<th>95% O₂</th>
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<td>Flag</td>
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B

![Bar chart showing the ratio of p53 (Ser-15) to p53 under 21% Oxygen and 95% Oxygen conditions for pBJ-vec, ATRwt, and ATRkd.](chart)

C

![Bar chart showing the ratio of p-p53 (ser-20) to p53 under 21% Oxygen, 24h and 95% Oxygen, 24h conditions for pBJ5-Ve, pBJ5-ATRwt, pBJ5-ATRkd.](chart)
Ratio of p-p53 (ser-392) to p53

- 21% Oxygen, 24h
- 95% Oxygen, 24h

D

Ratio of p-p53 (ser-37) to p53

- 21% Oxygen, 24h
- 95% Oxygen, 24h

E
Figure 6A

21% Oxygen, 24h  Triplicate
95% Oxygen, 24h  Triplicate
95% Oxygen, 24h  Triplicate

Caffeine, 10mM + p-Chk1(ser-345)

Chk1
**Figure 6B**

Wortmannin, 15 µM

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**Figure 6C**

UV, H₂O₂, µM

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<th>100</th>
<th>150</th>
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pChk1(ser-345) Chk1
Figure 7A

21% Oxygen, 24h

ATM+/+  ATM-/-

| Triplicate | Triplicate |

95% Oxygen, 24h

ATM+/+  ATM-/-

| Triplicate | Triplicate |

pChk1 (ser-345)

Chk1

Figure 7B

Control  UV, 100 mJ/cm²  H₂O₂, 100 µM

ATM+/+  ATM-/-

| Triplicate | Triplicate |

ATM+/+  ATM-/-

| Triplicate | Triplicate |

ATM+/+  ATM-/-

| Triplicate | Triplicate |

pChk1 (Ser-345)

Chk1
**Figure 8A**

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</table>

**Figure 8B**

![Graph showing relative densitometric units for pChk1 and pBJ-Vectors, pBJ-ATRwt, and pBJ-ATRkd under 95% and 21% oxygen conditions.](image)

- **pChk1(ser-345)**
- **Chk1**
- **Flag**

- **Relative Densitometric Units**
- **pChk1**

- **95% Oxygen**
- **21% Oxygen**
Table-1. Summary of phosphorylation of various p53-serine residues and Chk1 in A549, ATM, DNA-PK and ATRwt or ATRkd transfected cells.

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