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7. Cells (5×10^6) were washed in ice-cold phosphate-buffered saline and lysed in 700 μl of 10 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.2% dodecyl maltoside (Sigma), 5 mM EDTA, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 100 μM $NaVO_4$, and aprotinin (lysis buffer) (2 μg/ml). Freshly prepared cell lysates were incubated for 2 hours at 4°C on a rotator with 10 μl of hybridoma supernatants. Magnetic beads coated with antibodies to mouse immunoglobulin G (50 μl; Dynabeads M-450, DYNAL, Oslo, Norway) were added and the mixture was further rotated at 4°C for 2 hours. Immune complexes were collected using a magnetic stand (DYNAL) and washed three times with lysis buffer. For protein immunoblotting analysis, one-third of this mixture was loaded on an SDS-polyacrylamide gel, which was later blotted onto a nitrocellulose membrane (Nitrocell MFF, Pharmacia) as described (*10*). Immune complexes were washed twice with kinase buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 4 mM $MnCl_2$, 6 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM dithiothreitol, and 100 μM $NaVO_4$]. Substrate (200 ng to 1 μg), 10 μCi of γ[^{32}P]adenosine triphosphate (ATP), and ATP to a final concentration of 5 μM were added, and the reaction mixtures were incubated at 30°C for 3 to 15 min. After electrophoresis in SDS-polyacrylamide gels, the reaction products were visualized by autoradiography.
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Activation of the ATM Kinase by Ionizing Radiation and Phosphorylation of p53

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The p53 tumor suppressor protein is activated and phosphorylated on serine-15 in response to various DNA damaging agents. The gene product mutated in ataxia telangiectasia, ATM, acts upstream of p53 in a signal transduction pathway initiated by ionizing radiation. Immunoprecipitated ATM had intrinsic protein kinase activity and phosphorylated p53 on serine-15 in a manganese-dependent manner. Ionizing radiation, but not ultraviolet radiation, rapidly enhanced this p53-directed kinase activity of endogenous ATM. These observations, along with the fact that phosphorylation of p53 on serine-15 in response to ionizing radiation is reduced in ataxia telangiectasia cells, suggest that ATM is a protein kinase that phosphorylates p53 in vivo.

Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder characterized by clinical manifestations that include progressive cerebellar ataxia, neuronal degeneration, hypersensitivity to ionizing radiation (IR), premature aging, hypogonadism, growth retardation, immune deficiency, and an increased risk for cancer (*1*). The gene mutated in A-T, *ATM* (ataxia telangiectasia–mutated), encodes a 370-kD protein that is a member of a family of proteins related to phosphatidylinositol 3-kinase (PI-3-K) that have either lipid or protein kinase activity. The subset of this family with the greatest identity to ATM functions in DNA repair, DNA recombination, and cell-cycle control (*2, 3*). Cell lines derived from A-T patients exhibit hypersensitivity to IR and defects in several IR-inducible cell-cycle checkpoints, including a diminished irradiation-induced arrest in the G_1 phase of the cell-cycle mediated by the p53 tumor suppressor gene product (*4, 5*). In response to DNA damage, cells with wild-type ATM accumulate p53 protein and show a subsequent increase in p53 activity, whereas cells with defective ATM show a smaller increase in the amount of p53 protein in response to IR (*4, 6*). Therefore, ATM appears to act upstream of p53 in a signal transduction pathway initiated by IR.

IR induces rapid, de novo phosphorylation of endogenous p53 at two serine residues within the first 24 amino acids of the protein, one of which was identified as Ser^{15} (*7, 8*). Phosphorylation of p53 at Ser^{15} in response to DNA damage correlates with both the accumulation of total p53 protein as well as with the ability of p53 to transactivate downstream target genes in wild-type cells (*8*). Furthermore, phosphorylation of p53 on Ser^{15} in response to IR is diminished in cell lines derived from A-T patients, suggesting that ATM participates in this response (*8*).

The PI-3-K–related protein, DNA-activated protein kinase (DNA-PK) phosphorylates p53 in vitro at two different Ser-Gln motifs, Ser^{15} and Ser^{37} (*9*). However, cells with diminished DNA-PK activity still normally accumulate p53 protein and undergo G_1 arrest in response to IR (*10*). We tested whether ATM might also phosphorylate p53 on Ser^{15} and whether the activity of ATM toward p53 as a substrate is regulated by IR.

Most naturally occurring ATM mutant proteins are unstable (*11*). Because a catalytically inactive ATM mutant is a critical control for in vitro kinase assays, we constructed such a mutant that can be stably expressed. The putative kinase domain of ATM resides in the COOH-terminus of the protein. In related proteins, three critical amino acids within this domain are necessary for phosphotransferase activity (*2, 12*). Thus, a recombinant, FLAG peptide–tagged, wild-type ATM

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was used as a source of ATM protein, and a FLAG peptide–tagged, mutant ATM expression construct was generated in which two of the three critical amino acid residues required for catalysis were mutated (Asp^{2870} → Ala and Asn^{2875} → Lys) (*13*). Wild-type and mutant recombinant ATM proteins were individually expressed in 293T cells, and in vitro kinase activity was assessed (*14*). Equivalent amounts of wild-type (wt) and mutant (kd) ATM recombinant proteins were immunoprecipitated and incubated with [γ-^{32}P]adenosine triphosphate (ATP) and recombinant glutathione S-transferase (GST)–conjugated p53 protein containing the first 101 amino acids of p53 ($GSTp53_{1-101}$) (Fig. 1A). Only the wild-type enzyme phosphorylated $GSTp53_{1-101}$ (Fig. 1B).

Endogenous p53 becomes phosphorylated on Ser^{15} and one other serine residue within the first 24 amino acids of the protein in response to IR (*8*). We tested whether mutation of each of the four serine residues (S6, S9, S15, S20) within the first 24 amino acids of p53 altered the ability of ATM to phosphorylate the NH_2-terminus of p53. Recombinant ATM was immunoprecipitated and used to phosphorylate wt or mutant $GSTp53_{1-101}$. Wild-type recombinant ATM phosphorylated wt p53, Ser^6 →Ala (S6A), and S9A mutant p53, but not S15A mutant p53 protein (Fig. 1B). Similar results were obtained with synthetic peptides comprising the first 24 amino acids of p53 (*15*). Therefore, ATM or a closely associated kinase phosphorylates $GSTp53_{1-101}$ exclusively on Ser^{15} in vitro. Wild-type ATM kinase also showed autophosphorylation in this assay (Fig. 1A). Because mutation of Asp^{2870} and Asn^{2875} within the kinase domain of ATM abolished both phosphorylation of p53 and autophosphorylation of ATM, the kinase activity observed in these assays appears to be intrinsic to the ATM protein. The DNA-PK also phosphorylates Ser^{15} (*9*), but unlike DNA-PK, ATM was dependent upon the presence of Mn^{2+} and did not require the addition of exogenous DNA for activity (*15*).

ATR/FRP-1 (ataxia telangiectasia and rad3-related/FRAP-related protein 1), another PI-3-K–related family member, may share functional overlap with ATM in cell-cycle checkpoint function (*16, 17*). Conditional expression of catalytically inactive ATR/FRP-1 abrogates G_2-M cell cycle arrest in response to IR. Furthermore, overexpression of wild-type ATR/FRP-1 complements the defective IR-inducible S-phase checkpoint in A-T cells (*17*). Although ATM is required for rapid phosphorylation of Ser^{15} in response to IR in vivo, ATM appears not to be required when cells are exposed to other genotoxic agents, such as ultraviolet (UV) radiation (*8*). Thus, other cellular kinases must also phosphorylate p53 on Ser^{15} in vivo. FLAG-tagged recombinant wt ATR/FRP-1 also showed autophosphorylation in vitro that was dependent upon the integrity of the catalytic domain. Like ATM, ATR/FRP-1 also phosphorylated p53 on Ser^{15} in a Mn^{2+}-dependent manner (Fig. 1B), though ATR/FRP-1 had at least 20-fold less activity than ATM toward $GSTp53_{1-101}$ when assayed under identical experimental conditions (Fig. 1B). Thus, p53 appears to be a better substrate for ATM than ATR/FRP-1.

To test whether endogenous p53 required ATM for phosphorylation on Ser^{15} in cells treated with IR in vivo, we generated a monoclonal antibody specific for p53 phosphorylated at Ser^{15} (Fig. 2A). The p53 protein was immunoprecipitated from normal and A-T lymphoblasts either exposed to 5 Gy IR or treated with the proteosome inhibitor, acetyl-Leu-Leu-norleucinal (ALLN), which causes stabilization of p53 protein (*8*). Immunoblot analysis with the monoclonal antibody to phosphoserine-15 of p53 demonstrated that p53 became phosphorylated only in normal lymphoblasts exposed to IR (Fig. 2B) (*18*). Phosphoserine-15 was undetected in normal cells treated with ALLN, although they accumulated equivalent amounts of total p53 protein to those in irradiated cells. Phosphoserine-15 p53 was also undetected in the 1526 A-T line (Fig. 2B, upper panel). Thus, examination of radiation responses in ATM-mutant cells further supports this link between ATM and irradiation-induced phosphorylation of p53.

Activation of endogenous ATM was examined in two different normal lymphoblast cell lines exposed to 0 or 5 Gy IR (*19*). ATM immunoprecipitates were used to phosphorylate $GSTp53_{1-101}$ in vitro. Within 20 min after exposure to IR, ATM protein kinase activity toward $GSTp53_{1-101}$ was increased approximately twofold (Fig. 3, B and C). This appeared to be an increase in the specific activity of ATM because the amount of ATM protein did not change in response to IR (Fig. 3A). Kinase activity toward p53 substrate was minimal in immunoprecipitates from an A-T lymphoblast line (Fig. 3, A and B). The IR-induced activity associated with ATM was directed to Ser^{15}, because the immunoprecipitated endogenous ATM from irradiated cells increased phosphorylation of Ser^{15} in in vitro kinase assays (Fig. 3B). Therefore, ATM kinase appears to be activat-

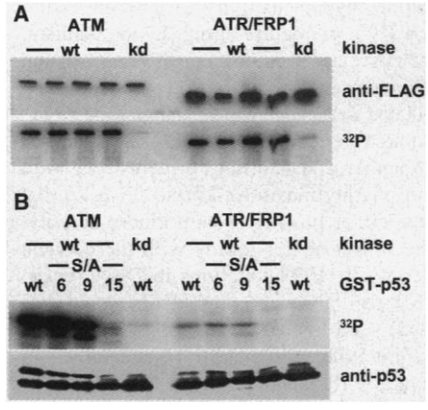

Fig. 1. Phosphorylation of Ser^{15} of p53 by ATM and ATR/FRP1 in vitro. We transfected 293T/17 cells with expression vectors encoding FLAG-tagged wild-type (wt) or catalytically inactive (kd) ATM or ATR/FRP1. After 48 hours, ATM or ATR was immunoprecipitated with antibody to FLAG and used in an in vitro kinase assay with [γ-^{32}P]ATP and either wt, S6A, S9A, or S15A $GSTp53_{1-101}$ as substrates (*14*). Proteins from each reaction were separated by SDS-PAGE (7% gel), transferred to nitrocellulose, and analyzed either on a PhosphorImager or by immunoblotting. **(A)** Amounts of FLAG-tagged ATM or ATR in each kinase reaction as measured by immunoblotting with anti-FLAG M2 (top panel) and amount of [γ-^{32}P]phosphate incorporated into ATM or ATR during the reaction (lower panel). **(B)** In vitro kinase assay with wt $GSTp53_{1-101}$ or various mutant $GSTp53_{1-101}$ proteins (S6A, S9A, or S15A) as substrates (top panel). Levels of substrate protein present in each reaction were determined by immunoblotting for p53 (lower panel). The upper immunoreactive band represents phosphorylated GSTp53 fusion protein. ATM did not phosphorylate GST alone. The same exposures are shown for ATM, ATR/FRP1, and corresponding substrate proteins in all panels shown in (A) and (B).

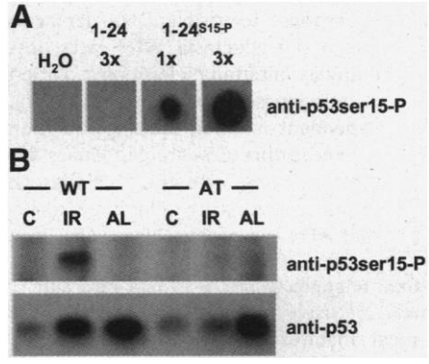

Fig. 2. Posttranslational modification of p53 on Ser^{15} in response to ionizing radiation requires ATM. **(A)** Monoclonal antibodies against a chemically synthesized p53 phosphoserine-15 peptide (amino acids 9 through 22) were used to immunoblot synthetic peptides (1×, 50 μg) consisting of the first 24 amino acids of p53 with ($1–24^{S15–P}$) or without (1–24) phosphoserine-15. **(B)** Normal WT (2184) or AT (1526) lymphoblasts were untreated (C), or were treated with 5 Gy IR (IR) or 20 μM ALLN (AL) for 90 min (*18*). The p53 was immunoprecipitated, subjected to SDS-PAGE (7.5% gel), transferred to nitrocellulose, and immunoblotted with the monoclonal antibody to phosphoserine-15 p53 (upper panel). Blots were then stripped and immunoblotted with antibodies to p53 (lower panel).

ed in response to IR and phosphorylates p53 on Ser[15].

Cells derived from A-T patients are not hypersensitive to UV irradiation (*1, 20*). Furthermore, such cells respond normally to UV with increased synthesis of p53, phosphorylation of p53 on Ser[15], and activation of the stress-activated SAP kinase (JNK) pathway (*6, 8, 21*). The kinase activity of ATM was not increased in UV-irradiated cells (Fig. 3C). Slight activation of ATM kinase was detected at more than 60 min after exposure, which may be due to signals generated by DNA strand breaks associated with DNA repair (*22*). These results confirm that ATM plays a minor role in the cellular UV response and suggest that another kinase other than ATM phosphorylates p53 on Ser[15] in response to UV irradiation.

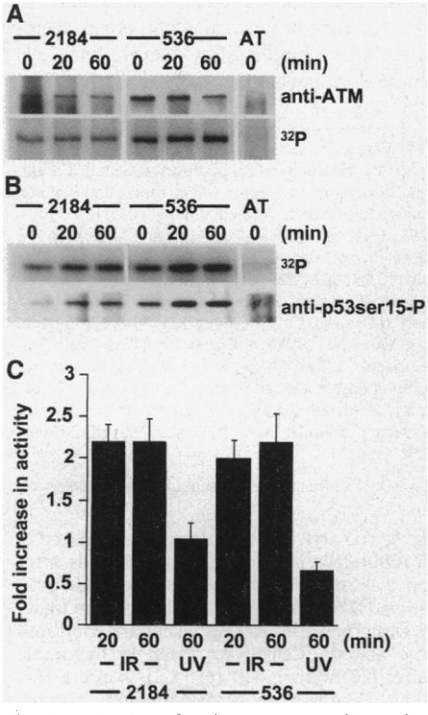

Fig. 3. Activation of endogenous ATM kinase by ionizing radiation in vivo. The 2184 or 536 individual normal lymphoblasts or 1526 AT lymphoblasts (AT) were either untreated or treated with 5 Gy IR and harvested 20 or 60 min later. ATM was immunoprecipitated and assayed (Fig. 1) with wild-type $GSTp53_{1-101}$ protein as a substrate (*19*). (**A**) Amounts of ATM present in each reaction were determined by immunoblotting with anti-ATM (Ab-3) (upper panel), and the amount of radiolabel incorporated into ATM during the kinase reaction was visualized with a PhosphorImager (lower panel). (**B**) Amounts of [γ-^{32}P]phosphate incorporated into $GSTp53_{1-101}$ during each reaction was visualized with a PhosphorImager (upper panel). Serine-15 phosphorylation of $GSTp53_{1-101}$ was determined by immunoblotting with anti-phosphoserine-15 p53 (lower panel). (**C**) The 2184 and 536 lymphoblasts were treated with IR or 10 J/m^2 UV radiation as above. Endogenous ATM was immunoprecipitated and used in an in vitro kinase assay with $GSTp53_{1-101}$ as substrate. The amount of ^{32}P-labeled $GSTp53_{1-101}$ was quantitated with a PhosphorImager and normalized to that obtained with immunoprecipitates from nonirradiated cells. Data are expressed as the mean ± standard error of five independent experiments.

Previous genetic and biochemical evidence implicated the ATM gene product in regulating the phosphorylation and induction of p53 in cells exposed to ionizing radiation (*4, 6, 8, 23*). Our results indicate that ATM is a protein kinase whose activity is increased by ionizing radiation and whose in vivo target may be Ser[15] of p53. This conclusion is consistent with the finding that ATM and p53 proteins directly interact with each other (*11*). The functional ramifications of radiation-induced Ser[15] phosphorylation remain to be clearly elucidated. However, phosphorylation of p53 on Ser[15] reduces binding of the mdm2 oncogene product to p53 in vitro (*7*), and binding of mdm2 to p53 promotes rapid degradation of p53 by targeting it for proteolytic degradation, thereby potentially controlling p53 protein levels (*24*). Because many of the clinical manifestations exhibited by A-T patients cannot be attributed to abnormal regulation of p53 alone, other important targets of the ATM kinase remain to be identified and characterized.

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13. The full-length cDNA encoding NH_2-terminal, FLAG-tagged wild-type ATM [Y. Ziv *et al.*, *Oncogene* **15**, 159 (1997)] was excised from pFB-YZ3 and subcloned into the Xho I site of pcDNA3 (Invitrogen) generating pcDNA-FLAG-ATMwt. To generate catalytically inactive ATM, a cDNA fragment encoding the PI-3-kinase–related domain of ATM [S. E. Morgan *et al.*, *Mol. Cell. Biol.* **17**, 2020 (1997)] was mutated by overlap polymerase chain reaction (PCR) substituting Asp[2870] with Ala and Asn[2875] with Lys. A cDNA fragment encoding the kinase domain was excised from wild-type ATM and replaced with a Bpu1101 I–Xho I fragment containing the mutations described above, generating pcDNA-FLAG-ATMkd.
14. We transiently transfected 293T cells with 10 μg of either pcDNA-FLAG-ATMwt, pcDNA-FLAG-ATMkd, pBJF-FRPwt, or pBJF-FRPkd (*16*) using calcium phosphate and harvested them 2 days later. Cells were lysed through sonication in TGN buffer [50 mM tris (pH 7.5), 50 mM glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM NaF, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mg pepstatin A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM dithiothreitol (DTT)] as described [G. J. Brunn *et al.*, *Science* **277**, 99 (1997)]. After centrifugation at 13,000*g*, 2 mg of extract was incubated with mouse immunoglobulin G and protein A/G Sepharose beads (Calbiochem). FLAG-tagged proteins were then immunoprecipitated with anti-FLAG M2 monoclonal antibody (Eastman Kodak) and protein A/G Sepharose beads. Immunoprecipitates were washed twice with TGN buffer, once with 100 mM tris (pH 7.5) plus 0.5 M LiCl, and twice with kinase buffer [10 mM Hepes (pH 7.5), 50 mM glycerophosphate, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 μM ATP, and 1 mM DTT]. Kinase reactions were initiated by resuspending washed beads in 30 μl of kinase buffer containing 10 μCi [γ-^{32}P]ATP and 1 μg $GSTp53_{1-101}$ and incubated for 30 min at 30°C. Proteins were electrophoretically separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and analyzed on a PhosphorImager. FLAG-tagged proteins and $GSTp53_{1-101}$ were subjected to immunoblotting with either anti-FLAG M2 antibody or monoclonal antibodies directed toward the NH_2-terminus of p53 (Ab-2 and Ab-6, Calbiochem) as described (*8*). $GSTp53_{1-101}$ fusion protein was made by amplifying cDNA encoding the first 101 amino acids of human p53 by PCR and subcloning the fragment into the Eco RI and Bam HI sites of pGEX-2T (Pharmacia). Serines 6, 9, or 15 were substituted with alanine, using the QuikChange Site-Directed Mutagenesis kit according to manufacturer's suggestions (Stratagene). $GSTp53_{1-101}$ wild-type and mutant proteins were individually expressed in bacteria and purified on glutathione-conjugated agarose beads.
15. C. E. Canman *et al.*, data not shown.
16. K. S. Keegan *et al.*, *Genes Dev.* **10**, 2423 (1996).
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18. Normal (2184) and AT (1526) Epstein-Barr virus immortalized human lymphoblasts were irradiated with a ^{137}Cs source or treated with 20 μm ALLN (Sigma) for 90 min. Cells were then harvested and lysed, and p53 was immunoprecipitated as described (*8*). Immunoprecipitates were resolved by nonreducing SDS-PAGE, transferred to nitrocellulose, and immunoblotted with monoclonal antibody to phosphoserine-15. Blots were stripped and reprobed with p53-specific monoclonal antibodies (Ab-2 and Ab-6).
19. Cells were irradiated with either IR or UV as described (*6*). Endogenous ATM was immunoprecipitated from 3 mg of lysate with ATM-specific rabbit polyclonal antibody (Ab-3, Calbiochem) and subjected to the in vitro kinase assay (*14*). The beads and reaction mixtures were separated, resolved by SDS-PAGE, and transferred onto Immobilon-P (Millipore) for ATM, or nitrocellulose for $GSTp53_{1-101}$. Radiolabeled proteins were visualized and quantitated on a PhosphorImager using ImageQuant software (Molecular Dynamics). Membranes were then immunoblotted with antibodies to ATM (Ab-3) or phosphoserine-15.
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