

Phosphorylation of Sp1 in Response to DNA Damage by Ataxia Telangiectasia-Mutated Kinase

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Abstract

Sp1, a transcription factor that regulates expression of a wide array of essential genes, contains two SQ/TQ cluster domains, which are characteristic of ATM kinase substrates. ATM substrates are transducers and effectors of the DNA damage response, which involves sensing damage, checkpoint activation, DNA repair, and/or apoptosis. A role for Sp1 in the DNA damage response is supported by our findings: Activation of ATM induces Sp1 phosphorylation with kinetics similar to H2AX; inhibition of ATM activity blocks Sp1 phosphorylation; depletion of Sp1 sensitizes cells to DNA damage and increases the frequency of double strand breaks. We have identified serine 101 as a critical site phosphorylated by ATM; Sp1 with serine 101 mutated to alanine (S101A) is not significantly phosphorylated in response to damage and cannot restore increased sensitivity to DNA damage of cells depleted of Sp1. Together, these data show that Sp1 is a novel ATM substrate that plays a role in the cellular response to DNA damage. (Mol Cancer Res 2007;5(12):1319–30)

Introduction

Sp1, one of the first gene-specific, metazoan transcription factors identified and cloned, is a ubiquitously expressed essential protein that regulates a variety of cellular and viral promoters (1–5). Sp1 binds to DNA elements known as GC boxes [5'-(G/T)GGGCGG(G/A)(G/A)(G/T)-3'] via three Cys₂His₂ zinc finger domains, and interacts with the general transcription machinery through two glutamine-rich transactivation domains, designated A and B (Fig. 1; refs. 3, 6–9). The majority of TATA-less genes have multiple Sp1 sites in the proximal promoter region, and more than half of expressed genes are TATA-less (10). As such, Sp1 plays a global role in controlling gene expression.

Sp1 activity is significantly regulated through posttranslational modifications, including phosphorylation, O-linked glycosylation, acetylation, and sumoylation (11–13). The most studied modification is phosphorylation; Sp1 is phosphorylated by several kinases *in vitro*, including DNA-dependent protein kinase (DNA-PK), casein kinase II, and cyclin A/cdk2, resulting in both positive and negative effects on transcription (11, 14–16). Responses to a variety of stimuli in a wide array of genes have been ascribed to Sp1, although the underlying mechanisms by which specificity is achieved have generally not yet been elucidated (17, 18). Phosphorylation of Sp1 also coincides with the transition from G₀ to G₁ (19) and the progression of viral infection (11, 20).

Several studies have also indirectly implicated Sp1 in the cellular response to DNA damage. In human cell lines exposed to ionizing radiation, Sp1 DNA-binding activity has been shown to increase in a transient and reversible manner (21, 22). Also, in cortical neurons, Sp1 DNA binding was shown to increase in response to oxidative stress, and reduction of Sp1 expression by antisense oligonucleotides decreased viability of neurons exposed to oxidative stress (23).

The human genome faces continuous threat of DNA damage by reactive oxygen species generated during aerobic respiration, cellular oxidase activity, and exposure to ionizing radiation (24). Reactive oxygen species-induced DNA damage includes small or bulky modifications to bases and sugars, interstrand and intrastrand cross-links, as well as single-strand breaks and double-strand breaks (DSB; refs. 24, 25). Molecular networks that rapidly sense and repair damage have evolved to maintain genomic stability and ensure cell survival (26).

Most threatening to genomic stability are DSBs, which activate the phosphatidylinositol 3-kinase-related kinases (PIKK), including ataxia-telangiectasia mutated (ATM), DNA-PK, and ATM and Rad3-related (ATR; ref. 27). Cells deficient in PIKKs exhibit accumulated oxidative damage, radiation sensitivity, and impaired cell cycle checkpoint activation in response to DNA damage (28). ATM kinase, which is defective in the hereditary cancer-prone disorder ataxia-telangiectasia (A-T), is activated by DSBs and phosphorylates a variety of proteins involved in the DNA damage response, leading to cell cycle checkpoint activation, DNA repair, altered gene expression patterns, and/or apoptosis (26). Among the ATM substrates are several transcription factors, including p53 (29), BRCA-1 (30), ATF2 (31), cyclic AMP-responsive element binding protein (32), E2F1 (33), and nuclear factor-κB regulators NEMO and IKK (34). ATR, which responds to bulky lesions and single-strand DNA generated by

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stalled replication forks or the DSB repair process, shares many substrates with ATM. The histone variant H2AX is phosphorylated by ATM, ATR, and DNA-PK over a large region of chromatin surrounding a DSB (35, 36).

Sp1-regulated genes are potentially regulated by ATM, based on gene expression profile comparisons after induction of genotoxic stress in cells encoding either wild-type or nonfunctional ATM (37). ATM function has also been related to Sp1-dependent expression of insulin growth factor-I receptor, as cells deficient in ATM exhibit decreased insulin growth factor-I receptor expression levels compared with normal fibroblasts (38). However, little is still known regarding the regulation of Sp1 in response to DNA damage. Here, we report that Sp1 is a novel substrate for ATM. The kinetics of Sp1 phosphorylation in response to DNA damage parallel those of H2AX, a marker for DSBs. We identify serine 101 of Sp1 as a critical ATM phosphorylation site and show that ATM-dependent phosphorylation of Sp1 modulates cellular survival in response to DNA damage.

Results

Sp1 Is Phosphorylated in Response to H₂O₂

ATM substrates possess SQ/TQ cluster domains (SCD) and are collectively referred to as SCD proteins (39). The most stringent classification requires a minimum of five SQ/TQ sites within a span of 50 residues with preferred phosphorylation sites generally preceded by a hydrophobic residue such as leucine (40, 41). The Sp1 sequence spanning residues 56 to 102 easily fulfills this criterion, containing five SQ/TQ sites within 47 amino acids (Fig. 1). Sp1 also contains a second SCD domain that meets the less stringent Traven classification (five SQ/TQ within 100 amino acids with a gap of <100 amino acids), possessing 9× SQ/TQ within a span of 147 residues (249–432). Interestingly, these domains reside within Sp1 transactivation domains A and B (42).

Based on the presence of putative SCDs in Sp1 and its reported association with DNA damage, we sought to determine whether Sp1 phosphorylation was stimulated by DNA damage. We initially explored this by treating cells with hydrogen peroxide. H₂O₂ is a relatively stable reactive oxygen species that diffuses freely into cells, and the resulting increase in intracellular reactive oxygen species production leads to the induction of varied DNA damage, including DSBs (25).

Normal human diploid fibroblasts (NHDF) in mid-log phase were exposed to H₂O₂, and total cell lysates were prepared after 1 h. Cellular lysates were assayed for Sp1 phosphorylation by immunoblot. Phosphorylation of Sp1 has been reported to induce a shift in electromobility of Sp1 from 95 to 105 kDa (11). Sp1 from untreated cells migrates in SDS-PAGE as a major species of 95 kDa and a minor species of 105 kDa. The shift to 105 kDa was found to be concentration dependent; it is detected in cells exposed to 50 μmol/L H₂O₂, and becomes the major Sp1 species in cells exposed to 200 μmol/L H₂O₂ (Fig. 2A, *top*). To verify the relationship between Sp1 phosphorylation and H₂O₂-induced DSB induction, we examined the PIKK-dependent phosphorylation of the histone variant H2AX, which occurs in response to DSB induction (36, 43). Immunoblot analysis of phosphorylated H2AX (γH2AX) revealed a concentration-dependent increase in γH2AX after H₂O₂ exposure (Fig. 2A, *middle*). To verify that the shift in Sp1 migration was due to phosphorylation, immunoprecipitated Sp1 from H₂O₂-treated cells was incubated with phosphatase and analyzed by Sp1 immunoblot. As shown in Fig. 2B, treatment with phosphatase resulted in the loss of the 105 kDa form and a corresponding increase in the 95 kDa form.

We next investigated the kinetics of Sp1 phosphorylation after H₂O₂ treatment. Extracts of NHDFs were prepared at various time points after exposure to 200 μmol/L H₂O₂. Phosphorylation was observed as quickly as 15 min post-treatment, reaching peak levels at 1 h (Fig. 2C, *top*). The change in the phosphorylation of Sp1 was also found to be transient, as the 95 kDa form of Sp1 became the predominant species by 8 h. Immunoblot analysis of γH2AX revealed rapid and transient kinetics of H2AX phosphorylation (corresponding to DSB induction and repair; ref. 44), which paralleled the rapid and transient kinetics of Sp1 phosphorylation (Fig. 2C, *middle*). These phosphorylation kinetics are consistent with those observed for other ATM substrates (45), suggesting that Sp1 may be a target of the ATM kinase pathway. Changing medium to remove H₂O₂ had no effect on the level or the kinetics of phosphorylation; therefore, the medium was not changed in subsequent experiments (data not shown).

To further establish a link between DSBs, activation of ATM, and phosphorylation of Sp1, cells were also treated with

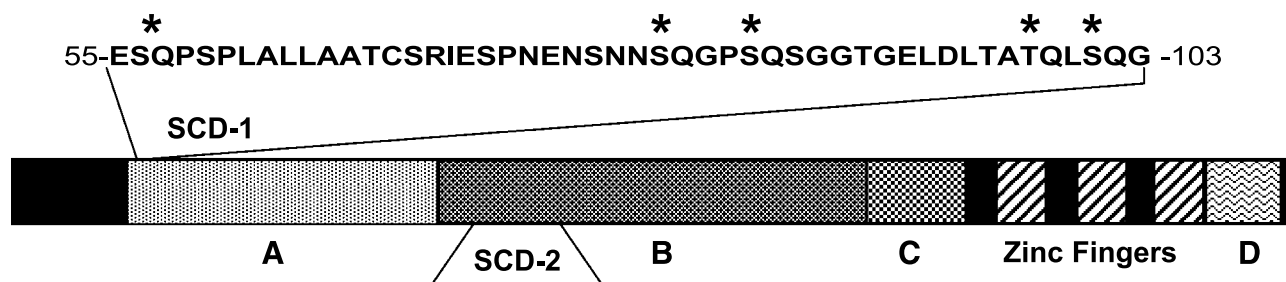


FIGURE 1. Schematic diagram of Sp1. Sp1 has been divided into five domains based on amino acid composition and functional analysis of deletion mutants (42). A and B are transactivation domains (ca. amino acids 85–220 and 261–495, respectively) that have serine/threonine-rich and glutamine-rich segments. SCDs within each of these are shown. The amino acid sequence of the SCD in the A domain is shown above. The C domain (ca. amino acids 496–610) is a highly charged transactivation domain; the DNA-binding domain (amino acids 615–708) contains three zinc finger motifs; and the D domain (ca. amino acids 708–785) mediates Sp1 multimerization and superactivation (76).

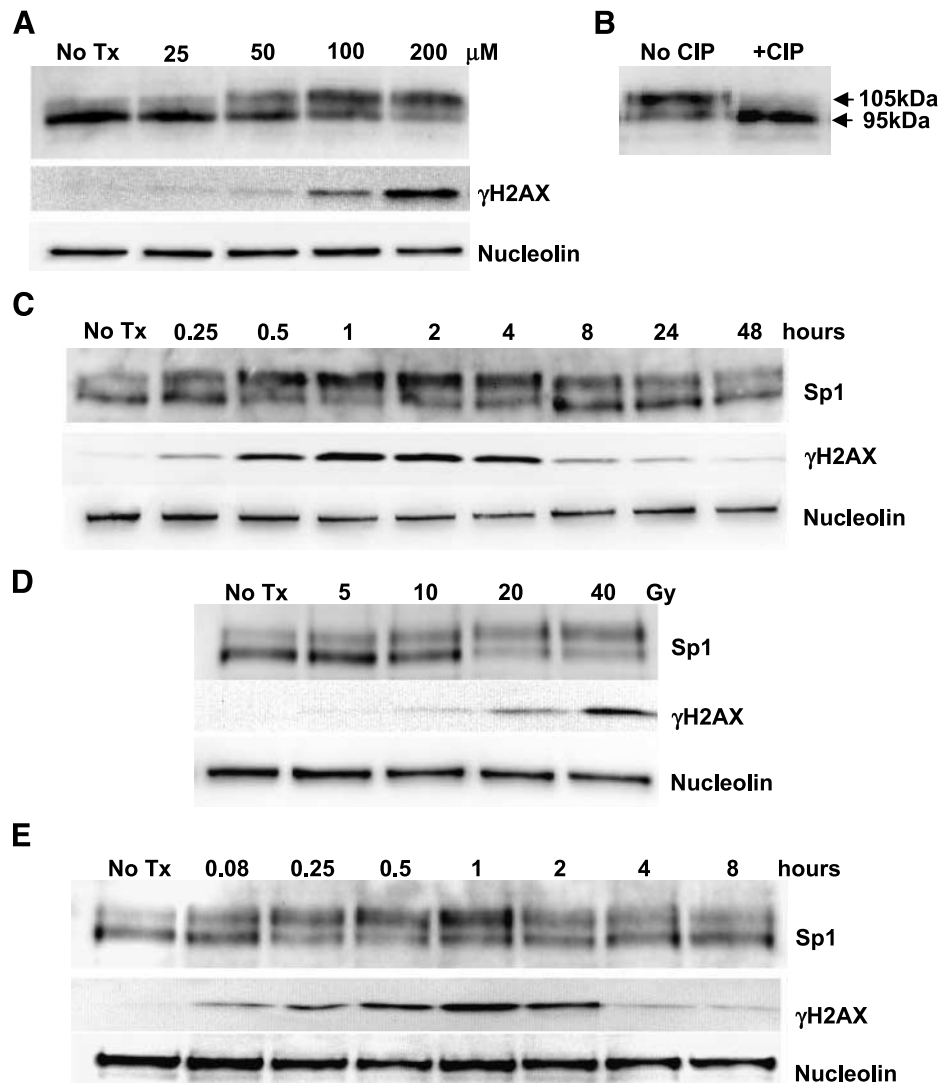


FIGURE 2. Dose-dependent modification of Sp1 in response to H_2O_2 and ionizing radiation. NHDF cells were treated as described followed by SDS-PAGE and immunoblot with the indicated antibody. **A.** Immunoblot of Sp1 (top), γ H2AX (middle), and nucleolin (bottom) prepared from NHDFs exposed to various concentrations of H_2O_2 . After 1 h, cells were harvested directly in SDS sample buffer. **B.** NHDF cells were exposed to 200 μ mol/L H_2O_2 for 1 h and lysed under nonreducing conditions. Immunoprecipitated Sp1 was incubated in the presence or absence of 6 units of calf intestinal phosphatase (CIP), followed by immunoblot as described in **A.** **C.** NHDFs exposed to 200 μ mol/L H_2O_2 for the indicated times and harvested directly in SDS sample buffer. Immunoblot was done with antibody specific to Sp1, γ H2AX, or nucleolin, as indicated. **D.** NHDF cells were treated with the indicated dose of ionizing radiation and harvested after 15 min. **E.** NHDF cells were treated with 20 Gy ionizing radiation and harvested at the indicated time points. Immunoblot was done with antibody specific to Sp1 (top), γ H2AX (middle), and nucleolin (bottom) as indicated.

ionizing radiation. As shown in Fig. 2D and E, exposure of NHDF cells to ionizing radiation induced the phosphorylation of Sp1, with greater radiation doses resulting in a shift to the 105 kDa form. Phosphorylation was detected within 5 min, with the 105 kDa form of Sp1 predominant until \sim 2 h after exposure. This transient shift in Sp1 phosphorylation paralleled the transient phosphorylation of H2AX.

The ATM Pathway Mediates the Phosphorylation of Sp1

Oxidative stress and ionizing radiation induce the PIKK family, ATM, ATR, and DNA-PK. To identify the signaling pathway(s) involved in mediating Sp1 phosphorylation in response to DNA damage, we tested the effect of wortmannin and KU55933, a specific ATM inhibitor (46), on peroxide-induced Sp1 phosphorylation. Wortmannin inhibits ATM but not ATR at 20 μ mol/L (27), whereas higher doses inhibit ATM, ATR, and DNA-PK. Phosphorylation of Sp1 and H2AX in response to H_2O_2 was markedly reduced in cells pretreated with 20 μ mol/L wortmannin and abolished in cells pretreated with 100 μ mol/L wortmannin (Fig. 3A). Pretreatment with

10 μ mol/L KU55933 also prevented Sp1 phosphorylation. These findings indicate that the ATM kinase is the primary mediator of peroxide-induced Sp1 phosphorylation, although other PIKK family members may also target Sp1 to a lesser extent.

To more directly assess the role of DNA-PK, which phosphorylates Sp1 *in vitro* (11), in the peroxide-induced phosphorylation of Sp1, we exposed human glioblastoma cell lines with and without functional DNA-PK to H_2O_2 and found a moderate induction of Sp1 phosphorylation in both cell types (Fig. 3B). These findings suggest that DNA-PK is dispensable for Sp1 phosphorylation in response to H_2O_2 .

Next, we compared Sp1 and H2AX phosphorylation in fibroblasts lacking functional ATM with wild-type fibroblasts from a related donor. In these primary ATM wild-type fibroblasts, Sp1 was found entirely in the 105 kDa form after a 1 h exposure to 25 μ mol/L H_2O_2 (Fig. 3C). H2AX phosphorylation was also induced, reaching maximal levels at 50 μ mol/L H_2O_2 . In contrast, the related primary fibroblasts lacking functional ATM did not induce significant Sp1

phosphorylation, and H2AX phosphorylation was detectable only at higher concentrations of H₂O₂. Taken together, our findings support the notion that the phosphorylation of Sp1 induced by H₂O₂ is primarily ATM dependent.

Sp1 Is Phosphorylated on S101 in Response to DNA Damage

PIKKs, like other kinases that reportedly phosphorylate Sp1, phosphorylate serine and/or threonine residues (47). As shown in Fig. 4A, Sp1 is primarily phosphorylated on serine(s) after H₂O₂ exposure. Based on the presence of two distinct SCDs and our phospho-amino acid results, mutations were made in nine serine residues within the two SCDs (36/56/81/85/101/291/296/313/431) of Sp1 either alone or in various combinations. To examine the phosphorylation of these residues in the context of DNA damage, HeLa cells were transfected with expression vectors encoding either HA-Sp1^{WT} or with Ser→Ala substitutions. Most of these mutations had little or no effect on Sp1 phosphorylation in response to DNA damage (data not shown). In contrast, the Sp1^{S101A} mutant remained largely at 95 kDa after H₂O₂ treatment whereas the HA-Sp1^{WT} shifted to 105 kDa as expected (Fig. 4B).

To verify that S101 is phosphorylated in response to DNA damage, a phosphospecific antibody that specifically recognizes phosphorylated S101 was generated by 21st Century Biochemicals, Inc. In immunoblot experiments, this antibody, designated γ Sp1¹⁰¹, detects Sp1 in cells treated with H₂O₂ but does not detect Sp1 in untreated cells (Fig. 4C). Further, in cells depleted of endogenous Sp1 by double-stranded

small interfering RNA (siRNA) oligonucleotides targeting the 3' untranslated region (UTR) of Sp1 mRNA, exogenously expressed HA-Sp1^{WT} is detected by γ Sp1¹⁰¹, whereas Sp1^{S101A} is not (Fig. 4D). Taken together, these data strongly support that S101 of Sp1 is phosphorylated in response to DNA damage.

Sp1 Depletion Renders Cells More Sensitive to DNA Damage

GC-rich regions of the genome are unusually sensitive to DNA damage by alkylating agents and represent more open regions of chromatin (48-51). Because Sp1 binds to GC boxes, we sought to determine the effect of Sp1 depletion on the induction of DSBs by ionizing radiation. NHDF cells were transfected with Sp1 siRNA, followed by treatment with low-dose ionizing radiation (0.5 Gy). At this dose, foci of γ H2AX around DSB sites are visible by indirect immunofluorescence (35). Cells were processed for immunofluorescence 10 min, 4 h, and 16 h after exposure. As shown in Fig. 5A, γ H2AX foci were observed 10 min and 4 h after exposure of cells depleted of Sp1 with siRNA to ionizing radiation, whereas foci were much less frequent and fewer in number in cells transfected with a nontargeting control siRNA. Foci were counted in at least 30 cells in a blinded fashion. An average of seven foci per cell were observed 10 min after treatment of cells depleted of Sp1 compared with less than two foci for the control cells (Fig. 5B). Analysis of data by Student's *t* test revealed that the number of γ H2AX foci observed in Sp1-depleted cells was significantly higher compared with cells transfected with

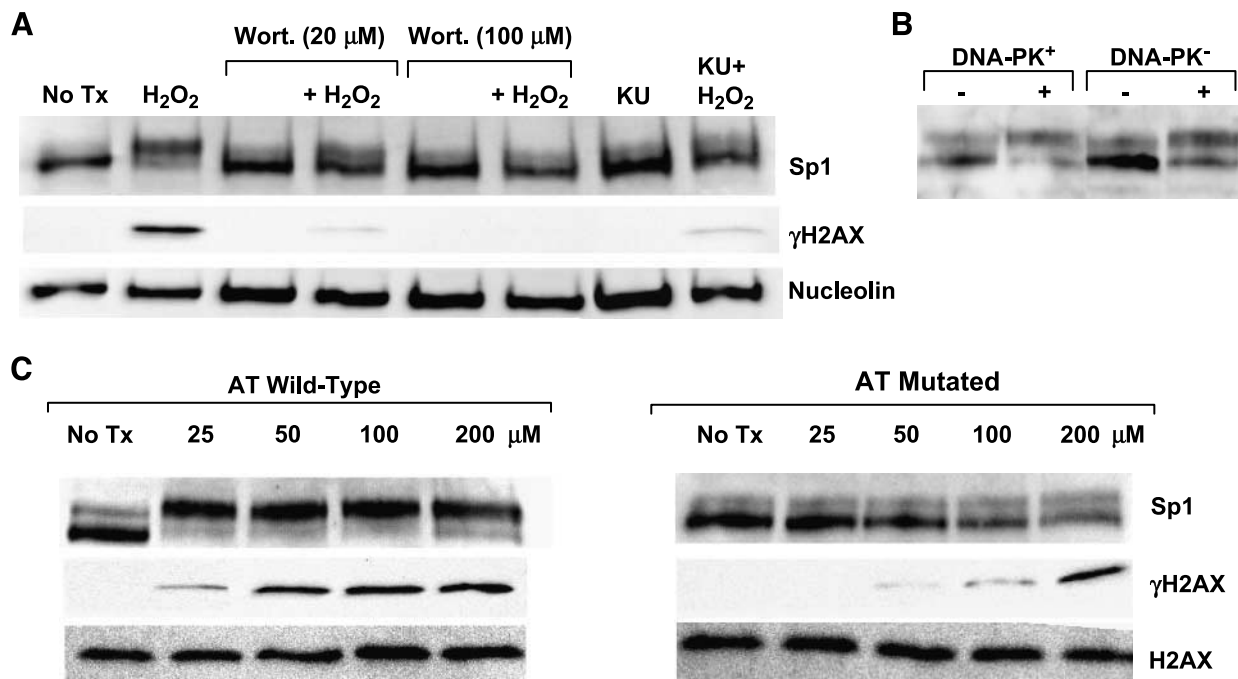


FIGURE 3. ATM dependence of H₂O₂-induced Sp1 phosphorylation. **A.** Immunoblot of Sp1 (top), γ H2AX (middle), and nucleolin (bottom) from NHDFs exposed to 200 μ mol/L H₂O₂ in the presence or absence of 20 or 100 μ mol/L wortmannin (Wort.) or 10 μ mol/L KU55933 (KU). **B.** Immunoblot of Sp1 from M059K (DNA-PK⁺) and M059J (DNA-PK⁻) glioblastoma cells untreated (-) or exposed to 200 μ mol/L H₂O₂ (+) for 1 h. **C.** GM03491 (AT wild-type fibroblasts) and GM02052 (AT mutant fibroblasts) were exposed to the indicated concentrations of H₂O₂ for 1 h, followed by direct lysis in SDS sample buffer, SDS-PAGE, and immunoblot with antibodies to Sp1, γ H2AX, or total H2AX, as indicated.

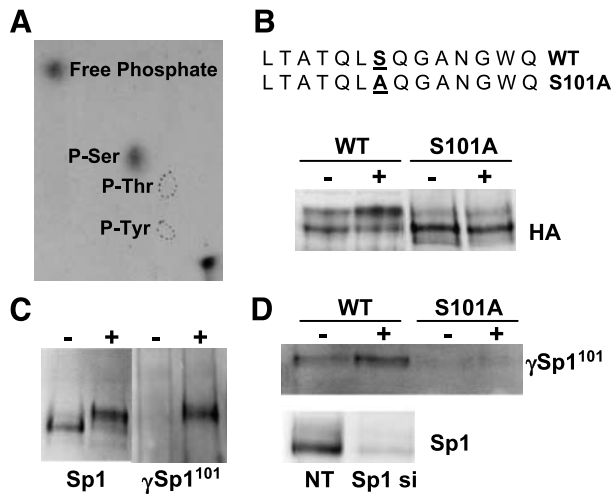


FIGURE 4. DNA damage–induced phosphorylation of Sp1 serine 101. **A.** Phospho–amino acid analysis of Sp1 from NHDFs exposed to 200 $\mu\text{mol/L}$ H_2O_2 for 1 h in the presence of $^{32}\text{P}[\text{P}_i]$. Immunoprecipitated Sp1 was resolved by SDS–PAGE, transferred to polyvinylidene difluoride membrane, hydrolyzed, spotted on cellulose-coated plates, and separated by two-dimensional electrophoresis. **B.** Serine 101 was mutated to Ala (S101A) by site-directed mutagenesis of a HA-tagged Sp1 expression construct. HeLa cells were transfected with either the wild-type or mutant expression constructs. Forty-eight hours after transfection, cells were untreated (–) or exposed to 400 $\mu\text{mol/L}$ H_2O_2 for 1 h (+) before lysis under nondenaturing conditions. Lysates were separated by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with HA-specific antibody. **C.** NHDFs were treated for 1 h with 200 $\mu\text{mol/L}$ H_2O_2 (+) or untreated (–), followed by lysis, SDS–PAGE, and immunoblot with polyclonal antibody to Sp1 (pAb581) or γSp1^{101} . **D.** NHDFs were depleted of endogenous Sp1 by siRNA [bottom, immunoblot of Sp1 from cells 72 h posttransfection with Sp1 siRNA or nontargeting (NT) siRNA]. Seventy-two hours after siRNA transfection, cells were transfected with HA–Sp1^{WT} or HA–Sp1^{S101A}. Forty-eight h after transfection, cells were untreated (–) or exposed to 200 $\mu\text{mol/L}$ H_2O_2 for 1 h (+), followed by direct lysis and immunoblot with the indicated antibodies.

nontargeting siRNA ($P < 0.01$ at 10 min and $P = 0.024$ at 4 h). As shown in Fig. 5C, γH2AX foci in U2OS were observed at 10 min, 4 h, and 16 h after exposure of cells depleted of Sp1 with siRNA to ionizing radiation with a similar pattern as seen in NHDF. Fewer foci were observed in cells transfected with nontargeting control siRNA. Foci were counted in at least 30 cells in a blinded fashion. An average of 36 foci per cell were observed 10 min after treatment of cells depleted of Sp1 compared with 14 foci for the control cells; U2OS cells depleted of Sp1 still maintained a high number of foci (an average of 10) 16 h after treatment, whereas the U2OS cells transfected with nontargeting control siRNA had an average of two foci after 16 h (Fig. 5D). The number of γH2AX foci observed in Sp1-depleted cells was significantly higher ($P < 0.001$) than in cells exposed to a nontargeting siRNA at all time points. Taken together, these data suggest that Sp1 may play a role in protecting DNA from the induction of DSBs by ionizing radiation.

To further examine whether Sp1 modulates the cellular response to DNA damage, the effect of Sp1 on cell survival after exposure to ionizing radiation or H_2O_2 was assessed by clonogenic survival assays in NHDF or U2OS cells that were depleted of Sp1 using siRNA. Transfection of double-stranded siRNA into NHDF and U2OS cells resulted in a 90%

knockdown of Sp1 protein levels 3 days posttransfection (Fig. 6, inset). Cell morphology and growth were not significantly perturbed by Sp1 siRNA (data not shown). Cells depleted of endogenous Sp1 exposed to 50 or 100 $\mu\text{mol/L}$ H_2O_2 showed decreased colony formation relative to cells transfected with a nontargeting siRNA (Fig. 6A and C). Similarly, cells depleted of Sp1 also showed decreased colony formation after exposure to 0.5, 1, 2.5, or 5 Gy ionizing radiation relative to a nontargeting control siRNA (Fig. 6B and D).

To determine whether the decreased cell viability detected by the colony survival assays resulted from an increase in apoptosis, terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assays were done in U2OS cells treated with siRNA compared with nontargeting control siRNA. U2OS cells were used in this assay because fibroblasts do not normally undergo apoptosis but enter senescence. U2OS cells were transfected with siRNA, followed by treatment with 400 $\mu\text{mol/L}$ H_2O_2 for 4 h. Cells were processed for immunofluorescence and, as shown in Fig. 7A, the number of TUNEL-positive cells (indicative of fragmented DNA) after treatment was significantly increased in cells depleted of Sp1. Quantification of these results revealed that 76% of the cells depleted of Sp1 with siRNA are TUNEL positive after H_2O_2 treatment, compared with 10% of cells transfected with nontargeting control siRNA (Fig. 7B). The significant ($P < 0.01$) increase in DNA fragmentation in cells depleted of Sp1 with siRNA corresponds to the increase in γH2AX foci and the decrease in cell viability resulting from depletion of Sp1 (Figs. 5 and 6).

Experiments were next done to determine whether the increased sensitivity of cells after Sp1 depletion by RNA interference was related to Sp1 phosphorylation. Colony survival assays were done in U2OS cells, which can be more effectively transfected with exogenous Sp1 (~60%) compared with fibroblasts. Sp1 is phosphorylated on serine 101 in U2OS cells exposed to varying concentrations of H_2O_2 in a manner similar to NHDF as seen by γSp1^{101} antibody (Fig. 8B). As shown in Fig. 8A, Sp1 depletion by Sp1 siRNA inhibited colony survival of U2OS cells in response to H_2O_2 in a concentration-dependent manner. Expression of wild-type Sp1 restored survival to the level observed with nontargeting RNA interference, rescuing cells from the effect of Sp1 depletion at every concentration of H_2O_2 tested. In contrast, expression of Sp1^{S101A} (which is not phosphorylated in response to damage) failed to rescue cells from the effects of Sp1 depletion on survival. Equivalent expression of wild-type Sp1 and Sp1^{S101A} is shown in Fig. 8B. These results show that the phosphorylation of Sp1 on serine 101 by ATM plays an important role in modulating the cellular response to DNA damage.

Discussion

These studies have established that Sp1, a global regulator of gene expression, is a target of the ATM-dependent DNA damage response pathway. Although the precise role of Sp1 phosphorylation in the DNA damage response is not entirely clear, the parallel kinetics of phosphorylation/dephosphorylation with H2AX, the increased sensitivity to DNA-damaging agents in cells depleted of Sp1, and the rescue of this sensitivity

with wild-type Sp1 but not the phosphorylation site mutant, Sp1^{S101A}, suggest that Sp1 is an early target of the DNA damage response pathway and may play a role in modulating the cellular response to DNA damage.

Sp1 is a member of the Sp/KLF family of transcription factors, which bind GC elements in a wide array of promoters (17). These factors compete for binding to the same elements depending on cellular conditions (22, 52-54). Sequence comparison among the Sp proteins [Sp2 (accession no. Q02086), Sp3 (accession no. Q02447), and Sp4 (accession no. Q02446)] reveals that Sp1 is the only member to contain consensus SCD (e.g., moreover, SCDs were not found in other KLF family members). Given that Sp1 is the only KLF family member containing SCDs, other KLF family members likely cannot compensate for the unique role of Sp1 in the DNA damage response, suggesting that this may explain the essential role of Sp1 accounting for the embryonic lethality of Sp1 knockout mice (4).

Identification of SCDs within the transactivation domains of Sp1 suggested that it was a likely substrate of PIKKs. Use of specific inhibitors and kinase-deficient cells revealed that treatment of cells with H₂O₂ or ionizing radiation induced ATM-dependent phosphorylation of Sp1. Our data do not rule out the possibility that other kinases may contribute to the overall phosphorylation. For example, phosphorylation of Sp1

by ATR is suggested by the delayed phosphorylation in the presence of the specific ATM inhibitor, KU55933, as well as by the phosphorylation at higher H₂O₂ concentrations in ATM mutated cells. Kinases downstream of ATM (e.g., Chk2) may also contribute to damage-induced phosphorylation of Sp1. Although DNA-PK has been reported to phosphorylate Sp1 *in vitro* (11) and in cells exposed to etoposide (55), the finding that Sp1 phosphorylation in response to H₂O₂ was not affected by the absence of DNA-PK suggests that it may not be involved (Fig. 3B).

Serine 101 is within one of the SCDs and mutation of this site to alanine abolishes peroxide-induced phosphorylation of Sp1. Although it is likely that other sites are phosphorylated in response to DNA damage, the significant reduction in phosphorylation in the S101A mutant suggests that S101 may serve to signal phosphorylation of additional Ser residues. Moreover, mutation of serine 101 to glutamic acid (S101E) to mimic phosphorylation results in a phosphorylation-dependent shift in the mobility of Sp1 from 95 to 105 kDa even in the absence of damage (data not shown).

Recently, a large-scale proteomic search based on the presence of previously identified SQ motifs phosphorylated by ATM revealed over 700 proteins phosphorylated by ATM and ATR, including many previously unknown ATM/ATR substrates (56). Proteins identified in this screen were found

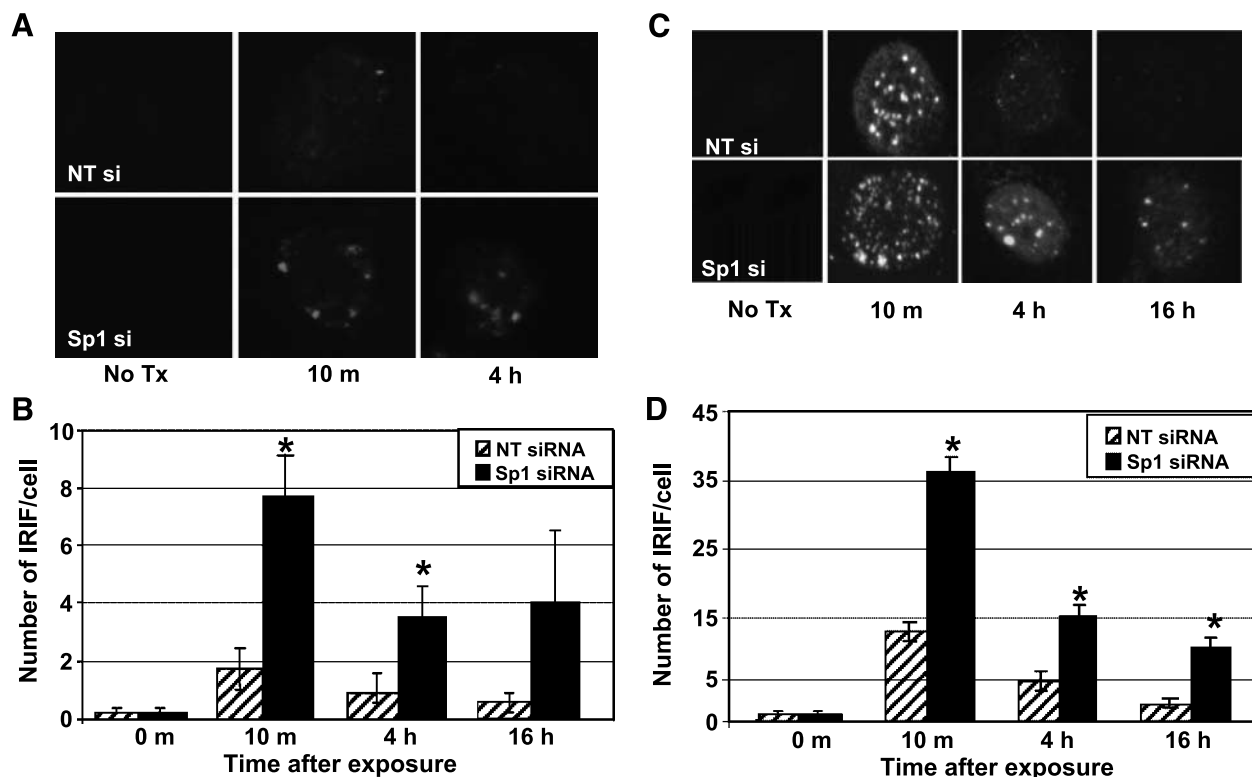


FIGURE 5. Effect of Sp1 on formation of ionizing radiation–induced γ H2AX foci. NHDF (**A** and **B**) or U2OS (**C** and **D**) cells were transfected with siRNA directed against the 3' UTR of Sp1 or nontargeting control siRNA as described in Materials and Methods. Seventy-two hours after transfection, cells were exposed to 0.5 Gy ionizing radiation and at the indicated time points after ionizing radiation exposure, cells were processed for indirect immunofluorescence and probed with antibodies to γ H2AX. **A** and **C**. Representative cells from each group. **B** and **D**. Quantitative analysis. Foci of γ H2AX were counted in at least 30 cells. Columns, average number of foci per cell; bars, SE. *, significant differences of $P < 0.01$ at 10 min and $P = 0.024$ at 4 h in **B** and $P < 0.001$ at all time points in **D**.

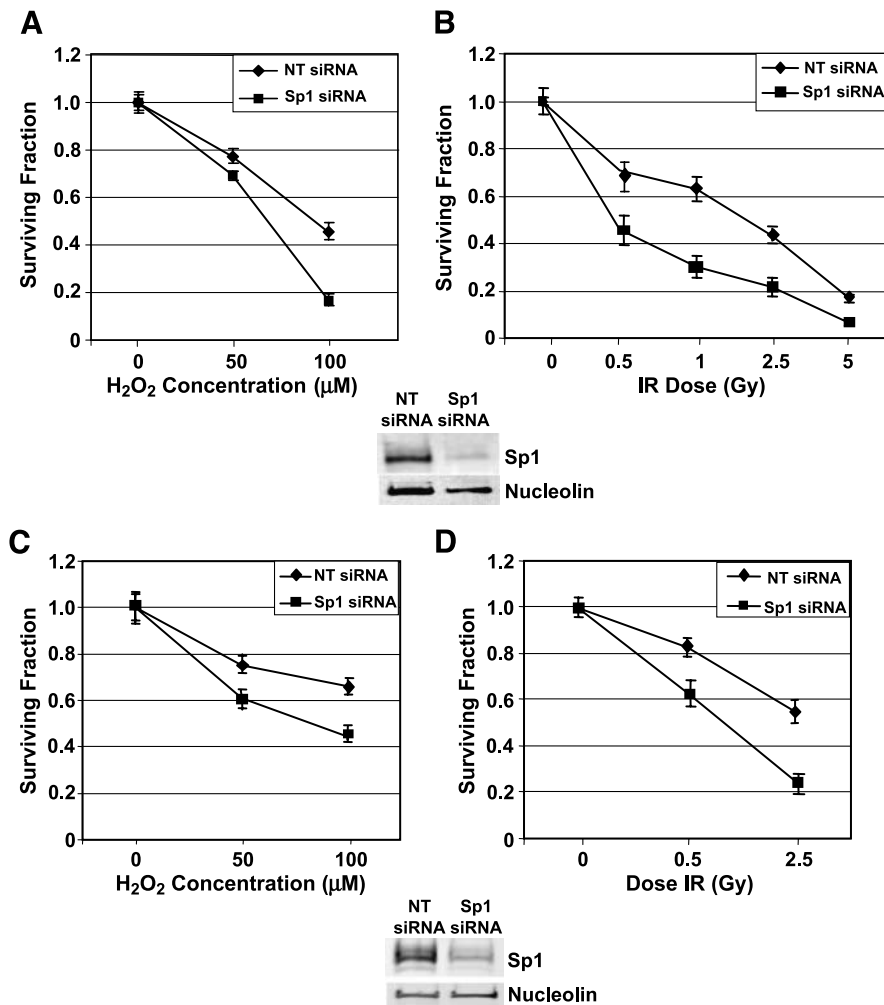


FIGURE 6. Sp1 knockdown decreases cell survival after H₂O₂ or ionizing radiation exposure. NHDFs (**A** and **B**) or U2OS (**C** and **D**) were transfected with siRNA directed against the 3' UTR of Sp1 or nontargeting control siRNA as described in Materials and Methods. Seventy-two hours after siRNA transfections, cells were exposed to 50 or 100 μmol/L H₂O₂ (**A** and **C**) or 0.5, 1, 2.5, or 5 Gy ionizing radiation (**B**) or 0.5 or 2.5 Gy ionizing radiation (**D**) followed by clonogenic survival assay. Western blots below each graph show Sp1 (*top*) and nucleolin (*bottom*) levels 72 h after transfection with Sp1 or nontargeting control siRNA in NHDF and U2OS cells.

to modulate the number of spontaneously formed DSBs, levels of γ H2AX after ionizing radiation exposure, cell cycle checkpoint activation, or levels of homologous recombination. These findings indicate that large networks of proteins are involved in modulating cellular survival after DNA damage. Interestingly, Sp1 as well as other transcription factors that have been shown to be ATM substrates were not identified in this study. The failure to detect these substrates may have resulted from the use of trypsin cleavage before mass spectrometry. In our experience and according to mass spectrometry protein cleavage prediction programs, such as Protein Prospector,¹ fragments containing the consensus ATM SQ/TQ sites including S101 are more than twice the size needed for efficient detection by mass spectrometry after cleavage of Sp1 with trypsin.

¹ <http://prospector.ucsf.edu>

Depletion of factors involved in the DNA damage response results in increased sensitivity to DNA damage, as shown in cells lacking functional ATM kinase or BRCA1 (57-60), as well as in cells deficient in NBS1, FANC-A, FANC-D, and MRE11 (61-67). Our results show that, like many other ATM/ATR substrates, depletion of Sp1 renders cells more sensitive to the effects of DNA damage. Moreover, the sensitivity is mediated by the phosphorylation of Sp1, as the phosphorylation site mutant failed to rescue cells depleted of endogenous Sp1. Although the mechanism whereby Sp1 modulates cell survival remains to be determined, we find an increased number of DSBs when cells deficient in Sp1 are exposed to ionizing radiation (Fig. 5), which suggests that the lack of Sp1 may leave GC-rich regions of the genome more vulnerable to DSBs.

The DNA damage response involves ATM-dependent changes in gene expression patterns (67-69). When cells with and without functional ATM were exposed to ionizing radiation, UV, and *t*-butyl hydroperoxide, several Sp1-regulated genes were found to be ATM dependent (37, 70). Studies

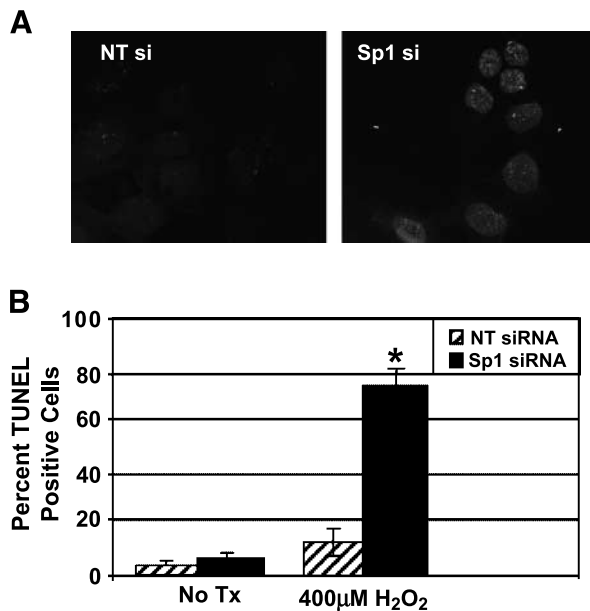


FIGURE 7. Sp1 knockdown increases cell death after H₂O₂ exposure. U2OS were transfected with siRNA directed against the 3' UTR of Sp1 or nontargeting control siRNA as described in Materials and Methods. Seventy-two hours after siRNA transfections, cells were exposed to 400 μmol/L H₂O₂ followed by TUNEL assay after 4 h. **A.** Representative cells from each group. **B.** Quantitative analysis. At least 300 cells were counted in each treatment group. Columns, average number of foci per cell; bars, SE. *, a significant difference ($P < 0.001$).

comparing the expression of the insulin growth factor receptor-1 in normal fibroblasts and AT-mutated fibroblasts showed decreased levels of insulin growth factor-1 receptor in AT-mutated cells and identified Sp1 as a potential mediator between ATM and insulin growth factor-1 receptor expression (38). In addition, ATM is induced by infection of cells with herpes simplex virus-1 and SV40 (71, 72), which also induce Sp1 phosphorylation (11, 20). Kim and DeLuca have shown that phosphorylated Sp1 purified from herpes simplex virus-1-infected cells has decreased ability to activate transcription in *in vitro* transcription assays and that the kinetics of the phosphorylation correlate with the expression of Sp1-independent viral late genes, suggesting a viral-mediated temporal regulation of gene expression. While this article was in review, another group reported that the phosphorylation of Sp1 during herpes simplex virus-1 infection was at S101 and was also seen in response to ionizing radiation (73). Together with our findings, Sp1 is a target of the ATM pathway. Moreover, phosphorylation of Sp1 by ATM may modulate Sp1-dependent gene expression (20). Studies are under way to examine the effect of Sp1 phosphorylation on Sp1-dependent gene expression using the S101A mutant.

This study places Sp1, once thought to be an unregulated, constitutive transcriptional activator, as a factor involved in the response to DNA damage. Sp1 joins a growing number of transcription factors targeted immediately after DNA damage and identification of S101 as a critical phosphorylation site leaves us poised to establish its role. Clearly, more work is needed to fully understand the role of Sp1 in the DNA damage response; however, we have provided evidence that it is a

substrate of ATM and that it is involved in the response evidenced by increased sensitivity to DNA damage. This work further extends the role of Sp1 beyond that of a constitutive transcription factor and provides evidence for a significant role in the response to DNA damage. Whether the phosphorylation of Sp1 by ATM induces changes in chromatin structure that affect sensitivity to DNA damage and/or gene expression or whether it has a more direct role in modulating gene expression requires further study.

Materials and Methods

Cell Lines

NHDFs (Clonetic) were cultured in DMEM (Cellgro, Mediatech, Inc.) with 10% fetal bovine serum (FBS, Equitech), 2 mmol/L L-glutamine (Cellgro, Mediatech), and 100 units/mL

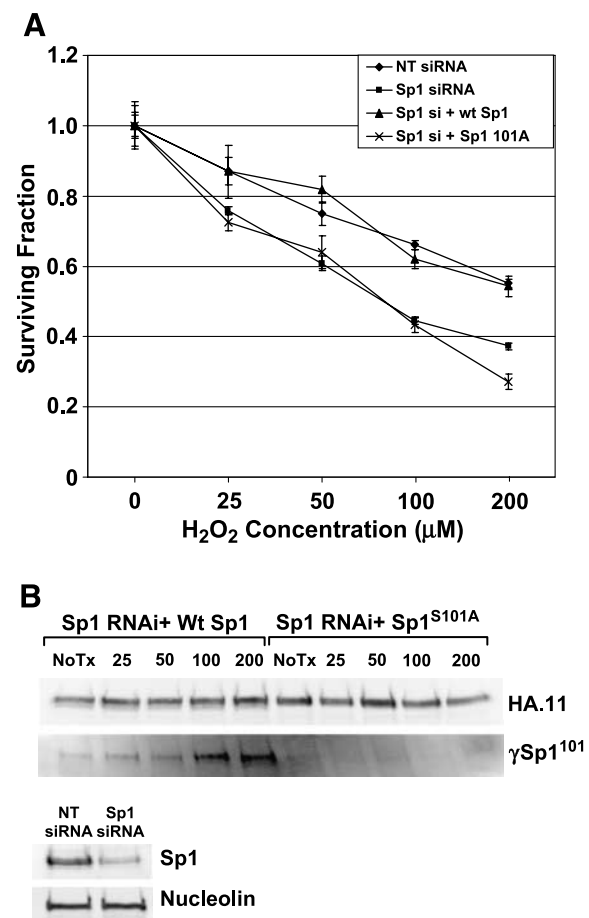


FIGURE 8. Sp1 complementation assay. **A.** U2OS cells were transfected with nontargeting control or Sp1 siRNA. Seventy-two hours after siRNA transfection, cells were transfected with Sp1^{WT} or Sp1^{S101A}. Forty-eight hours after transfection, cells were exposed to the indicated amount of H₂O₂ for 1 h. Cells were then plated at a density of 300 cells per 60-mm dish. After 11 d, discrete colonies were stained with crystal violet and counted in a blinded manner. Data are expressed relative to the number of colonies in the nontreated cells receiving the same DNAs. **B.** U2OS cells were exposed to the indicated concentrations of H₂O₂ for 1 h and harvested directly in SDS sample buffer. Immunoblot was done with antibody specific to HA.11 and γSp1¹⁰¹. Bottom inset, Western blot shows Sp1 (top) and nucleolin (bottom) levels 72 h after transfection with Sp1 or nontargeting control siRNA.

penicillin, and 100 µg/mL streptomycin (Pen/Strep, Sigma) in a 37°C humidified atmosphere of 10%CO₂, 90% air. ATM untransformed fibroblasts from a clinically affected 15-year-old female homozygous for the 103C>T transition in exon 5 of the *ATM* gene (GM02052, Coriell) and untransformed fibroblasts from a clinically unaffected sister of the proband (GM03491, Coriell) were cultured in MEM with Eagle-Earle salts (MEM, Cellgro, Mediatech) containing 15% FBS (Equitech), 2 mmol/L L-glutamine, 1× nonessential amino acids (Sigma), and Pen/Strep at 37°C in a humidified atmosphere of 5% CO₂, 95% air. The M059J human glioblastoma cell line (American Type Culture Collection) lacks DNA-dependent protein kinase activity whereas the M059K human glioblastoma cell line (American Type Culture Collection) from the same donor expresses normal levels of active DNA-dependent protein kinase. These cells were cultured in DMEM/Ham's F-12 50/50 mix (Cellgro, Mediatech) with 10% FBS (Equitech), 2 mmol/L L-glutamine, and Pen/Strep at 37°C in a humidified atmosphere of 10%CO₂, 90% air. The U2OS human osteosarcoma cell line (kindly provided by M. Murphy, Fox Chase Cancer Center, Philadelphia, PA) were maintained in DMEM containing 10% FBS, 2 mmol/L L-glutamine, and Pen/Strep at 37°C in a humidified atmosphere of 10% CO₂, 90% air. Human papilloma virus-transformed cervical epithelial cells (HeLa, University of North Carolina Lineberger Cancer Center Cell Culture Facility) were cultured in DMEM (Cellgro, Mediatech) containing 10% FBS (Equitech), 2 mmol/L L-glutamine (Cellgro, Mediatech), and Pen/Strep at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

Cell Treatments

H₂O₂ (30% w/w solution; Calbiochem) was added directly to the medium of exponentially growing cells. To deliver an accurate volume, it was sometimes necessary to dilute the H₂O₂ in sterile water by a factor of 10 or 100 immediately before adding it to the cell medium. Medium was not changed after H₂O₂ treatment. For cells additionally exposed to wortmannin (Biomol), this agent was dissolved in DMSO (10 mmol/L stock, 20 or 100 µmol/L final) and added to the cell medium 5 to 15 min before the addition of H₂O₂. KU55933 (kindly provided by Graeme Smith of KuDOS Pharmaceuticals Ltd.) was dissolved in DMSO (10 mmol/L stock, 10 µmol/L final) and added 1 h before addition of H₂O₂. For the experiments using ionizing radiation, exponentially growing cells were irradiated at room temperature using an X-ray source (dose rate of 3 Gy/min).

In vitro Phosphatase Assay

Sp1 was immunoprecipitated from NHDFs exposed to 200 µmol/L H₂O₂ for 1 h. Cells were lysed in ice-cold immunoprecipitation buffer (50 mmol/L Tris 8.0, 0.5% Igepal, 120 mmol/L NaCl, 1.5 mg/mL aprotinin, 0.01 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride). The chromatin was sheared by passing the lysate several times through a tuberculin syringe, and the lysate was cleared by centrifugation at 14,000 × g for 10 min at 4°C. Approximately 500 µg of lysate were mixed with 8 µg Sp1-specific antibody (PEP-2G, Santa Cruz Biotechnology). The mixture was

incubated for 1 h at 4°C with rocking, followed by the addition of protein G slurry (Sigma). After 2 h incubation at 4°C with rocking, immunocomplexes were washed thrice in immunoprecipitation buffer and incubated with or without 6 units calf intestinal alkaline phosphatase (Promega; 1 unit/µL) for 40 min at 30°C, according to the manufacturer's instructions. The reaction was stopped by centrifuging the protein G slurry, removing the supernatant, and adding 2× SDS sample buffer to the immune complexes. Sp1 electrophoretic mobility was detected by immunoblot using a Sp1-specific antibody.

Phospho-Amino Acid Analysis

NHDFs were plated at 1.5×10^6 cells in a 10-cm dish 24 h before the experiment. On the day of the experiment, the cells were washed thrice in 1× HBS. The cells were then incubated in phosphate-free DMEM supplemented with 10% dialyzed FBS for 2 h at 37°C. NHDFs were then exposed to 200 µCi ³²P-P_i (NEN) per milliliter of medium for 2.5 h. NHDFs were then treated with 200 µmol/L H₂O₂ for an additional hour. The cells were washed once in 1× HBS and then harvested in 400 µL of lysis buffer [10 mmol/L Tris (pH 7.4), 1 mmol/L DTT, 1% SDS]. The lysate was boiled at 95°C for 6 min and 800 µL of cold immunoprecipitation buffer was added [15 mmol/L Tris (pH 7.4), 230 mmol/L NaCl, 1.5% Triton X-100, 0.75% Igepal, 7.5 mmol/L EDTA, 0.02 mg/mL leupeptin, 2 mg/mL pepstatin A, 3 µg/mL aprotinin, 2 mmol/L phenylmethylsulfonyl fluoride, 15 mmol/L NaF, and 3 mmol/L NaVO₄]. The lysate was sheared with a tuberculin syringe and centrifuged at 14,000 × g for 15 min at 4°C. The supernatant was transferred to a new tube and rocked with Sp1-specific antibody (pAb581, polyclonal, made at Roswell Park Cancer Institute against amino acids 524-543) for 30 min at 4°C. Twenty-five microliters of protein A:Sepharose beads (Sigma) were added and rocked overnight at 4°C. Beads were washed thrice in 0.5 mL radioimmunoprecipitation assay buffer. Samples were boiled in 30 µL 2× SDS sample buffer for 5 min at 90°C. Samples were resolved on 6.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Blots were exposed to film overnight. The band corresponding to the signal on the developed film was cut from the polyvinylidene difluoride membrane. The excised membrane was washed in methanol once and four times in water and transferred to a tube containing 200 µL of boiling HCl (Sigma) and boiled for 1 h at 110°C. Supernatant was transferred to a new tube and dried and resuspended in 5 µL buffer (pH 1.9; 50 mL formic acid, 156 mL glacial acid, 1794 mL distilled H₂O) and with 5 µL phospho-amino acid standards (1 mg/mL phospho-serine, phospho-threonine, phospho-tyrosine) added. The sample was added to a nitrocellulose plate (EM Science) in 0.5 µL drops and run in Hunter Thin Layer Peptide Mapping System at 1.5 kV for 25 min. After air drying the plate, it was rewet in buffer (pH 3.5; 100 mL glacial acetic acid, 10 mL pyridine, 1890 mL dH₂O) and the plate was turned 90° counterclockwise and run in Hunter Thin Layer Peptide Mapping System at 1.3 kV for 20 min. The plate was allowed to air dry after the second run then sprayed with ninhydrin and baked at 65°C for 10 min to visualize standards. The plate was exposed to a phosphoscreen for 24 h.

Site-Specific Mutagenesis

Point mutations of various SQ sites located in the trans-activation domains of Sp1 were made using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The amino acid numbers are based on Sp1 sequence containing 785 amino acids (National Center for Biotechnology Information accession number NM_138473, NP_612482). The following primer sequences were used:

- S101A: 5'-CCTCACAGCCACACAACTTGCCACAGGGT-GCCAATGGC-3';
 S101E: 5'-GACCTCACAGCCACACAACTTGAACAGG-GTGCCAATGGCTGG-3';
 S291A: 5'-GCAGCTCTGGGGCCCCCAGGAGAGTGGC-3';
 S296A: 5'-CCCAGGAGAGTGGGCGCCACAGCCTGTCCAC-3';
 S36A: 5'GGTGGTGGTGCCTTTGCCACAGGCTCGAAGT-AGC-3';
 S56A: 5'-GGAGGGCAGGAGGCCCCAGCCATCCC-3';
 S131A: 5'-GCCAGCTTGGTATCAGCACAAGCCAGTCC-3';
 S431A: 5'-CCTTTACAACTCAAGCCATCGCCCCAGGA-ACCC-3';
 S81,85A: 5'-GAACAGCAACAACGCCCCAGGGCCCG-GCTCAGTCAGGGGAACAGGTGAGC-3'.

QuikChange PCR was done on Sp1 cDNA with a COOH-terminal HA-tag subcloned into the pFLAG-CMV2 plasmid (Sigma). PCR reactions were prepared according to the manufacturer's specifications. Recombinant colonies were screened for the appropriate mutation by fluorescent sequencing.

RNA Interference

We designed RNA oligonucleotides targeting the 3' UTR of Sp1 mRNA. The two targets are 3' UTR³⁴²⁹UCAGUG-GAAUUGUACAAGA³⁴⁴⁷ (siSp1 2) and 3' UTR⁴⁹⁷⁴GGA-AUCUUCUCCAGUAUGA⁴⁹⁹² (siSp1 1) of Sp1 mRNA (NCBI accession no. NM_138473). These were tested separately and together and 200 pmol (100 pmol of each siRNA) were used together to transfect 2×10^5 cells per well of a six-well plate in all experiments shown. As a nontargeting control siRNA, we altered siSp1 1 by one nucleotide: GGAAUCUUCUACCAGUAUGA. This change created a siRNA that did not significantly reduce Sp1 levels. These double stranded RNA oligonucleotides were synthesized by Dharmacon.

Transfections

NHDF, HeLa, or U2OS cells were transfected with wild-type and mutant Sp1 constructs in pFLAG-CMV2 using Metafectene Pro (Biontex) or Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturers' instructions. Approximately 48 h after transfection, cells were either exposed to H₂O₂ for 1 h or were left untreated. Cells were then harvested by direct lysis in 2.0× SDS sample buffer. For siRNA transfections, NHDF or U2OS cells were plated in six-well plates 24 h before transfection. Transfections were carried out using Oligofectamine Reagent (Invitrogen Corp) according to the manufacturer's instructions. Seventy-two hours after transfection, cells were assayed for the presence of Sp1 by immunoblot.

Immunoblot

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by either direct lysis of washed cells in 1.5× SDS sample buffer, or by nondenaturing cell lysis in 20 mmol/L Tris-HCl (pH 7.8), 100 mmol/L NaCl, 0.5% Igepal, 1 mmol/L EDTA, 1.5 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L DTT, 1 μg/mL pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L NaF, 5 mmol/L β-glycerophosphate, and 0.1 mmol/L Na₃VO₄ (each from Sigma). Cells lysed under nondenaturing conditions were incubated at 0°C to 4°C for 15 to 20 min and then transferred to microcentrifuge tubes. Chromatin was sheared by passing the lysate three to four times through a tuberculin syringe. Insoluble material was cleared by centrifugation at 14,000 × g for 10 min at 4°C. Following protein quantitation by the Bradford method, 10 to 20 μg of protein were used for SDS-PAGE. Samples were electrophoresed at 150 V in Tris glycine SDS running buffer [25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (pH 8.3)]. Following electrophoresis, proteins were transferred to nitrocellulose membrane (Schleicher and Schuell) in Tris acetate transfer buffer. Immunoblot was done by blocking membranes in 5% nonfat dried milk (Carnation) in PBS with 0.1% Tween 20 (PBST) followed by incubation with primary antibodies in 5% nonfat dried milk in PBST 14 to 18 h at 4°C with rocking with all antibodies, except γSp1¹⁰¹ in which bovine serum albumin (5%, Fraction V, Fisher Scientific) was used. Primary antibodies used for immunoblot include rabbit polyclonal antibodies specific for Sp1 (pAb581, made at Roswell Park Cancer Institute against amino acids 524-543), mouse monoclonal antibodies specific for γH2AX [phospho-histone H2AX (serine 139), clone JBW301; Upstate], total H2AX (Upstate), α-tubulin (Sigma), nucleolin (C23; Santa Cruz Biotechnology), and HA (HA.11; Covance). Phospho-Sp1^{S101} was made by 21st Century Biochemicals, Inc. Acetyl-DLTATQL[pS]QGANGK-amide produced a high-affinity polyclonal antibody that specifically recognized phosphorylated S101; this antibody was designated γSp1¹⁰¹. The primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) or goat anti-mouse IgG (Santa Cruz Biotechnology). Chemiluminescence was developed using Super Signal (Pierce).

Cytotoxicity Assays

Clonogenic assays were done in NHDFs as follows: 2.0×10^5 NHDFs were seeded in 30-mm dishes for transfection with RNA interference. Seventy-two hours after transfection, cells were exposed to 50 to 100 μmol/L H₂O₂ or 0.5 to 5 Gy ionizing radiation. One hundred NHDFs or 300 U2OS cells were seeded onto 60-mm dishes after exposure to H₂O₂ or ionizing radiation. The cell medium was changed every 3 days. Colonies, which formed between 18 and 24 days after plating for NHDF and 11 days after plating for U2OS, were fixed and stained with a crystal violet solution (0.5% in 20% ethanol) and then counted. Assays were done in triplicate. Clonogenic survival assays in U2OS cells were done as follows: 4.0×10^5 U2OS were seeded in 30-mm dishes and transfected with RNA interference. These cells were trypsinized and counted 72 h after RNA interference transfection and then

transfected with wild-type or S101A mutant Sp1 constructs. Cells were exposed to 25 to 200 $\mu\text{mol/L}$ H_2O_2 48 h after transfection. Three hundred cells were reseeded onto 60-mm dishes 2 h after exposure to H_2O_2 . The cell medium was changed every 3 days. Colonies, which formed 11 days after plating, were fixed and stained with a crystal violet solution (0.5% in 20% ethanol), and then counted. Assays were done in triplicate. Data were analyzed by Student's *t* test.

Immunofluorescence

NHDF or U2OS cells were plated onto glass coverslips 24 h before treatment with 0.5 Gy ionizing radiation. The *in situ* cell fractionation protocol for NHDF was adapted from ref. 74 with modifications. Briefly, cells were washed in ice-cold PBS twice and incubated in ice-cold cytoskeleton buffer [10 mmol/L PIPES (pH 6.8); 100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl_2 , 1 mmol/L EGTA] for 5 min, followed by incubation in ice-cold cytoskeleton stripping buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L MgCl_2 , 1% v/v Tween 40, 0.5% sodium deoxycholate] for 5 min. Cells were then washed in PBS, and fixed using Streck Tissue Fixative (Streck Laboratories) with 50 mmol/L EDTA (pH 5.7; ref. 75) for 30 min at room temperature. Cells were washed thrice in room temperature PBS and blocked in 10% FBS-PBS for 1 h at room temperature. Primary antibody, mouse monoclonal γH2AX (serine 139, Upstate Biotechnology), diluted 1:500 in 5% FBS-PBS solution was added and cells were incubated in this solution for 1 h. Cells were washed in PBS five times followed by the addition of secondary antibody, AlexaFlour594 donkey anti-mouse antibody, diluted 1:1,000 in 5% FBS-PBS for 2 h in the dark. Cells were washed five times with PBS. U2OS cells were washed in ice-cold PBS twice and fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. Cells were washed twice in PBS at room temperature and then incubated in 0.5% Triton X-100 in PBS for 1 h at room temperature. Cells were washed thrice in room temperature PBS and blocked in 1% FBS-PBS for 1 h at room temperature. Primary antibody, mouse monoclonal γH2AX (serine 139, Upstate Biotechnology), diluted 1:500 in 1% FBS-PBS solution was added and cells were incubated in this solution for 1 h. Cells were washed in PBS five times followed by the addition of secondary antibody, AlexaFlour594 donkey anti-mouse antibody, diluted 1:1,000 in 1% FBS-PBS for 2 h in the dark. Cells were washed five times with PBS. Slides were mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Labs).

TUNEL Assay

U2OS cells were plated onto glass coverslips 24 h before treatment with 400 $\mu\text{mol/L}$ H_2O_2 . Cells were incubated for 4 h and then processed for TUNEL staining following the manufacturer's protocol (Chemicon). Briefly, cells were washed twice in PBS and fixed in 1% paraformaldehyde in PBS for 10 min at room temperature. Cells were washed twice in PBS and incubated in 2:1 mixture of ethanol/acetic acid at -20°C for 5 min. Cells were washed twice in PBS and incubated in equilibration buffer for 10 s immediately followed by incubation with working strength terminal deoxynucleotidyl transferase enzyme for 1 h in a humidified chamber at 37°C .

Cells were then incubated in Working Strength Stop/Wash Buffer for 10 min at room temperature. Slides were mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Labs). Data were quantified by counting TUNEL-positive cells and analyzed by Student's *t* test.

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