E2F1 Uses the ATM Signaling Pathway to Induce p53 and Chk2 Phosphorylation and Apoptosis

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Abstract

The p53 tumor suppressor protein is phosphorylated and activated by several DNA damage-inducible kinases, such as ATM, and is a key effector of the DNA damage response by promoting cell cycle arrest or apoptosis. Deregulation of the Rb-E2F1 pathway also results in the activation of p53 and the promotion of apoptosis, and this contributes to the suppression of tumor development. Here, we describe a novel connection between E2F1 and the ATM DNA damage response pathway. In primary human fibroblasts lacking functional ATM, the ability of E2F1 to induce the phosphorylation of p53 and apoptosis is impaired. In contrast, ATM status has no effect on transcriptional activation of target genes or the stimulation of DNA synthesis by E2F1. Cells containing mutant Nijmegen breakage syndrome protein (NBS1), a component of the Mre11-Rad50 DNA repair complex, also have attenuated p53 phosphorylation and apoptosis in response to E2F1 expression. Moreover, E2F1 induces ATM- and NBS1-dependent phosphorylation of the checkpoint kinase Chk2 at Thr^{68}, a phosphorylation site that stimulates Chk2 activity. Delayed γH2AX phosphorylation and absence of ATM autophosphorylation at Ser^{1981} suggest that E2F1 stimulates ATM through a unique mechanism that is distinct from agents that cause DNA double-strand breaks. These findings identify new roles for several DNA damage response factors by demonstrating that they also participate in the oncogenic stress signaling pathway between E2F1 and p53. (Mol Cancer Res 2004;2(4):203–14)

Introduction

Loss of Rb tumor suppressor function leads to deregulated cell proliferation and contributes to the development of most human cancers. In response to Rb inactivation, the p53 tumor suppressor is activated, which results in increased apoptosis and the suppression of tumorigenesis (1–3). Experimental evidence demonstrates that the signaling pathway from Rb inactivation to p53 activation requires E2F1, a transcription factor negatively regulated by Rb (4, 5). Deregulation of E2F1, by overexpression or Rb inactivation, results in p53 accumulation and apoptosis (4–8). It had been widely speculated that the alternative reading frame (ARF) tumor suppressor, which inhibits Mdm2, is an important mediator of p53-dependent apoptosis induced by E2F1. This suggestion was based on the findings that the *ARF* gene is a transcriptional target of E2F1 and that ARF is required for p53 activation in response to other oncogenic signals (6, 9–12). However, several recent reports have made it clear that ARF is not required for p53-dependent apoptosis in response to Rb inactivation or E2F1 overexpression (13–16). Instead, the promotion of apoptosis by E2F1 correlates with an ARF-independent increase in p53 phosphorylation at multiple residues, including Ser^{15} and Ser^{20} (13, 14, 17).

Ataxia telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, extreme sensitivity to ionizing radiation, and strong predisposition to lymphoreticular malignancies. Some AT heterozygous carriers with dominant-negative mutations are also prone to developing cancers (*i.e.*, breast cancer; Ref. 18). The defective gene in this pleiotropic disease, *ATM*, encodes a large serine/threonine kinase belonging to the phosphatidylinositol 3-kinase family. Cells derived from AT patients are extremely sensitive to agents that cause DNA strand breaks such as ionizing radiation (IR), radiomimetic drugs, and topoisomerase inhibitors. Cells lacking ATM are also defective for a variety of cellular responses induced by these DNA damaging agents. For example, AT cells do not arrest in the G_1 phase of the cell cycle in response to IR and display radioresistant DNA synthesis (19, 20). T cells and neurons lacking ATM are also defective for the induction of apoptosis in response to DNA damage (21, 22).

Molecular studies have revealed that ATM is a critical component of an elaborate network of factors that senses DNA damage and other cellular stresses and transmits these signals to appropriate effector molecules that mediate cell cycle arrest, DNA repair, or apoptosis (for review, see Refs. 23, 24). To date, the proteins that directly sense DNA damage in mammalian cells are largely unidentified and the mechanism by which these sensors transmit DNA damage recognition to ATM remains to be elucidated. One recent report suggests that ATM is activated as a result of changes to chromatin structure rather than

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DNA damage *per se* (25). ATM responds primarily to double-strand breaks, while other forms of DNA damage, such as UV radiation-induced photoproducts, signal through the ATM- and Rad3-related (ATR) kinases (26). Two other kinases, Chk1 and Chk2, are phosphorylated and activated by ATR and ATM and are also critical for transmitting the DNA damage signal to effector molecules (27–30). A key effector molecule for the DNA damage response is p53. ATM and ATR directly phosphorylate p53 on Ser^{15} (31, 32) and mediate the phosphorylation of p53 on additional residues through the activation of other kinases, such as Chk1, Chk2, and Plk3 (33–36). These phosphorylation events stabilize p53 by blocking Mdm2 binding and can lead to further p53 modifications such as acetylation (37–39). ATM also directly phosphorylates Mdm2 and this further inhibits the interaction between p53 and Mdm2 (40).

E2F1 has also been identified as a direct target for phosphorylation by ATM and ATR (41). In response to DNA double-strand breaks, E2F1 becomes phosphorylated by ATM at Ser^{31}, a site not conserved in other E2F family members. Phosphorylation of Ser^{31} results in the accumulation of E2F1 due to increased protein stabilization (41). In the absence of ATM, E2F1 is not phosphorylated or stabilized following exposure to agents that cause DNA double-strand breaks (41). Phosphorylation of E2F1 at Ser^{31} creates a binding site for the BRCT domain-containing protein TopBP1 (42). Association of E2F1 with TopBP1 following DNA damage recruits E2F1 into DNA damage-induced foci that also contain BRCA1 (42). Phosphorylation of E2F1 at Ser^{31} in response to double-strand breaks is also associated with the recruitment of E2F1 to the *p73* gene promoter and the stimulation of p73 expression (43). Like its relative p53, p73 can promote apoptosis and it has been demonstrated that under some circumstances the induction of p73 contributes to the apoptotic activity of E2F1 (43–45).

Hypomorphic mutations in the *NBS1* gene cause Nijmegen breakage syndrome (NBS), a disease with similar characteristics to AT. The NBS1 protein is part of the Mre11-Rad50 DNA repair complex, which is involved in homologous recombination repair of double-strand breaks (46, 47). Like p53 and E2F1, NBS1 is phosphorylated by ATM in response to DNA damage (48–50). In addition to its role in DNA repair, NBS1 also appears to function in the ATM signal transduction network because the ability of ATM to phosphorylate Chk2 and some other targets is impaired in cells lacking NBS1 (30, 51, 52). Moreover, the NH_2 terminus of NBS1 directly interacts with the COOH terminus of E2F1, including the Rb binding and transactivation domains of E2F1 (53). The functional consequence of this physical interaction on the activities of NBS1 and E2F1 is unknown. In this study, we describe a novel functional relationship among E2F1, ATM, NBS1, Chk2, and p53 in which ATM and NBS1 are required for E2F1 to induce the phosphorylation of p53 and Chk2 and promote efficient apoptosis.

Results

Inactivation of Rb Results in E2F1 Accumulation and p53 Phosphorylation

E2F1 is required for the p53-dependent apoptosis that occurs in response to Rb inactivation (4, 5). We and others recently demonstrated that deregulated expression of E2F1 leads to the phosphorylation and subsequent accumulation of p53 (13, 14, 17). To examine whether acute Rb loss and resulting deregulation of endogenous E2F1 would also lead to p53 phosphorylation, we infected primary mouse adult fibroblasts (MAFs) containing floxed *Rb* alleles with a recombinant adenovirus expressing either green fluorescent protein (GFP; AdGFP) or Cre (AdCre). Infection with AdCre, but not AdGFP, resulted in inactivation of both functional *Rb* alleles and subsequent loss of Rb protein expression (Fig. 1). AdGFP infection did not affect total p53 levels or p53 phosphorylation at Ser^{15}, whereas AdCre-infected cells accumulated phosphorylated p53 concomitant with an increase in E2F1 protein levels (Fig. 1A). This coincided with the up-regulation of the E2F target genes *ribonucleotide reductase subunit 2* and *cyclin A* (data not shown).

The kinetics of p53 phosphorylation following the inactivation of *Rb* by Cre suggests that it was the loss of Rb, and not the function of the Cre recombinase, that led to the increase in Ser^{15} phosphorylation. To rule out the possibility of Cre-mediated DNA recombination leading to phosphorylation of p53 at Ser^{15}, MAFs containing floxed *Brg1* alleles were infected with AdCre. Although BRG1 can be detected in complex with Rb, inactivation of *Brg1* does not result in widespread deregulation of E2F transcriptional activity (data not shown). Inactivation of *Brg1* by Cre recombination did not result in an increase in p53 Ser^{15} phosphorylation (Fig. 1, C and D). Thus, p53 phosphorylation is specific for the inactivation of *Rb* and not the result of Cre activity. This finding is consistent with a recent report, which demonstrates that expression of the human papillomavirus E7 protein, which binds to Rb and releases free E2F, can also lead to increased phosphorylation of p53 (54). Thus, Rb inactivation and the deregulation of endogenous E2F1 lead to the accumulation and phosphorylation of p53 at Ser^{15}, which is similar to what is observed following overexpression of exogenous E2F1.

E2F1-Induced p53 Phosphorylation and Apoptosis Are Caffeine Sensitive

Previous studies have shown that ATM and ATR regulate p53 phosphorylation in response to various forms of damaged DNA as well as other stresses that do not involve DNA damage (25, 26, 31, 32). To determine whether ATM or ATR may serve as mediators of E2F1-induced p53 phosphorylation, we infected normal human fibroblast (NHF) cultures with a recombinant adenovirus expressing E2F1 (AdE2F1) in the presence of caffeine, a potent inhibitor of both ATM and ATR (55). To more easily observe phosphorylated p53 species, we coinfected cells with a low multiplicity of infection (MOI) of recombinant adenovirus expressing human p53 (Adp53). All subsequent experiments were performed in the absence of Adp53 and demonstrated that the phosphorylation of endogenous p53 mirrored the observed phosphorylation when Adp53 was used. Expression of E2F1 led to p53 accumulation and phosphorylation at Ser^{15} and Ser^{20}, which was inhibited by increasing doses of caffeine (Fig. 2A). Similar results were obtained using wild-type and *Arf* null primary mouse embryo fibroblasts (Ref. 40; data not shown). A control AdGFP did not induce p53 accumulation or phosphorylation. Inhibition of p53

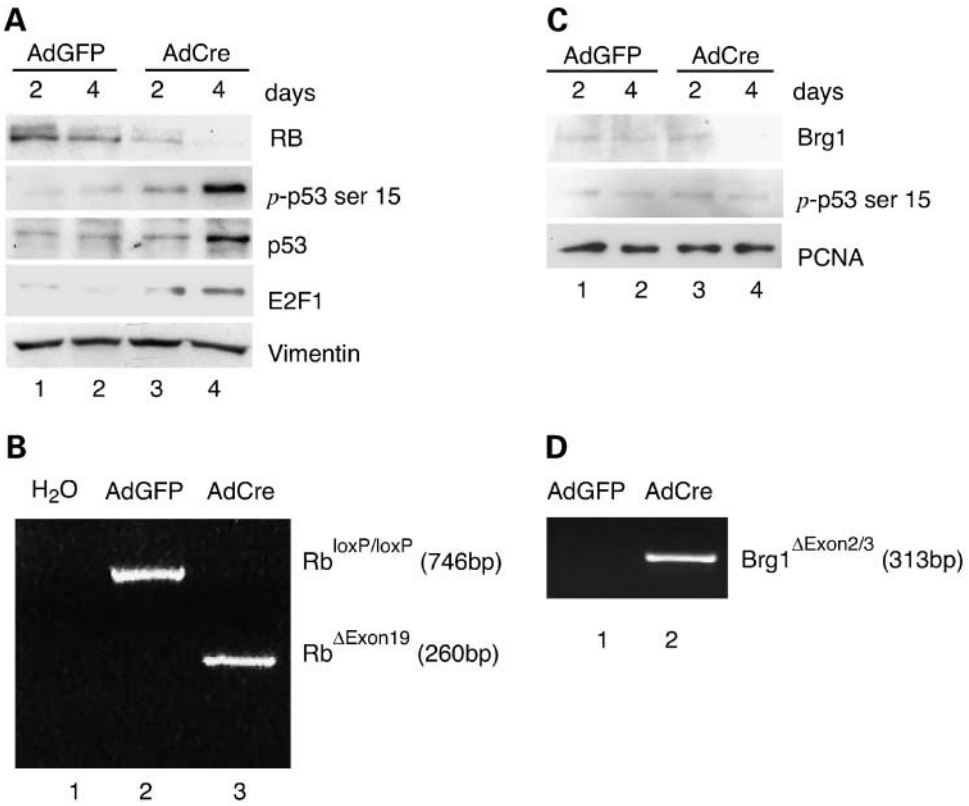

FIGURE 1. Rb inactivation induces the phosphorylation of p53. **A.** Primary MAFs containing floxed *Rb* alleles were infected with AdGFP (*lanes 1* and *2*) or AdCre (*lanes 3* and *4*). Cell protein was harvested 2 and 4 days postinfection and Western blot analysis was performed for Rb, p53, phospho-Ser[15] p53, E2F1, and vimentin. **B.** Genomic DNA was isolated from *Rb* floxed MAFs infected with AdGFP (*lane 2*) or AdCre (*lane 3*). PCR analysis was performed using primers spanning the floxed exon 19 allele. **C.** MAF cultures containing floxed *Brg1* alleles were infected with AdGFP (*lanes 1* and *2*) or AdCre (*lanes 3* and *4*). Cell protein was harvested 2 and 4 days postinfection and Western blot analysis was performed for BRG1, phospho-Ser[15] p53, and proliferating cell nuclear antigen. **D.** Genomic DNA was isolated from *Brg1* floxed MAFs infected with AdGFP (*lane 1*) or AdCre (*lane 2*). PCR analysis was performed using primers specific for the recombined *Brg1* allele.

phosphorylation by caffeine was not due to an effect on E2F1 expression levels, which remained constant or slightly increased throughout the dose curve (Fig. 2A). This finding confirms a recent study using an inducible estrogen receptor-E2F1 fusion construct that demonstrated inhibition of p53 phosphorylation by caffeine following E2F1 induction (17).

In cultured fibroblasts, the ability of E2F1 to induce apoptosis is dependent on functional p53 (8, 56). We therefore examined the ability of caffeine to suppress apoptosis by E2F1 in primary fibroblasts infected with recombinant adenoviruses. Forty-eight hours postinfection, 46% of AdE2F1-infected cells had an apoptotic nuclear morphology compared with only 11% of cells infected with AdGFP (Fig. 2B). In the presence of caffeine, the percentage of apoptotic cells deceased in a dose-dependent manner. This finding agrees with a previous report that also demonstrated inhibition of E2F1-induced apoptosis by caffeine (13). The ability of E2F1 to promote entry into S phase was not significantly affected by caffeine (Fig. 2C), which precludes a general inhibition of E2F1 activity by caffeine.

ATM Is Required for E2F1-Induced p53 Phosphorylation and Apoptosis

To determine if ATM is involved in p53 phosphorylation induced by E2F1, primary human fibroblasts from AT patients, which lack functional ATM, were obtained. In AT fibroblasts, p53 phosphorylation at Ser[15] and Ser[20] was absent in response to E2F1 expression (Fig. 3A). Similar results were observed when primary fibroblast cultures isolated from different AT patients were used (data not shown). This finding is consistent with the fact that ATM directly phosphorylates p53 on Ser[15] and indirectly regulates Ser[20] phosphorylation by activating other kinases (31–36). Notably, similar levels of total p53 protein were induced in normal and AT fibroblasts following expression of E2F1. The accumulation of unphosphorylated p53 in AdE2F1-infected AT cells is likely the result of alternative mechanisms that do not involve ATM such as up-regulation of ARF by E2F1. The finding that total p53 levels increase in the absence of phosphorylation in AT cells demonstrates that increased phosphorylation of p53 in E2F1-expressing normal fibroblasts is not simply a consequence of an increased pool of p53 protein being phosphorylated at basal levels. This is consistent with our previous findings that E2F1 activity increases the percentage of p53 that is phosphorylated (14).

In NHF cells, the percentage of apoptotic cells 48 h postinfection with AdE2F1 increased by a factor of 10 compared with infection with AdGFP (Fig. 3B). In contrast, AT cells exhibited a much lower apoptotic response to E2F1 expression, with a >2-fold increase in the percentage of apoptotic cells. DNA laddering confirmed the increase in apoptosis in normal fibroblasts compared with AT fibroblasts following AdE2F1 infection (Fig. 3C). Expression of the E2F1 transcriptional target p73, a p53 family member implicated in E2F1-induced apoptosis (44, 45), is increased to similar levels in normal and AT cells by AdE2F1 infection (Fig. 3D). This demonstrates that the impairment of apoptosis in AT cells is not due to a deficiency in transactivation of p73 by E2F1. The absence of ATM also did not affect up-regulation of cyclin E, another well-established transcriptional target of E2F1 (Fig. 3D). Consistent with this observation, E2F1 was capable of stimulating S-phase

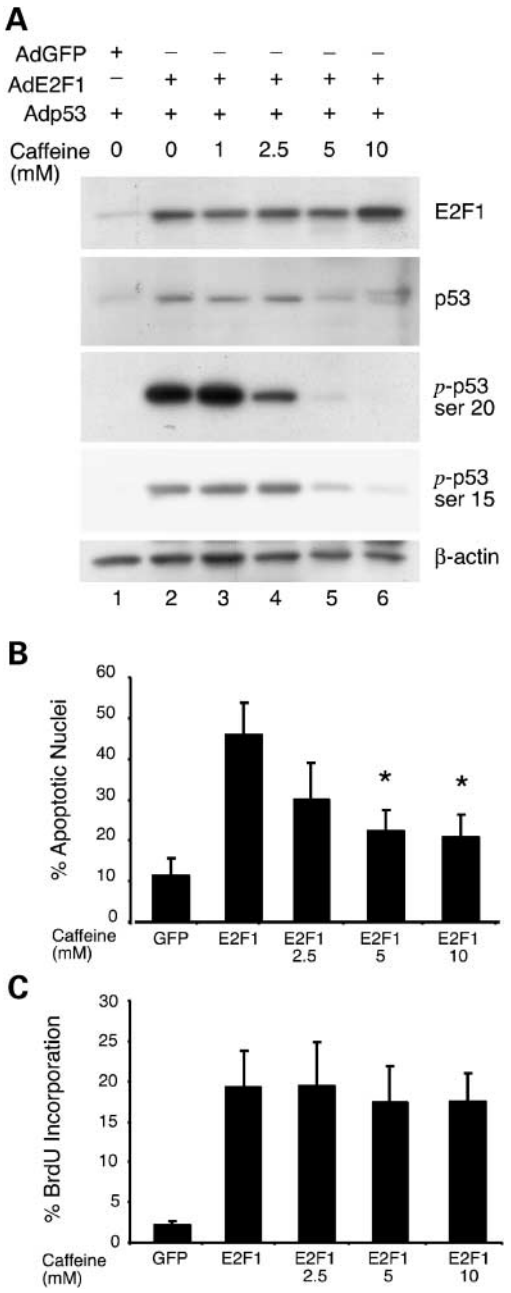

FIGURE 2. Inhibition of E2F1-induced p53 phosphorylation and apoptosis by caffeine. **A.** Western blot analysis of cell lysates (30 μg) from NHF cells infected with Adp53 (MOI of 25) and either AdGFP (*lane 1*) or AdE2F1 (*lanes 2*, *3*, *4*, and *5*) at a MOI of 50 in the presence of the indicated caffeine concentrations (*lanes 3*, *4*, and *6*). Cells were exposed to caffeine from the time of infection to the time of harvest. Protein was harvested 24 h postinfection and probed with antisera or antibodies directed against E2F1, p53, phospho-Ser^{20} p53, phospho-Ser^{15} p53, and β-actin as a loading control. **B.** NHF cells were serum starved for 24 h, infected with AdGFP or AdE2F1 (MOI of 200), and incubated without or with the indicated concentration of caffeine for 48 h. Cells with condensed or fragmented nuclei were scored as apoptotic and the average of three experiments is presented; *bars,* SE. *, $P < 0.05$, statistically significant difference as compared with AdE2F1 infection in the absence of caffeine (*t* test). **C.** Infections (MOI of 100) were performed as in **B**, with the exception of a 1 h BrdUrd incubation prior to fixation, 24 h postinfection. BrdUrd immunocytochemistry was performed and the percentage of BrdUrd-positive cells was determined for three separate experiments.

entry to a similar extent in control and AT fibroblasts (Fig. 3E). Thus, the absence of ATM specifically impairs E2F1's apoptotic activity but not its transcriptional and S-phase-promoting activities.

NBS1 Is Required for E2F1 to Induce p53 Phosphorylation and Apoptosis

Mutations in the *NBS1* gene cause NBS, a disease with similar characteristics to AT. The NBS1 protein, also known as nibrin, is part of the Mre11-Rad50 DNA repair complex and can also physically associate with E2F1 (46, 47, 53). In addition, it has been reported that phosphorylation of some ATM targets, such as Chk2, in response to IR is impaired in cells that lack NBS1 (30, 51, 52). To examine the role of NBS1 in the ATM-dependent phosphorylation of p53 induced by E2F1, we examined the response to E2F1 expression in human fibroblasts lacking functional NBS1. As in AT cells, total p53 levels were still induced by E2F1 in NBS cells, but the phosphorylation of p53 at Ser^{15} and Ser^{20} was greatly impaired (Fig. 4A). This implies that NBS1 is required for ATM to directly phosphorylate p53 on Ser^{15} and to indirectly induce the phosphorylation of p53 on Ser^{20} in response to E2F1 expression. The lack of NBS1 also impaired the promotion of apoptosis by E2F1 (Fig. 4, B and C) but did not significantly affect S-phase induction by E2F1 (Fig. 4D). These observations indicate that, like ATM, the lack of NBS1 specifically impairs p53 phosphorylation and apoptosis in response to E2F1 without a general inhibition of E2F1 activity.

Of note, NBS cells retain the ability to phosphorylate p53 in response to IR, albeit not as efficiently as NHF cells (Fig. 5A). This is consistent with several studies demonstrating that p53 accumulation and phosphorylation in NBS cells following exposure to IR still occurs but is delayed and reduced (52, 57–59). In contrast, we repeatedly observe a profound defect in p53 phosphorylation in NBS fibroblasts compared with normal fibroblasts in response to E2F1 overexpression. As expected, IR did not induce the phosphorylation of p53 Ser^{15} in AT cells (Fig. 5A), whereas Ser^{15} was phosphorylated in response to UV radiation, which signals through the ATR kinase, in cells lacking either NBS1 or ATM (Fig. 5B). This demonstrates that p53 is present and able to respond to stress signals that do not require ATM in each of the cell cultures used in these experiments.

E2F1 Up-Regulates and Induces the Phosphorylation of Chk2

Although it was originally believed that Chk2 mediates the ATM-dependent phosphorylation of p53 at Ser^{20} in response to double-strand breaks, more recent data demonstrate that p53 Ser^{20} phosphorylation occurs in the absence of Chk2 (60–62). Nonetheless, Chk2 does appear to participate in p53 activation and the promotion of apoptosis in response to IR (60, 63). To determine if Chk2 might be involved in p53 activation and the promotion of apoptosis by E2F1, the level and phosphorylation status of Chk2 was examined in response to E2F1 expression in normal, AT, and NBS fibroblasts. As can be seen in Fig. 6, AdE2F1 infection led to increased levels of total Chk2 protein in all three primary fibroblast cultures. This suggests

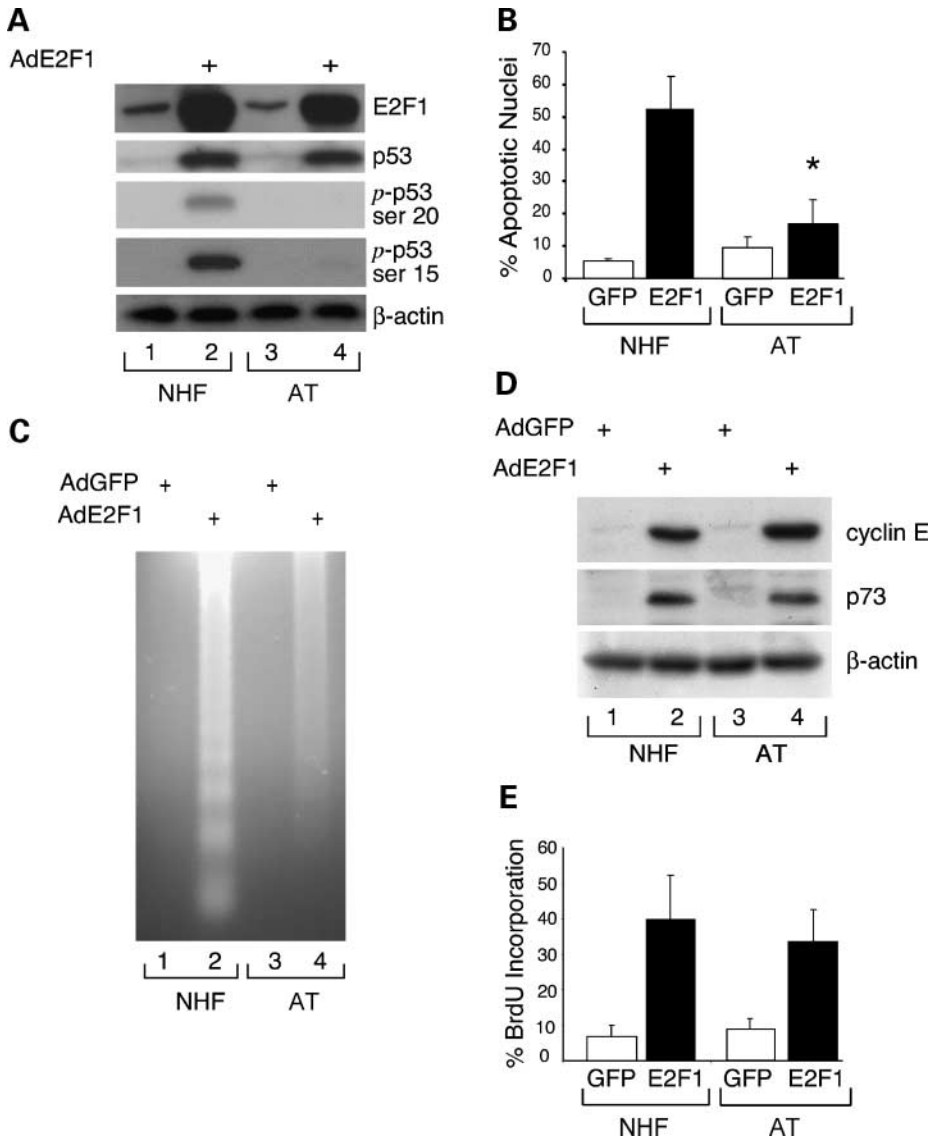

FIGURE 3. The absence of ATM impairs E2F1-induced p53 phosphorylation and apoptosis. **A.** Western blot analysis was performed using lysates from NHF cells (*lanes 1* and *2*) and AT fibroblasts (*lanes 3* and *4*) that were either mock infected (*lanes 1* and *3*) or infected (MOI of 100) with AdE2F1 (*lanes 2* and *4*). Cell lysates were made 48 h postinfection and probed for E2F1, p53, phospho-Ser^{20} p53, phospho-Ser^{15} p53, and β-actin. **B.** Normal (*NHF*) and AT human fibroblasts were infected with AdGFP or AdE2F1 (MOI of 200) after 24 h serum starvation. Forty-eight hours postinfection, cells were analyzed for apoptosis as described for Fig. 2B. Average of three independent experiments; *bars,* SE. *, $P < 0.01$, statistically significant difference (*t* test) in percentage of apoptotic nuclei in AT cells compared with NHF cells following AdE2F1 infection. **C.** A DNA laddering assay was performed on normal (*lanes 1* and *2*) and AT (*lanes 3* and *4*) fibroblasts infected (MOI of 200) with AdGFP (*lanes 1* and *3*) or AdE2F1 (*lanes 2* and *4*). **D.** Western blot analysis was performed using lysates (100 μg) from NHF cells (*lanes 1* and *2*) and AT fibroblasts (*lanes 3* and *4*) that were infected (MOI of 100) with AdGFP (*lanes 1* and *3*) or AdE2F1 (*lanes 2* and *4*). Membranes were probed with antibodies to p73, cyclin E, and β-actin. **E.** Infections (MOI of 100) were performed as in **B**, with the exception of a 1 h BrdUrd incubation prior to fixation, 24 h postinfection. BrdUrd immunocytochemistry was performed and the average percentage of BrdUrd-positive cells was determined for three separate experiments.

that Chk2 might be a transcriptional target for E2F1. As was observed with p53, phosphorylation of Chk2 at Thr^{68} occurred in AdE2F1-infected normal fibroblasts but was absent in AdE2F1-infected AT and NBS cells (Fig. 6A). This phosphorylation event is known to stimulate the kinase activity of Chk2 in response to double-strand breaks (27, 28). Our results agree with the established ATM dependence of Chk2 Thr^{68} phosphorylation and establish that NBS1 is also required for the induction of Chk2 phosphorylation induced by E2F1 (Fig. 6, *lane 4*). This result also agrees with earlier studies that suggest a dependence on NBS1 for phosphorylation of Chk2 by ATM (51, 52).

NHF cells were infected with AdE2F1, irradiated with IR, or exposed to cisplatin or etoposide to compare the level of Chk2 Thr^{68} phosphorylation induced by these different treatments. AdE2F1 infection, IR, and etoposide, but not cisplatin, induced the phosphorylation of Chk2 (Fig. 6B). All treatments increased the level of phosphorylated (Ser^{15}) p53 between 5- and 10-fold compared with untreated or AdGFP infection (Fig. 6B). Compared with IR and etoposide, AdE2F1 infection induced at least twice the level of phosphorylated Chk2 relative to the level of phosphorylated p53. This difference may in part be due to E2F1, but not DNA damaging agents, up-regulating the expression of Chk2 in addition to stimulating ATM-dependent Chk2 phosphorylation.

E2F1 Does Not Stimulate the ATM Pathway by Established Mechanisms

It has recently been demonstrated that E2F1 can transcriptionally activate the *ATM* gene promoter (54). Although simply increasing ATM levels would likely not be sufficient to stimulate the kinase activity of ATM, up-regulation of ATM could potentially contribute to the activation of the ATM pathway by E2F1. However, Western blot analysis demonstrated similar ATM protein levels among uninfected and AdE2F1-infected fibroblasts (Fig. 7).

In response to DNA double-stand breaks and some other forms of stress, ATM becomes autophosphorylated at Ser^{1981} (25). It has been proposed that this phosphorylation event stimulates the kinase activity of ATM by dissociating inactive ATM dimers and higher-order complexes (25). To determine if stimulation of the ATM pathway by E2F1 might also involve autophosphorylation of ATM at Ser^{1981}, antisera specific for phospho-Ser^{1981} was used in a Western blot of lysates from NHF cells either treated with IR or etoposide, both of which induce double-strand breaks, or infected with AdE2F1 (Fig. 7A). In contrast to IR and etoposide, overexpression of E2F1 did not induce appreciable levels of ATM Ser^{1981} phosphorylation. Nonetheless, AdE2F1 infection induced the phosphorylation of p53 at Ser^{15}, an event that is ATM dependent (Fig. 2), to an equivalent level compared with IR and etoposide. This result strongly suggests that the mechanism by which E2F1 stimulates ATM to phosphorylate p53 (and Chk2) differs from the mechanism used to activate ATM in response to double-strand breaks.

It has been demonstrated that the ATM signaling pathway is suppressed during infection with replication-competent adenovirus dependent on the viral E1b55K and E4orf6 viral gene products (64, 65). E1b55k/E4orf6 targets the Mre11-Rad50-NBS1 complex for degradation, preventing ATM Ser^{1981} autophosphorylation and ATM activation in response to replicating adenovirus or DNA double-strand breaks. The replication-incompetent adenoviral vectors used in these studies lack E1b55K and do not target NBS1 for degradation (data not shown). To verify that the adenoviral vectors do not block ATM autophosphorylation at Ser^{1981}, NHF cells infected with AdGFP or AdE2F1 were irradiated with IR. Infection with adenoviral vectors did not inhibit Ser^{1981} phosphorylation in response to IR and similar levels of phosphorylated ATM were observed in irradiated fibroblasts that were mock infected or infected with AdGFP or AdE2F1 (Fig. 7B, *lanes 5*, *6*, and *7*). As observed previously, AdE2F1 infection in the absence of IR stimulated the phosphorylation of p53 Ser^{15} but not ATM Ser^{1981} (Fig. 7B, *lane 4*), while AdE2F1 infection cooperated with IR to further enhance the level of phosphorylated p53 (Fig. 7B, *lane 7*).

Phosphorylation of histone H2AX (γH2AX) has become an established marker for DNA damage, particularly double-strand breaks. In response to double-strand breaks, H2AX rapidly localizes to sites of DNA damage and is phosphorylated in an ATM-dependent manner (66). Other cellular stresses that stimulate ATM kinase activity but do not cause DNA double-strand breaks, such as hypotonic conditions, do not induce the phosphorylation of H2AX (25). Phosphorylation of H2AX occurs within minutes of DNA damage with kinetics that are similar to or faster than the kinetics of p53 phosphorylation in

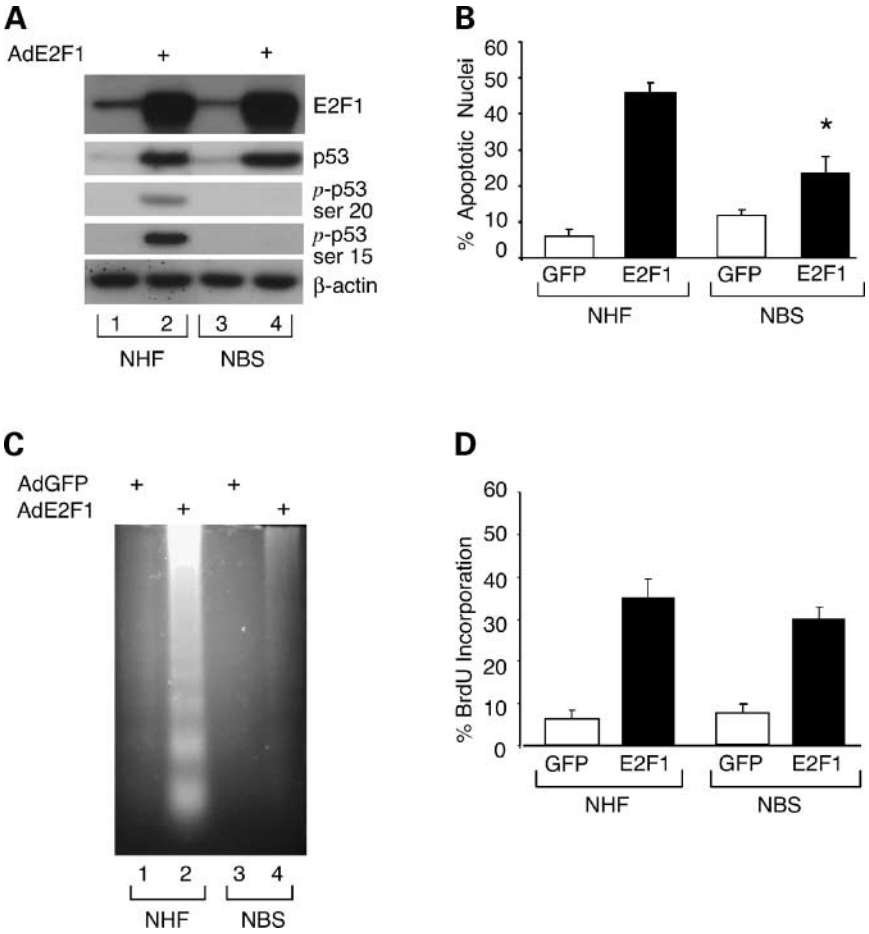

FIGURE 4. The absence of functional NBS1 impairs E2F1-induced p53 phosphorylation and apoptosis. **A.** Western blot analysis of lysates from normal (*lanes 1* and *2*) and NBS (*lanes 3* and *4*) human fibroblasts either mock infected (*lanes 1* and *3*) or infected (MOI of 100) with AdE2F1 (*lanes 2* and *4*). Cell lysates were made 48 h postinfection and probed for E2F1, p53, phospho-Ser^{20} p53, phospho-Ser^{15} p53, and β-actin. **B.** Normal (*NHF*) and NBS human fibroblasts were infected with AdGFP or AdE2F1 (MOI of 200) after 24 h serum starvation. Forty-eight hours postinfection, cells were scored for apoptosis as described in Fig. 2B. Average of three independent experiments; *bars,* SE. *, $P < 0.01$, statistically significant difference (*t* test) in percentage of apoptotic cells in NBS cells compared with NHF cells following AdE2F1 infection. **C.** A DNA laddering assay was performed on normal (*lanes 1* and *2*) and NBS (*lanes 3* and *4*) fibroblasts infected (MOI of 200) with AdGFP (*lanes 1* and *3*) or AdE2F1 (*lanes 2* and *4*). **D.** AdGFP and AdE2F1 infections and BrdUrd immunocytochemistry were performed on normal and NBS fibroblasts as described in Fig. 3C. The average of three independent experiments is presented.

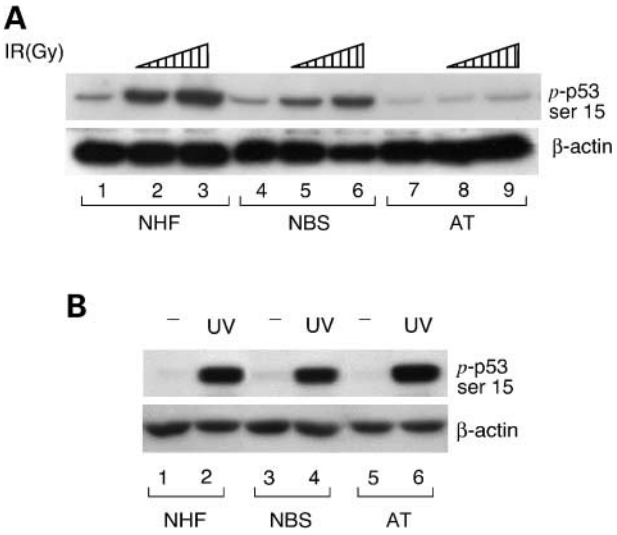

FIGURE 5. Phosphorylation of p53 in response to IR and UV in NBS and AT cells. **A.** Normal (*lanes 1*, *2*, and *3*), NBS (*lanes 4*, *5*, and *6*), and AT (*lanes 7*, *8*, and *9*) fibroblasts were treated with 0 (*lanes 1*, *4*, and *7*), 3 (*lanes 2*, *5*, and *8*), or 6 (*lanes 3*, *6*, and *9*) Gy IR. Cells were harvested 2.5 h postexposure and cell lysates were analyzed by Western blot using antibodies to phospho-Ser[15] p53 and β-actin. **B.** Normal (*lanes 1* and *2*), NBS (*lanes 3* and *4*), and AT (*lanes 5* and *6*) fibroblasts were mock treated (*lanes 1*, *3*, and *5*) or exposed to 100 J/m^2 UVB radiation. Cells were harvested 8 h postexposure and analyzed for phospho-Ser[15] p53 and β-actin as above.

response to double-strand breaks (67). If E2F1 induces DNA double-strand breaks to activate ATM and induce p53 phosphorylation, it would be expected that H2AX would be phosphorylated coincident with p53 in response to E2F1 expression.

As expected, H2AX was phosphorylated coincident with the phosphorylation of p53 in response to IR (Fig. 8A). Phosphorylation of p53 was observed as early as 24 h postinfection with AdE2F1, whereas histone H2AX phosphorylation was not detected until 96 h postinfection, at a time when apoptosis in these infected cells is widespread (Fig. 8A). Eventual histone H2AX phosphorylation is not unexpected, as it is has been observed to be phosphorylated during the later stages of apoptosis when genomic DNA becomes fragmented (68). This result further supports the conclusion that E2F1 does not induce DNA double-strand breaks as a mechanism for activating ATM.

ATM is also activated in response to reactive oxygen species (ROS) accumulation (69, 70). It has been suggested that overexpression of E2F1 induces apoptosis through the induction of ROS in NIH3T3 and Saos-2 cells (71). If this finding is universally true, it could explain how E2F1 stimulates the ATM pathway to promote apoptosis. To test this possibility, NHF cells were infected with either AdE2F1 or AdGFP as a control, and 24 h postinfection, cells were analyzed by flow cytometry following incubation with a dye sensitive to ROS. It was repeatedly found that expression of exogenous E2F1 did not induce ROS in AdE2F1-infected primary human fibroblasts (Fig. 8B). In contrast, a similar adenovirus vector expressing c-*myc* did induce significant levels of ROS in primary human fibroblasts (Fig. 8B), consistent with previous findings by others (71, 72). Taken together, these findings suggest that E2F1 stimulates ATM to phosphorylate p53 and Chk2 without inducing DNA double-strand breaks or ROS and that this occurs in the absence of autophosphorylation of ATM at Ser[1981].

Discussion

E2F1 Activation of p53 and the Promotion of Apoptosis

It had been widely speculated that ARF, a negative regulator of Mdm2, participates in the induction of p53-dependent apoptosis in response to deregulated E2F1 activity. This hypothesis was based on the findings that E2F1 transcriptionally activates the *ARF* gene (6, 9) and that apoptosis induced by Myc and E1A, two other oncogenic signals, is defective in cells lacking ARF (10, 11, 14). Nonetheless, several recent studies demonstrate that ARF is dispensable for apoptosis induced by deregulation of the Rb-E2F1 pathway. For example, inactivation of *Rb* leads to high levels of apoptosis in the central

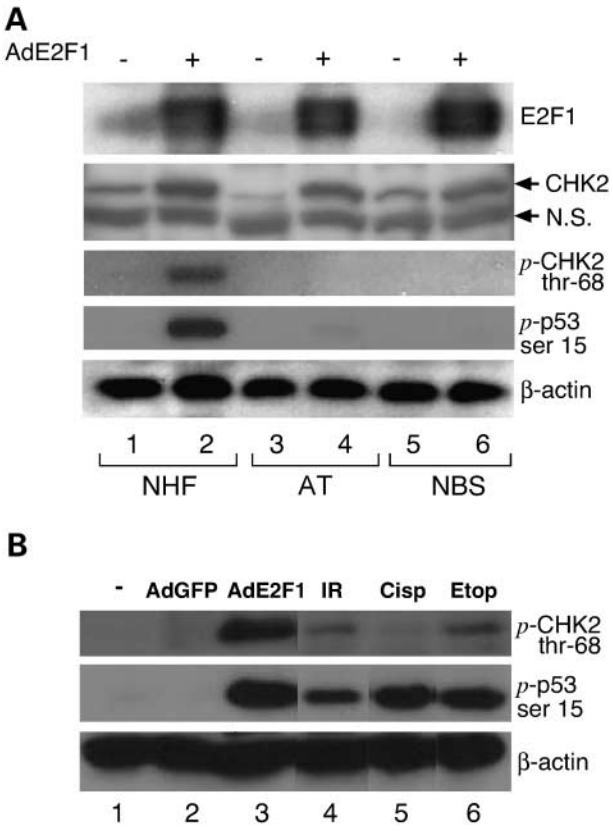

FIGURE 6. E2F1 induces the phosphorylation of Chk2 on Thr[68] in an ATM- and NBS1-dependent manner. **A.** Normal (*lanes 1* and *2*), AT (*lanes 3* and *4*), and NBS (*lanes 5* and *6*) fibroblasts were mock infected (*lanes 1*, *3*, and *5*) or infected (MOI of 100) with AdE2F1 (*lanes 2*, *4*, and *6*). and *6*). Forty-eight hours postinfection, cell lysates were collected and Western blot analysis was performed for E2F1, Chk2, phospho-Thr[68] Chk2, phospho-Ser[15] p53, and β-actin. *N.S.,* nonspecific band. **B.** NHF cells were untreated (*lane 1*), infected with AdGFP (*lane 2*) or AdE2F1 (*lane 3*), irradiated with 10 Gy IR (*lane 4*), or exposed to 25 μM cisplatin (*lane 5*) or 25 μM etoposide (*lane 6*). For IR, cells were harvested 2 h postirradiation. For infections and chemical exposures, cells were harvested 48 h following treatment. Western blot analysis was performed using antibodies or antisera for phospho-Thr[68] Chk2, phospho-Ser[15] p53, and β-actin.

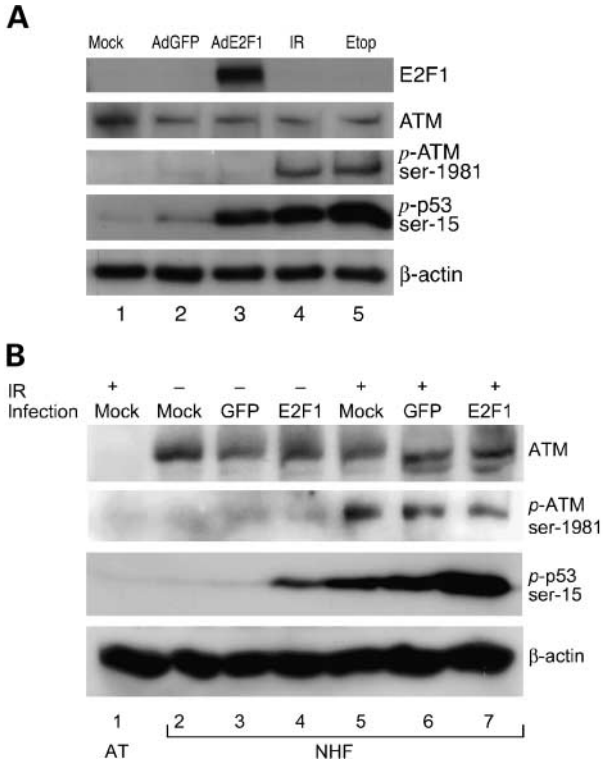

FIGURE 7. Ectopic E2F1 expression does not up-regulate ATM levels or induce ATM phosphorylation on Ser[1981]. **A.** NHF cells were untreated (*lane 1*), infected with AdGFP (*lane 2*) or AdE2F1 (*lane 3*) at MOI of 100, irradiated with 10 Gy IR (*lane 4*), or treated with 25 μM etoposide (*lane 5*). Cells were harvested 2 h postirradiation treatment, 24 h postexposure to etoposide, or 72 h postinfection with AdE2F1 or AdGFP. Western blot analysis was performed for E2F1, ATM, phospho-Ser[1981] ATM, phospho-Ser[15] p53, and β-actin as indicated. **B.** AT fibroblasts (*lane 1*) or NHF cells (*lanes 2*, *3*, *4*, *5*, *6*, and *7*) were either mock infected (*lanes 1*, *2*, and *5*) or infected at MOI of 100 with AdGFP (*lanes 3* and *6*) or AdE2F1 (*lanes 4* and *7*). Two hours prior to harvest, some cultures were irradiated with 10 Gy IR (*lanes 1*, *5*, *6*, and *7*). Cells were harvested 72 h postinfection and Western blot analysis was performed for ATM, phospho-Ser[1981] ATM, phospho-Ser[15] p53, and β-actin as indicated.

nervous system of developing embryos that is dependent on p53 and E2F1 (5, 73) but independent of ARF (16). We have found that ARF is also dispensable for apoptosis induced by the ectopic expression of E2F1 in either primary MEF cultures or transgenic epidermis *in vivo* (14). In fact, inactivation of *Arf* actually enhanced the ability of E2F1 to induce apoptosis in both of these systems. The ability of E2F1 to stimulate proliferation was also enhanced in the absence of ARF (14). Thus, ARF appears to function as a negative regulator of E2F1 activity, as has been suggested by others (74, 75), and not as a mediator of E2F1-induced apoptosis. This means that an alternative mechanism must exist for p53 activation in response to deregulated E2F1 activity.

A variety of stress stimuli, including DNA damage, stimulate p53 activity by inducing the phosphorylation of p53. We and others have found that deregulated E2F1 activity stimulates the phosphorylation of p53 on multiple residues, including Ser[15] and Ser[20] (13, 14, 17). The ability of E2F1 to stimulate p53 phosphorylation is independent of ARF and is sensitive to caffeine, a potent inhibitor of the ATM kinase (55). Consistent with this, the ability of E2F1 to stimulate p53 phosphorylation is impaired in human fibroblasts from AT patients. Moreover, cells lacking ATM are relatively resistant to apoptosis induced by E2F1. Based on these findings, we suggest a model in which activation of the ATM kinase is a critical event for p53-dependent apoptosis induced by E2F1 (Fig. 9). In addition to directly phosphorylating p53 on Ser[15], ATM may also stimulate p53 activity in response to E2F1 through the phosphorylation of additional targets such as Chk2, Mdm2, and Plk3 (28, 34, 36, 40). Indeed, we find that ectopic expression of E2F1 induces the ATM-dependent phosphorylation of Chk2 on Thr[68], a modification that stimulates the kinase activity of Chk2 (27, 28). Chk2 activation by E2F1 may promote p53 activity by an as yet undiscovered mechanism and promote apoptosis independent of p53 (60, 63). A possible role for ARF in p53 activation by E2F1 could be to increase the total pool of p53 protein that is available for activation by the ATM pathway through the inhibition of Mdm2. It is also possible that in some cellular contexts the ARF and ATM pathways are redundant and that either pathway is sufficient for p53 activation by E2F1. This would explain why p53- and E2F1-dependent apoptosis in SV40 T-antigen transgenic mice is unaffected when either *Arf* or *Atm* is inactivated alone (15, 76).

The phosphorylation of p53 on Ser[15] and Ser[20] and Chk2 on Thr[68] in response to E2F1 also requires a functional NBS1 protein. Moreover, E2F1-induced apoptosis is impaired in NBS cells, but S-phase entry induced by E2F1 is unaffected by the absence of NBS1. Thus, NBS1 is not generally required for E2F1 activity but is specifically required for E2F1's apoptotic function. It has been reported that NBS1 functions as a cofactor to allow ATM to phosphorylate and activate Chk2 and some other targets in response to DNA damage (30, 51, 52). This is consistent with our finding that Chk2 Thr[68] is not phosphorylated in response to E2F1 expression in NBS cells. On the other hand, we and others find that NBS1 is dispensable for the ATM-dependent phosphorylation of p53 at Ser[15] in response to IR, although this modification is reduced in NBS cells (52, 58, 59). In response to E2F1 expression, however, p53 Ser[15] phosphorylation is just as defective in NBS cells as it is in AT cells. This difference in requirement for NBS1 suggests that NBS1 might play a more critical role in the activation of ATM in response to E2F1 compared with IR. NBS1 has recently been shown to directly bind the COOH terminus of E2F1 (53). Future experiments will address whether a physical interaction between E2F1 and NBS1 is involved in NBS1 regulation of E2F1's apoptotic activity.

Relationship Between E2F1, ATM, and DNA Damage

Although not formally demonstrated, the data presented here suggest that E2F1 stimulates the kinase activity of ATM toward at least two of its targets, p53 and Chk2. What is unclear is how E2F1 activates ATM. ATM regulation is poorly understood and the molecular mechanism by which DNA damage activates ATM is also unknown. A recent report suggests that it is not DNA damage *per se* but rather widespread chromatin remodeling that activates ATM (25). It is therefore possible that deregulated E2F1 activity results in large-scale chromatin

remodeling as the mechanism of ATM activation, perhaps through transcriptionally activating many genes or inducing aberrant DNA synthesis. However, other E2F family members (*i.e.*, E2F2 and E2F3) also induce transcription and DNA synthesis with similar capacity as E2F1 but do not efficiently induce p53 phosphorylation or apoptosis (6, 13, 56). Moreover, stresses that activate ATM through chromatin remodeling without inducing DNA damage still induce autophosphorylation of ATM at Ser^{1981} (25), an event that appears to be absent in response to E2F1 overexpression. Although several pieces of data suggest that E2F1 activates ATM through a mechanism that is distinct from the mechanism used by DNA damage, it is still possible that deregulated E2F1 induces a type of DNA damage that is then recognized by the ATM signaling pathway.

ATM can directly phosphorylate E2F1 on Ser^{31} in response to DNA double-strand breaks, which results in E2F1 protein stabilization (41). Other E2F family members are not phosphorylated by ATM and, unlike E2F1, do not accumulate in response to DNA damage (41, 77, 78). Thus, not only does E2F1 appear to activate ATM but also ATM specifically

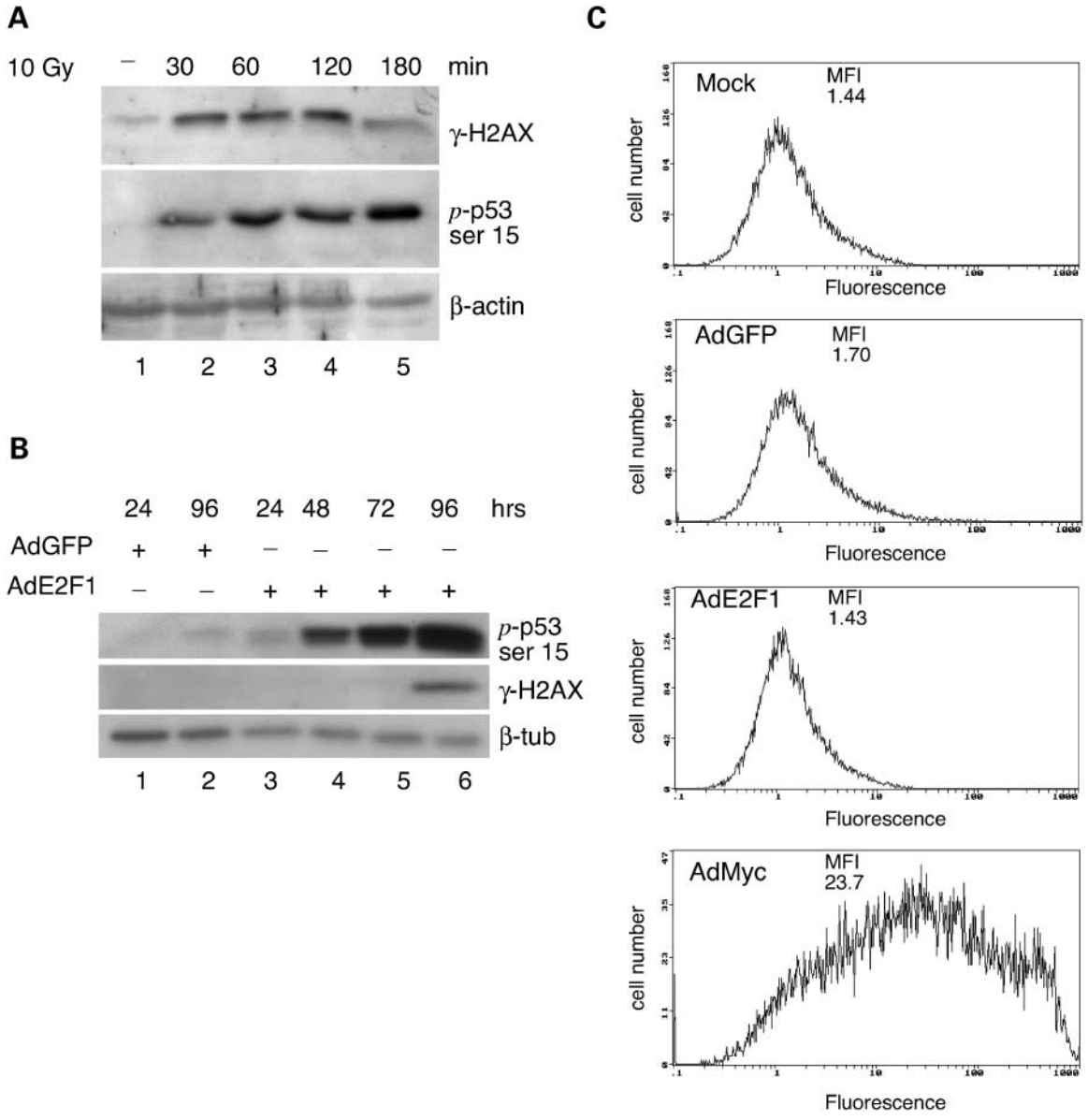

FIGURE 8. E2F1 does not induce timely H2AX phosphorylation or ROS accumulation. **A.** NHF cells were untreated (*lane 1*) or treated with 10 Gy IR and cells were harvested at 30 (*lane 2*), 60 (*lane 3*), 90 (*lane 4*), and 120 (*lane 5*) min postirradiation. Western blot analysis was performed on cell lysates for p53, phospho-Ser^{15} p53, γH2AX, and β-actin. **B.** NHF cells were infected (MOI of 100) with AdGFP (*lanes 1* and *2*) or AdE2F1 (*lanes 3*, *4*, *5*, and *6*) and lysates were made 24 (*lanes 1* and *3*), 48 (*lane 4*), 72 (*lane 5*), or 96 (*lanes 2* and *6*) h postinfection. Western blot analysis was performed for phospho-Ser^{15} p53, γH2AX, and β-tubulin. **C.** Serum-starved NHF cells were mock infected or infected (MOI of 100) with AdGFP, AdE2F1, or AdMyc. Twenty-four hours postinfection, cells were incubated with 1 μM Redox Sensor Red CC-1 (Molecular Probes) for 10 min, trypsinized, and analyzed by flow cytometry. *MFI,* mean fluorescence intensity.

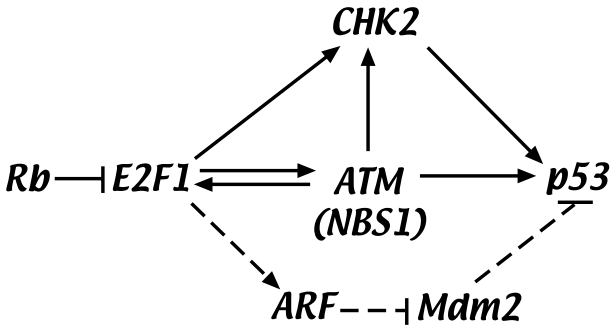

FIGURE 9. Schematic model depicting the signaling pathway between E2F1 and p53.

stabilizes E2F1 in response to DNA damage. This raises the possibility that E2F1 and ATM participate in a positive feedback loop that may amplify the ATM signaling pathway in response to both cell cycle deregulation and DNA damage. Interestingly, thymocytes lacking E2F1 are defective for ATM-dependent apoptosis in response to a radiomimetic drug (41). Although this result was originally interpreted as meaning that E2F1 is downstream of ATM in this apoptotic pathway, it is equally possible that E2F1 is important for amplifying the ATM response to DNA damage.

Potential Role of the E2F1-p53 Pathway in AT and NBS Cancer Predisposition

In addition to cerebellar ataxia, ocular telangiectasia, and severe immunodeficiency, AT patients are also predisposed to developing cancers, particularly lymphoma and leukemia. Moreover, at least some AT carriers (heterozygotes) with dominant-negative mutations are at increased risk for developing breast cancer (18). NBS is a disease with some of the same symptoms as AT, including immunodeficiency and cancer predisposition. Most human cancers have mutations or epigenetic events that result in the deregulation of the Rb-E2F1 pathway. Several experimental systems have demonstrated that inactivation of Rb or deregulated expression of E2F1 results in the activation of p53 and the induction of apoptosis (1–3, 7, 56). This p53-dependent apoptotic response serves to eliminate cells that might otherwise go on to form a tumor. The finding that E2F1-induced apoptosis is impaired in AT and NBS cells suggests that ATM and NBS1 are components of the signaling pathway that connects E2F1 to p53. If this tumor suppression mechanism is impaired in AT and NBS patients, this may contribute to their cancer predisposition.

Materials and Methods

Cells and Viruses

Generation of mice harboring floxed *Rb* (79) and *Brg1* (80) alleles has been described previously. Detection of the recombined *Rb* allele was performed using primers Rb212 and Rb18 as described previously (81). *Brg1* recombination was detected using primers TB82 and TG57 as described previously (80). Fibroblasts were isolated from the peritoneal fascia of mice at least 5 weeks old as follows. The peritoneum was excised, minced into small pieces, and dissociated by constant agitation for 40 min at 37°C in 0.2 mg/ml collagenase (type I; Sigma Chemical Co., St. Louis, MO) supplemented with 100 units DNase I (Roche, Indianapolis, IN). After washing in PBS, dissociated tissue was incubated for 20 min at 37°C in 0.25% trypsin (Life Technologies, Inc., Carlsbad, CA) with constant agitation. Isolated cells were then washed twice and plated in tissue culture dishes. Adult fibroblasts were infected with AdGFP or AdCre at $\sim 0.5 \times 10^8$ plaque-forming units/10 cm dish. Infection efficiency of >95% was confirmed by GFP visualization. AdGFP and AdCre viruses were obtained from the University of Iowa Gene Transfer Vector Core Facility (Iowa City, IA).

NHF dermal cells (Coriell Cell Repositories, Camden, NJ) were maintained in MEM with 2 mM glutamine, nonessential amino acids, and 15% fetal bovine serum. Cells from age-, sex-, and ethnicity-matched individuals GM007532 (wild-type) and GM002502 (from AT patient) were used for the AT experiments. Experiments were repeated in GM003492 (wild-type) and GM003487 (AT patient) cells with similar results. For the NBS experiments, age- and sex-matched cells GM008399 (wild-type) and GM007166 (NBS patient) were used. Recombinant adenoviruses expressing human E2F1, human p53, and GFP have been described (8, 56) and were a kind gift of Dr. Timothy Kowalik or were made using the AdEasy kit (Quantum Biotechnologies, Montreal, Canada).

Western Blot Analysis

Rb and *Brg1* floxed mouse embryo fibroblasts infected with AdGFP or AdCre were harvested by scraping in ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [150 mM NaCl, 1.0% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 13 mg/ml β-glycerophosphate, and 12 μg/ml sodium vanadate]. Insoluble material was removed by centrifugation (13,000 rpm, 10 min at 4°C) and soluble protein concentrations were determined by DC Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of protein from each lysate were boiled in SDS-PAGE sample buffer and resolved by SDS-PAGE. Specific proteins were detected by standard immunoblotting procedures using the following primary antibodies: p53 (Ab-3; Oncogene, La Jolla, CA), phospho-Ser^{15} p53 (9284; Cell Signaling, Beverly, MA), proliferating cell nuclear antigen (Santa Cruz Biotechnology, Santa Cruz, CA), Rb (PharMingen, San Diego, CA), E2F1 (Santa Cruz Biotechnology), and BRG1 (Santa Cruz Biotechnology). Vimentin antibody was a generous gift from Wallace Ip (Department of Cell Biology, University of Cincinnati, Cincinnati, OH).

Western blot analysis of primary human fibroblasts was performed essentially as described (14). Briefly, cells were plated at 4000 cells/cm^2 on 10 cm plates, allowed to recover overnight in complete media, and serum starved for 24 h prior to infection. Protein was harvested at time indicated post-infection or as described for IR and chemical treatments and probed with antisera or antibodies to E2F1 (C-20; Santa Cruz Biotechnology), p53 (Ab-3; Oncogene), phospho-Ser^{15} p53 (9284; Cell Signaling), phospho-Ser^{20} p53 (9287; Cell Signaling), Chk2 (H-300; Santa Cruz Biotechnology), phospho-Thr^{68} Chk2 (2661; Cell Signaling), β-actin (H-2350; Santa Cruz Biotechnology), β-tubulin (H-235; Santa Cruz Biotechnology),

cyclin E (M-20; Santa Cruz Biotechnology), p73 (Ab-2; Oncogene), NBS1 (NB100-143C1; Novas Biologicals, Littleton, CA), γH2AX (4411-PC-100; Trevigen, Gaithersburg, MD), and phospho-Ser[1981] ATM (200-301-400; Rockland, Gilbertsville, PA).

S-Phase Analysis

Cells were grown in plastic chamber slides and serum starved 24 h prior to infection with AdGFP or AdE2F1 at a MOI of 100. Cells were ethanol fixed 24 h postinfection following a 1 h incubation with 10 μM BrdUrd. Immunohistochemistry was performed using an antibody to BrdUrd (Molecular Probes, Eugene, OR) per manufacturer's protocol and cells were examined microscopically.

Apoptosis Assays

Cells were grown in plastic chamber slides and serum starved 24 h prior to infection with AdGFP or AdE2F1 at a MOI of 200. Forty-eight hours postinfection, cells were methanol fixed and stained with fluorescence medium containing 4′,6-diamidino-2-phenylindole. Cells with condensed and/or fragmented nuclei were scored as apoptotic. DNA laddering analysis was performed by collecting floating cells 72 h postinfection. DNA was obtained by incubating the floating cells in lysis buffer containing 20 μg/ml proteinase K at 55°C for 2 h followed by phenol/chloroform extraction. The samples were rinsed in 50% isopropanol in the presence of 1 μg/ml glycogen. After a final 70% ethanol rinse, samples were run on a 1.5% Tris-acetate EDTA gel at 3 V/cm.

ROS Accumulation Assay

NHF cells were mock infected or infected with AdGFP, AdE2F1, or AdMyc. Twenty-four hours postinfection, cells were incubated with 1 μM Redox Sensor Red CC-1 in DMSO (Molecular Probes) for 10 min and analyzed by flow cytometry.

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