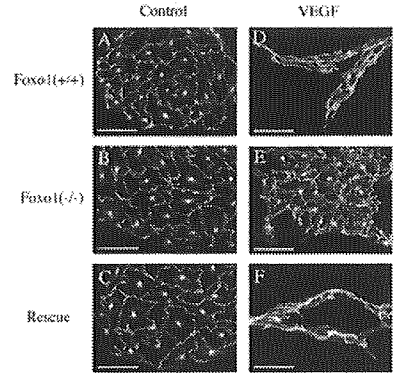


FIG. 6. **Immunolocalization of VE-cadherin on the endothelial cell colonies.** Endothelial cells derived from *Foxo1*$^{+/+}$ (*A* and *D*), *Foxo1*$^{-/-}$ (*B* and *E*), and *Foxo1*-rescued *Foxo1*$^{-/-}$ (*C* and *F*) ES cells were grown on OP9 feeder cells to form colonies in the absence (*A–C*) or presence (*D–F*) of exogenous VEGF-$A_{165}$. Colonies were then stained with anti-VE-cadherin antibody. The molecule localized along cell-cell junctions. The colonies shown are representative of those observed under each condition. *A–C*, round clusters of flat polygonal endothelial cells; *D* and *F*, a dispersed colony of spindle-like endothelial cells; *E*, a holly leaf-like colony of flat endothelial cells. *Scale bars* = 50 μm.

FOXO1 does not influence the proliferation and survival of endothelial cells.

We next examined the morphology of the endothelial cell colonies. In the absence of exogenous VEGF-$A_{165}$, there was no morphological difference between colonies generated from *Foxo1*$^{+/+}$ and *Foxo1*$^{-/-}$ endothelial cells. Most of the colonies were packed round clusters composed of flat polygonal endothelial cells (Fig. 5*C*, *panels a–f*). A small fraction of colonies consisted of a dispersed assemblage of flat endothelial cells (Fig. 5*C*, *panels c* and *f*). When 50 ng/ml VEGF-$A_{165}$ was added to the cultures, however, *Foxo1*$^{+/+}$ and *Foxo1*$^{-/-}$ endothelial cell colonies exhibited quite distinct morphological changes. As reported previously (25), VEGF-$A_{165}$ induced a morphological change in wild-type endothelial cells from a polygonal to a spindle-like shape (Fig. 5*C*, *panels g-i*). The elongated endothelial cells formed either scattered colonies or densely packed bundles. In contrast, ~30% of the *Foxo1*$^{-/-}$ endothelial cell colonies remained round even in the presence of exogenous VEGF-$A_{165}$ (Fig. 5*C*, *panels j* and *k*). They consisted of packed flat polygonal endothelial cell clusters surrounded by fibrous endothelial cells. Most of the other colonies were also made up of flat endothelial cells, but exhibited a holly leaf-like shape (Fig. 5*C*, *panel l*).

Finally, confocal microscopic analyses of endothelial cell colonies stained with anti-VE-cadherin antibody revealed that endothelial cells exhibited a similar flat polygonal shape regardless of *Foxo1* expression in the absence of exogenous VEGF-$A_{165}$ (Fig. 6, *A–C*). Stimulation with exogenous VEGF-$A_{165}$ induced elongation of *Foxo1*$^{+/+}$ endothelial cells, whereas *Foxo1*$^{-/-}$ endothelial cells remained flatten (Fig. 6, *D* and *E*). Endothelial cells of the latter genotype exhibited rather straight adherens junctions and appeared to overlap each other. Cell overlaps were also observed on elongated (VEGF-$A_{165}$-stimulated) wild-type endothelial cells, but were rarely found on unstimulated colonies. Despite repeated trials, we could isolate only one ES clone homozygous for the *Foxo1*-null allele. To exclude the possibility that the abnormal phenotype of *Foxo1*$^{-/-}$ endothelial cells is attributed to some genetic lesions irrelevant to the *Foxo1* gene, we restored expression of *Foxo1* in the differentiating *Foxo1*$^{-/-}$ ES cells by means of an endothelial cell-specific promoter/enhancer cassette. The coding sequence of *Foxo1* cDNA was inserted between the 2.5-kilobase pair 5′-flanking (26) and the 4-kilobase pair 3′-flanking sequences of the first exon of the *Cdh5* (VE-cadherin) gene. The 5′-flanking sequence was shown to confer endothelial cell-specific expression of a reporter gene in transgenic mice (26). The 3′-flanking sequence further enhanced the expression.[2] We introduced the expression cassette into the *Foxo1*$^{-/-}$ ES cells and isolated two rescued clones that gave similar results. Fig. 5*A* (*panel a*) shows the expression of *Foxo1* transcripts in a representative clone. Undifferentiated wild-type ES cells expressed the *Foxo1* gene, whereas neither the *Foxo1*$^{-/-}$ cells nor the rescued clone displayed the expression. In contrast, VE-cadherin$^{+}$ endothelial cells sorted from the differentiating rescued clone expressed *Foxo1* transcripts, as was the case with wild-type endothelial cells. The *Foxo1*-rescued endothelial cells elongated and displayed similar morphology compared with *Foxo1*$^{+/+}$ endothelial cells in response to VEGF-$A_{165}$ (Fig. 6*F*). As the *Cdh5* (VE-cadherin) promoter is specific to the endothelial cell lineage, this result further confirms the endothelial cell autonomous requirement of FOXO1 for the elongation response. These results suggest that the defective vascular development observed in the *Foxo1*$^{-/-}$ embryos is attributable to an abnormal response of endothelial cells to angiogenic stimuli, including VEGF-A.

## DISCUSSION

This study showed that targeted inactivation of the gene encoding FOXO1 resulted in defects in branchial arches and vascular remodeling in early mouse embryos and yolk sacs, which most likely caused the death of the embryos at around day 11. The expression of the *Foxo1* gene was temporarily observed in the branchial arches between E8.5 and E9.5. The *Crabp1* signal, a marker for neural crest cells, was detected as defined streams in the mutant embryos similar to the wild-type embryos at E9.5 despite the severe defects in the branchial arches (22). These findings suggest that FOXO1 is involved in the later stages of branchial arch formation from precursor cells or, alternatively, in the migration or survival of a restricted population of neural crest cells contributing to the formation of the first and second branchial arches at a specific stage. The defects in branchial arches potentially lead to the abnormal formation of branchial arch arteries, resulting in the accumulation of pericardial fluid and death.

The impaired vascular development observed in *Foxo1*-deficient mice may be due to a variety of potential abnormalities within the heterogeneous population of cells composing blood vessels. In this study, however, we focused on whether endothelial cells were affected in *Foxo1* mutants. Cytological examinations of endothelial cells derived *in vitro* from wild-type and *Foxo1*-deficient ES cells detected no differences in morphology and numbers of endothelial cell colonies formed on a stromal cell layer in the absence of exogenous VEGF. This indicates that FOXO1 is not a prerequisite for the regulation of proliferation and survival of endothelial cells supported by a low dose of VEGF and/or other factors derived from stromal cells (25). FOXO1 is phosphorylated by Akt activated through phosphatidylinositol 3-kinase and translocated into the cytosol from the nucleus with a resultant decrease in transcriptional activity (1). On the other hand, several lines of evidence indicate that activation of phosphatidylinositol 3-kinase is crucial for VEGF-mediated endothelial cell proliferation, survival, and migration (27). The tumor suppressor PTEN has been shown to down-regulate phosphatidylinositol 3-kinase signaling, and indeed, overexpression of PTEN inhibits the anti-apoptotic, proliferative, and chemotactic effects of VEGF (28, 29). Moreover, constitutively active Akt or dominant-negative PTEN can promote angiogenesis through an increase in mRNA levels of VEGF

[2] H. Hisatsune, unpublished data.

(27–29). These studies imply that activation of the phosphatidylinositol 3-kinase/Akt signaling system leads to promoted angiogenesis. Recently, it was reported that VEGF promotes survival and growth of endothelial cells via inhibition of FOXO family members through the phosphatidylinositol 3-kinase/Akt signaling system (30). However, *Foxo1*-deficient endothelial cells exhibited normal colony formation in response to a low dose of VEGF and an abnormal morphological response to a high dose of VEGF. Taken together, these findings imply that FOXO family members other than FOXO1 are involved in the responses to a low dose of VEGF such as growth and survival of *Foxo1*-deficient endothelial cells.

In contrast, the *Foxo1*-deficient endothelial cells showed markedly different responses compared with the wild-type endothelial cells in the presence of exogenous VEGF (50 ng/ml). It has been reported that the formation of blood vessels is impaired not only in homozygous VEGF-deficient embryos, but also in heterozygous VEGF-deficient embryos (31, 32). Therefore, the function of VEGF is clearly dependent upon its expression levels. The abnormal phenotype for blood vessel formation in *Foxo1*-deficient mice is similar to that in heterozygous VEGF-deficient mice in terms of the presence of endothelial cells, enlarged yolk sac vessels, and hypoplasia of the dorsal aorta (31, 32). Taken together, these results suggest that a dose of VEGF above a certain threshold and the ability of endothelial cells to properly respond to it are essential for normal vascular development and that products transcriptionally regulated by FOXO1 are required for the latter. Although it is not well understood how the behavior of individual endothelial cells accounts for the processes of vascular development, undoubtedly the morphological response of endothelial cells serves as a driving force for vascular remodeling *in vivo*. Thus, it is more likely that the impaired angiogenesis observed in the mutants was due to the abnormal response of endothelial cells to VEGF and/or other angiogenic growth factors. However, we cannot formally exclude the possibility that the defects in branchial arch arteries secondarily influenced vascular remodeling. It is necessary to elucidate whether endothelium-specific inactivation of *Foxo1* would lead to the same phenotype.

In this study, we found that the transcripts of connexin-37 and connexin-40 were remarkably decreased in *Foxo1*-deficient yolk sacs. It is known that these connexins are rich in endothelial cells and that double connexin-37/connexin-40-deficient mice have vascular abnormalities such as blood vessel dilation and congestion (33–35). Moreover, the transcript of ephrin-B2 was also reduced to 35% of the wild-type yolk sac. Ephrin-B2-null mice die *in utero* before E11.5 because of defects in the remodeling of the embryonic vascular system and display defects in angiogenesis by both arteries and veins in the capillary networks of heads and yolk sacs (35–37). The phenotypes are very similar to that of *Foxo1*-deficient mice. Since *Foxo1*-deficient mice have at least 35% of the ephrin-B2 transcripts of the wild-type mice and since heterozygous ephrin-B2-deficient mice show a normal phenotype (35–37), the ephrin-B2 reduction alone can hardly cause the vascular defects in *Foxo1*-deficient mice. A synergistic effect of some factors, including connexin-37, connexin-40, and ephrin-B2, may induce the abnormal phenotype. It is not clear whether the connexins and ephrin-B2 are direct target genes of *Foxo1* or whether the impairment of endothelial cells by *Foxo1* deficiency secondarily induces the reduced expression of the genes. Mukouyama *et al.* (38) showed similar losses of connexin-40 and ephrin-B2 in blood vessels of limb skin of mice lacking neurogenin-1 and neurogenin-2. They attributed the loss of arterial markers to loss of VEGF produced by peripheral nerves and showed that VEGF could induce connexin-40 and ephrin-B2 in endothelial cells *in vitro* (38). The *Foxo1*-deficient embryos showed the same loss of arterial markers and of connexin-37, a third known arterial marker. The data in Figs. 4 and 5 show that VEGF receptors were retained, but Fig. 6 shows the endothelial cells to be resistant to VEGF. Because restoring *Foxo1* restores sensitivity to VEGF, FOXO1 seems to be required for responsiveness to VEGF. Thus, the ability of the primary vascular plexus to respond to an arterialization signal mediated by VEGF may be blocked in *Foxo1*-deficient embryos.

Finally, defects in vascular remodeling and pericardial fluid accumulation similar to those described here have also been reported in embryos that bear mutations in components of the transforming growth factor-β signaling pathway, including the type I receptor (ALK5), ALK1, endoglin, Smad5, and so on (39–42). Remarkably, genetic analyses in *C. elegans* imply that the transforming growth factor-β signaling pathway and the insulin/DAF-2 signaling pathway converge on a common target, *daf-9*, which encodes a cytochrome P-450 related to vertebrate steroidogenic hydroxylases (43–45). Thus, it is possible that a component of the transforming growth factor-β signaling pathway directly or indirectly interacts with a component of the insulin/insulin-like growth factor-1 signaling pathway during angiogenesis in mammals. It remains to be examined whether or not the target genes controlled by the transforming growth factor-β signaling pathway are properly expressed in the *Foxo1*-deficient mice.

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# DNA-dependent Protein Kinase and Checkpoint Kinase 2 Synergistically Activate a Latent Population of p53 upon DNA Damage*

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**The role of the checkpoint kinase 2 (Chk2) as an upstream activator of p53 following DNA damage has been controversial. We have recently shown that Chk2 and the DNA-dependent protein kinase (DNA-PK) are both involved in DNA damage-induced apoptosis but not $G_1$ arrest in mouse embryo fibroblasts. Here we demonstrate that Chk2 is required to activate p53 *in vitro* as measured by its ability to bind its consensus DNA target sequence following DNA damage and is in fact the previously unidentified factor working synergistically with DNA-PK to activate p53. The gene mutated in ataxia telangiectasia is not involved in this p53 activation. Using wortmannin, serine 15 mutants of p53, DNA-PK null cells and Chk2 null cells, we demonstrate that DNA-PK and Chk2 act independently and sequentially on p53. Furthermore, the p53 target of these two kinases represents a latent (preexisting) population of p53. Taken together, the results from these studies are consistent with a model in which DNA damage causes an immediate and sequential modification of latent p53 by DNA-PK and Chk2, which under appropriate conditions can lead to apoptosis.**

In response to DNA damage induced by ionizing radiation (IR),[1] eukaryotic cells can activate cell cycle checkpoints or apoptosis. The p53 tumor suppressor mediates these cell responses (1–4); however, an understanding of the mechanism of its activation remains elusive. Upstream candidates include the gene mutated in ataxia telangiectasia (ATM) (5, 6), the DNA-dependent protein kinase (DNA-PK) catalytic subunit (7–9), and more recently, the evolutionarily conserved checkpoint kinases Chk1 and Chk2 (10–15).

Chk2 has been demonstrated to form stable complexes with p53 (16) and was proposed to activate p53 via its kinase activity toward Ser-20 on p53 (10, 11). Furthermore, Chk2 has been identified in a subset of Li Fraumeni patients with normal p53 alleles, making it a potential tumor suppressor protein (17–19). How endogenous Chk2 functions in this capacity has been the focus of many recent studies. Chk2−/− murine embryonic stem cells have been examined and reported to be unable to maintain IR-induced $G_2$ arrest, and murine Chk2−/− thymocytes and mouse embryo fibroblasts (MEFs) have been shown to have an attenuated apoptotic response (12). The role of Chk2 in the $G_1$ cell cycle checkpoint in the murine model has been controversial with some groups reporting its requirement, whereas others are reporting it as unnecessary for the p53-mediated $G_1$/S arrest (12–15).

The role of Chk2 in the human p53 responses has recently been questioned. Ahn *et al.* (20) have purified Chk2 from DNA damaged human cells and demonstrate that p53 phosphorylation is not enhanced after IR. Furthermore, following introduction of Chk2 short interfering RNA into three different human tumor cell lines, p53 was still found to be stabilized and active after IR. Concurrently, Jallepalli *et al.* (21) disrupted the Chk2 gene in human cancer cells and found that p53 Ser-20 phosphorylation, stabilization, and transcriptional activation as well as its cell cycle-mediated arrest and apoptotic responses remained intact. These two reports call into question the role of Chk2 in human cells and further question whether Chk2 lies upstream of the p53-dependent apoptotic response because it seems clear that it does lie upstream in the murine system. More recently, however, Craig *et al.* (22) have demonstrated that Chk2 is regulated by allosteric effects of p53 and that its kinase-dependent phosphorylation of p53 requires conformational docking sites on p53, lending evidence again to a role for human p53 as a target of Chk2 phosphorylation.

Because disruption of the p53 tumor suppressor is often found in many human cancers, understanding the mechanism of p53 activation is essential to our progress in the treatment of cancer. We have recently reported that DNA-PK and Chk2 are both required for p53-mediated apoptosis in MEFs and that they function in a pathway that uses latent p53 to mediate this response (8, 13). In this study, we used various approaches to establish the link between these two kinases in p53 activation following DNA damage. We show that Chk2 and DNA-PK act synergistically and in parallel to activate p53 (including human p53) as measured by its ability to bind its consensus DNA target sequence. The p53 target of these two kinases is a latent (preexisting) population of p53. The gene mutated in ATM is not involved in this p53 activation. Our results are consistent with a model in which the immediate actions of DNA-PK and Chk2 on latent p53 following DNA damage are the first events that dictate the subsequent cellular apoptotic response.

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[1] The abbreviations used are: IR, ionizing radiation; ATM, ataxia telangiectasia-mutated; DNA-PK, DNA-dependent protein kinase; Chk, checkpoint kinase; MEF, mouse embryo fibroblast; EMSA, electrophoretic mobility shift assay.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Preparation of Nuclear Extracts*—The two glioma cell lines M059K and M059J were grown in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum (Invitrogen). A-T lymphoblasts were obtained from ATCC and were cultured in Dulbecco's modified Eagle's medium/F-12 with 20% fetal bovine serum (Invitrogen). DNA-PK−/− or Chk2−/− mouse embryo fibroblasts were isolated from 12.5-day-old embryos and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were grown to 80% confluency and were then irradiated with a $^{137}$Cs irradiator at a rate of 2.5 grays min$^{-1}$ for 2 min. Nuclear extracts were prepared as described previously (13).

*Cell-free in Vitro Translation*—For cell-free *in vitro* translation, cytoplasmic extracts from M059K and M059J cells were prepared as reported previously (7). Wild-type and mutants of human p53 mRNA were synthesized by *in vitro* transcription using a T7 polymerase Megascript kit (Ambion), and human Chk2 mRNA was synthesized using the T7 polymerase kit and the vector containing wild-type human Chk2 sequence kindly provided by Dr. T. Halazonetis (Wistar Institute). The human Chk1 clone was a kind gift from Dr. Y. Sanchez (University of Cincinnati). *In vitro* translation reactions were carried out in a final volume of 50 μl containing the following components: 30 μl of cytoplasmic extract, 1 mM ATP, 0.5 mM GTP, 1 mg ml$^{-1}$ creatine phosphokinase (from ICN), 10 mM creatine phosphate, 40 mM hemin (from ICN), 80 mM KCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 5 μM of each amino acid (Promega), placental RNase inhibitor (RNA Guard, Amersham Biosciences), and either water (for mock translations) or p53 mRNA (for p53 translations). Translation reactions were incubated at 37 °C for 30 min followed by the addition of an equal volume of nuclear extract from mock-treated or γ-irradiated M059K or M059J cells (see above) that had been immunodepleted of endogenous p53 using the anti-p53 monoclonal antibody pAb421 preadsorbed onto inactivated *Staphylococcus* A (IgSorb, The Enzyme Center). Reaction mixtures were then subjected to electrophoretic mobility shift assay (EMSA) as described.

*DNA Binding Analysis by EMSA*—DNA binding was analyzed by EMSA using the $^{32}$P-labeled p53 consensus sequence 5′-AGCTTAGACATGCCTAGACATGCCAAGCT-3′ as described previously (7).

*Western Blotting*—For SDS-PAGE, protein samples were boiled for 5–10 min in protein sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 2% β-mercapto-ethanol, 0.01% bromphenol blue). Electrophoresis was carried out at room temperature with an applied current of 35 mA for ~3 h. Proteins were transferred to nitrocellulose membranes for 2 h at 80 V, 4 °C. The blot was then rinsed in Tris-buffered saline plus 0.2% Tween 20 and placed in blocking buffer with 5% nonfat milk powder in Tris-buffered saline plus 0.2% Tween 20 overnight. The blot next was incubated in primary antibody (FL393 (Santa Cruz Biotechnology), p53 Ser-15-phosphospecific antibody, and Chk2 Thr-68-phosphospecific antibody (Cell Signaling Technology)) at a dilution of 1:1000 in blocking buffer for 1 h. Following incubation with the primary antibody, the blot was thoroughly washed in blocking buffer. Anti-rabbit (Jackson Laboratories) IgG-horseradish peroxidase secondary antibody was used at 1:5000 dilution in blocking buffer and incubated at room temperature for 30–45 min followed by washing in blocking buffer. The blot was then subjected to chemiluminescence (ECL, Amersham Biosciences) and then exposed to Kodak X-Omat AR film. For protein loading control, a monoclonal antibody to actin or tubulin (Sigma) was used at 1:10,000 dilution and processing was carried out as described.

## RESULTS

*DNA-PK and Chk2 Are Both Required to Activate p53 DNA Binding in Vitro*—We have previously demonstrated that DNA-PK is necessary but not sufficient to activate p53 DNA binding *in vitro* and that an unknown factor present in DNA damaged cells is also required for p53 activation (7). More recently, we reported that both DNA-PK and Chk2 are involved in p53-dependent apoptosis and that the latent, rather than the inducible population of p53, is implicated in this process (8, 13). To determine whether the unknown factor that collaborated with DNA-PK in activating p53 *in vitro* was Chk2, we employed the same cell-free translation system used previously to show that DNA-PK acts upstream of p53 in promoting p53 sequence-specific binding (7). Accordingly, wild-type human p53 was translated in a cytoplasmic extract prepared from the human glioma cell line M059J, which lacks any DNA-PK activity (because of defective expression of DNA-PK catalytic subunit) (23). Nuclear extract from either DNA-PK-positive (M059K) or null (M059J) glioma cell lines (untreated or γ-irradiated) was then added to test whether the translated p53 could be activated for p53 binding. As previously reported (7), the irradiated glioma line with DNA-PK (M059K) activated p53 binding as did the glioma line lacking DNA-PK (M059J) if supplemented with purified DNA-PK (Fig. 1, *lanes 1–4*). Most importantly, whereas DNA-PK or Chk2 (*in vitro* translated) alone was unable to activate p53 (*lanes 5* and *6*), together they activated p53 binding to the same extent as with the irradiated nuclear extract (*lane 7*). The specificity of the Chk2 kinase in this role was confirmed because Chk1 kinase did not cooperate with DNA-PK to activate p53 (data not shown). The ability of DNA-PK to activate p53 in the absence of DNA damage in this case can be explained by the fact that DNA-PK is activated by the presence of DNA ends supplied by the consensus DNA sequence. Also, it has been shown recently that overexpression of Chk2 can result in autophosphorylation and trans-phosphorylation events that activate Chk2 in the absence of IR (24). Therefore, our experiment identifies the previously hypothesized "unknown" nuclear factor acting with DNA-PK to activate p53 DNA binding *in vitro* (13) as the checkpoint kinase Chk2. In addition, because all of the components involved are human in origin, our experiment supports a possible role of Chk2 upstream of human p53.

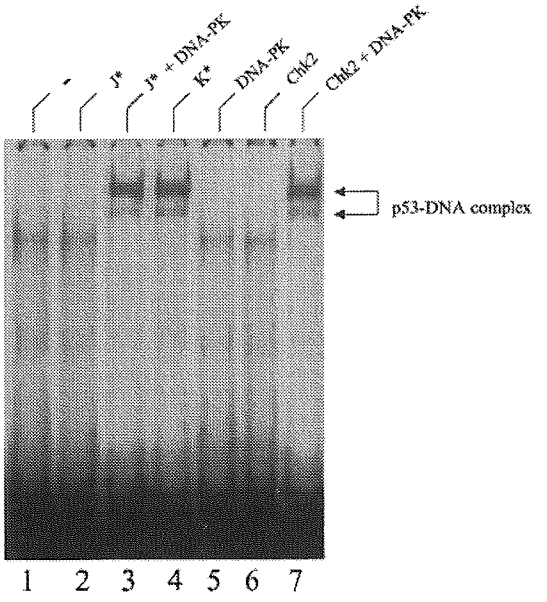


FIG. 1. **Co-requirement of DNA-PK and Chk2 for activation of p53 DNA binding *in vitro*.** Wild-type p53 was translated in cytoplasmic extracts from M059J cells. The following was then added to the reaction: *lane 1*, control (nothing added); *lane 2*, nuclear extract from γ-irradiated M059J (*J**) cells; *lane 3*, same as *lane 2* with the exception that purified DNA-PK holoenzyme was also added; *lane 4*, nuclear extract from γ-irradiated M059K (*K**) cells; *lane 5*, purified DNA-PK holoenzyme; *lane 6*, Chk2 (*in vitro* translated); and *lane 7*, purified DNA-PK holoenzyme and Chk2 in combination. Samples from these reactions were then tested for p53 sequence-specific DNA binding activity by EMSA.

*ATM Is Not Required for Activation of p53 Sequence-specific Binding in Vitro*—We previously reported that whereas both DNA-PK and Chk2 are required for activating latent p53-mediated apoptosis in MEFs, ATM is dispensable for this activity (8, 13). We now seek to determine whether the *in vitro* system required ATM to activate p53 DNA binding and establish whether this system reflects the observed apoptotic situation in MEFs.

To this end, nuclear cell extracts were prepared from an

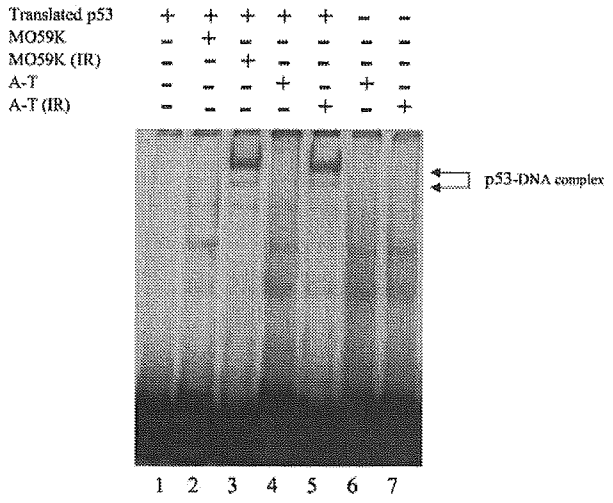


FIG. 2. **ATM is dispensable for *in vitro* activation of p53 DNA binding.** Wild-type 53 was synthesized in a cell-free translation system, and nuclear extracts from non-DNA-damaged or γ-irradiated (*IR*) M059K cells or A-T lymphoblasts were added. The reaction mixtures were then assayed for p53 sequence-specific binding by EMSA.

ataxia telangiectasia (A-T)-lymphoblastoid cell line and examined for their ability to activate p53 DNA binding in the cell-free translation system. The p53 in these A-T cells is highly unstable, and therefore, no endogenous p53 protein was detectable by EMSA (Fig. 2, *lanes 6* and *7*) or by Western blot (data not shown). However, nuclear extracts from IR-treated A-T cells, similar to those from IR-treated M059K cells, were able to activate *in vitro* translated p53 (Fig. 2, *lanes 2–5*). This finding suggests that ATM, although necessary for the stabilization of p53, is not required for the activation of p53 DNA binding. In an independent experiment, A-T cells were treated with leptomycin B to determine whether accumulation of p53 by stabilization alone was enough to activate p53 DNA binding and it was found that it was not (data not shown). This finding is consistent with our previous results, demonstrating that Chk2 functions independently of ATM to activate the p53-apoptotic response (13).

*DNA-PK and Chk2 Act Independently and Sequentially to Activate p53*—We then proceeded to determine whether DNA-PK and Chk2 act independently of one another or whether they are required for each other's action. A number of experimental approaches were taken to examine the sequence of events in the activation of p53 by DNA-PK and Chk2.

Using the *in vitro* system, the fungal metabolite wortmannin was employed to inhibit DNA-PK before and after the addition of various components in the pathway. As can be seen in Fig. 3*A*, DNA-PK activity is required to activate p53 because inhibition of DNA-PK prior to its incubation with p53 prevented p53 DNA binding (compare *lanes 3* and *4*). By contrast, if DNA-PK and p53 are allowed to interact and then DNA-PK is inhibited by wortmannin prior to Chk2 addition, p53 binding can still be observed (Fig. 3*A*, *lane 5*). This finding suggests that DNA-PK acts directly on p53 but it is not upstream of Chk2.

We have previously demonstrated that Ser-15 on latent p53 is a target of DNA-PK immediately following DNA damage and that this residue plays a role in IR-induced apoptosis of MEFs (8). To determine whether Ser-15 is also involved in the *in vitro* activation of p53 by DNA-PK, two Ser-15 mutants of p53, S15A and S15D, were tested in our *in vitro* activation assays. Because S15D mimics phospho-Ser-15, it was expected to be active even in the absence of DNA-PK, whereas S15A would be innately inactive. Unexpectedly, we found that for both mutants, Chk2 was absolutely required but DNA-PK was dispensable for activation of DNA binding (Fig. 3*B*). This finding suggests that conformational changes in p53 brought on by Ser-15 phosphorylation, rather than a difference in charge, are responsible for DNA-PK-induced activation of p53. The observation that, for both S15A and S15D mutants, Chk2 alone was able to activate DNA binding in the absence of DNA-PK also strongly argues for the independence of Chk2 from DNA-PK. It further implies that upon DNA damage, p53 is probably first acted on by DNA-PK before being further modified by Chk2.

We also altered a few other serine phosphorylation sites on p53 to see whether these mutations would affect p53 activation by DNA-PK and Chk2 in any way. These included two N-terminal phosphorylation sites, Ser-20 (a known target site of Chk2) and Ser-37 (another known DNA-PK target site), and two C-terminal phosphorylation sites, Ser-315 and Ser-392. None of these mutations was found to alleviate the dependence on DNA-PK or Chk2 for p53 activation (Fig. 3*C*). It is also interesting that the mutation of p53 at Ser-20, a known Chk2 target site, to either alanine (simulating the unphosphorylated state) or aspartic acid (simulating constitutive phosphorylation) had no effect on its requirement for Chk2, which in turn suggests that Ser-20 is not the target of Chk2 for p53 activation *in vitro*.

That DNA-PK and Chk2 probably act on p53 independently of each other was further suggested by the following observations. First, Chk2+/+ and Chk2−/− MEFs displayed similar levels of DNA-PK-dependent Ser-15 phosphorylation upon IR (Fig. 3*D*). Second, Chk2 phosphorylation at Thr-68, often used as a measure of Chk2 activation (25–28), was found to be immediate and at comparable levels between human cells with and without DNA-PK (M059K and M059J, respectively) upon γ-irradiation (Fig. 3*E*), These observations, combined with those from the wortmannin and S15A and S15D studies described above (Fig. 3, *A* and *B*, respectively) have led us to conclude that DNA-PK and Chk2 work sequentially and independently of one another in activating p53 upon DNA damage.

*Latent but Not Inducible p53 Requires Chk2 and DNA-PK for in Vitro DNA Binding*—We have recently reported that the *latent* population of p53 is sufficient to mediate the Chk2 and DNA-PK-directed p53 apoptotic response (7, 13). To determine whether it is the latent population of p53 whose DNA binding activity is regulated by DNA-PK and Chk2, wild-type, Chk2 null, and DNA-PK null MEFs were exposed to IR in the presence and absence of cycloheximide. Treatment of the cells with cycloheximide blocks translation of any new p53 protein and allows us to assess the DNA binding activity of preexisting (latent) p53. Fig. 4 clearly shows that the latent p53 population in wild-type MEFs are activated to bind DNA upon IR. In contrast, only the induced but not the latent population of p53 was activated in DNA-PK−/− cells. This finding is consistent with our previous contention that DNA-PK targets latent p53, whereas ATM targets induced p53 (8, 13). Likewise, an examination of wild-type *versus* Chk2−/− cells yielded the same results, suggesting that Chk2 is absolutely required for latent p53 binding to its consensus sequence (Fig. 4).

## DISCUSSION

Following DNA damage such as ionizing radiation, cell signaling events cause cell cycle arrest or apoptosis. The p53 tumor suppressor is central to these responses and has been shown to be a target of a number of kinases including ATM, ATR, DNA-PK, Chk1, and Chk2. Until recently, it has been strongly suggested that following IR, the Chk2 kinase was an upstream mediator of p53 cell cycle and apoptotic responses. With the more recent evidence questioning the role of Chk2 in human cancer cell lines, it is important to decipher the role, if any, that Chk2 plays in activating p53 responses.

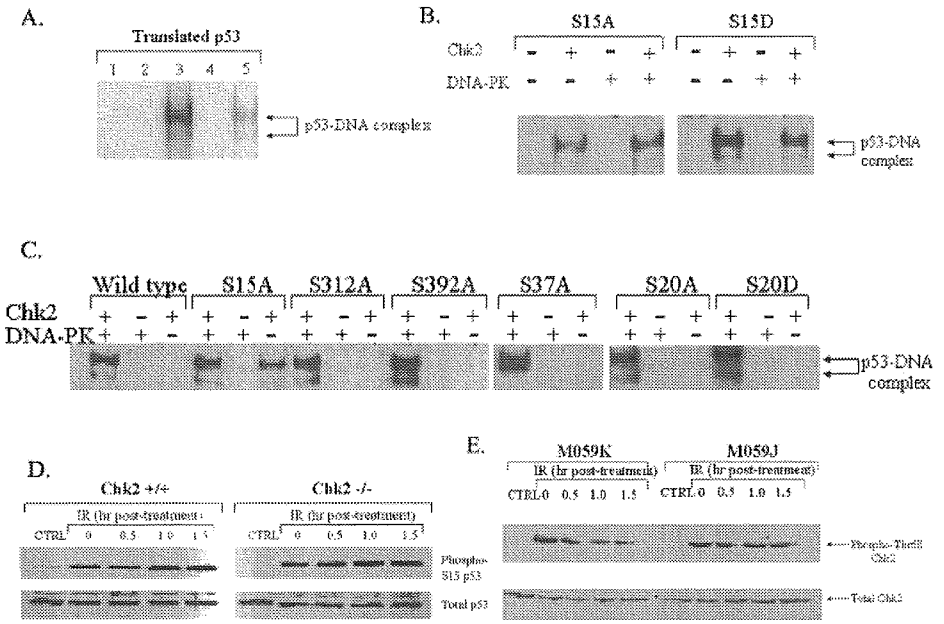


FIG. 3. **DNA-PK and Chk2 act independently and sequentially to activate p53.** *A*, effect of wortmannin on p53 activation by DNA-PK and Chk2. Wild-type p53 was translated *in vitro*. The following was then added to the reaction: *lane 1*, control (nothing added); *lane 2*, Chk2 (*in vitro* translated); *lane 3*, Chk2 and purified DNA-PK holoenzyme in combination; *lane 4*, DNA-PK that had been inactivated by wortmannin for 10 min followed by Chk2; *lane 5*, DNA-PK for 10 min, wortmannin for 10 min, and then Chk2. p53 DNA binding was then assayed by EMSA. *B*, Chk2 is required, but DNA-PK is dispensable for Ser-15 p53 mutant DNA binding. S15A or S15D p53 mutants were translated and assayed for their requirement of Chk2 and DNA-PK to induce DNA binding to the consensus sequence as assayed by EMSA. *C*, comparing the serine 15 modification (S15A) to those at other serine phosphorylation sites (S315A, S392A, S37A, S20A, and S20D) in terms of requirement for DNA-PK and Chk2 for activation. S15A requires only Chk2, whereas wild-type as well as all of the other serine mutants of p53 require both DNA-PK and Chk2 for activation. *D*, comparing DNA damage-induced phosphorylation on serine 15 of p53 in Chk2+/+ and Chk2−/− MEFs. Cells were exposed to γ-irradiation and harvested at various times thereafter. Following SDS-PAGE and transfer to nitrocellulose membrane, the membrane was probed with an anti-phosphoserine 15 antibody or for total p53 protein. *E*, comparing DNA damage-induced phosphorylation on threonine 68 of Chk2 in M059K and M059J cells. Cells were exposed to γ-irradiation and harvested at various times thereafter. Following SDS-PAGE and transfer to nitrocellulose membrane, the membrane was probed with an anti-phosphothreonine 68 antibody or for total Chk2 protein.

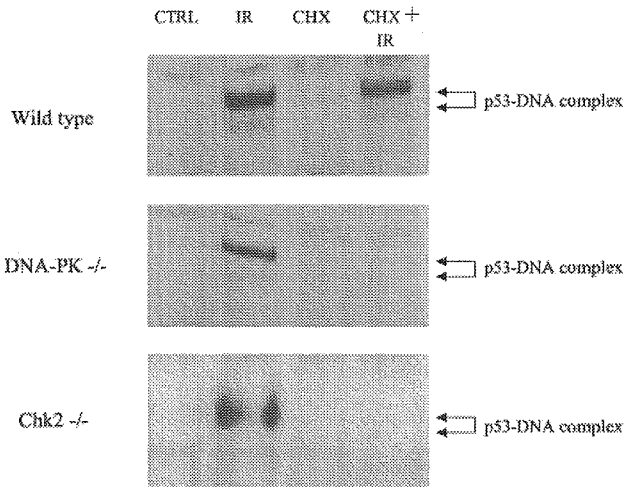


FIG. 4. **DNA-PK and Chk2 are required for activation of latent p53 DNA binding following DNA damage.** Wild-type, DNA-PK−/−, or Chk2−/− MEFs were mock-treated (*CTRL*), γ-irradiated (*IR*), cycloheximide-treated (*CHX*), or CHX-treated followed by IR (*CHR + IR*). Nuclear extracts were then prepared and assayed for p53 DNA binding by EMSA.

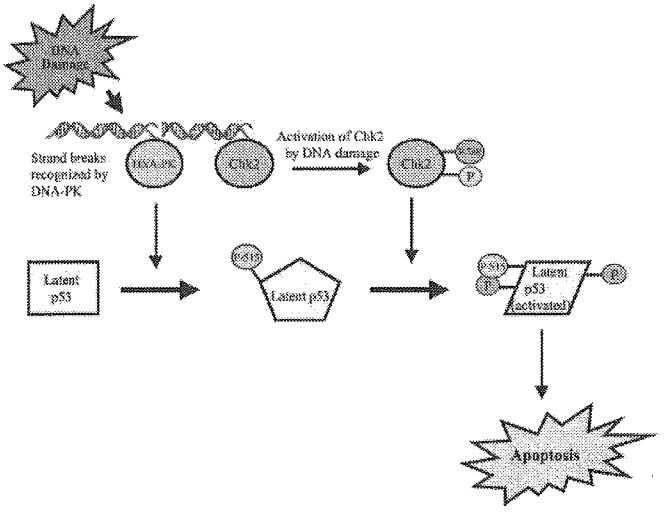


FIG. 5. **Model for activation pathway of latent p53 and apoptosis.** Immediately following DNA damage, DNA-PK is activated by strand breaks and phosphorylates a latent (preexisting) population of p53 on Ser-15. This induces a conformational change in the p53 protein such that it is recognized by the Chk2 kinase, which then phosphorylates (and activates) the latent p53. The severity and nature of DNA damage incurred dictates the extent of this latent p53 activation, which in many cell types probably represents the first obligatory event that leads to apoptosis. Under circumstances where cell growth arrest or DNA repair is the preferred outcome, a separate pathway (data not shown) involving inducible p53 and mediated by ATM, ATR, or other kinases comes into play.

To examine the role of Chk2 upstream of p53, we utilized an *in vitro* cell free translation system whereby p53 activation could be assessed by its ability to bind its consensus sequence This system was previously used to demonstrate that DNA-PK acts upstream of p53 in response to DNA damage and that an unknown factor (also activated by DNA damage) was also involved in this process. We now show that this unknown factor is Chk2. These two kinases work synergistically to activate p53 DNA binding that is demonstrable *in vitro*. Whether the p53 binding is an actual indication of p53 transcriptional activity or simply a readout for some conformation change in the p53 protein induced by Chk2 and DNA-PK is unclear at present. What is clear, however, is that Chk2 and DNA-PK together are

capable of activating p53 including human p53 *in vitro*.

The observation that human ATM cell extract was also capable of promoting p53 DNA binding is also consistent with our previous demonstration that Chk2 activates p53-mediated apoptosis independently of the ATM protein. Here again the results indicate that, although ATM is necessary to stabilize the p53 protein, the ability of Chk2 and DNA-PK to activate p53 does not require ATM. This also underscores the need to consider stabilization and activation of p53 as two separate events.

The independent nature of DNA-PK and Chk2 in activating p53 *in vitro* and the sequential order of their involvement were determined using a number of approaches. We used wortmannin to show that DNA-PK must act upon p53 since inhibition of its kinase activity completely abolished p53 binding. However, if DNA-PK was first allowed to interact with p53, the subsequent addition of wortmannin did not interfere with the activating effect of Chk2 that was added later. This indicates that DNA-PK is not required upstream of Chk2 to promote p53 activation. The demonstration that Ser-15 is phosphorylated by DNA-PK equally well in Chk2+/+ and Chk2−/− cells further attests to the complete independence of action of these two kinases.

Further evidence for a parallel and sequential mechanism of p53 activation by DNA-PK and Chk2 comes from the observation that the two p53 Ser-15 mutants, S15A and S15D, do not require DNA-PK and can be activated by Chk2 alone. Although this result is expected for the S15D mutant, which mimics phospho-Ser-15, it came initially as a surprise in the case of the S15A mutant. The most logical explanation for both of these mutants being able to bypass the DNA-PK requirement is that modification of serine 15 by any means (phosphorylation or mutation) leads to an altered conformation of p53, which is recognizable by Chk2. Indeed, previous work by Shieh *et al.* (29) shows that phosphorylation at the serine 15 site by DNA-PK alters the tertiary structure of p53. Very recently, Craig *et al.* (22) demonstrated that two peptides derived from the DNA binding domain of p53 could bind Chk2 and allosterically stimulate the phosphorylation of full-length p53. Based on our present findings, we contend that the reason why full-length p53 by itself cannot activate Chk2 is probably because these Chk2-docking sites on native p53 are cryptic and are exposed only after phosphorylation of Ser-15 by DNA-PK. Thus, the initial modification of p53 by DNA-PK is a prerequisite for its subsequent modification by Chk2 with the two events probably occurring quickly in tandem and resulting in full p53 activation.

Finally, our study suggests that it is the latent population of p53 that is activated by DNA-PK and Chk2 upon DNA damage. This finding is consistent with our previous observation that latent p53 becomes phosphorylated at serine 15 immediately upon IR treatment and that latent p53, rather than inducible p53, is sufficient to induce apoptosis in a DNA-PK-dependent and Chk2-dependent manner. Therefore, it therefore appears that the fate of the cell (cell growth arrest or apoptosis) is determined very early on, possibly immediately following DNA damage. Depending on the nature and extent of DNA damage, p53 that appears later on the scene (inducible p53) may then be modified by other kinases such as ATM or ATR and may have a function that is distinct from that of the latent population of p53.

In summary, results from our investigation are consistent with a model wherein upon DNA damage, DNA-PK, and Chk2 act synergistically and sequentially (with DNA-PK followed by Chk2) on a latent population of p53 (Fig. 5). The modified p53 manifests DNA binding activity *in vitro*, which may reflect the actual event *in vivo* or an altered conformational state that is unrelated to its DNA binding activity. In either case, the extent of such modifications on this latent population of p53 probably dictates the subsequent course of action and, depending on the cell context, can lead to apoptosis.

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# Stress-induced Premature Senescence in hTERT-expressing Ataxia Telangiectasia Fibroblasts*

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**In addition to replicative senescence, normal diploid fibroblasts undergo stress-induced premature senescence (SIPS) in response to DNA damage caused by oxidative stress or ionizing radiation (IR). SIPS is not prevented by telomere elongation, indicating that, unlike replicative senescence, it is triggered by nonspecific genome-wide DNA damage rather than by telomere shortening. ATM, the product of the gene mutated in individuals with ataxia telangiectasia (AT), plays a central role in cell cycle arrest in response to DNA damage. Whether ATM also mediates signaling that leads to SIPS was investigated with the use of normal and AT fibroblasts stably transfected with an expression vector for the catalytic subunit of human telomerase (hTERT). Expression of hTERT in AT fibroblasts resulted in telomere elongation and prevented premature replicative senescence, but it did not rescue the defect in $G_1$ checkpoint activation or the hypersensitivity of the cells to IR. Despite these remaining defects in the DNA damage response, hTERT-expressing AT fibroblasts exhibited characteristics of senescence on exposure to IR or $H_2O_2$ in such a manner that triggers SIPS in normal fibroblasts. These characteristics included the adoption of an enlarged and flattened morphology, positive staining for senescence-associated β-galactosidase activity, termination of DNA synthesis, and accumulation of p53, $p21^{WAF1}$, and $p16^{INK4A}$. The phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), which mediates signaling that leads to senescence, was also detected in both IR- or $H_2O_2$-treated AT and normal fibroblasts expressing hTERT. These results suggest that the ATM-dependent signaling pathway triggered by DNA damage is dispensable for activation of p38 MAPK and SIPS in response to IR or oxidative stress.**

Culture of primary cells for many generations eventually results in a loss of proliferative potential, a phenomenon referred to as replicative senescence or, more generally, as cellular senescence. Cellular senescence can also be induced by stressful conditions (1). Replicative senescence likely results from the shortening of telomeres to such an extent that the chromosome ends are not fully masked from recognition by the proteins responsible for double strand break repair. Whereas primary human fibroblasts, which lack telomerase activity, normally exhibit a finite life span in culture (2), ectopic expression of the catalytic subunit of human telomerase (hTERT)[1] in these cells restores telomerase activity, stabilizes telomere length, and prevents replicative senescence (3). Although rodent cells in culture also undergo senescence, this phenotype is attributable to unsuitable conditions (culture shock) or to growth arrest mediated by p53 and $p19^{Arf}$, not to telomere shortening *per se*. In contrast, the major signaling pathway responsible for senescence in human cells, including that due to telomere shortening, is mediated by RB and $p16^{INK4A}$ (4, 5).

The introduction of activated oncogenes into primary cells triggers defense responses that prevent cell proliferation. Some oncogenes, such as those for c-Myc and E2F1, trigger apoptosis (6, 7), whereas others, such as those for Ras and Raf, trigger a permanent and irreversible cell cycle arrest that is reminiscent of replicative senescence and termed oncogenic stress-induced senescence (8, 9). Such senescence-like growth arrest induced by oncogenic Ras is accompanied by the accumulation of growth inhibitors such as p53 and $p16^{INK4A}$ (8). The induction of premature senescence or apoptosis is important for tumor suppression in the presence of oncogenic stimuli.

Cells subjected to other types of sublethal stress also enter a state that closely resembles replicative senescence and is referred to as stress-induced premature senescence (SIPS) (10). Cells subjected to DNA damage induced by ultraviolet or x-radiation (10–13), to oxidative stress (induced by $H_2O_2$ or hyperoxia) (14–16), or to treatment with a histone deacetylase inhibitor (17) thus enter SIPS. Cells undergoing SIPS manifest all the major characteristics of replicatively senescent cells, including an enlarged flattened morphology and positive staining for senescence-associated (SA) β-galactosidase activity. Rapid shortening of telomeres is also associated with SIPS (10). Overexpression of antioxidant proteins in human fibroblasts slows the rate of telomere shortening and extends their life span (18). Accelerated telomere shortening caused by oxidative stress has thus been suggested as a cause of SIPS (10, 19, 20). However, no difference in SIPS induction was detected between parental and hTERT-expressing human fibroblasts after exposure to $H_2O_2$ or to ultraviolet or x-radiation, and telomere shortening was not apparent in the hTERT-expressing cells

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[1] The abbreviations used are: hTERT, catalytic subunit of human telomerase; SIPS, stress-induced premature senescence; SA, senescence-associated; MAPK, mitogen-activated protein kinase; AT, ataxia telangiectasia; IR, ionizing radiation; PD, population doubling; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; BrdUrd, bromodeoxyuridine; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; RB, retinoblastoma.

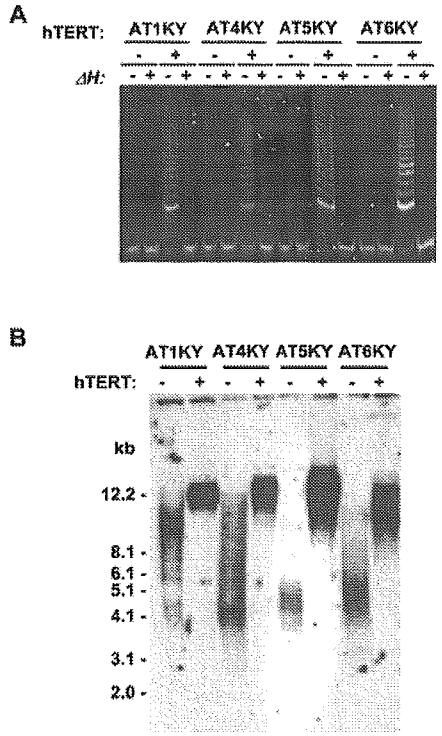


FIG. 1. **Establishment of hTERT-expressing AT fibroblasts.** *A*, telomerase activity of parental and hTERT-expressing AT fibroblasts. AT fibroblasts (AT1KY, AT4KY, AT5KY, and AT6KY) were infected with a retrovirus encoding hTERT and subjected to selection by culture in the presence of puromycin. The drug-resistant and expandable cells as well as the corresponding parental cells (1000 per assay) were analyzed for telomerase activity with the TRAP assay. As a negative control, samples were incubated for 5 min at 85 °C to inactivate telomerase before the assay (*lanes ΔH*). *B*, telomere length of parental and hTERT-expressing AT fibroblasts. Genomic DNA was digested with HinfI and RsaI and then subjected to the TRF assay with the telomeric oligonucleotide probe $(TTAGGG)_4$. The positions of DNA size markers are indicated on the *left*.

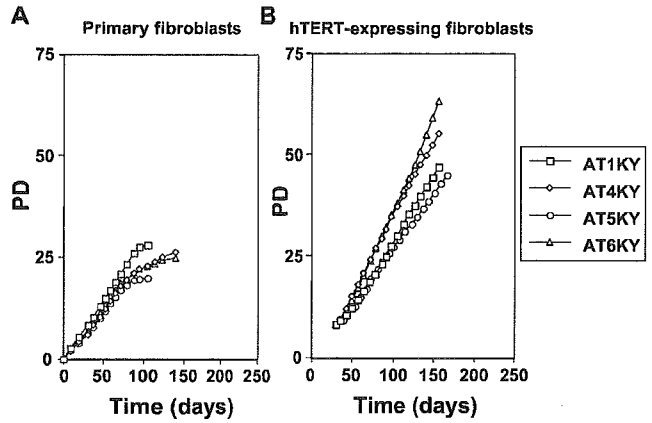


FIG. 2. **Proliferation of parental (*A*) and hTERT-expressing (*B*) AT fibroblasts.** Cells were plated at a density of $2 \times 10^5$ per 95-mm dish and passaged (after 7–9 days) before they achieved confluence. The number of cells was determined at each passage, and the PD value was calculated. For parental cells, time zero was defined as the time of initial plating; for hTERT-expressing cells, it was defined as the beginning of retroviral infection.

during SIPS induction (21). These observations thus indicate that, in contrast to replicative senescence, SIPS cannot be prevented by hTERT-mediated telomere elongation (21). They further suggest that SIPS is triggered not only by telomere shortening but also by nonspecific genome-wide DNA damage. Senescence induced by oncogenic Ras is prevented by inhibition of the activity of the p38 (stress-activated) isoform of mitogen-activated protein kinase (p38 MAPK), suggesting that activation of p38 MAPK is essential for oncogenic stress-induced senescence (22). The activation of p38 MAPK also contributes to the onset of senescence induced by telomere shortening, oxidative stress, culture shock, or activation of Ras-Raf signaling (23). However, the signaling pathway responsible for p38 MAPK activation during SIPS induction is not well characterized.

Ataxia telangiectasia (AT) is an autosomal recessive disorder characterized by progressive neurological degeneration, telangiectasia, growth retardation, specific immunodeficiency, high sensitivity to ionizing radiation (IR), an increased incidence of malignancy, and premature aging of the skin and hair (24, 25). Mitotic cells, such as fibroblasts and lymphoblasts, from individuals with AT exhibit a variety of anomalies in culture include an increased sensitivity to IR and radiomimetic agents, radioresistant DNA synthesis, chromosomal instability, a reduced life span, and an increased rate of telomere loss (26–31). ATM, the product of the gene mutated in AT patients, regulates the phosphorylation of many proteins important in cell cycle checkpoints triggered by DNA damage and in DNA repair, including c-Abl, NBS1, BRCA1, Chk2, p53, MDM2, FANCD2, and SMC1 (32–34). ATM thus plays a central role in the response to DNA damage by contributing to the $G_1$, S, and $G_2$-M cell cycle checkpoints (33–35). For example, ATM phosphorylates and activates Chk2 in response to IR, resulting in the stabilization and activation of p53, the induction of $p21^{WAF1}$ ($p21^{CIP1}$) expression, and cell cycle arrest at $G_1$ phase (36–40).

The premature replicative senescence of AT fibroblasts is rescued by telomere elongation achieved by ectopic expression of hTERT (41, 42), as is that of fibroblasts from individuals with Werner's (premature aging) syndrome (43) or with Nijmegen breakage syndrome (44). ATM has recently been shown to contribute to telomere maintenance and to senescence signaling originating from telomeres (45, 46). In addition to its role as a damage sensor for cell cycle checkpoints and DNA repair, ATM mediates up-regulation of ATF3, a target of p38 MAPK, in cells exposed to IR (47), suggesting that it acts as a trigger for stress-related MAPK signaling by activating c-Abl (32). ATM has thus been proposed to function as a stress sensor in SIPS (48). It has not been possible to study SIPS in AT cells, however, because of their premature replicative senescence. To evaluate the possible role of ATM in SIPS, we therefore established hTERT-expressing AT fibroblasts and examined whether SIPS is induced in these cells.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Infection*—Primary human dermal fibroblasts established from a normal individual (YMM) or from individuals with AT (AT1KY, AT2KY, AT4KY, AT5KY, AT6KY, and AT1OS) were cultured under a humidified atmosphere of 5% $CO_2$ at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen, Rockville, MD) supplemented with 15% heat-inactivated fetal bovine serum (Invitrogen) (49). The retroviral vector pMX-puro-hTERT (23) and Phenix-A retroviral packaging cells were kindly provided by F. Ishikawa (Kyoto University), T. Kitamura (University of Tokyo), and G. P. Nolan (Stanford University). For retrovirus production, we transfected Phenix-A cells with pMX-puro-hTERT with the use of FuGENE 6 (Roche Applied Science, Mannheim, Germany). The resulting retroviruses were used to infect fibroblasts, which were then selected in medium containing puromycin (0.5 μg/ml) (Sigma, St. Louis, MO). To examine the kinetics of cell proliferation, we plated hTERT-expressing and parental primary fibroblasts ($2 \times 10^5$ cells) in 95-mm-diameter culture dishes and determined the cell number with a Coulter Counter (Beckman Coulter, Fullerton, CA) at each passage. The number of population doublings (PD value) was calculated from $PD = \log(N_f/N_0)/\log 2$, where $N_f$ is the final cell number and $N_0$ is the initial number of seeded cells.

*Determination of Telomerase Activity and Telomere Length*—Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP) with the use of a TPAPeze kit (Intergen, Purchase,

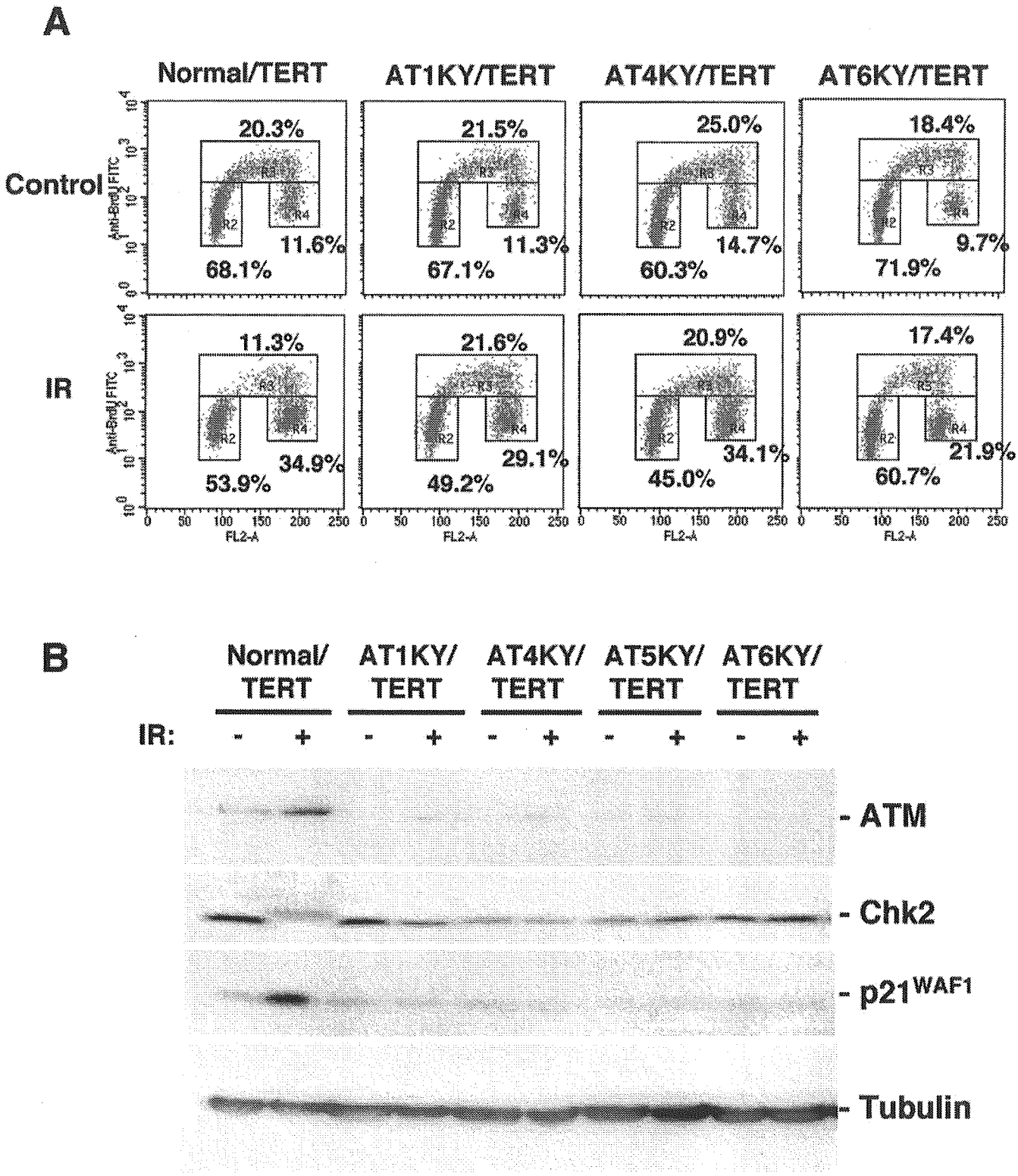


FIG. 3. **Defective $G_1$ checkpoint response to IR in hTERT-expressing AT fibroblasts.** *A*, cell cycle analysis. Asynchronous normal/TERT or AT/TERT cells were exposed (or not) to 4 Gy of x-radiation (1.6 Gy/min) and then cultured for 7 h in the absence and 1 h in the presence of 10 μM BrdUrd. The cells were then fixed, stained with both fluorescein isothiocyanate-conjugated antibodies to BrdUrd and propidium iodide, and analyzed by flow cytometry. *B*, analysis of signaling via the ATM-Chk2-p53-$p21^{WAF1}$ pathway. hTERT-expressing normal and AT fibroblasts were irradiated as in *A* and, after incubation for 2 h, lysed and subjected to immunoblot analysis with antibodies to the indicated proteins.

NY). Telomere length was measured with a terminal restriction fragment (TRF) assay. Genomic DNA (5 μg) was thus digested with HinfI and RsaI, and the resulting fragments were subjected to Southern blot analysis with a $^{32}$P-labeled telomeric oligonucleotide probe, $(TTAGGG)_4$; hybridization was performed for 12–15 h at 37 °C in a solution containing 0.75 M NaCl, 30 mM sodium citrate, and 1% SDS. Signals were visualized by autoradiography.

*Cell Cycle Analysis*—Cells were labeled for the indicated times with 10 μM bromodeoxyuridine (BrdUrd), fixed with 70% ethanol, and stained with a fluorescein isothiocyanate-conjugated mouse monoclonal antibody to BrdUrd (BD Pharmingen, San Diego, CA) and with propidium iodide (Sigma). The cellular content of DNA was determined by

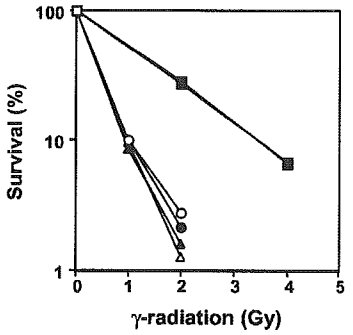


FIG. 4. **Hypersensitivity of hTERT-expressing AT fibroblasts to IR.** Parental (*open symbols*) or hTERT-expressing (*closed symbols*) normal (*squares*), AT2KY (*circles*), or AT4KY (*triangles*) fibroblasts were exposed to the indicated doses of γ -radiation. Two weeks thereafter, the cells were fixed and stained by crystal violet, and colonies containing >50 cells were scored as being derived from viable clonogenic cells.

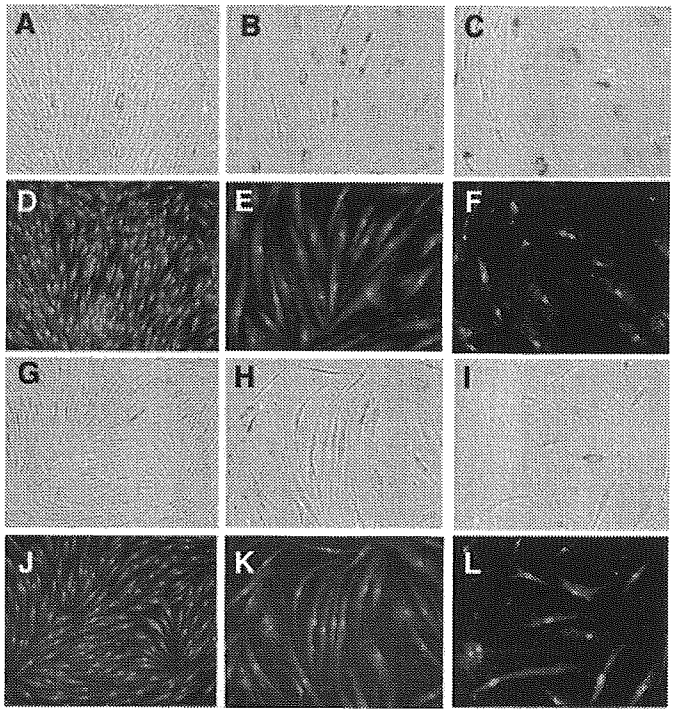


FIG. 5. **Morphology and SA β-galactosidase activity of hTERT-expressing normal and AT fibroblasts after exposure to SIPS inducers.** Normal/TERT (*A–F*) or AT6KY/TERT (*G–L*) cells were left untreated (*A, D, G,* and *J*), exposed to 55 Gy of x-radiation (*B, E, H,* and *K*), or treated with 500 μM $H_2O_2$ for 2 h (*C, F, I,* and *L*). After 10 days, the cells were stained for SA β-galactosidase activity (*A–C* and *G–I*) and counterstained with propidium iodide (*D–F* and *J–L*).

flow cytometry with a FACSCalibur instrument, and analysis of the resulting data was carried out with CELL Quest software (BD Biosciences, San Jose, CA).

*Clonogenic Assay*—Clonogenic assays were performed as described previously (26). Cells in the exponential phase of growth were plated on 95-mm culture dishes, incubated for 24 h, and then irradiated at room temperature, at the indicated doses, with a $^{137}Cs$ γ-ray source at a rate of 1.143 Gy/min. The number of cells per dish was chosen to ensure that ~100 colonies would survive the particular treatment. After 2 weeks, cells were fixed with methanol:acetic acid (3:1, v/v) and stained with crystal violet. Only colonies containing >50 cells were judged to be derived from viable clonogenic cells.

*Induction and Analysis of SIPS*—For x-irradiation, cells ($5 \times 10^5$) were seeded in 95-mm dishes, incubated for 3 days, and exposed to 55 Gy of x-radiation at a rate of 7.32 Gy/min (21). For $H_2O_2$ treatment, cells were exposed to 500 μM $H_2O_2$ for 2 h at 37 °C. All cells were then washed twice with phosphate-buffered saline, cultured for 10 days in culture medium, fixed, stained for SA β-galactosidase activity as described (50), and counterstained with propidium iodide.

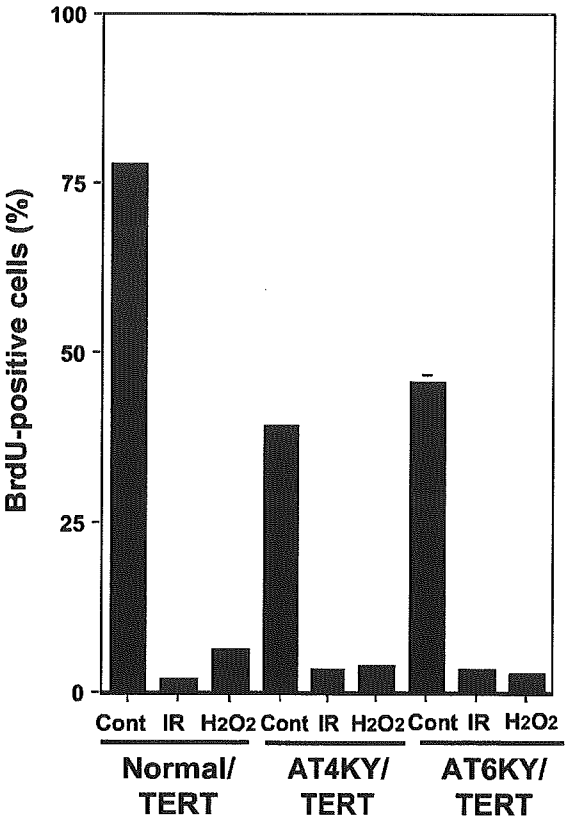


FIG. 6. **DNA synthesis in hTERT-expressing normal and AT fibroblasts after exposure to SIPS inducers.** Normal/TERT, AT4KY/TERT, or AT6KY/TERT cells were exposed (or not) to x-radiation or $H_2O_2$ as in Fig. 5 and then cultured for 36 h in the absence and 36 h in the presence of BrdUrd. Cells were fixed, and BrdUrd incorporation was determined by flow cytometry. Data are means ± S.D. from three independent experiments.

*Immunoblot Analysis*—Cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Nonidet P-40 (Sigma), 5 mM EDTA, 0.1 M NaF, and a mixture of protease inhibitors (Complete, Roche Applied Science). The protein concentration of the lysate was determined with the BCA protein assay reagent (Pierce, Rockford, IL), after which samples (50 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis with mouse monoclonal antibodies to ATM (2C-1; GeneTex, San Antonio, TX), sheep polyclonal antibodies to Chk2 (Upstate Biotechnology, Lake Placid, NY), mouse monoclonal antibodies to p53 (Ab-6; Oncogene Research, Cambridge, MA), mouse monoclonal antibodies to $p21^{WAF1}$ (F-5; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibodies to α -tubulin (B-5-1-2, Sigma), rabbit polyclonal antibodies to $p16^{INK4A}$ (C-20, Santa Cruz Biotechnology), rabbit polyclonal antibodies to p38 MAPK (Santa Cruz Biotechnology), or mouse monoclonal antibodies to phosphorylated ($Thr^{180}$ and $Tyr^{182}$) p38 MAPK (28B10, Santa Cruz Biotechnology). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and the ECL Plus system (Amersham Bioscience, Piscataway, NJ).

## RESULTS

*Establishment of hTERT-expressing AT Fibroblasts*—Primary fibroblasts derived from individuals with AT exhibit a variety of abnormalities, including defective cell cycle checkpoint function in response to DNA damage as well as rapid shortening of telomere length and consequent premature replicative senescence. To examine the role of ATM in SIPS, we established primary fibroblasts from a normal individual (normal/TERT) and from AT patients (AT/TERT) that stably express hTERT as a result of infection with a recombinant retrovirus. The cells were selected and expanded as polyclonal populations. Telomerase activity was detected by the TRAP assay in AT1KY/TERT, AT2KY/TERT, AT4KY/TERT, AT5KY/

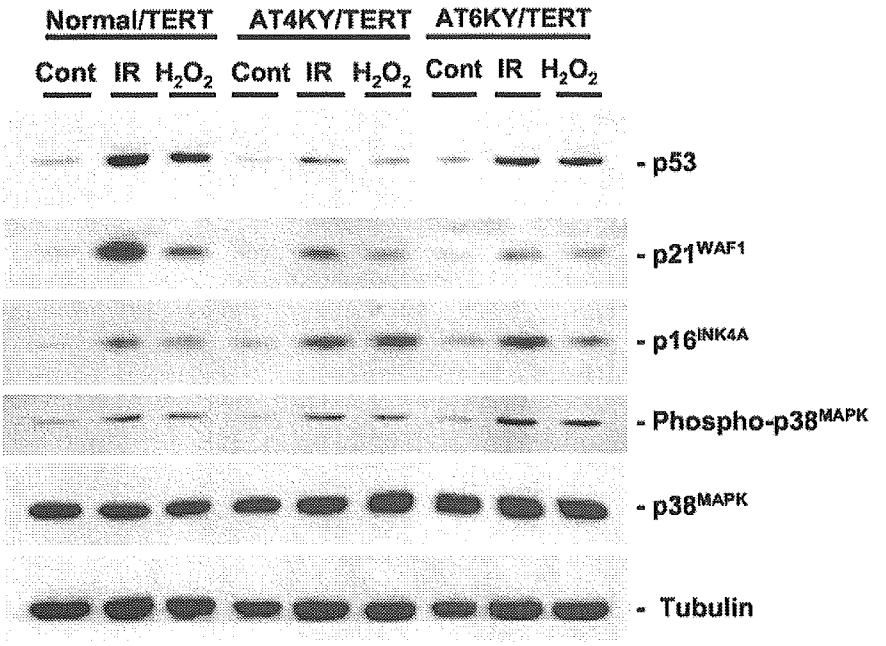


FIG. 7. **Expression and phosphorylation of senescence-related proteins in hTERT-expressing normal and AT fibroblasts after exposure to SIPS inducers.** Cells were exposed (or not) to x-radiation or $H_2O_2$ as in Fig. 5, cultured for 6 days, lysed, and subjected to immunoblot analysis with antibodies to the indicated proteins.

TERT, AT6KY/TERT, and AT1OS/TERT cells as well as in normal/TERT cells but not in the corresponding parental cells (Fig. 1*A*; data not shown). Reverse transcription and polymerase chain reaction analysis also revealed the presence of hTERT mRNA in AT/TERT and normal/TERT cells but not in the corresponding parental cells (data not shown). The TRF assay showed that the telomere length of AT/TERT cells was extended to >12 kb, compared with ~4–11 kb for parental AT cells (Fig. 1*B*). Whereas parental AT cells underwent replicative senescence after ~24 PDs (Fig. 2*A*), AT/TERT cells survived for >70 to 100 PDs (Fig. 2*B*, data not shown). These results indicated that the telomere elongation induced by expression of hTERT allowed AT cells to overcome premature replicative senescence.

*Defective Checkpoint Function in AT/TERT Cells Exposed to IR*—AT fibroblasts show defective cell cycle checkpoint responses to DNA damage induced by IR (51) or to oxidative stress (52). To examine whether the abnormality of the $G_1$ cell cycle checkpoint in AT fibroblasts was rescued by hTERT expression, we exposed asynchronous normal/TERT or AT/TERT cells to IR and determined the numbers of cells in $G_1$, S, and $G_2$-M phases of the cell cycle 8 h thereafter by flow cytometry. The proportion of normal/TERT cells in S phase (BrdUrd-positive) was greatly reduced 8 h after irradiation, whereas IR had no effect on the proportion of AT/TERT cells in S phase (Fig. 3*A*). The IR-induced $G_1$ checkpoint is triggered by sequential signaling by ATM, Chk2, p53, and $p21^{WAF1}$ (40). Although ATM was detected in normal/TERT cells, this protein was not apparent in AT/TERT cells by immunoblot analysis (Fig. 3*B*). The IR-induced phosphorylation and activation of Chk2, as revealed by a decrease in the electrophoretic mobility of the protein, were also evident in normal/TERT cells but not in AT/TERT cells (Fig. 3*B*). Consistent with these observations, up-regulation of $p21^{WAF1}$ in response to IR was apparent in normal/TERT cells but not in AT/TERT cells (Fig. 3*B*). Activation of the ATM-Chk2-p53-$p21^{WAF1}$ signaling pathway by IR was thus impaired in the AT/TERT cells. Like primary AT fibroblasts, AT/TERT cells also exhibit a defective $G_1$ cell cycle checkpoint as a result of their ATM deficiency.

*Radiosensitivity of AT/TERT Cells*—AT fibroblasts are markedly more sensitive to IR than are normal cells. To examine whether hTERT expression affected the radiosensitivity of AT cells, we performed a clonogenic survival assay after exposure of AT/TERT, normal/TERT, and the corresponding parental cells to various doses of IR. Consistent with previous report, both AT/TERT cells and primary AT fibroblasts were hypersensitive to IR in comparison with normal fibroblast and normal/TERT cells (Fig. 4) (41). ATM is thus essential for signaling that results in DNA repair after exposure of cells to IR.

*SIPS in AT/TERT Fibroblasts*—We have shown that, like that of the parental cells, the response of AT/TERT cells to DNA damage is impaired. If the DNA damage signaling pathway also contributes to SIPS, then AT/TERT cells would also be expected to be resistant to SIPS. To examine this possibility, we compared the responses of normal/TERT and AT/TERT fibroblasts to SIPS inducers (21). 5 or 10 days after exposure to IR or $H_2O_2$, normal/TERT cells exhibited a senescence phenotype, including a flattened and enlarged morphology (Fig. 5, *A–F*, data not shown). Similar morphological changes were also evident in AT4KY/TERT and AT6KY/TERT cells (Fig. 5, *G–L*, data not shown). Furthermore, normal/TERT cells stained intensely for SA β-galactosidase activity10 days after exposure to IR or $H_2O_2$ (Fig. 5, *A–C*), as did AT4KY/TERT and AT6KY/TERT cells (Fig. 5, *G–I*, data not shown).

We also examined DNA synthesis in the treated cells by measuring BrdUrd incorporation. The proportion of BrdUrd-positive cells was reduced by >95% 3 days after exposure of normal/TERT, AT4KY/TERT, or AT6KY/TERT cells to IR or to $H_2O_2$ (Fig. 6). These results thus indicate that, despite the defects in cell cycle checkpoint and other DNA damage responses in AT/TERT cells, SIPS was induced similarly in AT/TERT cells and normal/TERT cells.

*Induction or Phosphorylation of Senescence-associated Proteins in AT/TERT Cells*—To investigate the molecular mechanism of SIPS induction, we examined the expression and modification of senescence-associated proteins in normal/TERT and AT/TERT fibroblasts. The abundance of p53, $p21^{WAF1}$, and $p16^{INK4A}$ has been known to be increased in senescent cells. The accumulation of p53 and $p21^{WAF1}$ was also observed in normal/TERT cells 6 days after the induction of SIPS by IR or $H_2O_2$ (Fig. 7). Furthermore, the accumulation of p53 and $p21^{WAF1}$ was evident in AT4KY/TERT and AT6KY/TERT cells after exposure to these SIPS inducers, albeit to a slightly lesser extent than that apparent in normal/TERT cells. Although $p16^{INK4A}$ plays an important role in cellular senescence in human cells, the accumulation of this protein was not previ-

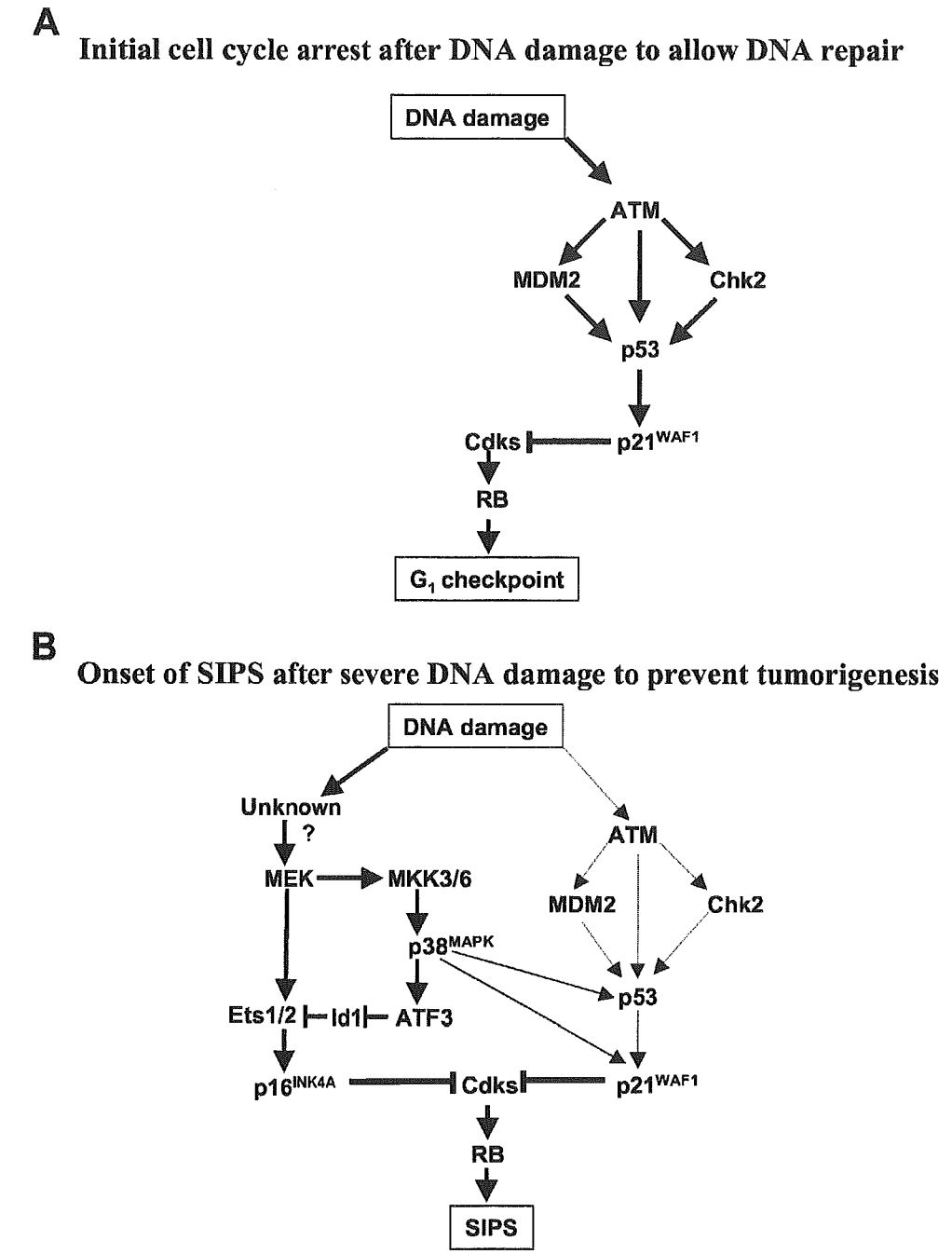


FIG. 8. **Model for two distinct stages of tumor suppression in human fibroblasts subjected to DNA damage.** *A*, the early response to DNA damage involves cell cycle arrest mediated by activation of the $G_1$ checkpoint to allow time for DNA repair. The major pathway responsible for activation of the $G_1$ checkpoint includes ATM-dependent activation of p53, p53-dependent induction of the Cdk inhibitor $p21^{WAF1}$, and a reduction in the phosphorylation level of RB. *B*, SIPS is triggered several days after DNA damage to prevent the accumulation of genetic mutations when the damage proves too severe for repair. The unrepaired DNA damage is likely recognized by an unknown sensor, and the stress signal is then transduced by a MAPK cascade, including MEK, MKK3 or MKK6, and p38 MAPK. Activated p38 MAPK induces ATF3 and represses Id1 expression, the latter effect contributing to Ets1- or Ets2-dependent induction of $p16^{INK4A}$.

ously detected during SIPS in human fibroblasts (21, 53). Similarly, we detected only a small increase in the abundance of $p16^{INK4A}$ in normal/TERT fibroblasts 6 days after SIPS induction (Fig. 7). The expression of $p16^{INK4A}$ was increased to a greater extent in AT/TERT fibroblasts than in normal/TERT cells after exposure to IR or $H_2O_2$. The activation of p38 MAPK mediates replicative senescence and senescence induced by Ras-Raf signaling (23). p38 MAPK is phosphorylated on $Thr^{180}$ and $Tyr^{182}$ and thereby activated by the kinases MKK3 and MKK6 (54). We therefore examined the phosphorylation status of p38 MAPK in normal/TERT and AT/TERT cells with antibodies specific for the enzyme phosphorylated on these residues. The phosphorylation of p38 MAPK was induced in normal/TERT, AT4KY/TERT, and AT6KY/TERT cells by IR or $H_2O_2$ treatment (Fig. 7). These SIPS inducers did not affect the abundance of p38 MAPK. Our results thus indicate that the signaling pathways responsible for SIPS are intact in AT/TERT cells.

## DISCUSSION

Cells that have been subjected to sublethal stress, such as that caused by radiation-induced DNA damage or oxidants, undergo SIPS. The molecular mechanisms by which cells monitor the effects of such stress and decide to undergo SIPS,

however, have remained unclear. Given that ATM plays a pivotal role as a sensor of DNA damage for cell cycle checkpoints and DNA repair, it has also been a prominent candidate for the stress sensor in SIPS (48). We have examined this possibility in the present study by characterizing SIPS in hTERT-expressing AT fibroblasts.

Expression of certain oncoproteins, such as E2F1 and c-Myc, promotes the accumulation of reactive oxygen species and consequent DNA damage (55, 56). These cellular oncoproteins, as well as the adenoviral oncoprotein E1A, induce apoptosis by a p53-dependent pathway (6, 7, 57). Such apoptosis is blocked by treatment of the cells with caffeine, an inhibitor of the kinase activities of ATM and the related protein ATR, suggesting that the oncoprotein-induced damage is recognized by ATM or ATR (58). These observations thus also suggested that ATM or a related protein might be the stress sensor for SIPS (48).

Consistent with previous observations (41, 42), we have now shown that expression of hTERT in AT fibroblasts rescued the premature replicative senescence phenotype of these cells. However, like the parental cells, the hTERT-expressing AT fibroblasts still exhibited hypersensitivity to IR, probably because of an impairment in DNA repair activity due to the loss of ATM. Moreover, in contrast to telomerase-positive normal fibroblasts, hTERT-expressing AT fibroblasts did not arrest in $G_1$ phase of the cell cycle in response to DNA damage. Consistent with these observations, IR did not induce either a shift in the electrophoretic mobility of Chk2, which reflects phosphorylation on $Thr^{68}$ and kinase activation (59), or expression of $p21^{WAF1}$ in AT/TERT fibroblasts, indicating that the Chk2-dependent activation of p53 in response to DNA damage was not rescued in AT fibroblasts by telomere elongation. These results thus suggested that the replicative senescence induced by telomere shortening and growth arrest in response to DNA damage are mediated by different sensors.

In contrast to the defective response to DNA damage in AT/TERT fibroblasts, the elicitation of SIPS in these cells by either $H_2O_2$ or x-radiation did not differ substantially from that in normal/TERT cells, suggesting the existence of an ATM-independent pathway for SIPS. We therefore propose the existence of distinct mechanisms for the immediate cell cycle arrest and for SIPS in response to DNA damage (Fig. 8). Upon experiencing DNA damage, cells initially arrest cell cycle progression by ATM-dependent activation of the $G_1$ checkpoint to gain time for DNA repair (Fig. 8*A*). The major pathway responsible for triggering the $G_1$ checkpoint involves the activation of p53 by ATM, MDM2, and Chk2; the p53-mediated induction of the gene for the Cdk inhibitor $p21^{WAF1}$; and a reduction in the level of RB phosphorylation that results from Cdk inhibition. If the DNA damage cannot be repaired within a relatively short time window (several days), the cells undergo SIPS, which is independent of ATM, to prevent accumulation of genetic mutations (Fig. 8*B*). The unrepaired DNA damage is thus likely recognized by an unknown sensor, and the stress signal is then transduced by a MAPK cascade, possibly mediated by MEK, MKK3 or MKK6, and p38 MAPK (22), resulting in the induction of ATF3 (60). ATF3 induced by p38 MAPK has been shown to repress transcription of the gene for Id1 (61). Activated MEK also induces $p16^{INK4A}$ expression through Ets1- or Ets2-dependent transcription (62). Given that Id1 inhibits the induction of $p16^{INK4A}$ through direct interaction with Ets1 or Ets2 (62), the p38 MAPK pathway also likely contributes to $p16^{INK4A}$ induction during stress signaling. Indeed, activation of p38 MAPK by MKK3 or MKK6 has been shown to increase the amount of $p16^{INK4A}$ mRNA (22). Consistent with previous observations (21, 53), we detected only a small increase in the abundance of $p16^{INK4A}$ in normal/TERT fibroblasts undergoing SIPS. In contrast, the increase in the amount of $p16^{INK4A}$ was markedly greater in similarly treated AT/TERT fibroblasts. Therefore, the defect in the induction of $p21^{WAF1}$ due to the lack of ATM in AT/TERT cells might be compensated for by p38 MAPK-dependent induction of $p16^{INK4A}$. Although up-regulation of ATF3 was not previously observed within 8 h after exposure of AT fibroblasts to IR (47), cells in which the ATM signaling pathway is inoperative appear to be capable of undergoing SIPS in response to severe DNA damage in a manner dependent on the p38 MAPK-$p16^{INK4A}$ pathway.

The accumulation of p53 was apparent in AT/TERT fibroblasts during SIPS. An alternative, ATM-independent pathway, possibly mediated by ATR or a related kinase, might thus be responsible for this p53 accumulation. Given that p38 MAPK also phosphorylates p53 on $Ser^{33}$ and $Ser^{46}$ in the $NH_2$-terminal activation domain and thereby regulates the transactivation activity of p53 (63, 64), p38 MAPK might contribute to the p53 accumulation in AT/TERT fibroblasts during SIPS. In addition, p38 MAPK has been shown to induce the stabilization of $p21^{WAF1}$ by phosphorylation on $Ser^{130}$ (65). The accumulation of the Cdk inhibitors $p16^{INK4A}$ and $p21^{WAF1}$ induced by p38 MAPK might thus contribute to SIPS and to tumor suppression. Indeed, disruption of the p38 MAPK gene by homologous recombination or inactivation of p38 MAPK by overexpression of Ppm1D, a phosphatase that inhibits the activity of p38 MAPK, increases tumor formation in mice (66). Furthermore, mice that lack both MKK3 and MKK6 and are therefore not able to activate p38 MAPK are also prone to tumorigenesis (67). These observations thus highlight the importance of p38 MAPK-mediated signaling for tumor suppression.

Consistent with our observations on SIPS, telomere dysfunction induced by inhibition of telomeric repeat binding factor 2 has also been shown to result in replicative senescence through an ATM-independent pathway in AT fibroblasts, indicating that ATM is not required for telomere-derived senescence signaling (68). Our results indicate that the ATM-mediated signaling pathway triggered by DNA damage is not required for p38 MAPK activation and consequent cellular senescence. The cell cycle checkpoint function of ATM is thus dispensable for the major pathway responsible for the triggering of SIPS.

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# Forum Review

# FOXO Transcription Factors in Cell-Cycle Regulation and the Response to Oxidative Stress

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## ABSTRACT

**Mammalian forkhead members of the class O (FOXO) transcription factors, including FOXO1, FOXO3a, and FOXO4, are implicated in the regulation of a variety of cellular processes, including the cell cycle, apoptosis, DNA repair, stress resistance, and metabolism. FOXO proteins are negatively regulated by the phosphatidylinositol 3-kinase–Akt signaling pathway, which is activated by growth factors and cytokines. Recent studies indicate that the activities of FOXO proteins are also regulated by oxidative stress, which induces their phosphorylation, translocation to the nucleus, and acetylation–deacetylation. Similar to the tumor suppressor p53, FOXO is activated by stress and induces the expression of genes that contribute to cell-cycle arrest, suggesting that it also functions as a tumor suppressor.** *Antioxid. Redox Signal.* 7, 752–760.

## INTRODUCTION

THE GENOME OF CELLS is continually damaged by environmental insults, such as ultraviolet light (UV) and ionizing radiation; by oxidative stress, such as that attributable to reactive oxygen species derived from oxidative metabolism; and, in dividing cells, by errors in DNA replication and mitosis. Organisms have evolved mechanisms that maintain genomic integrity by inducing cell-cycle arrest in response to DNA damage. Cell-cycle checkpoints at the $G_1$-S and $G_2$-M transitions are thus responsive to DNA damage and constitute a major mechanism for genomic surveillance (43, 75). These checkpoints allow the cell time to repair DNA damage before resumption of cell-cycle progression, or, if the damage is too extensive, they trigger cellular senescence or apoptosis. Incomplete repair of DNA damage as a result of defective checkpoint operation leads to damage accumulation over time and, eventually, to age-related conditions such as cancer.

The FOXO (forkhead member of the class O) family of forkhead transcription factors comprises three functionally related proteins—FOXO1 (also known as forkhead in rhabdomyosarcoma, or FKHR) (22), FOXO3a (FKHR-like 1, or FKHRL1) (1, 26), and FOXO4 (acute lymphocytic leukemia–1 fused gene from chromosome X, or AFX) (4)—that are vertebrate orthologues of the *Caenorhabditis elegans* (*C.elegans*) transcription factor DAF-16 (38, 49). The genes for the three mammalian proteins of this family were initially identified as sites of chromosomal breakpoints in human cancers (1, 4, 22, 26). The DNA-binding domains of the transcription factors PAX3 or PAX7, which are important in neuromuscular differentiation, are thus fused to the transactivation domain of FOXO1 in some rhabdomyosarcomas (22). Similarly, the DNA-binding domain of MLL (mixed-lineage leukemia), which functions as a positive regulator of Hox genes during embryonic development, is fused to the transactivation domains of FOXO3a or FOXO4 in certain leukemias (1, 4). These fusions alter the transactivation properties of the respective proteins and are thereby thought to contribute to carcinogenesis. FOXO proteins participate in regulation of the cell cycle, apoptosis, DNA repair, and detoxification by either inducing or suppressing the expression of target genes. Furthermore, the intracellular localization of these proteins is regulated by oxidative stress, as well

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as by a phosphatidylinositol 3-kinase (PI3K)–mediated signaling pathway. In this review, we focus on the role of FOXO proteins in regulation of the cell cycle and in the cellular response to oxidative stress.

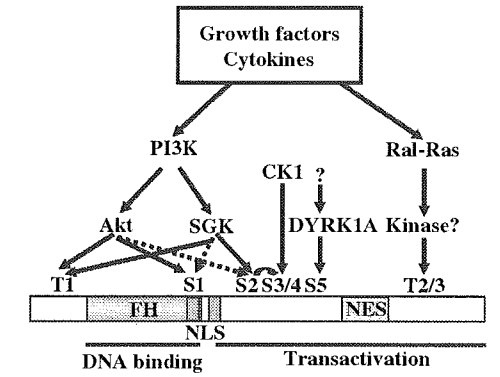
## SIGNALING PATHWAYS THAT REGULATE FOXO PROTEINS

Regulation of the subcellular localization and transactivation activity of FOXO proteins is achieved primarily by post-translational modifications, such as phosphorylation and acetylation. Genetic studies in *C. elegans* have indicated that the forkhead transcription factor DAF-16 functions downstream of Akt (also known as protein kinase B, or PKB) in a PI3K signaling pathway (50). Certain single-gene mutations, such as *daf-2* [which affects the gene for the insulin and insulin-like growth factor–1 (IGF-1) receptor] and *age-1* (catalytic subunit of PI3K), increase adult life span and promote constitutive dauer formation in *C. elegans* (18, 30). Such mutants also exhibit an increased resistance to a variety of environmental insults, including oxidative stressors and UV (27, 37, 39, 45, 69), and these longevity and stress resistance phenotypes are dependent on DAF-16 (30, 65). DAF-16 possesses four consensus sequences (RXRXXS/T) for phosphorylation by Akt (50), three of which are conserved in the mammalian FOXO proteins, suggesting that mammalian FOXO family members are also regulated by the PI3K-Akt signaling pathway.

In mammals, the PI3K-Akt signaling pathway is activated by a variety of cytokines and growth factors, including insulin and IGF-1, and it regulates various cellular processes, such as cell proliferation and survival (11, 14, 70). The constitutive activation of this signaling pathway leads to the development of tumors both through deregulation of cell-cycle progression and through an increase in cellular resistance to proapoptotic signals. The activation of PI3K triggered by the binding of growth factors or cytokines to their receptors results in the production of phosphatidylinositol 3,4,5-trisphosphate ($PIP^3$), which provides a membrane binding site for the serine-threonine kinase Akt. The translocation of Akt to the plasma membrane leads to its activation through phosphorylation by 3'-phosphoinositide–dependent kinase 1 (PDK1) (11, 14, 70). The tumor suppressor PTEN (phosphatase and tensin homologue on chromosome 10), which functions as a lipid 3'-phosphatase, reduces the amount of $PIP_3$, with the result that PI3K signaling is turned off (12).

The three highly conserved Akt recognition sequences in FOXO1, FOXO3a, and FOXO4 are designated T1, S1, and S2 (Fig. 1). Akt indeed phosphorylates FOXO proteins, and these three sites become phosphorylated in a PI3K-dependent manner in a variety of mammalian cells in response to stimulation with cytokines or growth factors such as insulin and IGF-1 (3, 6, 34, 53, 62, 64).

The serum- and glucocorticoid-induced kinase (SGK), which is structurally related to Akt, also phosphorylates FOXO proteins (7). SGK is also activated by PI3K- and PDK1-mediated signaling in response to extracellular stimuli and has been suggested to play a role in cell-cycle progression, although it is not recruited to the plasma membrane by $PIP_3$ (7, 10, 32, 51). Although Akt and SGK are able to phosphorylate the same




| | T1 | S1 | S2 | S3 | S4 | S5 | T2/T3 |
|---|---|---|---|---|---|---|---|
| FOXO1: | T24 | S256 | S319 | S322 | S325 | S329 | |
| FOXO3a: | T32 | S253 | S315 | S318 | S321 | S325 | |
| FOXO4: | T28 | S193 | S258 | S261 | S264 | S268 | T447T451 |

**FIG. 1. Phosphorylation of FOXO proteins.** The phosphorylation of FOXO proteins is regulated by growth factor–activated signaling pathways, including those mediated by PI3K-Akt and by Ras-Ral. The general phosphorylation sites, T1 to T3 and S1 to S5, that are phosphorylated by various kinases are indicated. The specific amino acid positions corresponding to these sites are also shown for human FOXO1, FOXO3a, and FOXO4. The S1 site is located within the nuclear localization sequence (NLS) and its phosphorylation might inhibit the nuclear import of FOXO. The positions of the forkhead (FH) domain, nuclear export sequence (NES), and DNA binding and transactivation domains are also indicated.

sites (T1, S1, S2) of FOXO, these kinases differ in their efficacies in this regard (7). Although T1 is similarly phosphorylated by both kinases, Akt preferentially targets S1, whereas SGK preferentially phosphorylates S2. The biological consequences of this difference between Akt and SGK remain to be determined in mammals. Although both Akt and SGK are able to phosphorylate DAF-16 in *C. elegans*, SGK appears to play the predominant role in the control of development, stress resistance, and longevity, whereas Akt is more important in the regulation of dauer formation (25). It is thus possible that the difference in FOXO phosphorylation site preferences between Akt and SGK results in the activation of distinct cellular responses by these kinases in mammals.

Activation of the PI3K-Akt pathway also triggers indirectly the phosphorylation of FOXO proteins on sites in addition to T1, S1, and S2. Both $Ser^{322}$ (S3) and $Ser^{325}$ (S4) of human FOXO1 thus become phosphorylated in IGF-1–stimulated cells as a result of phosphorylation of the S2 site ($Ser^{319}$) by Akt (54). Phosphorylation of $Ser^{319}$ generates a consensus sequence for phosphorylation of $Ser^{322}$ by casein kinase 1 (CK1). In turn, phosphorylation of $Ser^{322}$ generates a consensus site for phosphorylation of $Ser^{325}$ by CK1. In contrast, phosphorylation of $Ser^{329}$ (S5) of FOXO1 is unaffected by IGF-1 signaling. Instead, this residue appears to be phosphorylated constitutively by DYRK1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) (72). Inhibition of the PI3K-Akt signaling pathway or mutation (to alanine) of the phosphorylation sites of FOXO targeted by Akt or CK1 thus does not influence the phosphorylation status of S5.

The phosphorylation of FOXO is also affected by the Ras-Ral signaling pathway, which targets $Thr^{447}$ (T2) and $Thr^{451}$ (T3) in the COOH-terminal region of human FOXO4 (17). A mutant protein in which both of these threonine residues are replaced by alanines still translocates from the nucleus to the cytoplasm in response to Akt signaling, but its transactivation activity is greatly impaired. Although normal activation of endogenous Ral increases the transactivation activity of FOXO, activation of Ral by oncogenic Ras appears to enhance the inhibition of FOXO activity by Akt. It remains to be determined whether this regulation characterized for FOXO4 is also operative for FOXO1 and FOXO3a. The region surrounding the T2 and T3 sites of FOXO4 is not well conserved in the other FOXO family members, however.

## REGULATION OF FOXO LOCALIZATION BY THE PI3K-AKT SIGNALING PATHWAY

Although each newly synthesized FOXO protein is imported into the nucleus through interaction of its nuclear localization sequence (NLS) with the nuclear import machinery (5), stimulation of cells with growth factors such as IGF-1 or expression of constitutively active forms of PI3K or Akt results in nuclear exclusion of FOXO (3, 6, 34, 53, 62, 64). In contrast, serum deprivation or inhibition of PI3K or of Akt results in the relocation of FOXO from the cytoplasm to the nucleus. The PI3K-Akt signaling pathway thus regulates nucleocytoplasmic shuttling of FOXO. Mutational analysis has shown that the phosphorylation of FOXO proteins on the three sites (T1, S1, S2) targeted by Akt promotes their exclusion from the nucleus and consequent inhibition of target gene transcription (3, 6, 34, 53, 62, 64).

Nuclear export is a highly regulated process that involves various accessory proteins such as Crm1, which binds to the nuclear export sequence (NES) of the target protein. All members of the FOXO family contain a consensus NES. Crm1 was shown to be required for the nuclear export of FOXO by the observation that treatment of cells with leptomycin B, an inhibitor of Crm1, blocks this process irrespective of the status of the phosphorylation sites targeted by Akt (3, 5). Activation of Akt at the plasma membrane of growth factor–stimulated cells is followed by translocation of the kinase into the nucleus, where it phosphorylates FOXO on the T1, S1, and S2 sites (5, 8). Phosphorylation of FOXO proteins by Akt generates consensus sequences [RSXp(S/T)XP] for the binding of 14-3-3 (73) and induces their association with 14-3-3. 14-3-3 proteins regulate the cell cycle and prevent apoptosis by controlling the nucleocytoplasmic distribution of signaling molecules with which they interact. They recognize and bind both of the phosphorylated sites surrounding $Thr^{32}$ (T1) and $Ser^{253}$ (S1) of human FOXO3a (8). Interaction of 14-3-3 with FOXO is not sufficient to trigger the nuclear export of FOXO, however. Deletion of the putative NES motif in FOXO3a thus inhibits its nuclear export in spite of the presence of intact Akt phosphorylation sites and the binding of 14-3-3 (8). Conversely, an intact NES also is not sufficient for nuclear export, given that mutation (to alanine) of the Akt phosphorylation sites of FOXO and consequent prevention of 14-3-3 binding result in retention of FOXO within the nucleus even of cells that have been stimulated with IGF-1 (8). Instead, subsequent phosphorylation of S3 and S4 by CK1 facilitates the interaction of FOXO that has already been phosphorylated by Akt with the nuclear export machinery, including the Ran GTPase and Crm1. Thus, mutation (to alanine) of the CK1 phosphorylation sites of FOXO1 prevented its binding to Ran (54). Moreover, the binding of 14-3-3 to phosphorylated FOXO3a appears to prevent the latter from reentering the nucleus, given that $Ser^{253}$ (S1) is located within the NLS (8). The DYRK1A phosphorylation site (S5) is adjacent to the S4 site phosphorylated by CK1. Although S5 is constitutively phosphorylated, its phosphorylation appears to contribute to the subcellular localization of FOXO because its mutation (to alanine) in FOXO1 increases the transactivation activity and nuclear localization of this protein in nonstimulated cells (72).

Taken together, these various observations indicate that newly synthesized FOXO accumulates in the nucleus as a result of interaction of its NLS with the nuclear import machinery, binds to its cognate consensus sequence in the promoters of target genes, and modulates gene expression in cells not exposed to growth factors or cytokines. In response to cell stimulation, however, activated Akt (or SGK) phosphorylates three sites (T1, S1, S2) within FOXO, which results in the phosphorylation of two additional sites (S3, S4) by CK1. The phosphorylated protein binds to 14-3-3 and the nuclear export machinery (Crm1, Ran) and thereby exits the nucleus. The transactivation activity of FOXO is thus regulated by its nucleocytoplasmic shuttling, which is in turn controlled by FOXO phosphorylation (Fig. 2).

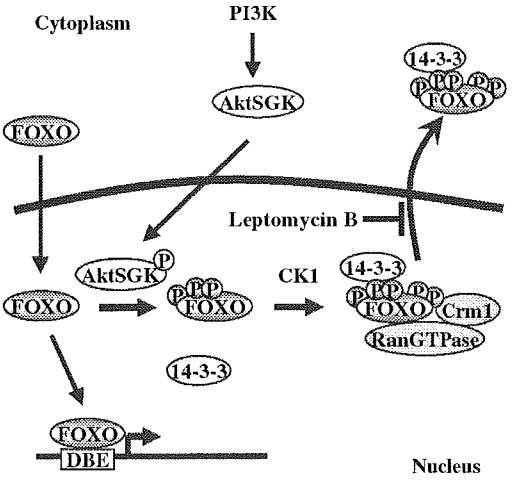



**FIG. 2. Regulation of nucleocytoplasmic shuttling of FOXO by the PI3K-Akt signaling pathway.** Newly synthesized FOXO molecules are imported into the nucleus, where they interact with the FOXO binding motif (DBE) present in the promoters of target genes and thereby regulate gene expression. Growth factor stimulation triggers the activation of PI3K and Akt (or SGK) and the subsequent translocation of activated Akt to the nucleus. Phosphorylation of FOXO by activated Akt induces its interaction with 14-3-3, as well as its further phosphorylation by CK1, resulting in the nuclear export of the FOXO–14-3-3 complex mediated by Crm1 and the Ran GTPase in a manner sensitive to leptomycin B.

## FOXO TARGET GENES INVOLVED IN CELL-CYCLE REGULATION

Progression of the cell cycle is tightly controlled by intracellular and extracellular signals. In normal dividing cells, cell-cycle progression is regulated by the balance between the amounts and activities of cyclin–CDK (cyclin-dependent kinase) complexes, such as cyclin D–CDK4, cyclin E–CDK2, and cyclin B–CDK1, and those of their inhibitors (CKIs) such as $p21^{Cip1}$ and $p27^{Kip1}$ (29, 57–59). Growth factors trigger various early events that promote the $G_0$-$G_1$ transition of the cell cycle; these events include up-regulation of cyclin D expression and $p27^{Kip1}$ degradation, which result in consecutive activation of the cyclin D–CDK4 and cyclin E–CDK2 complexes and progression into S phase. Activation of the PI3K-Akt signaling pathway appears to be required for the entry of quiescent cells into the cell cycle. Activation of this pathway was shown to be sufficient to induce DNA synthesis in serum-deprived fibroblasts (31). Negative regulation of FOXO by the PI3K-Akt signaling pathway was thus also implicated in cell-cycle regulation. Indeed, expression of a constitutively active form of FOXO that is resistant to phosphorylation by Akt led to cell-cycle arrest or apoptosis as a result of induction or suppression of the transcription of genes involved in cell-cycle regulation (Fig. 3). A combination of comprehensive microarray analysis of gene expression and the determination of the canonical DNA consensus sequence for FOXO binding (TTGTTTAC) has facilitated the identification of FOXO target genes (21).

Ectopic expression of FOXO4, FOXO3a, or FOXO1 blocks cell-cycle progression at $G_1$ phase by inducing the expression of $p27^{Kip1}$(17, 42, 47, 61, 63). This CKI binds to the cyclin E–CDK2 complex and inhibits its activity, thereby preventing entry of cells into S phase (55). FOXO directly activates transcription of the $p27^{Kip1}$ gene, whose promoter contains multiple consensus FOXO binding motifs (42, 47). FOXO also increases the expression of a reporter gene controlled by the $p27^{Kip1}$ gene promoter (42, 47). In addition, FOXO has been shown to increase the stability of the $p27^{Kip1}$ protein (47).

FOXO also contributes to regulation of the exit of cells from the cell cycle into a state of quiescence ($G_0$) (36). The abundance of p130, a member of the retinoblastoma protein family, is low in cycling cells, but increases as cells exit the cell cycle (23). In $G_0$ cells, p130 is hypophosphorylated and binds to the transcription factor E2F-4, and this complex is thought to repress the expression of genes required for reentry of cells into the cell cycle, thereby maintaining the quiescent state (60). FOXO binds directly to consensus binding motifs in the promoter of the p130 gene and activates its transcription, resulting in the accumulation of p130 protein (36). The PI3K-Akt signaling pathway thus controls the abundance of p130 during the cell cycle via regulation of FOXO. FOXO also binds and activates the promoter of the cyclin G2 gene and thereby increases the amount of cyclin G2 protein (41). Cyclin G2 is an unconventional cyclin that is highly expressed in quiescent cells, but undergoes marked down-regulation in a manner dependent on activation of the PI3K-Akt signaling pathway as cells enter the cell cycle (2, 28).

FOXO has been implicated in a $p27^{Kip1}$-independent mechanism of inhibition of cell-cycle progression by the observation that forced expression or conditional activation of FOXO leads to reduced expression of cyclins D1 and D2 and consequent cell-cycle arrest even in the absence of $p27^{Kip1}$ (52, 56). Chromatin immunoprecipitation assays have revealed that FOXO1 binds to the promoter of the cyclin D1 gene (52). A mutant form of FOXO1 that was not able to recognize the canonical FOXO binding motif or to induce $p27^{Kip1}$ gene expression was still able to suppress the expression of cyclins D1 and D2 (56), suggesting that FOXO might bind to the promoters of the cyclin D1 and D2 genes indirectly by interacting with other transcription factors and modulating their activities. Alternatively, the regulation of cyclin D gene expression by FOXO might be mediated by the direct binding of FOXO to a promoter element other than the classical FOXO binding motif. Indeed, DNA microarray analysis in *C. elegans* has suggested the existence of an additional consensus sequence that might be recognized by FOXO (46).

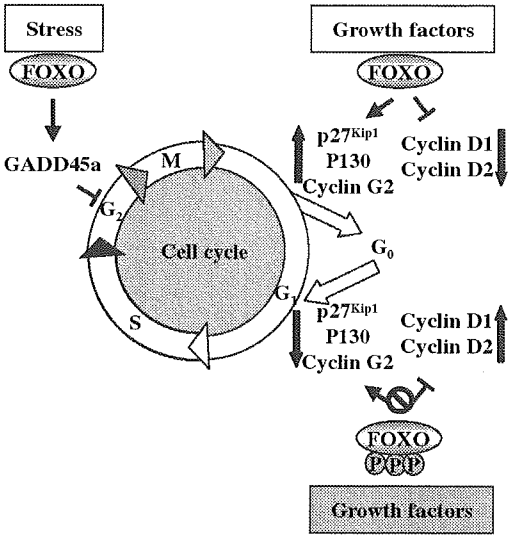



**FIG. 3. Cell-cycle regulation by FOXO-mediated activation or suppression of gene expression.** In the absence of growth factors, activated FOXO both induces expression of the genes for $p27^{Kip1}$, p130, and cyclin G2 (all of which inhibit cell-cycle progression) and suppresses that of the genes for cyclins D1 and D2 (both of which promote cell-cycle progression), thereby rendering cells quiescent ($G_0$ phase). In the presence of growth factors, activation of the PI3K-Akt signaling pathway induces the nuclear exclusion of FOXO and thereby prevents its effects on these target genes. The expression of cyclins D1 and D2 is thus up-regulated and that of $p27^{Kip1}$, p130, and cyclin G2 is down-regulated, resulting in the reentry of cells into the cell cycle. In response to oxidative stress, FOXO is activated and induces the expression of genes, such as that for GADD45a, that inhibit $G_2$-M progression, thereby triggering cell-cycle arrest at $G_2$ phase.

In summary, in the absence of growth factor–induced PI3K-Akt signaling, the sustained activity of FOXO results in up-regulation of the expression of $p27^{Kip1}$, p130, and cyclin G2, as well as in inhibition of the expression of cyclins D1 and D2, thereby ensuring maintenance of the quiescent state. Stimulation of cells with growth factors and the consequent activation of the PI3K-Akt signaling pathway result in the phosphoryla-