Atm Deficiency Affects Both Apoptosis and Proliferation to Augment Myc-Induced Lymphomagenesis

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Abstract

Myc oncoproteins are commonly activated in malignancies and are sufficient to provoke many types of cancer. However, the critical mechanisms by which Myc contributes to malignant transformation are not clear. DNA damage seems to be an important initiating event in tumorigenesis. Here, we show that although Myc does not directly induce double-stranded DNA breaks, it does augment activation of the Atm/p53 DNA damage response pathway, suggesting that Atm may function as a guardian against Myc-induced transformation. Indeed, we show that *Atm* loss augments Myc-induced lymphomagenesis and impairs Myc-induced apoptosis, which normally harnesses Myc-driven tumorigenesis. Surprisingly, *Atm* loss also augments the proliferative response induced by Myc, and this augmentation is associated with enhanced suppression of the expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Therefore, regulation of cell proliferation and $p27^{Kip1}$ seems to be a contributing mechanism by which Atm holds tumor formation in check. (Mol Cancer Res 2007;5(7):705–11)

Introduction

Resolving the precise mechanisms by which oncogenes such as Myc promote tumorigenesis is a major thrust of oncology research. At one level, this involves a hyperproliferative response provoked by Myc, because when Myc is overexpressed, it accelerates the rates of cell proliferation (1, 2) and increases the numbers of cells in cycle *in vivo* (3-5). This enhanced proliferative response is held in check by the universal cyclin-dependent kinase inhibitor $p27^{Kip1}$ and by the Arf-p53 pathway, which also mediates Myc-induced apoptosis; bypass of these inhibitory steps are common denominators of cancer (5-11). However, Myc overexpression also sensitizes cells to agents that induce DNA damage and leads to genomic instability (12-16), suggesting that alterations in the DNA damage and/or DNA repair pathways might contribute to mutations augmented by Myc activation.

A key arbiter of the DNA damage pathway is the *ataxia-telangiectasia mutated* (*ATM*) gene, a member of the phosphatidylinositol-3-OH kinase superfamily. ATM is activated by DNA damage and phosphorylates key substrates that arrest the cell cycle in the G_1 (e.g., p53), S, and G_2-M phases of the cell cycle (17). Ataxia-telangiectasia patients with loss-of-function mutations in *ATM* suffer from cerebellar degeneration causing ataxia and are prone to development of malignancies, particularly lymphomas (18). Furthermore, sporadic, generally missense, inactivating mutations of *ATM*, with loss of the wild-type *ATM* allele, have been reported in mantle cell lymphoma, T-cell prolymphocytic leukemia, and B-cell chronic lymphocytic leukemia (19). Finally, *Atm* deficiency in mice results in the development of thymic lymphoma (20, 21). Collectively, these data all indicate that ATM functions as a bona fide tumor suppressor.

Atm phosphorylates and activates p53 (17) and p53 mediates Myc-induced apoptosis (7, 8, 22-24). Further, Myc has been suggested to directly provoke DNA damage (14, 25) and sensitize cells to irradiation (15), suggesting potential interplay between Myc and Atm in tumorigenesis. Indeed, in epithelial cells, *Atm* deficiency has been shown to cooperate with Myc in the development of oral and skin tumors, and in this scenario *Atm* loss impairs Myc-induced apoptosis (25). Here, we report that although Myc does not directly induce DNA strand breaks in proliferating cells *ex vivo* or in B cells *in vivo*, it does augment the Atm/p53 DNA damage pathway. Importantly, the Atm tumor suppressor is shown to harness Myc-induced lymphomagenesis at two levels: by impairing Myc-induced apoptosis and by augmenting its proliferative response, with the latter linking Atm to the control of $p27^{Kip1}$ cyclin-dependent kinase inhibitor.

Results

Myc Augments the DNA Damage Response to Irradiation

To initially assess the possible interplay between Myc, Atm, and the DNA damage response, primary early-passage human diploid foreskin fibroblasts were transduced with a control MSCV-IRES-puro retrovirus, which expresses the gene for *puromycin resistance* (*puro*) in *cis* from an internal ribosome entry site (IRES), or with the MSCV-*Myc*-ER^{TAM}-IRES-*puro*

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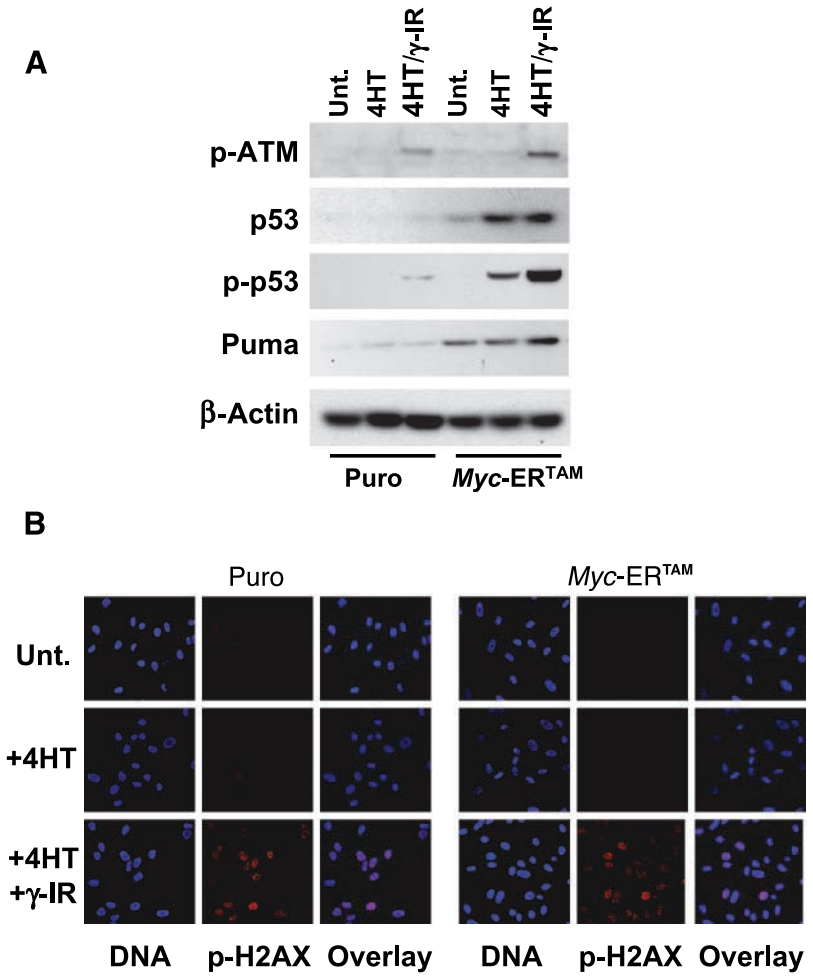

FIGURE 1. Myc augments the DNA damage signaling pathway in normal human diploid foreskin fibroblasts. **A.** Primary early-passage cultures of human foreskin fibroblasts were transduced with the MSCV-IRES-puro retrovirus or with the MSCV-*Myc*-ER^{TAM}-IRES-puro retrovirus, and puromycin-resistant pools of cells were then left untreated (*Unt.*) or treated with 1 μmol/L 4-hydroxy tamoxifen (*4HT*) for 24 h. Half of these cells were then treated with 5 Gy of ionizing radiation (*γ-IR*). After 4 h, samples were then evaluated for their levels of phospho-Ser^{1981} ATM, p53, phospho-Ser^{15} p53, Puma, and β-actin by immunoblotting. **B.** Vector-only– (*Puro*) or *Myc*-ER^{TAM}–expressing human foreskin fibroblasts (*Myc-ER^{TAM}*) were treated with 4-hydroxy tamoxifen (1 μmol/L) for 24 h, followed by 5-Gy ionizing radiation, and harvested after 4 h. Following fixation and blocking procedures, double-strand breaks in DNA were detected by incubating with anti–Ser^{139}-H2AX antibody (*p-H2AX*). Cells were then stained with a Cy3 antirabbit secondary antibody. DNA was visualized with 4′,6-diamidino-2-phenylindole (*DNA*). Representative of three separate experiments.

retrovirus (7) that additionally encodes a chimeric form of c-Myc fused to the estrogen binding domain of an estrogen receptor (ER) modified to selectively bind to the estrogen receptor agonist tamoxifen (TAM). In the presence of tamoxifen, the *Myc*-ER^{TAM} fusion protein relocalizes to the nucleus and activates Myc transcriptional programs (26). Puromycin-resistant cells were expanded in culture, treated with tamoxifen to activate *Myc*-ER^{TAM}, and were then exposed to 5-Gy ionizing radiation. The effects of Myc on ATM activation were then monitored by evaluating Ser^{1981} phosphorylation of ATM, a marker of ATM activation (27), and by evaluating the levels of p53 protein and damage-dependent p53 phosphorylation on Ser^{15} (28, 29).

As expected, ionizing radiation induced the Ser^{1981} phosphorylation of ATM and the Ser^{15} phosphorylation of p53 in control, vector-only–expressing fibroblast cells (Fig. 1A). By contrast, activation of Myc alone did not induce ATM phosphorylation, but it was sufficient to induce p53 and Ser^{15} p53 phosphorylation (Fig. 1A). The expression of the BH3-only p53 transcription target Puma, a proapoptotic Bcl-2 family member (30-32), was also elevated in *Myc*-ER^{TAM}–expressing human foreskin fibroblasts (Fig. 1A), even without activation of the *Myc*-ER^{TAM} transgene, which is likely due to its leaky activation. Notably, however, Myc activation did augment Ser^{1981} ATM phosphorylation in response to ionizing radiation (Fig. 1A); therefore, Myc overexpression does not seem to induce direct DNA damage per se in proliferating human foreskin fibroblasts, but rather enhances activation of the ATM-p53 pathway following DNA damage.

Damage that provokes double-strand breaks in DNA triggers Ser^{139} phosphorylation of H2AX, which then accumulates at discrete foci in the nuclei of damaged cells (33). As expected, exposure of both vector-only– and Myc-ER^{TAM}–overexpressing cells to ionizing radiation led to the formation of discrete H2AX foci (Fig. 1B). However, Myc activation alone, which fails to induce ATM phosphorylation (Fig. 1A), also failed to induce H2AX foci (Fig. 1B). Therefore, Myc overexpression alone can provoke some (e.g., Ser^{15} phosphorylation of p53), but not all, hallmarks of the DNA damage response.

Myc Augments Ser^{15} p53 Phosphorylation In vivo

Precancerous B cells of Eμ-*Myc* transgenic mice, a mouse model of human Burkitt lymphoma that bears *MYC/Immunoglobulin* translocations (34), are exquisitely sensitive to ionizing radiation (15), suggesting that some aspects of the DNA damage pathway might also be activated by Myc

overexpression *in vivo*. Indeed, compared with expression in B cells from wild-type littermate mice, there were marked increases in the steady-state levels of p53 and Ser^{15} phosphorylated p53 in both spleen- and bone marrow–derived $B220^+$ B cells from precancerous Eμ-*Myc* transgenic mice. Increased levels of Puma were also detected in Eμ-*Myc* splenic B cells (Fig. 2A). However, similar to the effects observed during Myc activation in human fibroblasts (Fig. 1B), H2AX foci were not detected in $B220^+$ B cells isolated from spleen of precancerous Eμ-*Myc* transgenic mice, and only a few foci-positive cells were detected in bone marrow–derived Eμ-*Myc* $B220^+$ B cells (Fig. 2B).

Atm Functions as a Guardian against Myc-Induced Lymphomagenesis

Ataxia-telangiectasia patients, who have loss of function mutations in *ATM*, are highly prone to develop lymphoid leukemia and lymphoma during childhood (35), and *Atm*-deficient mice spontaneously develop malignant thymic lymphomas by 4 to 6 months of age (20, 21). Because evidence of activation of DNA damage signaling pathways has been detected in precancerous cells of mice and men (36, 37), we reasoned that loss of ATM might accelerate tumorigenesis in the face of an oncogenic insult. To test this hypothesis, $Atm^{+/-}$ mice were crossed with Eμ-*Myc* transgenic mice, which develop clonal, lethal pre–B or B-cell lympholeukemia by 3 to 6 months of age (34). Eμ-*Myc*;$Atm^{+/-}$ F1 mice were then mated with $Atm^{+/-}$ mice, and Eμ-*Myc*;$Atm^{+/+}$, Eμ-*Myc*;$Atm^{+/-}$, Eμ-*Myc*;$Atm^{-/-}$, and $Atm^{-/-}$ littermates were followed for their course of disease. As expected, non-transgenic $Atm^{-/-}$ mice died of thymic lymphoma with a median survival time of 160 days, whereas Eμ-*Myc*;$Atm^{+/+}$ mice died of pre-B and B-cell lymphoma with a median survival of 120 days (Fig. 3). Notably, although no effects of *Atm* haploinsufficiency were evident (median survival, 127 days), all Eμ-*Myc*;$Atm^{-/-}$ mice developed aggressive, rapid-onset pre-B and B-cell lympholeukemia (mean survival, 69 days; Fig. 3). Therefore, Atm indeed serves as a guardian against Myc-induced lymphomagenesis.

Loss of Atm Impairs Myc Apoptotic Responses

Myc accelerates the rate of cell proliferation and augments apoptosis in precancerous B cells of Eμ-*Myc* transgenic mice (3-5, 38), and thus loss of cell cycle regulators such as $p27^{Kip1}$ or of apoptotic regulators such as Arf or p53 dramatically increases the rate of lymphoma development (7-9). The acceleration of pre-B and B-cell lymphoma development in Eμ-*Myc*;$Atm^{-/-}$ transgenic mice could result from effects of *Atm* deficiency on Myc-induced apoptosis, Myc-induced proliferation, or both. To assess the apoptotic response, we evaluated B cells derived from precancerous (4-week-old) Eμ-*Myc*;$Atm^{+/+}$, Eμ-*Myc*;$Atm^{-/-}$, $Atm^{-/-}$, and wild-type mice cultured in interleukin-7–containing medium on S17 stromal cells (15), which selectively allows for the outgrowth of $B220^+$ B cells from bone marrow (39). *Atm* deficiency alone had little effect on spontaneous apoptosis in primary B-cell cultures, whereas rates of spontaneous apoptosis of Eμ-*Myc*;$Atm^{+/+}$ B cells were, as expected (7), quite high (Fig. 4A). Notably, rates of spontaneous apoptosis of Eμ-*Myc*;$Atm^{-/-}$ were comparable with those of wild-type B cells (Fig. 4A), indicating that *Atm* deficiency can effectively inhibit the Myc-induced apoptotic response.

Myc-induced apoptosis in primary B cells is governed by activation of the Arf-p53 pathway and also through suppressing the expression of antiapoptotic Bcl-2 family members such as $Bcl\text{-}X_L$ and Bcl-2 (7, 15, 40). We therefore directly compared the expression of these regulators and of Puma in magnetic-activated cell sorted $B220^+$ B cells from the spleens or bone marrows of Eμ-*Myc*;$Atm^{+/+}$, Eμ-*Myc*;$Atm^{-/-}$, $Atm^{-/-}$, and wild-type weanling-aged (4-week-old) littermates. As expected, Eμ-*Myc*;$Atm^{+/+}$ B cells expressed higher levels of $p19^{Arf}$, p53, and Puma and reduced levels of $Bcl\text{-}X_L$ and Bcl-2, compared with the $B220^+$ B cells from the bone marrows or spleens of nontransgenic $Atm^{-/-}$ or wild-type littermates (Fig. 4B). *Atm* deficiency affected both arms of Myc's apoptotic response because Eμ-*Myc*;$Atm^{-/-}$ B cells expressed both reduced levels

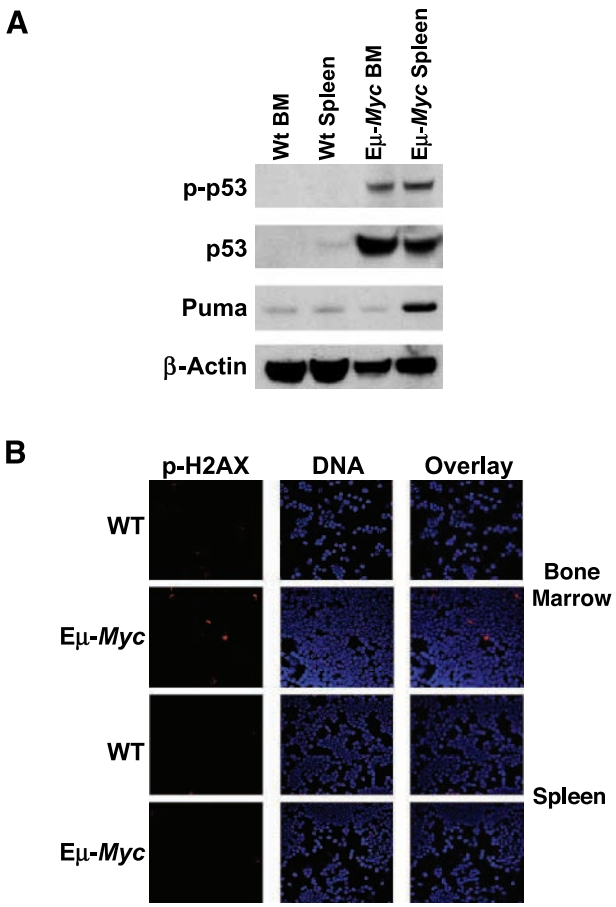

FIGURE 2. Myc augments the DNA damage signaling pathway *in vivo*. **A.** $B220^+$ spleen- and bone marrow–derived B cells from 4-wk-old Eμ-*Myc* transgenic mice and their wild-type (*Wt*) littermates were evaluated for their levels of phospho-Ser^{15} p53, p53, and Puma by Western blot. **B.** B cells derived from either the bone marrow or spleen of wild-type or Eμ-*Myc* mice were analyzed for the presence of double-strand breaks in DNA by incubating with anti–Ser^{139}-H2AX antibody (*p-H2AX*). Cells were then stained with a Cy3 antirabbit secondary antibody. DNA was visualized with 4′,6-diamidino-2-phenylindole (*DNA*). Representative of three separate experiments.

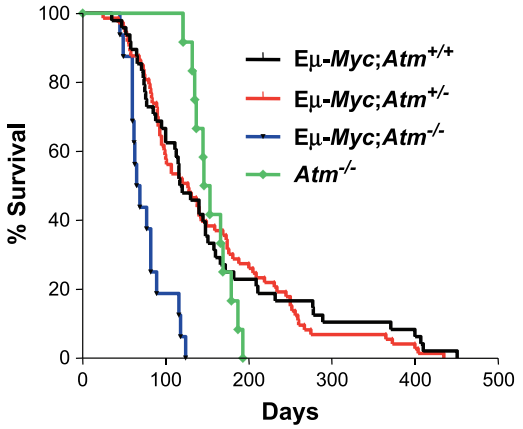

FIGURE 3. Atm functions as a guardian against Myc-induced lymphomagenesis. Eμ-*Myc* transgenic mice (on a C57BL/6 background) were crossed with C57BL/6 $Atm^{+/-}$ mice. F1 Eμ-*Myc*;$Atm^{+/-}$ mice were then mated with $Atm^{+/-}$ mice, and Eμ-*Myc*;$Atm^{+/+}$ (n = 40; *black line*), Eμ-*Myc*;$Atm^{+/-}$ (n = 66; *red line*), Eμ-*Myc*;$Atm^{-/-}$ (n = 13; *blue line*), and $Atm^{-/-}$ (n = 14; *green line*) littermates were followed for their course of lymphoma development. Median survival time was 120 d for Eμ-*Myc*;$Atm^{+/+}$, 127 d for Eμ-*Myc*;$Atm^{+/-}$, and 69 d for Eμ-*Myc*;$Atm^{-/-}$ (P = 0.003) transgenic mice. Median survival time of $Atm^{-/-}$ littermates was 160 d. Lymphomas that arose in all Eμ-*Myc* transgenics were pre-B or immature B-cell lymphoma, whereas only thymic lymphoma arose in $Atm^{-/-}$ mice.

of p53, $p19^{Arf}$, and Puma and increased levels of Bcl-X_L (and of Bcl-2 in splenic B cells) compared with wild-type Eμ-*Myc* transgenics (Fig. 4B).

Atm Deficiency Augments Myc Proliferative Response

Myc accelerates the rate of cell proliferation through its ability to provoke degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (5, 6). Loss of $p27^{Kip1}$ markedly accelerates the rate of Myc-induced lymphomagenesis in the Eμ-*Myc* mouse model (9), whereas *E2f1* or *Cks1* deficiency disables Myc's ability to suppress $p27^{Kip1}$ and thus impairs lymphomagenesis (3, 5). To assess the potential effects of *Atm* deficiency on Myc-induced proliferation, weanling wild-type Eμ-*Myc* and Eμ-*Myc*;$Atm^{-/-}$ littermates were injected with bromodeoxyuridine (BrdUrd) and, after 12 h, the S-phase indices of their bone marrow and splenic B220+ B cells were determined by flow cytometry. As expected (3-5), the proliferative indices of both immature (bone marrow) and mature (spleen) B cells were augmented in Eμ-*Myc* B cells (Fig. 5). Surprisingly, this proliferative response was even higher in B cells from Eμ-*Myc*;$Atm^{-/-}$ mice (Fig. 5). Finally, the markedly high rates of proliferation of Eμ-*Myc*;$Atm^{-/-}$ cells were associated with drastically reduced levels of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor (Fig. 4D). Therefore, Atm seems to hold Myc-induced transformation of B cells in check by compromising both the apoptotic and proliferative responses induced by Myc.

Discussion

Myc has been recognized to trigger genomic instability for a number of years (12, 13, 16), yet precisely how this occurs has not been resolved. Under some scenarios, this phenomenon has been linked to the ability of Myc to directly induce double-strand breaks in DNA and γ-H2AX foci (41), which may or may not occur through augmenting levels of reactive oxygen species (14, 41) or via direct activation of Atm (42). However, in other settings, Myc does not induce reactive oxygen species (43) and, as shown here, Myc overexpression in normal proliferating human fibroblasts and B cells *in vivo* does not measurably induce ATM activation or γ-H2AX foci. Nevertheless, Myc still clearly sensitizes cells to apoptosis following DNA damage (15) and augments activation of ATM in response to γ-ionizing radiation (Fig. 1A). Further, loss of *Atm*, a key regulator of the DNA damage response, impairs Myc-induced apoptosis and also augments Myc proliferative response. Thus, *Atm* loss markedly accelerates lymphoma development in Eμ-*Myc* transgenic mice, a model of human Burkitt lymphoma (34).

Several previous observations predict that disabling the Atm-p53 pathway would enhance Myc-induced tumorigenesis by specifically impairing Myc apoptotic response: (*a*) loss of p53 function augments Myc-induced transformation by impairing its apoptotic response (7, 8, 23, 24); (*b*) blocking apoptosis by modulation of Bcl-2 family members also augments Myc-induced tumorigenesis (32, 40, 44-47); (*c*) ATM is a protein kinase that signals to p53 and is required for optimal p53 induction after ionizing radiation (17); (*d*) loss of *Atm* leads to decreased apoptosis of immature thymocytes and brain cells in mice exposed to irradiation (48); and (*e*) inactivation of *Atm* in a transgenic mouse model overexpressing Myc in squamous epithelial tissues leads to decreased apoptosis and rapid onset of tumor development (25). Similar to studies of Myc-driven tumorigenesis in keratinocytes (25) and to very recent findings by Shreeram et al. (49) evaluating the effects of the Atm-p53 pathway downstream of the Wip1 phosphatase in Eμ-*Myc* mice, we also observed that *Atm* deficiency disables Myc apoptotic response. Notably, here we have shown that this is specifically associated with reductions in the degree of activation of the Arf-p53 pathway in the precancerous state as well as to unexpected effects of *Atm* deficiency on the expression of Bcl-2 and Bcl-X_L in these at-risk cells (Fig. 4B). Precisely how *Atm* deficiency would dampen these two apoptotic pathways is not clear but could conceivably be due to the effects of *Atm* loss on other checkpoints that have ancillary effects on these pathways. Finally, these findings suggest that alterations which disable the Arf-p53 pathway and often accompany lymphoma development in Eμ-*Myc* mice would be more infrequent in an *Atm*-null context, as suggested by others (49), but this issue will have to be addressed using large cohorts of these lymphomas.

A surprising finding of our studies was that *Atm* deficiency also augments Myc-driven proliferation, which is also rate limiting for lymphoma development in Eμ-*Myc* mice (3, 5, 9). Myc accelerates the rate of cell proliferation (1, 2), at least in part, through its ability to provoke degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (5, 6), and the combined effect of Myc overexpression and *Atm* deficiency effectively abolishes $p27^{Kip1}$ expression and drives high rates of cell proliferation. Precisely how *Atm* deficiency affects the Myc-to-$p27^{Kip1}$ pathway is also not clear, but, at one level, this might involve the effects of *Atm* loss on E2f1 expression and/or activity because proper thresholds of E2f1 are necessary for Myc to suppress $p27^{Kip1}$ expression in Eμ-*Myc* B cells (3). The

dual tumor suppressor mechanism (apoptosis and growth arrest) suggested here for ATM is similar to the scenario for p53, where both its proapoptotic (8, 50) and antiproliferative (51) effects have been shown to contribute to its tumor-suppressive properties.

Materials and Methods

Mice and Cell Culture

Atm$^{+/-}$ mice on a C57BL/6;Svj129 mixed background (kindly provided by Dr. Peter McKinnon; ref. 48) were crossed with Eμ-*Myc* transgenic mice (C57BL/6 background; ref. 34) to generate F1 Eμ-*Myc*;*Atm*$^{+/-}$ mice. These mice were then crossed to *Atm*$^{+/-}$ mice to generate Eμ-*Myc*;*Atm*$^{+/+}$, Eμ-*Myc*;*Atm*$^{+/-}$, Eμ-*Myc*;*Atm*$^{-/-}$, and *Atm*$^{-/-}$ offspring and these littermates were followed for their course of disease.

Primary bone marrow–derived pre-B-cell cultures were generated from 4- to 6-week-old wild-type, Eμ-*Myc*;*Atm*$^{+/+}$, Eμ-*Myc*;*Atm*$^{-/-}$, and *Atm*$^{-/-}$ littermates as previously described (40). B cells were maintained in culture on S17 stromal cells (S17 stroma kindly provided by Dr. Kenneth Dorshkind, University of California at Los Angeles, Los Angeles, CA; ref. 39) in medium containing interleukin-7 (10 units/mL) as described (40).

Normal human foreskin fibroblasts (American Type Culture Collection) were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Retroviral infections were done as previously described (7, 15).

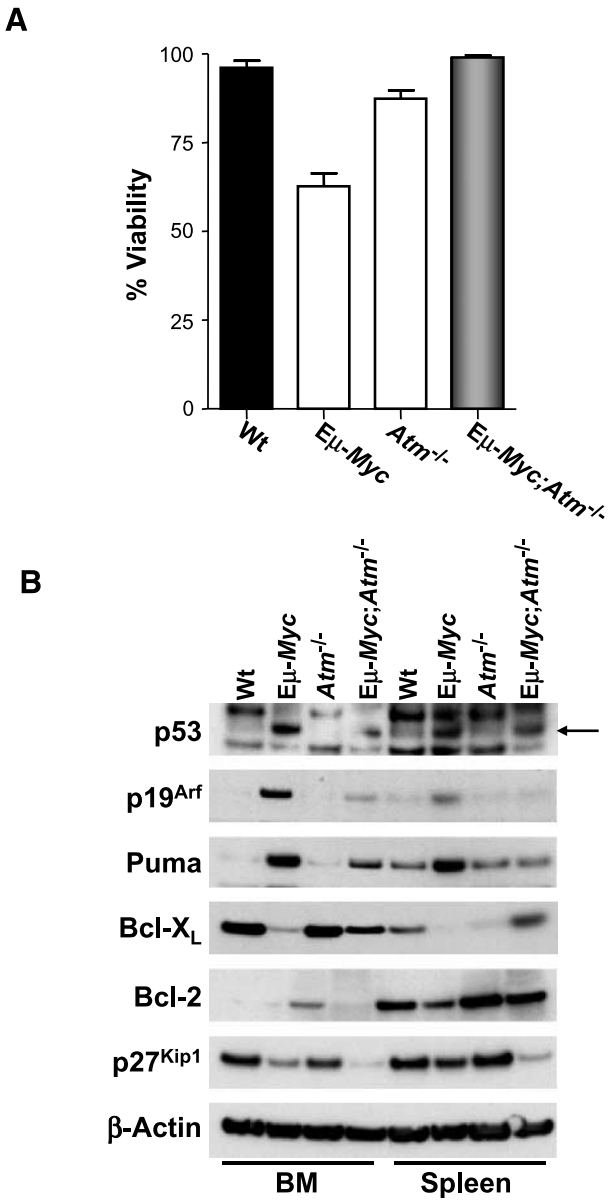

FIGURE 4. *Atm* loss impairs Myc apoptotic pathways. **A.** Primary B cells were grown on S17 stroma and in media supplemented with interleukin-7 from 4-week-old nontransgenic (wild-type), Eμ-*Myc, Atm*$^{-/-}$, and Eμ-*Myc*;*Atm*$^{-/-}$ mice. The percent of spontaneous apoptosis was determined by Annexin V-FITC. Columns, mean of three independent B-cell cultures for the indicated mice; bars, SD. **B.** Lysates were prepared from bone marrow– and spleen-derived B cells from 4-week-old nontransgenic (wild-type), Eμ-*Myc, Atm*$^{-/-}$, and Eμ-*Myc*;*Atm*$^{-/-}$ mice and were evaluated for their levels of p53, p19^{Arf}, Puma, Bcl-X$_L$, Bcl-2, p27^{Kip1}, and β-actin by Western blot.

Fluorescence-Activated Cell Sorting and Magnetic-Activated Cell Sorting of B Cells

To obtain bone marrow and splenic B cells, single-cell suspensions were prepared, followed by a red cell lysis using an ammonium chloride/potassium bicarbonate solution. Cell suspensions were then incubated with B220 microbeads and enriched by magnetic-activated cell sorting according to the manufacturer's instructions (Miltenyi Biotech). Rates of proliferation of B220$^+$ cells were determined using a flow kit as described by the manufacturer (BD Biosciences PharMingen). Briefly, animals were injected i.p. with 100 μL of 10 mg/mL BrdUrd in sterile PBS. Animals were sacrificed 12 h postinjection and B220$^+$ cells from bone marrow and spleen were harvested. For the BrdUrd proliferation assays, 1×10^6 cells were used. Following incubation, cells were washed, resuspended in PBS, and analyzed by fluorescence-activated cell sorting.

Apoptosis Assays

To assess the effects of *Atm* deficiency on Myc-induced apoptosis, bone marrows from individual 4-week-old wild-type, Eμ-*Myc*;*Atm*$^{+/+}$, Eμ-*Myc*;*Atm*$^{-/-}$, and *Atm*$^{-/-}$ littermates were cultured on S17 stroma in interleukin-7 medium as described (40). After 10 days in culture, the rates of spontaneous apoptosis of the pre-B cells in these cultures were determined by staining 5×10^5 cells with Annexin V-FITC antibody (Annexin V-Fluor Kit, Roche Applied Sciences) and propidium iodide as previously described (4). Following incubation, cells were washed, resuspended in PBS, and analyzed by fluorescence-activated cell sorting.

Protein Analyses

Protein extracts from magnetic-activated cell sorted B cells and human foreskin fibroblasts were prepared as previously described (7). Proteins (25 or 50 μg per lane) were electrophoretically separated on 4% to 12% SDS-PAGE gels (Invitrogen), transferred to membranes (Protran, Schleicher & Schuell), and blotted with antibodies specific for Puma, β-actin (Sigma), p27^{Kip1}, Bcl-X$_L$, Bcl-2, p53, phospho-Ser^{15} p53 (Cell Signaling), p19^{Arf} (kindly provided by Dr. Charles Sherr), and

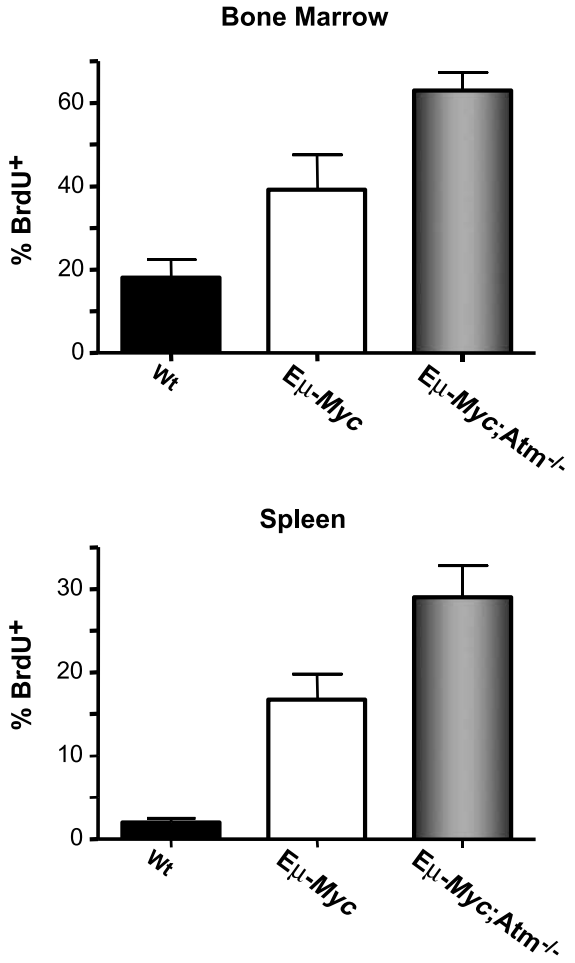

FIGURE 5. *Atm* loss augments Myc-induced proliferation *in vivo*. Four-week-old nontransgenic (wild-type), Eμ-*Myc*, and Eμ-*Myc*;*Atm*$^{-/-}$ littermates were injected with BrdUrd (*BrdU*) and, after 12 h, the percentage of B220^{+} B cells (derived from both bone marrow and spleen) in S phase was determined by fluorescence-activated cell sorting. Columns, mean of three independent experiments from the indicated mice; bars, SD.

phospho-Ser^{1981} ATM (Abcam). Following incubation with primary antibodies, the blots were then incubated with appropriate antimouse or antirabbit immunoglobulin secondary antibodies (Amersham). Bound immunocomplexes were detected by enhanced chemiluminescence (Amersham) or ECL SuperSignal (Pierce).

Immunofluorescence Analyses

For immunofluorescence analysis of levels of Ser^{139} phosphorylated H2AX, cytospins of 5×10^4 B220^{+} B cells isolated from 4-week-old wild-type or Eμ-*Myc* transgenic mice, or from human fibroblasts (control or *Myc*-ER^{TAM}-expressing) grown on glass coverslips, were prepared. Slides were fixed with 1:1 (v/v) methanol-acetone for 10 min at −20°C and were air dried. After blocking with 10% fetal bovine serum/PBS, slides were incubated for 1 h at room temperature with a polyclonal antibody specific for phospho-Ser^{139} histone γ-H2AX [1:100 dilution in 1% fetal bovine serum/PBS (Trevigen)]. Following 10 washes in PBS, primary antibody binding was visualized with an antirabbit antibody conjugate incubated at room temperature for 30 min. Coverslips and slides were washed 10 times before being mounted with Fluoromount-G antifade reagent containing 4′,6-diamidino-2-phenylindole (Southern Biotechnology) and were analyzed by confocal microscopy.

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References

1. Shichiri M, Hanson KD, Sedivy JM. Effects of c-myc expression on proliferation, quiescence, and the G_0 to G_1 transition in nontransformed cells. Cell Growth Differ 1993;4:93–104.

2. Lutz W, Stohr M, Schurmann J, Wenzel A, Lohr A, Schwab M. Conditional expression of N-myc in human neuroblastoma cells increases expression of α-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 1996;13:803–12.

3. Baudino TA., Maclean KH, Brennan J, et al. Myc-mediated proliferation and lymphomagenesis, but not apoptosis, are compromised by E2f1 loss. Mol Cell 2003;11:905–14.

4. Nilsson JA, Keller UB, Baudino TA, et al. Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. Cancer Cell 2005;7:433–44.

5. Keller UB, Old JB, Dorsey FC, et al. Myc targets Cks1 to provoke the suppression of p27Kip1, proliferation and lymphomagenesis. EMBO J 2007;26:2562–74.

6. Vafa O, Wade M, Kern S, et al. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell 2002;9:1031–44.

7. Eischen CM, Weber JD, Roussel MF, Sherr CJ, Cleveland JL. Disruption of the ARF-Mdm2-53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev 1999;13:2658–69.

8. Schmitt CA, McCurrach ME, de Stanchina E, Wallace-Brodeur RR, Lowe SW. INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. Genes Dev 1999;13:2670–7.

9. Martins CP, Berns A. Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. EMBO J 2002;21:3739–48.

10. Sherr CJ. Principles of tumor suppression. Cell 2004;116:235–46.

11. Finch A, Prescott J, Shchors K, et al. Bcl-xL gain of function and p19 ARF loss of function cooperate oncogenically with Myc *in vivo* by distinct mechanisms. Cancer Cell 2006;10:113–20.

12. Mai S, Fluri M, Siwarski D, Huppi K. Genomic instability in MycER-activated Rat1A-MycER cells. Chromosome Res 1996;4:365–71.

13. Felsher DW, Bishop JM. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci U S A 1999;96:3940–4.

14. Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B. Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. EMBO J 1996;15:6595–604.

15. Maclean KH, Keller UB, Rodriguez-Galindo C, Nilsson JA, Cleveland JL. c-Myc augments γ irradiation-induced apoptosis by suppressing Bcl-X(L). Mol Cell Biol 2003;23:7256–70.

16. Louis SF, Vermolen BJ, Garini Y, et al. c-Myc induces chromosomal rearrangements through telomere and chromosome remodeling in the interphase nucleus. Proc Natl Acad Sci U S A 2005;102:9613–8.

17. Kastan MB, Bartek J. Cell cycle checkpoints and cancer. Nature 2004;432:316–23.

18. Shiloh Y, Kastan MB. ATM: genome stability, neuronal development, and cancer cross paths. Adv Cancer Res 2001;83:209–54.

19. Boultwood J. Ataxia telangiectasia gene mutations in leukaemia and lymphoma. J Clin Pathol 2001;54:512–6.

20. Barlow C, Hirotsune S, Paylor R, et al. Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell 1996;86:159–71.

21. Liyanage M, Weaver Z, Barlow C, et al. Abnormal rearrangement within the α/δ T-cell receptor locus in lymphomas from Atm-deficient mice. Blood 2000;96: 1940–6.

22. Hermeking H, Eick D. Mediation of c-Myc-induced apoptosis by p53. Science 1994;265:2091–3.

23. Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev 1994;8:2817–30.

24. Zindy F, Eischen CM, Randle DH, et al. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev 1998;12:2424–33.

25. Pusapati RV, Rounbehler RJ, Hong S, et al. ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. Proc Natl Acad Sci U S A 2006; 103:1446–51.

26. Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res 1995;23:1686–90.

27. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421: 499–506.

28. Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev 1997;11:3471–81.

29. Canman CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998;281:1677–9.

30. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7:683–94.

31. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001;7:673–82.

32. Jeffers JR, Parganas E, Lee Y, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003;4: 321–8.

33. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000;10:886–95.

34. Adams JM, Harris AW, Pinkert CA, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 1985;318:533–8.

35. McConville CM, Stankovic T, Byrd PJ, et al. Mutations associated with variant phenotypes in ataxia-telangiectasia. Am J Hum Genet 1996;59: 320–30.

36. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–70.

37. Gorgoulis VG, Vassiliou LV, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005;434:907–13.

38. Jacobsen KA, Prasad VS, Sidman CL, Osmond DG. Apoptosis and macrophage-mediated deletion of precursor B cells in the bone marrow of Eμ-myc transgenic mice. Blood 1994;84:2784–94.

39. Collins LS, Dorshkind K. A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. J Immunol 1987;138:1082–7.

40. Eischen CM, Woo D, Roussel MF, Cleveland JL. Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. Mol Cell Biol 2001;21:5063–70.

41. Ray S, Atkuri KR, Deb-Basu D, et al. MYC can induce DNA breaks *in vivo* and *in vitro* independent of reactive oxygen species. Cancer Res 2006; 66:6598–605.

42. Pauklin S, Kristjuhan A, Maimets T, Jaks V. ARF and ATM/ATR cooperate in p53-mediated apoptosis upon oncogenic stress. Biochem Biophys Res Commun 2005;334:386–94.

43. Packham G, Ashmun RA, Cleveland JL. Cytokines suppress apoptosis independent of increases in reactive oxygen levels. J Immunol 1996;156: 2792–800.

44. Strasser A, Harris AW, Cory S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 1990;348:331–3.

45. Egle A, Harris AW, Bouillet P, Cory S. Bim is a suppressor of Myc-induced mouse B cell leukemia. Proc Natl Acad Sci U S A 2004;101:6164–9.

46. Hemann MT, Zilfou JT, Zhao Z, Burgess DJ, Hannon G, Lowe SW. Suppression of tumorigenesis by the p53 target PUMA. Proc Natl Acad Sci U S A 2004;101:9333–8.

47. Hemann MT, Bric A, Teruya-Feldstein J, et al. Evasion of the p53 tumor surveillance network by tumor-derived myc mutants. Nature 2005;436:807–11.

48. Herzog KH, Chong MJ, Kapsetaki M, Morgan JI, McKinnon PJ. Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system. Science 1998;280:1089–91.

49. Shreeram S, Hee WK, Demidov ON, et al. Regulation of ATM/p53-dependent suppression of myc-induced lymphomas by Wip1 phosphatase. J Exp Med 2006;203:2793–9.

50. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions *in vivo*. Cancer Cell 2002;1:289–98.

51. Liu G, Parant JM, Lang G, et al. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat Genet 2004;36:63–8.

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