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Short Telomeres Limit Tumor Progression In Vivo by Inducing Senescence

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SUMMARY

Telomere maintenance is critical for cancer progression. To examine mechanisms of tumor suppression induced by short telomeres, we crossed mice deficient for the RNA component of telomerase, *mTR*$^{-/-}$, with *Eμ-myc* transgenic mice, an established model of Burkitt's lymphoma. Short telomeres suppressed tumor formation in *Eμ-myc* transgenic animals. Expression of *Bcl2* blocked apoptosis in tumor cells, but surprisingly, mice with short telomeres were still resistant to tumor formation. Staining for markers of cellular senescence showed that pretumor cells induced senescence in response to short telomeres. Loss of *p53* abrogated the short telomere response. This study provides in vivo evidence for the existence of a *p53*-mediated senescence mechanism in response to short telomeres that suppresses tumorigenesis.

INTRODUCTION

Burkitt's lymphoma is a highly aggressive, rapidly growing, and lethal cancer. Tumors that arise are uniformly associated with translocations that activate the c-*myc* oncogene. A transgenic mouse model of Burkitt's lymphoma in which c-*myc* is expressed in B cells (Adams et al., 1985) provides an excellent model to understand the biology of lymphoma and to test mechanisms that may limit tumor growth. Understanding the pathways that interrupt tumor growth will allow the development of specific therapies for lymphoma.

Cells contain two major intrinsic pathways of tumor suppression, apoptosis and senescence, that can be activated by multiple stimuli (Lowe et al., 2004). Telomerase inhibition was proposed as a potential cancer therapy when telomere shortening was first described in human cells (Harley et al., 1990). Telomerase is critical for telomere length maintenance; when telomerase is absent or blocked, progressive telomere shortening occurs with each cell division. In primary cultures of human cells, telomere shortening results in dysfunctional telomeres, which trigger a DNA damage response that ultimately leads to an irreversible state of cellular senescence (Bodnar et al., 1998; d'Adda di Fagagna et al., 2003). In mouse cells, short telomeres also initiate a DNA damage response (Hao et al., 2004); however, in response, these cells do not enter senescence (Blasco et al., 1997; Parrinello et al., 2003). In vivo short telomeres induce apoptosis in multiple highly proliferative tissues, including testis germ cells and lymphocytes (Hemann et al., 2001a; Lee et al., 1998). Senescent cells were detected in a subset of hepatocytes that were induced to proliferate via partial hepatectomy in mice with dysfunctional telomeres (Lechel et al., 2005; Satyanarayana et al., 2003). However, senescence was not observed in quiescent hepatocytes in animals with acute telomere dysfunction due to telomere uncapping (Denchi et al., 2006). Strong evidence has accumulated that short telomeres indeed limit tumor growth. Crosses of *mTR*$^{-/-}$ mice to tumor-prone models demonstrate that the short

SIGNIFICANCE

Telomerase inhibition has potential therapeutic benefits for cancer treatment. Experimental systems indicate that short telomeres impair tumor formation by inducing apoptosis. Therefore, blocking apoptosis was expected to block the tumor-suppressive effect of short telomeres. However, we found that, despite efficient inhibition of apoptosis, short telomeres retained potent tumor-suppressive signaling in *Eμ-myc*-induced lymphomas. This response required *p53* and showed hallmarks of cellular senescence. Thus, telomerase inhibition can engage multiple *p53*-dependent tumor suppressor pathways and may have therapeutic potential for tumors that have lost specific *p53* effector functions but retain wild-type *p53*.

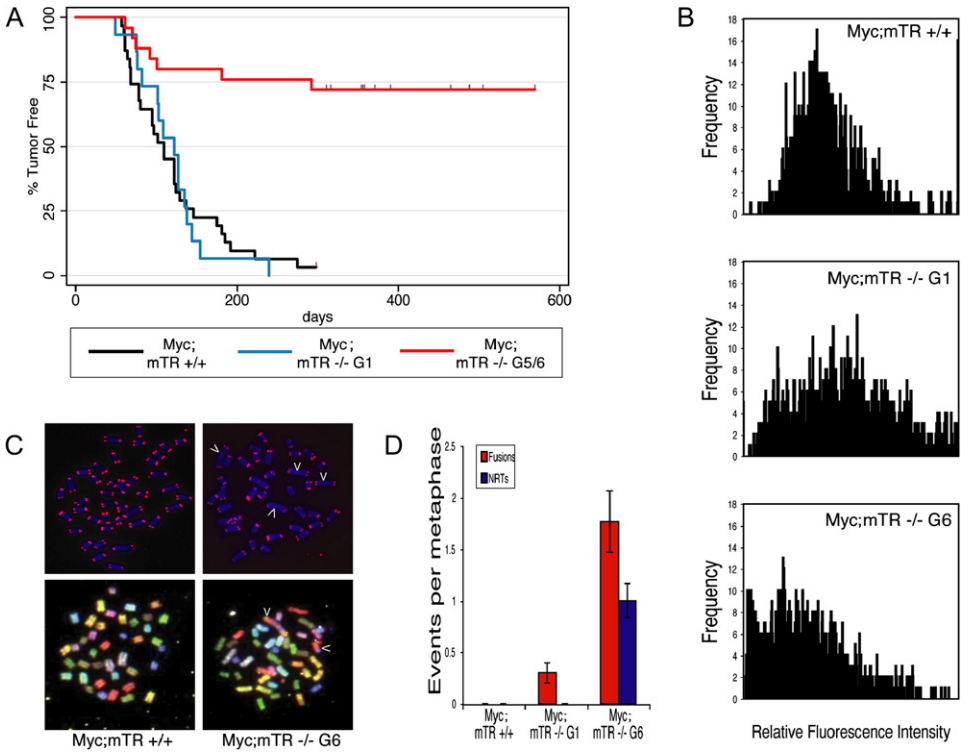

Figure 1. Short Telomeres Decrease Penetrance of Tumor Formation and Initiate Genomic Instability

(A) Kaplan-Meier survival analysis showing lymphoma onset in *Myc;mTR*$^{+/+}$ (black, n = 31), *Myc;mTR*$^{-/-}$ G1 (blue, n = 18), and *Myc;mTR*$^{-/-}$ G5/6 (red, n = 25) cohorts.

(B) Quantitative fluorescence in situ hybridization (Q-FISH) of tumor cell metaphase spreads from *Myc;mTR*$^{+/+}$ (top), *Myc;mTR*$^{-/-}$ G1 (middle), and *Myc;mTR*$^{-/-}$ G6 (bottom). Ten metaphases each from three separate lymphomas are represented for each genotype.

(C) Representative Q-FISH (top) and spectral karyotypes (SKY)(bottom) for *Myc;mTR*$^{+/+}$ (left) and *Myc;mTR*$^{-/-}$ G6 (right) tumors. Arrowheads indicate end-to-end chromosome fusions and nonreciprocal translocations in Q-FISH and SKY images, respectively.

(D) Quantitation of chromosome abnormalities. The number of chromosome end-to-end fusions (red bars) and nonreciprocal translocations (blue bars) per metaphase are shown (ten metaphases per tumor, n = 3). Error bars indicate standard error.

telomere response significantly limits tumor formation (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999; Qi et al., 2003, 2005; Rudolph et al., 2001; Wong et al., 2003). In these experiments, decreased tumor formation correlated with an increase in DNA damage, chromosome instability, and apoptosis. However, senescence was not reported in these studies. Taken together, these observations raise the question whether short telomere-induced senescence may act as a tumor suppressor mechanism.

The p53 tumor suppressor protein is the major mediator of the DNA damage response, apoptosis, and senescence. Consistent with this role, *p53* deficiency abrogates many of the cellular responses to short telomeres (Chin et al., 1999). In addition, short telomeres stimulated the formation of tumors in *p53*-deficient mice and caused the occurrence of tumor types that are not normally associated with this tumor-prone mouse model (Artandi et al., 2000). The loss of *p53* confers multiple advantages to uncontrolled proliferation, including loss of the DNA damage checkpoint, genome stability, senescence, and apoptosis (Vogelstein et al., 2000). It is therefore unclear which p53 effector functions are important for tumor suppression in response to short telomeres.

In the work presented here, we demonstrate that short telomeres suppress tumorigenesis in a mouse model of Burkitt's lymphoma and examine the requirement of apoptosis in mediating this response. We found that, surprisingly, inhibition of apoptosis did not abrogate the reduction in lymphoma formation in response to short telomeres. After long latency, microlymphomas that did appear displayed multiple markers of senescence. Genetic evidence showed that *p53* was absolutely required for tumor suppression. We conclude that short telomeres activate a *p53*-dependent cellular senescence pathway that limits tumor formation in vivo.

RESULTS

The *Eμ-myc* transgenic mouse is an established model of Burkitt's lymphoma. Overexpression of the *Myc* oncogene in B cells leads to B cell lymphoma with a median onset of 4–6 months (Adams et al., 1985). To assess the impact of short telomeres on lymphomagenesis, we generated *Eμ-myc* transgenic mice that were deficient for the RNA component of telomerase (Blasco et al., 1997) (*Eμ-myc;mTR*$^{-/-}$). To obtain *Eμ-myc* transgenic mice

with short telomeres, we intercrossed *Eμ-myc;mTR*$^{-/-}$ mice for six successive generations in the absence of telomerase. This breeding strategy allows for the comparison of *Eμ-myc* transgenic mice that are wild-type for telomerase (*Myc;mTR*$^{+/+}$), those that lack telomerase but have long telomeres (*Myc;mTR*$^{-/-}$ G1), and those that lack telomerase but have short telomeres (*Myc;mTR*$^{-/-}$ G5/6).

To determine the effect of short telomeres on tumor formation, we monitored cohorts of *Myc;mTR*$^{+/+}$, *Myc;mTR*$^{-/-}$ G1, and *Myc;mTR*$^{-/-}$ G5/6 mice for lymphoma onset. Consistent with previous studies (Adams et al., 1985), *Myc;mTR*$^{+/+}$ and *Myc;mTR*$^{-/-}$ G1 animals developed fully penetrant B cell lymphoma, and the latency of lymphoma onset was indistinguishable between the two cohorts (Figure 1A). In contrast, only 7 of 25 *Myc;mTR*$^{-/-}$ G5/6 animals developed lymphoma over the course of the study ($p < 10^{-4}$, Figure 1A). As expected, telomere length was significantly shorter in *Myc;mTR*$^{-/-}$ G5/6 tumors compared to *Myc;mTR*$^{+/+}$ and *Myc;mTR*$^{-/-}$ G1 tumors (Figure 1B). Further, these tumors displayed a high frequency of chromosome end-to-end fusions and nonreciprocal translocations (Figures 1C and 1D). In contrast, *Myc;mTR*$^{+/+}$ and *Myc;mTR*$^{-/-}$ G1 tumors showed no nonreciprocal translocations and only one independent, but clonal, end-to-end chromosome fusion. These observations indicate that short telomeres induce extensive genomic instability and are a potent inhibitor of tumorigenesis in *Eμ-myc* transgenic mice.

Previous studies in telomerase-deficient mice have suggested that the apoptotic pathway is critical for short telomere tumor suppression (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999; Qi et al., 2003, 2005; Rudolph et al., 2001; Wong et al., 2003). To directly test the requirement of apoptosis for short telomere-mediated tumor suppression, we disabled apoptotic signaling by expressing the *Bcl2* oncogene using an adoptive transfer protocol (Figure 2A). We cultured bone marrow, enriched for hematopoietic stem cells, from young, nonlymphomic *Myc;mTR*$^{+/+}$, *Myc;mTR*$^{-/-}$ G1, and *Myc;mTR*$^{-/-}$ G5/6 animals. Whole bone marrow was infected with a murine stem cell retrovirus (MSCV) that expresses *Bcl2* and EGFP from a bicistronic message (Schmitt et al., 2002). Adoptive transfer of *Bcl2* expressing *Eμ-myc* bone marrow into lethally irradiated syngeneic recipient animals results in rapid lymphoma onset approximately 6 weeks posttransplant (Schmitt et al., 2002). Disabling apoptosis results in a rapid onset of tumors during *Eμ-myc* lymphomagenesis (Schmitt et al., 2002). As expected, transplantation of *Bcl2*-positive HSCs derived from *Myc;mTR*$^{+/+}$ and *Myc;mTR*$^{-/-}$ G1 animals resulted in rapid tumor onset in all recipient animals in both genotypes (8 of 8 *Bcl2;Myc;mTR*$^{+/+}$ and 7 of 7 *Bcl2;Myc;mTR*$^{-/-}$ G1 mice were lymphoma bearing by 42 days posttransplant Figure 2B). These animals harbored highly aggressive tumors displaying complete effacement of the cervical lymph nodes and infiltration of tumor cells into adjacent salivary gland. Surprisingly, animals transplanted with *Bcl2*-positive *Myc;mTR*$^{-/-}$ G5/6 bone marrow failed to develop palpable tumors for more than 100 days posttransplant (0 of 3 *Myc;mTR*$^{-/-}$ G5 [129 days] and 0 of 5 *Myc;mTR*$^{-/-}$ G6 [100 days]; Figure 2B). Histological evaluation of lymphatic tissues from these animals showed small encapsulated tumor masses in the cervical lymph nodes with little infiltration into salivary gland (Figure 5B). To confirm that *Bcl2* was blocking apoptosis in the tumor cells in these animals, we used a terminal UTP nick end labeling (TUNEL) protocol to examine the extent of apoptosis in lymphomas from *Bcl2*-expressing mice. Apoptotic levels were significantly reduced in *Bcl2*-expressing

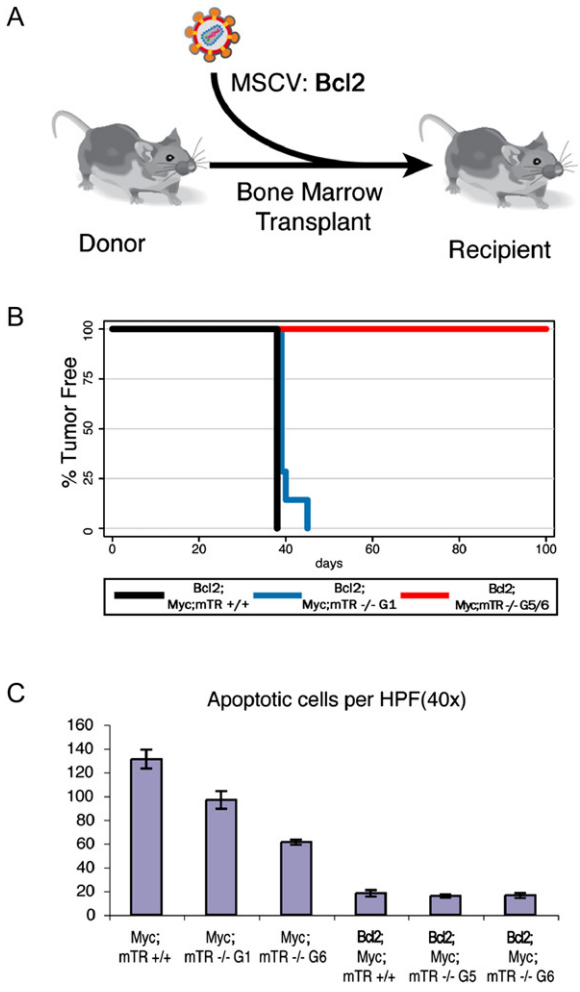

Figure 2. Short Telomeres Suppress Lymphoma Formation despite Abrogation of the Apoptotic Program

(A) Experimental bone marrow transplant scheme. Bone marrow from donors of defined genotypes was infected with *Bcl2*-expressing murine stem cell virus prior to transplantation into lethally irradiated wild-type recipient animals. Iconography reproduced by kind permission of New Science Press from *Immunity* (DeFranco et al., 2007).

(B) Kaplan-Meier survival analysis of *Myc;mTR*$^{+/+}$ (black, n = 8), *Myc;mTR*$^{-/-}$ G1 (blue, n = 7), and *Myc;mTR*$^{-/-}$ G5/6 (red, n = 3[G5], n = 5 [G6]) cohorts.

(C) TUNEL analysis of tumor masses from *Bcl2;Myc;mTR*$^{+/+}$, *Bcl2;Myc;mTR*$^{-/-}$ G5, and *Bcl2;Myc;mTR*$^{-/-}$ G6. Error bars indicate standard error.

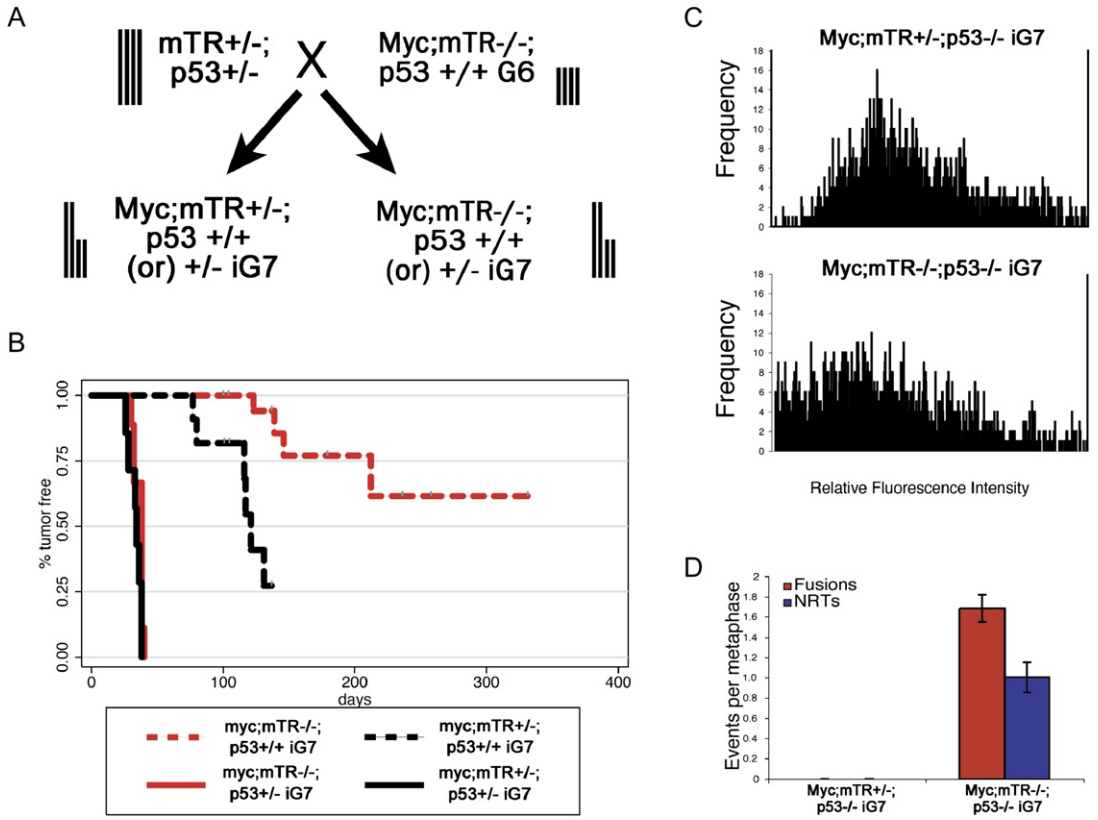

Figure 3. Short Telomeres Block Tumor Formation in a p53-Dependent Manner

(A) Intergenerational breeding scheme. Vertical bars represent telomere lengths.

(B) Kaplan-Meier survival analysis of *Myc;mTR*$^{+/-}$;*p53*$^{+/+}$ iG7 (black dashed line, n = 11), *Myc;mTR*$^{-/-}$;*p53*$^{+/+}$ iG7 (red dashed line, n = 20), *Myc;mTR*$^{+/-}$;*p53*$^{+/-}$ iG7 (black solid line, n = 7), and *Myc;mTR*$^{-/-}$;*p53*$^{+/-}$ iG7 (red solid line, n = 9) cohorts. *Myc;p53*$^{+/-}$ animals invariably develop *Myc;p53*$^{-/-}$ tumors at ~40 days. Short telomeres promote chromosomal abnormalities in p53-deficient lymphomas.

(C) Q-FISH analysis of metaphase spreads from *Myc;mTR*$^{+/-}$;*p53*$^{-/-}$ (top) and *Myc;mTR*$^{-/-}$;*p53*$^{-/-}$ (bottom) lymphomas. Ten metaphases each from three separate lymphomas are represented for each genotype.

(D) Quantitation of chromosome abnormalities. The number of chromosome end-to-end fusions (red bars) and nonreciprocal translocations (blue bars) per metaphase are shown (ten metaphases per tumor, n = 3). Error bars indicate standard error.

lymphomas compared to spontaneously arising *Eμ-myc* lymphomas that do not express *Bcl2* (Figure 2C). Importantly, the amount of apoptotic cells was similar in all *Bcl2*-positive lymphomas tested (Student's t test; p = 0.6 for *Myc;mTR*$^{+/+}$ to *Myc;mTR*$^{-/-}$ G6 comparison). These data indicate that short telomeres effectively abolished tumor progression even when apoptosis was blocked.

Decreased tumor formation in the absence of apoptosis suggested that another mechanism is limiting tumor growth. p53 regulates multiple pathways that act to suppress tumor formation, including apoptosis and senescence (Lowe et al., 2004; Vogelstein et al., 2000). To genetically test whether *p53* is required for the short telomere-induced suppression of lymphoma, we crossed mice doubly heterozygous for *mTR* and *p53* (*mTR*$^{+/-}$;*p53*$^{+/-}$) to *Myc;mTR*$^{-/-}$ G6 mice (Figure 3A). This cross results in intergenerational G7 (iG7) littermates that all have half-long and half-short telomeres and are either telomerase positive (*mTR*$^{+/-}$) or telomerase negative (*mTR*$^{-/-}$). The *mTR*$^{-/-}$ iG7 mice have short telomeres that mimic *mTR*$^{-/-}$ G6 mice (Feldser et al., 2006; Hemann et al., 2001b; Qi et al., 2003). *Eμ-myc* transgenic animals that are heterozygous for *p53* (*p53*$^{+/-}$) invariably develop *p53*-deficient lymphomas due to loss of the remaining wild-type *p53* allele (LOH) (Schmitt et al., 1999). As expected, *Myc;mTR*$^{+/-}$;*p53*$^{+/-}$ iG7 mice formed tumors rapidly. Interestingly, *Myc;mTR*$^{-/-}$;*p53*$^{+/-}$ iG7 mice also formed tumors with similar rapid onset as their telomerase-positive counterparts; all animals developed lymphoma by day 42 after birth (Figure 3B). Despite similar rates of lymphoma onset, *mTR*$^{-/-}$ iG7 tumors had significantly shorter telomeres, high rates of chromosome end-to-end fusions, and numerous nonreciprocal translocations, whereas their telomerase-positive (*mTR*$^{+/-}$) counterparts had none (Figure 3D). These genetic experiments indicate that short telomere-induced tumor suppression requires the p53 tumor suppressor pathway and that loss of *p53* abrogates the cellular response to telomere dysfunction.

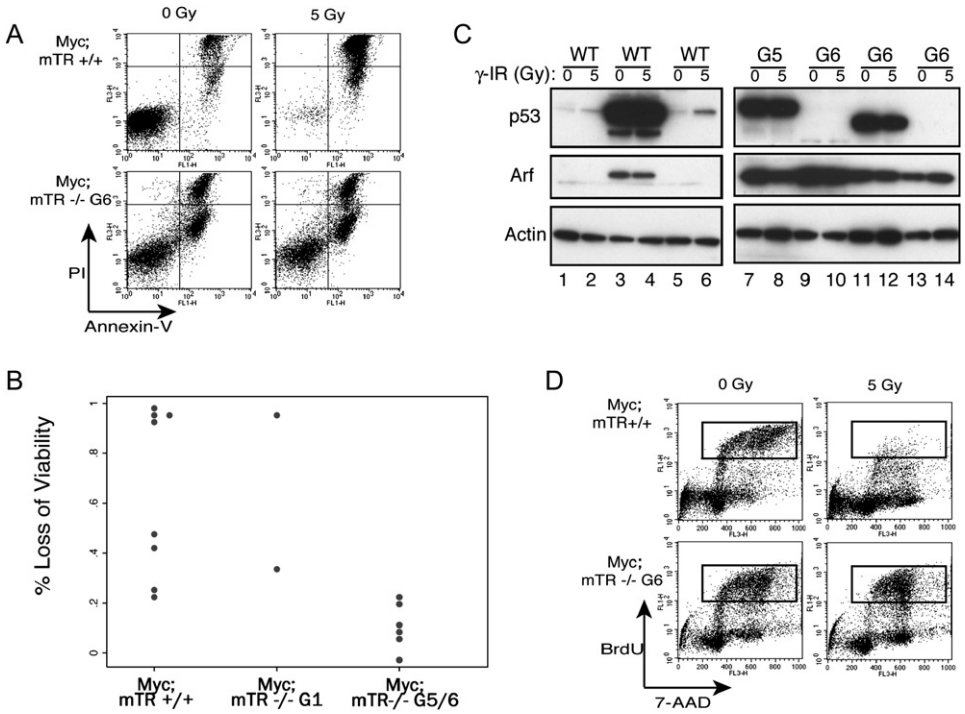

Figure 4. Short Telomeres in Spontaneous Lymphomas from *Eμ-Myc;mTR*$^{-/-}$ G5/6 Select for Mutations in *p53*

(A) Loss of apoptotic induction in tumor cells derived from *Myc;mTR*$^{-/-}$ G5/6 mice. Six hours after 0 or 5 Gy γ-IR treatment, cells were stained with Annexin-V FLUOS (FL-1) and propidium iodide (FL-3) and processed by flow cytometry. Representative tumors from *Myc;mTR*$^{+/+}$ (top) and *Myc;mTR*$^{-/-}$ G6 (bottom) are shown. Sensitivity to γ-IR is shown by loss of viable cells (lower left quadrant) after irradiation.

(B) Summary of the percent of viable cells, as described in (A), for all tumors analyzed.

(C) Immunoblot of nonirradiated or irradiated (5 Gy γ-IR) tumor cell lysates for p53, Arf, and Actin. Representative tumors from *Myc;mTR*$^{+/+}$ (left panel) and *Myc;mTR*$^{-/-}$ G5/6 (right panel). Normal p53 induction is shown by comparing lanes 1 and 2, and 5 and 6, while overexpression of mutant p53 is shown in lanes 3–4, 7–8, and 11–12. Complete loss of p53 protein is shown in lanes 9–10, and 13–14. Arf expression is seen only in samples with suspected *p53* mutations (Eischen et al., 1999).

(D) Radioresistant DNA synthesis in lymphomas with *p53* mutations. Cells were treated with 5 Gy γ-IR. After 1 hr, BrdU was added to culture medium, then 6 hr postirradiation cells were fixed and stained with anti-BrdU antibody and subjected to FACS analysis (FL-3 = 7-AAD signal and FL-1 = anti-BrdU FLUOS) (the boxed area indicates cells in S phase).

In addition to these genetic data, several lines of evidence suggest that loss of *p53* is critical for tumor development in the presence of short telomeres. First, tumors that are derived from *Myc;mTR*$^{-/-}$ G5/6 animals (Figure 1) are unable to elicit a G1 checkpoint in response to γ-IR, and these tumors also do not initiate apoptosis after γ-IR treatment (Figures 4A, 4B, and 4D). Second, immunoblot analysis showed that, in these tumors, p53 was either undetectable regardless of γ-IR treatment or grossly overexpressed and uninducible (Figure 4C). Finally, p19^{ARF} was overexpressed in all tumors from *Myc* G5/6 animals (6 out of 6 lymphomas tested, χ^2 test $p = 0.01$ compared to *Myc;mTR*$^{+/+}$). These observations are consistent with p53 inactivation in the tumors, either by deletion or by point mutation, which impairs p53 degradation (Eischen et al., 1999). This implies that, for tumors to grow in the presence of short telomeres, the p53 pathway must be inactivated.

The requirement for *p53*, but not apoptosis, implies that *p53*-mediated cellular senescence may be responsible for tumor suppression. To determine whether short telomeres could be blocking tumor progression by initiating senescence, we analyzed microlymphomas from *Bcl2;Myc;mTR*$^{-/-}$ G5/6 animals (Figure 2) for proliferation defects and the presence of senescence markers. We scored mitotic indices from tissue sections derived from *Bcl2;Myc;mTR*$^{+/+}$ and *Bcl2;Myc;mTR*$^{-/-}$ G5/6 lymphomas. Compared to *Bcl2;Myc;mTR*$^{+/+}$ sections, *Bcl2;Myc;mTR*$^{-/-}$ G5/6 sections had significantly fewer mitotic figures per high-power field (Figure 5A; $p < 0.0001$), indicating that short telomeres negatively affect cellular proliferation. Next, *Bcl2;Myc* lymphomas were analyzed for the presence of markers that are specifically upregulated in senescent tissues (Collado and Serrano, 2006). *Bcl2*-expressing *Myc;mTR*$^{-/-}$ G5/6 microlymphomas stained positive for SA-βgal and displayed both p16^{INK4a} and p15^{INK4b} immunohistochemical staining. In contrast, *Bcl2*-expressing *Myc;mTR*$^{+/+}$ lymphomas did not stain for any of these markers (Figure 5B). These staining patterns indicate that *Bcl2*-expressing *Myc;mTR*$^{-/-}$ G5/6 microlymphomas were indeed senescent. Taken together with the failure of these lymphomas to progress, these data indicate that

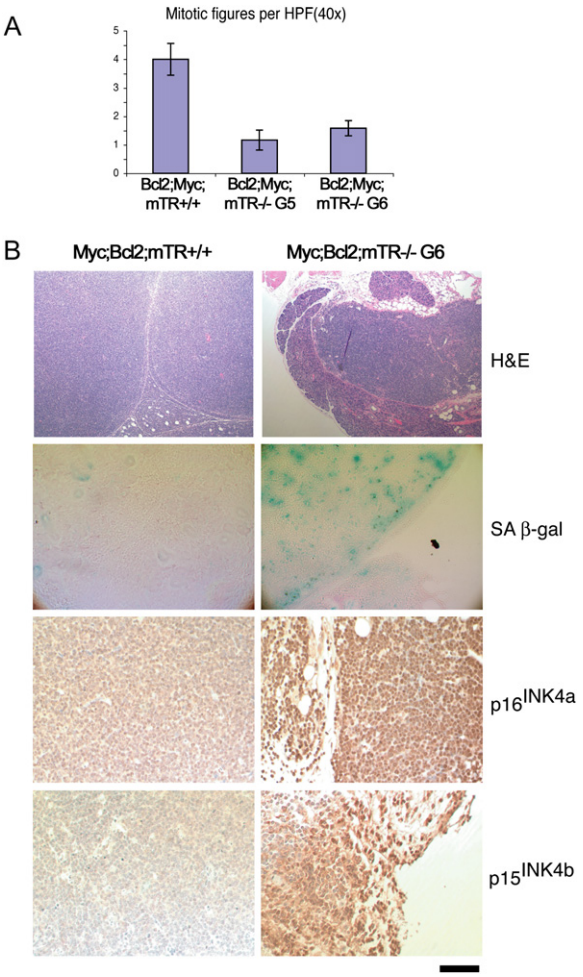

Figure 5. Microlymphomas from *Bcl2;Myc;mTR*$^{-/-}$ G5/6 Animals Are Senescent

(A) Mitotic indices for *Bcl2;Myc;mTR*$^{+/+}$, *Bcl2;Myc;mTR*$^{-/-}$ G5, and *Bcl2;Myc;mTR*$^{-/-}$ G6. Error bars indicate standard error.

(B) Histological analysis of *Bcl2;Myc;mTR*$^{+/+}$ and *Bcl2;Myc;mTR*$^{-/-}$ G6 lymphomas. Top: H&E stain showing complete effacement of cervical lymph node in *Bcl2;Myc;mTR*$^{+/+}$ and small encapsulated microlymphoma surrounded by salivary glands in *Bcl2;Myc;mTR*$^{-/-}$ G6. Senescence-associated β-gal activity and p16^{INK4a} and p15^{INK4b} immunostain specifically associated with *Bcl2;mTR*$^{-/-}$ G6 microlymphomas. Scale bar represents 250 μm for low-power H&E stained images (top panels) and 100 μm for all other images.

short telomeres can induce senescence in vivo to effectively abolish tumor progression.

DISCUSSION

Understanding cellular mechanisms that may limit tumor growth could provide new therapeutic approaches to cancer therapy. Telomere shortening, through the inhibition of telomerase, has been proposed as a potential cancer treatment. Here we show that short telomeres can block lymphoma induction in a mouse model of Burkitt's lymphoma. Short telomeres have been shown to reduce tumor formation in several other mouse tumor models (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999; Qi et al., 2003, 2005; Rudolph et al., 2001; Wong et al., 2003); however, in all of those systems, apoptosis was thought to be responsible for the decreased incidence of tumor formation. We found that blocking apoptosis still resulted in tumor suppression. *p53* was required for the tumor reduction due to short telomeres, and direct evidence indicated that a *p53*-mediated senescence program was activated in the small tumors that did form. These results indicate that short telomeres can mediate a *p53*-dependent senescence program that limits tumor formation in vivo.

Telomere-mediated senescence in tumors may not have been detected in previous studies because it likely is initiated in only a few cells. Recently, cellular senescence induced by oncogene expression has been established as a tumor suppressor mechanism in vivo (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). Activation of cellular senescence in these instances occurs in all cells in the initiating tumor mass, thus facilitating detection of senescence markers. In contrast, senescence induced by short telomeres may occur initially in only a few cells in which telomeres become critically short. This may impede the detection of short-telomere-mediated senescence during tumor formation.

Short telomeres can engage both the apoptosis and the senescence pathways. *p53* is necessary to carry out both apoptotic and senescence pathways (Figure 6). The requirement of the loss of *p53* for the growth of the *Eμ-myc* lymphomas with short telomeres suggests both apoptosis and senescence play roles in limiting tumor growth. If only one tumor suppressor pathway, apoptosis or senescence, were necessary for short-telomere-mediated tumor suppression, we would have expected mutations in other components of these pathways to also allow tumor formation in these mice. For example, if disruption of apoptosis were the only *p53* effector function required for tumor formation in the presence of short telomeres, we would have expected overexpression of *Bcl2* to confer a selective growth advantage regardless of telomere length. Consistent with this, we speculate that loss of p16 pathway components in tumors that express *Bcl2* would be resistant to the tumor-suppressive effects of short telomeres (Figure 6).Tumor suppressor programs induced by *p53* are context dependent. Restoration of *p53* function in *p53* null tumors induces tumor regression in multiple tumor types (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). However, *p53* restoration induces apoptosis in lymphomas (Martins et al., 2006; Ventura et al., 2007) and senescence in sarcomas (Ventura et al., 2007) and liver carcinomas (Xue et al., 2007). It is unclear whether *p53*-induced senescence or apoptosis correlates with cell type, oncogenic lesion, or both. We show here that, in a tumor type that is predisposed to *p53*-mediated apoptosis, short telomeres can effectively redirect the *p53* tumor suppressor response to induce senescence. It would be interesting if short telomeres could, likewise, alter *p53*-mediated senescence programs to induce apoptosis in tumor types predisposed to undergo senescence.

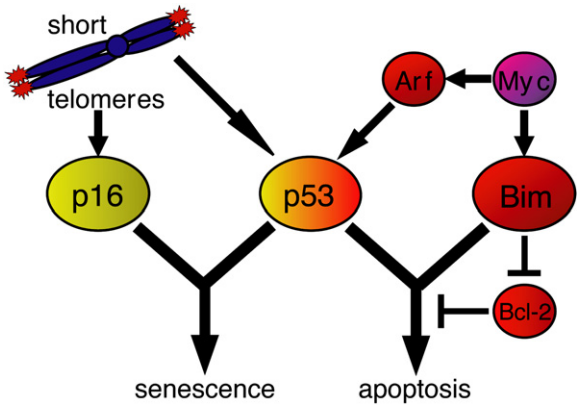

Figure 6. Tumor Suppressor Signaling Downstream of Short Telomeres and *Myc*

The *myc* oncogene negatively regulates its effects on cell proliferation by inducing both Arf and Bim tumor suppressors. Arf activity results in stabilization of the p53 protein, whereas *Bim* is directly transcribed by myc and induces apoptosis by inhibiting Bcl2. Activation of both Arf and Bim is required for *myc*-induced apoptosis. Apoptotic signaling can also be disrupted by overexpression of the downstream antiapoptotic *Bcl2* oncogene. The short telomere response signals through both p16 and p53 tumor suppressor pathways, which carry out senescence and/or apoptotic tumor suppressor functions. The p53 tumor suppressor lies at the crossroads of senescence and apoptosis and is required for both programs.

Short telomeres and *p53* deficiency have profound effects on tumor formation. First, short telomeres promote transformation of *p53*-deficient mouse embryo fibroblasts (Chin et al., 1999). Second, short telomeres contribute to mortality of tumor-prone *p53* null mice by increasing tumor formation and facilitating tumorigenesis in tissues not usually prone to tumor formation in this system (Artandi et al., 2000; Jacks et al., 1994). Here we used a mouse tumor model in which the initiating mutation, the *Myc* transgene, is unrelated to *p53* and found that the growth of tumors with short telomeres requires *p53* loss. This requirement for *p53* has implications for the clinical application of telomerase inhibitors. First, the fact that short telomeres can induce both apoptosis and senescence to limit tumorigenesis indicates that both of these pathways can be targeted in cancer therapy. However, the data also imply that telomerase inhibition will be most effective in tumors that have an intact p53 pathway. Alternatively, telomerase inhibition may be combined with approaches that reinstate p53 function or other mechanisms to activate senescence or apoptosis programs in tumors. The lymphoma model described here thus provides an excellent system to understand the genetic requirements that will guide the development of more sophisticated approaches to targeting telomerase in cancer therapy.

EXPERIMENTAL PROCEDURES

Lymphoma Monitoring and Analysis

The Institutional Animal Care and Use Committee at the Johns Hopkins University approved all animal procedures. *Eμ-myc* transgenic animals were inspected twice weekly for lymph node enlargement by palpation and for overall signs of distress. Animals were considered lymphomic when tumor mass was easily palpated no more than 1 cm in diameter. Lymphoma-bearing animals were euthanized, and tumor masses were dissociated through nylon mesh into single-cell suspensions and cultured on mitomycin C-treated 3T3 feeder layers in B cell media (45% DMEM [Invitrogen], 45% IMDM [Invitrogen], 10% FBS [Hyclone], Pen/Strep/Glutamine [Invitrogen]).

Chromosome Analysis

Metaphase spreads were generated by arresting exponentially growing cultures with 0.5 μg/ml KaryoMax (Gibco) 2 hr before hypotonic swelling in 0.075 M KCl and fixation in 3:1 methanol:acetic acid. Fixed cells were dropped onto glass slides over a steaming water bath. Telomere length was determined by hybridization of metaphases with Cy3 $(CCCTAA)_3$ PNA probes (PE Biosystems) as described (Lansdorp et al., 1996). Spectral karyotyping was performed on metaphases as described (Liyanage et al., 1996). Images were obtained using IP-Lab software on a Zeiss Axioscope microscope.

Immunoblot, Apoptosis, and Cell-Cycle Analysis

Exponentially growing lymphoma cells (2×10^6) were grown in 6-well plates. Cells were exposed to 0 or 5 Gy γ-IR from a cesium source (Gamma Cell) prior to analyses. For immunoblot analysis, cells were lysed in RIPA buffer containing protease inhibitors (Roche), denatured in SDS loading buffer, separated on a 4%–12% gradient Tris-acetate gel (Novex), transferred to PVDF (MilliPore), and probed with anti-p53 (CM5 Novocastra), anti-Arf (Ab80 Abcam), and anti-actin (Sigma). For apoptosis analysis, cells were harvested, washed in PBS, and then stained with an Annexin-V FLUOS staining kit (Roche) per the manufacturer's instructions. For BrdU incorporation, BrdU was added to culture medium 1 hr postirradiation, and analysis was performed 6 hr after γ-IR. Cells were harvested and analyzed using an in situ cell proliferation kit (Roche) per the manufacturer's instructions.

Bone Marrow Transplantation Protocol and Retrovirus Production

Donor animals (6- to 10-week-old on C57BL/6J background) were primed with 5-FU (150 mg/kg) 4 days prior to bone marrow harvest. Marrow was harvested from tibia and femur by flushing with Hank's balanced salt solution with a 23-gauge needle. After red cell lysis, cells were cultured in IMDM with 18% FBS (Hyclone) 4% WEHI-3 conditioned media, 10 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml mSCF (Fitzgerald). Ecotropic retrovirus was produced using Phoenix cells (G. Nolan, Stanford) by transfecting 12 μg MSCV:*Bcl2* (Schmitt et al., 2002) into 2×10^6 cells with 20 μl Lipofectamine 2000 (Invitrogen) overnight. Virus was collected 24 and 48 hr posttransfection and used immediately to infect bone marrow cells four times by 90 min spinfection (1000 g). Recipient animals (C57BL/6J, Jackson Labs) were lethally irradiated with 9 Gy total body γ-IR (Gamma Cell cesium source) and transplanted with 2.5×10^5 bone marrow cells.

In Situ Apoptosis and Mitosis

Formalin-fixed tissue sections were stained using an in situ cell death kit (Roche) per the manufacturer's instructions and counterstained with DAPI. Mitotic cells were counted based on morphology, and apoptotic cells were identified by Cy-3 label after TUNEL reaction.

Histology and Immunohistochemistry

Tissues harvested from lymphoma-bearing animals were fixed in 4% neutral buffered formalin and then sectioned into 5 μm thick sections for H&E and immunochemical stain. Prior to antibody hybridization, antigens were retrieved by boiling slides for 10 min in 10 mM citrate solution. Positive staining was routinely assessed by comparing serial sections exposed to either specific primary antibodies or antibody dilution buffer only (negative controls). All subsequent steps were identical. $p16^{Ink4a}$ antibody (F-12 Santa Cruz; 1:100) and $p15^{INK4b}$ antibody (Ab-6 Lab Vision; 1:50) were visualized with a Vectastain Elite kit

(Vector Labs). For SAβ-gal activity, tissues were snap frozen in OCT mounting media (Sakura), and 12 μm sections were stained as described (Dimri et al., 1995).

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