

Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis

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Mdm2 harnesses the p53 tumor suppressor, yet loss of one *Mdm2* allele in *Mdm2*^{+/-} mice has heretofore not been shown to impair tumor development. Here we report that *Mdm2* haplo-insufficiency profoundly suppresses lymphomagenesis in Eμ-*myc* transgenic mice. *Mdm2*^{+/-}-Eμ-*myc* transgenics had greatly protracted rates of B cell lymphoma development with life spans twice that of wild-type transgenic littermates. Impaired lymphoma development was associated with drastic reductions in peripheral B cell numbers in *Mdm2*^{+/-}-Eμ-*myc* transgenics, and primary pre-B cells from *Mdm2*^{+/-}-Eμ-*myc* transgenics and *Mdm2*^{+/-} littermates were extremely susceptible to spontaneous apoptosis. Loss of *p53* rescued all of the effects of *Mdm2* haplo-insufficiency, indicating they were *p53* dependent. Furthermore, half of the lymphomas that ultimately emerged in *Mdm2*^{+/-}-Eμ-*myc* transgenics harbored inactivating mutations in *p53*, and the majority overcame haplo-insufficiency by overexpressing Mdm2. These results support the concept that Mdm2 functions are rate limiting in lymphomagenesis and that targeting Mdm2 will enhance *p53*-mediated apoptosis, compromising tumor development and/or maintenance.

Keywords: apoptosis/lymphoma/Mdm2/Myc/p53

Introduction

Mdm2 (murine double minute 2, termed Hdm2 in humans) is amplified in 10% of all human malignancies (Momand *et al.*, 1998), and Mdm2 protein is frequently overexpressed in murine and human lymphomas through other mechanisms (Watanabe *et al.*, 1996; Eischen *et al.*, 1999). Mdm2's oncogenic properties are due in part to its ability to inactivate the *p53* tumor suppressor (reviewed in Momand *et al.*, 2000). Mdm2 is an E3 ubiquitin ligase that inhibits *p53* by blocking *p53* transcriptional activity (Momand *et al.*, 1992), ubiquitylating *p53* (Honda and Yasuda, 1999) and shuttling *p53* from the nucleus into the cytoplasm where it is degraded by the proteasome (Freedman and Levine, 1998; Roth *et al.*, 1998). *Mdm2*-null mice die shortly after implantation due to unregulated *p53* activity (de Rozières *et al.*, 2000), as the lethality of

Mdm2-null mice is rescued by crossing them to *p53*-null mice (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). *Mdm2* is a direct transcriptional target of *p53*, and therefore Mdm2 is the critical component of a negative feedback loop that regulates *p53* activity (Wu *et al.*, 1993). However, Mdm2's regulation of *p53* is also controlled by the ARF tumor suppressor. ARF binds to and can sequester Mdm2 in the nucleolus (Tao and Levine, 1999; Weber *et al.*, 1999). However, nucleolar relocalization of Mdm2 by ARF is not essential to inhibit Mdm2 (Korgaonkar *et al.*, 2002), as ARF also blocks Mdm2 ubiquitin ligase activity, *p53* degradation and transcriptional inactivation of *p53* (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998; Honda and Yasuda, 1999).

Both ARF and *p53* mediate apoptosis in response to hyperproliferation signals from oncogenes, including Myc, E2F-1, E1A and Ras (reviewed in Sherr, 1998). Consequently, inactivation of ARF or *p53* occurs frequently in cancers that overexpress oncogenes, including the majority of B cell lymphomas that arise in Eμ-*myc* transgenic mice engineered to overexpress Myc in the B cell compartment (Eischen *et al.*, 1999; Schmitt *et al.*, 1999). In Eμ-*myc* transgenics, a mouse model of human non-Hodgkin's lymphoma, inactivation of *p53* and ARF occurred in 28% and 24%, respectively, of the B cell lymphomas that emerged (Eischen *et al.*, 1999). Furthermore, loss of *ARF* or *p53* confers resistance to Myc-induced apoptosis and accelerates lymphoma development in Eμ-*myc* transgenics (Eischen *et al.*, 1999; Jacobs *et al.*, 1999; Schmitt *et al.*, 1999), highlighting the importance of ARF and *p53* in inhibiting oncogene-induced tumorigenesis.

Despite the essential role ARF and *p53* play in Myc-initiated lymphomagenesis, little is known about Mdm2's role in lymphoma development. A number of reports have shown that lymphomas in both humans and Eμ-*myc* transgenic mice frequently overexpress Mdm2 protein without gene amplification (Momand and Zambetti, 1997; Eischen *et al.*, 1999, 2001b). Furthermore, enforced expression of Mdm2 in transgenic mice where *mdm2* was under the control of its own promoter results in late-onset of lymphomas and sarcomas (Jones *et al.*, 1998). Although these findings suggest that Mdm2 expression can contribute to lymphoma development, it was unclear whether Mdm2 plays an essential role during lymphomagenesis and whether this was linked to Mdm2's ability to harness *p53*. Here we show that *Mdm2* haplo-insufficiency has profound and surprising effects in impairing lymphoma development in Eμ-*myc* transgenic mice. The inhibition of B cell lymphomagenesis by *Mdm2* haplo-insufficiency was due to a marked increase in *p53*-dependent apoptosis of B cells, resulting in severely reduced numbers of peripheral B cells in *Mdm2*^{+/-}-Eμ-*myc* transgenics. Finally, the lymphomas that ultimately did

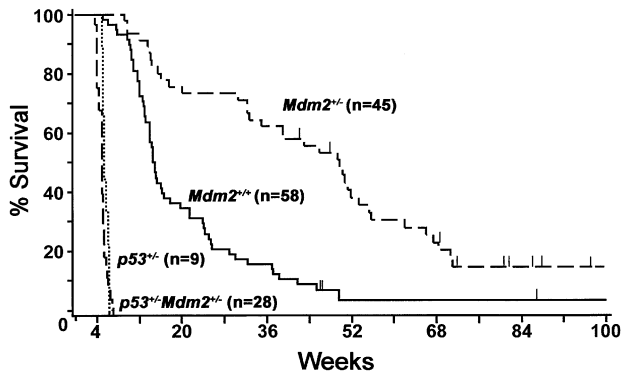


Fig. 1. Myc-induced lymphomagenesis is inhibited by *Mdm2* haplo-insufficiency. Kaplan–Meier survival curves of *Mdm2*^{+/-}*Eμ-myc* transgenic, *Mdm2*^{+/+}*Eμ-myc* transgenic, *p53*^{+/-}*Eμ-myc* transgenic and *p53*^{+/+}*Mdm2*^{+/-}*Eμ-myc* transgenic mice. The average survivals are 44.3, 20.6, 5.6 and 5 weeks, respectively (log-rank test, $P < 0.001$). n , the number of mice in each group. Vertical lines indicate ages of surviving mice. Three (3/58) *Mdm2*^{+/-}*Eμ-myc* transgenic mice and nine (9/45) *Mdm2*^{+/+}*Eμ-myc* transgenic mice are still alive, whereas none of the *p53*^{+/-}*Eμ-myc* and *p53*^{+/+}*Mdm2*^{+/-}*Eμ-myc* transgenics is alive. The Kaplan–Meier curves are right-censored because the study was terminated before all of the animals were sacrificed. Therefore, although 20% (9/45) of the *Mdm2*^{+/-}*Eμ-myc* transgenic mice are still alive at 70 weeks, the Kaplan–Meier estimate at 70 weeks is lower because there were fewer animals at risk of death at 70 weeks. Lymphoma was documented in all of the animals.

arise in *Mdm2*^{+/-}*Eμ-myc* mice preferentially suffered mutations that inactivated p53, and also compensated for *Mdm2* haplo-insufficiency by overexpressing Mdm2. Therefore, Mdm2 functions are rate limiting in tumorigenesis and targeting Mdm2 in even a quantitative fashion may prove efficacious in cancer therapy.

Results

Mdm2 haplo-insufficiency inhibits Myc-induced lymphomagenesis

Eμ-myc transgenic mice develop pre-B and/or B cell lymphoma (Adams *et al.*, 1985), and half of these lymphomas overexpress Mdm2 protein (Eischen *et al.*, 1999, 2001b). This observation suggests that Mdm2 overexpression is selected for during lymphoma development and that Mdm2 may be necessary for and/or facilitate Myc-induced lymphomagenesis. Therefore, we postulated that a decrease in Mdm2 expression would inhibit lymphoma development. To test this issue, we crossed *Mdm2* heterozygous mice (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995) to *Eμ-myc* transgenics and evaluated whether there were any effects of *Mdm2* haplo-insufficiency on Myc-induced lymphomagenesis. Strikingly, lymphoma development was drastically delayed in *Mdm2*^{+/-}*Eμ-myc* transgenics. The *Mdm2*^{+/-}*Eμ-myc* transgenics had an average survival of 44.3 weeks, more than twice that of the 20.6 week average survival for the *Mdm2*^{+/+}*Eμ-myc* transgenic littermates (log-rank test $P < 0.001$; Figure 1). Furthermore, 20% (9/45) of the *Mdm2*^{+/-}*Eμ-myc* transgenics failed to develop lymphoma (and are still alive), whereas only 5% (3/58) of the *Mdm2*^{+/+}*Eμ-myc* transgenics remained disease free. These results support the hypothesis that Mdm2 functions are

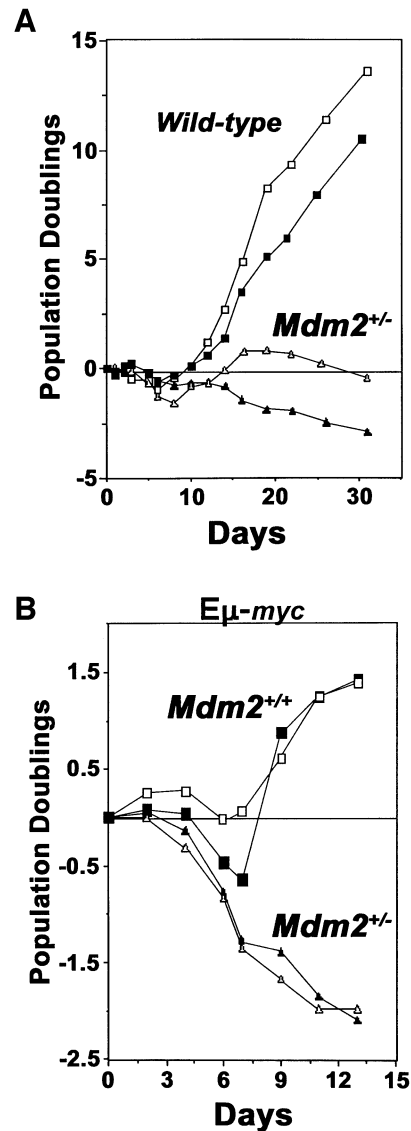


Fig. 2. *Mdm2* heterozygous bone marrow is sensitive to spontaneous and Myc-induced apoptosis. Bone marrow from two *Mdm2*^{+/-} mice [(A), triangles], two wild-type littermates (A, squares), two *Mdm2*^{+/-}*Eμ-myc* transgenics [(B), triangles], and two *Mdm2*^{+/+}*Eμ-myc* transgenic littermate controls (B, squares) prior to any detectable lymphoma was placed into IL-7-containing medium (day 0). Cells were counted on the indicated days. Pre-B cell growth was calculated as net population doublings at the indicated intervals. Viability was determined by Trypan Blue dye exclusion and apoptosis was verified by PI staining.

rate limiting for tumor development during Myc-induced lymphomagenesis.

The B cell lymphomas that arose in the *Mdm2*^{+/-}*Eμ-myc* transgenic mice were typical of those that arise in *Eμ-myc* transgenics, with the majority having a large diffuse cell lymphoma and expressing IgM (data not shown). Both wild-type *Eμ-myc* and *Mdm2*^{+/-}*Eμ-myc* transgenics also developed splenomegaly, with spleens 10–20 times the normal size, and both genotypes had frequent metastases to the lungs. Surprisingly, many of the lymphomas that ultimately emerged in the *Mdm2*^{+/-}*Eμ-myc* transgenics failed to localize to peripheral lymph nodes. In wild-type *Eμ-myc* transgenics, B cell lymphomas usually arise in the peripheral lymph nodes in the neck, front and back legs

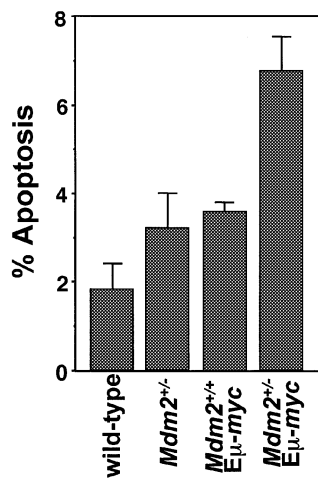


Fig. 3. Increased apoptosis in splenocytes from *Mdm2*^{+/-}Eμ-myc transgenics. Disaggregated, ethanol fixed splenocytes from the indicated genotypes prior to any detectable disease were stained with PI and analyzed on a FACScan. Sub-G₁ DNA was quantified from three separate mice of each genotype with CellQuest software. Each bar is an average and error bars represent 1 SD.

and can also be observed in the mesenteric lymph nodes in the abdomen and occasionally in the thymus (Adams *et al.*, 1985; Harris *et al.*, 1988). In contrast, the primary site of lymphoma development in the majority (58%, 21/36) of *Mdm2*^{+/-}Eμ-myc transgenics appeared to be in the liver and mesenteric lymph nodes, followed by the thymus (data not shown), especially in older mice. The underlying cause for altered sites of lymphomagenesis is unclear, as *Mdm2* has not been reported to regulate lymphocyte homing. Nonetheless, although many of the lymphomas arose in atypical regions, they were typical B cell lymphomas as determined by FACS analysis (B220⁺, CD19⁺) and/or immunohistochemistry (B220⁺) (data not shown). Therefore, along with a delay in tumor onset, there was also a difference in the sites of B cell lymphomagenesis in the *Mdm2* haplo-insufficient Eμ-myc transgenics.

***Mdm2* haplo-insufficiency predisposes B cells to apoptosis**

Our previous studies demonstrated a crucial role for ARF- and p53-dependent apoptosis in inhibiting lymphoma development in Eμ-myc transgenic mice (Eischen *et al.*, 1999, 2001b). We therefore addressed whether the profound delay in lymphoma development in *Mdm2*^{+/-}Eμ-myc transgenic mice was due to an altered susceptibility of *Mdm2*^{+/-} B cells to Myc-induced apoptosis. We attempted to address this issue by infecting bone marrow-derived primary pre-B cells isolated from *Mdm2*^{+/-} and wild-type littermates with a retrovirus encoding a 4-hydroxytamoxifen (HT)-inducible myc fusion protein (termed Myc-ER^{TAM}, described in the Materials and methods; Littlewood *et al.*, 1995) that we have previously utilized (Zindy *et al.*, 1998; Eischen *et al.*, 1999, 2001b,c). Surprisingly, however, *Mdm2*^{+/-} pre-B cells had very high apoptotic indices (<40% viable at day 19) and failed to grow *ex vivo*, whereas wild-type pre-B cells from littermate controls were viable (>85% at day 19) and readily expanded in culture (Figure 2A). This finding was unexpected, as *Mdm2*^{+/-} mice have been reported to have

normal numbers of B cells and no obvious lymphocyte defects (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995), but clearly *Mdm2*^{+/-} pre-B cells are highly susceptible to apoptosis when cultured *ex vivo*.

Since overexpression of Myc by retroviral infection of *Mdm2*^{+/-} B cells was not feasible, we assessed effects of Myc overexpression in bone marrow from 10- to 12-week-old *Mdm2*^{+/-}Eμ-myc and wild-type Eμ-myc transgenic littermates prior to any detectable disease. The bone marrow cells were cultured in interleukin (IL)-7-containing medium, as described previously (Eischen *et al.*, 1999). Not surprisingly, *Mdm2*^{+/-}Eμ-myc transgenic pre-B cells were unable to grow *ex vivo* (Figure 2B), due to very high rates of spontaneous apoptosis. In contrast, pre-B cells from wild-type Eμ-myc transgenics, after a normal initial lag period (Eischen *et al.*, 1999), proliferated over the same time frame (Figure 2B). Therefore, *Mdm2* haplo-insufficient B cells are prone to undergo apoptosis *ex vivo*, particularly when Myc is overexpressed.

To determine whether *Mdm2* haplo-insufficiency had similar effects when combined with Myc overexpression *in vivo*, we directly measured the apoptotic index in splenocytes of *Mdm2*^{+/-}Eμ-myc transgenics and *Mdm2*^{+/+}Eμ-myc transgenics prior to any detectable disease, and also in *Mdm2*^{+/-} and wild-type mice. Splenocytes from both *Mdm2*^{+/+}Eμ-myc transgenics and *Mdm2*^{+/-} mice had a higher apoptotic index than splenocytes from wild-type controls (Figure 3). Importantly, there was also a greater percentage of apoptotic splenocytes in *Mdm2*^{+/-}Eμ-myc transgenics, compared with the percentage of apoptotic splenocytes in wild-type Eμ-myc transgenics (Figure 3). Therefore, loss of one *Mdm2* allele alone increases the susceptibility of B cells to apoptosis, and this response is augmented in cells overexpressing Myc.

To address potential effects of *Mdm2* haplo-insufficiency on lymphocyte populations, we performed detailed phenotyping of B cells in the spleens of *Mdm2*^{+/-}Eμ-myc transgenics. Normally, mature B cells (IgM⁺, CD19⁺, B220⁺) account for 45–50% of the cells in a mouse spleen, and the B cell numbers from wild-type and *Mdm2*^{+/-} spleens were in accord with this value (Figure 4A). As previously reported (Adams *et al.*, 1985; Langdon *et al.*, 1986), there was a population of B cell precursors IgM⁻/CD19⁺ (8.8%), and slightly reduced numbers (34%) of mature IgM⁺/CD19⁺ B cells, in the spleens of the Eμ-myc transgenics (Figure 4A). In contrast, *Mdm2*^{+/-}Eμ-myc transgenics had profoundly reduced numbers of splenic B cells. Only 5–18% of the cells in the spleens of *Mdm2*^{+/-}Eμ-myc transgenics were IgM⁺ B cells, and these mice completely lacked peripheral IgM⁻/CD19⁺ B cell precursors (Figure 4A). There were also modest increases (36% versus 23%) in the percentage of splenic T cells (CD3⁺) in the *Mdm2*^{+/-}Eμ-myc transgenic mice (Figure 4B). However, this may simply reflect the decreased levels of B cells in the spleens of *Mdm2*^{+/-}Eμ-myc transgenics. Therefore, *Mdm2* haplo-insufficient Eμ-myc transgenic mice have very few splenic B cells, and this may have also contributed to the delayed lymphoma development in these mice.

To determine whether the decreased B cell numbers in the spleens of *Mdm2*^{+/-}Eμ-myc transgenics was also reflected in the numbers of circulating lymphocytes, we

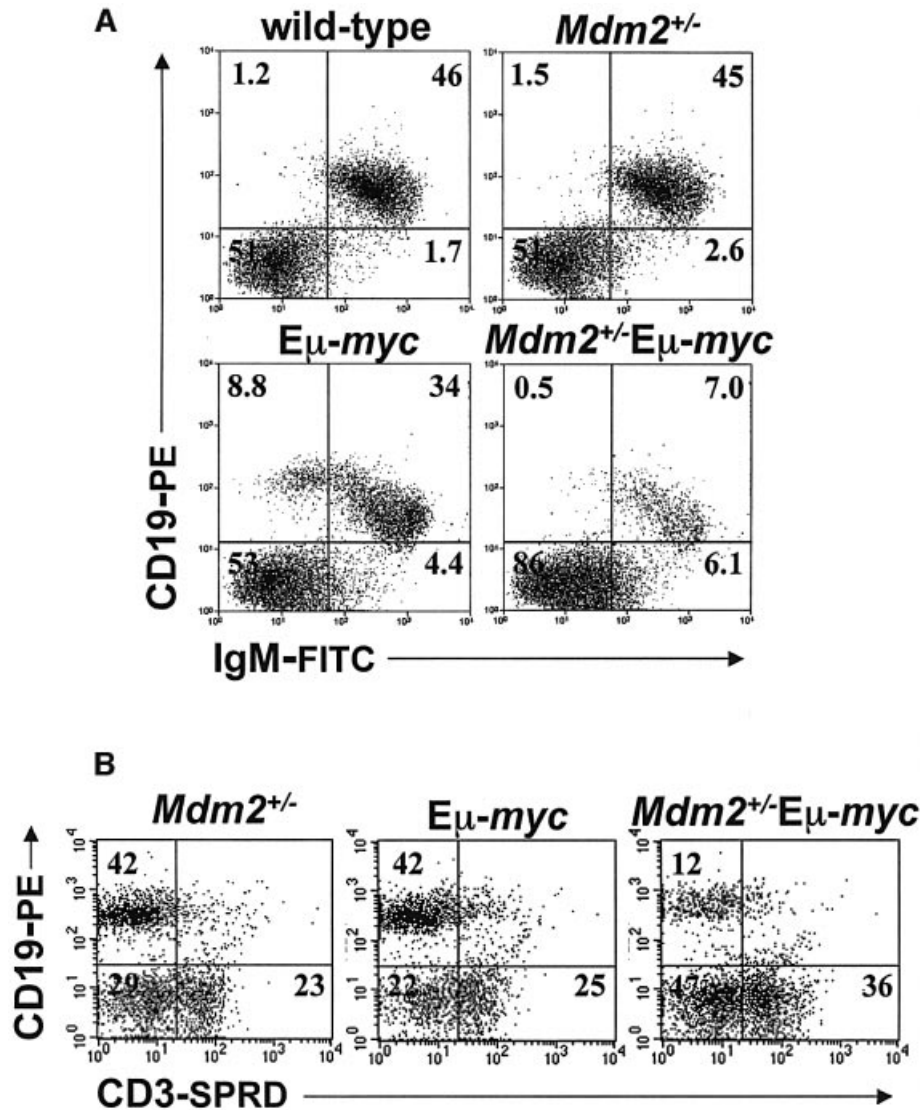


Fig. 4. *Mdm2*^{+/-}*Eμ*-myc transgenics have drastically reduced numbers of B cells. (A and B) Splenic cells from mice of the indicated genotypes prior to any detectable disease were stained with fluorescent antibodies specific for B cells (IgM, CD19) and T cells (CD3) and subjected to flow cytometry phenotype analysis. The location of the quadrant axes was determined from isotype controls, and the numbers in the quadrants are the percentage of cells in each of those quadrants. Data are representative of five separate experiments.

quantitated peripheral lymphocytes from whole blood of mice prior to any detectable disease. Strikingly, *Mdm2*^{+/-}*Eμ*-myc transgenic mice had less than half the number of circulating lymphocytes (2750 lymphocytes/ μ l) compared with wild-type *Eμ*-myc transgenic controls (6170 lymphocytes/ μ l), which were similar to wild-type controls (6800 lymphocytes/ μ l; Figure 5). Notably, the number of peripheral blood lymphocytes in *Mdm2*^{+/-}*Eμ*-myc transgenics was also appreciably lower than the peripheral blood lymphocyte numbers in non-transgenic *Mdm2*^{+/-} mice (5310 lymphocytes/ μ l; Figure 5). Therefore, there is a combined inhibitory effect of Myc overexpression and *Mdm2* haplo-insufficiency on peripheral lymphocyte numbers *in vivo*.

The effects of *Mdm2* haplo-insufficiency are *p53* dependent

Since loss of one allele of *Mdm2* leads to B cell apoptosis *ex vivo* and *in vivo*, and *Mdm2*-null embryos appear to die from constitutive *p53* activation (de Rozières *et al.*, 2000),

we predicted that the propensity of the *Mdm2*^{+/-} B cell precursors to undergo apoptosis, particularly when Myc was overexpressed, was due to dysregulated *p53*. To test this issue, we evaluated pre-B cells from *p53*^{-/-}, *p53*^{-/-}*Mdm2*^{+/-} and *p53*^{-/-}*Mdm2*^{-/-} mice. Both *Mdm2*^{+/-} and *Mdm2*^{-/-} pre-B cells lacking *p53* proliferated *ex vivo* and grew at rates similar to those of *p53*-null pre-B cells (Figure 6A). All pre-B cells lacking *p53*, regardless of their *Mdm2* status, were >95% viable and proliferated more rapidly than wild-type pre-B cells (Figure 6A), as previously reported for primary *p53*-null or *p53*/*Mdm2* double-null cells (McMasters *et al.*, 1996; Eischen *et al.*, 1999). Therefore, the spontaneous apoptosis of the *Mdm2*^{+/-} pre-B cells (Figure 2A) was *p53* dependent.

To determine whether *p53* loss also influenced the sensitivity of *Mdm2*-deficient B cells to Myc-induced apoptosis, we infected pre-B cells lacking *p53* alone or also lacking *Mdm2* with a retrovirus encoding Myc-ER^{TAM} or vector-only virus. As expected (Eischen *et al.*, 1999), the rates of Myc-induced apoptosis following the addition

of 4-HT was markedly impaired in *p53*-null pre-B cells. Importantly, the rates of Myc-induced apoptosis were comparable in *p53*-null and *Mdm2/p53* double-null pre-B cells following the addition of 4-HT (Figure 6B). Thus, *p53* loss blocks the inherently high rates of spontaneous and Myc-induced apoptosis of *Mdm2*-deficient pre-B cells, and *Mdm2* haplo-insufficiency impairs Myc-induced lymphomagenesis by augmenting *p53*-dependent apoptosis.

***p53* is preferentially inactivated in *Mdm2*^{+/-}*Eμ-myc* transgenic lymphomas**

p53 and ARF are inactivated in 28% and 24%, respectively, of lymphomas arising in *Eμ-myc* transgenics (Eischen *et al.*, 1999). If *Mdm2* haplo-insufficiency augments *p53* activation in pre-cancerous transgenic B cells, then one would predict that many of the lymphomas that ultimately arise in *Mdm2*^{+/-}*Eμ-myc* transgenics would harbor mutations in *p53*. To address this issue, we analyzed the *p53*, ARF and *Mdm2* status in the lymphomas that arose in *Mdm2*^{+/-}*Eμ-myc* transgenics. Importantly, half (48%, 11/23) of the *Mdm2*^{+/-}*Eμ-myc* lymphomas had sustained *p53* mutations, which is detected as greatly increased levels of *p53* protein and concomitant elevated ARF protein (Figure 7; Table I). The sites of the *p53* mutations in these lymphomas, as determined by sequence analysis, were within the DNA binding core domain, a hot spot for *p53* mutations (data not shown). *p53* is infrequently deleted in wild-type *Eμ-myc* lymphomas (Eischen *et al.*, 1999), and similarly Southern blot analysis demonstrated that only one lymphoma from an *Mdm2*^{+/-}*Eμ-myc* transgenic had deleted *p53* (Table I). However, *p53* can also be inactivated by *Mdm2* overexpression (Momand *et al.*, 2000), and *Mdm2* protein is overexpressed in half of all lymphomas that arise in wild-type *Eμ-myc* transgenics (Eischen *et al.*, 1999). The percentage of lymphomas overexpressing *Mdm2* protein was even higher in the

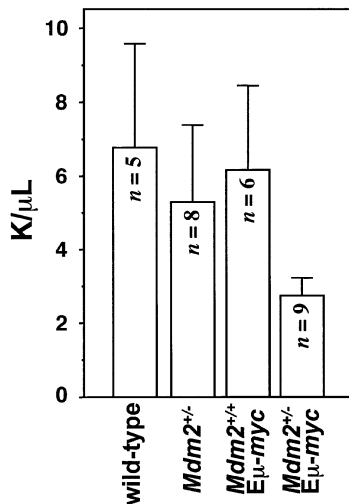


Fig. 5. *Mdm2*^{+/-}*Eμ-myc* transgenics have reduced numbers of circulating lymphocytes. Peripheral blood lymphocytes were counted from retro-orbital eye bleeds from wild-type, *Mdm2*^{+/-}, *Mdm2*^{+/-}*Eμ-myc* transgenic and *Mdm2*^{+/-}*Eμ-myc* transgenic mice prior to any detectable disease. *n*, number of animals that were analyzed from each genotype. Error bars represent 1 SD. K/μl = 1000 cells/μl.

lymphomas that arose in *Mdm2*^{+/-}*Eμ-myc* transgenics (65%, 15/23), and *Mdm2* was also overexpressed in many of the lymphomas that had mutated *p53* (Figure 7; Table I). Therefore, *Mdm2* overexpression is selected for during Myc-induced lymphomagenesis, and this occurs at a higher frequency when *Mdm2* is haplo-insufficient.

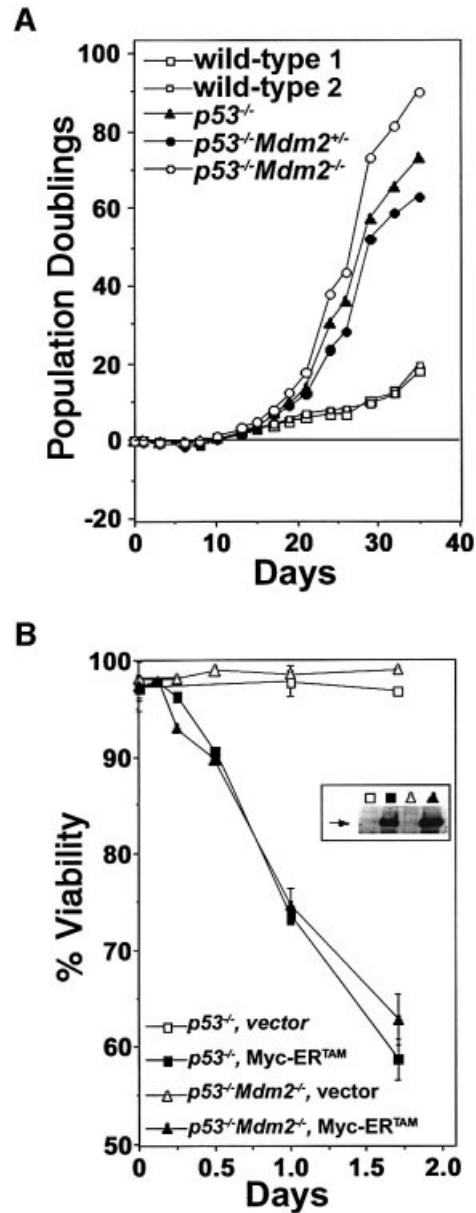


Fig. 6. Loss of *p53* inhibits apoptosis of *Mdm2*^{+/-} pre-B cells. (A) Bone marrow cells from *p53*^{-/-}, *p53*^{-/-}*Mdm2*^{+/-}, *p53*^{-/-}*Mdm2*^{+/-} (prior to any detectable disease) and two wild-type littermates were placed into IL-7 containing medium (day 0) and pre-B cell growth, calculated as net population doublings, was determined at the indicated intervals. (B) 4-HT was added to the indicated primary pre-B cell cultures to activate Myc-ER^{TAM}, and their viability was determined at intervals thereafter by Trypan Blue dye exclusion. Apoptosis was confirmed by analysis of subdiploid DNA content after staining with PI. Steady-state levels of apoptosis in the wild-type primary pre-B cells are indicated at the 0 h time point. Data points are an average of at least three separate experiments, and error bars represent 1 SD. (B, inset) The protein levels of Myc-ER^{TAM} in the indicated pre-B cells was determined by immunoblotting with an antibody specific for Myc. Arrow denotes location of Myc-ER^{TAM}.

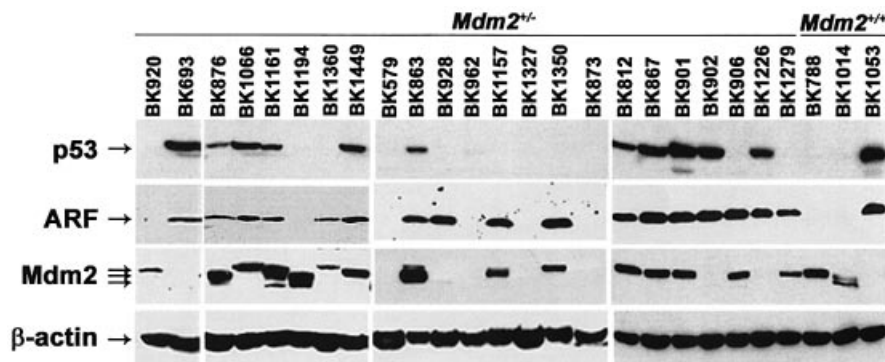


Fig. 7. Analysis of p53, ARF and Mdm2 status in lymphomas arising in *Mdm2*^{+/-}*Eμ-myc* transgenic mice. Western blot analysis: levels of p53 (top panel), p19^{ARF} (second panel), Mdm2 (third panel) and β-actin (bottom panel) protein in whole-cell extracts of lymphomas from the indicated *Eμ-myc* transgenic mice were assessed by immunoblotting with antibodies specific to each protein. The three arrows next to Mdm2 indicate the location of the three isoforms of Mdm2 protein. Lymphomas from *Mdm2*^{+/-}*Eμ-myc* transgenics were run as controls for p53, ARF and/or Mdm2 protein expression.

Table I. p53, ARF and Mdm2 expression in lymphomas from *Mdm2*^{+/-}*Eμ-myc* transgenics

Genetic alteration	Percent of lymphomas ^a
p53 mutation ^b	48 (11)
p53 deletion ^c	4 (1)
ARF deletion ^c	4 (1)
ARF overexpression ^d , p53 wild type	26 (6)
Mdm2 overexpression ^d only	4 (1)
No detectable alteration in Mdm2, p53 or ARF	13 (3)
Mdm2 overexpression ^c , and p53 ^{b,c} or ARF ^c inactivation	60 (14)
Alteration in Mdm2 ^d , p53 ^{b,c} or ARF ^c expression	87 (20)

^aNumbers in parentheses indicate numbers of tumors out of 23 total lymphomas analyzed.

^bMutations were determined by sequencing *p53* cDNA.

^cDeletions were determined by Southern blot.

^dOverexpression was determined by western blot.

Notably, biallelic deletion of *ARF*, as detected by Southern blots, was only observed in one of the *Mdm2*^{+/-}*Eμ-myc* transgenic lymphomas (Table I). However, one-quarter of the lymphomas overexpressed ARF and had reduced levels of wild-type p53 (Figure 7; Table I), suggesting that the regulation of ARF expression and/or the feedback control of p53–Mdm2–ARF pathway was affected in these tumors. Overall, these findings point to a key role for Mdm2 in regulating the rate of Myc-induced tumorigenesis, and bypass of haplo-insufficiency effects selects for events that preferentially disable p53 and/or result in Mdm2 overexpression.

To further test whether the inhibition of lymphomagenesis in *Mdm2*^{+/-}*Eμ-myc* transgenics was due to augmenting the p53 apoptotic response, we also generated *Mdm2*^{+/-}*Eμ-myc* transgenics deficient in *p53*. Lymphoma rapidly developed in *p53*^{+/-}*Mdm2*^{+/-}*Eμ-myc* transgenics, which had an average survival of 5 weeks, a rate similar to that of *p53*^{+/-}*Eμ-myc* transgenics, which had an average survival of 5.6 weeks (Figure 1). The B cell lymphomas that arose in both of these genotypes were typical and arose in the usual peripheral lymph nodes (data not shown). Furthermore, the second allele of *p53* was deleted in

90% (19/21) of the *p53*^{+/-}*Mdm2*^{+/-}*Eμ-myc* transgenic lymphomas analyzed by Southern blot (data not shown), which is characteristic of lymphomas arising in *p53*^{+/-}*Eμ-myc* transgenics (Schmitt *et al.*, 1999). Interestingly, the remaining allele of *p53* was mutated in the other two (2/21) *p53*^{+/-}*Mdm2*^{+/-}*Eμ-myc* transgenic lymphomas analyzed, which has not previously been reported for *p53*^{+/-}*Eμ-myc* transgenic lymphomas. Therefore, p53 was inactivated in 100% (21/21) of the *p53*^{+/-}*Mdm2*^{+/-}*Eμ-myc* transgenic lymphomas. In conclusion, in *Eμ-myc* transgenic mice, the combined haplo-insufficiency of *p53* and *Mdm2* has the same effect as haplo-insufficiency of *p53* alone. *p53*^{-/-}*Eμ-myc* transgenics with or without *Mdm2* could not be generated, presumably due to their death *in utero*, as previously reported (Hsu *et al.*, 1995). Thus, a deficiency in *p53* is dominant and overrides any negative effects on cell growth or survival that *Mdm2* haplo-insufficiency has upon disease progression in *Eμ-myc* transgenic mice.

Discussion

The p53 tumor suppressor pathway is a critical checkpoint that inhibits lymphoma development by triggering apoptosis. This is underscored by the fact that *Eμ-myc* transgenics lacking the tumor suppressor *p53* or *ARF* develop B cell lymphomas at an accelerated rate and that there are inhibitory effects of *p53* or *ARF* loss on Myc-induced apoptosis (Eischen *et al.*, 1999; Schmitt *et al.*, 1999). Furthermore, 80% of the B cell lymphomas that arose in *Eμ-myc* transgenics have alterations in p53, ARF and/or Mdm2 (Eischen *et al.*, 1999). The current study reveals even more complexity on the regulation of the p53 pathway during Myc-induced lymphomagenesis, and extends what was previously known about Mdm2's regulation of p53. Strikingly, the findings presented here establish that one allele of *Mdm2* is insufficient to inhibit p53 activity induced by Myc overexpression *in vivo*. Specifically, the results demonstrate that a certain threshold of Mdm2 is essential for B cell survival, as loss of a single allele of *Mdm2* renders B cells susceptible to p53-dependent apoptosis and inhibits Myc-induced B cell transformation. The profound effects of *Mdm2* haplo-insufficiency on B cell survival and tumor development in

$E\mu$ -myc transgenics therefore establishes the concept that a specific threshold of Mdm2 protein is essential for Mdm2 to harness p53-dependent apoptosis. Thus, it appears likely that Mdm2's enzymatic activity as a ubiquitin ligase is rate limiting and is required for tumor development.

Mdm2 haplo-insufficiency targets the p53 pathway during Myc-induced lymphomagenesis

The profound effects of *Mdm2* haplo-insufficiency on B cell apoptosis and transformation were surprising, as *Mdm2* heterozygous mice have no obvious phenotype and have normal life spans, and their frequency of tumor development is not different than wild-type mice (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Moreover, all cells in *Mdm2*^{+/-} mice should still have one functional *Mdm2* allele, and lymphocyte defects in *Mdm2*^{+/-} mice have not previously been reported (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Nonetheless, *Mdm2* haplo-insufficiency clearly augments the apoptotic program in precursor B cells *ex vivo*, even when Myc was not overexpressed. However, *in vivo* there were only modest increases in the apoptotic index of B cells in *Mdm2*^{+/-} mice and little or no difference in peripheral B cell numbers. These observations suggest that the *in vivo* environment must provide survival signals for *Mdm2*^{+/-} B cells that are lacking in tissue culture. The culture of primary cells is inherently stressful, and consequently the ARF-p53 pathway is activated (Sherr and DePinho, 2000). Therefore, the culture of cells that are more susceptible to p53-mediated apoptosis, such as *Mdm2*^{+/-} pre-B cells, should result in the apoptosis of these cells, and this scenario was indeed evident.

In B cells, Myc triggers at least two apoptotic pathways and both of these are inactivated during Myc-induced lymphomagenesis (Eischen *et al.*, 1999, 2001c). First, Myc provokes p53-dependent apoptosis by inducing the expression of ARF, which disables Mdm2 function and thus augments p53 activity (Wagner *et al.*, 1994; Zindy *et al.*, 1998; Eischen *et al.*, 1999). Secondly, Myc suppresses the expression of the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L, and this occurs even in cells lacking ARF and/or p53 (Eischen *et al.*, 2001a,c). Importantly, 80% of the lymphomas that arise in wild-type $E\mu$ -myc transgenics harbor alterations in ARF, Mdm2 or p53, whereas half of these lymphomas also disable the Bcl-2/Bcl-X_L pathway (Eischen *et al.*, 2001a,c). However, there are links between these two apoptotic pathways. For example, the loss of *bax*, a pro-apoptotic Bcl-2 family member that is induced by p53 (Miyashita and Reed, 1995) and antagonizes the function of Bcl-2 and Bcl-X_L (Korsmeyer 1999), selectively eliminates the need for p53 mutations that usually arise in one-quarter of all lymphomas of $E\mu$ -myc transgenics, without altering the percentage of tumors bearing deletions in ARF (Eischen *et al.*, 2001b). Here we report the opposite scenario occurs in transgenics that are *Mdm2* haplo-insufficient, as lymphomas arising in these mice had an increased frequency of p53 mutations and Mdm2 overexpression, whereas ARF deletions were only rarely observed. Specifically, half of the *Mdm2*^{+/-} $E\mu$ -myc lymphomas had sustained p53 mutations, twice the percentage of p53 mutations in lymphomas that arise in wild-type $E\mu$ -myc transgenics (24%) (Eischen *et al.*, 1999), and 65% (15/23)

of lymphomas overexpressed Mdm2 protein, which is usually only overexpressed in half of the lymphomas of wild-type $E\mu$ -myc transgenics. In contrast, biallelic deletion of ARF, which occurs in one-quarter of $E\mu$ -myc lymphomas, was only detected in one of the lymphomas that emerged in *Mdm2*^{+/-} $E\mu$ -myc transgenics, suggesting that loss of ARF was not able to inhibit p53-mediated apoptosis in *Mdm2*^{+/-} $E\mu$ -myc transgenic B cells. Thus, *Mdm2* haplo-insufficient B cells bypassed apoptosis during Myc-induced lymphomagenesis by selectively provoking mutations that directly or indirectly disable p53.

Mdm2 functions are rate limiting for lymphomagenesis

Mdm2^{+/-} mice do not have an increased frequency of cancer development and T cell lymphoma development in *p53/Mdm2*-double null mice is comparable to those just lacking p53 (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). However, *Mdm2/p53/ARF*-triple null mice do have an altered tumor spectrum when compared with *p53/ARF*-double null mice (Weber *et al.*, 2000), indicating that *Mdm2* loss can indeed affect tumor development. Further, *Mdm2* haplo-insufficiency also influences thymoma development in the context of p53 deficiency, as *Mdm2*^{+/-}*p53*^{-/-} mice develop fewer thymomas and more sarcomas than mice null for both *Mdm2* and p53, or p53 alone (McDonnell *et al.*, 1999). Thus, *Mdm2* haplo-insufficiency, rather than total loss of *Mdm2*, also impairs T cell lymphomagenesis, a scenario akin to what we observe in $E\mu$ -myc transgenics, where there are little effects of *Mdm2* loss when combined with loss of p53 on B cell growth and tumor latency, but there are striking inhibitory effects on B cell lymphomagenesis when *Mdm2* is haplo-insufficient. Moreover, *Mdm2* haplo-insufficiency also somehow alters the sites of lymphoma development in $E\mu$ -myc transgenics. Therefore, reductions in *Mdm2* expression can influence both the site and rate of tumor development, and this is particularly manifest during lymphomagenesis.

Mdm2 protein is overexpressed in half of all B cell lymphomas arising in $E\mu$ -myc transgenic mice (Eischen *et al.*, 1999, 2001b). Furthermore, Hdm2 (the human Mdm2 homolog) expression is elevated in up to half of human non-Hodgkin's lymphomas (Watanabe *et al.*, 1996), and Hdm2 overexpression correlates with disease progression (Pagnano *et al.*, 2001). Collectively, these results suggest that Mdm2/Hdm2 overexpression is selected for during lymphomagenesis. In support of this notion, the percentage of lymphomas overexpressing Mdm2 is even higher (65%) in tumors derived from *Mdm2*^{+/-} $E\mu$ -myc transgenics, where *Mdm2* haplo-insufficiency is such a formidable barrier to tumor development. Mdm2 overexpression in this scenario and others is usually not due to amplification, as *Mdm2* is rarely amplified in leukemia or lymphoma (Moland *et al.*, 1998), and we have never observed *Mdm2* amplification in any of the lymphomas of $E\mu$ -myc transgenic mice (our unpublished data). Therefore, the data strongly support the concept that Mdm2 expression is necessary to block the p53-dependent apoptotic effects of Myc, and Mdm2 overexpression is an important regulator that allows tumor cells that overexpress Myc to survive. However, our findings here and in wild-type $E\mu$ -myc transgenics (Eischen *et al.*, 1999, 2001b) have shown that Mdm2 is

also overexpressed in many of the lymphomas that have mutant p53. This strongly suggests that Mdm2 may also have targets other than p53 that are harnessed during lymphomagenesis, and other important targets of Mdm2 that have been implicated in cancer include E2F-1, DP-1, p300/CBP and/or pRb (reviewed in Momand *et al.*, 2000), TSG101 (Li *et al.*, 2001), Numb (Juven-Gershon *et al.*, 1998), and/or MTBP (Boyd *et al.*, 2000). Therefore, although these studies certainly reveal the need for a proper threshold of Mdm2 protein to inhibit p53-dependent apoptosis, Mdm2 appears to also provide benefits to tumor cells by inactivating other targets that impair tumorigenesis.

Materials and methods

Transgenic and knockout mice

The Eμ-*myc* transgenic mouse strain (congenic C57Bl/6) was kindly provided by Drs Alan Harris (Walter & Eliza Hall Institute, Melbourne, Australia) and Charles Sidman (University of Cincinnati, Cincinnati, OH). The *p53^{+/-}Mdm2^{+/-}* (C57Bl/6 × 129/Sv) (Montes de Oca Luna *et al.*, 1995) mice were generously provided by Dr Guillermina Lozano (M.D. Anderson Cancer Center, Houston, TX). The *p53^{+/-}Mdm2^{+/-}* mice were crossed to the Eμ-*myc* transgenics to generate F₁s. The F₁s were then crossed to generate F₂ *Mdm2^{+/-}Eμ-*myc**, *Mdm2^{+/+}Eμ-*myc**, *p53^{+/-}Mdm2^{+/+}Eμ-*myc** and *p53^{+/-}Mdm2^{+/-}Eμ-*myc** transgenics, and *p53^{+/-}Mdm2^{+/+}*, *p53^{+/-}Mdm2^{+/-}*, *p53^{+/-}Mdm2^{+/-}*, *Mdm2^{+/-}* and wild-type mice. These mice were followed and used for all analyses. A Kaplan–Meier analysis was performed. The Kaplan–Meier plot (Figure 1) includes mice that were still alive at the time the plot was generated, and therefore some of the cases are right-censored because the study was terminated before death occurred. A log-rank test was performed to determine the statistical significance of the survival between the different genotypes of Eμ-*myc* transgenic mice.

Immunohistochemistry

Tissues and tumors from *Mdm2^{+/-}Eμ-*myc** transgenics were fixed in 10% buffered formalin, paraffin embedded and sectioned (5 μm). Immunohistochemistry on sections of spleen and/or tumor was performed, as described previously (Eischen *et al.*, 2002) with an antibody against B220 (PharMingen, San Diego, CA).

Isolation, culture, and infection of primary pre-B cells

Primary pre-B cell cultures were generated from the bone marrow of 9- to 15-week-old wild-type, *Mdm2^{+/-}*, *p53^{+/-}*, *p53^{+/-}Mdm2^{+/-}*, *p53^{+/-}Mdm2^{+/-}*, *Mdm2^{+/+}Eμ-*myc** transgenic and *Mdm2^{+/+}Eμ-*myc** transgenic mice, as described previously (Eischen *et al.*, 1999). Briefly, within 14 days bone marrow cultured in IL-7 containing medium results in >98% pure populations of pre-B cells (CD19⁺, B220⁺, CD43⁻, IgM⁻) as determined by phenotype analysis using fluorescent B-cell-specific antibodies and FACS analysis. All antibodies used for phenotypic analyses were from Southern Biotechnology (Birmingham, AL) or PharMingen. For retroviral infections, MSCV-Myc-ER^{TAM}-IRES-GFP or control MSCV-IRES-GFP retroviruses were generated and used to infect primary pre-B cells as described previously (Eischen *et al.*, 1999). [Myc-ER^{TAM} was previously called Myc-ERTM (Littlewood *et al.*, 1995), but due to the confusion that TM denotes trademark instead of tamoxifen, TM was changed to TAM.] Myc-ER^{TAM} was activated in MSCV-Myc-ER^{TAM}-IRES-GFP-infected pre-B cells by adding 1 μM 4-HT (Sigma, St Louis, MO), which binds to the modified estrogen receptor hormone binding domain (ER) of the Myc-ER^{TAM} fusion protein (Littlewood *et al.*, 1995). This results in the release of Myc-ER^{TAM} from heat shock protein complexes and translocation of Myc-ER^{TAM} into the nucleus, where it activates transcription (Littlewood *et al.*, 1995). Addition of 4-HT to uninfected or GFP control virus infected cells had no effect on pre-B cell growth or viability.

Viability and apoptosis assays

Cell viability following explantation of bone marrow into IL-7 containing medium or after the addition of 1 μM 4-HT to the culture medium of pre-B cells infected with the retrovirus encoding Myc-ER^{TAM} was determined at specific intervals by Trypan Blue dye exclusion. For apoptosis measurements, whole spleens were disaggregated, filtered through a 100 μm nylon filter and then fixed in 70% ethanol prior to propidium

iodide (PI) staining of DNA, whereas pre-B cells were PI stained without ethanol fixation. All PI-stained samples were analyzed on a FACScan and quantitation of fragmented (sub-G₁) DNA was performed with CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Peripheral blood lymphocyte counts

For peripheral blood lymphocyte counts, blood was collected by retro-orbital eye bleeds from wild-type, *Mdm2^{+/-}*, *Mdm2^{+/+}Eμ-*myc** transgenic and *Mdm2^{+/-}Eμ-*myc** transgenic mice into EDTA treated tubes to inhibit coagulation. Each blood sample (20 μl) was analyzed on a Hemavet 3700 (Drew Scientific, Oxford, CT) cell counter.

Phenotype analysis

Whole spleens from wild-type, *Mdm2^{+/-}*, *Mdm2^{+/+}Eμ-*myc** transgenic and *Mdm2^{+/-}Eμ-*myc** transgenic mice were harvested, minced and strained through a 100 μm nylon filter. Approximately 500 000 splenocytes per sample were incubated with one to three fluorescently labeled antibodies (CD19-PE, IgM-FITC, B220-CyChrome, CD3-SRPD and/or fluorescent isotype controls) for 30–45 min at 4°C. Cells were washed with phosphate-buffered saline and immediately analyzed on a MoFlo (Cytomation, Fort Collins, CO) or FACSCalibur (BD Immunocytometry Systems) instrument. CellQuest software was used to analyze data generated on the FACSCalibur.

Western blotting

Primary pre-B cells or pre-B/B cell lymphomas (3–5 mm³ chunk) were lysed as described previously (Zindy *et al.*, 1998). Briefly, cell pellets or small tumor chunks were sonicated (2 × 7 s) in ice cold lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 1 mM phenylmethylsulfonyl fluoride, 0.4 U/ml aprotinin, 1 mM NaF, 10 mM β-glycerophosphate and 0.1 mM sodium orthovanadate). Following sedimentation of undissolved cellular material by centrifugation (4°C, 7 min, 14 000 r.p.m.; Sorvall Biofuge), the protein in the supernatant was quantified with the Bio-Rad Protein Assay Reagent (Hercules, CA). Equal amounts of protein (200 μg per lane) were then separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Dassel, Germany), and blotted with antibodies specific for p19^{ARF} (GeneTex, San Antonio, TX; Quelle *et al.*, 1995), p53 (Ab-7; Calbiochem, La Jolla, CA), Mdm2 (C-18; Santa Cruz, Inc., Santa Cruz, CA), Myc (06-340; Upstate Biotechnology, New York, NY) and β-actin (Sigma). Membranes were incubated with HRP-linked secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) followed by either ECL (Amersham) or Supersignal (Pierce, Rockford, IL) to detect bound immunocomplexes.

Southern blotting

Following isolation from lymphomas emerging in *Mdm2^{+/-}Eμ-*myc** transgenic mice, 15 μg of genomic DNA was digested with *Afl*II or *Bam*HI. Digested DNAs were electrophoretically separated in 0.7–1.0% agarose gels, transferred to Nytran (Schleicher & Schuell) membranes, and then probed with radioactive cDNAs coding *ARF* (exon 1β) (*Afl*II digested) and *p53* (exons 2–10) (*Bam*HI digested).

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