Apaf-1 and caspase-9 do not act as tumor suppressors in myc-induced lymphomagenesis or mouse embryo fibroblast transformation

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Based on experiments with cultured fibroblasts, the apoptosis regulators caspase-9 and Apaf-1 are hypothesized to function as tumor suppressors. To investigate their in vivo role in lymphomagenesis, an IgH enhancer-driven c-myc transgene was crossed onto Apaf-1−/− and caspase-9−/− mice. Due to perinatal lethality, Eμ-myc transgenic Apaf-1−/− or caspase-9−/− fetal liver cells were used to reconstitute lethally irradiated recipient mice.

Surprisingly, no differences were seen in rate, incidence, or severity of lymphoma with loss of Apaf-1 or caspase-9, and Apaf-1 was not a critical determinant of anticancer drug sensitivity of c-myc–induced lymphomas. Moreover, loss of Apaf-1 did not promote oncogene-induced transformation of mouse embryo fibroblasts. Thus, Apaf-1 and caspase-9 do not suppress c-myc–induced lymphomagenesis and embryo fibroblast transformation.

Introduction

Programmed cell death, or apoptosis, is a process which removes redundant, damaged, or infected cells and has a vital role in development, tissue homeostasis, and defense against pathogens (Strasser et al., 2000). Genetic and biochemical studies have uncovered a functional network of cell death regulators (Hengartner, 2000). These regulators include aspartate-specific cysteine proteases (caspases), which dismantle cells by cleaving vital structural proteins, by unleashing latent enzymes that degrade DNA, and by activating engulfment by phagocytes. Studies with knockout and transgenic mice have shown that mammals have two distinct apoptosis signaling pathways (Strasser et al., 2000). One pathway is activated by “death receptors” (a subgroup of the TNF-R family) and requires caspase-8 and its activator FADD. The other is initiated by certain developmental cues and cytotoxic stress, and it is regulated by pro- and antiapoptotic members of the Bcl-2 family. Biochemical studies have shown that in the Bcl-2–regulated pathway release of cytochrome c from mitochondria promotes activation of caspase-9 by its adaptor Apaf-1 (Wang, 2001). Experiments with knockout mice showed that caspase-9 and its activator, Apaf-1, are essential for normal brain development and suggested that these molecules were also required for DNA damage or cytotoxic drug-induced apoptosis of fibroblasts and thymocytes (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998).

Defects in the control of cell death cause an overgrowth of cells that may lead to tumor formation or autoimmune disease (Strasser et al., 2000). For example, overexpression of the antiapoptotic protein Bcl-2 promotes the development of lymphoma in humans and mice, particularly in combination with oncogenic mutations that deregulate cell cycle control, such as enforced c-myc expression (Mufti et al., 1983; Vaux et al., 1988; Strasser et al., 1990). Based on a report that

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Abbreviations used in this paper: C9DN, caspase-9 dominant-negative; MEF, mouse embryo fibroblast; PI, propidium iodide.
mouse embryo fibroblasts (MEFs) lacking Apaf-1 or caspase-9 displayed reduced c-myc–induced cell death and enhanced myc plus mutant ras-mediated transformation, it has been hypothesized that Apaf-1 and caspase-9 function as tumor suppressors (Soengas et al., 1999). However, this experimental system does not study the progress of tumorigenesis as it occurs in vivo. To investigate the role of Apaf-1 and caspase-9 in tumorigenesis in vivo, an immunoglobulin heavy chain gene enhancer-driven (Eμ) c-myc transgene, which provokes the development of B cell lymphomas (Adams et al., 1985; Harris et al., 1988), was introduced onto the Apaf-1−/− and caspase-9−/− backgrounds. Our work showed that Apaf-1 and caspase-9 do not function as tumor suppressors in myc-induced lymphomagenesis and that Apaf-1 is not a critical determinant of the responsiveness of myc-induced lymphomas to chemotherapeutic drugs and γ-irradiation. Furthermore, we were unable to reproduce the findings that loss of Apaf-1 enhances oncogene-induced transformation of MEFs. These results demonstrate that cell death inducers other than Apaf-1 and caspase-9 play critical roles as tumor suppressors in cells overexpressing c-myc.

Results

Loss of Apaf-1 or caspase-9 does not accelerate Eμ-myc transgene-induced lymphomagenesis

An Eμ-myc transgene was introduced onto the Apaf-1−/− and caspase-9−/− backgrounds. Because most Apaf-1−/− or caspase-9−/− mice die around embryonic day 16.5 (E16.5) due to brain malformation (Ceconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998), fetal liver cells from Eμ-myc transgenic Apaf-1−/− or caspase-9−/− E14.5 embryos (n > 10 C57BL/6(Ly5.2) background) were used to reconstitute lethally irradiated C57BL/6(Ly5.1) mice. As controls, we reconstituted recipients with fetal liver cells from E14.5 wild-type, Eμ-myc transgenic, or Eμ-myc/Eμ-bcl-2 double transgenic embryos. Recipient mice were examined for preneoplastic abnormalities and for rate and incidence of lymphoma. Mice were killed when noted to be sick, and lymphomas were studied histologically and by staining with cell surface marker–specific mAbs. Polymorphisms at the Ly5 locus allowed verification that recipients were efficiently reconstituted with donor-derived hemopoietic cells and that lymphomas were derived from donor cells.

Six cohorts of mice were reconstituted and followed: Eμ/myc/Apaf-1+/+, n = 60 mice for 44 wk (mean); Eμ/myc/Apaf-1−/−, n = 51 for 61 wk; Eμ/myc/Apaf-1−/−, n = 39 for 52 wk; Eμ/myc/caspase-9−/−, n = 34 for 44 wk; Eμ/myc/caspase-9−/−, n = 10 for 62 wk; and Eμ/myc/Eμ-bcl-2, n = 22 for 19 wk. Immunofluorescent staining with antibodies to Ly5.1 and Ly5.2 and FACS® analysis demonstrated that for 61 out of 66 recipient mice tested, reconstitution of the hemopoietic system was >80%. Surface immunostaining of tumor cells from Eμ/myc/Apaf-1+/+, Eμ/myc/Apaf-1−/−, and Eμ/myc/caspase-9−/− stem cell–reconstituted mice confirmed that the majority were donor-derived pre–B cell (Ly5.2 B220 B220−sIgM+) or B cell (Ly5.2 B220−sIgM−) lymphomas (Table SI, available at http://www.jcb.org/cgi/content/full/jcb.200310041/DC1). Lymphomas were transplantable into nonirradiated C57BL/6 recipient mice as follows: Eμ-myc/Apaf-1+/+, n = 7/7; Eμ-myc/Apaf-1+/−, n = 6/6; Eμ-myc/Apaf-1−/−, n = 8/8; and Eμ-myel Eμ-bcl-2, n = 3/3. Apaf-1 genotype was confirmed by PCR, and lack of Apaf-1 protein expression was confirmed by Western blotting (Fig. 1, A and B).

No differences were seen with the loss of Apaf-1 in the severity of Eμ-myc lymphoma, as determined by peripheral blood lymphocytosis or spleen size. An increase in leukemic transformation was seen more frequently in mice with Bcl-2 overexpression although the splenomegaly was similar in all genotypes (Fig. 2). The severity of multi-organ involvement by lymphoma was determined by histologic analysis of bone marrow, spleen, lymph nodes, liver, lung, kidney, and heart (scored blinded as to genotype of section), and no increase in severity was observed for either the heterozygote or homozygote deficiency for either Apaf-1 or caspase-9 (unpublished data).
Sick animals were killed and autopsied and, with the exception of a small number of poorly reconstituted mice morbid with anemia or infection, were found to suffer from Eμ–myc lymphoma. The rate of lymphoma onset in mice reconstituted with an Eμ–myc/Eμ–bcl-2 double transgenic system was delayed (50% survival: 57 wk; Fig. 3 A) compared with that observed for unmanipulated C57BL/6 Eμ–myc transgenic mice (50% survival: 14 wk; unpublished data). Despite the increase in tumor latency seen in the reconstituted system, which was also reported by Schmitt et al. (2002), and for which the reason is presently unknown, marked acceleration of lymphomagenesis was seen for mice reconstituted with an Eμ–myc/Eμ–bcl-2 hematopietic system (50% survival: 10 wk, Kaplan-Meier analysis log-rank P = 0.001 for Eμ–myc/Eμ–bcl-2 vs. Eμ–myc; Fig. 3 A). In contrast, loss of one or both alleles of Apaf-1 did not increase the rate or incidence of lymphoma (50% survival: Eμ–myc/Apaf-1+/- 57 wk; Eμ–myc/Apaf-1+/- 53 wk; and Eμ–myc/Apaf-1+/- 59 wk; Kaplan-Meier analysis log-rank P = 0.9 for Eμ–myc/Apaf-1+/- vs. Eμ–myc/Apaf-1+/-; Fig. 3 A). Similarly, loss of one or both alleles of caspase-9 did not enhance lymphomagenesis (50% tumor-free survival: Eμ–myc caspase-9+/- 57 wk; Eμ–myc caspase-9+/- 52 wk; and Eμ–myc caspase-9+/- 54 wk; Kaplan-Meier analysis log-rank P = 0.9 for Eμ–myc/caspase-9+/- vs. Eμ–myc/caspase-9+/-; Fig. 3 B). When all causes of mortality were included in the analysis (overall survival), we also found no increase in, or acceleration of, death with loss of Apaf-1 or caspase-9 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200310041/DC1).

No loss of heterozygosity, determined by PCR as loss of the wild-type allele (Fig. 1), was observed for Apaf-1 in either lymphomas (0/19) or cell lines derived from these tumors (0/6), indicating that loss of Apaf-1 is not selected for, nor is haplo-insufficiency limiting in, c-myc–induced lymphoma development in vivo (Fig. 3) or for growth of lymphoma cell lines in culture (Fig. 4).

Loss of Apaf-1 does not render Eμ–myc lymphomas resistant to anticancer therapy

It has been hypothesized that Apaf-1 and caspase-9 are not only required for tumor suppression but are also essential for γ-radiation– or chemotherapeutic drug–induced apoptosis of tumor cells (Soengas et al., 1999; Jia et al., 2001). Therefore, we generated several independent cell lines from Eμ–myc lymphomas that were either Apaf-1+/- or Apaf-1+/− and exposed them to a range of cytotoxic stimuli. Variability in response to apoptotic stimuli was seen for all genotypes, suggesting that the immortalizing process in vitro selects for a range of death-response phenotypes; the range of variation was similar between lymphomas that were Apaf-1+/-, Apaf-1+/-, or Apaf-1−/−. Compared with Eμ–myc/Apaf-1+/- lymphoma-derived cell lines, a small reduction in sensitivity to etoposide and γ-radiation was observed for Eμ–myc/Apaf-1−/− lymphoma lines at 24 h, however, by 72 h no difference in cell survival was apparent with loss of Apaf-1 (Fig. 4). As previously shown for nontransformed Apaf-1−/− and caspase-9−/− lymphocytes (Marsden et al., 2002), the dying Eμ–myc/Apaf-1−/− lymphoma cells exhibited classical features of apoptosis, such as early surface exposure of phosphatidylserine (detected by staining with Annexin V; unpublished data). These results indicate that Apaf-1 is not
Loss of Apaf-1 does not cause an increase in Eμ-μc transgene-induced B lymphoid cellularity

We also examined whether loss of Apaf-1 had an effect on the preneoplastic B cell phenotype induced by Eμ-μc transgene expression. This was done by measuring total leukocyte numbers and numbers of B lymphocytes in blood, bone marrow, spleen, and lymph nodes in mice reconstituted with Eμ-μc/Apaf-1+/+ or Eμ-μc/Apaf-1−/− fetal liver cells 12 wk after transplantation (range = 5–27, and median and mean = 12 wk), an earlier time than most reconstituted mice succumbed to lymphoma (range = 5–77, median = 27, and mean = 31 wk). Absence of significant numbers of tumor cells in all mice used for these analyses was proven by showing that transplanting 10^6 spleen cells into nonirradiated histocompatible mice did not cause lymphoma (unpublished data). No differences in pre–B cell (B220-sIgM−) or B cell (B220-sIgM+) numbers were found between mice reconstituted with Eμ-μc/Apaf-1+/+ or Eμ-μc/Apaf-1−/− stem cells (Fig. 5). As previously reported (Langdon et al., 1986), Eμ-μc expression caused a reduction in numbers of mature B cells in bone marrow compared with wild-type mice; this is thought to be a consequence of the proapoptotic and differentiation inhibiting effects of μc in B lymphocytes.

Loss of Apaf-1 does not inhibit μc-enhanced apoptosis in B lymphoid cells

Deregulated μc expression increases susceptibility of lymphocytes and many other cell types to a broad range of apoptotic stimuli (Pelengaris et al., 2002). Therefore, we FACS®-sorted preneoplastic Eμ-μc/Apaf-1+/+ and Eμ-μc/Apaf-1−/− pre–B cells and B cells from reconstituted animals and investigated whether loss of Apaf-1 rendered them resistant to cytokine withdrawal, dexamethasone, etoposide, or γ-irradiation in culture. A modest (<1.5-fold) reduction in sensitivity to these stress stimuli was observed for pre–B cells at 6, 24, and 48 h (P < 0.05 at 24 h, t test); however, by 72 h, no consistent difference in cell death was apparent with loss of Apaf-1 (Fig. 6, A–D). These conclusions were based on short-term survival assays. A more stringent measurement of cell survival is to determine whether cells retain colony forming potential. As pro/pre–B
Loss of Apaf-1 does not enhance oncogene-induced transformation of MEFs

As aforementioned, the hypothesis that Apaf-1 and caspase-9 function as tumor suppressor genes was based on the observation that the loss of these proteins increased colony formation of MEFs infected with viruses encoding the oncogenes myc plus or minus mutant ras (Soengas et al., 1999). We performed similar experiments with the adenoviral oncogene E1A or c-myc, plus or minus mutant ras, but found no increase in oncogene-induced colony formation of early passage MEFs with loss of Apaf-1 (Fig. 7). In short-term assays, MEFs lacking Apaf-1, Bax, or p53 or expressing Bcl-xL were resistant to apoptosis induction in response to serum withdrawal or UV-C (Fig. 7 A), and similar results were obtained with etoposide, actinomycin D, staurosporine, doxorubicin, cisplatin, and taxol (not depicted). However, in longer-term studies requiring cellular transformation, loss of Apaf-1 did not result in a growth advantage (Fig. 7 B). In contrast, a large increase in colony formation was observed when MEFs from p53 or Bax-deficient mice were infected with E1A plus mutant ras- or c-myc plus mutant ras-containing viruses, but not when Apaf-1−/− MEFs were used (Fig. 7 B). All MEFs grew at comparable rates under standard cell culture conditions (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200310041/DC1), and the oncogenic proteins were expressed at similar levels in each of the MEFs infected with oncogene-containing retroviruses (Fig. S2 B). Comparable results were obtained in three independent experiments performed with separately derived sets of MEFs of each genotype. In addition, the inability of the Apaf-1 deficiency to enhance transformation in focus assays was observed in MEFs from two independently generated knockout mouse lines (Cecconi et al., 1998; Yoshida et al., 1998; unpublished data). The ability of p53 and Bax to suppress cell transformation in this system is consistent with observations that induction of apoptosis is critical for p53’s tumor suppressive effects (Schmitt et al., 2002) and that such apoptosis can be activated via Bax (McCurrach et al., 1997; Zhang et al., 2000). Therefore, under the conditions we used, a block to apoptosis upstream of the mitochondria can promote transformation, which is not seen when the defect is at the level of Apaf-1.

Discussion

Based on experiments with MEFs engineered to express the oncogene c-myc with or without oncogenic ras, it was proposed that Apaf-1 and caspase-9 function as critical tumor suppressors (Soengas et al., 1999). We performed the first examination of the role of Apaf-1 and caspase-9 in tumorigenesis in whole animal experiments and found that these proteins are not critical for suppressing myc-induced lymphomagenesis. Moreover, we found that Apaf-1 is not essential for myc-enhanced apoptosis and responsiveness of myc-induced lymphomas to anticancer therapy. Finally, we have been unable to reproduce the results that indicated a role for Apaf-1 in suppressing oncogene-induced transformation in MEFs.

One possible explanation for the differences between our results and those published previously (Soengas et al., 1999) could be that we used freshly isolated Apaf-1−/− MEFs (passage 2 or 3) and a rapid selection procedure for infected cells using cotransduction techniques rather than sequential selection, which minimizes the risk of up-regulating growth-promoting genes. In contrast, the more protracted transduction and sequential selection procedure used in the previous study (Soengas et al., 1999) may have resulted in significant opportunities for up-regulation of or, less likely, mutation of growth-promoting genes in the MEFs tested. The accumulation of growth-promoting changes may in some way be en-
hanced on an Apaf-1<sup>-/-</sup>-background, implying an indirect growth-promoting effect of loss of function of this gene, which may have altered the composition of the MEFs used in the Soengas et al. (1999) work.

It is possible that constant loss of a molecule (e.g., by germ-line deletion) may allow developmental compensation to occur. In this case, different phenotypes may result from constitutive loss compared with acute loss of the same molecule, although this should not account for differences between this paper and the Soengas et al. (1999) paper because the MEFs used in both sets of experiments originated from mice with germ-line deletion of Apaf-1. Although compensation by proteins with similar function is a theoretical caveat on this paper, we have not yet found evidence for up-regulation of potentially compensatory molecules for Apaf-1 and caspase-9. This is in spite of an extensive analysis being performed in lymphoid cells from wild-type, Apaf-1<sup>-/-</sup>, and caspase-9<sup>-/-</sup> mice, the cell type relevant for this model of tumorigenesis, comparing expression levels and activation of caspase-2, -3, -6, -7, -8, or -9 after exposure to a range of death stimuli (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200310041/DC1; and see Fig. 4 A in Marsden et al., 2002). In contrast, compensatory caspase activation has been reported in an analysis of caspase-9<sup>-/-</sup>-hepatocytes (Zheng et al., 2000); cell type-specific differences in the regulation of caspase expression and/or activation may account for the differences observed in the two works.

The E<sub>K</sub>-myc lymphoma model has been used to demonstrate the tumor suppressor activity of p53 (Hsu et al., 1995), Arf (Eischen et al., 1999; Schmitt et al., 1999), and proapoptotic BH3-only Bcl-2 family member Bim (O’Connor et al., 1998; unpublished data), including the effect of haplo-insufficiency of these molecules. Thus, for at least two bona fide tumor suppressor genes involved in abrogation of apoptosis in E<sub>K</sub>-myc lymphoma via the Bcl-2-regulated apoptotic pathway, the use of this model has been firmly established.

There have been no previous studies describing the effects of loss of Apaf-1 or caspase-9 on E<sub>K</sub>-myc-induced lymphomagenesis or lymphomagenesis in general. Retroviral transduction of a caspase-9 dominant-negative (C9DN) construct into E<sub>K</sub>-myc/p53<sup>3/3</sup>-stem cells was used to investigate the role of caspase-9 in lymphomagenesis (Schmitt et al., 2002). Using as the endpoints of analysis selection for cells expressing the C9DN or retention of the wild-type p53 allele, an effect of C9DN was observed; however, this was not sufficient to accelerate lymphomagenesis, the more stringent and meaningful parameter for analysis. Indeed, this dominant-negative allele of caspase-9 is suspected to have targets in addition to caspase-9 because it was shown to block apoptotic pathways, such as Fas signaling (Srinivasula et al., 1998), that are intact in caspase-9-deficient cells (Hakem et al., 1998; Kuida et al., 1998). Therefore, it is possible that the effects of C9DN on E<sub>K</sub>-myc-induced lymphomagenesis on a p53<sup>3/3</sup>-background may have resulted from factors other than blocking caspase-9 function.

The other evidence for a tumor suppressor role reported to date for Apaf-1 was in human melanoma (Soengas et al., 2001). A collection of early and metastatic melanomas and melanoma cell lines in which a low rate of p53 mutation was observed was analyzed, and, in a significant proportion, Apaf-1 was found to be silenced by gene methylation. Apaf-1 deficiency correlated with poor response to chemotherapy and its reexpression increased sensitivity to cytotoxic drugs. However, these data are correlutive and no studies have been reported that demonstrated that Apaf-1 loss could accelerate tumorigenesis in a mouse model of melanoma development. Moreover, in neuroblastoma, a tumor examined specifically because of its similarity with melanoma, as both arise from neural crest progenitor cells and some show N-myel gene amplification, Apaf-1 and caspase 9 were found to be present and active in all specimens examined (Teitz et al., 2002). Thus, although it is possible that Apaf-1 has a cell type–restricted role in tumor suppression, to date, there is no proof for this.

In conclusion, our studies investigated the possible roles of Apaf-1 and caspase-9 as tumor suppressors in an in vivo model relevant for tumorigenesis. We found no evidence to support a proposed role for either Apaf-1 or caspase-9 as critical tumor suppressors in c-myc–induced lymphomagenesis in the mouse, nor did we find evidence that Apaf-1 is a critical determinant of anticancer drug sensitivity of c-myc–induced lymphomas. The reason why Bcl-2 synergizes potentially with c-myc in tumorigenesis (Strasser et al., 1990) and blocks myc-enhanced apoptosis (Bissonnette et al., 1992; Fanidi et al., 1992; Strasser et al., 1996) but loss of Apaf-1 or caspase-9 do not, is most likely due to the fact that Bcl-2 inhibits not only Apaf-1–mediated caspase-9 activation but also other apoptotic processes. Our previous work has indicated that the Bcl-2 protein family regulates activation of caspases that initiate apoptosis by acting upstream of mitochondrial membrane disruption (Marsden et al., 2002). However, there is also evidence for caspase-independent processes regulated by Bcl-2 that might play a role in apoptosis induced by deregulated protooncogene expression (McCarthy et al., 1997), including preservation of mitochondrial membrane integrity and membrane potential (Green and Reed, 1998). Indeed, Bcl-2 has been shown to block apoptosis in cells lacking Apaf-1 (Haraguchi et al., 2000).

Thus, the apoptotic regulators, Apaf-1 and caspase-9, do not have an essential suppressive role in myc-induced lymphomagenesis, nor does Apaf-1 have an essential role in MEFs transformation as previously ascribed. Other regulators of myc-induced apoptosis acting as tumor suppressors need to be identified and mechanisms of cell specificity determined.

### Materials and methods

#### Mice

Apaf-1<sup>-/-</sup>-myc transgene was introduced onto the Apaf-1<sup>-/-</sup> (Cocconi et al., 1998) provided by P. Gruss and F. Cocconi, Max Plank Institute, Goettingen, Germany) and caspase-9<sup>-/-</sup>-background (Kuida et al., 1998; provided by K. Kuida, Vertex, Inc., Cambridge, MA). Fetal liver cells from E<sub>K</sub>-myc transgenic Apaf-1<sup>+/+</sup> or Apaf-1<sup>-/-</sup> or E<sub>K</sub>-myc transgenic caspase-9<sup>+/+</sup> or caspase-9<sup>-/-</sup> E14.5 embryos were used to reconstitute lethally irradiated C57BL/6Ly5.1 mice. As controls, recipients were reconstituted with fetal liver cells from E14.5 wild-type, E<sub>K</sub>-myc transgenic, or E<sub>K</sub>-myc/E<sub>K</sub>-myc double transgenic (Strasser et al., 1990) embryos (provided by J. Adams, S. Cory, and A. Harris, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). All mouse strains used as donors were on an inbred C57BL/6Ly5.2 genetic background or had been backcrossed for >10 generations. As a further control, we also reconstituted mice with wild-type, Apaf-1<sup>-/-</sup>, and caspase-9<sup>-/-</sup> E14.5 fetal liver cells, and the majority of these recipients remained healthy for at least 70 wk, with the exception of two animals that developed radiation-induced T cell lymphomas (in comparison, two animals expressing a myc transgene also developed radiation-induced T cell lymphomas).
3-week-old mice and E14.5 embryos were genotyped by PCR analysis of DNA extracted from tails or heads as previously described (Marsden et al., 2002). For timed pregnancies, the morning when vaginal plugs were detected was taken as day 0. Day 14.5 pregnant mice were killed by cervical dislocation, embryos were removed, and fetal livers were dissected under a microscope. Fetal liver cell suspensions were prepared in balanced salt solution (BSS) containing 0.5% FCS. For lymphocytes, 10^6 cells were injected into lethally irradiated (2 × 5.5 Gy; 3-h interval) C57BL/6-Ly5.1 mice. To prevent infections, the transplanted animals were initially provided with water containing neomycin (Sigma-Aldrich).

**Lymphoma analysis**

Rate and incidence of lymphoma in cohorts of recipient mice were compared by log-rank test and Kaplan-Meier analysis using the StatView® software. The cause of death was attributed to E1A transgene plating at limiting dilution. The bone marrow by immunofluorescent staining and FACS® sorting (Cy5-Ra3-682, anti-CD45R-B220; Cy5-1D9, anti-CD19; biotin-ACK4, anti-c-kit plus R-PE-streptavidin and FITC-labeled M170, anti-Mac-1; and RB6-8C5, anti-Gr-1 and TER119) and cultured for 7–10 d on an ST-2 feeder cell layer in simple medium containing 100 U/ml IL-7. For induction of apoptosis, cells were exposed for 6 h to graded doses of etoposide (VP-16) or actinomycin D (10–2 × 10^3 C cells) for 10 min. Then, cell cycle analysis was performed by flow cytometry.

**MEF culture and transformation**

All MEFs (provided by T. Mak; University of Toronto, Toronto, Canada) were maintained in full DM and were used between passage 2 and 3. For long-term proliferation assays, 5,000 MEFs were resuspended in full DM (normal glucose) supplemented with 100 μg/ml hygromycin B plus 0.5 μg/ml puromycin and plated per 35-mm dish. Retroviral vectors were generated by transient cotransfection (calciu/m sulfate precipitation technique) of the respective vector plasmid and pJCl-Eco (provided by I.M. Verma, The Salk Institute, La Jolla, CA) in 293T cells. Virus-containing supernatants were collected 48 and 72 h after transfection, clarified by filtration, and frozen until further use. Transductions were conducted in the presence of 5 μg/ml polybrene (Sigma-Aldrich). The vector plasmids pBabePuro.H-ras (G12V), pLPC.E1A, pWZL.H1E1A, pBabePuro.myc, and pWZL.H.myc were provided by S.W. Lowe and G. Hannon (Cold Spring Harbor Laboratory). The vector plasmids pBabehygro.H-ras and pBabePuro.bcl-xl were generated by subcloning the respective cDNA into pBabe. All inserts were verified by sequencing. The fraction of apoptotic cells was determined by flow cytometry after staining with FITC-conjugated Annexin V (Calbiochem) and PI. Whole cell extracts were generated by resuspending cells in lysis buffer (50 mM Hepes, 250 mM NaCl, 5 mM EDTA, and 0.1% NP-40) on ice, followed by centrifugation. After transfer into nitrocellulose membranes (Amersham Biosciences), primary antibody binding was detected by ECL (Pierce Chemical Co.). Primary antibodies were used mouse anti-p53 (pAb 122, pAb 240; BD Biosciences), anti-E1A (Oncogene Research Products), anti-H-ras (Oncogene Research Products), anti-Myc (Santa Cruz Biotechnology, Inc.), and antiactin (clone C4; ICN).

**Online supplemental material**

Analysis of (Fig. S1 A) mice reconstituted with Eµ-myc/Apaf-1<sup>-/-</sup>, Eµ-myc/Apaf-1<sup>-/-</sup>-Ex-E <small>µ</small>-myc/Eµ-bcl-2<sup>-/-</sup>, or (Fig. S1 B) Eµ-myc/caspase-9<sup>-/-</sup>, Eµ-myc/caspase-9<sup>-/-</sup>-Ex-E <small>µ</small>-myc/Eµ-bcl-2<sup>-/-</sup> stem cells counting all deaths (rather than only the deaths due to myc lymphoma; Fig. S3, A and B) demonstrates that loss of Apaf-1 or caspase-9 does not accelerate mortality caused by Eµ-myc transgenes. MEFs that are either wild type, p53<sup>-/-</sup>, bax<sup>+/−</sup> (provided by S. Korsmeyer, Dana Farber Laboratories, Boston, MA), or Apaf-1<sup>-/-</sup> grew at similar rates in culture (Fig. S2 A). After infection with oncogene-containing retroviruses, MEFs that were wild type, p53<sup>-/-</sup>, bax<sup>+/−</sup>, or Apaf-1<sup>-/-</sup> all expressed similar levels of E1A plus mutant ras or myc plus mutant ras proteins (Fig. S2 B).

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