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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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Key words: apoptosis; cancer; Bcl-2; Apaf-1; caspase

Abbreviations used in this paper: C9DN, caspase-9 dominant-negative; MEF, mouse embryo fibroblast; PI, propidium iodide.

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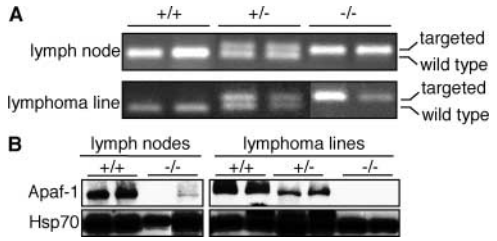

Figure 1. **Genotyping of lymphomas and lymphoma-derived cell lines.** (A) Apaf-1 allele-specific PCR and (B) Western blot analysis of lymphomas and lymphoma cell lines. A small amount of Apaf-1 protein was detected in some *Eμ-myc*/Apaf-1$^{-/-}$ lymphoma samples, most likely due to contaminating recipient-derived stromal tissue, as no Apaf-1 protein was detected in the cell lines derived from these lymphomas.

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 (Cecconi 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.

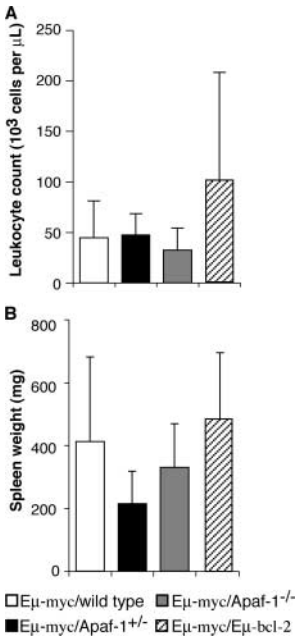

Figure 2. **Hematologic parameters of mice with lymphoma.** (A) Total peripheral blood white cell count from mice with lymphoma at time of sacrifice. (B) Spleen weight from mice with lymphoma. Data represent arithmetic means ± SD of 3–12 mice of each genotype.

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$^+$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$^{+/-}$, $n = 4/4$; *Eμ-myc* /Apaf-1$^{-/-}$, $n = 8/8$; and *Eμ-myc* / *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).

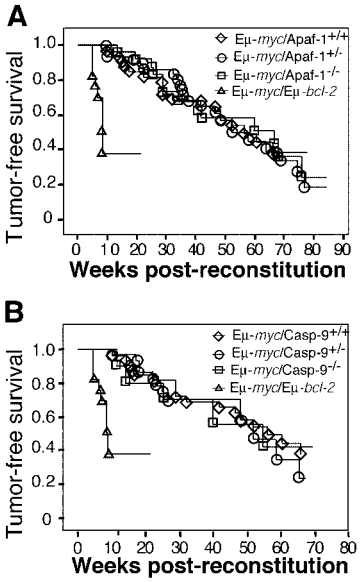

Figure 3. ***Eμ-myc* lymphoma onset is not affected by loss of Apaf-1 or caspase-9.** Fetal liver cells from *Eμ-myc* transgenic Apaf-$1^{+/-}$ or Apaf-$1^{-/-}$ or *Eμ-myc* transgenic caspase-$9^{+/-}$ or caspase-$9^{-/-}$ E14.5 embryos were used to reconstitute lethally irradiated mice. As controls, recipients were reconstituted with fetal liver cells from E14.5 wild-type, *Eμ-myc* transgenic, or *Eμ-myc*/*Eμ-bcl*-2 double transgenic embryos. Mice were killed when noted to be sick and were found to suffer from *Eμ-myc* lymphoma, with the exception of a small number of poorly reconstituted mice morbid with anemia or infection. (A and B) Kaplan-Meier analysis of tumor-free survival (weeks).

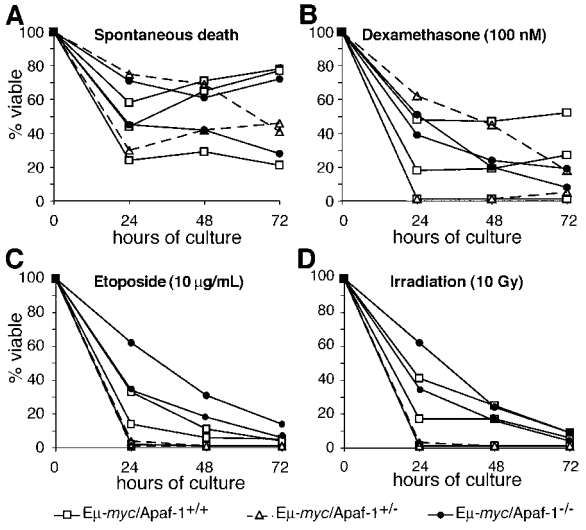

Figure 4. **Sensitivity of lymphoma-derived cell lines to apoptotic stimuli.** Stable cell lines were derived from fresh lymphomas and sorted by flow cytometry using a modified FACSII® or FACStar. For cell death analysis, viable cells (negative for PI) were cultured in DME at a concentration of $0.2–0.5 \times 10^6$ cells/ml in 96-well flat bottom microtiter plates. Medium alone (A), 100 nM dexamethasone (B), 10 μg/ml etoposide (C,) and 10 Gy γ-irradiation (D). *Eμ-myc* (open square), *Eμ-myc*/Apaf-$1^{+/-}$ (triangle, hashed line), *Eμ-myc*/Apaf-$1^{-/-}$ (closed circle). Two to three independently derived lines per genotype.

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* /Apaf-$1^{+/+}$ hemopoietic 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 a *Eμ-myc*/*Eμ-bcl*-2 hemopoietic 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

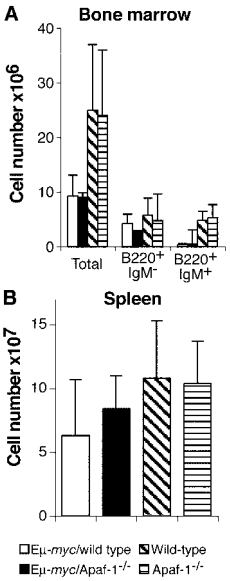

Figure 5. **Hematologic parameters of preneoplastic mice.** (A) Bone marrow cellularity (per 2 femurs) and B cell and pre–B cell counts. (B) Spleen cellularity. Data represent the arithmetic means ± SD from four to eight mice of each genotype.

limiting for cancer therapy–induced apoptosis and killing of *Eμ-myc* lymphomas.

Loss of Apaf-1 does not cause an increase in *Eμ-myc* 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-myc* 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-myc*/Apaf-$1^{+/+}$ or *Eμ-myc*/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-myc*/Apaf-$1^{+/+}$ or *Eμ-myc*/Apaf-$1^{-/-}$ stem cells (Fig. 5). As previously reported (Langdon et al., 1986), *Eμ-myc* 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 *myc* in B lymphocytes.

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

Deregulated *c-myc* 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-myc*/Apaf-$1^{+/+}$ and *Eμ-myc*/Apaf-$1^{-/-}$ pre–B cells and B cells from reconstituted

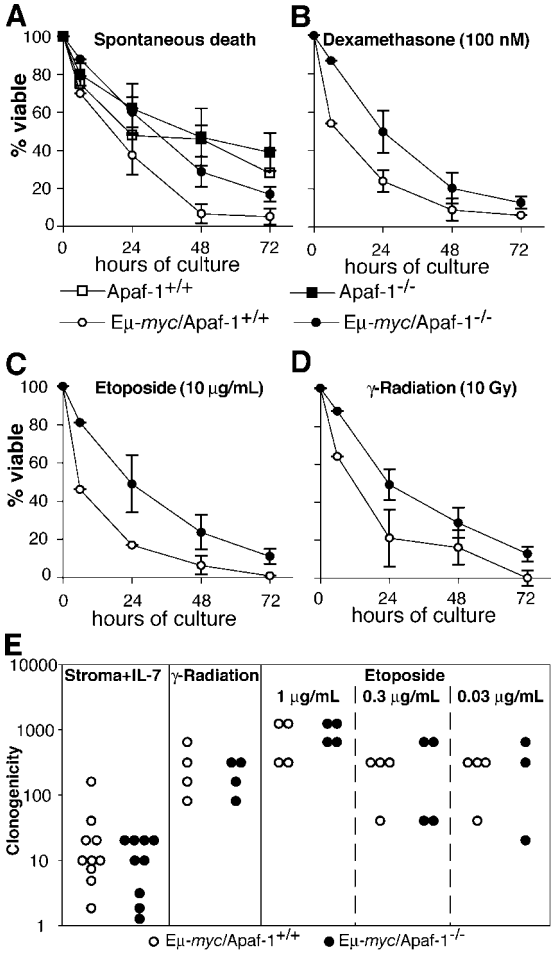

Figure 6. **Sensitivity of preneoplastic B lymphoid cells to apoptotic stimuli.** Pre–B cells ($B220^+sIg^-$) and B cells ($B220^+sIg^+$) were purified from bone marrow and lymph nodes, respectively, of recipient mice showing no signs of lymphoma by immunofluorescent staining and sorting on a FACStar, DIVA, or MoFlo. (A–D) Sensitivity of preneoplastic B cells to the following apoptotic stimuli: culture in the absence of cytokines (A), 100 nM dexamethasone (B), 10 μg/ml etoposide (VP16; C), and 10 Gy γ-irradiation (D). *Eμ-myc* (open circle), *Eμ-myc*/Apaf-$1^{-/-}$ (closed circle), Apaf-$1^{+/+}$ (open square), and Apaf-$1^{-/-}$ (closed square). Cells were cultured and viability was determined over 6–72 h as described in Fig. 4 legend. (E) The colony-forming potential of pro/pre–B cells from the bone marrow of C57BL/6-Ly5.1 mice reconstituted with *Eμ-myc* or *Eμ-myc*/Apaf-$1^{-/-}$ fetal liver stem cells in limiting dilution after no treatment or after exposure to 2.5 Gy γ-radiation or 1.0, 0.3, 0.03 μg/ml etoposide (VP16). B cell colonies consisting of at least 20 cells were scored blinded as to genotype using an inverted microscope at day 10, and the cloning frequency was determined using limiting dilution analysis. Clonogenicity is less than or equal to the value shown. Data represent arithmetic means ± SD of 3–10 mice of each genotype.

animals and investigated whether loss of Apaf-1 rendered them resistant to cytokine withdrawal, dexamethasone, etoposide, or γ-radiation 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

cells can be grown as colonies on stromal cells in the presence of IL-7 (Rolink et al., 1991), we investigated whether loss of Apaf-1 had an effect on clonogenic survival of *Eμ-myc* pro/pre–B cells. *Eμ-myc*/Apaf-$1^{+/+}$ pro/pre–B cells formed colonies at a frequency of 1/14 ± 12 (Fig. 6 E) and loss of Apaf-1 did not alter this (*Eμ-myc*/Apaf-$1^{-/-}$ 1/12 ± 8; P = 0.5). When colony forming cells were exposed to γ-radiation (2.5 Gy) or a 6-h treatment with etoposide (0.3–1.0 μg/ml), the clonal frequency decreased by 10–80-fold, and loss of Apaf-1 did not afford any protection (P = 0.6; Fig. 6 E). Thus, the protection against apoptosis seen at early time points (24–48 h) in short-term survival assays could be compensated for in the longer term by other apoptotic regulators. Collectively, these results demonstrate that Apaf-1 plays no, or only a minor, role in cell death that is enhanced by deregulated *c-myc* expression.

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-x_L 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, doxorubicine, 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.

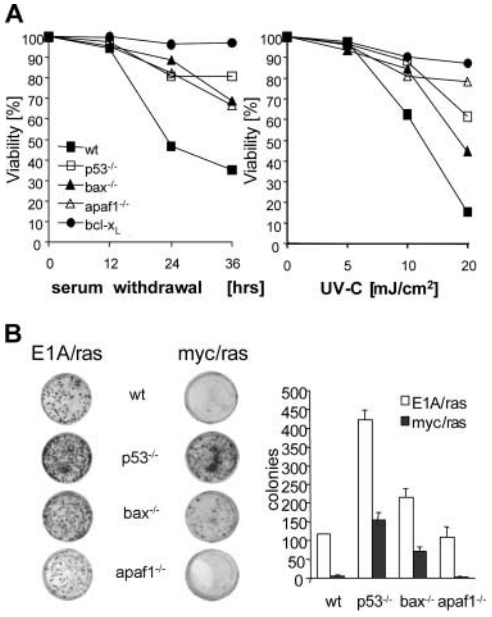

Figure 7. **Loss of Apaf-1 protects MEFs against rapid p53-dependent apoptosis, but not against p53-dependent suppression of long-term proliferative survival.** (A) Early passage MEFs of the indicated genotypes were retrovirally transduced to express E1A plus mutant *ras* or E1A, mutant *ras,* and Bcl-x_L. Apoptotic cell death was assessed by staining with FITC-coupled Annexin V plus PI and flow cytometry at the indicated time points after serum withdrawal or 24 h after UV radiation, and the mean fractions of Annexin V^-/PI^- viable cells are given. (B) Early passage MEFs of the indicated genotypes were retrovirally transduced to express the indicated combinations of oncogenes. 2 d after transduction, the cells were seeded in triplicate at low density in selection medium. After 7 d culture in selection medium, cells were fixed, stained, and, the resulting colonies, enumerated. Mean value ± SD of three independent experiments.

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-$1^{-/-}$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 germline 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 germline 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-$1^{-/-}$, and caspase-$9^{-/-}$ 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-$9^{-/-}$ 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 *Eμ–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 *Eμ-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 *Eμ-myc*–induced lymphomagenesis or lymphomagenesis in general. Retroviral transduction of a caspase-9 dominant-negative (C9DN) construct into *Eμ-myc/p53*$^{+/-}$ 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 *Eμ-myc*–induced lymphomagenesis on a p53$^{+/-}$ 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 correlative 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-*myc* 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 potently 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

An *Eμ-myc* transgene was introduced onto the Apaf-$1^{-/-}$ (Cecconi et al., 1998; provided by P. Gruss and F. Cecconi, Max Planck Institute, Goettingen, Germany) and caspase-$9^{-/-}$ background (Kuida et al., 1998; provided by K. Kuida, Vertex, Inc., Cambridge, MA). Fetal liver cells from *Eμ-myc* transgenic Apaf-$1^{+/-}$ or Apaf-$1^{-/-}$ or *Eμ-myc* transgenic caspase-$9^{+/-}$ or caspase-$9^{-/-}$ E14.5 embryos were used to reconstitute lethally irradiated C57BL/6(Ly5.1) mice. As controls, recipients were reconstituted with fetal liver cells from E14.5 wild-type, *Eμ-myc* transgenic, or *Eμ-myc*/*Eμ-bcl-2* 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/6(Ly5.2) genetic background or had been backcrossed for >10 generations. As a further control, we also reconstituted mice with wild-type, Apaf-$1^{-/-}$, and caspase-$9^{-/-}$ 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-wk-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 containing 10% BSS-FCS. For injection, 2×10^6 cells in BSS were injected into lethally irradiated (2×5.5Gy, 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. Cause of death was attributed to *Eμ-myc* lymphoma if a combination of the following suggestive features were noted: presence of enlarged spleen and/or lymph nodes, histologic evidence of invasive lymphoma, lymphocyte count $>20 \times 10^6$/ml, FACS® profile consistent with $Ly5.2^+$ donor-derived lymphoma, and growth of lymphoma cells in nonirradiated transplant recipients. For a small number of mice the cause of death was inadequate hemopoietic reconstitution (early death defined as <28 d after reconstitution or late death if later than 28 d after reconstitution with spleen <50 mg and/or evidence of severe anemia), recipient-derived T cell lymphoma (presumed secondary to irradiation), or infection.

Mice were killed when noted to be sick, peripheral blood was harvested from the orbital plexus, lymph node and spleen suspensions were prepared, and other organs were harvested. Lymphomas were studied by staining with cell surface marker-specific mAbs followed by flow cytometry. Antibodies used were A201.7 anti-Ly5.1, 5.450.15.2 anti-Ly5.2, RA3–6B2 anti-CD45R(B220), 11/26C anti-IgD, 5.1 anti-IgM (μ heavy chain), T24.3.2.1 anti-Thy 1, H129 anti-CD4, YTS169 anti-CD8, MI/70 anti-Mac 1, RB6–8C5 anti-Gr-1, and Ter119 antierythroid cell surface marker. These antibodies were purified from hybridoma supernatant on protein G–Sepharose columns (Amersham Biosciences) and conjugated to FITC, R-phycoerythrin (R-PE), cychrome 5 (Cy5), or biotin according to the manufacturer's (Molecular Probes) instructions. Bound biotinylated antibodies were revealed by R-PE–labeled streptavidin (Caltag). Stained cells were analyzed on a FACScan® (Becton Dickinson), live and dead cells being discriminated by staining with 2 μg/ml propidium iodide (PI; Sigma-Aldrich) and by their forward and side light scattering properties. Stable cell lines were derived from fresh lymphomas by culturing in the high glucose version of DME supplemented with 250 μM asparagine, 50 μM 2-mercaptoethanol, and 10% FCS. Genotyping of lymphomas and lymphoma cell lines was performed by PCR as described in Mice section. Cell extracts were Western blotted as described previously (Marsden et al., 2002) with mAbs as follows: rat anti–Apaf-1 mAb 18H2 (Alexis Biochemicals), rat anti–mouse caspase-2 mAb 10C6 (O'Reilly et al., 2002), rat anti–mouse caspase-8 mAb 1G12, mouse anti–caspase-9 mAb (gift of Y. Lazebnik, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and mouse anti–HSP-70 mAb (gift of R. Anderson, Peter MacCallum Cancer Centre, Melbourne, Australia) or polyclonal rabbit anti–mouse caspase-1 antibody (gift of P. Vandenabeele, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium). HRP-conjugated goat anti–mouse Ig or goat anti–rat Ig antibodies were used as secondary reagents, and bound antibodies were revealed by ECL.

Stable lymphoma cell lines were sorted by flow cytometry using a modified FACSII® or FACStar (Becton Dickinson). For cell death analysis, viable cells (negative for PI) were cultured in DME at a concentration of $0.2–0.5 \times 10^6$ cells/ml in 96-well flat bottom microtiter plates (Falcon). Cytotoxic stimuli used included the following: $10^{-8}–10^{-6}$ M dexamethasone (Sigma-Aldrich), 0.01–1.0 μg/ml etoposide (VP16; David Bull Laboratories), and γ-radiation (1–10 Gy from a ^{60}Co source). Cells were cultured for 24, 48, or 72 h, and the fraction of viable cells was determined by staining with 2 μg/ml PI and FITC-coupled Annexin V followed by flow cytometric analysis.

Analysis of preneoplastic phenotype in reconstituted mice

Recipient mice showing no signs of lymphoma were killed and their organs were harvested (cellularity refers to the total cell number in an organ). Spleen cells (2×10^6) were injected intraperitoneally into nonirradiated C57BL/6 mice to ensure no tumor development in the subsequent 3 mo. pre–B cells ($B220^+sIg^-$) and B cells ($B220^+sIg^+$) were purified from the bone marrow and lymph nodes, respectively, by immunofluorescent staining and sorting on a FACStar, DIVA (Becton Dickinson), or MoFlo (DakoCytomation). Cells were cultured and viability determined over 6–72 h as described in Lymphoma analysis. Donor-derived pro/pre–B cells ($c\text{-}kit^+B220^+CD19^+sIg^-Mac\text{-}1^-Gr\text{-}1^-Ter119^-Ly5.1^-$) were purified from the bone marrow by immunofluorescent staining and FACS® sorting (Cy5-RA3-6B2, anti–CD45R-B220; Cy5-1D9, anti-CD19; biotin-ACK4, anti–c-kit plus R-PE–streptavidin and FITC-labeled MI/70, 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 (VP16) or γ-radiation (2.5 Gy) before plating at limiting dilution. B cell colonies consisting of at least 20 cells were scored blinded as to genotype using an inverted microscope at day 10, and the cloning frequency was determined using limiting dilution analysis.

MEF culture and transformation

All MEFs (provided by T. Mak, University of Toronto, Toronto, Canada) were maintained in full DME and were used between passage 2 and 3. For long-term proliferation assays, 5,000 MEFs were resuspended in full DME (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 (calcium phosphate precipitation technique) of the respective vector plasmid and pCL-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, cleared 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, pWZLH.E1A, pBabePuro.myc, and pWZLH.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-coupled Annexin V (Calbiochem) and PI. Whole cell extracts were generated by resuspending cell pellets in lysis buffer (50 mM Hepes, 250 mM NaCl, 5 mM EDTA, and 0.1% NP-40) on ice, followed by centrifugation. After transfer onto nitrocellulose membranes (Amersham Biosciences), primary antibody binding was detected by ECL (Pierce Chemical Co.). Primary antibodies used were 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-$1^{+/+}$, *Eμ-myc*/Apaf-$1^{+/-}$, *Eμ-myc*/Apaf-$1^{-/-}$, *Eμ-myc*/*Eμ-bcl-2*, or (Fig. S1 B) *Eμ-myc*/caspase-$9^{+/+}$, *Eμ-myc*/caspase-$9^{+/-}$, *Eμ-myc*/caspase-$9^{-/-}$, or *Eμ-myc*/*Eμ*-bcl-2 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 *c-myc* overexpression.

MEFs that are either wild type, $p53^{-/-}$, $bax^{-/-}$ (provided by S. Korsmeyer, Dana Farber Laboratories, Boston, MA), or Apaf-$1^{-/-}$ grew at similar rates in culture (Fig. S2 A). After infection with oncogene-containing retroviruses, MEFs that were wild type, $p53^{-/-}$, $bax^{-/-}$, or Apaf-$1^{-/-}$ all expressed similar levels of E1A plus mutant ras or myc plus mutant ras proteins (Fig. S2 B).

Western blot analysis demonstrated that loss of Apaf-1 or caspase-9 does not cause a compensatory increase in expression or activation (after γ-radiation) of caspase-1, -2, -8, or -9 (in Apaf-$1^{-/-}$ cells) in thymocytes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200310041/DC1.

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References

Adams, J.M., A.W. Harris, C.A. Pinkert, L.M. Corcoran, W.S. Alexander, S. Cory, R.D. Palmiter, and R.L. Brinster. 1985. The c-*myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature.* 318:533–538.

Bissonnette, R.P., F. Echeverri, A. Mahboubi, and D.R. Green. 1992. Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. *Nature.* 359:552–554.

Cecconi, F., G. Alvarez-Bolado, B.I. Meyer, K.A. Roth, and P. Gruss. 1998. Apaf-1 (CED-4 homologue) regulates programmed cell death in mammalian development. *Cell.* 94:727–737.

Eischen, C.M., J.D. Weber, M.F. Roussel, C.J. Sherr, and J.L. Cleveland. 1999. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev.* 13:2658–2669.

Fanidi, A., E.A. Harrington, and G.I. Evan. 1992. Cooperative interaction between c-*myc* and *bcl*-2 proto-oncogenes. *Nature.* 359:554–556.

Green, D.R., and J.C. Reed. 1998. Mitochondria and apoptosis. *Science.* 281: 1309–1311.

Hakem, R., A. Hakem, G.S. Duncan, J.T. Henderson, M. Woo, M.S. Soengas, A. Elia, J.L. de la Pompa, D. Kagi, W. Khoo, et al. 1998. Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell.* 94:339–352.

Haraguchi, M., S. Torii, S. Matsuzawa, Z. Xie, S. Kitada, S. Krajewski, H. Yoshida, T.W. Mak, and J.C. Reed. 2000. Apoptotic protease activating factor 1 (Apaf-1)–independent cell death suppression by Bcl-2. *J. Exp. Med.* 191:1709–1720.

Harris, A.W., C.A. Pinkert, M. Crawford, W.Y. Langdon, R.L. Brinster, and J.M. Adams. 1988. The Em-*myc* transgenic mouse: a model for high-incidence spontaneous lymphoma and leukemia of early B cells. *J. Exp. Med.* 167:353–371.

Hengartner, M.O. 2000. The biochemistry of apoptosis. *Nature.* 407:770–776.

Hsu, B., M.C. Marin, A.K. El-Naggar, L.C. Stephens, S. Brisbay, and T.J. McDonnell. 1995. Evidence that c-*myc* mediated apoptosis does not require wild-type p53 during lymphomagenesis. *Oncogene.* 11:175–179.

Jia, L., S.M. Srinivasula, F.T. Liu, A.C. Newland, T. Fernandes-Alnemri, E.S. Alnemri, and S.M. Kelsey. 2001. Apaf-1 protein deficiency confers resistance to cytochrome c-dependent apoptosis in human leukemic cells. *Blood.* 98: 414–421.

Kuida, K., T.F. Haydar, C.-Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M.S.-S. Su, P. Rakic, and R.A. Flavell. 1998. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell.* 94:325–337.

Langdon, W.Y., A.W. Harris, S. Cory, and J.M. Adams. 1986. The c-*myc* oncogene perturbs B lymphocyte development in Em-*myc* transgenic mice. *Cell.* 47:11–18.

Marsden, V., L. O'Connor, L.A. O'Reilly, J. Silke, D. Metcalf, P. Ekert, D.C.S. Huang, F. Cecconi, K. Kuida, K.J. Tomaselli, et al. 2002. Apoptosis initiated by Bcl–2-regulated caspase activation independently of the cytochrome *c*/Apaf–1/caspase–9 apoptosome. *Nature.* 419:634–637.

McCarthy, N.J., M.K.B. Whyte, C.S. Gilbert, and G.I. Evan. 1997. Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. *J. Cell Biol.* 136:215–227.

McCurrach, M.E., T.M.F. Connor, C.M. Knudson, S.J. Korsmeyer, and S.W. Lowe. 1997. Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. *Proc. Natl. Acad. Sci. USA.* 94:2345–2349.

Mufti, G.J., T.J. Hamblin, D.G. Oscier, and S. Johnson. 1983. Common ALL with pre-B-cell features showing (8;14) and (14;18) chromosome translocations. *Blood.* 62:1142–1146.

O'Connor, L., A. Strasser, L.A. O'Reilly, G. Hausmann, J.M. Adams, S. Cory, and D.C.S. Huang. 1998. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J.* 17:384–395.

O'Reilly, L.A., P. Ekert, N. Harvey, V. Marsden, L. Cullen, D.L. Vaux, G. Hacker, C. Magnusson, M. Pakusch, F. Cecconi, et al. 2002. Caspase-2 is not required for thymocyte or neuronal apoptosis even though cleavage of caspase-2 is dependent on both Apaf-1 and caspase-9. *Cell Death Differ.* 9:832–841.

Pelengaris, S., M. Khan, and G. Evan. 2002. c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer.* 2:764–776.

Rolink, A., A. Kudo, H. Karasuyama, Y. Kikuchi, and F. Melchers. 1991. Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells *in vitro* and *in vivo*. *EMBO J.* 10:327–336.

Schmitt, C.A., M.E. McCurrach, E. de Stanchina, R.R. Wallace-Brodeur, and S.W. Lowe. 1999. *INK4a/ARF* mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev.* 13:2670–2677.

Schmitt, C.A., J.S. Fridman, M. Yang, E. Baranov, R.M. Hoffman, and S.W. Lowe. 2002. Dissecting p53 tumor suppressor functions in vivo. *Cancer Cell.* 1:289–298.

Soengas, M.S., R.M. Alarcón, H. Yoshida, A.J. Giaccia, R. Hakem, T.W. Mak, and S.W. Lowe. 1999. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science.* 284:156–159.

Soengas, M.S., P. Capodieci, D. Polsky, J. Mora, M. Esteller, X. Opitz-Araya, R. McCombie, J.G. Herman, W.L. Gerald, Y.A. Lazebnik, et al. 2001. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature.* 409: 207–211.

Srinivasula, S.M., M. Ahmad, T. Fernandes-Alnemri, and E.S. Alnemri. 1998. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol. Cell.* 1:949–957.

Strasser, A., A.W. Harris, M.L. Bath, and S. Cory. 1990. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl*-2. *Nature.* 348:331–333.

Strasser, A., A.G. Elefanty, A.W. Harris, and S. Cory. 1996. Progenitor tumours from Em-*bcl*-2-*myc* transgenic mice have lymphomyeloid differentiation potential and reveal developmental differences in cell survival. *EMBO J.* 15: 3823–3834.

Strasser, A., L. O'Connor, and V.M. Dixit. 2000. Apoptosis signaling. *Annu. Rev. Biochem.* 69:217–245.

Teitz, T., T. Wei, D. Liu, V. Valentine, M. Valentine, J. Grenet, J.M. Lahti, and V.J. Kidd. 2002. Caspase-9 and Apaf-1 are expressed and functionally active in human neuroblastoma tumor cell lines with 1p36 LOH and amplified MYCN. *Oncogene.* 21:1848–1858.

Vaux, D.L., S. Cory, and J.M. Adams. 1988. *Bcl*-2 gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. *Nature.* 335:440–442.

Wang, X. 2001. The expanding role of mitochondria in apoptosis. *Genes Dev.* 15: 2922–2933.

Yoshida, H., Y.-Y. Kong, R. Yoshida, A.J. Elia, A. Hakem, R. Hakem, J.M. Penninger, and T.W. Mak. 1998. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell.* 94:739–750.

Zhang, L., J. Yu, B.H. Park, K.W. Kinzler, and B. Vogelstein. 2000. Role of BAX in the apoptotic response to anticancer agents. *Science.* 290:989–992.

Zheng, T.S., S. Hunot, K. Kuida, T. Momoi, A. Srinivasan, D.W. Nicholson, Y. Lazebnik, and R.A. Flavell. 2000. Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. *Nat. Med.* 6:1241–1247.