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Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die

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Apoptosis after growth factor withdrawal or drug treatment is associated with mitochondrial cytochrome *c* release and activation of Apaf-1 and caspase-9. To determine whether loss of Apaf-1, caspase-2, and caspase-9 prevented death of factor-starved cells, allowing them to proliferate when growth factor was returned, we generated IL-3–dependent myeloid lines from gene-deleted mice. Long after growth factor removal, cells lacking Apaf-1, caspase-9 or both caspase-9 and caspase-2 appeared healthy, retained intact plasma membranes, and did not expose phosphatidylserine. However, release of cytochrome *c* still occurred, and they failed to form clones when IL-3 was restored. Cells lacking caspase-2 alone had no survival advantage. Therefore, Apaf-1, caspase-2, and caspase-9 are not required for programmed cell death of factor-dependent cells, but merely affect its rate. In contrast, transfection with Bcl-2 provided long-term, clonogenic protection, and could act independently of the apoptosome. Unlike expression of Bcl-2, loss of Apaf-1, caspase-2, or caspase-9 would therefore be unlikely to enhance the survival of cancer cells.

Introduction

During apoptosis of mammalian cells after removal of serum or growth factors, proteins such as cytochrome *c* and Diablo/Smac are released from the mitochondria, apoptosomes containing Apaf-1 and caspase-9 are formed, and effector caspases become active and cleave their substrates. Apoptosis due to growth factor withdrawal can usually be inhibited by Bcl-2 (Vaux et al., 1988).

Programmed cell death in the worm *Caenorhabditis elegans* has many similarities. It requires direct binding of the Apaf-1–like adaptor protein CED-4 to the caspase CED-3 (Chinnaiyan et al., 1997; Irmler et al., 1997; Seshagiri and Miller, 1997), and does not occur in worms with a gain of function mutation of the Bcl-2 homologue CED-9 (Hengartner and Horvitz, 1994). CED-9 interacts directly with CED-4 to inhibit apoptosis (Spector et al., 1997). These observations suggested that Apaf-1 and caspase-9 might be essential for cell death in mammals, just as CED-4 and CED-3 are in the worm, and that Bcl-2 would prevent apoptosis in mammals by directly binding to and inhibiting Apaf-1 just as CED-9 binds to and inhibits CED-4.

However, this simple scheme is complicated by the finding that neither Bcl-2 nor Bcl-x binds to Apaf-1 (Moriishi et al., 1999). Furthermore, although most mice lacking genes for Apaf-1 or caspase-9 die in the perinatal period due to neuronal overgrowth, some develop normally and reproduce (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998). These experiments, and those showing that programmed cell death of lymphoid cells occurs normally in Apaf-1– and caspase-9–deficient mice (Marsden et al., 2002), raised the possibility that another caspase, such as caspase-2 (Lassus et al., 2002) may compensate to cause apoptosis in the absence of caspase-9.

We wished to determine whether myeloid cells undergo apoptosis normally in the absence of Apaf-1 and caspase-9, and if so whether also deleting caspase-2 would prevent cell death. In addition, we wanted to test whether Bcl-2 could

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Abbreviation used in this paper: PI, propidium iodide.

function in the absence of the apoptosome and caspase-2. For the apoptotic stimulus we first used growth factor withdrawal because it does not depend on direct toxic effects as do chemotherapeutic drugs or irradiation, and can readily be reversed by readdition of growth factor. We then tested whether these observations also applied when apoptosis was induced by the chemotherapeutic agents etoposide and doxorubicin.

IL-3–dependent myeloid cell lines were established from *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, *caspase-2* $^{-/-}$, and *caspase-9* $^{-/-}$; *caspase-2* $^{-/-}$ mice, and were tested in both short-term cell death assays as well as clonogenic survival assays to see whether any apparent survival advantage conferred by the absence of Apaf-1 or caspases would allow cells to proliferate when IL-3 was restored. To test whether Bcl-2 requires Apaf-1, caspase-9, or caspase-2 to function, we enforced stable expression of Bcl-2. We found that, although cells lacking Apaf-1, caspase-9 or both caspase-9 and caspase-2 appeared protected from apoptosis induced by IL-3 withdrawal or cytotoxic drugs, there was no enhancement of clonogenic survival over wild-type cells unless Bcl-2 was also overexpressed. Our data suggest Apaf-1 and caspase-9 are required for rapid, efficient apoptosis but not for commitment to cell death and that loss of Apaf-1 or caspase-9 does not enhance long-term survival of cells.

Results

Cell death is delayed in Apaf-1 $^{-/-}$, caspase-9 $^{-/-}$, and caspase-9 $^{-/-}$; caspase-2 $^{-/-}$ IL-3–dependent cell lines

Growth factor withdrawal-induced apoptosis can be blocked by Bcl-2 (Vaux et al., 1988), and is associated with release of cytochrome *c* from mitochondria, and sequential activation of Apaf-1 and caspase-9 (Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998). To investigate the requirement for Apaf-1, caspase-2, and caspase-9 in growth factor withdrawal-induced cell death, we generated multiple, independently derived, clonal, IL-3–dependent, promyeloid cell lines from mice lacking either *Apaf-1, caspase-2*, *caspase-9*, or both *caspase-2* and *caspase-9*. These lines were produced by transforming E14 fetal liver cells with a Hox 2.4 expressing retrovirus in the presence of high amounts of IL-3, as described previously (Perkins and Cory, 1993). Individual clones selected from soft agar were maintained in liquid culture in the presence of IL-3, and absence of the relevant proteins was confirmed by Western blot (Fig. 1 G and not depicted).

When cultured in the absence of IL-3, many more *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$; *caspase-2* $^{-/-}$ cells appeared alive at each time point when viability was determined by exclusion of propidium iodide (PI; Fig. 1). Although there was some variation between individual clones, the wild-type and *caspase-2* $^{-/-}$ cells died significantly more rapidly than the *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$; *caspase-2* $^{-/-}$ double knockout cells and *caspase-2* $^{-/-}$ cells even more rapidly than wild-type cells. Nevertheless, it was also clear that even in the absence of Apaf-1 or caspase-9 some cells died over the 10-d duration of the experiments. These data show that in the absence of Apaf-1 or caspase-9 ~10 times more cells survived when viability was measured by uptake of PI.

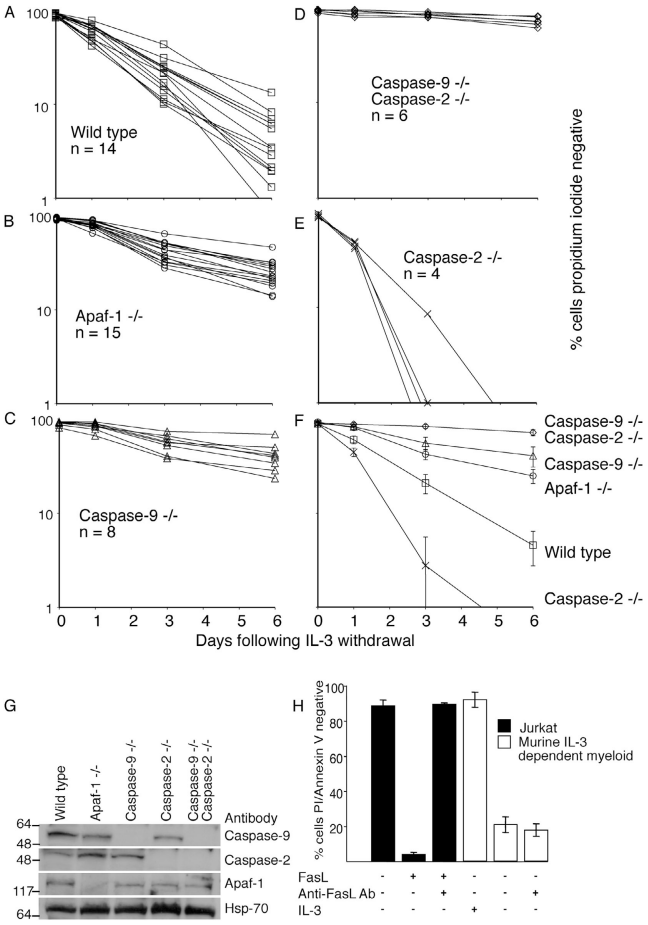

Figure 1. **Deficiency of Apaf-1, caspase-9, or both caspase-9 and caspase-2 provides short-term protection against IL-3 withdrawal.** Multiple independent clones of wild-type (A), *Apaf-1* $^{-/-}$ (B), *caspase-9* $^{-/-}$ (C), *caspase-2* $^{-/-}$ (E), or *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ (D) IL-3–dependent cell lines were cultured in the absence of IL-3 and viability determined at the indicated times by propidium iodide (PI) exclusion using flow cytometry. The values represent the means of *n* independent clones in two to three independent experiments. (F) The pooled arithmetic means ± 2 SEM of clones of each genotype is shown. (G) Western blot of representative clones of each of wild-type, *Apaf-1* $^{-/-}$, and *caspase-9* $^{-/-}$, *caspase-2* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cell lines. Probing with antibody to Heat shock protein 70 (Hsp 70) was used as a loading control. (H) Wild-type IL-3–dependent cells (open bars) were cultured in the presence or absence of IL-3 and a mouse-specific Fas ligand blocking antibody. Closed bars show Jurkat cells with and without mouse Fas ligand and the Fas ligand blocking antibody. Viability was determined by Annexin V expression and PI exclusion by flow cytometry. The results for wild-type IL-3–dependent cells show the mean of two independent clones in three independent experiments and the results for Jurkat cells shows the mean of an experiment done in triplicate. Error bars are SEM.

To determine whether apoptosis in response to IL-3 withdrawal involved Fas ligand signaling as has been reported previously (Le-Niculescu et al., 1999), wild-type cells were incubated in the presence or absence of IL-3 and an antibody that blocked mouse Fas ligand signaling (Fig. 1 H). Fas ligand treated Jurkat cells (and SKW6 cells; unpublished data) were used as a control to demonstrate the antibody could inhibit Fas ligand induced apoptosis. When IL-3 was removed, the same percentage of cells were Annexin V posi-

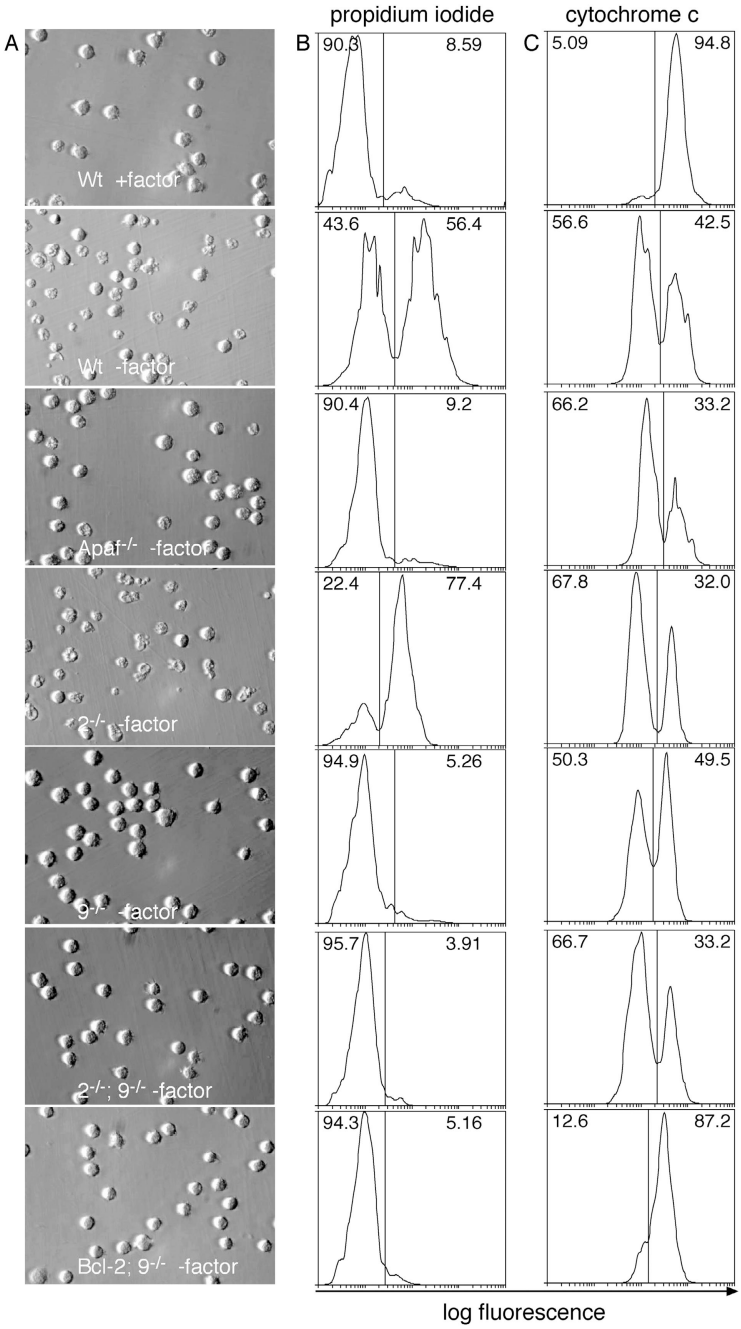

Figure 2. **After 24 h without IL-3, *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells appear healthy, exclude PI but have released cytochrome *c* from mitochondria.** (A) Light microscopy of cells cultured with or without IL-3 for the indicated genotype. Wild-type and *caspase-2* $^{-/-}$ cells show similar changes, with marked cell shrinkage and loss of refractivity whereas *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells appear healthy. (B) PI uptake determined by flow cytometry. Increasing fluorescence (FL-3 channel) indicates PI uptake by cells that have lost membrane integrity. The majority of *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells exclude PI 24 h after withdrawal of IL-3. (C) Intracellular cytochrome *c* staining assessed by flow cytometry (FL-1 channel). Loss of cytochrome *c* from mitochondria is indicated by a shift of fluorescence to the left. *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells lose cytochrome *c* like wild-type and *caspase-2* $^{-/-}$ cells, despite excluding PI. Bcl-2 overexpression (shown here in Bcl-2; *caspase-9* $^{-/-}$ cells) prevents cytochrome *c* release. Multiple clones of cells of all genotypes were examined with and without IL-3, and typical results are shown.

tive and/or had lost membrane integrity in the presence or absence of the blocking antibody, indicating death after IL-3 withdrawal does not require Fas ligand signaling in these cells. Furthermore, IL-3–dependent FDC-PI cells resistant to Fas-induced apoptosis because they overexpress the viral caspase-8 inhibitor crmA or a dominant negative FADD construct, were equally sensitive to IL-3 withdrawal as were wild-type cells, whereas cells overexpressing Bcl-2 were protected (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200312031/DC1).

Many of the signs of apoptosis do not manifest in cells lacking Apaf-1 or caspase-9

Fig. 2 A shows the microscopic appearance of the cells 24 h after growth factor withdrawal. Although many of the wild-type and *caspase-2* $^{-/-}$ cells were shrunken and displayed plasma membrane blebbing, the *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells looked as healthy as the cells cultured with factor. Less *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells exposed phosphatidylserine on the membrane surface as indicated by Annexin V staining (unpublished data). Western blots of lysates from wild-type, *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells (Fig. 3) showed that much more caspase-3, caspase-7 and ICAD processing occurred in wild-type cells than in those lacking Apaf-1 or caspase-9. These data suggest that many of the morphological changes associated with apoptosis in response to growth factor withdrawal are dependent on caspase-9 and Apaf-1. This appears to be cell type–dependent because thymocytes from *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$

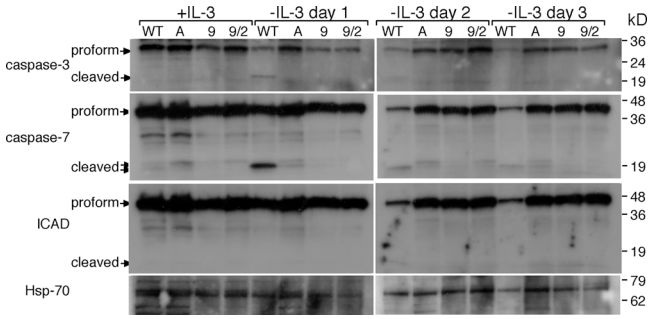

Figure 3. **Diminished caspase activity in IL-3–starved *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells.** Lysates from cells cultured in the presence or absence of IL-3 over a 3-d period were separated by SDS PAGE on 4–20% gradient gels and immunoblotted with antibodies to the indicated proteins. Activation of caspase-3 and caspase-7 is indicated by the loss of the full-length protein and, in the case of caspase-7, by the appearance of the processed p10 fragment. The cleavage of ICAD is indicated by the loss of the full-length protein. The ICAD cleavage fragment could not be observed. Levels of Hsp70 are shown as a loading control.

mice showed DNA degradation, caspase-7, PARP and ICAD processing, as well as cleavage of a fluorogenic caspase substrate in response to various apoptotic stimuli; although in most instances, this was reduced compared with that observed in control cells (Marsden et al., 2002).

To determine whether cytochrome *c* was still released in the absence of Apaf-1 or caspase-9, we stained plasma membrane–permeabilized, IL-3–starved cells with an antibody to cytochrome *c* and analyzed the cells by flow cytometry. As shown in Fig. 2 C, although cells lacking Apaf-1 or caspase-9 appeared normal when growth factor was removed, cytochrome *c* had been released from the mitochondria.

These data show that the downstream events associated with caspase-9 activation are greatly reduced in factor-starved *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells, just as they were in *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ MEFs (Cecconi et al., 1998; Hakem et al., 1998). However, the fact that cytochrome *c* was still released from the IL-3 deprived *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ cells made us question whether they were still committed to die, despite their healthy appearance.

Short-term survival of *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ cells does not translate into long-term, clonogenic survival

To determine whether the survival advantage of *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ cells observed after IL-3 withdrawal would also permit long-term survival, thereby allowing more cells to proliferate when cytokine was returned, we starved cells of IL-3 for increasing time periods and then transferred them to soft agar with abundant growth factor, and counted the number of colonies that formed (Fig. 4). Factor-starved *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, or *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ double knockout lines were no more able to generate colonies than wild-type cells when transferred to soft agar with IL-3 (Fig. 4 B), even though 10-fold more *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ cells excluded PI at the time they were plated in agar (Fig. 4 A). These results show that although the absence of Apaf-1 or caspase-9 significantly delays certain morphological changes associated with apoptosis, these lines were not growth factor independent, and were normally committed to programmed cell death when IL-3 was withdrawn.

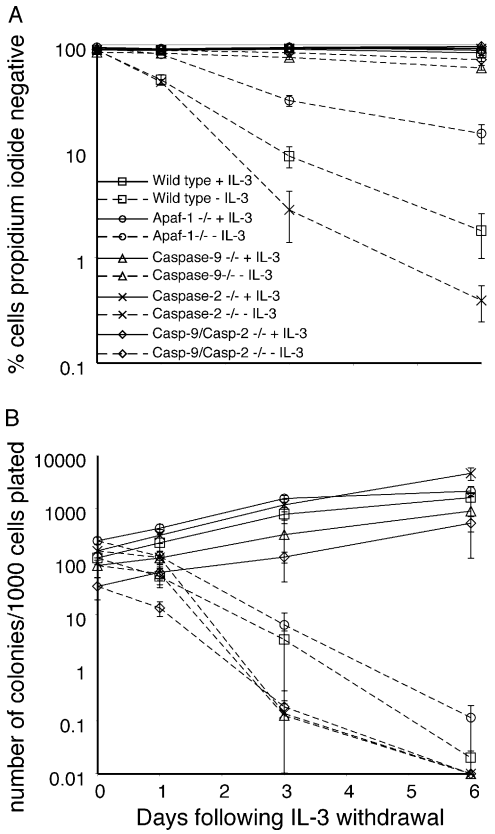

Figure 4. ***Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells are committed to die after IL-3 withdrawal.** Wild-type, *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, *caspase-2* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells were cultured in the presence (solid line) or absence (dashed line) of IL-3 for the indicated times. (A) Analysis of PI exclusion at each time point with and without growth factor. (B) Varying dilutions of cells were cultured in soft agar with abundant IL-3 after the indicated period of IL-3 deprivation, and the number of colonies formed was counted after 21 d. The y axis indicates the number of colony forming units per 1,000 cells originally plated. Values shown are the means of at least two independent clones for each genotype from four independent experiments. Error bars are ± SEM.

The fact that *caspase-9* $^{-/-}$ myeloid and lymphoid cells (Marsden et al., 2002) can still undergo programmed cell death raised the possibility that another caspase was responsible. Although evidence from RNA interference experiments suggested that this might be caspase-2 (Lassus et al., 2002), our experiments on *caspase-2* $^{-/-}$ and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells revealed no role for caspase-2 in cell death in either short or long-term clonogenic survival assays, or any redundancy with caspase-9 (Figs. 1, 2, and 4).

Bcl-2 promotes clonogenic survival of *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ cells

Because Bcl-2 has been shown to promote clonogenic survival of growth factor–deprived cells, we tested whether it could still do so in the absence of Apaf-1 or caspase-9. Multiple independent clones were established that stably overex-

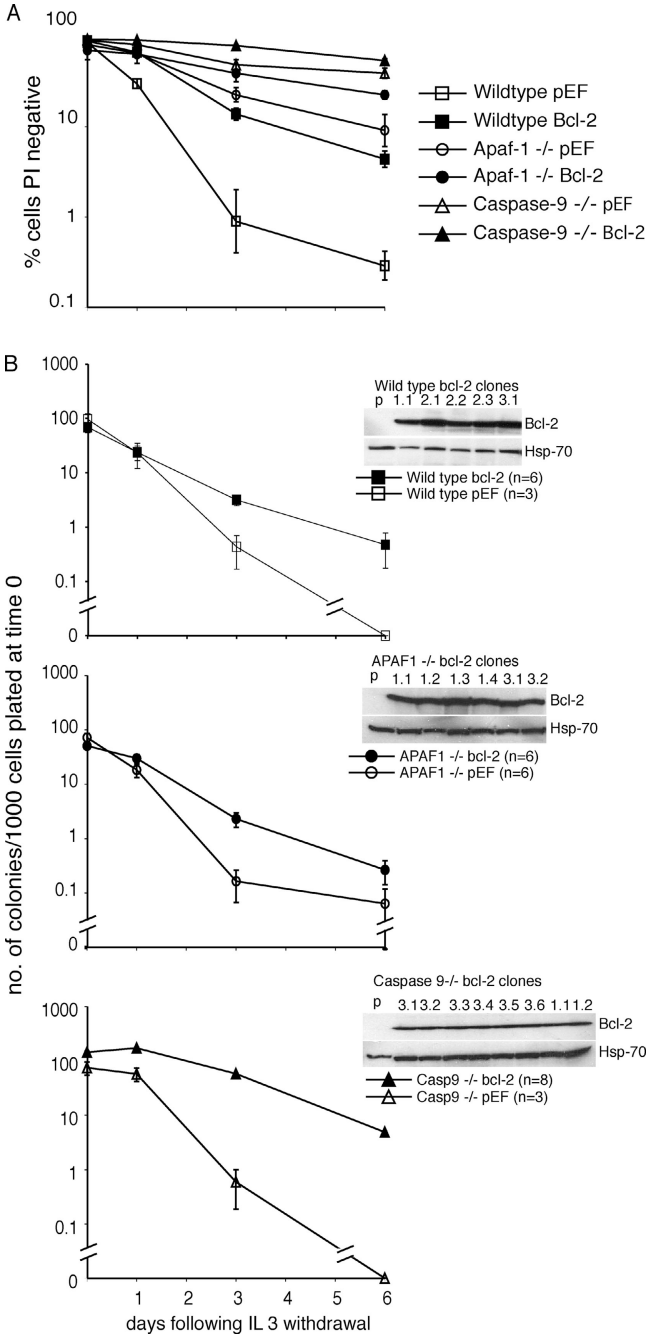

Figure 5. **Expression of Bcl-2 provides protection against IL-3 withdrawal-induced apoptosis and promotes clonogenic survival.** Cells of the indicated genotype containing either empty vector (pEF) or Bcl-2 expression construct were cultured in the absence of IL-3 for the indicated times. (A) Viability determined by PI exclusion using flow cytometry. (B) Varying dilutions of cells were cultured in soft agar with abundant IL-3 following the indicated period of IL-3 deprivation and the number of colonies formed counted after 21 d. The y axis represents the number of colony forming units per 1,000 cells originally plated. Western blots show the levels of Bcl-2 expression in the cell lines that were examined (five representative wild-type lines are shown). The value *n* represents the number of independent clones tested for each genotype. The values shown are the means ± SEM from three independent experiments.

press Bcl-2 (confirmed by Western blot; Fig. 5). When these cells were cultured in the absence of IL-3, and their viability determined by exclusion of PI, Bcl-2 was able to increase

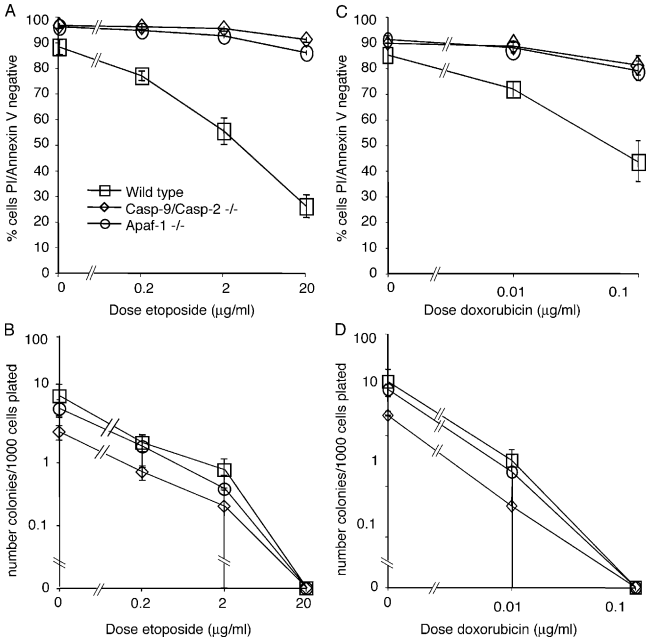

Figure 6. ***Apaf-1*** $^{-/-}$ **and *caspase-9*** $^{-/-}$***; caspase-2*** $^{-/-}$ **cells appear viable when treated with etoposide or doxorubicin, but are committed to die.** Wild-type, *Apaf-1* $^{-/-}$, and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells were treated with the indicated doses of etoposide (A and B) or doxorubicin (C and D) for 24 h. Viability was determined by Annexin V staining and PI exclusion using flow cytometry (A and C), and clonogenic survival was determined by plating in soft agar and counting the number of colonies after 21 d (B and D). The viability curves show the mean ± SEM of two independent clones of each genotype in three independent experiments. The clonal assays show mean ± SEM of two independent clones of each genotype in two independent experiments.

survival of wild-type, *Apaf-1* $^{-/-}$, and *caspase-9* $^{-/-}$ cells (Fig. 5 A). Furthermore, in Bcl-2 overexpressing lines derived from the same gene-deleted parental clones, we found that Bcl-2 promoted clonogenic survival after IL-3 withdrawal (Fig. 5 B). These results show that Bcl-2 can prevent cell death, and can do so independently of Apaf-1 or caspase-9, which is consistent with its ability to prevent cytochrome *c* release from the mitochondria (Fig. 2 C, bottom).

Apaf-1 $^{-/-}$ and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells show short-term resistance to cytotoxic drugs but not clonogenic survival

Some cell types from *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$ animals showed typical apoptotic morphology in response to a range of death stimuli, including cytotoxic drugs (Marsden et al., 2002). To determine whether the short-term protection of the cell lines depended on the death stimulus used, we treated *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells with etoposide or doxorubicin and determined viability after 24 h (Fig. 6). Compared with wild-type cells, both *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells survived treatment with these agents as determined by Annexin V-Fluos/PI uptake (Fig. 6, A and C). This result was similar to that observed after IL-3 withdrawal. To determine whether this short-term protection translated into clonogenic survival, the cells were plated in soft agar after washing the drug from the culture

(Fig. 6, B and D). *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells were no more able to form colonies than wild-type cells, even though many more *Apaf-1* $^{-/-}$ and *caspase-9* $^{-/-}$*; caspase-2* $^{-/-}$ cells appeared viable. These data show that, as was the case in IL-3 withdrawal induced apoptosis, the absence of Apaf-1 or caspase-9 delayed certain morphological changes associated with apoptosis, but these lines nevertheless remained committed to programmed cell death after treatment with cytotoxic drugs.

Discussion

Although either too much or too little apoptosis has been associated with a multiplicity of diseases, the clearest example where abnormalities of apoptosis are primary and causative is in the case of certain cancers. Translocations involving the *bcl-2* gene in follicular lymphoma led to the identification of Bcl-2 (Tsujimoto et al., 1984, 1985), and its recognition as the first component of the apoptosis mechanism (Vaux et al., 1988). Transgenic mice expressing Bcl-2 formally confirmed that inhibition of cell death could lead to the development of cancer (Strasser et al., 1990).

Correlative evidence also exists implicating other components of the apoptotic mechanism in cancer. For example, loss of expression of Apaf-1 has been associated with melanoma, Apaf-1 has been reported to be required for apoptosis triggered by the tumor suppressor gene p53 (Soengas et al., 1999, 2001), and expression of the caspase inhibitory IAP protein ML-IAP has been associated with melanoma (Vucic et al., 2000).

Development of a cancer requires the survival of a clone of cells capable of further reproduction. For an apoptosis inhibitor to enhance tumor development, it must allow cells to retain their clonogenic potential. Bcl-2, which acts upstream of the mitochondria, can promote clonogenic protection. We wished to determine whether inhibiting events downstream of the mitochondria, such as activation of caspase-9 by Apaf-1, would also promote long-term clonogenic survival. By testing IL-3–dependent cell lines lacking genes for Apaf-1 or caspase-9 and transfecting them with Bcl-2, we also sought to determine whether Bcl-2 could act independently of Apaf-1.

Unlike Marsden et al. (2002), who found that lymphoid cells underwent apoptosis relatively normally in the absence of Apaf-1 or caspase-9, we found that the appearance of classical hallmarks of apoptosis, including exposure of phosphatidylserine, membrane blebbing, cleavage of caspases and their substrates, and uptake of PI, were markedly delayed in myeloid cells lacking Apaf-1 or caspase-9. Nevertheless, absence of Apaf-1 or caspase-9 did not increase the number of surviving cells that could form clones after IL-3 was restored, indicating that in these cells Apaf-1 and caspase-9 act after the cell death commitment point to enhance the rate of cell demolition, but that these molecules do not determine whether cells will ultimately die. These experiments illustrate the critical importance of clonal assays in cell death research, because measuring cell death by morphology, uptake of vital dyes, exposure of phosphatidylserine, activation of caspases or cleavage of their substrates, will not necessarily reveal whether a cell is committed to die.

Overexpressing Bcl-2 in the *Apaf-1* and *caspase-9* null cells demonstrated that Bcl-2 requires neither Apaf-1 nor caspase-9 to function, and acts before the commitment point, to provide clonogenic protection. The ability of Bcl-2 to provide clonal protection independently of Apaf-1 and caspase-9 in factor-dependent cells responding to a physiological death stimulus extends earlier work showing that Bcl-2 was capable of giving short- and long-term protection to Apaf-1 null embryonic stem cells treated with chemotherapeutic agents (Haraguchi et al., 2000). Furthermore, deficiency of either Apaf-1 or caspase-9 did not enhance lymphomagenesis in c-myc transgenic mice, nor contribute to oncogenic transformation of fibroblasts (Scott et al., 2004). Collectively, these observations question the ability of Apaf-1 or caspase-9 to act as tumor suppressor genes.

Deletion of *caspase-2* did not inhibit cytochrome *c* release from mitochondria or confer any short- or long-term survival advantage, suggesting caspase-2 is not required for apoptosis resulting from growth factor withdrawal in our cell lines. Indeed, *caspase-2* $^{-/-}$ lines on average exhibited apoptotic changes even more rapidly than wild-type lines in response to growth factor withdrawal. If the presence of caspase-2 does somehow delay appearance of some of the markers of apoptosis, this is unlikely to be physiologically important, because *caspase-2* $^{-/-}$ cells commit to die at the same rate as wild-type cells as revealed by clonogenic assays, and *caspase-2* $^{-/-}$ mice are indistinguishable from wild-type or heterozygous littermate controls (O'Reilly et al., 2002).

There have been suggestions that Fas ligand signaling contributes to death of neuronal cells after withdrawal of a survival factor (KCl; Le-Niculescu et al., 1999). In IL-3–dependent myeloid lines, a Fas-ligand blocking antibody did not reduce apoptosis after IL-3 withdrawal. Furthermore, IL-3–dependent FDC-P1 cells overexpressing a FADD dominant negative construct or crmA remained as susceptible to IL-3 withdrawal as control cells (Fig. S1), indicating no role for Fas ligand in growth factor withdrawal-induced apoptosis.

Although these experiments show that Bcl-2 can provide long-term clonogenic protection, they do not reveal how it acts. Several possibilities present themselves. The cells lacking Apaf-1 might have died from inadequate mitochondrial respiratory function (Gottlieb et al., 2002); they may have died as a result of substrate deprivation and autophagy; or they might have died because of activation of caspases that do not require Apaf-1 for their activation. In the first two scenarios Bcl-2 would protect the cells by preventing cytochrome *c* release or in some way maintaining mitochondrial respiratory function, whereas in the third possibility Bcl-2 would protect the cells by preventing caspase activation.

Materials and methods

Cell lines and culture

Apaf-1 $^{-/-}$ (gift from F. Cecconi, Universita Tor Vergata, Rome, Italy, and P. Gruss, Max Planck Institute of Biophysical Science, Göttingen, Germany) and *caspase-9* $^{-/-}$ mice (gift from K. Kuida, Genomic Pharmacology, Vertex Pharmaceuticals, Cambridge, MA; 3450; Cecconi et al., 1998; Kuida et al., 1998) originally derived from 129/sv ES cells and backcrossed over 10 times to C57BL/6, *caspase-2* $^{-/-}$ mice (129/sv; O'Reilly et al., 2002), offspring of intercrossed *caspase-9* $^{+/-}$ and *caspase-2* $^{-/-}$ mice, and their wild-type littermates, were used as sources of fetal liver for production of IL-3–dependent cell lines as described previously (Perkins and

Cory, 1993). In brief, suspensions of E14 fetal liver from wild-type, *Apaf-1* $^{-/-}$, *caspase-9* $^{-/-}$, *caspase-2* $^{-/-}$, and *caspase-9* $^{-/-}$; *caspase-2* $^{-/-}$ embryos were cocultured with cells expressing a Hox 2.4 retrovirus in the presence of a high concentration of IL-3 containing supernatant (5–10%) derived from an IL-3–producing hybridoma cell line. This promotes the conditional immortalization of IL-3–dependent promyeloid lines. After 5 d, nonadherent cells were cultured in soft agar and then a further 10–14 d later compact colonies were individually selected and put back into liquid culture containing IL-3. Rarely, a cell line would differentiate after 2–3 wk in culture (indicated by changed morphology and becoming adherent). Such lines were not used. Each line was tested for IL-3 dependence as indicated by inhibition of proliferation in the absence of IL-3. No clones that maintained a suspension phenotype but continued to proliferate in the absence of IL-3 were encountered. Cells were maintained in DME supplemented with 10% FCS and 3% IL-3 supernatant.

Plasmids and transfection

To generate cells expressing empty vector or Bcl-2, 10^7 cells were washed in balanced salt solution and then resuspended in 400 μl of balanced salt solution and 20% FCS, and electroporated with 10–15 μg of either empty pEF vector or pEF containing a human Bcl-2 construct (Huang et al., 1997) linearized with Fsp-1 (New England Biolabs, Inc.). Cells were then divided into three aliquots (to ensure independent clones) and cultured in soft agar with 3 μg/ml puromycin. After 2–3 weeks, puromycin resistant colonies were selected and tested for Bcl-2 expression by flow cytometry as described previously (Ekert et al., 1999) and by Western blot.

Clonal assays and viability

To assay IL-3 withdrawal induced cell death, cells were washed and suspended in IL-3–deficient media at a density of 10^6 cells/ml and plated in 200 μl aliquots in 96 well plates. Cell viability was determined by PI exclusion using flow cytometry (Becton Dickinson). In assays using etoposide or doxorubicin, cells were plated at a density of 10^5 cells/ml in 48 well plates in normal growth media. Drug was added at the indicated doses. After 24 h, a 100-μl aliquot was used to determine viability by Annexin V-Fluos/PI staining (Roche) as described in the manufacturer's protocol. The remainder of the culture was used in a clonogenic assay (see below).

Fas ligand was generated as described previously (Knight et al., 2001). Jurkat cells were treated with Fas ligand at a 1:20 dilution for 6 or 24 h. A blocking antibody (mouse monoclonal anti-Fas ligand antibody; clone 3C82; Qbiogene) was used at 5 μg/ml.

For clonal assays, cells were washed twice in PBS and then plated at a density of 2×10^4 or 5×10^4 with or without IL-3 in 1 ml of media in 24 well plates. Separate wells were harvested at each time point. At the indicated times, the cells were removed, counted, viability determined by PI uptake by flow cytometry and various dilutions plated in soft agar. After 21 d, the number of colonies was counted and the number of colony forming units per 1,000 cells plated at time 0 calculated. For clonal assays using cytotoxic drugs, 500 μl (half) of the culture was washed in 10 ml of DME and the cells then resuspended in 500 μl of normal media. 25 and 250 μl of each culture was plated in soft agar and the number of colonies counted at 21 d as before.

Cell lysis and Western blotting

Cells were lysed in 50 mM Tris-HCl, pH 7.5, 1% SDS, 0.5 mM EDTA, 1 mM DTT, and immediately boiled for 10 min. The lysate was then centrifuged at 13,000 rpm and the supernatant diluted 1:5 in RIPA buffer plus protease inhibitors. Lysates were run either on precast 4–20% gradient gels or 10% gels (Gradipore) and then transferred to nitrocellulose (Hybond-N). The antibodies used were: mouse-specific anti–caspase-9 (Cell Signaling), mouse monoclonal anti–caspase-7 (a gift from Y. Lazebnik, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), anti–caspase-3 (Cell Signaling), rat monoclonal anti–Apaf-1 (clone 18H2; gift from L. O'Reilly, Walter and Eliza Hall Institute for Medical Research), mouse anti–human Bcl-2 (DakoCytomation), rabbit anti–mouse ICAD (BD Biosciences), and anti–Hsp-70 (gift from W. Welch and R. Anderson, Peter McCallum Cancer Institute, Melbourne, Australia).

Intracellular cytochrome *c* staining

Cytochrome *c* release was assayed using a method described previously (Waterhouse and Trapani, 2003). Cells cultured with and without IL-3 were washed in PBS and were resuspended in 200 μl digitonin (120 μg/ml) in buffer (KCl 75 mM, sucrose 250 mM, NaH_2PO_4 1 mM, Na_2HPO_4 8 mM). After a 15-min incubation on ice, formaldehyde was added to a final concentration of 4% in a total volume of 400 μl and the cells were incubated at RT for 1 h. They were then pelleted, washed in PBS, and resuspended in blocking buffer (3% BSA, 0.05% saponin in PBS) and tubes rotated at 4°C for 1 h. Anti–cytochrome *c* antibody (BD Biosciences) was added (1/200) and the cells were rotated overnight at 4°C. The cells were pelleted, washed once in PBS and rotated in blocking buffer containing 1:100 of anti–mouse Ig G FITC (Amersham Biosciences) at 4°C for 1 h. Cells were pelleted, washed once in PBS, and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). Debris was excluded from analysis by gating for intact cells using forward and side scatter parameters.

Image acquisition

Microscopy was performed on a microscope (model IX70; Olympus) using Hoffmann differential contrast microscopy and a 40X objective. The image was acquired using a SPOT camera (model 1.4.0; Diagnostic Instruments) and SPOT software (version 2.2) and saved as TIFF files. The images were imported into Freehand MX (Macromedia) for the compilation of the figure (Fig. 2) and saved as a JPEG file.

Online supplemental material

In Fig. S1, FDC-P1 cells were stably transfected with pEF empty vector or pEF vector containing human Bcl-2, dominant negative FADD, or crmA. Cells were cultured in RPMI with 10% FCS and 0.1% IL-3 supernatant. For IL-3 withdrawal experiments, cells were washed three times in PBS and cultured in RPMI with 10% FCS over 3 d. Viability was determined by PI uptake using flow cytometry. Four independent clones of each construct were used in three independent experiments. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200312031/DC1.

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References

Cecconi, F., G. Alvarezbolado, B.I. Meyer, K.A. Roth, and P. Gruss. 1998. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell.* 94:727–737.

Chinnaiyan, A.M., K. Orourke, B.R. Lane, and V.M. Dixit. 1997. Interaction of CED-4 with CED-3 and CED-9 - a molecular framework for cell death. *Science.* 275:1122–1126.

Ekert, P.G., J. Silke, and D.L. Vaux. 1999. Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. *EMBO J.* 18:330–338.

Gottlieb, E., S.M. Armour, and C.B. Thompson. 2002. Mitochondrial respiratory control is lost during growth factor deprivation. *Proc. Natl. Acad. Sci. USA.* 99:12801–12806.

Hakem, R., A. Hakem, G.S. Duncan, J.T. Henderson, M. Woo, M.S. Soengas, A. Elia, J.L. Delapompa, 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.

Hengartner, M.O., and H.R. Horvitz. 1994. Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. *Nature.* 369:318–320.

Huang, D., S. Cory, and A. Strasser. 1997. Bcl-2, Bcl-X_L and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene.* 14:405–414.

Irmler, M., K. Hofmann, D. Vaux, and J. Tschopp. 1997. Direct physical interaction between the *Caenorhabditis elegans* death proteins CED-3 and CED-4. *FEBS Lett.* 406:189–190.

Knight, M.J., C.D. Riffkin, A.M. Muscat, D.M. Ashley, and C.J. Hawkins. 2001. Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. *Oncogene.* 20:5789–5798.

Kuida, K., T.F. Haydar, C.Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M. 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.

Lassus, P., X. Opitz-Araya, and Y. Lazebnik. 2002. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science.* 297:1352–1354.

Le-Niculescu, H., E. Bonfoco, Y. Kasuya, F.X. Claret, D.R. Green, and M. Karin. 1999. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol. Cell. Biol.* 19:751–763.

Marsden, V.S., L. O'Connor, L.A. O'Reilly, J. Silke, D. Metcalf, P.G. 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.

Moriishi, K., D.C. Huang, S. Cory, and J.M. Adams. 1999. Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. *Proc. Natl. Acad. Sci. USA.* 96:9683–9688.

O'Reilly, L., P. Ekert, N. Harvey, V. Marsden, L. Cullen, D. 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.

Perkins, A., and S. Cory. 1993. Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene. *EMBO J.* 12:3835–3846.

Scott, C.L., M. Schuler, V.S. Marsden, A. Egle, M. Pellegrini, D. Nesic, S. Gerondakis, S.L. Nutt, D.R. Green, and A. Strasser. 2004. Apaf-1 and caspase-9 do not act as tumour suppressors in myc-induced lymphomagenesis or mouse embryo fibroblast transformation. *J. Cell Biol.* 164:89–96.

Seshagiri, S., and L.K. Miller. 1997. *Caenorhabditis elegans* CED-4 stimulates CED-3 processing and CED-3-induced apoptosis. *Curr. Biol.* 7:455–460.

Soengas, M.S., R.M. Alarcon, 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 tumour 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.

Spector, M.S., S. Desnoyers, D.J. Hoeppner, and M.O. Hengartner. 1997. Interaction between the *C. elegans* cell-death regulators CED-9 and CED-4. *Nature.* 385:653–656.

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.

Tsujimoto, Y., L.R. Finger, J. Yunis, P.C. Nowell, and C.M. Croce. 1984. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science.* 226:1097–1099.

Tsujimoto, Y., J. Cossman, E. Jaffe, and C.M. Croce. 1985. Involvement of the Bcl-2 gene in human follicular lymphoma. *Science.* 228:1440–1443.

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.

Vucic, D., H.R. Stennicke, M.T. Pisabarro, G.S. Salvesen, and V.M. Dixit. 2000. ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. *Curr. Biol.* 10:1359–1366.

Waterhouse, N.J., and J.A. Trapani. 2003. A new quantitative assay for cytochrome c release in apoptotic cells. *Cell Death Differ.* 10:853–855.

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.