Antiapoptotic BCL-2 is required for maintenance of a model leukemia

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Summary

Resistance to apoptosis, often achieved by the overexpression of antiapoptotic proteins, is common and perhaps required in the genesis of cancer. However, it remains uncertain whether apoptotic defects are essential for tumor maintenance. To test this, we generated mice expressing a conditional *BCL-2* gene and constitutive *c-myc* that develop lymphoblastic leukemia. Eliminating BCL-2 yielded rapid loss of leukemic cells and significantly prolonged survival, formally validating BCL-2 as a rational target for cancer therapy. Loss of this single molecule resulted in cell death, despite or perhaps attributable to the presence of other oncogenic events. This suggests a generalizable model in which aberrations inherent to cancer generate tonic death signals that would otherwise kill the cell if not opposed by a requisite apoptotic defect(s).

Introduction

A myriad of genetic changes accompanies the evolution of a normal cell to a cancer cell. These changes confer many abnormal properties, including invasion and metastasis, cell cycle aberrations, growth factor-independent proliferation, telomere preservation, angiogenesis, genomic instability, and resistance to apoptosis (Green and Evan, 2002; Hanahan and Weinberg, 2000; Schmitt, 2003). These aberrations constitute modern molecular targets for developing cancer therapeutics. Yet, we have only started to test which of the genetic changes selected for during tumorigenesis are actually required to maintain that cancer (Chin et al., 1999; Felsher and Bishop, 1999; Gunther et al., 2003; Huettner et al., 2000; Pelengaris et al., 1999).

BCL-2, the cardinal antiapoptotic oncogene, is located at the chromosomal breakpoint of t(14;18) bearing human follicular B cell lymphoma (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). Overexpressed BCL-2 opposes the apoptotic death of cells and mice bearing a *Bcl*-2-Ig minigene recapitulating the t(14;18) progress from follicular hyperplasia to high grade lymphoma (Hockenbery et al., 1990; McDonnell et al., 1989; McDonnell and Korsmeyer, 1991; Vaux et al., 1988). High levels of BCL-2 expression have been found in many cancers, including follicular lymphoma (Gaulard et al., 1992), chronic lymphocytic leukemia (Schena et al., 1992), acute myelogenous leukemia (Andreeff et al., 1999), myeloma (Harada et al., 1998), small cell lung cancer (Ben-Ezra et al., 1994), and melanoma (Leiter et al., 2000).

The BCL-2 family of proteins is comprised of multidomain antiapoptotic proteins (including BCL-2), multidomain proapoptotic proteins (BAX, BAK), and the proapoptotic BH3-only proteins (including BID, BAD, and BIM) (Danial and Korsmeyer, 2004). In response to selected death signals, individual proapoptotic BH3-only members are activated. Much of the intrinsic pathway of apoptosis is focused at the surface of mitochondria, where BH3-only proteins either directly or indirectly induce the oligomerization of proapoptotic BAX and BAK (Letai et al., 2002; Wei et al., 2001). Subsequently, the mitochondrial outer membrane is permeabilized, resulting in the release of cytochrome *c*, which forms an apoptosome complex with Apaf-1 and initiator Caspase 9 to induce the activation of effector caspases (Li et al., 1997). By their proteolytic activity, the effector caspases cause widespread cellular dysfunction and cell death. Antiapoptotic members like BCL-2 can disrupt this apoptotic program by binding and sequestering BH3-only proteins, protecting BAX and BAK from oligomerization (Cheng et al., 2001).

Many of the abnormal properties of cancer cells mentioned above violate physiological checkpoints within cells that theoretically would result in the generation of tonic death signals. This suggests that eliminating an antiapoptotic influence such as BCL-2 from such cancers might unmask inherent death signals and result in cell death. To test whether BCL-2 is necessary

SIGNIFICANCE

Cancer cells possess many abnormalities that generate apoptotic signals. Moreover, genetic changes that impede apoptosis (such as p53 loss or BCL-2 overexpression) result in cancer and are often found in cancer, supporting a role for blocking apoptosis during tumorigenesis. Here we show that an apoptotic defect is also necessary for the unique stage of tumor maintenance. We demonstrate that elimination of overexpressed BCL-2 rapidly induces apoptosis and remission of a B-lymphoblastic leukemia. This finding credentials BCL-2 as a legitimate therapeutic target. Furthermore, it suggests that inherent, tonic death signals would kill cancer cells if the apoptotic defect is eliminated.

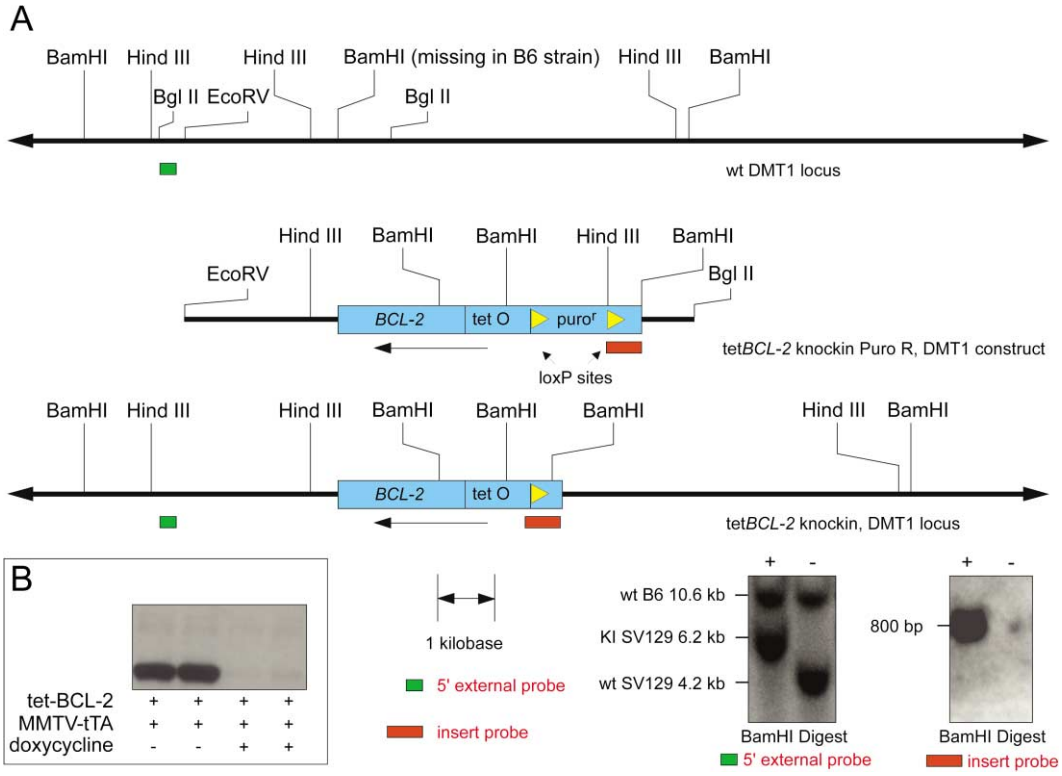

Figure 1. Construction of a conditional BCL-2 transgenic mouse

A: A conditional BCL- 2 allele. A human BCL-2 cDNA under the control of a minimal CMV promoter adjacent to a heptamer of the tet operon (tet-BCL-2) locus was targeted into the DNA methyltransferase I (DMT1) locus. Shown is a schematic of the wt DMT1 locus, the 4 kb EcoRV to Bgl II targeting construct, and final *tet BCL-2* knockin DMT1 locus following cre-mediated deletion of the selectable marker. At bottom are representative Southern blots of correctly integrated (+) and nontargeted (−) clones.

B: Doxycycline eliminates BCL-2 expression. Extracts from dissociated splenocytes from doubly transgenic mice were subjected to immunoblot for BCL-2. Left two lanes are from mice that received no doxycycline; right two lanes are from mice that received 200 μg/ml in drinking water for 2 weeks prior to sacrifice.

for maintaining as well as developing a cancer, we generated a mouse model in which the expression of BCL-2 is conditional.

Results

A conditional BCL-2 trangene is tightly controlled by doxycycline

We obtained germline transmission of a knockin allele in which a human *BCL-2* cDNA under the control of multimerized tet-operons (Gossen et al., 1995) adjacent to a minimal CMV promoter was targeted to the DNA methyltransferase1 (DMT1) locus (tet-*BCL-2*) (Figure 1A). The DNA methyltransferase 1 (DMT1) locus was chosen for its high rate of integration and consistent expression patterns (Li et al., 1992). *tet-BCL*-2 mice were intercrossed with a line bearing the tetracycline transactivator (tTA) under control of the MMTV promoter (MMTV-tTA), known to express tTA in spleen as well as epithelial tissues (Hennighausen et al., 1995; Huettner et al., 2000). Progeny bearing both alleles (doubly transgenic) expressed BCL-2 in splenocytes and bone marrow cells as well as in epithelial cells, including mammary, sebaceous, salivary, basal epidermal cells, thymic epithelium, and testes (data not shown). BCL-2 expression was effectively ablated when doxycycline was added to the drinking water (Figure 1B). At 18 months of age, doubly transgenic mice did not develop malignancies and demonstrated normal survival (not shown).

tet-*BCL-2*/MMTV-tTA/Eμ-*myc* mice develop a lymphoblastic leukemia

Since BCL-2 oncogenesis requires additional aberrations, we introduced deregulated *c-myc*, since this is a naturally occurring secondary event known to complement BCL-2 in mouse and human lymphomagenesis (Gauwerky et al., 1988; McDonnell and Korsmeyer, 1991; Schmitt et al., 2000; Strasser et al., 1993; Vaux et al., 1988). Mice singly transgenic for Eμ-myc develop a bulky B cell lymphoma, with a relatively normal white blood count (WBC) until late in the course of malignancy (Adams et al., 1985). In a nonconditional model, mice expressing both BCL-2 and c-*myc* under the control of the immunoglobulin enhancer (Eμ) develop an immature lymphoblastic leukemia within days of birth (Strasser et al., 1990). Similarly, our triply transgenic (tet-*BCL-2*/MMTV-tTA/Eμ-*myc*) mice uniformly developed lymphoblastic leukemia by 2 weeks of age (WBC ~50,000/μl). Over weeks, the WBC rose to 2×10^5 to 2×10^6 per microliter. The bone marrow was replaced with lymphoblasts expressing human BCL-2 (Figures 2C–2F), which also infiltrated the spleen (Figures 2A and 2B), resulting in marked splenomegaly (spleen weight 5- to 10-fold > wt mice). Lymphoblasts effaced the

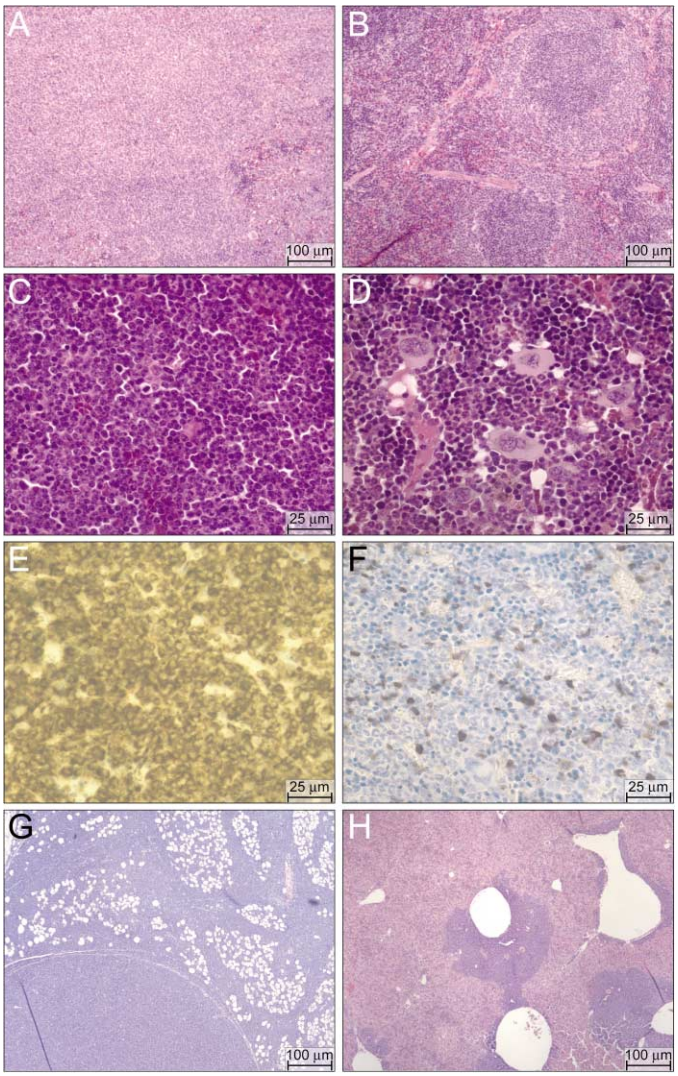

Figure 2. Leukemic cell involvement of spleen, bone marrow, lymph node, and liver

A: Splenic white and red pulp from tet-*BCL*-2/MMTV-tTA/Eμ-*myc* mouse is replaced by leukemic infiltrate with evidence of extramedullary hematopoiesis (lower right), hematoxylin and eosin (H+E).
B: Normal appearance of spleen in tet-*BCL*-2/MMTV-tTA transgenic mouse.
C: Bone marrow from tet-*BCL*-2/MMTV-tTA/Eμ-*myc* mouse is replaced with leukemic cells, H+E. Compare with normal bone marrow in **D**.
D: Normal bone marrow from tet-*BCL*-2/MMTV-tTA mouse displaying trilineage hematopoiesis, H+E.
E: Immunohistochemistry detects human BCL-2 in leukemic cells of bone marrow from tet-*BCL*-2/MMTV-tTA/Eμ-myc mouse.
F: Human BCL-2 is detected only in a minority of cells in bone marrow from tet-*BCL*-2/MMTV-tTA mouse.
G: Leukemia cells from tet-*BCL*-2/MMTV-tTA/Eμ-*myc* mouse efface lymph node architecture and invade adjacent fat, H+E.
H: Leukemic cells from tet-*BCL*-2/MMTV-tTA/Eμ-*myc* mouse invade liver, H+E.

lymph node architecture (Figure 2G) and often infiltrated other tissues including the liver (Figure 2H).

Immunophenotypic analysis revealed that all leukemic cells were of B lymphocyte origin. The majority of leukemias represented a continuum from pro-B cell ($CD43^+$, $B220^+$, $CD19^+$, $CD4^-$, HSA^+ [Fraction C]) to pre-B cells ($CD43^-$, $B220^+$, $CD19^+$, $CD4^-$, HSA^+ [Fraction D]) (Figure 3A). Four of 14 characterized leukemias were less mature pre-pro-B cells ($CD43^+$, $B220^+$, $CD19^-$, $CD4^+$, HSA^- [Fraction A1]) (Figure 3A; Allman et al., 1999; Hardy and Hayakawa, 2001). To further assess the stage of differentiation as well as clonality of the leukemias, we assessed the configuration of immunoglobulin (Ig) heavy chain gene loci. Pre-pro-B cell leukemias displayed a germline pattern, as expected, whereas the pro-B to pre-B cell stage leukemias demonstrated gene rearrangements indicative of monoclonal or oligoclonal expansions of cells (Figure 3B). As a further assessment of clonality and genomic instability, spectral karyotypes (SKY) were performed. A representative karyotype of a pro-/pre-B cell stage leukemia revealed clonal abnormalities and evidence for genomic instability, including extra chromosomes 6 and 12 (Figure 3C).

BCL-2 is required for leukemia maintenance

To assess whether BCL-2 is required to maintain lymphoblastic leukemia, we followed a matched cohort of 5- to 7-week-old triply transgenic mice with leukemia, 14 of which received doxycycline and 14 of which remained untreated. Administration of doxycycline reduced the level of BCL-2 in peripheral mononuclear cells within 3 days (Figure 4A) and resulted in a dramatic reduction in the WBC (Figures 4B–4D). Mice treated with doxycycline, with only one exception, normalized their WBC over 10 days of treatment (Figure 4B). A subsequent cohort of mice revealed that the bone marrow was no longer replaced by leukemic lymphoblasts and demonstrated the resumption of normal trilineage hematopoiesis (Figures 4E and 4F). Moreover, within 2 weeks of treatment, the spleen returned to normal (Figure 4G).

Apoptosis of lymphoblasts was noted in the bone marrow of mice 48 hr following initiation of doxycycline (Figures 5A and 5B). Addition of doxycycline to cultured primary leukemia cells from the bone marrow of triply transgenic mice resulted in apoptotic death, providing evidence for a cell-autonomous effect of eliminating BCL-2 (Figure 5C). Leukemic mice that demonstrated overt signs of illness (lethargy, hunched posture, tachypnea, and loss of skin turgor) resumed normal activity when the WBC returned to normal. However, the WBC did not decline in the untreated cohort, all of which died within 9 weeks of initiating the study (Figures 4B and 6A). The doxycycline-treated mice demonstrated a substantially improved median survival of 145 days versus 82 days for the untreated cohort and a significantly altered survival plot ($p = 0.000123$; Figure 6A). While all untreated mice died by just over 100 days of age, five doxycycline-treated mice survived >200 days, and one has survived longer than 1 year. Comparison of these cohorts demonstrates that elimination of BCL-2 expression clearly resulted in remission of the leukemia and prolonged survival of the mice.

The late deaths of doxycycline-treated triply transgenic mice resulted from a bulky B cell lymphoma, essentially indistinguishable in phenotype, histology, WBC, and tissue distribution to that developing in singly transgenic *Eμ-myc* mice (Adams et al., 1985). Moreover, the time of onset of these tumors and the median survival of doxycycline-treated triply transgenic mice (145 days) is also comparable to that of the 15 singly transgenic *Eμ-myc* mice (146 days) followed in our study, and their survival curves do not differ significantly ($p = 0.55$; Figure 6A). These late-onset B cell lymphomas would be expected since the constitutive *c-myc* transgene expression continues unabated, as it is not responsive to doxycycline. Only an occasional late-onset

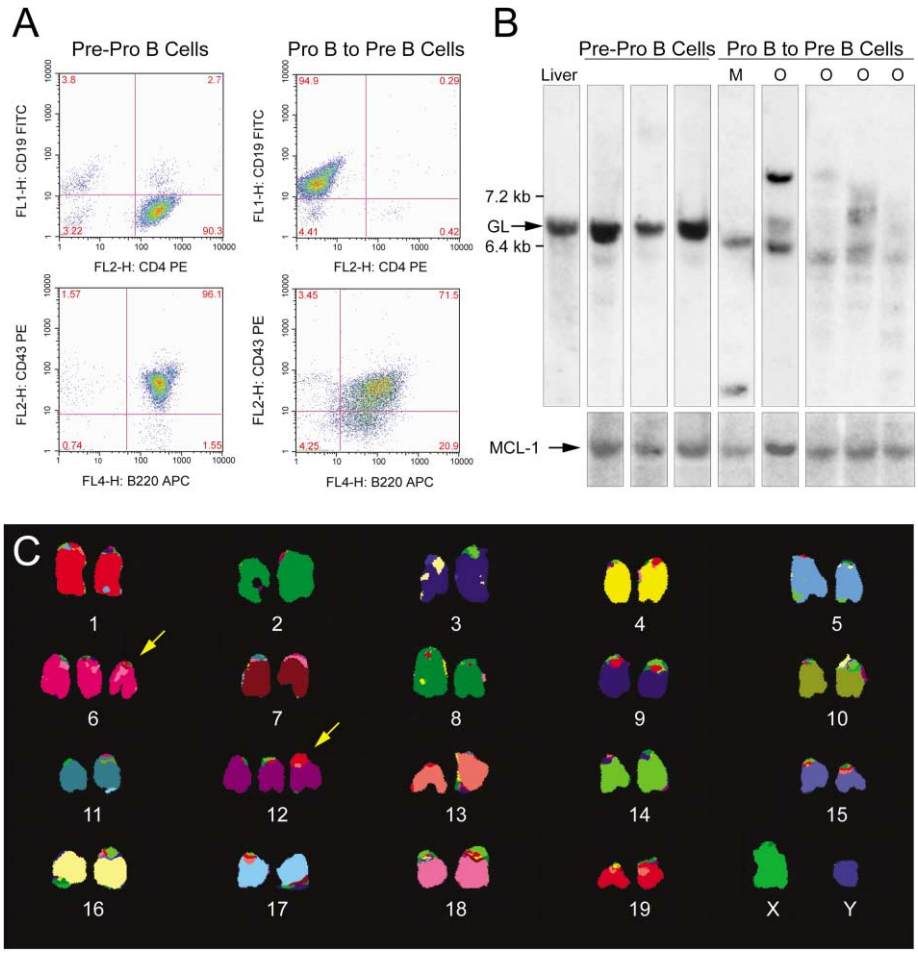

Figure 3. Leukemias are clonal lymphoblastic leukemias

A: Immunophenotype of representative leukemias. A pre-pro [Fraction A1] ($CD43^+$, $B220^+$, $CD19^-$, $CD4^+$, HSA^-) and a pro to pre [Fraction C-D] ($CD43^{var}$, $B220^+$, $CD19^+$, $CD4^-$, HSA^+) leukemia are shown.

B: Southern analysis of immunoglobulin heavy chain configuration of leukemias. Three $B220^+$/$CD19^-$/$CD4^+$ leukemias demonstrate germline (GL) configuration; four $B220^+$/$CD19^+$/$CD4^-$ leukemias demonstrate a recombined pattern. One displays a monoclonal (M) and four an oligoclonal (O) pattern. As a control for DNA quality and loading, the blot was stripped and reprobed for MCL-1, shown at bottom.

C: Spectral karyotype of a leukemia demonstrating a clonal abnormality. Shown is the dominant karyotype: 42, XY, +6, +12.

lymphoma in doxycycline-treated mice possessed human BCL-2 expression, ruling out an escape from doxycycline regulation as the explanation for progression in the majority of tumors (Figure 6B). A unique Ig gene rearrangement, distinct from the original tumor, was noted in a late-onset lymphoma of a doxycycline-treated mouse supporting the emergence of a clonally distinct tumor (Figure 6E). In summary, the late-onset B cell lymphomas that follow ablation of BCL-2 expression resemble *Eμ-myc* driven lymphomagenesis. This is consistent with the emergence of independent lymphomas, although a clonal relationship with the initial leukemia can not be completely excluded with available technologies. Such *Eμ-myc*-expressing tumors could be expected to acquire a complementary aberration in apoptosis; however, these spontaneous, secondary events would not be under the control of doxycycline.

The transplantable leukemia is susceptible to doxycycline ablation of BCL-2

The transplantability of a tumor is a standard measure of its malignant potential. Three cohorts of sublethally irradiated (650 rad) recipient mice received from 1.0×10^6 to 2.5×10^6 bone marrow cells from triply transgenic donor mice with pre-pro-B or pre-B cell leukemia. In each cohort, bone marrow cells from leukemic mice, when injected into irradiated recipient mice, transferred a lethal lymphoblastic leukemia (Figures 6C and 6D). Treatment with doxycycline once again eliminated the leukemic cells from the blood (Figure 6C) and substantially extended survival in all the recipient groups (Figure 6D).

Differential sensitivity of mitochondria from lymphoblastic leukemias versus nonmalignant FL5.12 cells to BAX oligomerization and cytochrome *c* release

The rapid induction of apoptosis that proved coordinate with the elimination of BCL-2 in the triply transgenic lymphoblasts prompted us to assess whether overexpressed BCL-2 was sequestering BH3-only proteins that were chronically activated by myc-driven oncogenesis. To test this, we purified mitochondria from FL5.12 (a nonmalignant pro-B lymphocyte line) and from triply transgenic pro-B lymphoblastic leukemia cells to compare their differential susceptibility to apoptosis. An "activating" BH3 domain (BIM) induced the activation and oligomerization of BAX and BAK and resulted in release of cytochrome *c* from normal FL5.12 mitochondria and leukemia mitochondria (Figures 7A and 7B; Letai et al., 2002; Wei et al., 2001). In contrast, a "sensitizing" BH3 domain (BAD) cannot activate BAX or BAK directly, but does bind antiapoptotic BCL-2 with high affinity and will displace other activator BH3 peptides present in the BCL-2 pocket (Letai et al., 2002; Wei et al., 2001). Whereas FL5.12 mitochondria are unaffected, the mitochondria from the lymph-

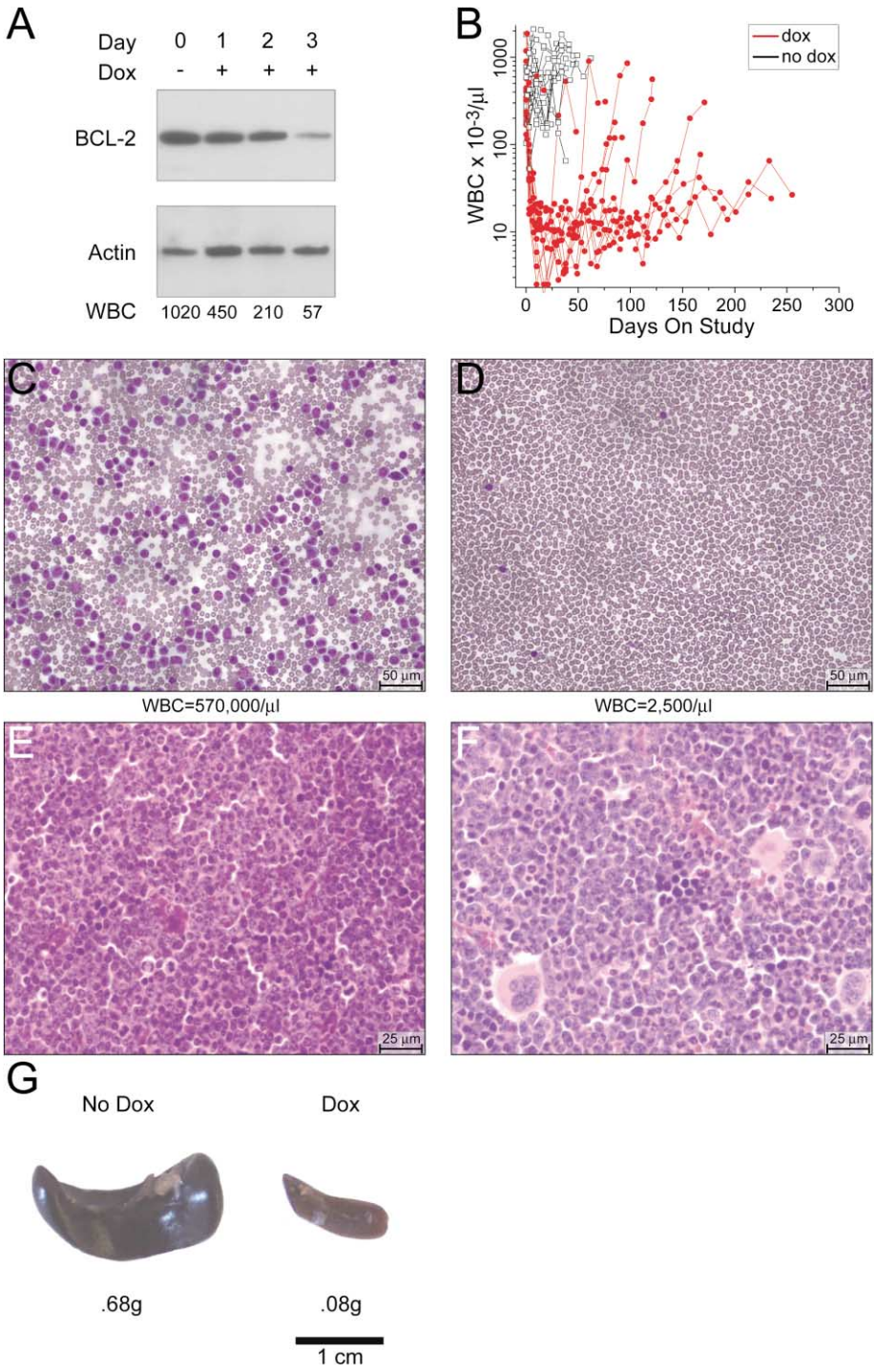

Figure 4. Removal of BCL-2 induces remission of leukemia and improves survival

A: Anti-human BCL-2 immunoblot. The four lanes demonstrate decreasing BCL-2 levels in circulating mononuclear cells over 3 days following doxycycline administration, in correlation with the WBC ($\times 10^{-3}/\mu l$).

B: Loss of BCL-2 normalizes the WBC in the 28 mouse cohort.

C and D: Peripheral blood smear of a triply transgenic mouse before (**C**) and 4 weeks after (**D**) treatment with doxycycline.

E: Bone marrow of leukemic mouse demonstrating replacement by a monotonous lymphoblast population with frequent mitotic figures (H+E).

F: Bone marrow of previously leukemic mouse 14 days after initiation of doxycycline treatment (H+E). Normal proportions of megakaryocytes, erythroid progenitors, and granulocyte precursors are now present.

G: Spleens from triply transgenic littermates at 7 weeks of age, in which the mouse on the right was given doxycycline 500 µg/ml in drinking water starting at 5 weeks of age.

oblastic leukemia cells release cytochrome *c* in response to sensitizing BAD BH3 as well as activating BIM BH3 (Figures 7A and 7B). Consistent with this, the sensitizing BAD BH3 domain induces the oligomerization of BAX in mitochondria from lymphoblastic leukemia cells, but not the mitochondria from normal pro-B cells (Figure 7B). These data support the hypothesis that in the leukemic cells, but not the nonmalignant FL5.12 cells, BCL-2, is sequestering significant quantities of chronically activated BH3 proteins.

Discussion

Abnormalities in genes affecting apoptosis have been found in nearly every cancer, suggesting they may be necessary to develop cancer (Green and Evan, 2002; Hanahan and Weinberg, 2000; Schmitt, 2003). Consequently, the apoptotic pathway including BCL-2 is a prominent target for drug development. A principal mechanism whereby BCL-2 protects cells includes binding and sequestering "BH3-only" molecules, thereby pre-

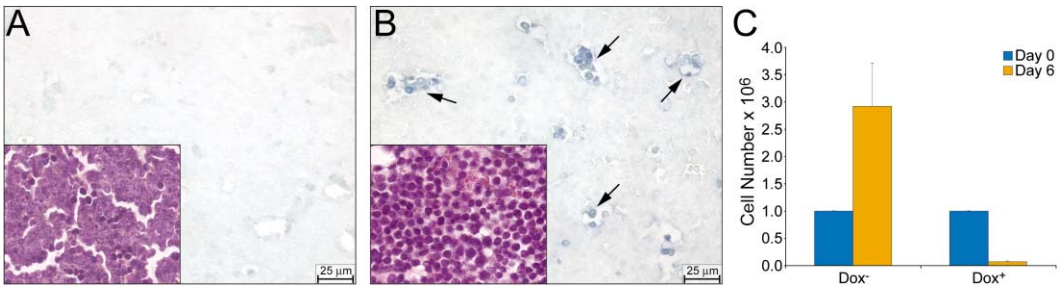

Figure 5. Leukemic cells die an apoptotic death when BCL-2 expression is eliminated

A: TUNEL staining of bone marrow from a leukemic mouse, methyl green counterstain.
B: TUNEL staining of a leukemic mouse 48 hr after addition of doxycycline to drinking water. Arrows indicate selected loci of TUNEL-positive leukemic cells undergoing apoptotic death. Insets for **A** and **B** contain an H+E stained serial section demonstrating replacement of marrow with leukemic cells. The mean number of TUNEL-positive cells per 40× field from the untreated (7.9) and doxycycline-treated (77.3) marrows differ with a p value of <.001.
C: Requirement for BCL-2 is autonomous to leukemia cells. Under normal culture conditions, the cell count more than tripled over 6 days. Treatment with doxycycline (1 μg/ml) induced death of >90% of the cultured cells as determined by trypan blue dye exclusion.

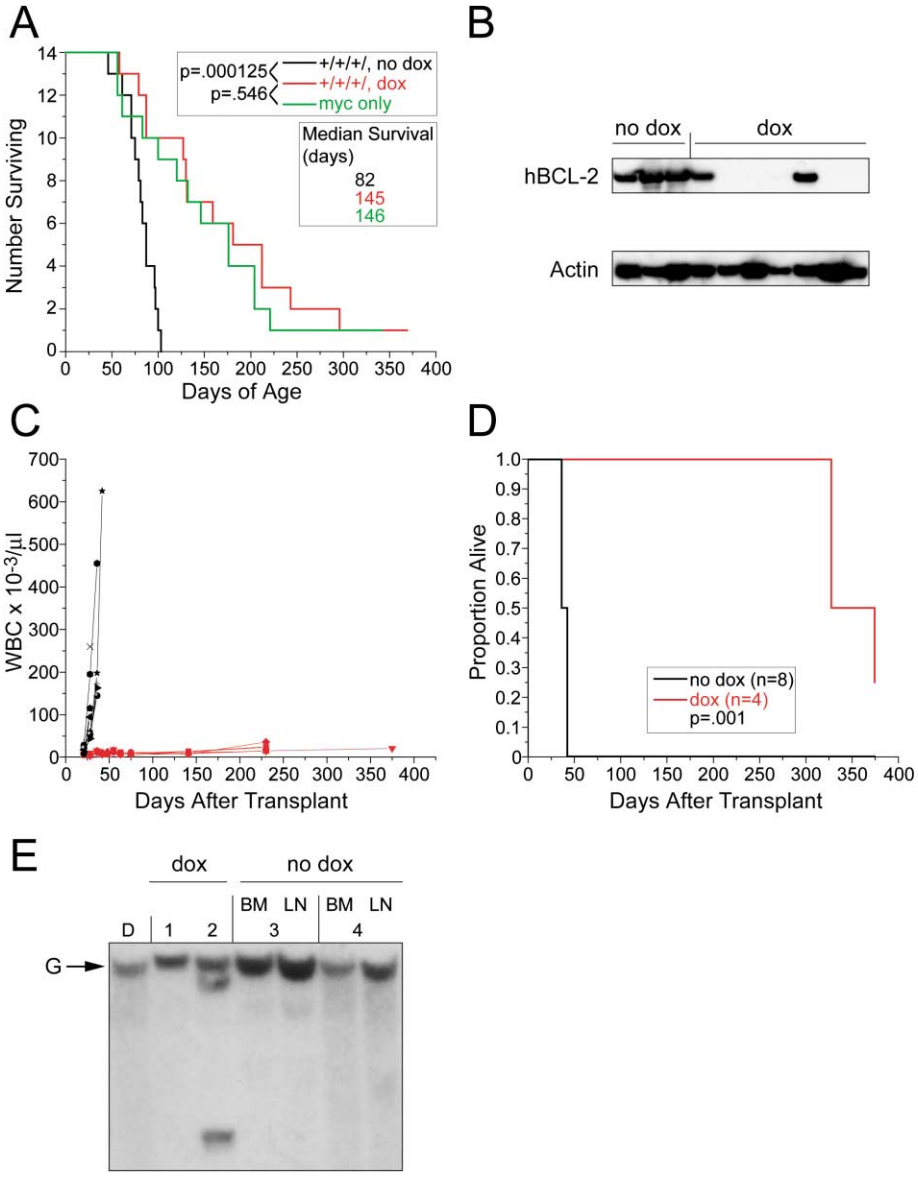

Figure 6. Elimination of BCL-2 expression extends survival of leukemic mice

A: Kaplan-Meier survival plot of treated and untreated cohorts, demonstrating significant survival advantage following ablation of BCL-2 expression (p = 0.000125). Leukemic mice treated with doxycycline have a survival curve that is indistinguishable from the myc-only cohort.
B: Lymphomas arising in doxycycline-treated mice usually lack human BCL-2 transgene expression. First three lanes contain lysates of leukemias from untreated triply transgenic mice. Next 7 lanes contain lysates of a lymphomas found in mice treated with doxycycline. BCL-2 immunoblot shown at top, actin immunoblot shown below.
C: The leukemia is transplantable, and the leukemia is prevented by doxycycline treatment. Bone marrow cells from a leukemic mouse were injected intravenously into 13 sublethally irradiated (650 rad) recipients. WBC of untreated mice (black symbols) or mice treated with doxycycline (500 μg/ml) administered in the water on the day of transplantation (red symbols) are presented.
D: Transplanted mice die of leukemia, while doxycycline-treated recipients display prolonged survival. Shown is one representative cohort of three independent transplant experiments that received 2.4 × 10^6 bone marrow cells from a donor with pre-B cell leukemia. Irradiated control mice that were not transplanted survived >1 year.
E: Emergence of a distinct clone in a doxycycline-treated late-onset B cell lymphoma. Southern blot of immunoglobulin heavy chain gene configuration. Lane D, germline configuration of donor leukemic bone marrow. Lanes 1 and 2, bone marrows of late-onset lymphoid malignancies in doxycycline-treated transplant recipients. Lane 2 shows a new clonal rearrangement. Lanes 3 and 4, bone marrow (BM) and lymph node (LN) from untreated recipient mice that rapidly succumbed to leukemia and display the germline configuration of the donor cells.

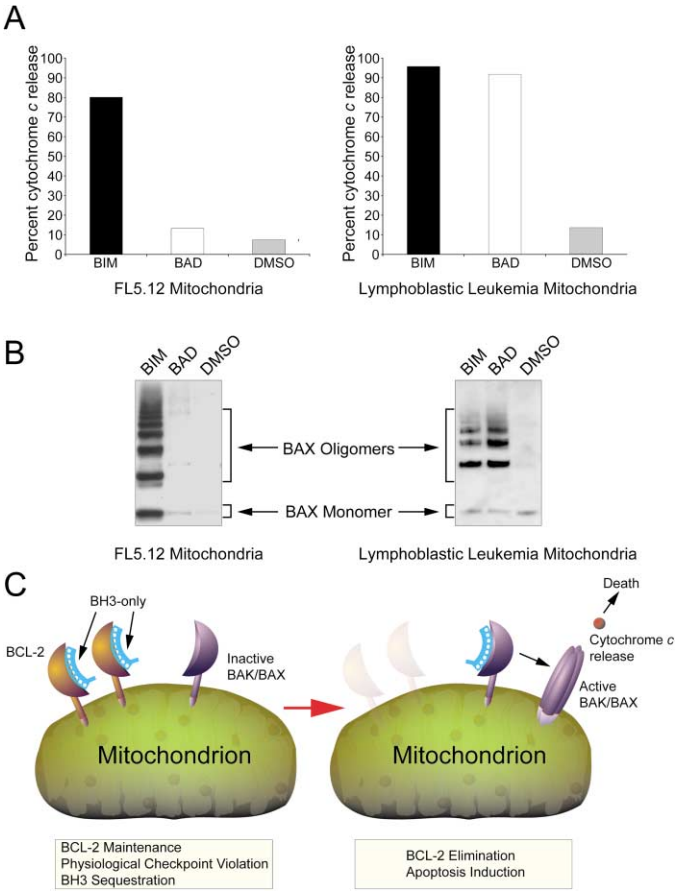

Figure 7. Models of BCL-2 dependence in cancer

A: Mitochondria from cancer cells are selectively sensitized to BCL-2 antagonism. Mitochondria were isolated from a nonmalignant pro-B cell line (FL5.12) or from a mouse pro-B lymphoblastic leukemia. Mitochondria were treated with 10 μM (leukemia) or 30 μM (FL5.12) BIM BH3 or 100 μM BAD BH3 peptides or a solvent only 1% DMSO central. The sensitizer BAD BH3 promotes cytochrome c release only from the malignancy-derived mitochondria. Shown are representative examples of at least three independent experiments.

B: Mitochondria treated as in **A** were examined for oligomerization of BAX, which correlates with cytochrome c release. Following peptide treatment and BMH crosslinking, an immunoblot against BAX was performed.

C: Schematic representation of the requirement for BCL-2 in tumor maintenance. Left, overexpressed antiapoptotic BCL-2 is required for tumor maintenance. Right, removal (or antagonism) of BCL-2 releases BH3-only molecules resulting in the activation, oligomerization of multidomain proapoptotic BAK or BAX, the permeabilization of the mitochondrial outer membrane, cytochrome c release, and apoptotic death. Genetic aberrations inherent to cancer cells violate physiological checkpoints that chronically activate BH3-only molecules, which are bound and sequestered by BCL-2, protecting BAK, BAX from activation.

venting the activation of the lethal proapoptotic effectors BAX and BAK (Cheng et al., 2001; Wei et al., 2001). Accordingly, considerable efforts are underway to develop peptidomimetics that mimic BH3 domains (Letai et al., 2002; Wang et al., 2000b) to screen and refine small molecules that occupy the BCL-2 pocket (Belli et al., 2003; Kim et al., 2001; Tzung et al., 2001; Wang et al., 2000a) or to regulate *BCL-2* by antisense approaches (Waters et al., 2000). Our model provides strong support for the heretofore untested supposition that antagonism of BCL-2 would prove toxic to cancer cells. *Bcl-2*-null mice, while they display developmental kidney defects and loss of lymphocytes and melanocytes over time, do suggest that pharmacologic inhibition of BCL-2 may be tolerated by normal tissues for defined periods (Veis et al., 1993). Other models conditional for the expression of transforming oncogenes including MYC (Felsher and Bishop, 1999; Pelengaris et al., 1999), RAS (Chin et al., 1999), BCR-ABL (Huettner et al., 2000), and WNT (Gunther et al., 2003) have witnessed tumor regression when the oncogene expression was eliminated. The *BCL-2* conditional allele examined here provides the first clear evidence that an apoptosis regulator is required for tumor maintenance.

Why is the lymphoblastic leukemia here so singularly dependent upon BCL-2? One model would hold that the initiating oncogenic event is sacrosanct, a version of a synthetic lethal mutation (Hartwell et al., 1997). The overexpression of BCL-2, like the t(14;18) in human follicular lymphoma, occurs first. Subsequent complementary mutations would reside in other pathways and actually depend on the presence of BCL-2, making BCL-2 inviolate. A second, more broadly applicable model argues that inherent to the transformation process, all cancers violate physiological checkpoints that would normally trigger their demise, unless neutralized by an essential apoptotic defect(s) (Figure 7C). The latter model predicts that whether acquired early or late, aberrations in apoptosis constitute a vulnerable target for therapeutic intervention that might prove common to all cancers. For example, BAD sensitizing BH3 domain, which binds BCL-2, initiates a displacement reaction that results in the activation of BAX on the mitochondria from malignant cells, but not nonmalignant counterparts (Figures 7A and 7B). This provides a plausible explanation as to why the ablation of BCL-2 is singularly effective in inducing apoptosis and remission of leukemia. Either elimination of BCL-2 or its inhibition by a BH3 mimetic would release BH3-only proteins, constitutively present in cancer cells, which are capable of activating BAX, BAK. Our data support a model in which cancer cells are subject to tonic death signaling, which must be opposed by an apoptotic defect to maintain cancer cell survival. In the case of this mouse model, it is likely that MYC itself is responsible for some of the death signaling, either directly or indirectly inducing "BH3-only" genes (Iaccarino et al., 2003). The leukemia model here provides an initial proof of concept that a cancer can be killed by correcting an apoptotic defect.

Experimental procedures

Production of transgenic mice

A DNA construct containing DMT targeting sequences, a minimal CMV promoter, and the tet operator sequences was electroporated into an SV129/Jae ES cell line. The selectable marker was removed by transient expression of cre-recombinase. Correctly targeted ES cells were injected into blastocysts. Chimeric progeny were bred to B6 mice and the targeted allele propagated through the germline.

Monitoring leukemia

Leukemia was diagnosed by examination of peripheral blood smears (Dip Quick, JorVet J-322). White blood counts (WBC) were determined by an automated Coulter counter or confirmed by manual counts, which gave comparable results. Manual counts were performed by examining peripheral blood from a razor-nicked tail diluted in buffer (saponin 0.3%, Hoechst 33258 1 μg/ml, EDTA 25 mM in phosphate-buffered saline) using a hemacytometer.

Histological analysis and immunohistochemistry
Tissue samples were fixed in formalin and embedded in paraffin. BCL-2 immunoperoxidase staining used the DAKO #M0887 mouse monoclonal anti-human BCL-2 antibody.

Leukemic cell culture
Leukemic cells from bone marrow were grown in IMDM supplemented with 10% fetal calf serum, IL-7 10 ng/ml, FLT-3 ligand 5 ng/ml, and SCF 10 ng/ml.

TUNEL staining
Formalin-fixed, paraffin-embedded sections were stained using the In Situ Death Detection Kit, AP (Roche).

Immunoblot analysis
Lysates of cells or mitochondria were size fractionated on NuPAGE 4%–12% Bis-Tris polyacrylamide gels (Invitrogen). Human BCL-2 was detected using the 6C8 hamster anti-human BCL-2 monoclonal antibody (Hockenbery et al., 1990). Analysis of Bax oligomers by BAX crosslinking was performed as previously described (Letai et al., 2002). Cytochrome *c* was detected using a mouse monoclonal antibody (Pharmingen 556433). BAX was detected using a rabbit polyclonal antibody (N-20, Santa Cruz).

Southern blot
Genomic DNA was prepared from leukemia cells. 10 μg of DNA was digested with EcoRI and electrophoresed through a 0.8% agarose gel, transferred to a positively charged nylon membrane, and hybridized with a radiolabeled 450 bp Hind III to NgoM IV fragment of the IgJH region (Adams et al., 1985).

Spectral karyotype
For SKY analysis, metaphase spreads were prepared from manually dissociated leukemic cells cultured overnight in RPMI 1640 with 15% fetal calf serum in the presence of growth factors (IL-7, 10 ng/ml; IL-4, 5 ng/ml; SCF, 10 ng/ml; FLT-3 ligand, 5 ng/ml) and Colcemid (50 ng/ml). Cells were swelled gently in .068 M KCl and then fixed in methanol/acetic acid (3:1). Whole chromosome paints (Spectral Imaging, Carlsbad, CA) were used per the supplier's recommendations. Images were acquired with an Olympus microscope and Applied Imaging (Santa Clara, CA) GENUS software.

FACS
Cells stained with fluorescently conjugated antibodies were analyzed on a FACSCalibur (Becton-Dickinson).

Statistics
p values were obtained by log-rank test or two-sample t test as appropriate (S-PLUS 2000, MathSoft, Inc.).

Cytochrome *c* release
Release of cytochrome *c* from mitochondria was performed by quantitation of cytochrome *c* in pellet and supernatant fractions following treatment. Quantitation was performed either by ELISA (MCTCD, R & D systems) or by densitometric analysis of cytochrome *c* immunoblot.

BMH crosslinking
1,6 bismaleimidohexane crosslinking of BAX was performed as previously described (Letai et al., 2002).

Mitochondria
Mitochondria were prepared as previously described (Letai et al., 2002).

Peptides
Synthesis and sequence of BH3 peptides was described previously (Letai et al., 2002).

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References

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

Allman, D., Li, J., and Hardy, R.R. (1999). Commitment to the B lymphoid lineage occurs before DH-JH recombination. J. Exp. Med. *189*, 735–740.

Andreeff, M., Jiang, S., Zhang, X., Konopleva, M., Estrov, Z., Snell, V.E., Xie, Z., Okcu, M.F., Sanchez-Williams, G., Dong, J., et al. (1999). Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid. Leukemia *13*, 1881–1892.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., and Korsmeyer, S.J. (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell *41*, 899–906.

Belli, B., Deckwerth, T., Al Assaad, S., Martineau, D., Armstrong, R., Tomaselli, K., Bruncko, M., Elmore, S., Fesik, S.W., Hajduk, P., et al. (2003). A-385358, a potent BCL-XL/BCL-2 antagonist induces apoptosis in tumor cell lines. Proc. AACR *44*.

Ben-Ezra, J.M., Kornstein, M.J., Grimes, M.M., and Krystal, G. (1994). Small cell carcinomas of the lung express the Bcl-2 protein. Am. J. Pathol. *145*, 1036–1040.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell *8*, 705–711.

Chin, L., Tam, A., Pomerantz, J., Wong, M., Holash, J., Bardeesy, N., Shen, Q., O'Hagan, R., Pantginis, J., Zhou, H., et al. (1999). Essential role for oncogenic Ras in tumour maintenance. Nature *400*, 468–472.

Cleary, M.L., and Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. USA *82*, 7439–7443.

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205–219.

Felsher, D.W., and Bishop, J.M. (1999). Reversible tumorigenesis by MYC in hematopoietic lineages. Mol. Cell *4*, 199–207.

Gaulard, P., d'Agay, M.F., Peuchmaur, M., Brousse, N., Gisselbrecht, C., Solal-Celigny, P., Diebold, J., and Mason, D.Y. (1992). Expression of the bcl-2 gene product in follicular lymphoma. Am. J. Pathol. *140*, 1089–1095.

Gauwerky, C.E., Haluska, F.G., Tsujimoto, Y., Nowell, P.C., and Croce, C.M. (1988). Evolution of B-cell malignancy: pre-B-cell leukemia resulting from MYC activation in a B-cell neoplasm with a rearranged BCL2 gene. Proc. Natl. Acad. Sci. USA *85*, 8548–8552.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*, 1766–1769.

Green, D.R., and Evan, G.I. (2002). A matter of life and death. Cancer Cell *1*, 19–30.

Gunther, E.J., Moody, S.E., Belka, G.K., Hahn, K.T., Innocent, N., Dugan,

K.D., Cardiff, R.D., and Chodosh, L.A. (2003). Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev. *17*, 488–501.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Harada, N., Hata, H., Yoshida, M., Soniki, T., Nagasaki, A., Kuribayashi, N., Kimura, T., Matsuzaki, H., and Mitsuya, H. (1998). Expression of Bcl-2 family of proteins in fresh myeloma cells. Leukemia *12*, 1817–1820.

Hardy, R.R., and Hayakawa, K. (2001). B cell development pathways. Annu. Rev. Immunol. *19*, 595–621.

Hartwell, L.H., Szankasi, P., Roberts, C.J., Murray, A.W., and Friend, S.H. (1997). Integrating genetic approaches into the discovery of anticancer drugs. Science *278*, 1064–1068.

Hennighausen, L., Wall, R.J., Tillmann, U., Li, M., and Furth, P.A. (1995). Conditional gene expression in secretory tissues and skin of transgenic mice using the MMTV-LTR and the tetracycline responsive system. J. Cell. Biochem. *59*, 463–472.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature *348*, 334–336.

Huettner, C.S., Zhang, P., Van Etten, R.A., and Tenen, D.G. (2000). Reversibility of acute B-cell leukaemia induced by BCR-ABL1. Nat. Genet. *24*, 57–60.

Iaccarino, I., Hancock, D., Evan, G., and Downward, J. (2003). c-Myc induces cytochrome c release in Rat1 fibroblasts by increasing outer mitochondrial membrane permeability in a Bid-dependent manner. Cell Death Differ. *10*, 599–608.

Kim, K.M., Giedt, C.D., Basanez, G., O'Neill, J.W., Hill, J.J., Han, Y.H., Tzung, S.P., Zimmerberg, J., Hockenbery, D.M., and Zhang, K.Y. (2001). Biophysical characterization of recombinant human Bcl-2 and its interactions with an inhibitory ligand, antimycin A. Biochemistry *40*, 4911–4922.

Leiter, U., Schmid, R.M., Kaskel, P., Peter, R.U., and Krahn, G. (2000). Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. Arch. Dermatol. Res. *292*, 225–232.

Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S., and Korsmeyer, S.J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell *2*, 183–192.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479–489.

McDonnell, T.J., and Korsmeyer, S.J. (1991). Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature *349*, 254–256.

McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J.P., and Korsmeyer, S.J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell *57*, 79–88.

Pelengaris, S., Littlewood, T., Khan, M., Elia, G., and Evan, G. (1999). Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. Mol. Cell *3*, 565–577.

Schena, M., Larsson, L.G., Gottardi, D., Gaidano, G., Carlsson, M., Nilsson, K., and Caligaris-Cappio, F. (1992). Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. Blood *79*, 2981–2989.

Schmitt, C.A. (2003). Senescence, apoptosis and therapy—cutting the lifelines of cancer. Nat. Rev. Cancer *3*, 286–295.

Schmitt, C.A., Rosenthal, C.T., and Lowe, S.W. (2000). Genetic analysis of chemoresistance in primary murine lymphomas. Nat. Med. *6*, 1029–1035.

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

Strasser, A., Harris, A.W., and Cory, S. (1993). E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. Oncogene *8*, 1–9.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C.M. (1985). The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science *229*, 1390–1393.

Tzung, S.P., Kim, K.M., Basanez, G., Giedt, C.D., Simon, J., Zimmerberg, J., Zhang, K.Y., and Hockenbery, D.M. (2001). Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. Nat. Cell Biol. *3*, 183–191.

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

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229–240.

Wang, J.L., Liu, D., Zhang, Z.J., Shan, S., Han, X., Srinivasula, S.M., Croce, C.M., Alnemri, E.S., and Huang, Z. (2000a). Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc. Natl. Acad. Sci. USA *97*, 7124–7129.

Wang, J.L., Zhang, Z.J., Choksi, S., Shan, S., Lu, Z., Croce, C.M., Alnemri, E.S., Korngold, R., and Huang, Z. (2000b). Cell permeable Bcl-2 binding peptides: a chemical approach to apoptosis induction in tumor cells. Cancer Res. *60*, 1498–1502.

Waters, J.S., Webb, A., Cunningham, D., Clarke, P.A., Raynaud, F., di Stefano, F., and Cotter, F.E. (2000). Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. J. Clin. Oncol. *18*, 1812–1823.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727–730.