Expression of *bbc3*, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals

Jia-wen Han*, Cathy Flemington*, Anne B. Houghton*, Zhengming Gu†, Gerard P. Zambetti†, Robert J. Lutz*, Li Zhu‡§, and Thomas Chittenden*¶

*ImmunoGen, Inc., 128 Sidney Street, Cambridge, MA 02139; †Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38105; and ‡Clontech Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, CA 94303

Edited by Suzanne Cory, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, and approved August 2, 2001 (received for review April 27, 2001)

BH3-only proteins function at a proximal point in a conserved cell death pathway by binding, through their BH3 domains, to other Bcl-2 family members and triggering mitochondrial events associated with apoptosis. Here, we describe a strongly pro-apoptotic BH3-only protein, designated Bbc3, whose expression increases in response to diverse apoptotic stimuli. *bbc3* mRNA levels were induced by exposure to DNA-damaging agents and by wild-type p53, which mediates DNA damage-induced apoptosis. p53 transactivated *bbc3* through consensus p53 binding sites within the *bbc3* promoter region, indicating that *bbc3* is a direct target of p53. Additionally, *bbc3* mRNA was induced by p53-independent apoptotic stimuli, including dexamethasone treatment of thymocytes, and serum deprivation of tumor cells. Insulin-like growth factor-1 and epidermal growth factor, growth factors with broad anti-apoptotic activity, were each sufficient to suppress Bbc3 expression in serum-starved tumor cells. These results suggest that the transcriptional regulation of *bbc3* contributes to the transduction of diverse cell death and survival signals.

The Bcl-2 family proteins regulate the cellular response to apoptotic stimuli (1, 2). Members of this family can function to either suppress or promote cell death, and are characterized by the presence of up to four conserved amino acid motifs, termed Bcl-2 homology (BH) domains (1, 2). The BH3 domain is a uniquely important functional element within the pro-apoptotic class of Bcl-2-related proteins, mediating their ability to dimerize with other Bcl-2-related proteins, and promote apoptosis (3, 4). The function of BH3 as a cell death domain has been further revealed by an emerging group of "BH3-only" proteins, which share only BH3 in common with other Bcl-2 family members. This class of proteins, which includes bcl-2-interacting killer (Bik), bcl-x_L/bcl-2-associated death promoter (Bad), BH3-interacting domain death agonist (Bid), Bcl-2-interacting mediator of cell death (Bim), egg-laying-defective-1 (Egl-1), and harakiri (Hrk), uses BH3 for binding to anti-apoptotic members of the Bcl-2 family and for inducing apoptosis (4).

Biochemical and genetic evidence indicates that BH3-only proteins function at a point upstream of Bcl-2 in a cell death pathway conserved in both vertebrates and invertebrates (4, 5). BH3 proteins have been shown to localize to mitochondria after apoptotic stimuli, where they bind to Bcl-2 family members and induce mitochondrial events associated with apoptosis, including the release of cytochrome *c* into the cytosol (4). The functions of the BH3 proteins Bad and Bid are regulated by phosphorylation and proteolytic activation, respectively, in response to extrinsic cell survival/cell death stimuli (6–8). Through posttranslational modifications, Bad and Bid transduce signals originating at cell surface receptors to a Bcl-2-regulated, mitochondrial apoptosis control point. In *Caenorhabditis elegans*, the BH3-only protein Egl-1 operates at the most proximal point in a genetically defined pathway required for all programmed cell deaths (9). Egl-1 binds to the nematode Bcl-2 counterpart, Ced-9, and antagonizes its function, similar to the function ascribed to mammalian BH3 proteins. By contrast to Bad and Bid, the activity of Egl-1 appears to be regulated primarily through transcriptional control mechanisms. The *egl-1* gene is active specifically in cells that are destined to die during development, and genetic studies have identified transcription factors upstream of *egl-1* that control its expression in certain cell lineages (10–12).

There is a remarkable degree of structural and functional conservation between the genes that control the cell death pathway in nematodes and mammals (5). The essential contribution of Egl-1 and its transcriptional regulation to programmed cell death in *C. elegans* strongly implies that apoptosis in mammalian cells may depend on the transcriptional control of gene(s) encoding BH3-only proteins. Potentially, the identification of such genes would be important for delineating how diverse, seemingly unrelated apoptotic stimuli connect to a common cell death pathway in mammalian cells. Moreover, disregulation of the mechanisms that control transcription of BH3-only genes in this class may contribute to defects in apoptosis in diseases such as cancer.

In keeping with the analogy to Egl-1, we have identified a mammalian pro-apoptotic BH3-only protein, Bbc3 (for *B*cl-2 *b*inding *c*omponent 3), whose mRNA is induced in response to diverse apoptotic stimuli, including DNA damage, glucocorticoid treatment, and growth factor deprivation. Analysis of the *bbc3* promoter region revealed that *bbc3* is a direct target for transactivation by the p53 tumor suppressor, which mediates apoptosis in response to DNA damage. Independently of p53, serum and anti-apoptotic growth factors such as insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) strongly suppressed *bbc3* mRNA expression. Our data suggest that the regulation of *bbc3* mRNA levels, and hence the pro-apoptotic activity of this BH3-only protein, is a common target in diverse cell death-signaling pathways.

Materials and Methods

Isolation of a *bbc3* cDNA. A 1.6-kb *bbc3* cDNA encoding the full-length Bbc3 ORF (accession no. U82987) was isolated in a yeast two-hybrid screen by using a GAL4 DNA-binding domain/Bcl-2 fusion protein and a human lymphocyte cDNA library (Matchmaker system, CLONTECH). Analysis of a homologous human expressed sequence tag (accession no. AI784404) identified additional 5′ untranslated sequences, yielding an assembled 1.9-kb

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BH3, Bcl-2 homology domain 3; PI 3-kinase, phosphatidylinositol 3-kinase; Bik, bcl-2-interacting killer; Bad, bcl-x_L/bcl-2-associated death promoter; Bid, BH3-interacting domain death agonist; Hrk, harakiri; IGF-1, insulin-like growth factor 1; HA, hemagglutinin tag; FT, Flag epitope tag; β-gal, β-galactosidase; PDGF, platelet-derived growth factor; Bim, Bcl-2-interacting mediator of cell death; Egl-1, egg-laying-defective-1.

Data deposition: The human *bbc3* cDNA, protein, and promoter sequences have been deposited in the GenBank database (accession nos. U89287, AAB51243, and AF411827, respectively).

§Present address: Genetastix Corp., 780 Montague Expressway, Suite 303, San Jose, CA 95131.

¶To whom reprint requests should be addressed. E-mail: tom.chittenden@immunogen.com.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

cDNA that matches the *bbc3* message size detected by Northern analysis. A cDNA encoding the murine Bbc3 ORF was isolated by PCR using cDNA from M1p53ts cells.

Cell Lines. All cell lines used were obtained from American Type Culture Collection (ATCC), unless otherwise noted. HeLa/Bcl-x_L cells (gift of C. Vater, ImmunoGen) were generated by stable transfection of HeLa cells with a vector encoding a Flag-epitope-tagged Bcl-x_L. Murine myeloid leukemia M1 cells containing a temperature-sensitive p53-val135 mutant (M1p53ts), and *E1A/ras*-transformed $p53^{-/-}$ mouse embryonic fibroblasts containing a p53-estrogen receptor fusion, have been described previously (13, 14).

Plasmid Constructs and Cell Death Assays. The 1.6-kb bbc3 cDNA was subcloned into the pcDNA3 vector (Invitrogen) for expression of an untagged form of Bbc3. The Bbc3 ORF was amplified by PCR and cloned into pcDNA3, incorporating either an amino terminal influenza hemagglutinin tag (HA) or Flag epitope tag (FT). Oligonucleotide-directed mutagenesis was used to introduce alanine substitutions at amino acids 141, 144, and 147, to generate the pcDNA3/HA-Bbc3-ala plasmid. Amino acids 141–150 were deleted to generate both HA and FT forms of Bbc3-ΔBH3 in pcDNA3, and a *bbc3* segment encoding amino acids 136–185 was amplified by PCR and cloned into pcDNA3 to generate the FT-BH3/50 construct. All Bbc3 constructs were verified by DNA sequencing. The effect of Bbc3 expression on cell viability was tested in Rat-1 cells by using a transient transfection assay, as previously described (15).

Binding Assays. Cos7 cells were transiently transfected with HA-Bbc3 and FT-Bcl-x_L expression plasmids, using the Lipofectamine procedure (GIBCO/BRL). Cell lysates were prepared, and coimmunoprecipitation assays were performed as described previously (15). The Bcl-x_L competition binding assay was performed as previously described (16), by using synthetic BH3 peptides comprising residues 133–152 of Bbc3, and residues 70–89 of bcl-2 antagonist/killer (Bak).

Generation of a Bbc3 Monoclonal Antibody and Western Blot Analysis. A mouse monoclonal antibody, KM140, was made against a recombinant glutathione *S*-transferase (GST)-Bbc3 fusion protein. The KM140 epitope was mapped to residues 73–76 by probing a blot with arrayed overlapping Bbc3 peptides (Research Genetics, Huntsville, AL). Cells were plated at 3–10 $\times$ 10^6 cells per 100-mm dish in either DMEM containing 10% FBS, or serum-free medium (0% FBS, 0.1% BSA). Cell lysates were prepared, electrophoresed (100 to 200 μg of protein per lane) on SDS/4–20% polyacrylamide reducing gels, and analyzed by Western blotting as described previously (15).

Northern Blot Analysis. Total RNA was isolated from mouse thymocytes (RNeasy system, Qiagen, Chatsworth, CA). Poly(A)$^+$ RNA (FastTract system, Invitrogen) was prepared in all other experiments. Ten micrograms of total RNA or 3 μg of poly(A)$^+$ RNA per lane was denatured and separated by electrophoresis in 1% formaldehyde agarose gels. Northern blotting was performed following standard protocols.

Bbc3 Reporter Constructs and Luciferase Reporter Assay. A human P1 genomic DNA library (Genome Systems, St. Louis) was screened with a 5′ end *bbc3* cDNA PCR product, yielding two P1 *bbc3* genomic DNA clones. A 2.0-kb *Bam*HI genomic DNA fragment immediately upstream of the 5′ end of *bbc3* cDNA was identified by Southern blot analysis, subcloned, and sequenced. The 3′ *Bam*HI site present in the 2.0-kb fragment is located 35 bp upstream of the 5′ end of the 1.9-kb *bbc3* cDNA. The 2.0-kb *Bam*HI fragment, representing the candidate *bbc3* promoter region, was cloned into the pGL3Luc-Basic luciferase reporter vector (Promega) to generate pGL3/2.0. Unique *Eco*RI and *Pst*I sites within the 2.0-kb fragment yielded a 0.9-kb *Eco*RI/*Bam*HI fragment and a 0.17-kb *Pst*I/*Bam*HI fragment, which were cloned into pGL3Luc-Basic to generate pGL3/0.9 and pGL3/0.17, respectively. Nucleotide substitutions were introduced into the putative p53 binding site in pGL3/0.9 by oligonucleotide-directed mutagenesis, to generate pGL3/0.9mut.

Luciferase assays were performed by using the Dual-Light System (Tropix, Bedford, MA) for the combined detection of luciferase and β-galactosidase (β-gal). Saos-2 cells were transfected with Superfect (Qiagen) in six-well plates by using 1.8 μg of luciferase reporter plasmid, and 0.025 μg of a cytomegalovirus promoter expression plasmid, pRC/CMV (Invitrogen), encoding either wild-type p53 or mutant p53-SN22/23 (17). The β-galactosidase reporter pCMVβ-gal (Promega) was included (0.1 μg) as an internal control for transfection efficiency. Luciferase and β-galactosidase activities were measured 24 h after transfection. Transfections of HeLa/Bcl-x_L cells were performed in six-well plates by using 1.8 μg luciferase reporter plasmid and 0.1 μg pCMVβ-gal. Cell culture medium was changed to either DMEM containing 20% serum, or DMEM without serum, 24 h after transfection, and luciferase/β-galactosidase levels were measured after an additional 24 h of incubation. All reporter assays yielded similar results in at least three independent experiments.

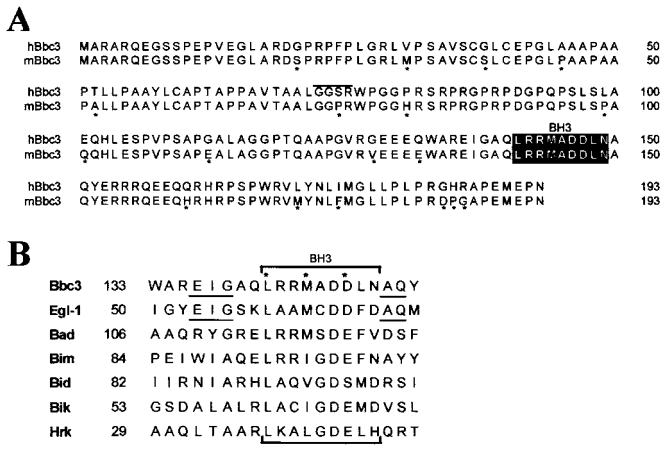

Fig. 1. Bbc3 aa sequence. (*A*) Alignment of human and mouse Bbc3 proteins, showing BH3 (shaded), KM140 antibody binding site (line), and nonidentical residues (*). (*B*) BH3 domains of Bbc3 and other BH3-only family members. Additional sequences shared by Bbc3 and Egl-1 are underlined; * indicates Bbc3 residues mutated to alanine in the BH3-ala mutant.

Results

Bbc3 Is a Novel BH3-Only, Pro-Apoptotic Protein. A yeast two-hybrid interaction screen was carried out by using Bcl-2 as bait to identify cellular proteins that bind to Bcl-2. Three classes of strongly interacting clones were isolated by screening a human lymphocyte cDNA library: two were cDNA clones encoding Bik and Bad, previously identified BH3-only proteins that bind to Bcl-2, and the third was a novel gene, designated *bbc3*, for *B*cl-2 *b*inding *c*omponent 3. Northern blot analysis of a panel of normal human tissue RNA samples (CLONTECH) detected expression of the 1.9-kb *bbc3* mRNA in peripheral blood lymphocytes, but failed to detect *bbc3* mRNA in other adult tissues (data not shown). This *bbc3* cDNA sequence was originally deposited in GenBank in 1997 (accession no. U82987), but was annotated at that time with a deduced amino acid sequence in a +1 register relative to the correct Bbc3 ORF. Bbc3 also interacted with the Bcl-2-related cell death suppressor, Bcl-x_L, but not with the pro-apoptotic proteins Bik and

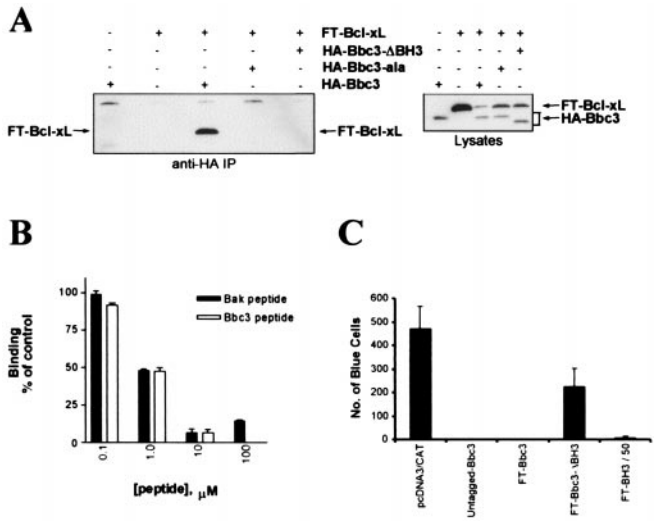

Fig. 2. Bbc3 is a pro-apoptotic BH3-only protein. (*A*) BH3-dependent coimmunoprecipitation of Bbc3 with Bcl-x_L. HA-Bbc3 or Bbc3-BH3 mutant constructs were cotransfected with FT-Bcl-x_L into Cos7 cells, lysates were immunoprecipitated with anti-HA antibody, and bound Bcl-x_L was detected by Western blotting with anti-flag antibody (*Left*). Expression of proteins in cell lysates was confirmed by Western blotting with anti-HA plus anti-flag antibodies (*Right*). (*B*) Binding of the Bbc3 BH3 domain to Bcl-x_L. Binding of GST-Bcl-x_L to an immobilized Bak BH3 peptide was measured by ELISA (triplicate samples ± SD), in the presence of the indicated concentrations of a soluble synthetic Bbc3 BH3 or Bak BH3 peptide. (*C*) Bbc3 triggers cell death through its BH3 domain. A control (pcDNA3CAT) or Bbc3 expression constructs were cotransfected with a β-galactosidase marker plasmid into Rat-1 cells. Cell death is measured by the reduction in the number of blue (β-galactosidase expressing) cells 24 h posttransfection (mean of triplicates ± SD).

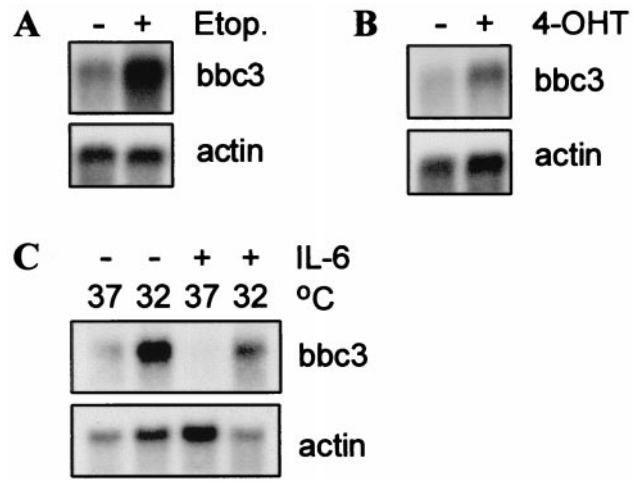

Fig. 3. Induction of *bbc3* mRNA by p53. Northern blots were hybridized with a murine *bbc3* cDNA probe, followed by rehybridization with an actin probe. (*A*) NIH 3T3 cells were treated with etoposide (50 μM) for 6 h. (*B*) Wild-type p53 function was induced in a E1a/Ras/p53MER mouse fibroblast cell line by treatment with 4-hydroxytamoxifen (4-OHT, 100 nM) for 5 h. (*C*) Wild-type p53 function was induced in M1p53ts cells by shifting to 32°C for 6 h, in the presence or absence of IL-6 (12.5 ng/ml).

Bak in the two-hybrid assay (not shown). The *bbc3* cDNA encodes a protein of 193 aa, harboring a candidate BH3 domain (Fig. 1*A*) that shares the most similarity to Egl-1 and Bad. Several residues immediately adjacent to the more highly conserved core BH3 residues are identical in Bbc3 and Egl-1 (Fig. 1*B*). Murine Bbc3 shares greater than 90% overall amino acid identity with human Bbc3, including perfect conservation within the BH3 regions.

We examined whether Bbc3 exhibited functional properties that define BH3-only proteins. Immunofluorescence and cell fractionation studies demonstrated that Bbc3 localizes to mitochondrial membranes (not shown), as has been described for other BH3-only proteins (4). In transiently transfected cells, an HA epitope-tagged form of Bbc3 coimmunoprecipitated with Flag epitope-tagged Bcl-x_L (Fig. 2*A*), confirming that Bbc3 can bind to Bcl-x_L in mammalian cells. Alanine substitution of three conserved residues within the BH3 domain of Bbc3 (HA-Bbc3ala), or a 10-aa deletion within BH3 (HA-Bbc3ΔBH3), destroyed the ability of Bbc3 to bind to Bcl-x_L both in transfected cells (Fig. 2*A*) and *in vitro* (not shown). Competition binding assays demonstrated that a 20-aa synthetic peptide encompassing the Bbc3 BH3 domain (residues 133 to 152) bound to recombinant GST-Bcl-x_L *in vitro* with an affinity comparable to a previously characterized Bak BH3 peptide (ref. 16; Fig. 2*B*). Therefore, the BH3 domain of Bbc3 is both necessary and sufficient for binding to Bcl-x_L.

Previously characterized BH3-only proteins exhibit pro-apoptotic activity that is dependent, at least in part, on their respective BH3 domains (4). To test the ability of Bbc3 to induce apoptosis, untagged and Flag-epitope tagged Bbc3 (FT-Bbc3) expression plasmids were transiently cotransfected with a β-galactosidase marker plasmid into Rat-1 cells (Fig. 2*C*). Bbc3 showed remarkably potent cell death-promoting activity in this assay, as detected by the almost complete elimination of β-gal-marked cells at 24 h posttransfection (Fig. 2*C*) in comparison with 400–500 β-gal-marked cells observed with the control plasmid (pcDNA3/CAT). Deletion of BH3 domain residues reduced, but did not completely eliminate, the cell-killing activity of Bbc3 (FT-Bbc3ΔBH3, Fig. 2*C*). Also, a truncated form of Bbc3 comprising 50 aa of the BH3 region (residues 136–185) retained significant pro-apoptotic function (FT-BH3/50, Fig. 2*C*). The pro-apoptotic activity of Bbc3 in this assay could be suppressed by cotransfection with a dominant-negative form of caspase-9 or Bcl-x_L at high molar ratio (not shown). Together, the BH3-dependent dimerization and pro-apoptotic activities of Bbc3 indicate that it functions in a mechanistically similar fashion to previously characterized BH3-only proteins.

***bbc3* mRNA Levels Are Induced by DNA Damage and p53.** To evaluate whether the activity of *bbc3* might be under transcriptional control, we examined the expression of *bbc3* in response to apoptotic stimuli likely to involve new mRNA/protein synthesis. Exposure of murine NIH 3T3 cells to the DNA-damaging drug etoposide led to the rapid induction of *bbc3* mRNA levels (Fig. 3*A*), before the onset of apoptosis. Cell death induced by etoposide and other DNA-damaging drugs in murine fibroblasts is mediated, at least in part, by the p53 tumor suppressor (18, 19). Therefore, we next examined whether p53 was sufficient to induce *bbc3* mRNA levels, by using cell lines with conditional p53 function. Wild-type p53 activity can be specifically induced by the addition of 4-hydroxytamoxifen (4-OHT) to *E1a/ras*-transformed $p53^{-/-}$ mouse embryo fibroblasts that express a p53-estrogen receptor fusion protein (13). Activation of p53 by exposure to 4-OHT in these cells was sufficient to induce *bbc3* mRNA levels, preceding the onset of cell death (Fig. 3*B*). A second cell line, murine M1p53ts cells, expresses a temperature-sensitive mutant p53 that is inactive at 37°C but assumes wild-type conformation and activity at 32°C (14). *bbc3* mRNA levels were significantly induced after wild-type p53 activation by temperature shift to 32°C (Fig. 3*C*). IL-6, which suppresses apoptosis induced by p53 in M1 cells (14), did not prevent induction of *bbc3* mRNA at 32°C (Fig. 3*C*). Thus, wild-type p53 is sufficient to induce *bbc3*, and elevated *bbc3* levels are not a response to the onset of cell death, *per se*.

***bbc3* Is a Direct Transcriptional Target of p53.** Considering the rapid activation of *bbc3* mRNA by p53, we examined whether p53 acts directly on the *bbc3* promoter. Comparison of the *bbc3* cDNA to the draft human genome sequence indicated that the *bbc3* gene is

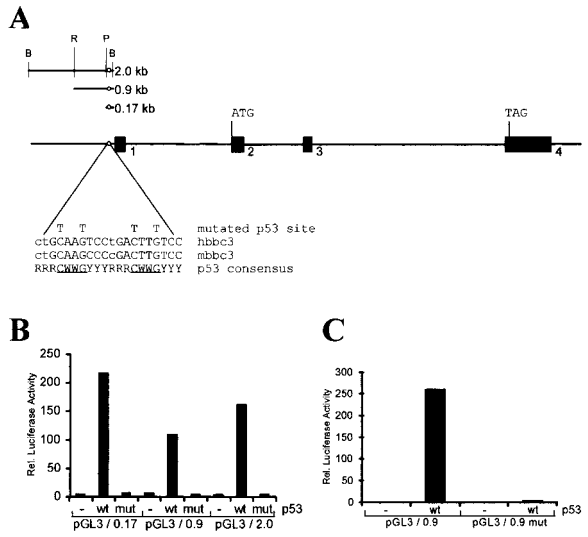

Fig. 4. Wild-type p53 directly activates the *bbc3* promoter. (*A*) Schematic of the *bbc3* gene structure, and cloned segments of the candidate *bbc3* promoter region. Restriction sites for *Bam*HI, *Pst*I, and *Eco*RI are indicated by B, P, and R, respectively. The putative p53 binding sites in the human and mouse *bbc3* promoter regions are shown, together with the consensus p53 binding site, and substitution mutations introduced into the human site. (*B*) Transactivation of the *bbc3* promoter by wild-type p53. The *bbc3* promoter region fragments shown in *A* were cloned into a luciferase reporter plasmid and cotransfected with plasmids expressing either wild-type p53 (wt) or mutant p53 (mut) into Saos-2 cells. Luciferase activity (measured in triplicate) was normalized to an internal transfection efficiency control (β-galactosidase). (*C*) Mutation of the p53 binding site abolishes transactivation of the *bbc3* promoter by p53. Transfections were performed as in *B*, with *bbc3* promoter reporter plasmids containing an intact (pGL3/0.9) or mutated (pGL3/0.9mut) p53 binding site, in the presence or absence of wild-type p53.

comprised of four exons on chromosome 19 (accession no. AC008532). P1 genomic DNA clones encompassing the *bbc3* gene were isolated, and DNA segments encoding exon 1 and sequences immediately 5′ to exon 1 were characterized by subcloning and DNA sequence analysis. Notably, a DNA sequence motif that is an excellent match to the consensus DNA binding site defined for p53 (20) was identified 150 bp upstream of the 5′ end of the *bbc3* cDNA (Fig. 4*A*) and is conserved in the mouse *bbc3* genomic locus (accession no. AC073741).

To test whether this candidate *bbc3* promoter region is transactivated by p53, genomic DNA segments encompassing either 2.0, 0.9, or 0.17 kb of DNA sequences immediately 5′ to *bbc3* exon 1 (Fig. 4*A*) were subcloned into the promoter-less luciferase reporter vector, pGL3. Each of these constructs containing the predicted p53 binding site element was strongly transactivated by cotransfection with a plasmid-encoding wild-type p53 in p53-deficient Saos-2 cells (Fig. 4*B*). No transactivation was detected on cotransfeciton with a plasmid expressing a mutant p53 that is defective for transactivation function (Fig. 4*B*). To determine whether the putative p53 binding site mediates transactivation of the *bbc3* promoter by p53, four single nucleotide substitution mutations were introduced to eliminate its potential recognition by p53 (shown in Fig. 4*A*). Whereas the 0.9-kb *bbc3* promoter region with the unaltered p53 binding site was strongly transactivated, the same 0.9-kb region harboring the mutated site failed to be transactivated by wild-type p53 (pGL3/0.9mut, Fig. 4*C*). These results demonstrate that *bbc3* is a direct target for p53 transactivation through a p53 binding site within the *bbc3* promoter region.

Glucocorticoid Treatment and Serum Deprivation Also Induce Bbc3. We next examined whether a broader range of apoptotic stimuli regulates *bbc3* expression, including cell death pathways that are not mediated by p53. In primary murine thymocytes, exposure to ionizing radiation triggers apoptosis through a p53-dependent pathway, whereas treatment with the glucocorticoid dexamethasone induces rapid cell death through a p53-independent pathway (21, 22). Consistent with our finding that *bbc3* is a direct p53 target, γ-irradiation of thymocytes led to the induction of *bbc3* mRNA in parallel with the mRNAs for the previously characterized p53 targets, *ei24* (23), *bax* (24), and *p21* (ref. 25; Fig. 5*A*). *bbc3* mRNA failed to be induced after irradiation of p53-null thymocytes (data not shown). Significantly, treatment of thymocytes with dexameth-

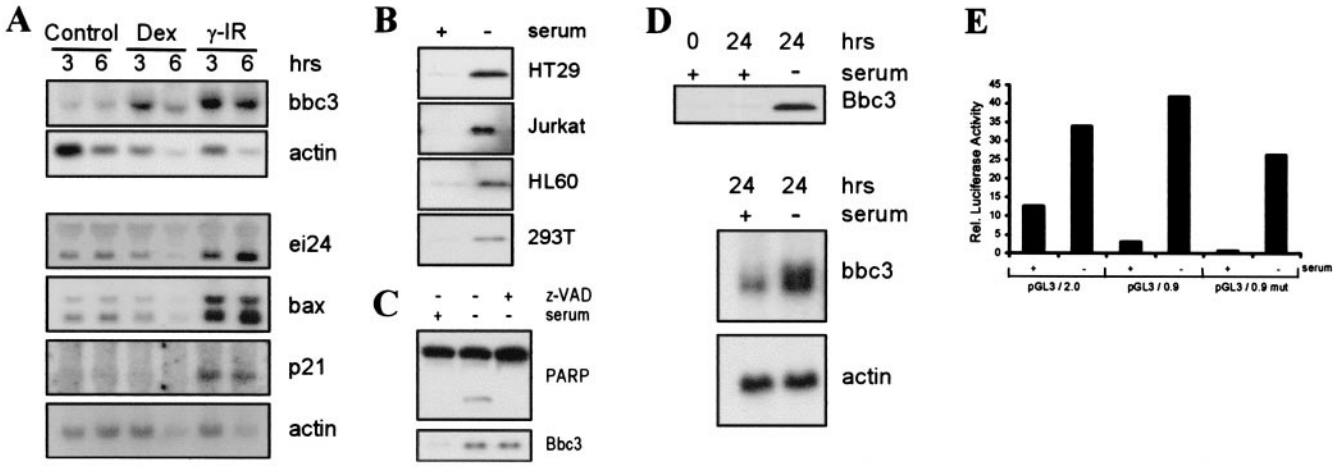

Fig. 5. Bbc3 expression is regulated by glucocorticoid treatment and serum withdrawal. (*A*) Northern blot analysis of primary murine thymocytes treated with 1 μM dexamethasone (Dex) or 5 Gy ionizing radiation (γ-IR). RNA samples were prepared 3 or 6 h after treatment, and mRNA for *bbc3* and actin was detected by sequential probing of the Northern blot (upper two blots). A separate blot prepared from the same RNA samples was probed for *ei24*, *bax*, *p21*, and actin, sequentially (lower four blots). (*B*) Immunoblot analysis of Bbc3 protein expression in human tumor cell lines, cultured in the presence or absence of serum for 24 h. (*C*) Jurkat cells were cultured in media without serum for 24 h, in the absence or presence of the caspase inhibitor z-VAD (100 μM). Bbc3 and poly(ADP-ribose) polymerase (PARP) were detected by Western blot analysis. (*D*) HT29 cells grown in the presence of serum (time 0) were incubated in the presence (+) or absence (−) of serum for 24 h. Bbc3 protein and mRNA expression was detected by Western analysis (*Top*) and by Northern analysis (*Middle* and *Bottom*). (*E*) Activation of the *bbc3* promoter by serum withdrawal. The indicated *bbc3* promoter luciferase reporter plasmids were transfected into HeLa-Bcl-x_L cells. At 24 h after transfection, cells were incubated for an additional 24 h in the presence of 20% serum (+) or absence of serum (−). Luciferase activity was measured and normalized for transfection efficiency.

asone also induced *bbc3* mRNA expression, in marked contrast to the other p53 target genes (Fig. 5*A*). Thus, *bbc3* mRNA levels can be induced in the same cell type by both p53-dependent and p53-independent cell death pathways.

Deprivation of serum is a strong pro-apoptotic stimulus for many cultured tumor cell lines, triggering cell death or sensitizing cells to other apoptotic stimuli. We examined the effect of serum deprivation on *bbc3* mRNA expression and Bbc3 protein levels, by using a monoclonal antibody generated against recombinant human Bbc3. This anti-Bbc3 antibody detects epitope-tagged and "untagged" forms of Bbc3 expressed after transfection of cells, and detects an endogenous 28-kDa band in Western blot analysis, which precisely comigrates with ectopically expressed Bbc3 (not shown). A striking increase in Bbc3 protein levels was observed on serum deprivation in multiple human tumor cell lines (Fig. 5*B*), including cell lines that lack functional p53. Addition of the broad-spectrum caspase inhibitor, z-VAD, to serum-starved Jurkat cells, prevented cleavage of the caspase substrate PARP but had no impact on the induction of Bbc3 protein levels (Fig. 5*C*). Therefore, the induction of Bbc3 precedes caspase activation, and is not simply a response to the engagement of the cell death program. In the human colon cancer cell line HT29, a dramatic increase in Bbc3 protein levels was observed 24 h after serum withdrawal (Fig. 5*D Upper*). Elevation of Bbc3 protein levels was observed within 4 h after serum withdrawal in these cells, whereas levels of other Bcl-2 and BH3-only family members tested, including Bax, Bcl-x_L, Bak, Bid, and Bad, were not induced by serum withdrawal (not shown). Coordinate changes in *bbc3* mRNA levels were observed (Fig. 5*D Lower*), suggesting that the elevation of Bbc3 expression by serum deprivation occurs principally at a transcriptional level.

To further define the mechanisms by which growth factors control *bbc3* expression, we tested whether the *bbc3* promoter region directly responds to serum deprivation. *bbc3* promoter/luciferase reporter constructs were tested for their activity in transfected HeLa/Bcl-x_L cells, cultured in either the presence or absence of serum. HeLa/Bcl-xL cells were used in these experiments because of their relatively high transfection efficiency, and Bcl-x_L expression prevents high levels of cell death that otherwise occur after serum withdrawal. Luciferase reporter constructs containing 2.0 kb and 0.9 kb of the *bbc3* promoter region were both significantly activated when transfected cells were cultured in serum-free conditions (Fig. 5*E*). By contrast, the activity of a pGL3/SV40 promoter control luciferase construct was reduced significantly in the absence of serum (not shown), representing a more typical response of promoters to serum deprivation. Mutation of the p53 binding site in the *bbc3* promoter region did not prevent activation by serum withdrawal (pGL3/0.9mut; Fig. 5*E*), confirming that this effect does not require p53. These findings indicate that the *bbc3* promoter region is activated in response to serum deprivation, and provide further evidence that growth factors control *bbc3* expression at a transcriptional level.

Growth Factors with Anti-Apoptotic Activity Suppress Bbc3 Expression. The increase in Bbc3 caused by serum withdrawal in HT29 cells was "reversible," in that Bbc3 protein levels were markedly reduced 24 h after the readdition of serum (Fig. 6*A Upper*), which correlated with changes in *bbc3* mRNA levels (Fig. 6*A Lower*). The influence of serum on Bbc3 expression led us to test whether the addition of specific growth factors would be sufficient for suppressing Bbc3 levels. Addition of either IGF-1 or epidermal growth factor, growth factors with broad anti-apoptotic activity, strongly inhibited Bbc3 protein expression in serum-starved HT29 cells (Fig. 6*B*), while having no effect on Bax or Bcl-x_L. A third growth factor, platelet-derived growth factor (PDGF), failed to suppress Bbc3 levels in this assay; however, it was not determined whether PDGF receptors are expressed and activated by PDGF in HT29 cells.

In many settings, anti-apoptotic signaling by the IGF-1 receptor requires the activation of phosphatidylinositol 3-kinase (PI 3-kinase; refs. 26 and 27). Addition of the PI 3-kinase-specific inhibitor LY294002 impaired the ability of IGF-1 to suppress Bbc3 levels in serum-starved HT29 cells (Fig. 6*C*). LY294002 treatment also significantly impaired the ability of serum to suppress Bbc3 protein levels in the same context (Fig. 6*D*), suggesting that PI 3-kinase is required for serum and IGF-1 to suppress expression of Bbc3. Together, these results raise the possibility that suppression of *bbc3*, a strongly pro-apoptotic BH3-only gene, is an important component of anti-apoptotic signaling by growth factor receptors.

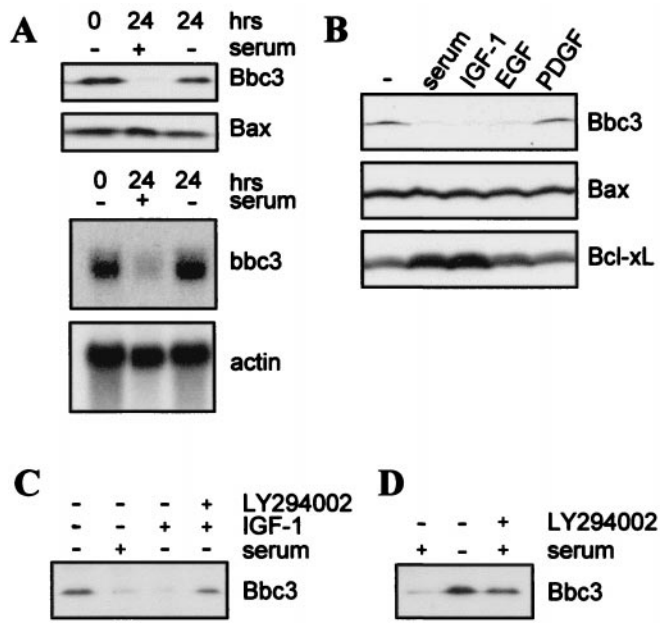

Fig. 6. Anti-apoptotic growth factors repress Bbc3 expression. (*A*) HT29 cells were incubated for one day in the absence of serum (time 0), and then cultured in the presence (+) or absence (−) of serum for 24 h. Bbc3 protein was detected by Western blot, with an additional probing of the blot for Bax expression (upper two blots); *bbc3* mRNA levels were detected by Northern analysis (lower two blots). (*B*) HT29 cells were incubated for one day in the absence of serum (−), and were then incubated in media containing either serum, 100 ng/ml IGF-1, 50 ng/ml epidermal growth factor (EGF), or 25 ng/ml PDGF, for 24 h. Bbc3 was detected by Western blot analysis, followed by sequential probing of the blot with anti-Bax and anti-Bcl-x_L antibodies. (*C*) HT29 cells were cultured as in *B* and then incubated for 24 h in media containing either serum, 100 ng/ml IGF-1, or 100 ng/ml IGF-1 in the presence of 50 μM LY294002 (added 1 h before IGF-1). Bbc3 was detected by Western blot analysis. (*D*) HT29 cells grown in the presence of serum (lane 1), were then incubated for 24 h in the absence of serum, or in the presence of serum supplemented with 50 μM LY294002.

Discussion

We present evidence in this study that *bbc3* is a strongly pro-apoptotic BH3-only gene that is subject to transcriptional regulation by multiple cell death-signaling pathways. The established role of *egl-1* in *c. elegans* programmed cell death (5) provides compelling reasons to predict that transcriptional control of analogous BH3-only genes will, likewise, contribute to cell death regulation in mammalian cells. In total, our results suggest that *bbc3* is a strong candidate for such a mammalian BH3-only gene. *bbc3* gene expression is activated by at least three apoptotic stimuli, including DNA damage, glucocorticoid treatment, and growth factor deprivation, constituting a broad transcriptional response thus far unique among mammalian cell death regulatory genes. By analogy to *egl-1*, *bbc3* may prove to be induced in other contexts as well, for example, during developmental cell deaths. We propose that the control of Bbc3 expression serves to transduce cell death signals originating from diverse stimuli to common mitochondrial apoptotic events regulated by the Bcl-2 family.

Precisely how independent cell death signals converge on the regulation of *bbc3* expression remains to be determined. After DNA damage, however, induction of *bbc3* mRNA levels can be accounted for by the direct binding and transactivation of the *bbc3* promoter by p53. While our manuscript was in preparation, two groups simultaneously reported the identification of a gene identical to *bbc3*, designated *PUMA*, through screens for p53 target genes by using microarray and serial analysis of gene expression (SAGE) approaches (28, 29). The *bbc3* cDNA identified here corresponds to the *PUMA* cDNA structure isolated by Yu *et al*. (29), whereas cDNAs isolated by Nakano and Vousden (28) encode an alternate first exon. All three groups demonstrated that *PUMA/bbc3* is a direct target of p53 and candidate effector of p53-mediated apoptosis. A gene encoding a different BH3-only protein, Noxa, was previously shown to be transactivated by p53 (30), as has the pro-apoptotic Bcl-2 homolog, Bax (24). Thus, p53 may promote apoptosis by targeting multiple effectors that use the BH3 death domain. The relative contribution of these targets to apoptosis in different settings awaits the results of gene knockout studies.

Our findings link the control of *bbc3* mRNA and protein expression to signaling by anti-apoptotic growth factor receptors. Removal of serum induces Bbc3 expression and promotes apoptosis; addition of serum or specific growth factors suppresses both Bbc3 expression and cell death. Two other BH3 family proteins, Hrk and Bim, have been reported to be regulated by withdrawal of cytokines in murine hematopoietic cells (31, 32). Based on our results, *bbc3* may comprise an important downstream target for the anti-apoptotic function of growth factor receptors, such as the IGF-1 receptor, in human tumor cell lines. Survival signaling by the IGF-1 receptor proceeds, at least in part, through the sequential activation of PI 3-kinase and the serine/threonine kinase Akt, followed by the rapid phosphorylation and inactivation of the BH3-only protein Bad (33). The inhibition of Bbc3 expression mediated by the IGF-1 receptor may provide an additional, sustained anti-apoptotic signal by reducing the effective levels of BH3 in cells. Mechanistically, regulation of *bbc3* expression by growth factor receptors also appears to require PI 3-kinase activity, although the relevant downstream transcription factors involved have not yet been identified. Candidates include certain members of the forkhead family, which are inactivated by PI 3-kinase-dependent pathways and have been shown to transactivate several pro-apoptotic targets, including the BH3-only gene, *bim* (34, 35).

Disregulation of the mechanisms that normally control the transcription of BH3-only genes such as *bbc3* may contribute to the evolution and/or survival of tumor cells. We speculate that several anti-apoptotic lesions that frequently occur in tumor cells are likely to alter the control of *bbc3* expression. The loss of p53 function may prevent the activation of *bbc3* that would normally follow DNA damage. Constitutive activation of PI 3-kinase-dependent pathways, for example, by loss of the PTEN lipid phosphatase or autocrine activation of the IGF-1 receptor (27), may lead to the inappropriate suppression of *bbc3* activity. In keeping with the analogy to Egl-1, a transcription factor that controls *egl-1* expression in a small number of *C. elegans* programmed cell deaths, Ces2, has a mammalian counterpart, E2A/HLF, implicated in the development of leukemia (36). E2A/HLF, the product of a recurrent translocation in leukemia, has potent anti-apoptotic properties that have been attributed to the suppression of an as yet unidentified downstream mammalian BH3-only gene (36, 37). Thus, defining the cell type or stimulus-specific mechanisms that regulate the transcription of *bbc3* and other BH3-only genes should provide new insights into how apoptosis is controlled normally and disregulated in tumor cells.

We would like to acknowledge Dong Yin for the isolation of the *bbc3* cDNA, Greg Gallo for its initial characterization, Kate Maes for antibody generation, and Carol Vater for providing HeLa/Bcl-x_L cells. We thank our colleagues at ImmunoGen for helpful scientific discussions. This work was supported in part by a research collaboration with BioChem Pharma, National Institutes of Health/National Cancer Institute Grant CA63230 (to G.P.Z), and the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

1. Adams, J. M. & Cory, S. (1998) *Science* **281,** 1322–1326.
2. Korsmeyer, S. J. (1999) *Cancer Res.* **59,** 1693s–1700s.
3. Chittenden, T., Harrington, E. A., O'Connor, R., Flemington, C., Lutz, R. J., Evan, G. I. & Guild, B. C. (1995) *Nature (London)* **374,** 733–736.
4. Huang, D. C. & Strasser, A. (2000) *Cell* **103,** 839–842.
5. Horvitz, H. R. (1999) *Cancer Res.* **59,** 1701s–1706s.
6. Zha, J., Harada, H., Yang, E., Jockel, J. & Korsmeyer, S. J. (1996) *Cell* **87,** 619–628.
7. Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. (1998) *Cell* **94,** 481–490.
8. Li, H., Zhu, H., Xu, C. J. & Yuan, J. (1998) *Cell* **94,** 491–501.
9. Conradt, B. & Horvitz, H. R. (1998) *Cell* **93,** 519–529.
10. Conradt, B. & Horvitz, H. R. (1999) *Cell* **98,** 317–327.
11. Metzstein, M. M., Hengartner, M. O., Tsung, N., Ellis, R. E. & Horvitz, H. R. (1996) *Nature (London)* **382,** 545–547.
12. Metzstein, M. M. & Horvitz, H. R. (1999) *Mol. Cell* **4,** 309–319.
13. Vater, C. A., Bartle, L. M., Dionne, C. A., Littlewood, T. D. & Goldmacher, V. S. (1996) *Oncogene* **13,** 739–748.
14. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A. & Oren, M. (1991) *Nature (London)* **352,** 345–347.
15. Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G. & Lutz, R. J. (1995) *EMBO J.* **14,** 5589–5596.
16. Zhou, X. M., Liu, Y., Payne, G., Lutz, R. J. & Chittenden, T. (2000) *J. Biol. Chem.* **275,** 25046–25051.
17. Lin, J., Chen, J., Elenbaas, B. & Levine, A. J. (1994) *Genes Dev.* **8,** 1235–1246.
18. Levine, A. J. (1997) *Cell* **88,** 323–331.
19. Vousden, K. H. (2000) *Cell* **103,** 691–694.
20. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W. & Vogelstein, B. (1992) *Nat. Genet.* **1,** 45–49.
21. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. & Jacks, T. (1993) *Nature (London)* **362,** 847–849.
22. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. & Wyllie, A. H. (1993) *Nature (London)* **362,** 849–852.
23. Gu, Z., Flemington, C., Chittenden, T. & Zambetti, G. P. (2000) *Mol. Cell. Biol.* **20,** 233–241.
24. Miyashita, T. & Reed, J. C. (1995) *Cell* **80,** 293–299.
25. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. & Vogelstein, B. (1993) *Cell* **75,** 817–825.
26. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R. & Greenberg, M. E. (1997) *Science* **275,** 661–665.
27. O'Connor, R., Fennelly, C. & Krause, D. (2000) *Biochem. Soc. Trans.* **28,** 47–51.
28. Nakano, K. & Vousden, K. H. (2001) *Mol. Cell* **7,** 683–694.
29. Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W. & Vogelstein, B. (2001) *Mol. Cell* **7,** 673–682.
30. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T. & Tanaka, N. (2000) *Science* **288,** 1053–1058.
31. Sanz, C., Benito, A., Inohara, N., Ekhterae, D., Nunez, G. & Fernandez-Luna, J. L. (2000) *Blood* **95,** 2742–2747.
32. Shinjyo, T., Kuribara, R., Inukai, T., Hosoi, H., Kinoshita, T., Miyajima, A., Houghton, P. J., Look, A. T., Ozawa, K. & Inaba, T. (2001) *Mol. Cell. Biol.* **21,** 854–864.
33. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M. E. (1997) *Cell* **91,** 231–241.
34. Datta, S. R., Brunet, A. & Greenberg, M. E. (1999) *Genes Dev.* **13,** 2905–2927.
35. Dijkers, P. F., Medemadagger, R. H., Lammers, J. W., Koenderman, L. & Coffer, P. J. (2000) *Curr. Biol.* **10,** 1201–1204.
36. Inaba, T., Inukai, T., Yoshihara, T., Seyschab, H., Ashmun, R. A., Canman, C. E., Laken, S. J., Kastan, M. B. & Look, A. T. (1996) *Nature (London)* **382,** 541–544.
37. Inukai, T., Inoue, A., Kurosawa, H., Goi, K., Shinjyo, T., Ozawa, K., Mao, M., Inaba, T. & Look, A. T. (1999) *Mol. Cell* **4,** 343–352.