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G_0 Function of BCL2 and BCL-x_L Requires BAX, BAK, and p27 Phosphorylation by Mirk, Revealing a Novel Role of BAX and BAK in Quiescence Regulation*

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BCL2 and BCL-x_L facilitate G_0 quiescence by decreasing RNA content and cell size and up-regulating p27 protein, but the precise mechanism is not understood. We investigated the relationship between cell cycle regulation and the anti-apoptosis function of BCL2 and BCL-x_L. Neither caspase inhibition nor abrogation of mitochondria-dependent apoptosis by BAX and BAK deletion fully recapitulated the G_0 effects of BCL2 or BCL-x_L, suggesting that mechanisms in addition to anti-apoptosis are involved in the cell cycle arrest function of BCL2 or BCL-x_L. We found that BCL2 and BCL-x_L expression in *bax*$^{-/-}$ *bak*$^{-/-}$ cells did not confer cell cycle effects, consistent with the G_0 function of BCL2 and BCL-x_L being mediated through BAX or BAK. Stabilization of p27 in G_0 in BCL2 or BCL-x_L cells was due to phosphorylation of p27 at Ser^{10} by the kinase Mirk. In *bax*$^{-/-}$ *bak*$^{-/-}$ cells, total p27 and p27 phosphorylated at Ser^{10} were elevated. Re-expression of BAX in *bax*$^{-/-}$ *bak*$^{-/-}$ cells and silencing of BAX and BAK in wild type cells confirmed that endogenous BAX and BAK modulated p27. These data revealed a novel role for BAX and BAK in the regulation of G_0 quiescence.

BCL2 is the prototypical apoptosis regulator, which classically functions as an oncogene in hematopoietic lineages by prolonging survival (1–3). In other systems, including mammary tumor and hepatocellular carcinogenesis models, BCL2 plays a tumor suppressing role by inhibiting proliferation (4–8). Therefore, BCL2 can be both oncogenic, because of its anti-apoptotic activity, and tumor suppressive, by blocking proliferation. The coexistence of the anti-apoptosis and the anti-proliferative functions is best illustrated in *BCL2* transgenic mice with expression in lymphoid cells. Mice with transgenic *BCL2* expression in B or T cells have increased propensity to develop lymphomas (1–3). At the same time, *BCL2* transgenic T cells exhibit anti-proliferative properties, including smaller size, less RNA content, and delay in activation-induced cell cycle entry (9, 10). The mechanistic relationship between the oncogenic and tumor-suppressive functions of BCL2 is not well understood.

Cellular quiescence, or the G_0 state, is characterized by significantly decreased ribosomal RNA synthesis and lowered total cellular protein content resulting in smaller cell size, compared with cycling cells. G_0 parameters can be measured by pyronin Y staining of polyribosomal RNA and forward scatter by flow cytometry (FSC),[4] as well as the marked increase in the protein level of the cdk inhibitor p27. Functionally, cells retained in quiescence take longer to exit G_0 and enter G_1, exhibiting an anti-proliferative phenotype. Previous studies have shown that expression of BCL2 or BCL-x_L significantly lengthens the time for a cell to reach the S phase from the arrested state (9, 11–14). We and others have found that BCL2 and BCL-x_L are unable to delay cell cycle entry in *p27*$^{-/-}$ cells, identifying p27 as a key molecule in the ability of BCL2 or BCL-x_L to exert cell cycle effects (15, 16). Assessment of cell size and RNA content during cell cycle arrest and re-entry suggested that BCL2 or BCL-x_L expression retained cells in G_0 (17, 18), which is a major cause of delayed cell cycle entry (18). These anti-proliferative functions of BCL2 (and BCL-x_L) are thought to be responsible for the tumor-suppressive effect of BCL2 in selected contexts.

BCL2 overexpression is a major pathogenic mechanism in lymphomas and leukemias. The identification of BCL2 at the major breakpoint of t(14;18) lymphomas, which resulted in overexpression of BCL2, was key in the discovery of apoptosis inhibition as an oncogenic mechanism (19, 20). Increased expression of BCL-x_L is also a well documented mechanism of therapy resistance. Thus, experiments using overexpression of BCL2 or BCL-x_L are physiologically relevant to cancer, especially hematopoietic malignancies. Published data on *bcl2*$^{-/-}$ T cells demonstrating hastened cell cycle entry provided evidence that regulation of quiescence and cell cycle entry are physiologic functions of endogenous BCL2 (9). When studying BCL2 as an oncogene, it is important to understand its cell cycle effects, which must be considered together with its

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[4] The abbreviations used are: FSC, forward scatter by flow cytometry; MEF, mouse embryonic fibroblast; WT, wild type; z, benzyloxycarbonyl; fmk, fluoromethyl ketone; HA, hemagglutinin; DMSO, dimethyl sulfoxide; BrdU, bromodeoxyuridine; siRNA, small interfering RNA; C9DN, caspase 9 dominant negative mutant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

anti-apoptosis functions to fully understand the oncogenic role of BCL2.

An important question in understanding the dual role of BCL2 and BCL-x_L is whether the cell cycle function is distinct from the anti-apoptotic function or whether the cell cycle activity is a consequence of enhanced survival. In support of the latter possibility, the ability of BCL2 or BCL-x_L to raise the threshold at which cells die because of a lack of growth factors may allow cells to further decrease their size and RNA content, which are characteristic of G_0. However, increased survival is unlikely to be the only reason for the observed G_0 effects of BCL2 and BCL-x_L, because transgenic *BCL2* or *BCL-x_L* T cells and contact-inhibited BCL2 or BCL-x_L-expressing fibroblasts also display characteristics of further entry into G_0 when growth factors are not limiting and apoptosis stimuli are absent (10, 15, 18). Our published data showed that BCL2 and BCL-x_L delayed cell cycle entry stimulated by serum or Myc, but not by E2F1, while protecting Myc-induced and E2F1-induced cell death equally effectively, demonstrating that the cell cycle function of BCL2 and BCL-x_L is not always a direct consequence of apoptosis inhibition (15). Structure-function analysis to separate the cell cycle function from the apoptosis function was undertaken, but an extensive mutant analysis of BCL2 or BCL-x_L failed to separate the two functions into different protein domains. Instead, mutations that affected the ability of BCL2 or BCL-x_L to inhibit apoptosis also attenuated the cell cycle activity of BCL2 or BCL-x_L, suggesting that the two functions are mediated through the same target(s) of BCL2 or BCL-x_L (18). Here, we further investigated the relationship between regulation of G_0 quiescence and inhibition of apoptosis by BCL2 or BCL-x_L.

To distinguish whether the cell cycle activity of BCL2 and BCL-x_L is a direct consequence of their anti-apoptotic function or is an additional function of BCL2 and BCL-x_L, we inhibited cell death by means other than BCL2 or BCL-x_L to see whether other methods of enhancing survival can also lead to the same cell cycle effects as seen in BCL2- or BCL-x_L-expressing cells. First, we inhibited caspase-dependent cell death and found that the cell cycle phenotype of BCL2 or BCL-x_L was not fully recapitulated. Then we asked whether *bax*$^{-/-}$ *bak*$^{-/-}$ cells, in which mitochondrial apoptosis is essentially eliminated, exhibited the same cell cycle characteristics as cells with BCL2 or BCL-x_L overexpression. We found that the cell cycle function of BCL2 or BCL-x_L depended on the presence of BAX and BAK, and *bax*$^{-/-}$ *bak*$^{-/-}$ cells have altered p27 regulation, unveiling a role for BAX and BAK in G_0 control.

EXPERIMENTAL PROCEDURES

Cell Culture—Medium containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum was used for WT and *bax*$^{-/-}$ *bak*$^{-/-}$ MEFs (originated in Dr. Craig Thompson's lab), WT and p27S10A knock-in MEFs (gift of Dr. James Roberts), bim$^{-/-}$ MEFs (gift of Dr. Hisashi Harada), Rat1MycER cells, and the human osteosarcoma line 143B. Medium containing 10% calf serum was used for NIH3T3 cells. All of the media contained 2 mM L-glutamine and 100 units/ml penicillin/streptomycin. Where indicated, MG132 was added to a final concentration of 10 μM, z-VAD was added to 40 μM, the final concentration of cycloheximide was 10 μg/ml, and actinomycin D was 1 μg/ml.

Retroviral Infection and Transfection—BCL-x_L, BCL2, caspase 9 dominant negative (from Dr. Lawrence Boise), or HA-BAX (from Dr. Sandra Zinkel) cDNAs were introduced into cells by retroviral infection. pBabe(puro), pWzl(neo), pMSCV(puro), or pMSCV-iRES-EGFP constructs containing full-length BCL2 (human) or BCL-x_L (mouse) or C9DN were transfected into BOSC cells using calcium phosphate or Lipofectamine 2000. Two days later, viral supernatants were used to infect fibroblasts or MEFs four times at 2-h intervals. The cells were selected in media containing puromycin (2–4 μg/ml) or G418 (0.75 mg/ml) 48 h after infection, or green fluorescent protein-positive cells were sorted on a FACS-Aria.

Cell Cycle Analysis—The cells were plated in six-well dishes. For serum starvation, the next day the cells were washed three times with phosphate-buffered saline and cultured in medium containing 0.05% fetal bovine serum for Rat1MycER cells or 0.75% calf serum for NIH3T3 or MEF cells. After 72 h, Rat1MycER cells were stimulated with 1 μM 4-hydroxytamoxifen, and NIH3T3 cells were stimulated with 10% calf serum. For contact inhibition, the cells were allowed to reach confluence and maintained for 5 days in confluence. To stimulate re-entry into cell cycle, contact-inhibited cells were trypsinized and replated at low density. At the indicated times, the cells were collected and resuspended in Krishan's reagent (0.1 mg/ml propidium iodide, 0.02 mg/ml RNase A, 0.3% Nonidet P-40, and 0.1% sodium citrate) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). For BrdU incorporation, at the indicated times, 20 μM BrdUrd was added to the media for 30 min. The cells were harvested and fixed in ice-cold 70% ethanol, treated with 4 N HCl, neutralized by 0.1 M borax, washed with phosphate-buffered saline containing 0.5% bovine serum albumin, and incubated sequentially with anti-BrdUrd antibody (Becton Dickinson) and fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Sigma) in the presence of 0.5% bovine serum albumin and 0.5% Tween 20. The cells were resuspended in phosphate-buffered saline containing propidium iodide and RNase A and analyzed by flow cytometry. The data were analyzed using Cell Quest.

RNA/DNA Staining—Simultaneous 7-amino-actinomycin D and pyronin Y staining was performed according to the protocol of Darzynkiewicz (21) and as previously described (18). The percentage of reduction in either pyronin Y staining or forward scatter is the difference in mean fluorescence between growing and arrested cells divided by mean fluorescence of growing cells, times 100. For each graph, at least three independent experiments were performed.

Immunoblots—The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The antibodies used for immunoblotting were: H-5 (Santa Cruz) or 13.6 (gift from Dr. Lawrence Boise) for BCL-x_L, 6C8 for human BCL2 (PharMingen), 652 for BAX (from Dr. Stanley Korsmeyer), 06-536 for BAK (Upstate), K25020 (Transduction Labs) and C-19 (Santa Cruz) for p27, and sc-12939-R for pS10p27 (Santa Cruz). The antibodies for caspase 9 and caspase 3 were

from Cell Signaling. Polyclonal Mirk antibody was from Dr. Eileen Friedman's laboratory.

Real Time PCR—Syber Green mixture (Applied Biosystems) and *i*cycler (Bio-Rad) was used for p27 real time PCR. The primers used were: p27 sense, 5′-CAG CTT GCC CGA GTT CTA; p27 antisense, 5′-GGG GAA CCG TCT GAA ACA; GAPDH sense, 5′-ACC ACA GTC CAT GCC ATC AC; and GAPDH antisense, 5′ TCC ACC ACC CTG TTG CTG TA. The p27 PCR product was 282 nucleotides, and the GAPDH product was 432 nucleotides. The p27 PCR product was assigned a valued based on the GAPDH concentration curve. The *p* values were determined using a two-tailed, equal variance Student's *t* test.

Knockdown—For Mirk knockdown, siRNA for human Mirk (GCCUGGUAUUUGAGCUGCUGUCCUA) and control siRNA (GCCUGUAUUAGCGGUUCUGCGUCUA) were purchased from Invitrogen. For knockdown of mouse Mirk, a SMART pool from Dharmacon was used (CUGAUGAACCAGCAUGAUAUU, CAACAGAGCCUACCGAUACUU, GACCAGAUGAGCCGUAUUGUU, and GGACAAAGGAACUCAGGAAUU). Transfections were performed according to the Lipofectamine 2000 protocol. Briefly, per 0.15×10^6 cells in a 6-well dish, 250 pmol of siRNA were diluted in 250 μl of Opti-MEM, and 5 μl of Lipofectamine 2000 were diluted in 250 μl of Opti-MEM. Following a 15-min incubation, the diluted siRNAs and diluted Lipofectamine 2000 were gently mixed and incubated for 15 min at room temperature. The siRNA-Lipofectamine 2000 complexes were added to the cells and incubated for 16 h. The growth medium was replaced, and the cells were allowed to contact inhibit. For BAX and BAK knockdown, BAX and BAK siGenome Smartpool siRNAs were purchased from Dharmacon and transfected using Lipofectamine 2000 according to the manufacturer's protocol.

RESULTS

z-VAD-fmk Partially Delayed Cell Cycle Entry but Did Not Affect G_0 or Elevate p27—To test whether increasing survival by caspase inhibition results in the same cell cycle arrest phenotype as seen in BCL2- or BCL-x_L-expressing cells, we treated cells during cell cycle arrest with the general caspase inhibitor z-VAD-fmk. NIH3T3 cells stably expressing BCL-x_L or empty vector were arrested for 3 days in the presence of z-VAD or DMSO. Although the complete absence of serum induced fibroblast cell death, cells arrested in G_0/G_1 without significant death (consistently <10%) in the presence of low serum (0.75% for NIH3T3 cells). To test the effect of caspase inhibition during arrest only, z-VAD was applied during incubation in low serum and washed out before the readdition of 10% serum for cell cycle entry. Following cell cycle stimulation with serum, control cells treated with DMSO began to show a significant rise in S phase cells by 10 h, whereas BCL-x_L-expressing cells remained in G_0/G_1 until 14–16 h when a small increase in the S phase became apparent. The cells treated with z-VAD during arrest showed entry into S phase at 12 h, which is intermediate between control and BCL-x_L-expressing cells (Fig. 1*A*). z-VAD rescued cell death in the absence of any serum almost as efficiently as BCL-x_L, confirming that caspase inhibition did not fully replicate the cell cycle delay phenotype of BCL-x_L despite efficient inhibition of apoptosis (Fig. 1*B*).

To determine whether z-VAD-treated cells exhibited the same G_0 parameters as BCL2- or BCL-x_L-expressing cells, we stained cells for polyribosomal RNA with pyronin Y and analyzed cells in G_0/G_1 by gating on cells with 2 n DNA content (18). BCL-x_L cells exhibited a significant reduction in RNA content following cell cycle arrest, as measured by the difference in mean fluorescence intensity between growing cells and arrested cells (40%, $p < 0.001$), compared with DMSO controls, whereas RNA content of z-VAD-treated cells did not change after cell cycle arrest ($p = 0.327$) (Fig. 1*C*). Cell size change, as measured by mean FSC, in z-VAD-treated cells after arrest was no different from DMSO controls ($p = 0.74$), in comparison with a significant FSC reduction in BCL-x_L-expressing cells (20%, $p < 0.001$) (Fig. 1*D*). Therefore, inhibition of cell death by z-VAD did not reproduce the G_0 characteristics of BCL-x_L cells.

BCL2- and BCL-x_L-expressing cells display markedly increased levels of p27 during cell cycle arrest, and the BCL2 and BCL-x_L-dependent cell cycle re-entry delay phenotype requires the presence of p27 (15, 16). To determine whether p27 elevation was solely the result of cell death inhibition, we compared p27 levels in control cells and z-VAD-treated cells during arrest (Fig. 1*E*). As expected, BCL-x_L-expressing cells showed increased p27 levels compared with DMSO-treated vector-control cells. However, z-VAD-treated vector cells exhibited levels of p27 comparable with DMSO-treated vector cells, indicating that inhibition of cell death alone did not lead to increased p27. Thus, z-VAD treatment resulted in partial cell cycle delay not accompanied by p27 elevation, and z-VAD did not affect G_0.

To test the effect of caspase inhibition on cell cycle arrest in another cell type, Rat1MycER cells stably expressing BCL2 or vector were arrested in the presence of z-VAD-fmk or DMSO and stimulated to re-enter the cell cycle by 4-hydroxytamoxifen. BCL2 expression effectively inhibited Myc-induced S phase entry, as expected. z-VAD treatment of vector cells had no effect on Myc-induced cell cycle entry (Fig. 1*F*), whereas it inhibited cell death almost as well as BCL2 (Fig. 1G). z-VAD-treated vector cells arrested with RNA content and cell size comparable with DMSO-treated vector cells (data not shown). These results showed that caspase inhibition did not exert an effect on G_0 or delay cell cycle entry in MycER cells. Taken together with the results from NIH3T3 cells (Fig. 1, *A–E*), we conclude that increasing survival through caspase inhibition cannot be the sole mechanism of the cell cycle function of BCL2 and BCL-x_L.

Caspase 9 Dominant Negative Mutant Expression Did Not Mimic BCL2 or BCL-x_L Expression during Cell Cycle Exit or Entry—Because BCL2 and BCL-x_L regulate mitochondrial release of cytochrome *c*, which binds Apaf-1 and pro-caspase 9 to form the apoptosome, we used a caspase 9 dominant negative mutant (C9DN) to test whether the inhibition of the apoptosome affects cell cycle arrest (22). Stable C9DN expression and function in NIH3T3 cells were confirmed by Western blotting for caspase 9 and the absence of cleaved caspase 3 after apopto-

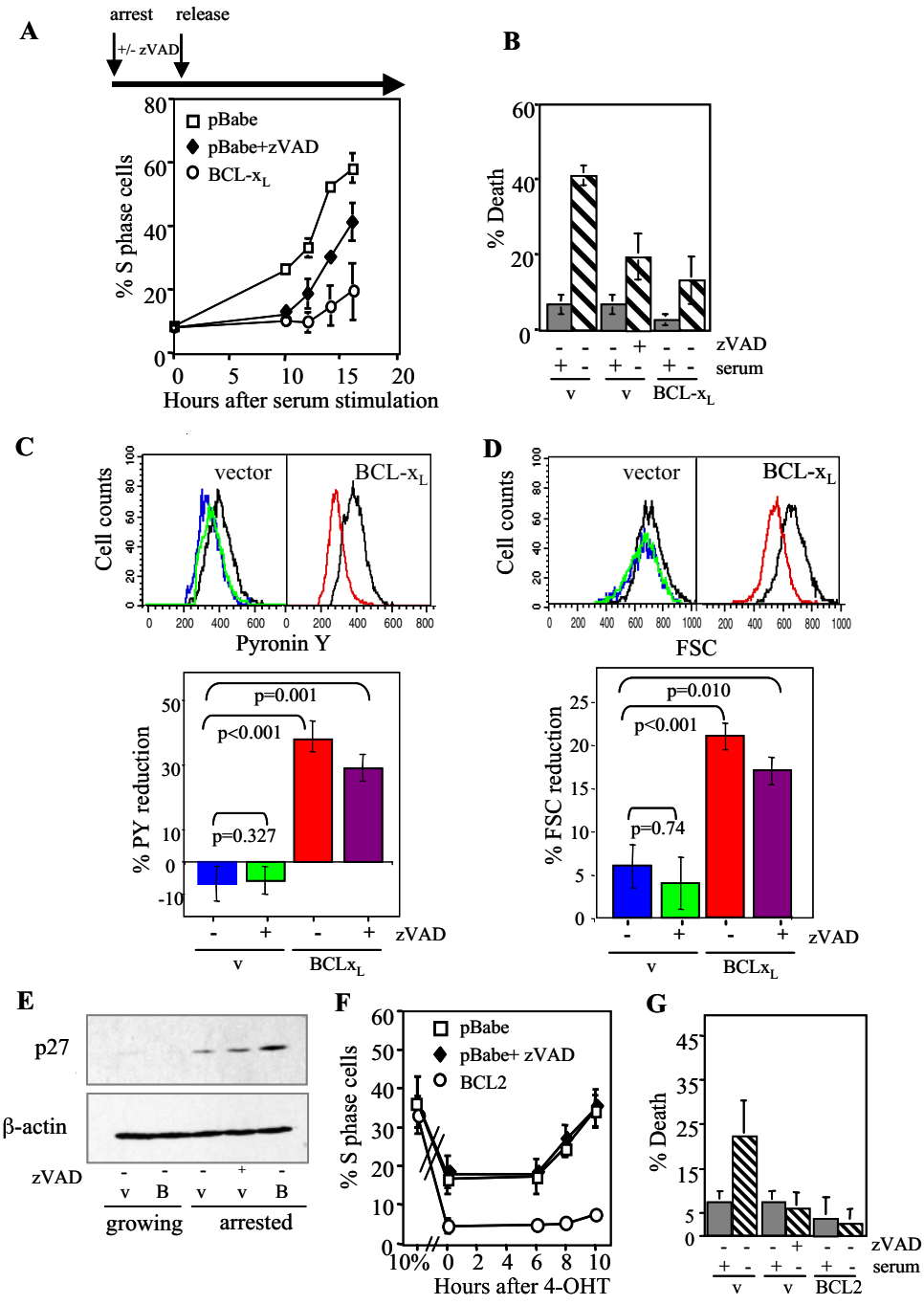


FIGURE 1. **z-VAD partially delays serum-induced, not Myc-induced, cell cycle entry but does not decrease RNA content, cell size, or increase p27.** *A*, NIH3T3 pBabe or BCL-x_L cells were arrested in 0.75% % serum in the presence of z-VAD-fmk (40 μM) or DMSO for 3 days and then were stimulated to enter cell cycle by the readdition of 10% serum. The percentage of S phase cells was obtained by propidium iodide staining for DNA content. *B*, the ability of z-VAD (40 μM), DMSO, or BCL-x_L to rescue cell death in the complete absence of serum after 24 h was compared by trypan blue exclusion. *v*, vector. *C–E*, cycling and arrested NIH3T3 cells treated with z-VAD or expressing BCL-x_L were collected and assayed for RNA content by pyronin Y staining (*C*), cell size by FSC (*D*; *black*, asynchronously growing cells; *blue*, DMSO-treated arrested vector cells; *green*, z-VAD-treated arrested vector cells; *red*, arrested BCL-x_L cells; *v*, vector), and p27 protein levels by Western blotting (*E*; *v*, vector; *B*, BCL-x_L). *F*, Rat1MycER pBabe or BCL2 cells were cultured in 0.1% serum and treated with z-VAD or DMSO for 3 days, and then stimulated with 1 μM 4-hydroxytamoxifen (*4-OHT*). S phase cells were obtained by propidium iodide staining. *G*, the ability of z-VAD, DMSO, or BCL2 to rescue Rat1MycER cell death in the complete absence of serum after 24 h was assessed by trypan blue exclusion. Except for cell death assays, all other assays were gated on live cells. For *C* and *D*, representative FACS plots are shown in the *top panels*, and the average percentage of reduction in mean fluorescence in arrested cells relative to growing cells with standard deviation of at least three experiments are graphed in the *lower panels*.

sis induction, respectively, as well as by inhibition of cell death in serum starvation (Fig. 2*A*). Cell cycle kinetics of C9DN cells was intermediate between control and BCL-x_L-expressing cells (Fig. 2*B*). Consistent with z-VAD data, the decrease in RNA content following arrest in C9DN cells was not statistically different from vector cells (20%, $p = 0.154$), whereas BCL-x_L expressing cells showed significantly more reduction in RNA content than vector cells (42%, $p = 0.012$) (Fig. 2*C*). The decrease in mean FSC of C9DN cells after arrest tended to be greater than vector cells, but the difference did not reach statistical significance ($p = 0.112$) (Fig. 2*D*). The level of p27 in arrested C9DN cells was similar to vector cells, whereas BCL-x_L-expressing cells up-regulated p27 to a much higher level (Fig. 2*E*).

To test the effect of caspase 9 inhibition in another system of cell cycle arrest and re-entry, C9DN was expressed in Rat1MycER cells (Fig. 3, *A* and *B*). C9DN expression had no effect on RNA content, cell size reduction, or p27 levels during cell cycle exit (Fig. 3, *D–F*). Whereas C9DN had an intermediate effect on serum-induced cell cycle entry, it had no effect on Myc-induced G_0 to S transition (Fig. 3*C*). Thus, the cell cycle effects of BCL-x_L or BCL2 during cell cycle arrest and re-entry could not be fully reproduced by C9DN expression in either NIH3T3 or Rat1MycER fibroblasts.

The data from Figs. 1–3 showed that even though z-VAD and C9DN both effectively inhibited apoptosis, neither changed cell cycle arrest, as measured by pyronin Y staining, FSC, or p27 level, in the manner of BCL2 or BCL-x_L expression. The only cell cycle effect of z-VAD or C9DN was partial delay of cell cycle re-entry under some circumstances. Although BCL2 or BCL-x_L expression inhibited apoptosis and retained cells in G_0, caspase inhibition effectively protected cells against apoptosis but did not result in cells entering a quiescence state.

$Bax^{-/-}$ $Bak^{-/-}$ Cells Exhibit Altered Cell Cycle Arrest and Are Insensitive to the Cell Cycle Effects of BCL2 or BCL-x_L Expression—BCL2 and BCL-x_L regulate mitochondria-mediated apoptosis, which includes both caspase-dependent and caspase-independent cell death. One reason that the inhibition of caspases did not pro-

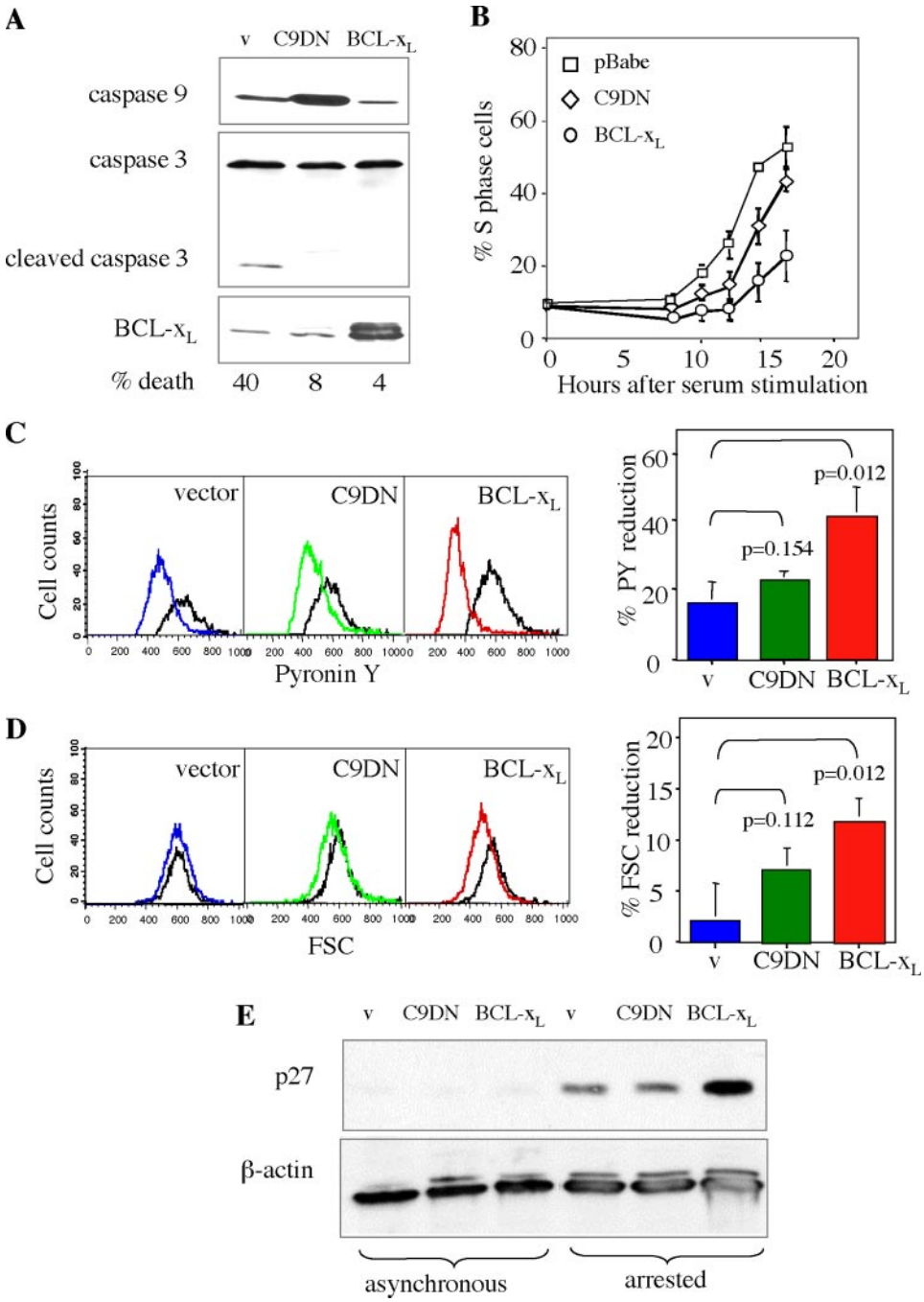


FIGURE 2. **C9DN partially delays cell cycle entry but does not affect measurements of G_0 arrest in NIH3T3 cells.** *A*, lysates of cells overexpressing dominant negative caspase 9 (*C9DN*) or BCL-x_L induced to die by serum withdrawal for 24 h were immunoblotted for caspase 9 and caspase 3. *B*, S phase progression of cells arrested in 0.75% serum for 3 days and stimulated to enter cell cycle by 10% serum readdition were measured by propidium iodide staining. Asynchronously growing and arrested NIH3T3 cells were collected and assayed for RNA content by pyronin Y staining (*C*), cell size by FSC (*D*; *black*, asynchronously growing cells; *blue*, arrested vector control cells; *green*, arrested C9DN cells; *red*, arrested BCL-x_L cells), and p27 protein levels by Western blotting (*E*). Except for cell death assays, all of the cell cycle measurements were gated on live cells. For *C* and *D*, representative FACS profiles are shown, and graphs with standard errors show the percent change in mean fluorescence of at least three different experiments.

mote G_0 arrest could be that caspase-independent pathways of cell death continued to function. To inhibit all cell death emanating from the mitochondria, we used cells lacking both BAX and BAK (23, 24). Wild type and *bax*$^{-/-}$ *bak*$^{-/-}$ mouse embryo fibroblasts (DKO MEFs) were plated at high density and allowed to reach confluence (Fig. 4). No significant cell death was observed in contact-inhibited cultures (Fig. 4*A*, *table*). We compared contact-inhibited *bax*$^{-/-}$ *bak*$^{-/-}$ MEFs with wild type cells expressing BCL2 or BCL-x_L in terms of pyronin Y staining, FSC, and p27 levels. We found that the reductions in cell size and RNA content of *bax*$^{-/-}$ *bak*$^{-/-}$ cells during density arrest were intermediate between wild type cells expressing vector and wild type cells expressing BCL2 or BCL-x_L, indicating that the absence of BAX and BAK was not equivalent to BCL2 overexpression in terms of cell cycle arrest (Fig. 4*A*). Thus, simply increasing survival does not result in accentuated G_0. Moreover, expression of BCL2 or BCL-x_L in DKO MEFs consistently failed to reduce pyronin Y staining after contact inhibition (Fig. 4*A*, compare *white bars*, *bax*$^{-/-}$ *bak*$^{-/-}$ *v* and BCL2 or *v* and BCL-x_L). BCL2 or BCL-x_L expression also did not change cell size of DKO MEFs (data not shown). These results indicated that the cell cycle arrest activity of BCL2 and BCL-x_L requires BAX and/or BAK.

Bax$^{-/-}$ *Bak*$^{-/-}$ *Cells Express Higher Level of p27*—Next, we examined p27 levels in *bax*$^{-/-}$ *bak*$^{-/-}$ DKO cells. In wild type MEFs, BCL2 or BCL-x_L expression elevated p27 levels during contact inhibition, as expected (Fig. 4*B*, *lanes 1–4*, *left* and *right panels*). Corresponding to the lack of a cell cycle arrest phenotype in DKO cells, neither BCL2 nor BCL-x_L expression elevated p27 levels in DKO cells after 5 days of contact inhibition (Fig. 4*B*, *lanes 7* and *8*, *both panels*). p27 levels also did not change appreciably in vector alone DKO cells before and after cell cycle arrest (Fig. 4*B*, *lanes 5* and *6*, *both panels*). Even after prolonged density arrest (up to 3 weeks), BCL2 expressing DKO MEFs still did not up-regulate p27 relative to control cells (Fig. 4*C*).

Compared with asynchronously growing wild type cells, growing DKO cells consistently expressed more p27 protein (Fig. 4*B*, compare *lanes 1* to *5* and *lanes 3* to *7*, *both panels*), suggesting that BAX and/or BAK are required to maintain p27 at a low level in cycling cells. To determine whether the higher

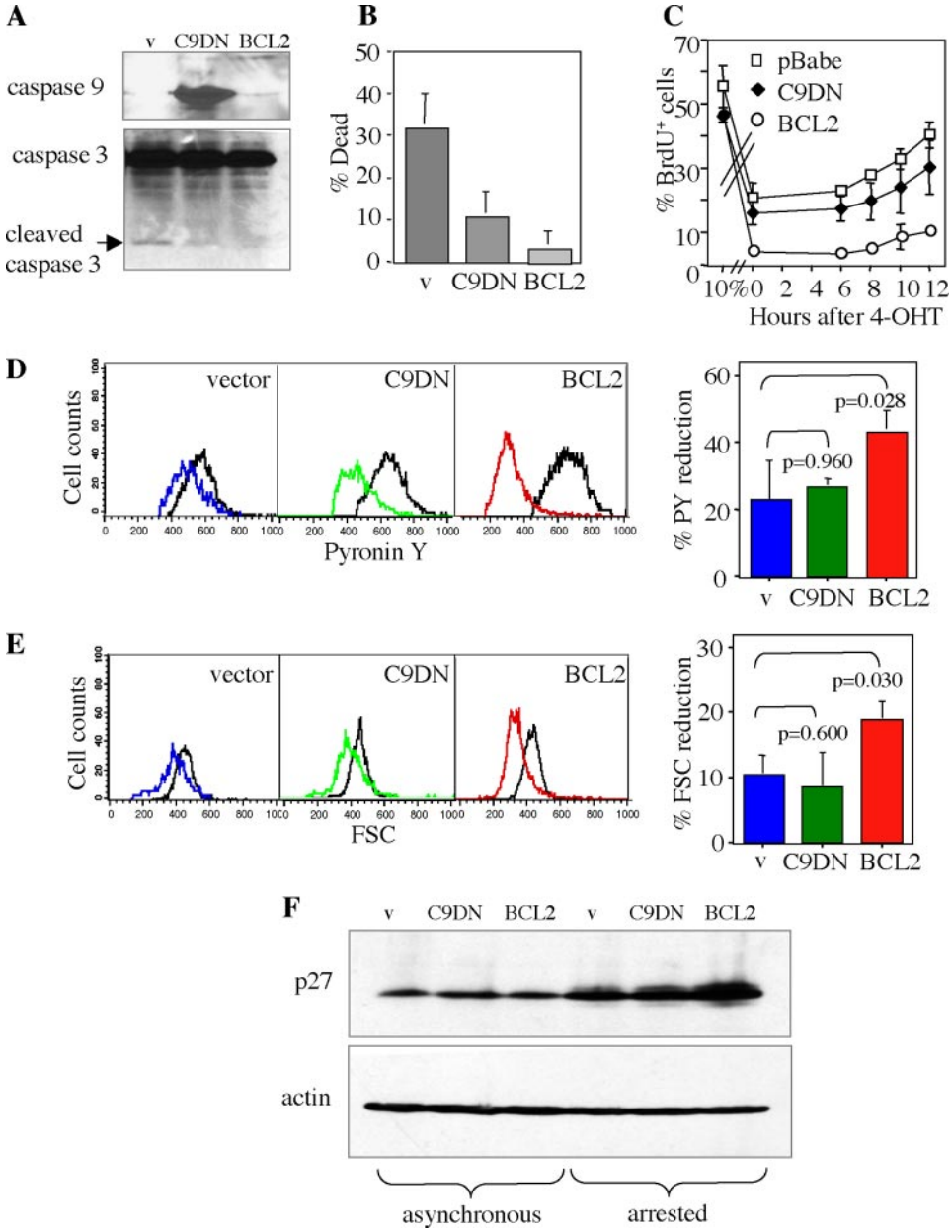


FIGURE 3. **C9DN does not affect cell cycle entry or G_0 arrest in Rat1MycER cells.** *A*, lysates of cells overexpressing C9DN or BCL2 induced to die by serum withdrawal for 24 h were immunoblotted for caspase 9 and caspase 3. *B*, cell death of Rat1MycER cells expressing vector, C9DN, or BCL2 after complete serum deprivation for 24 h was assessed by trypan blue exclusion. *C*, cells were arrested in 0.05% fetal calf serum medium for 3 days and stimulated to enter cell cycle by 1 μM 4-hydroxytamoxifen addition, and S phase cells were measured by BrdU pulse labeling. Asynchronously growing and arrested Rat1MycER cells were assayed for RNA content by pyronin Y staining (*D*), cell size by FSC (*E*; *black*, asynchronously growing cells; *blue*, arrested vector control cells; *green*, arrested C9DN cells; *red*, arrested BCL2 cells), and p27 protein levels by Western blotting (*F*). Except for cell death assays, all of the cell cycle measurements were gated on live cells.

level of p27 in DKO cells affected cell cycle progression, wild type and DKO cells expressing BCL2 or not were contacted inhibited and then released by replating at low density and followed for cell cycle re-entry. Compared with wild type MEFs, DKO cells were slower to reach the S phase. BCL2 delayed S phase re-entry of wild type MEFs but had no effect on the progression to the S phase of DKO MEFs, consistent with the inability of BCL2 to further increase the p27 level, which was already elevated in DKO MEFs (Fig. 4*D*).

Thus, inhibition of essentially all mitochondria-mediated apoptosis by BAX and BAK deletion did not completely phenocopy BCL2 or BCL-x_L overexpression in cell cycle activities. Instead, the results in Fig. 4 indicated that the cell cycle arrest function of BCL2 and BCL-x_L required the presence of BAX and/or BAK. Moreover, in addition to activating apoptosis, BAX and BAK have a role in cell cycle correlated with the up-regulation of p27.

BCL2/BCL-x_L Stabilizes p27 Protein—We have previously shown that BCL2 and BCL-x_L were unable to delay cell cycle entry in *p27*$^{-/-}$ cells (15, 16). Here, we asked whether p27 was also required for the cell cycle arrest function of BCL2 and BCL-x_L. Although BCL-x_L expression reduced pyronin Y staining in wild type MEFs during arrest, pyronin Y staining of growing and arrested cultures was the same between vector and BCL-x_L-expressing *p27*$^{-/-}$ MEFs, indicating that in the absence of p27, BCL-x_L expression does not lead to further decrease in ribosomal RNA synthesis during cell cycle arrest (Fig. 5*A*). Similar results were obtained with forward scatter. Thus, in addition to the delay of cell cycle entry, the G_0 function of BCL-x_L is also dependent on p27.

p27 protein levels are regulated by translation and ubiquitin-mediated degradation (25). Western analysis indicated that p27 levels increased between 8 and 16 h after removal of serum from the culture medium for both vector and BCL2-expressing cells, but p27 ultimately rose to a higher level in BCL2-expressing cells in cell cycle arrest (Fig. 5*B*). Similar data were obtained for BCL-x_L-expressing cells. To determine whether the increase in p27 protein was due to decreased degradation, we arrested vector and BCL2-expressing cells in the presence of cycloheximide, which inhibited new protein synthesis, and followed the levels of previously synthesized p27 while cells were subjected to arrest. In the absence of new protein synthesis, existing p27 protein degraded over time, as expected (Fig. 5*C*, *left brace*). However, in cells expressing BCL-x_L subjected to arrest in low serum, p27 protein synthesized before the cells were switched to low serum was stable over 4 days in low serum (Fig. 5*C*, *right brace*). These results indicated that BCL2 and BCL-x_L caused post-translational stabilization of p27. We found that stabilization of p27 was

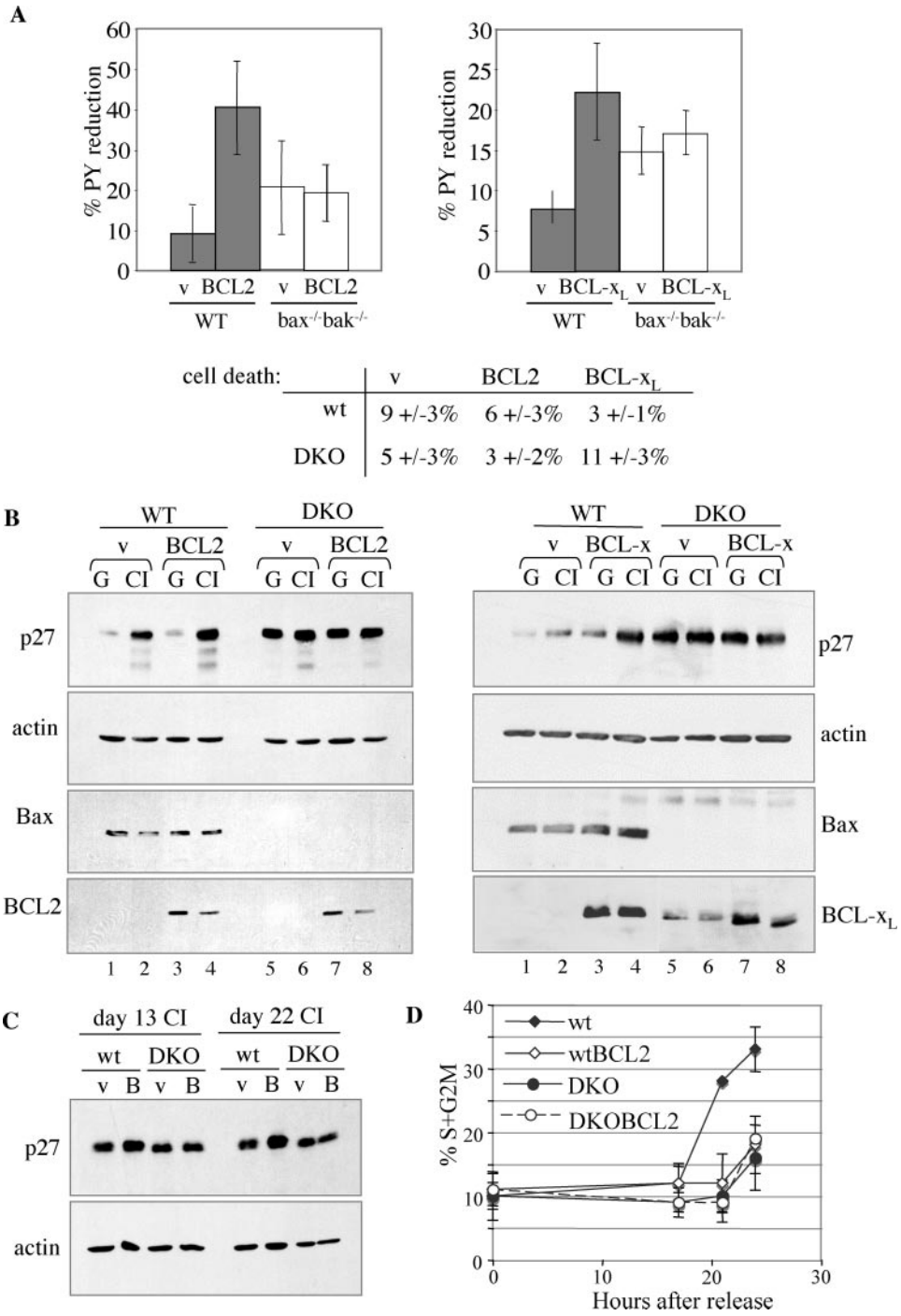
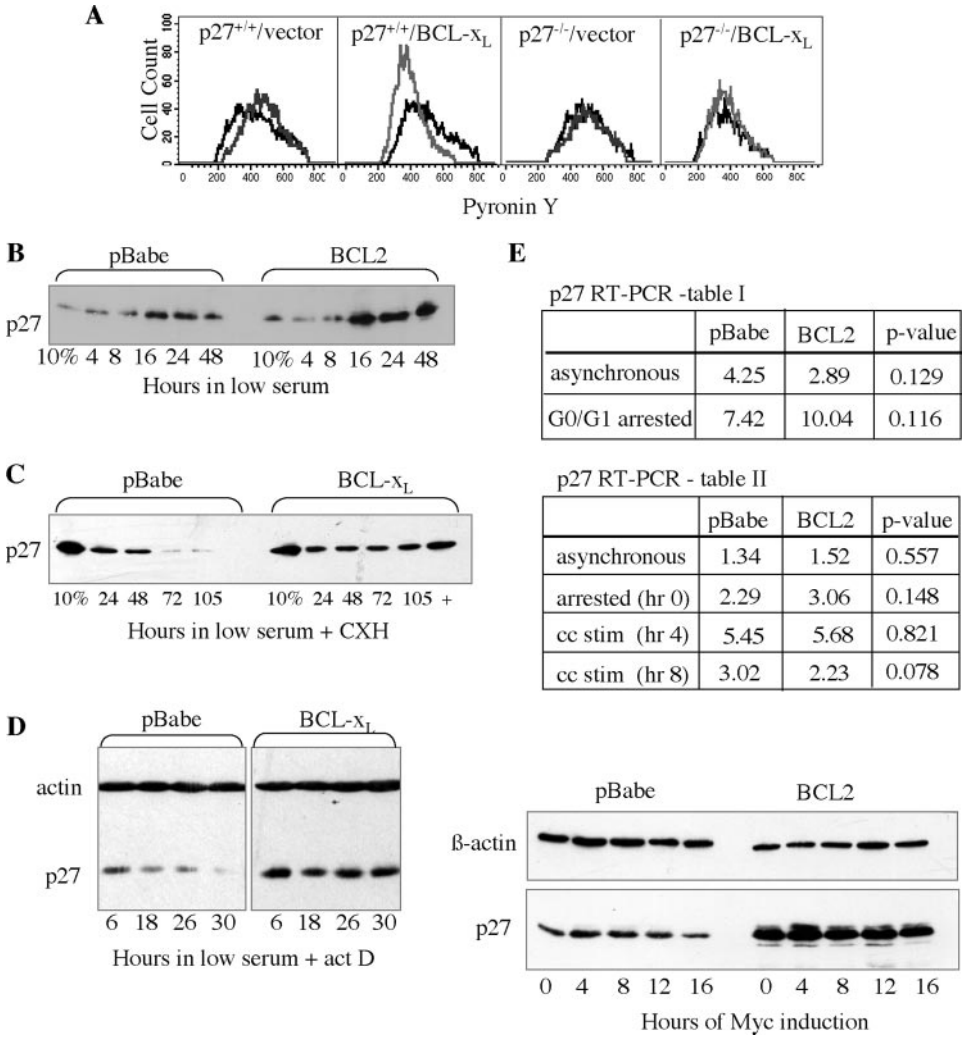


FIGURE 4. **Deficiency in BAX and BAK exhibit intermediate G_0 arrest.** *A*, $bax^{-/-}$ $bak^{-/-}$ MEFs harboring vector (*v*) or BCL2 (*left panel*) or BCL-x_L (*right panel*) were assayed for RNA content by pyronin Y staining. The percentage of reductions in mean PY fluorescence from growing cells to contact-inhibited cells are shown. The table below shows percentages of cell death in contact-inhibited cultures. The errors represent at least three experiments. *B*, p27, BAX, and BCL2 or BCL-x_L were immunoblotted for wild type and $bax^{-/-}$ $bak^{-/-}$ (*DKO*) MEFs asynchronously growing and contact-inhibited for 5, 13, or 22 days (*C*). *G*, growing; *CI*, contact-inhibited; *v*, vector; *B*, BCL2. *D*, contact-inhibited wild type and DKO MEFs expressing vector or BCL2 were released from G_0 arrest by replating at low density. The percentage of cells in S phase over time was obtained by propidium iodide staining and FACS analysis of cell cycle profiles.

not caused by alterations in the induction of either SCF^{skp2} ubiquitin ligase or Cks1 protein, which regulate p27 degradation at the G_1-S transition (data not shown) (26–28). We also found no evidence to suggest that differences in the synthesis of p27 protein were responsible for the observed increase in p27 protein levels in BCL2 or BCL-x_L cells (data not shown).

To determine whether increased transcription contributed to high p27 protein levels in BCL2 or BCL-x_L cells, we first assayed the level of p27 when transcription was inhibited by actinomycin D. In vector control cells, p27 protein levels fell in the absence of new RNA synthesis, as expected (Fig. 5*D*, *left panel*). However, in cells expressing BCL-x_L, p27 protein levels remained relatively unchanged even when inhibition of RNA synthesis was applied, consistent with stabilization of p27 protein by BCL-x_L (Fig. 5*D*, *right panel*). Next, we performed real time PCR of p27 on RNA from cells during asynchronous growth, arrest, and cell cycle entry. Although p27 protein showed the expected difference between BCL2-expressing and vector-containing cells (Fig. 5*E*, *bottom panel*, *hour 0*), replicate RNA samples showed no significant difference in real time PCR between BCL2 and vector cells in growing compared with arrested cultures (Fig. 5*E*, Table I) or during cell cycle entry (Fig. 5*E*, Table II), confirming that the reason for p27 elevation is not likely due to different levels of p27 RNA. These experiments using $p27^{-/-}$ cells, RNA, and protein synthesis inhibitors, as well examination of p27 RNA, indicated that BCL2 or BCL-x_L mediated G_0 arrest through stabilization of p27.

The G_0 Function of BCL-x_L Requires Phosphorylation of p27 Ser^{10}—p27 levels during cell cycle are regulated by phosphorylation. During G_0, phosphorylation at Ser^{10} leads to stabilization of p27 in the nucleus (29–31). We asked whether BCL2 or BCL-x_L might regulate p27 phosphorylation at Ser^{10}. Indeed, Western blotting showed that $Ser(P)^{10}$ was increased in BCL-x_L-expressing MEFs during arrest, compared with control cells, paralleling the increase in total p27 (Fig. 6*A*, compare *lanes 2* and *4*). To determine whether p27 Ser^{10} phosphorylation was required for BCL-x_L up-regulation of p27, BCL-x_L was expressed in MEFs with a knock-in allele of phosphorylation-deficient p27 with Ser^{10} mutated to alanine



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p27 RT-PCR -table I

	pBabe	BCL2	p-value
asynchronous	4.25	2.89	0.129
G0/G1 arrested	7.42	10.04	0.116

p27 RT-PCR - table II

	pBabe	BCL2	p-value
asynchronous	1.34	1.52	0.557
arrested (hr 0)	2.29	3.06	0.148
cc stim (hr 4)	5.45	5.68	0.821
cc stim (hr 8)	3.02	2.23	0.078

FIGURE 5. **The G_0 effect of BCL2 or BCL-x_L is mediated through p27 stability.** *A*, representative FACS profiles of pyronin Y staining of growing or arrested vector- or BCL-x_L-expressing $p27^{+/+}$ and $p27^{-/-}$ MEFs are shown (*black*, asynchronously growing cells; *gray*, arrested vector control cells; *gray*, arrested BCL-x_L cells). *B*, lysates of pBabe vector and BCL-x_L expressing NIH3T3 cells collected at the indicated times after switching to 0.75% serum were immunoblotted for p27. Lysates of pBabe- and BCL-x_L-expressing cells collected at the times indicated during cell cycle arrest in the presence of10 μg/ml cycloheximide (*CXH*) (*C*) or 1 μg/ml actinomycin D (*act D*) (*D*) were immunoblotted for p27. The *lanes* in *C* labeled as 10% represent the level of p27 at the time of switch to low serum, and serve as time 0 for both *C* and *D*, because the same starting cultures were divided for either cycloheximide or actinomycin D treatment. *E*, BCL2 expression does not alter p27 RNA levels during arrest or cell cycle entry. Real time PCR of p27 in asynchronously growing and arrested pBabe and BCL2 cells (*Table I*) or during asynchronous growth, arrest (*hour 0*), and *hour 4* or *8* of cell cycle stimulation (*cc stim*) (*Table II*). The *numbers* in the *tables* represent duplicates and are normalized to GAPDH. The Western blot below confirms p27 protein elevation in the samples used for real time PCR.

(S10A) (29). In contrast to wild type MEFs expressing vector or BCL-x_L, p27 protein levels did not change in either vector- or BCL-x_L-expressing p27S10A MEFs subjected to arrest (Fig. 6*A*, *lanes 5–8*), demonstrating that stabilization of p27 by BCL-x_L required phosphorylation of Ser^{10}.

Consistent with the lack of p27 up-regulation, p27S10A MEFs expressing BCL-x_L failed to show significant reductions in RNA content as measured by pyronin Y staining or in cell size during cell cycle arrest (Fig. 6*B*). In addition, cell cycle re-entry of BCL-x_L-expressing wild type MEFs was delayed, as expected (Fig. 6*C*, *left panel*), but in S10A MEFs, the kinetics of S phase entry were the same between vector and BCL-x_L-expressing cells (Fig. 6*C*, *right panel*). Thus, examination of RNA content, cell size, p27 levels, and progression to S phase all indicated that BCL-x_L was unable to exert cell cycle effects in the absence of p27 Ser^{10} phosphorylation. Failure of BCL-x_L to promote G_0 arrest in p27S10A cells strongly suggested a mechanistic role for p27 Ser^{10} phosphorylation in the cell cycle arrest function of BCL-x_L.

Up-regulation of p27 by BCL-x_L Requires Mirk—The kinases regulating phosphorylation of p27 at Ser^{10} include hKIS and Mirk (29, 32, 33). Ser^{10} phosphorylation by hKIS has been associated with exit from G_0 and cellular proliferation, whereas Mirk is a G_0 kinase that is a more likely candidate for the increased phosphorylation of p27 observed here. Mirk/Dyrk1B is a member of the Dyrk/minibrain family of arginine-directed serine/threonine kinases (33–35). Mirk is present at low levels in most normal tissues and is activated by the MAPK kinase MKK3 (36). Mirk protein levels are elevated during contact inhibition and diminish as cells enter the cell cycle (34). To determine whether increased p27 Ser^{10} phosphorylation in arrested BCL2- or BCL-x_L-expressing cells was due to higher levels of Mirk, growing and contact-inhibited cells were immunoblotted for Mirk. We found that contact-inhibited BCL-x_L cells have much higher levels of Mirk protein than contact-inhibited vector control cells, corresponding to higher p27 Ser^{10} phosphorylation and increased total p27 (Fig. 7*A*, compare *lanes 2* and *4*).

To assess whether Mirk up-regulation was functionally important in the G_0 function of BCL-x_L, Mirk was knocked down by siRNA transfection. Knockdown of Mirk in BCL-x_L expressing NIH3T3 cells resulted in significantly reduced total p27 and Ser^{10}-phosphorylated p27, in comparison with cells treated with control siRNA (Fig. 7*B*, *left panel*). An even more pronounced decrease in p27 expression was found with Mirk knockdown in human 143B cells, in which Ser^{10} phosphorylation became undetectable (Fig. 7*B*, *right panel*). These findings indicate that promotion of G_0 by BCL-x_L was dependent on Mirk induction and phosphorylation of p27 at Ser^{10} by Mirk.

$Bax^{-/-}$ $Bak^{-/-}$ Cells Express Increased p27 Ser^{10} Phosphorylation—Because p27 was elevated in *$bax^{-/-}$ $bak^{-/-}$* DKO cells (Fig. 4), we asked whether phosphorylation of p27

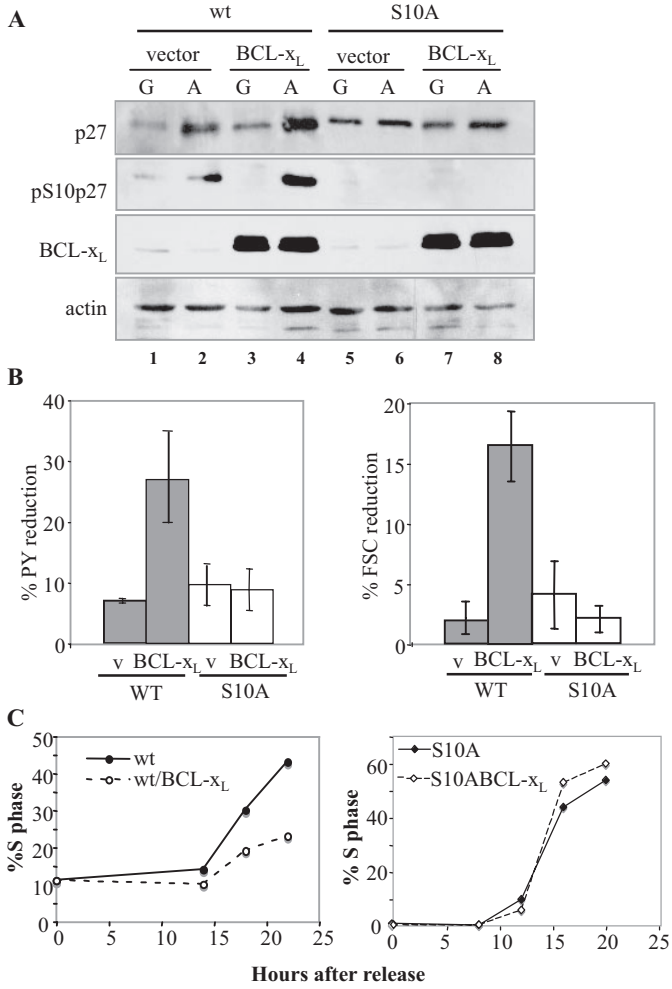

FIGURE 6. **Up-regulation of p27 by BCL-x_L requires phosphorylation of p27 at Ser[10].** Asynchronously growing (*lanes G*) and arrested (*lanes A*) vector or BCL-x_L-expressing wild type MEFs and MEFs with a knock-in allele of p27S10A (S10A) were assayed for total p27 protein level and p27 Ser[10] phosphorylation by immunoblotting (*A*), reduction in RNA content by pyronin Y staining (*left*) and cell size by FSC (*right*) (*B*), and percentage of S phase cells after release from arrest (*C*).

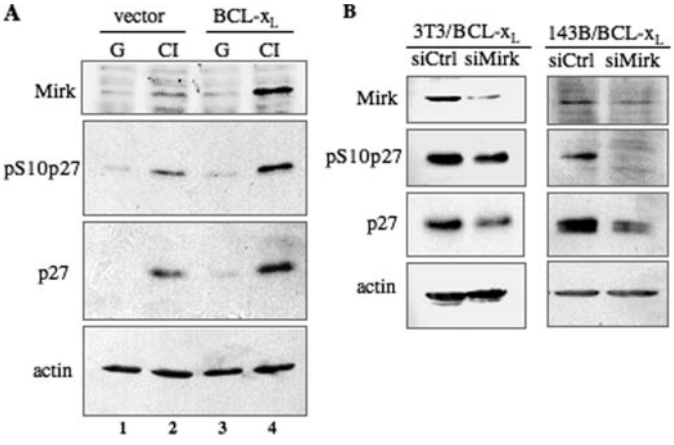

FIGURE 7. **Up-regulation of p27 by BCL-x_L is due to Ser[10] phosphorylation by Mirk.** *A*, asynchronously growing (*G*) and contact-inhibited (*CI*) vector- or BCL-x_L-expressing NIH3T3 cells were immunoblotted for Mirk, Ser[10]-phosphorylated-p27, and total p27. *B*, lysates from NIH3T3 and 143B cells expressing BCL-x_L subjected to contact inhibition were immunoblotted for Mirk, Ser[10]-phosphorylated p27, and total p27.

Ser[10] was increased in DKO cells. Comparing asynchronously growing and contact-inhibited wild type and $bax^{-/-}$ $bak^{-/-}$ DKO MEFs, more Ser(P)[10] was present in cycling $bax^{-/-}$ $bak^{-/-}$ cells, concurrent with the higher total p27 level (Fig. 8*A*, *lanes 1* and *5*). However, whereas phosphorylation of Ser[10] was markedly increased in wild type cells with BCL2 expression, Ser(P)[10] was not further increased in BCL2-expressing DKO cells in contact inhibition (Fig. 8*A*, compare *lanes 3* and *4* with *lanes 7* and *8*). Similarly, whereas p27 protein was increased during arrest in wild type MEFs expressing BCL-x_L, total and Ser[10]-phosphorylated p27 protein were already abundant in DKO MEFs and did not further increase significantly with BCL-x_L expression in arrest (Fig. 8*A*, compare *lanes 9* and *10* with *lanes 11* and *12*). Phosphorylation of Ser[10] correlated with total p27 level, suggesting that elevation of p27 in $bax^{-/-}$ $bak^{-/-}$ DKO cells was due to increased Ser[10] phosphorylation. In addition, in the absence of BAX and BAK, BCL2 or BCL-x_L expression could not further increase p27 Ser[10] phosphorylation.

To test the role of BAX in the maintenance of the p27 level, HA-BAX was re-expressed in $bax^{-/-}$ $bak^{-/-}$ DKO cells. Although p27 and Ser[10]-phosphorylated p27 levels were significantly increased in contact-inhibited wild type MEFs expressing BCL-x_L (Fig. 8*B*, *lanes 5* and *6*), p27 levels were high in DKO cells whether they were growing or arrested (*lanes 1* and *2*); however, p27 levels were significantly lower in BAX add-back cells, accompanied by lower Ser[10] phosphorylation (*lanes 3* and *4*). The decrease in p27 resulting from re-expression of HA-BAX provided evidence that higher p27 levels in DKO cells was due to the loss of BAX. The total p27 level did not increase significantly in contact-inhibited cells ectopically expressing HA-BAX. Slight variable elevation of p27 Ser[10] phosphorylation could be seen in $bax^{-/-}$ $bak^{-/-}$ cells stably expressing HA-BAX, but this change was insignificant compared with the robust increase of p27 Ser[10] phosphorylation in wild type cells expressing BCL-x_L (Fig. 8*B*, *lanes 3* and *4* with *lanes 5* and *6*). This result indicated that high BAX expression prevented the up-regulation of p27 during cell cycle arrest. Similar results were obtained with the re-expression of BAK in DKO cells (data not shown).

Because Mirk was necessary for the up-regulation of p27 by BCL-x_L, we asked whether Mirk played a role in the elevation of p27 in $bax^{-/-}$ $bak^{-/-}$ DKO cells. Immunoblotting showed that although Mirk was readily detectable in control DKO cells, it was present at a much lower level in DKO cells re-expressing HA-BAX, consistent with decreased Ser[10]-phosphorylated and total p27 (Fig. 8*C*).

To assess the functional consequence of lowered p27 levels caused by re-expression of BAX in DKO cells, S phase re-entry was measured. The cells were released from contact inhibition by replating at low density and pulse-labeled with BrdU at consecutive time points (Fig. 8*D*). Although the increase in BrdU+ uptake in DKO cells was delayed compared with wild type cells, DKO cells expressing HA-BAX exhibited an accelerated rise in the percentage of BrdU+ cells, suggesting that these cells underwent G_1 arrest when reaching confluence rather than G_0 arrest. These findings indicate that lowering of p27 by BAX has a functional cell cycle effect. In repeated

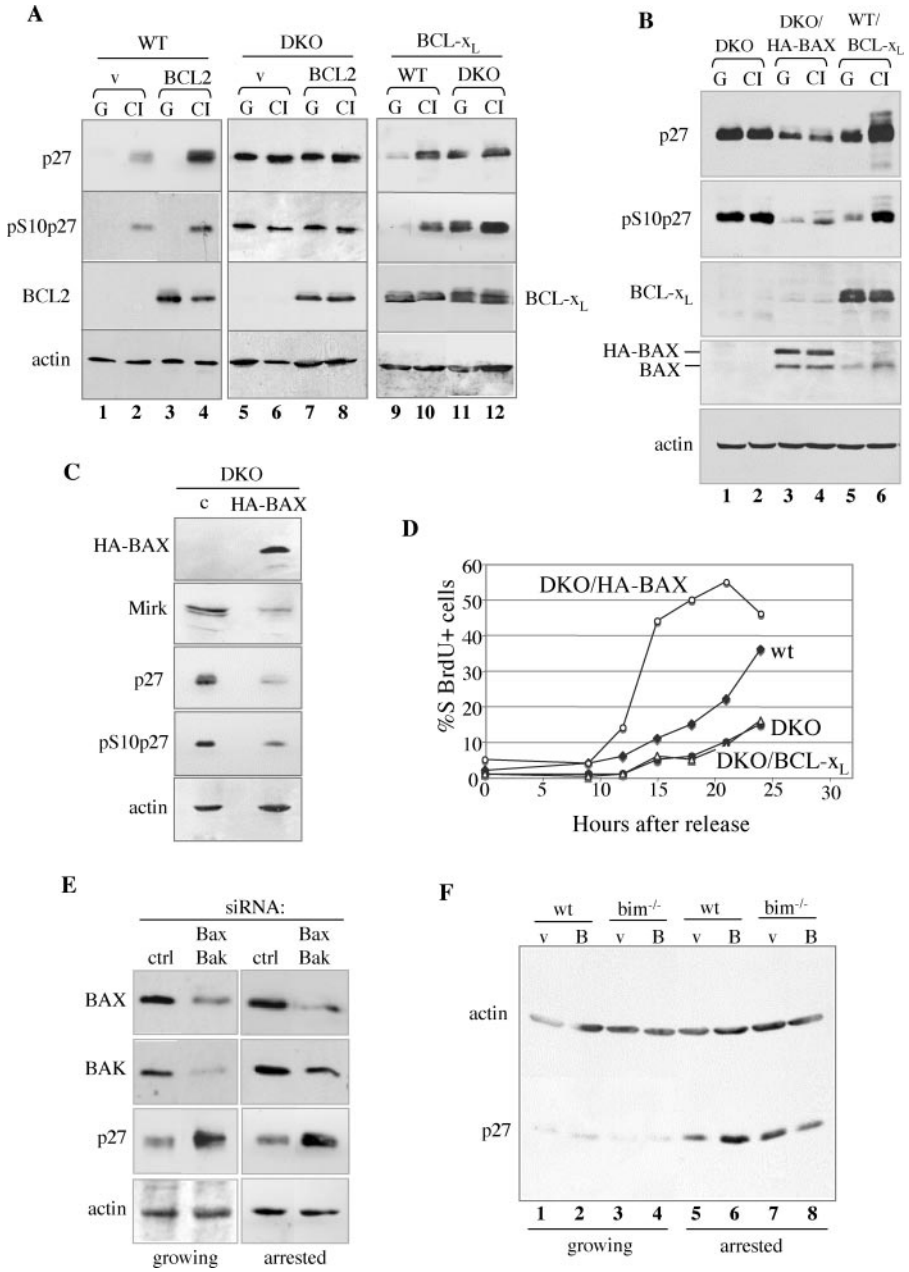

FIGURE 8. **BAX and BAK regulate p27 Ser[10] phosphorylation level.** *A*, asynchronously growing (*G*) and contact-inhibited (*CI*) WT and *bax*$^{-/-}$ *bak*$^{-/-}$ MEFs (*DKO*) expressing vector, BCL2, or BCL-x_L were immunoblotted for total p27and p27 phosphorylated at Ser[10]. *B*, DKO MEFs with or without reintroduced HA-BAX and WT MEFs expressing BCL-x_L were contact-inhibited and immunoblotted for p27, Ser[10]-phosphorylated p27, and BAX. The *lower band* on the BAX immunoblot of the HA-BAX-expressing lysates (*lanes 3* and *4*) migrating with endogenous BAX is likely to be either the result of proteolysis or translation initiation at the second methionine (the initiation methionine for BAX). *C*, control or DKO MEFs re-expressing HA-BAX were immunoblotted for BAX, Mirk, Ser[10]-phosphorylated p27, and total p27. *D*, cell cycle re-entry rate was compared between DKO/HA-BAX, DKO/BCL-x_L, DKO, and WT cells by replating contact-inhibited cells at low density and following BrdU incorporation over time. *E*, WT MEFs transfected with control siRNA or siRNA against BAX and BAK were immunoblotted for BAX, BAK, and p27. siRNA-transfected cells were collected either as growing cultures (growing) or were collected after cells reached confluence (arrested). *F*, asynchronously growing or arrested WT and *bim*$^{-/-}$ MEFs expressing vector (*lanes v*) or BCL2 (*lanes B*) were immunoblotted for p27 and β-actin.

assays, DKO/HA-BAX cells did not exhibit reproducible changes in RNA content or cell size in contact-inhibited cultures, consistent with the inability of DKO/HA-BAX cells to enter a quiescent state.

BAX and BAK Are Required for Suppression of p27—To further confirm that the increase of p27 in DKO cells was due to a lack of BAX or BAK and not other unrelated changes in DKO cells, BAX and BAK expression was silenced. Wild type cells transfected with siRNAs against BAX and BAK were compared with cells transfected with control siRNA for p27 protein expression (Fig. 8*E*). Immunoblotting confirmed that BAX and BAK protein were significantly lowered in siRNA-treated cells. BAX and BAK silencing was sufficient to raise the p27 level, both in asynchronously growing cultures and when the cells were allowed to reach confluence (Fig. 8*E*, *left* and *right panels*). Knockdown of either BAX or BAK alone also caused elevation of p27, but the effect was less dramatic and less consistent than when both BAX and BAK were knocked down. Elevation of p27 in cells with acutely decreased BAX and BAK expression indicated a causal inverse relationship between BAX, BAK expression, and p27 protein level.

Thus, the absence of BAX and BAK resulted in constitutively elevated Mirk phosphorylation of p27 Ser[10] and total p27 level, whereas the presence of ectopic BAX lowered basal p27 protein and blocked entry into G_0, revealing a role for BAX and BAK in the regulation of cell cycle arrest.

Because the apoptotic activity of BAX and BAK can be activated by BH3-only members of the BCL2 family, such as BIM (37, 38), it is possible that BH3 molecules also play a role in cell cycle regulation. We examined p27 levels in *bim*$^{-/-}$ MEFs and found that the p27 level was not altered in growing *bim*$^{-/-}$ MEFs compared with wild type MEFs and that the expected p27 increase in G_0 was unaffected in vector only cells (Fig. 8*F*, compare *lanes 1* and *5* with *lanes 3* and *7*). However, in the absence of BIM, the p27 level during arrest was not further increased by BCL2 expression (Fig. 8*F*, compare *lanes 5* and *6* with *lanes 7* and *8*). Thus, the up-regulation of p27 by BCL2, and likely BCL-x_L, involves interaction with BIM, which may ultimately affect the activity of BAX and BAK during cell cycle arrest.

DISCUSSION

To address whether the cell cycle effects of BCL2 and BCL-x_L are mere consequences of enhanced survival or whether they represent additional functions of BCL2 and BCL-x_L, we inhibited cell death by means other than BCL2 or BCL-x_L expression and assayed whether this led to the same cell cycle arrest phenotype as that caused by BCL2 or BCL-x_L expression. Pharmacologic and genetic inhibition of caspases, which promoted survival nearly as well as BCL2 or BCL-x_L, led to only a partial delay of cell cycle reminiscent of BCL2 or BCL-x_L expression, but not other features of G_0 arrest, including reduction in RNA and cell size or p27 elevation (Figs. 1–3). The partial effects of z-VAD and C9DN on cell cycle entry are consistent with evidence that caspases are involved in the regulation of cell cycle in addition to apoptosis (39–41) but also indicate that caspase inhibition is not sufficient to reproduce the cell cycle arrest effects seen in BCL2 and BCL-x_L cells. Rather, results from z-VAD and dominant caspase 9 experiments suggest that caspase-independent mechanisms are also responsible for the cell cycle effects of BCL2 and BCL-x_L.

The mitochondrial death program is activated by BAX or BAK, and to date, the most effective way of blocking apoptosis other than BCL2 or BCL-x_L overexpression is deletion of BAX and BAK (23, 24). If the G_0 phenotype of BCL2 and BCL-x_L cells simply reflects the selection of a population of cells that survive better in serum starvation or contact inhibition, then $bax^{-/-}$ $bak^{-/-}$ cells, which do not undergo mitochondria-mediated apoptosis, should exhibit the same G_0 characteristics as BCL2 and BCL-x_L cells. We found that although $bax^{-/-}$ $bak^{-/-}$ cells were more "arrested" than wild type cells, they did not phenocopy BCL2 or BCL-x_L cells in the regulation of RNA content, cell size, or p27 level (Fig. 4).

The finding that BCL2 and BCL-x_L were unable to drive cells further into G_0 in $bax^{-/-}$ $bak^{-/-}$ cells suggests that the cell cycle function of BCL2 and BCL-x_L is dependent on BAX and/or BAK. This implies that BAX and/or BAK normally exert a cell cycle function. Indeed, the base-line p27 level was higher in $bax^{-/-}$ $bak^{-/-}$ cells, suggesting that a physiologic function of BAX and BAK is to keep p27 levels low. Supporting this conclusion are the reversal of p27 elevation by re-expression of BAX in $bax^{-/-}$ $bak^{-/-}$ cells and the increase in p27 by knockdown of BAX and BAK in wild type cells (Fig. 8). Consistent with this, a role for BAX in promoting proliferation was shown in BAX transgenic mice with expression in the T cell lineage, which also displayed lowered p27 expression (42). Despite constitutively increased levels of p27, $bax^{-/-}$ $bak^{-/-}$ cells still cycle and arrest, albeit with somewhat slower kinetics. Cell cycle control in these cells must utilize molecules other than p27, either other cdk inhibitors or other pathways. BCL2 has no effect on these other pathways, as indicated by the lack of a cell cycle arrest phenotype by BCL2 or BCL-x_L overexpression in these cells.

Throughout the experiments presented here, when p27 was not elevated, cells failed to enter G_0. Previously, we reported that p27 elevation is necessary for cell cycle delay by BCL2 and BCL-x_L (15). The results here show that up-regulation of p27 is also critical for the G_0 function of BCL2 and BCL-x_L. p27 is regulated predominantly by post-translational mechanisms but also at the transcriptional and translational levels. We found no evidence that p27 was regulated transcriptionally or translationally in BCL2 or BCL-x_L cells. Instead, the stability of p27 was markedly increased in BCL2 or BCL-x_L cells during quiescence.

Stability of p27 in G_0 has been reported to be mediated by a ubiquitin ligase system through phosphorylation of p27 at Ser^{10} (29, 31, 43). Increased p27 in BCL2 or BCL-x_L cells and the inability of BCL-x_L to exert its G_0 function in MEFs in which p27 Ser^{10} is mutated to alanine indicates that BCL2, BCL-x_L, BAX, and/or BAK regulate the stability of p27 through phosphorylation of p27 at Ser^{10}, the major phosphorylation site of p27 (Fig. 6). In support of this result, we found that stabilization of p27 was not caused by alterations in the induction of either SCF^{skp2} ubiquitin ligase or Cks1 protein, which regulate p27 degradation at the G_1-S transition through Thr^{187} phosphorylation (data not shown) (26–28).

We identified Mirk as a kinase responsible for the BCL2/BCL-x_L-mediated increase in Ser^{10} phosphorylation, which is in keeping with the known function of Mirk as a G_0 kinase. The finding that BCL2/BCL-x_L could not up-regulate p27 in the absence of Mirk indicated that the G_0 function of BCL2/BCL-x_L required the pathway involving Mirk and p27. Similarly, the suppression of the Mirk level by BAX expression implies that a normal function of BAX is to keep cells in cycle. How Mirk is regulated by expression of the BCL2 family is unknown, but the mechanism is probably indirect, considering that Mirk is a nuclear enzyme and BCL2 family members are mostly cytoplasmic. Mirk is not the only kinase known to phosphorylate p27 Ser^{10}. The kinase hKIS also phosphorylates this site, but hKIS activity is associated with degradation of p27 by the Kipl ubiquitylation-promoting complex ubiquitin ligase as cells leave G_0 to re-enter G_1 (44, 45). Thus, hKIS is an unlikely candidate kinase for the promotion of G_0 by BCL2 or BCL-x_L, although we do not have evidence to exclude this possibility.

Increased phosphorylation of p27 Ser^{10} in cycling $bax^{-/-}$ $bak^{-/-}$ cells suggested that the presence of BAX or BAK normally either suppresses p27 Ser^{10} phosphorylation during cell cycle or is involved in the degradation of Ser^{10}-phosphorylated p27. Caspase inhibition did not result in increased p27 levels; therefore, the ability BAX or BAK to modulate p27 is independent of caspase activation. The causative role of BAX in maintaining a low level of p27 in cycling cells was confirmed by re-expression of BAX in $bax^{-/-}$ $bak^{-/-}$ cells and by silencing of BAX and BAK in wild type cells. In our hands, BAX appears to play a stronger modulatory role on p27 levels, whereas re-expression of BAK had a less pronounced effect on p27 and S phase kinetics (data not shown). We found that single knockdown of either BAX or BAK resulted in variable p27 levels, but simultaneous knockdown of both BAX and BAK consistently raised p27 protein expression. This suggests that both BAX and BAK regulate p27, and endogenous BAX and BAK share redundancy in cell cycle regulation.

Our experiments revealed an unexpected function of endogenous BAX and/or BAK, which is to prevent p27 accumulation and maintain p27 at low levels necessary for cell cycle. The finding that BCL2 and BCL-x_L require the presence of BAX or BAK to promote G_0 revealed that what has been observed as the

anti-proliferative effect of BCL2 and BCL-x_L may actually reflect the cell cycle function of BAX and BAK. This provides an explanation for the inability to genetically separate the survival and the cell cycle functions of BCL2 and BCL-x_L.

We propose a mechanism for quiescence regulation by BCL2, BCL-x_L, BAX, and BAK in which BAX and BAK normally exert a negative effect on p27 phosphorylation at Ser^{10}, maintaining p27 at a low level, whereas BCL2 and BCL-x_L oppose this negative effect of BAX and BAK to cause accumulation of p27 through phosphorylation by Mirk. An unanswered question is why deletion of BAX and BAK is not the same as BCL2 or BCL-x_L expression in terms of cell cycle arrest. If BCL2 and BCL-x_L simply inactivated BAX and BAK, then the p27 level in BCL2 or BCL-x_L cells should be the same as in $bax^{-/-}$ $bak^{-/-}$ cells, but in fact, p27 level in arrested BCL2 or BCL-x_L cells exceeded the level in $bax^{-/-}$ $bak^{-/-}$ cells (Fig. 8*B*). It is not clear whether this was due to cellular adaptation or whether there are required dynamic changes in BAX or BAK activity that cannot occur in $bax^{-/-}$ $bak^{-/-}$ cells, for example due to activities of BH3 molecules.

We did not detect a cell cycle arrest phenotype in $bim^{-/-}$ cells, but the inability of BCL2 to exert an effect on p27 in $bim^{-/-}$ cells suggests the possibility that BIM activation of BAX and BAK keeps p27 level in check even during cell cycle arrest. The high level of BCL2 sequesters BIM, so that BAX and BAK are not activated, and the p27 level rises unrestrained. Why this scenario would only operate during arrest but not growing cells requires further investigation.

$bax^{-/-}$ $bak^{-/-}$ cells exhibit features of quiescence, whereas BAX-overexpressing cells fail to arrest in G_0. Thus, BAX and BAK negatively regulate quiescence through suppression of p27 phosphorylation, but the precise mechanism is unknown and may be indirect. Our results are based on immortalized, but not oncogene-transformed, $bax^{-/-}$ $bak^{-/-}$ MEFs, and could not be further tested *in vivo* at this point since $bax^{-/-}$ $bak^{-/-}$ animals do not exist due to lethality. Taken together, in addition to their well known role in apoptosis, our data are indicative of a role of BAX and BAK in the regulation of cellular quiescence *versus* proliferation.

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