2007

Bcl-2 related ovarian killer, Bok, is cell cycle regulated and sensitizes to stress-induced apoptosis

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Bcl-2 Related Ovarian Killer, Bok, Is Cell Cycle Regulated And Sensitizes To Stress-Induced Apoptosis

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Graduate School
University of South Florida

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Date of Approval
June 28, 2007

Keywords: E2F1, flavopiridol, promoter, luciferase assay, chromatin immunopresipitation

Dedication

I would like to dedicate this work to my parents María C. Medina and José A. Rodríguez for all their support and love during this journey. Mom, you always told me to believe in God because he is the only one who is going to help me pass this challenge, and also told me how important it is in taking care of my family because at the end of the day, that is what really maters. Dad you are my rock, you never hide the reality of how hard it is to accomplish my goal and you told me that anything in this life is not easy to obtained specially a good education. Because of your support, now I am graduating with a PhD. Thank you mom and dad for teaching me how important it is to have a career and be grateful for all the good things I have in my life. To my sister Michelle J. Rodriguez thank you for being the best sister and teaching me how to be patient with my self and the rest of the people, thank you for all the advice you gave me during this time. To my in-laws, Luisa A. Cruz and Carlos Reyero for always been there for me and help me with the children. Also thank you for you're daughter Carole C. Reyero Cruz who is my lovely wife because with out her I wouldn't be the person I am now. To Carole for always been there even during the very stressful times. Thank you for your unconditional love and for making me a better person. Last but not least, to my beautiful children Adrian A. and Ryan L. Rodríguez who make my life complete and joyful. You guys are, with out a doubt, the base of my will to succeed!

Acknowledgments

First I will like acknowledge my mentor Dr. Cress for all of his support, advice and guidance and thank him for believing in me when not even I was believing in myself. I feel fortunate to have him as mentor in science as well as in life. Also my committee Dr. Chellappan, Dr. Haura and Dr. Wright for taking time from their busy schedule to advice and review my work. In addition I like to acknowledge Jaime L. Matta, PhD. from the Ponce School of Medicine in Ponce, Puerto Rico for being a great advisor as well as the chairman of my committee.

This work was supported by funds from the National Cancer Institute (CA90489, W.D.C.), Minority Supplement to CA090489 (J.M.R.), Department of Defense (National Functional Genomics Pilot Project 12-12990-01-01, W.D.C.) and by the Molecular Biology, Flow Cytometry, Analytical Microscopy, and the Molecular Imaging Core Facilities of the H. Lee Moffitt Cancer Center and Research Institute.

I also acknowledge Michele A. Glozak, Yihong Ma, Scott N. Freeman and Rachel C. Haviland for their friendship, scientific input and review.

Note to Reader

The original of this document contains color that is necessary for understanding the data. The original dissertation is on file with the USF library in Tampa, Florida.

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Bcl-2 Related Ovarian Killer, Bok, is Cell Cycle Regulated and Sensitizes to Stress-Induced Apoptosis

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ABSTRACT

Bok/Mtd (Bcl-2-related ovarian killer/Matador) is considered a pro-apoptotic member of the Bcl-2 family. Though identified in 1997, little is known about its biological role. We have previously demonstrated that Bok mRNA is upregulated following E2F1 over-expression. In the current work, we demonstrate that Bok RNA is low in quiescent cells and rises upon serum stimulation. To determine the mechanism underlying this regulation, we cloned and characterized the mouse Bok promoter. We find that the mouse promoter contains a conserved E2F binding site (-43 to –49) and that a Bok promoter-driven luciferase reporter is activated by serum stimulation dependent on this site. Chromatin immunoprecipitation assays demonstrate that endogenous E2F1 and E2F3 associate with the Bok promoter *in vivo*. Surprisingly, we find that H1299 cells can stably express high levels of exogenous Bok. However, these cells are highly sensitive to chemotherapeutic drug treatment. Taken together these results demonstrate that Bok represents a cell cycle-regulated pro-apoptotic member of the Bcl-2 family, which may predispose growing cells to chemotherapeutic treatment.

Chapter 1: Introduction

E2F Family of Transcription Factors

The E2F family of transcription factors has key roles in regulating the G1/S transition [67,79,85,90]. There are nine E2F members identified, so far [12,20,24,42,62,89,114,132]. This family can be divided into three distinct groups based on both structure and function. E2F1, 2 and 3A make up the first distinct group. Structurally, a long N-terminal region, of unclear function, distinguishes these E2Fs (Fig 1). They also contain a cyclin A binding domain important for their down regulation in S phase [72,76,144]. At the C-terminus, each possesses a potent transcriptional activation domain that contains an Rb binding motif [1,44,69,78,78]. Functionally, these E2Fs appear necessary for cell cycle progression [1,57,90], they are primarily expressed at the G1/S boundary [1,27,52,59,65,79,92,116] and they potently drive S phase when expressed in otherwise quiescent rodent fibroblast [22,67,75,85].

In contrast, members of the second group of E2Fs (3B, 4 and 5) lack the N-terminal region (Fig 1) and are expressed ubiquitously through the cell cycle [134]. They can activate transcription of G1/S genes when over expressed in rodent fibroblast, particularly E2F3B [43], but do so less efficiently than E2F1-3A [22,85]. These E2Fs appear essential to maintain growth arrest [31,109] and contribute to differentiation [103,109]. Mechanistically these E2Fs may primarily serve to tether Rb to E2F-regulated promoters

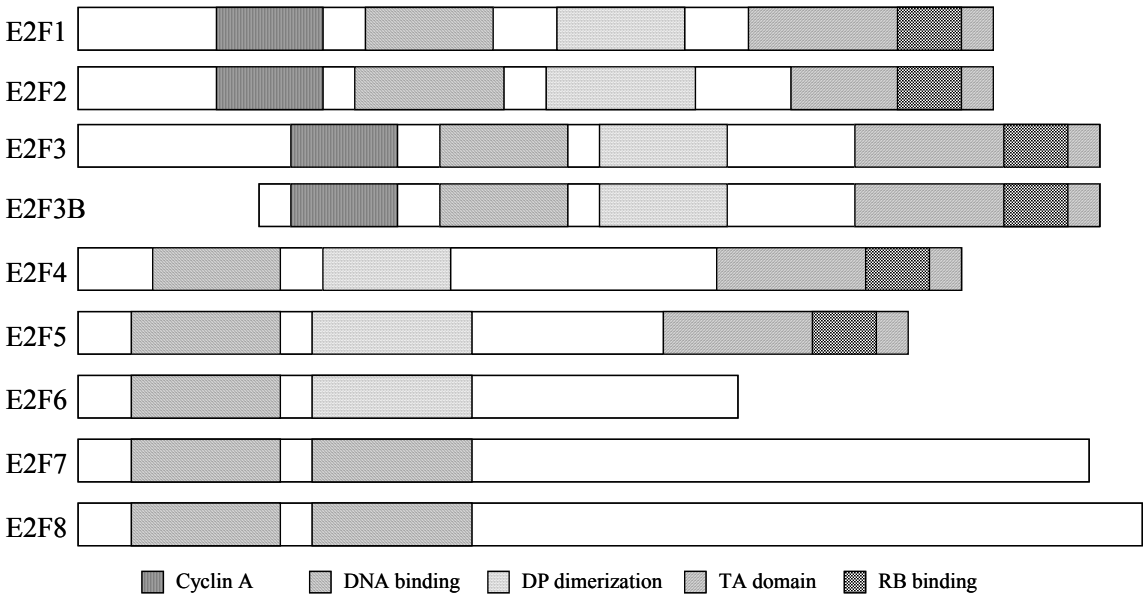


Figure 1. Schematic representation of the E2F family of proteins. Shaded boxes indicate important and conserved domains. What all E2F's have in common is the DNA binding domain. E2F1-3 are the activating E2F's, where as E2F3B-8 are implicated in growth repression.

during G0 [31,103], and may also serve to generate an initial pulse of E2F activity that is subsequently amplified by activating the transcription of the more potent E2F1, 2 and 3A.

Finally, E2F6, 7 and 8 represent the third group (Fig 1). These E2Fs appear to lack the transcriptional activation/Rb binding domain present in other E2Fs and serve exclusively to repress transcription via interaction with transcriptional repressors [12,20,24,83,89,97,132]. For example, E2F6 binds to transcriptional co-repressors due to its ability to bind polycomb protein molecules and generally serves to repress growth [97].

E2F Role in Cell Cycle

Progression through the cell cycle is regulated by many proteins, which include cyclins, cyclin dependent kinases (CDK), cyclin dependent kinase inhibitors (CKI), E2F family members and the retinoblastoma protein (pRb) family members among others [2,6,9,14,23,32,36,41,45,66,71,73,95,98,106,115,120,121,124,127,128,131,133,146,147]. In a resting cell, hypophosphorylated pRb and its family members p107 and p130, bind and inactivate the E2F transcription factors forming the checkpoint during the G1/S boundary. This checkpoint regulates the transition between cell proliferation and terminal differentiation. Studies in mouse fibroblast with deleted pRb, p107 or p130 suggest their important role in the arrest of the G1 phase of the cell cycle [15]. When a cell receives mitogenic signals by growth factors, cyclins become upregulated and form complexes with CDKs. The main regulators of the G1/S transition are the D type cyclins and their binding partners CDK4/6 (Fig 2). After mitotic stimulation, cyclin D/CDK4 and cyclinD/CDK6 complex hyperphosphorylates pRb family leading to the release and activation of E2F. The E2F

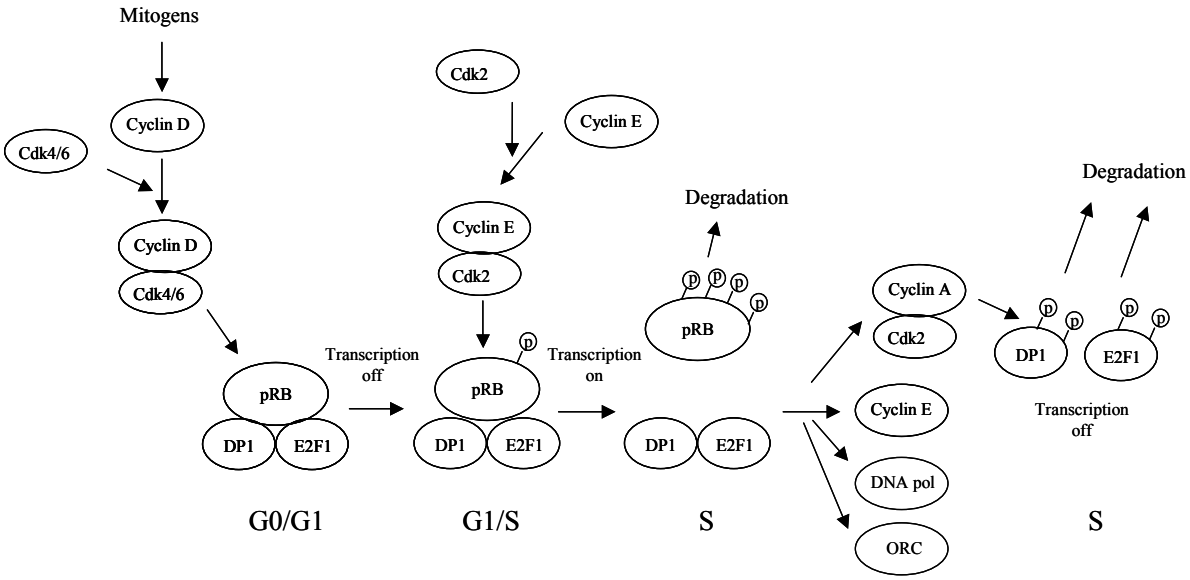


Figure 2. Role of E2F in cell cycle control. After mitogenic stimulation (top left), cyclin D/CDK 4/6 lays the initial phophorylation on RB. Cyclin E/CDK 2 then continues to phosphorylate RB and this leads to the hyperphosphorylation and degradation of RB. Thus, E2F's are free to transactivate genes required for DNA synthesis. Once in S phase, cyclin A/CDK 2 in a negative feedback loop targets E2F's for degradation.

transcription factor then mediates cell cycle-dependent expression of genes important for DNA synthesis such as thymidylate synthase (TS), dihydrofolate reductase (DHFR) and DNA polimerase α [21]. Though *in vitro* E2F1 can bind DNA as a homodimer, in cells E2F binds promoters as a heterodimer with a memberof the DP family [104,149,150]. Association with a DP protein significantly increases its sequence-specific binding to its target genes. After S phase induction Cyclin A/CDK2 down regulates E2F by phosphorylating it and targeting it for degradation [125] (Fig 2). Down regulation of E2F is important for cell survival because otherwise the cell would undergo apoptosis. In addition to the regulation of genes required for cell cycle progression E2Fs also regulate genes involved in growth arrest, differentiation and apoptosis.

Another layer of regulation involves CKI, which mediated growth arrest through the inhibition of the phosphorylation of pRB and the stabilization of p53; and are therefore involved in tumor suppression. The CKIs are grouped in two families, the INK4 and the Cip/Kip family [13,40,117]. The first family, the INK4 (inhibitors of cdk4) proteins, is composed of 4 members and selectively inhibits CDK4 and CDK6. The four INK4 inhibitors are $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, and they do not bind any other CDKs. The second family, Cip/Kip inhibitors (CDK interacting protein/Kinase inhibitory protein) are $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, and in contrast to the INK4 family, they are not as selective in their activity, and are able to inhibit cyclin E/CDK2 and cyclin A/CDK2.

Role of E2F1 in Apoptosis

The most striking functional difference between E2F family members is the unique ability of E2F1 to induce apoptosis and our laboratory and others have demonstrated this role of E2F1 by over-expressing it in tissue culture cells and measuring apoptosis [1,10,22,28,29,33,50,51,53,58,64,81,82,99,100,105,113,122,123,143]. Physiologically, E2F1's role in apoptosis is suggested by experiments showing that mice deficient in E2F1 develop tumors in the reproductive tract, lung and lymphatic system, presumably for the lack of apoptosis [26,139,148]. E2F1 can induce apoptosis by both p53-dependent [1,63,100,112] and p53-independent pathway [80,123] (Fig 3).

In tissue culture, over-expression of E2F1 leads to the increase of p53 and subsequent apoptosis. One of the molecular pathways this is achieved is by the direct transcriptional activation of p14ARF gene (p19ARF in mouse) by E2F1 [5,48,82,129,130]. Accumulation of p14ARF leads to its interaction with the Hdm2 (Mdm2 in mouse) E3 ubiquitin ligase. The binding between p14ARF and Hdm2 inhibits the ability of Hdm2 to target p53 for degradation. As a net consequence the E2F1 increase in p14ARF levels leads to stabilization and activation of p53. In addition, E2F1 can lead to the accumulation of a p53 relative by directly transactivating p73. p73 is a homolog of p53 that regulate the p53 promoter [82,136]. p73 transactivates some of the same targets genes as p53 [68,82,119] and also has the ability to induce apoptosis in mouse embryonic fibroblast that are deficient of p53 [61] indicating a tumor control mechanism that runs parallel. Additionally E2F1 can elevate the activity of the ataxia-telangiectasia-mutated kinase [49] promoter and induces an increase in the ATM mRNA and protein. In turn ATM leads to

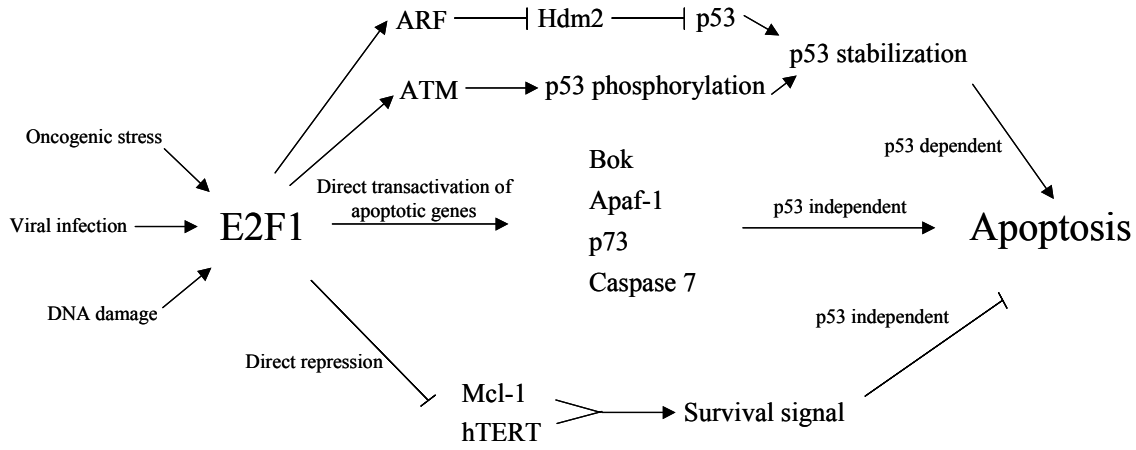


Figure 3. E2F1 pathways towards apoptosis. E2F1 is the best inducer of apoptosis among the E2F family and it does this trough a p53-dependent (top lanes), and p53-independent (bottom lanes) pathways. E2F1 can lead to the stabilization of p53 by transactivating ARF, which leads to the inhibition of Hdm-2 (a protein that targets p53 for degradation), or by inducing an ATM-dependent phosphorylation of p53. In addition, E2F1 can induce apoptosis independently of p53 protein by directly transactivating apoptotic genes or directly repressing survival genes.

the phosphorylation and stabilization of p53 as well as E2F1 (in a positive feedback loop) in response to genotoxic stress [105].

Many cancer cells evade apoptosis by deregulating or mutating the tumor suppressor gene p53. Most chemotherapeutic agents work by inducing apoptosis incancer cells, but in many cancer cells the apoptotic induction of p53 is not functional. In the other hand E2F1 is not found mutated in human cancers and it can induce apoptosis in a p53-independent manner [19,47,61]. Thus, E2F1 and the apoptotic proteins that it induces are excellent targets that might be used in cancer chemotherapy.

Figure 3 also highlights the fact that E2F1 can induce apoptosis independent of p53 by transactivating genes involve apoptosis and repressing genes involved in survival pathways. Our lab showed in a microarray and Northen blot analysis that E2F1 can repress the survival genes such as myeloid cell leukemia-1 (Mcl-1), amyloid-β precursor protein binding protein 2(APP-BP2), programmed cell death 4 (PDCD4), and carnitine palmitoyltransferace I (CPT-I) [87]. On top of that, E2F1 can activate genes involved in the induction of apoptosis such as Bok, apoptosis protease-activating factor 1 (Apaf-1), and caspase 7 [102,111]. Increase E2F1 activity leads to the release of cytochrome C from the mitochondria to the cytoplasm as a consequence of the action of the aforementioned genes. Thus, E2F1 can tip the balance between pro-survival and pro-apoptotic genes towards cell death.

Bcl-2 Family of Proteins

Apoptosis or programmed cell death is an important process for the maintenance of tissue homeostasis and the prevention of diseases such as cancer. A number of targets in E2F-regulated cell death have been identified and these include members of the Bcl-2 family [19,25,39]. The Bcl-2 family of proteins consists of different anti- and pro-apoptotic members that mediate cytochrome C release from mitochondria and thus play important roles in the "decision" step of the intrinsic apoptotic pathway [16,108]. All members of the Bcl-2 family are characterized by containing at least one of the four Bcl-2 homology domain (BH). Traditionally anti-apoptotic members, contain all four BH domains, where as pro-apoptotic members contain only three or less. Within the pro-apoptotic members there is a subgroup that contain only one BH domain (the BH3-only members), which is presume as a critical death domain in the pro-apoptotic [91]. Bok, a pro-apoptotic member of the Bcl-2 family, was first cloned in a yeast two hybrid screen of an ovarian cDNA library for proteins that interacted with Mcl-1, BHRF1 and Bfl-1 [55]. The mouse homolog (Mtd) was identified bioinformatically [60]. Bok contains Bcl-2 homology domains (BH1, 2, 3) and can heterodimerize with Mcl-1, BHRF-1 and Bfl-1, but not Bcl-2 or Bcl-xl [54,55,60]. Bok can induce apoptosis in a variety of cell types [7,54,55,60,126] and this activity is inhibited by Mcl-1, BHRF-1 and Bfl-1, but not Bcl-2 or Bcl-xl. There have been reports that Bok has a nuclear export signal within its BH3 domain and that Bok localizes to the nucleus as well as the cytoplasm [4]. They also showed that accumulation of Bok in the nucleus increases Bok's apoptotic activity. In the present work, we investigated the transcriptional regulation of Bok and its potential roles in cell cycle. We find that Bok is an E2F-regulated gene activated by serum stimulation that localizes mainly in the

cytoplasm, and that it may function as a checkpoint sensitizing growing cells to stress-induced apoptosis.

Chemotherapeutic Agents

One of the hallmarks of cancer is the limitless replicative potential, which is not under strict regulatory control as in a normal cell. Cancer cells lose the ability to respond to contact inhibition. In addition cancer cells bypass cell cycle checkpoints and apoptosis that otherwise a normal cell will undergo after "sensing" an imbalance or an uncontrolled regulation of cell division. Most of the chemotherapeutic agents developed target this characteristic of a rapidly dividing cancer cell. The first chemotherapeutic agent was discovered by accident during World War I when Mustard gas was used as a chemical warfare agent. The observation that people that were exposed to this gas had low blood cell count intrigued scientist and motivated them to study it further. During the decade of the 1940s, patients with lymphomas were given the drug intravenously (instead of inhaling the irritating gas) and scientist saw a remarkable improvement, although temporary [101,137]. Thereafter many studies have focus on discovering or developing other chemical agents to kill rapidly dividing cells such as cancer cells. Most of the chemotherapeutic agents can be classified as alkalating agents, antimetabolites, kinase inhibitors or topoisomerase inhibitors. In my research I used the chemotherapeutic agent Flavopiridol, which is a kinase inhibitor, and VP-16 (a.k.a. etoposide) that is a topoisomerase II inhibitor because they function via E2F1. We believe that Flavopiridol inhibits cyclin A/cdk 2's ability to phosphorylate E2F1 and target it for degradation, leading to its stabilization and consequent transactivation of apoptotic target genes, where

as VP-16 induces cell death by stabilizing topo II-double stranded breaks complex, which leads to the accumulation of ATM kinase, and subsequent phophorylation and stabilization of E2F1.

Flavopiridol

Flavopiridol is one of the most studied CDK inhibitors. A semi-synthetic N-methylpiperidinyl chlorophenyl flavone alkaloid compound originally isolated from the leaves of *Amora rohituka*. It was first intended to be used as an inhibitor of EGFR, however upon examination it was found to inhibit the cell replication CDKs at a far lower concentration. At nanomolar concentrations, Flavopiridol was shown to inhibit CDK4 and CDK6 [11,93], the main kinases known to regulate the G1/S transition and E2F1 activity, among others. Its been shown in clinical trials that combination of Flavopiridol treatment and other chemotherapeutic drugs, such as docetaxel, can increase apoptosis in cancer cells [34]. Flavopiridol induces cell cycle arrest in G1 *in vivo* and *in vitro* [96,107,142]. It is cytotoxic to cells synthesizing DNA and can induce apoptosis in a p53-independent manner [118]. Flavopiridol stabilizes E2F1 protein levels in a dose-dependent manner and the inverse effect is seen on the Mcl-1 levels [86]. One of the proposed mechanisms in which Flavopiridol can lead to apoptosis is by antagonizing cyclin/cyclin dependent kinase 2's ability to target E2F1 for degradation. This leads to E2F1 stabilization and subsequent reduction in Mcl-1, a pro-survival protein, and presumably the accumulation of pro-apoptotic E2F1 target genes such as Bok, p73, caspases and others. In this study we assess the importance of Bok, an E2F1 target gene, in the sensitivity of Flavopiridol-induced apoptosis. We found that higher expression of the Bok protein, the faster the

induction of apoptosis by Flavopiridol. This observation suggests that assessing a patient's levels of Bok might predict the outcome response of the flavopiridol treatment.

VP-16

Topoisomerase II is a ubiquitously express enzyme that regulates the winding of DNA [30,77]. It removes the knots and tangles generated during DNA replication and transcription, through the creation of double-stranded breaks in the double helix. VP-16 is a chemotherapeutic agent that targets topoisomerase II enzyme [3,8,38,84,94,151], and has been used for several types of cancer including lung, prostate, ovarian and testicular cancer. VP-16 works by stabilizing a covalent enzyme-cleaved DNA complex. After treatment with VP-16, cells accumulate enzyme-cleaved DNA complexes, which results in the generation of permanent DNA strand breaks that in turn trigger recombination/repair pathways and mutagenesis. The massive accumulation of these breaks can overwhelm the cell and can trigger the initiation of death pathways. Thus, VP-16 converts topoisomerase II from an essential enzyme to a potent cellular toxin that fragments the genome.

Experimental Procedures

Cloning the Bok promoter- Approximately $5x10^5$ plaques from a *Sau*3A I partially digested 129SV mouse genomic library in λFIXII (Stratagene) were screened in duplicate with a mixture of Bok cDNA probes. The probes consisted of full-length human Bok cDNA (nt 247-882 of NM_032515) (human and mouse sequences are 88% identical in the coding region) and a 3' UTR mouse Bok probe (nt 940-1430 of NM_016778). Screening was performed in 50% formamide and filters were washed at high stringency. Ten positive plaques were identified and rescreened in secondary and tertiary screens using the same combination of probes. Following plaque purification and a quaternary screen, seven purified positive plaques were identified. Plate lysates were prepared from these seven clones to serve as phage stocks. The phage stocks were titered, then used to prepare plate lysates to extract the phage DNA. Phage DNA was extracted using the Qiagen MIDI lambda kit according to manufacturer's specifications. NotI digestion of the phage DNA indicated that each clone had a different sized insert, each in the ~15-20 kb range.

Each of the phage DNAs was digested with a panel of restriction enzymes, then loaded on duplicate 0.8% agarose gels. The digested DNA was Southern blotted overnight to Immobilon Ny+ membranes. The duplicate blots were hybridized to each of the Bok probes individually to roughly map the 5' and 3' ends of the inserts. Comparison of the hyridization to each of the probes revealed similar, but not identical, patterns of hybridizing bands. This indicated that the clones were unique.

Each of the phage DNAs was then digested with NotI to excise the entire insert for cloning into pBluescript (pBS). In addition, based on differential hybridization patterns, phage DNAs was also digested with XhoI or SstI to subclone smaller fragments into pBS. Bluescript clones containing inserts were sequenced with T3 and T7 promoter primers using the Moffitt Cancer Center Molecular Biology Core Facility. Sequences were BLASTed against the mouse genome database to confirm the ends of each clone. Each clone matched an area of the Mus musculus chromosome 1 genomic contig NT_039173.2. Overlapping clones covering the entire Bok locus are shown in Figure 1. Clones 8N1 (approximately 15 kb, 8090408- (the 3' and has not been determined due to suboptimal sequencing), 11N6 (16.2 kb, 8083204-8099427) and 15N9 (16.8 kb, 8089917-8106754) contain the entire phage insert. In particular, clone 11N6 contains the entire Bok coding region and will be used to prepare the targeting construct.

Plasmids-Mouse Bok promoters were generated by digestion of pBS-13S2 with *Sst* I and ligated into pGL3 basic. Initial PCR primers were design to amplify 331 bp (-244/+87) of our sequenced Bok promoter, which are numbered relative to the transcriptional start site. The forward (192 F) and reverse (141 R) PCR primers for the Bok promoter were 5'-GGTACCAGAACTTGTGCTGGCCTTTCT-3' and 5'-AAGCTTAGTTCTGGTTTCAGGACCCGC-3', respectively. The forward primer added a *Kpn I* site, and the reverse added a *Hin*d *III* site to facilitate sub-cloning. The E2F binding site mutant of the Bok promoter was generated by site-directed mutagenesis with PCR. The initial reaction was done using 192 F and 192 R (5'-TCCGCCGGTCTTCC*AT*CGCGC-3'); a second reaction used primer 141 F (5'-

CGCG*AT*GGAAGACCGGCGGA-3') and 141 R. The PCR products from these reactions, 192 bp and 141 bp respectively, were band purified, phenol/chloroform extracted and ethanol precipitated. They were then resuspended in water, combined, and used as template in another PCR reaction using the flanking primers 192 F and 141 R. The resulting PCR product was inserted in pCRII-TOPO, followed by digestion with *Kpn* I and *Hin*d *III* (to excise PCR insert). Insert was run in a 1% agarose gel and band purified using QIAquick gel extraction kit (Quiagen) and ligated to pGL3 luciferase vector. The E2F1 mutant constructs, E2F1 (1-284) and E2F1 (Eco 132) have been previously described [17,18].

Cell culture- Mouse NIH 3T3 fibroblasts were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% calf serum. The H1299 lung cancer cell line was cultured in DMEM supplemented with 5% fetal bovine serum. H1299 cells that constitutively express Flag-Bok fusion protein were obtained by transfecting with pcDNA3-Flag-Bok (a gift from Gabriel Nunez, Univ. of Michigan) and selecting for transformants in 400 μg/ml G418. G418-resistant lines were screened for expression of Flag-Bok. Adenoviruses were described previously [18,87] and were tittered by plaque assay. Cell cycle parameters were measured by fixing cells with 70% ethanol-PBS, staining with propidium iodide (PI) and analyzing by FACS, using ModFit.

Biochemical assays- Transfections were performed using LipofecAMINE PLUS™ Reagent from Invitrogen with test DNA totaling 2.85 μg of DNA per 60-mm dish. Transfections included 100 ng of expression plasmids (pcDNA3-based vectors), 2.5

μg of test construct firefly luciferase reporter plasmid (pGL3, Promega), and 250 ng of renilla luciferase reporter plasmid (pRL-TK, Promega). Cells were harvested 48 hrs after transfection, and luciferase assays were performed using the Dual-Luciferase Reporter Assay System following the manufacturer's protocol (Promega). Experiments were done in duplicate or triplicates, and the relative activities and standard deviation values were determined. To control for transfection efficiency, firefly luciferase values were normalized to the values for renilla luciferase. Western blots were performed as previously described [18,86] using monoclonal antibody against Flag epitope (F3165, Sigma) or against PARP antibody (Cell signaling 9542). Western blots were stripped and re-probed with an antibody to actin (A5441, Sigma) to ensure equivalent loading.

RT-PCR-Isolation of total RNA was done using the RNeasy mini kit (Qiagen 74104) as recommended by manufacturer. Total RNA was primed with random hexamers and cDNA created using SuperScrip™ First Strand Synthesis System for RT-PCR (Invitrogen 11904-018). PCR primers were designed to amplify 490 bp. The forward and reverse primers were 5'-CGCTCGCCCACAGACAAGGAG-3' and 5'-TCTGTGCTGACCACACACTTG-3'.

Chromatin Immunoprecipitation- ChIP assays were performed as previously described [18,37,86,110,138-140]. Briefly, asynchronously growing NIH 3T3 cells were treated with formaldehyde to create protein-DNA cross-links, and the cross-linked chromatin was then extracted, diluted with ChIP buffer, and sonicated. Sonicated chromatin was

divided into equal samples for immuno-precipitation. Antibodies used included E2F1 (sc-193X), E2F3 (sc-878X), and IgG (sc-2027) (from Santa Cruz Biotechnology).

Chapter 2: Characterizing the Bok Promoter

Identification of Bok as a Potential E2F1 Target

In a previous microarray screen [87], we identified Bok as a potential E2F1 target gene. To confirm this observation, we tested if over-expression of E2F1 would correlate with increased expression of Bok mRNA. NIH 3T3 cells were brought to quiescence by 48-hrs incubation in 0.5% calf serum. Cells were then stimulated with 10% fetal calf serum or were infected with ten plaque-forming units of the indicated adenovirus per cell. Fig. 4 highlights the observation that Bok mRNA is very low in quiescent NIH3T3 fibroblasts (lane 3), but is highly induced following infection with an E2F1-expressing adenovirus (lane 1). Lane 4 reveals that serum treatment, which stimulates quiescent cells to enter S phase, also elevated Bok message (lane 4), suggesting that Bok is E2F and cell cycle regulated. As a control we wanted to determine the cell cycle status of the treated cells (Fig 4) by harvesting half of the samples and fixing the NIH 3T3 cells with 70% ethanol-PBS, stained with PI and analyzed by FACS. Figure 5 demostrate that NIH 3T3 cells were brought to quiescence by 48-hrs incubation in 0.5% calf serum (accumulation in G0/G1), and upon stimulation with 10% fetal calf serum or infection with ten plaque-forming units of E2F1 adenovirus, cells progress through the cell cycle, in contrast to empty-vector control virus.

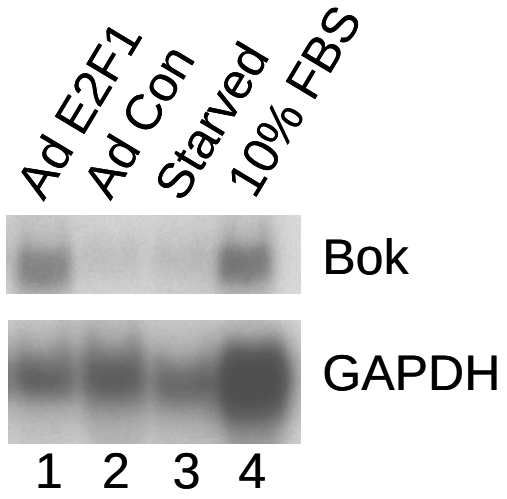


Figure 4. Bok mRNA is activated by E2F1 or serum stimulation. NIH 3T3 cells were brought to quiescence by 48-hrs incubation in 0.5% calf serum. Cells were then stimulated with 20% fetal calf serum or were infected with ten plaque-forming units of the indicated adenovirus per cell. Total RNA was harvested after 24 hrs (serum) or 30 hrs (virus). Twenty microgram of RNA were subjected to Northern analysis using the indicated cDNA probes.

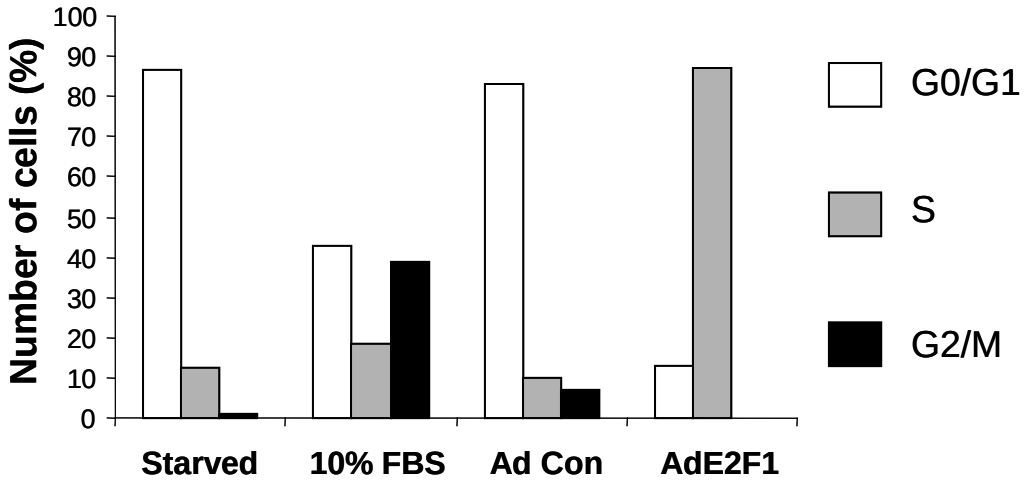


Figure 5. Cell cycle status of NIH 3T3. NIH 3T3 cells were brought to quiescence by 48-hrs incubation in 0.5% calf serum. Cells were then stimulated with 10% fetal calf serum or were infected with ten plaque-forming units of the indicated adenovirus per cell. Cells were harvested after 24 hrs (serum) or 30 hrs (virus). NIH 3T3 cells were fixed with 70% ethanol-PBS, stained with PI and analyzed by FACS.

The Bok Promoter Contains a Conserved E2F Binding Site Between Mouse and Human.

To understand how Bok is regulated in an E2F/cell cycle-dependent manner, we compared the genomic sequences of human (AC110299) and mouse Bok (NT_039173). To obtain authentic Bok genomic sequence from mouse, we screened a lambda phage library using a mixture of human cDNA probes and mouse UTR Bok probes. Fig 6 shows a schematic of the various clones obtained. One of the sub-clones, 13S2, which contains the first two Bok exons and over 900 bp of upstream promoter region, was sequenced. Comparison of the mouse and human Bok 5' regions (shown in Fig. 7) revealed significant sequence homology within the first exon (non-coding) and in a region –244 upstream of the putative transcriptional start site in mouse [141].

Crude deletion analysis localized the promoter to –244/+87 (not shown). Potentially important motifs within this region include numerous SP1 binding sites and, most importantly, a conserved E2F1 consensus-binding site. We used PCR to generate a luciferase reporter vector using the mouse genomic sequence from –244/+87. To examine the role of the conserved E2F1 site spanning from position –43 to –49, we also generated a mutated version of the –244/+87 construct in which the E2F1 site was rendered nonfunctional. Fig. 8 shows a schematic representation of the constructs generated. They differ in that the consensus E2F binding site CGCG*CG*GGAAGACCGGCGGA (wild type) is changed to CGCG*AT*GGAAGACCGGCGGA (mutant).

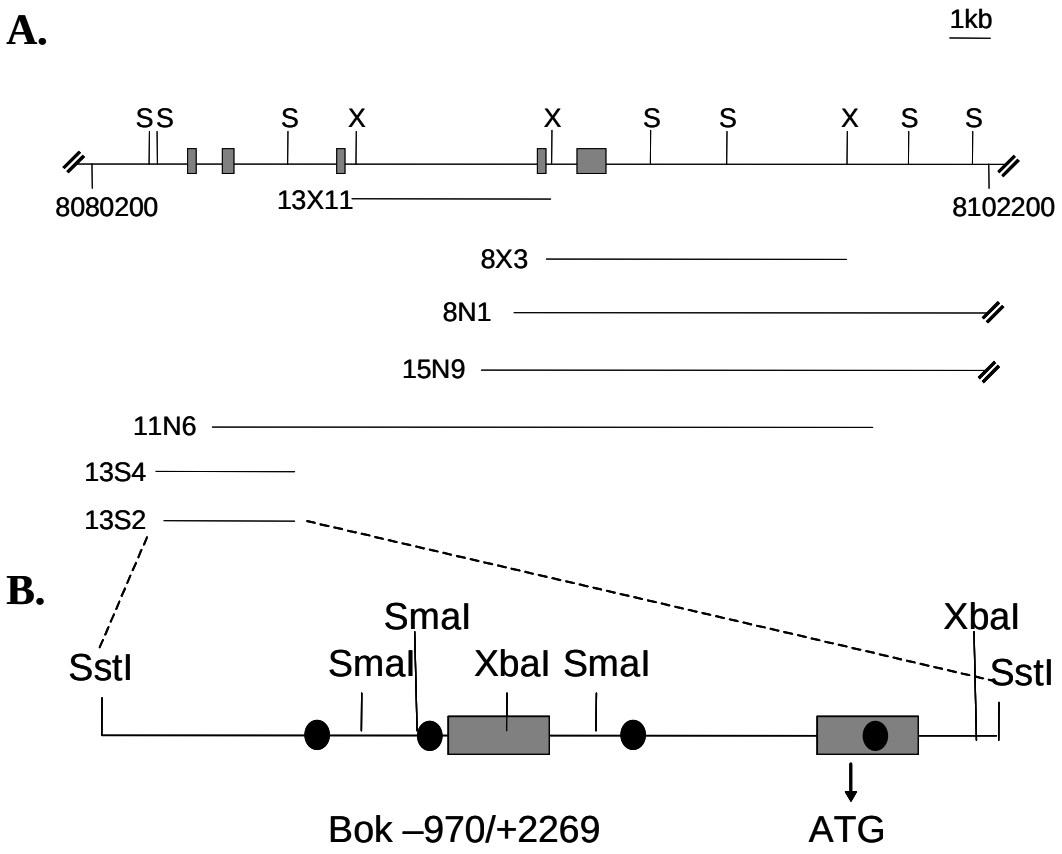
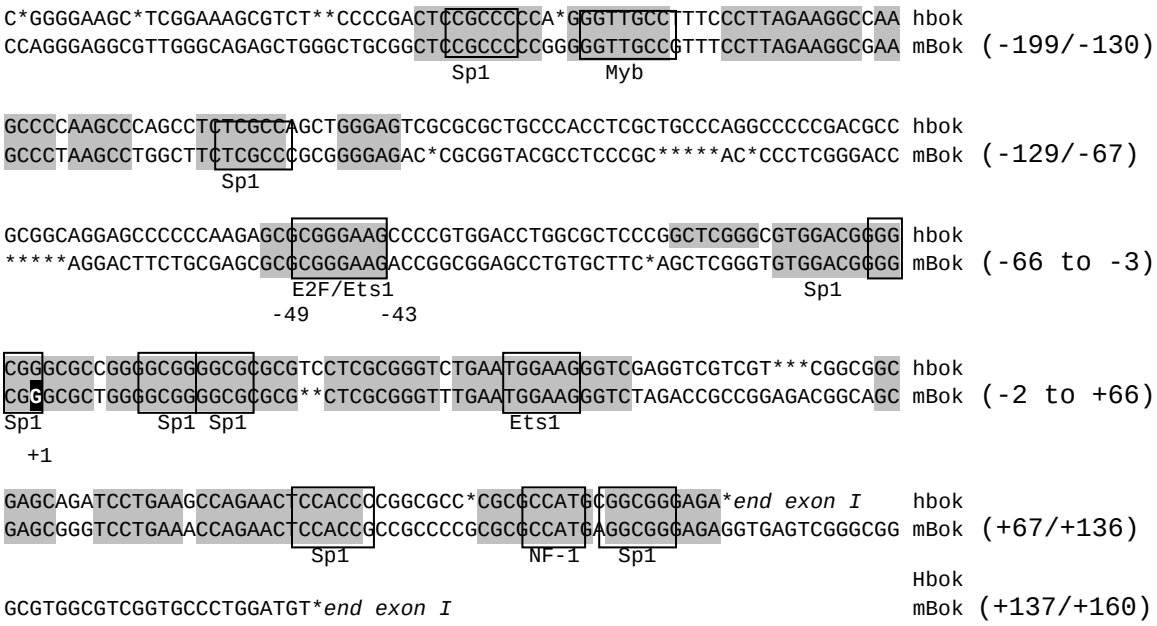


Figure 6. Overlapping subclones in pBS encompassing the entire Bok genomic locus. (A) Subclones were excised from the phage clones with *Sst* I (S), *Xho* I (X) or *Not* I (N). *Not* I subclones represent the entire insert of the phage clones, whereas *Sst* I and *Xho* I subclones contain only part of the original phage clone. Numbering is relative to the *Mus musculus* chromosome 1 genomic contig NT_039173.2, which contains the Bok locus. Solid boxes indicate exons. Exon 1 is noncoding. The ATG start codon is located at position 8083483 in exon 2. The stop codon is located at position 8092198 in exon 5. (B) The pBS-13S2 was further subcloned into pGL3 luciferase vector using *Sst* I, *Sma* I or *Xba* I. These subclones contain the Bok promoter region and the longest four putative E2F binding sites marked by black circles.

Figure 7. Evolutionary conserved E2F binding site. An alignment between the mouse (NT_039173) and human (AC110299) Bok gene sequences using MegAlign (DNASTAR, Inc) showed a conserved putative E2F binding site that extends from position –42 to –49 relative to the putative transcriptional start site in the mouse sequence. Shaded blocks indicate sequence identity of at least five base pairs. Boxed areas indicate putative transcription factor binding sites identified by MatInspector (Genomatix). The highlighted G at +1 in the mouse sequence indicates the putative transcription start site based on NCBI annotations (1).


C*GGGGAAGC*TCGGAAAGCGTCT**CCCCGACTCCGCCCCCA*GGGTTGCCTTTCCTTAGAAGGCCAA hbok
CCAGGGAGGCGTTGGGCAGAGCTGGGCTGCGGCTCCGCCCCCGGGGGGTTGCCGTTTCCTTAGAAGGCGAA mBok (-199/-130)
Sp1 Myb
GCCCCAAGCCCAGCCTCTCGCCAGCTGGGAGTCGCGCGCTGCCCACCTCGCTGCCCAGGCCCCCGACGCC hbok
GCCCTAAGCCTGGCTTCTCGCCCGCGGGGAGAC*CGCGGTACGCCTCCCGC*****AC*CCCTCGGGACC mBok (-129/-67)
Sp1
GCGGCAGGAGCCCCCCAAGAGCGCGGGAAGCCCCGTGGACCTGGCGCTCCCGGCTCGGGCGTGGACGGGG hbok
*****AGGACTTCTGCGAGCGCGCGGGAAGACCGGCGGAGCCTGTGCTTC*AGCTCGGGTGTGGACGGGG mBok (-66 to -3)
E2F/Ets1 Sp1
-49 -43
CGGGCGCCGGGGCGGGGCGCGCGTCCTCGCGGGTCTGAATGGAAGGGTCGAGGTCGTCGT***CGGCGGC hbok
CGGGCGCTGGGGCGGGGCGCGCG**CTCGCGGGTTTGAATGGAAGGGTCTAGACCGCCGGAGACGGCAGC mBok (-2 to +66)
Sp1 Sp1 Sp1 Ets1
+1
GAGCAGATCCTGAAGCCAGAACTCCACCCCGGCGCC*CGCGCCATGCGGCGGGAGA*end exon I hbok
GAGCGGGTCCTGAAACCAGAACTCCACCGCCGCCCCGCGCGCCATGAGGCGGGAGAGGTGAGTCGGGCGG mBok (+67/+136)
Sp1 NF-1 Sp1
Hbok
GCGTGGCGTCGGTGCCCTGGATGT*end exon I mBok (+137/+160)

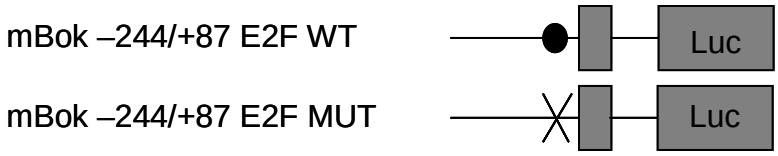


Figure 8: Schematic representation of the Bok promoter. Site-directed mutagenesis assay was used to mutate the putative E2F binding site. The Bok promoter containing wild type (closed circle) or mutated (X) E2F binding site are shown. These fragments were then cloned into pGL3.

Regulation of the Bok Promoter Throughout Cell Cycle

To characterize the activity of the cloned Bok promoter throughout the cell cycle, NIH 3T3 cells were transfected with Bok –244/+87 WT or MUT promoter/reporter. Cells were brought to quiescence by incubation with 0.5% calf serum for 48 hrs and were then serum stimulated with 10% fetal calf serum and harvested every 6 hrs. In parallel, cells were fixed with 70% ethanol-PBS, stained with PI and analyzed by FACS to determine cell cycle status. Fig. 9A shows that the activity of the WT Bok promoter is maximal at 6 and 12 hrs after addition of serum corresponding to the mid to late G1 phase of the cell cycle (Fig 9B). This pattern of regulation is very typical of an E2F1-regulated gene. In contrast, the activity of the MUT Bok promoter is unaffected by serum addition. This supports the conclusion that the conserved E2F binding site at -49/ -43 is central to the cell cycle regulation of Bok.

Activation of the Bok Promoter is Not Specific to E2F1.

E2F1 is the most potent inducer of apoptosis amongst the E2F family of proteins and appears essential for E2F-induced apoptosis [22,74]. Since Bok is a known pro-apoptotic protein, we anticipated that E2F1 might be a specific activator of Bok. To test this idea, we compared the ability of various E2Fs to induce the Bok luciferase reporter. We co-transfected the wild type (Bok –244/+87 WT) promoter, or the E2F site mutant (Bok –244/+87 MUT) in the presence and absence of exogenous E2F proteins (Fig 10A). E2Fs 1, 2 and 3B expression each led to promoter activation. This result suggests that

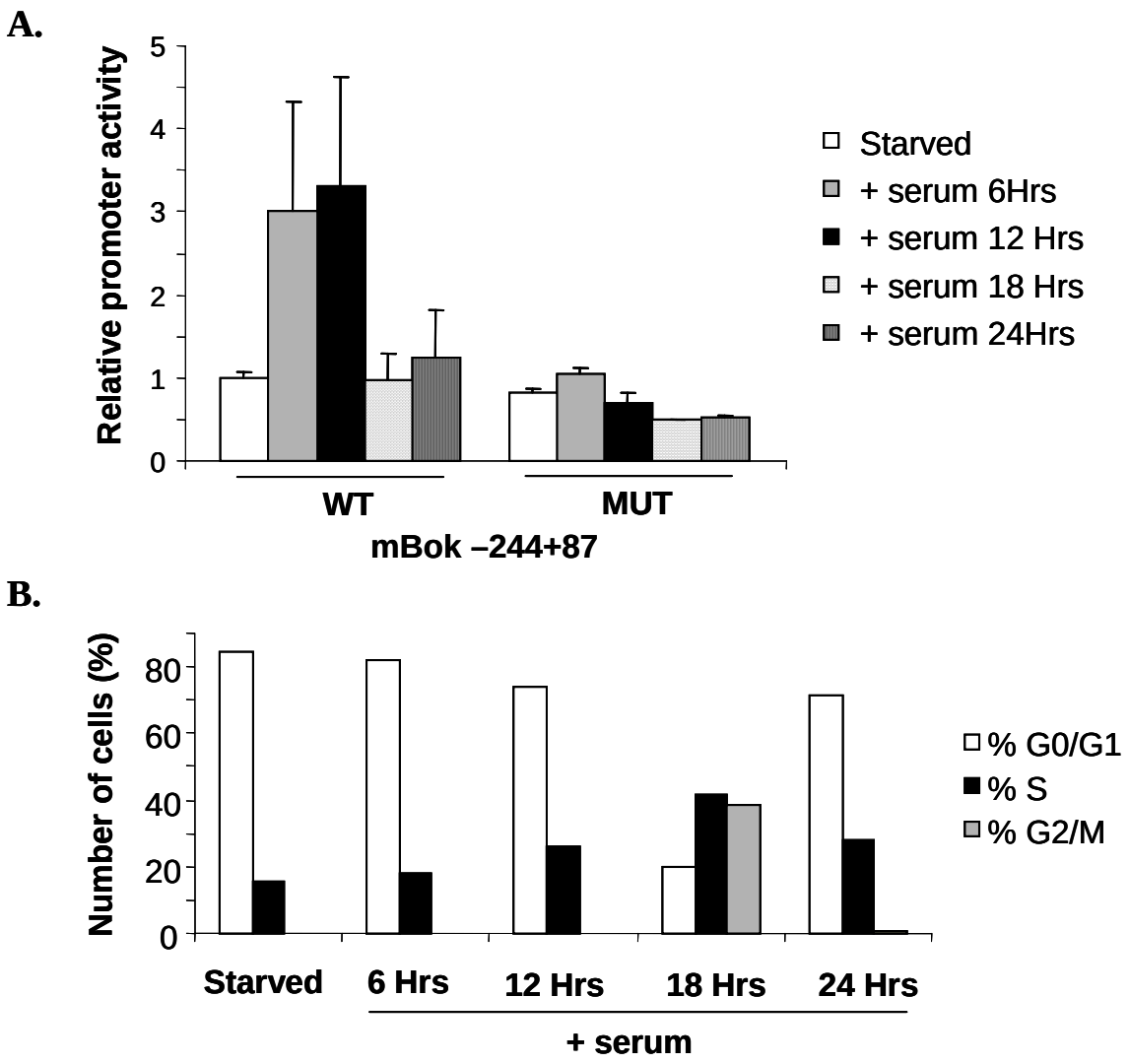


Figure 9: The Bok promoter is activated by addition of serum dependent upon a conserved E2F binding site. (A) NIH 3T3 cell were transfected with the WT or MUT –244/+87 Bok promoter luciferase construct and then brought to quiescent by 48-hrs incubation with 0.5% calf-serum. Following starvation cells were stimulated with 10% fetal calf serum and harvested every 6-hrs and assayed for luciferase activity. (B) Cells cycle progression of NIH 3T3 cells after treatment as above. Cells were fixed with 70% ethanol-PBS, stained with PI and analyzed by FACS. Luciferase assay was performed three time in triplicates each. p values were less than 0.05 at 6 and 12 hrs after serum treatment.

activation of Bok is not specific to E2F1. The growth-repressing members of the E2F family E2F4, 5 and 6 did not significantly activate the promoter and neither did two E2F1 mutants. $E2F1_{1-283}$ is a C-terminally truncated version of E2F1 [18] that does not have a transcriptional activation domain, indicating that activation of the Bok promoter requires the activation domain. Likewise, the DNA binding E2F1 mutant, Eco 132 [17], was unable to activate transcription. Thus, DNA binding is required for activation of the Bok promoter.

Since E2F1 and E2F3B were the most potent activators of the Bok promoter in the comparison of Fig. 5A, we focused experiments comparing E2F1, E2F3A and E2F3B. Together Fig. 10A and 10B reveal that E2F3A is the most potent inducer of the Bok promoter followed by E2F3B, E2F1 and E2F2. Although the importance of this pattern of activity is not certain, it is clear that E2F1 is unlikely to be the sole regulator of Bok. The observation that over-expression of E2Fs can stimulate the MUT Bok reporter suggests that additional functional E2F binding sites may exist in the promoter, if E2F levels are sufficiently high.

The Bok promoter is Not Activated by p53 Expression.

A recent report suggested that Bok was a p53 target and that Bok was an essential mediator of p53-mediated apoptosis during treatment with chemotherapuetic drugs [145]. In their study they evaluated the role of caspases and new protein synthesis in the induction of the intrinsic pathway of apoptosis. They demonstrated that if protein synthesis was inhibited by treatment of cyclohexamide they would block the induction of apoptosis by

the DNA damaging agent VP-16. They also demonstrated that the activity of the tumor suppressor p53 was also necessary for apoptosis induction by VP-16. They then investigated what pro-apoptotic members of the Bcl-2 family were up-regulated by VP-16, in which Bok and Noxa were identified. Furthermore, experiments with RNAi targeting exogenous Bok ans Noxa demonstrated their importance in the apoptosis induction after VP-16 treatment. Taken together their results suggested that p53 activity and new protein synthesis was required for apoptosis induction after DNA damage by VP-16 and that decreasing the expression of exogenous Bok and Noxa significantly protected from cell death after VP-16 treatment. In their discussion they strongly suggested the possibility of p53 elements on the promoter of Bok and Noxa that would account for the activation of these proteins. However, sequence analysis did not reveal a p53 element in the Bok promoter, calling to question whether Bok is indeed a direct p53 target. To test this, we cotransfected NIH 3T3 cells with our –244/+87 WT Bok construct in the presence or absence of p53 expression (Fig 11A). In this experiment we can conclude that E2F1 is a stronger inducer of the Bok promoter than p53. However, we do not exclude the possibility of other p53 binding sites further upstream or downstream of the E2F binding site that are not present in our Bok promoter construct (-244/+87). In addition, we are also limited by our control, a p53-regulated reporter that was induced two-fold under identical conditions, similar to the induction of the Bok promoter (Fig 11B). Future experiments with larger Bok promoter constructs and additional controls such as a Bax promoter construct could proof or disproof the hypothesis that the Bok promoter is regulated by the tumor suppressor p53.

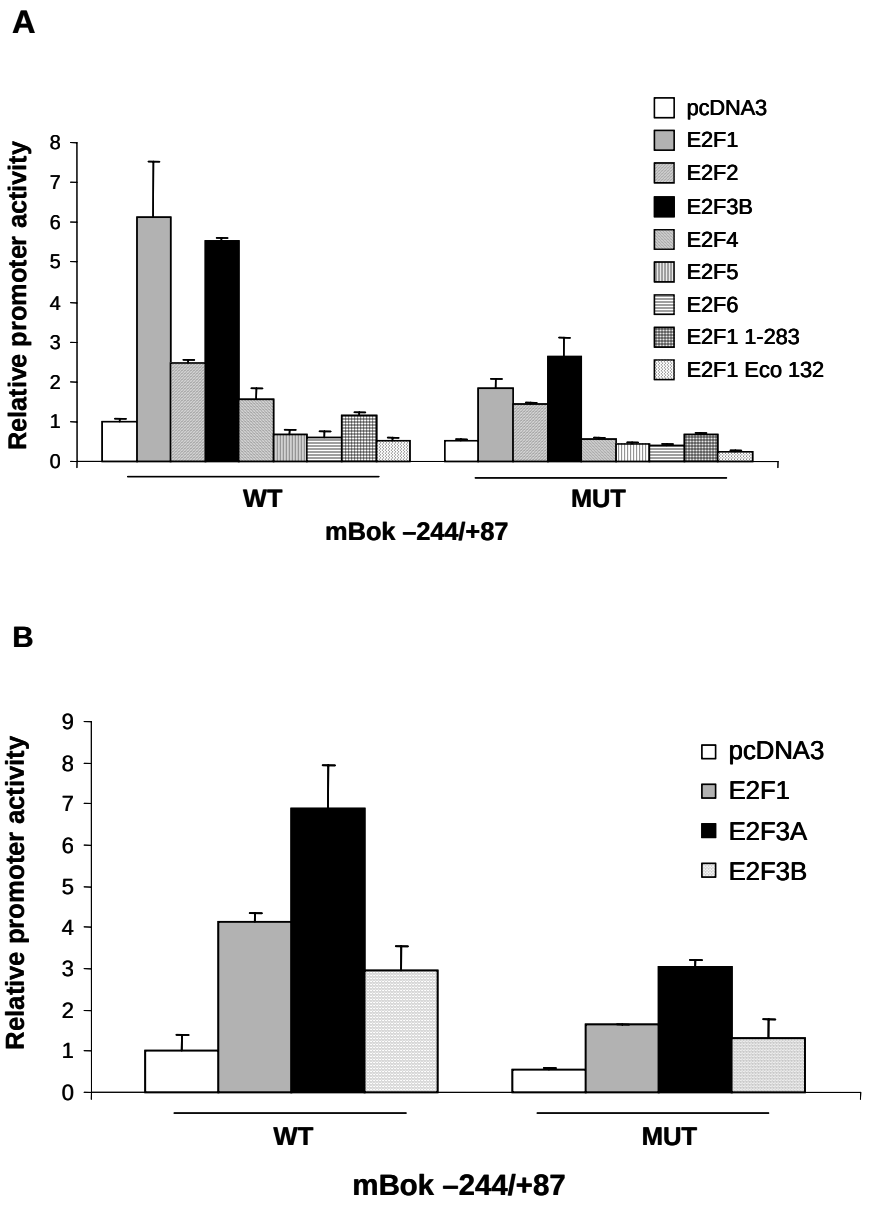


Figure 10: S phase promoting members of the E2F family activate the Bok promoter. (A) E2F binding site MUT and WT Bok promoters were co-transfected with expression vectors for different members of the E2F family and their ability to activate the Bok promoter was measured. (B) Same as in A except focusing on strongest S phase promoting E2Fs. E2F3A is the most potent activator of the Bok promoter. Experiments were performed in twice in triplicates each. p values were less than 0.05 for E2F-1, -2, -3a and -3b.

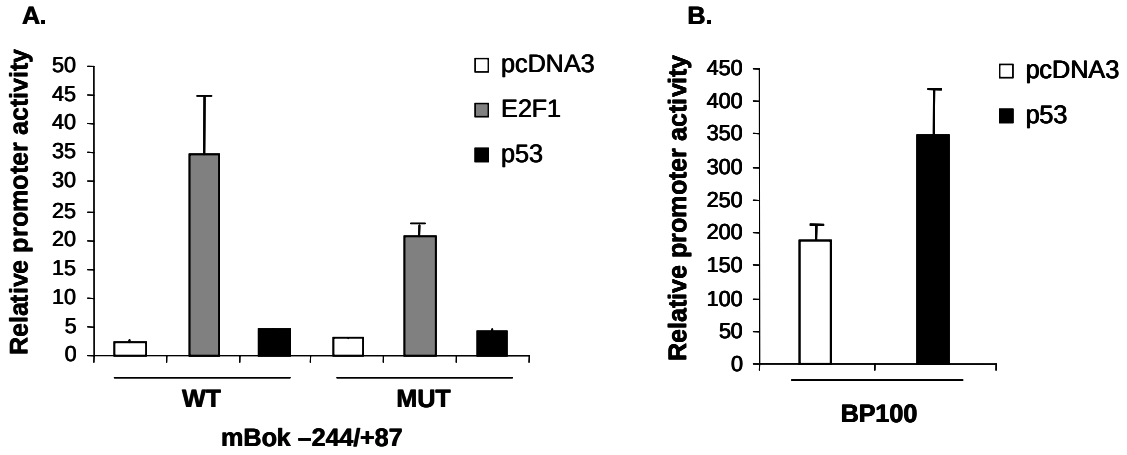


Figure 11. p53 protein does not activate the Bok promoter. (A) E2F binding site MUT and WT Bok promoters were co-transfected with either E2F1 expression vector or p53 expression vector and measure their ability to activate the Bok promoter. (B) As a control p53 was co-expressed with a known p53 regulated promoter, BP100. Experiment was performed twice in triplicates each. p values were less than 0.05.

E2F1 and E2F3 Associate With the Bok Promoter *in vivo*.

In light of the fact that E2F1 and E2F3A potently activate the Bok promoter in context of a luciferase reporter, we wanted to determine whether E2Fs associate with the Bok promoter *in vivo*. For this, we turned to chromatin immunoprecipitation assay of asynchronous NIH 3T3 cells. As shown in Fig. 12, using Bok specific oligonucleotide primers that span –244 to +87 of the murine Bok gene, E2F1 and E2F3 each associate with the Bok promoter *in vivo*, in agreement with the aforementioned luciferase result. The fact that immunoprecipitation with a control antibody (anti-IgG) results in absence of signal from the Bok promoter, demonstrates the specificity of the interaction between E2Fs and the Bok promoter. In addition, the lower panel in Fig. 12 reveals that the murine albumin promoter, which does not possess E2F sites and has been shown not to associate with E2F (98), is not immunoprecipitated with E2F antibodies under identical conditions.

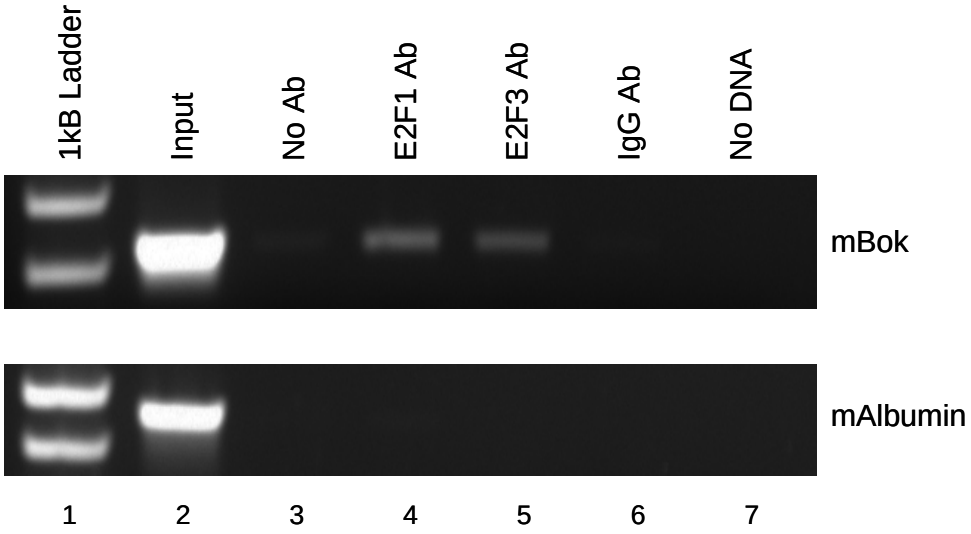


Figure 12. E2F1 and E2F3 associate with the Bok promoter *in vivo*. Asynchronously growing NIH 3T3 were subject to chromatin immunoprecipitation analysis with antibodies against E2F1 (lane 4), E2F3 (lane 5), or IgG (lane 6). Following DNA purification, samples were subject to PCR with primers designed to amplify the Bok promoter or the albumin promoter as control.

Chapter 3: Functional Relevance of Bok

In chapter two we demonstrate that E2F1 binds to the Bok promoter and leads to its transcriptional activation. Since E2F1 is the most potent inducer of apoptosis amongst the E2F family, it was interesting to see that other members of this family also regulate the Bok promoter. Furthermore, the observation that Bok mRNA increases after serum stimulation and that the Bok promoter is cell cycle regulated, suggested to us that Bok might have an unprecedented role in cell cycle. In this chapter we will investigate the role of Bok in cell cycle, E2F1-induced apoptosis and stress-induced apoptosis. We will use RNA interference to deplete cells from Bok and test its effect in the aforementioned context

Bok d-siRNA Shuts Down the Expression of Bok.

To determine the functional effect of increased Bok expression, we created H1299 cells lines that constitutively express a Flag epitope-tagged version of Bok. Expression of the introduced Flag-Bok transgene was confirmed via RT-PCR and Western blot (Fig. 13A and 13B). Surprisingly, constitutive expression of Flag-Bok did not necessarily induce spontaneous apoptosis in these cells, and several lines were developed. Clone #8 expressed the highest level of Flag-Bok and was this used for subsequent experiments.

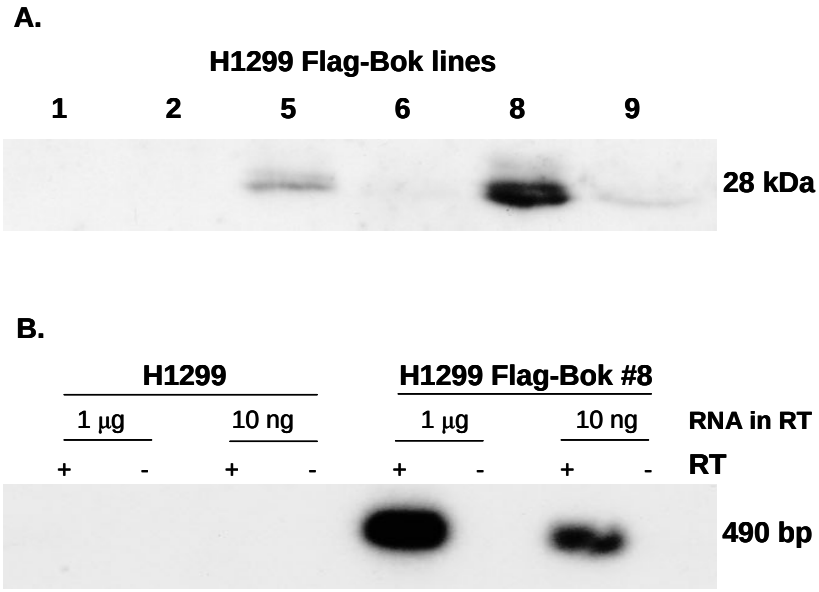


Figure 13. Stable over-expression of Flag-Bok protein. Flag-Bok expressing H1299 cell lines were generated by transfection with pcDNA3-Flag-Bok followed by selection with G418 (see Methods). G418-resistant colonies emerged with same efficiency as control pcDNA3. Of the first six lines emerging from this screen three expressed Flag-Bok as measured by anti-Flag Western blot. Clone #8 was used for subsequent experiments.

In order to block Bok expression we decided to turn to RNAi, and we utilize an approach that would target the most of the Bok mRNA. Using plasmid pcDNA3 Flag-Bok as template, we PCR amplified a 561 bp fragment of Bok and that PCR product cloned it into pCRII-TOPO vector. We then transformed this plasmid into bacteria, grew it up and screened for insert orientation. We finally identified one plasmid with each orientation. Using a RiboMax^{TM} Large Scale RNA production System under the T7 promoter we *in vitro* transcribed and produced milligram quantities of RNA from both, the sense and the antisense strand of Bok. We then continue and combine 60 μg of each RNA strand and let them anneal by heating up to 65˚C and letting it slowly cool down to room temperature. As control we ran an aliquot on a 4% agarose gel to make sure the RNAs anneal and form a single band ~561 bp (not shown). After annealing we performed the Dicer reaction (Block IT^{TM} Dicer kit from Invitrogen) followed by purification. With this experiment we generated a pool of ~21 bp diced-small interfering RNA (d-siRNA) (Figure 14, lanes 2 and 3) directed to many different parts of the of the Bok mRNA transcript. We then tested if these Bok d-siRNAs shut down the expression of Bok on H1299 cell lines that constitutively express Bok (Flag-Bok). Figure 15 shows Western blot analysis against Flag Bok protein after transfection of Bok d-siRNA at 24, 48 and 72 hours post transfection.

Bok is Not Necessary for E2F1-Induced Apoptosis.

We have shown that E2F1 overexpression increases Bok mRNA (Fig 4). For this reason we hypothesize that Bok is down stream of E2F1 and being a pro-apoptotic member of the Bcl-2 family it might be an important player in E2F1-induce apoptosis. In

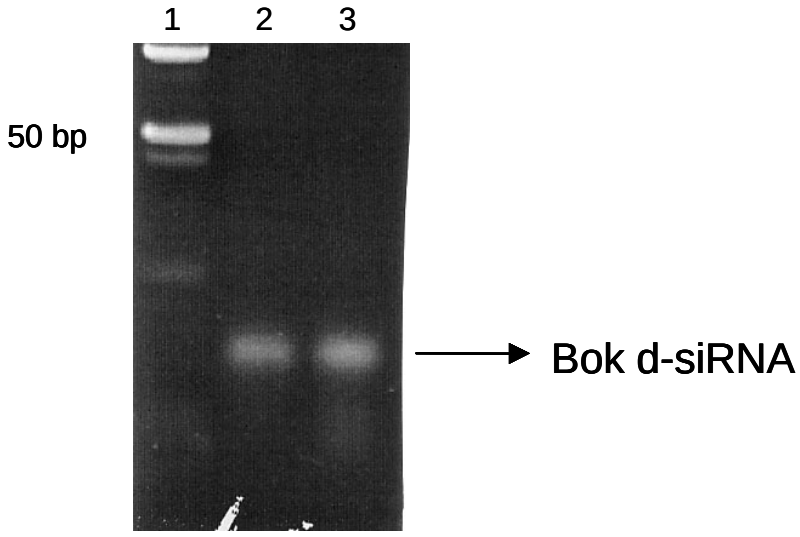


Figure 14. Generation of Bok d-siRNA. Bok d-siRNA was produced by *in vitro* transcription of the sense and anti-sense strand of Bok cDNA. The product was then purified, mixed and heated to 65° followed by slow cool down in order to anneal both ssRNA. The 561 bp dsRNA was then diced using Invitrogen's Dicer enzyme kit. This generated a pool of 21-23 bp dsRNAi that was visualized in a 4% agarose gel after the reaction (lane 2) and after purification (lane 3). Lane 1 is a 50bp ladder for size comparison.

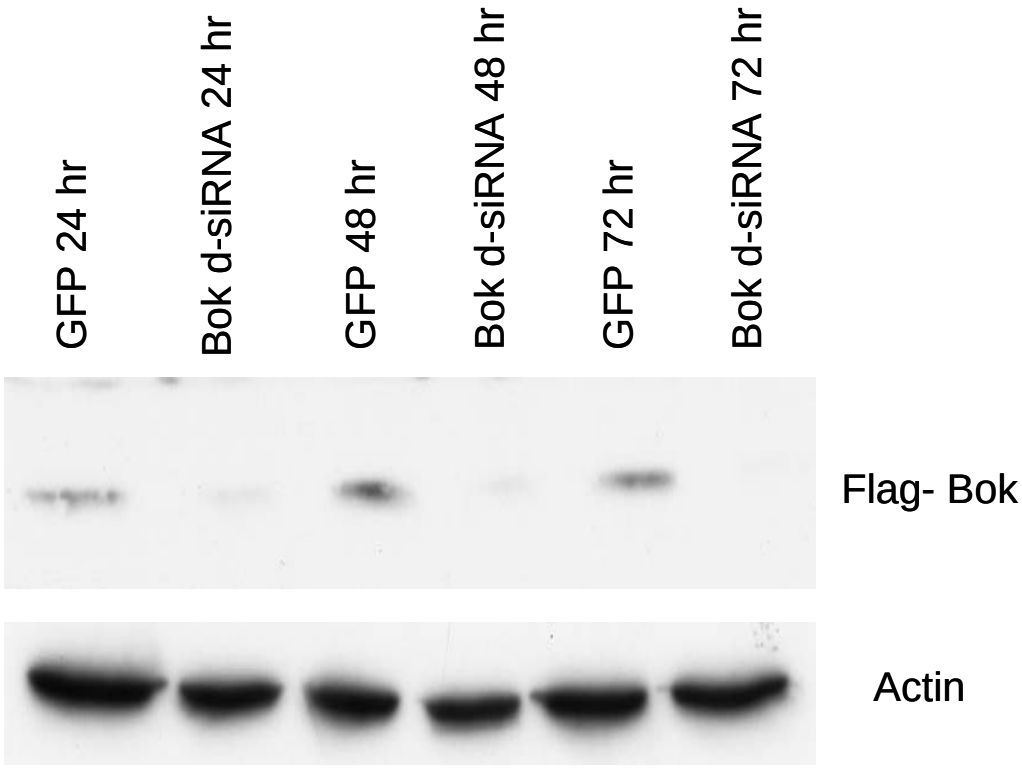


Figure 15. Bok d-siRNA shuts down the expression of Bok. H1299 Flag-Bok cell line were mock transfected with GFP or transfected with Bok d-siRNA at 0 and 24 hrs. Cells were harvested at the indicated times and 100 μg of cell lysate was run on a 12 % SDS-PAGE. Western blot was against Flag-Bok and actin as a control.

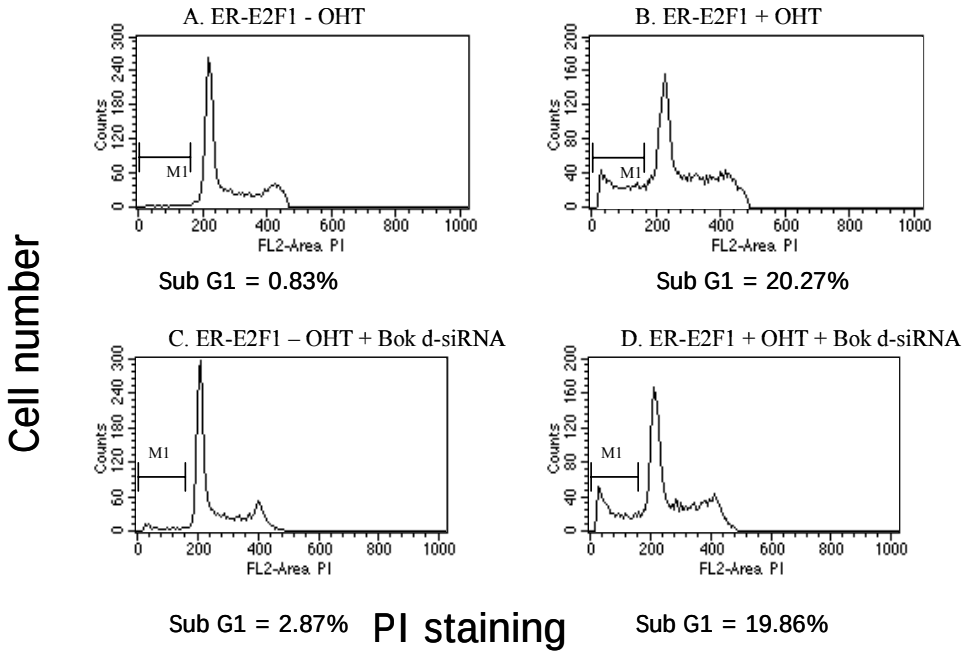


Figure 16. Bok deficiency does not block E2F1-induced apoptosis. This experiment was done in H1299 cells that stably express ER-E2F1 fusion protein. A. Mock transfected. B. Mock transfected and treated with OHT. C. Transfected with Bok d-siRNA. D. Transfected with Bok d-siRNA and treated with OHT. Note that there is no difference between B. and D. therefore Bok is not necessary for E2F1 induced apoptosis.

order to test this hypothesis, we used a cell line that stably expresses ER-E2F1 fusion protein. When these cells are treated with 4-Hydroxytamoxifen (OHT) they undergo apoptosis induced by E2F1 following its nuclear re-localization. Cells were mock transfected or transfected with Bok d-siRNA and after 24, 48 and 72 hrs post-transfection they were harvested, fixed with 70 % ethanol-PBS, stained with PI and sub-G1 content measured by FACS analysis. We expected that cells lacking Bok by virtue of Bok d-siRNA would be more resistant to E2F1-induce apoptosis. Surprisingly Bok deficient cells were not significantly resistant to the induction of apoptosis by E2F1 (Figure 16). This suggests that Bok is not an essential player in the induction of apoptosis by E2F1, at least in the limited context of our experimental model.

Bok is Not Necessary for Cell Cycle Progression.

We were very surprised by the observation that Bok message is up regulated by E2F1 as well as by serum, since Bok is an apoptotic protein. However, this pattern of regulation suggested that Bok plays a role in cell cycle control. The idea that Bcl-2 family members regulate cell cycle progression is not new since there have been reports that Bcl-2 negatively regulates cell cycle progression by increasing the activity of $p27^{Kip1}$ [135], which leads to the formation of a repressive E2F4/p130 complex that in turn block cell cycle progression through the G1/S boundary.

To determine if transient Bok deficiency would affect cell cycle progression, H1299 cells were transiently transfected with Bok RNAi and cell cycle distribution was determined. Since endogenous Bok is difficult to detect with current antibodies it was

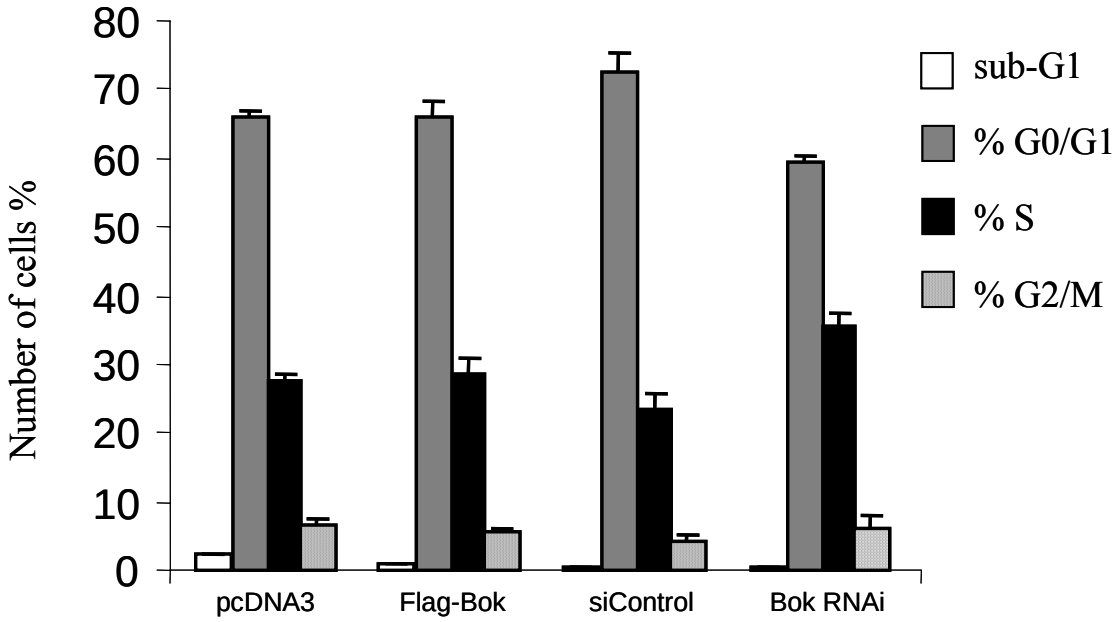


Figure 17. Bok is not required for cell cycle progression. The pattern of Bok regulation suggested that Bok might have an important role in the progression of cell cycle. However, neither over-expression nor depletion of Bok significantly affected the cell cycle compared to control counterparts. Experiment was performed more than three times and representative data is shown. p values were more than 0.05.

necessary to use a surrogate cell line to demonstrate that the Bok RNAi was functional. Having established that the Bok RNAi was active (Fig 15), the parental H1299 cell line was transfected with the Bok siRNA and cell cycle distribution was measured. Figure 17 shows a cell cycle analysis of cells that were mock transfected with pcDNA3-empty vector, transfected with Flag-Bok plasmid, control RNAi or Bok d-siRNA. After repeating this experiment several times we did not find any significant evidence that Bok is required for cell cycle progression.

Bok Localizes to the Cytoplasm

The mechanism by which the Bcl-2 family of proteins induce cell death is not completely understood, yet a key component is the activation of caspases. The Bcl-2 family of proteins regulate the activation of caspases by controling the release of cytochrome C from the mitochondria; which with Apaf-1 and procaspase 9 form the apoptosome. In the traditional view, pro- and anti-apoptotic members of the Bcl-2 family are found as heterodimeric proteins in the cytosol. After stress such as DNA damage, pro-apoptotic proteins such as Bok, Bax, and Bak, release anti-apoptotic members and oligomerizes or heterodimerizes with other pro-apoptotic members in the mitochondrial membrane. It is propose that the pro-apoptotic proteins form “pores” in the mitochondrial membrane bringing about the release of cytochrome C, and this event triggers the apoptosis cascade.

In the traditional view Bok should be cytosolic, however a recent report from Bartholomeusz et al. [4] suggests that Bok contains a nuclear export sequence (NES)

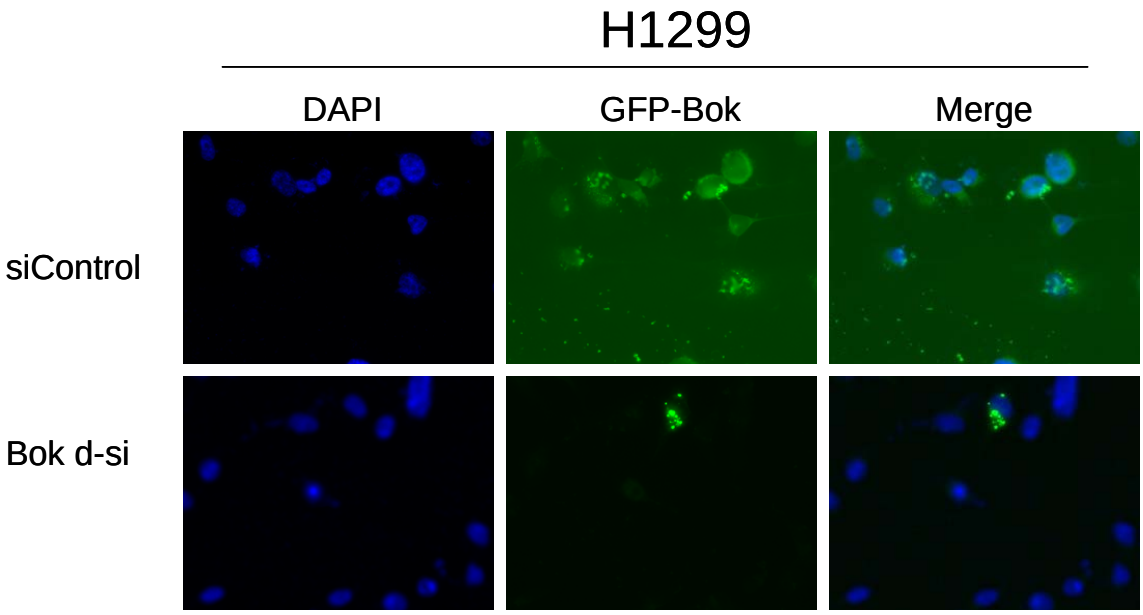


Figure 18. Bok localizes to the cytoplasm. H1299 cells were co-transfected with GFP-Bok and either Bok d-siRNA or control RNA and cell were then visualized by fluorescent microscopy. Over-expression of GFP-Bok demonstrate that Bok is mainly cytoplasmic. Top row, separation from blue (DAPI) and green (GFP-Bok). As expected co-transfection with Bok d-siRNA abrogated the expression of Bok.

and is also found in the nucleus, where its apoptotic activity is enhanced. In order to determine where does Bok localizes we turned to fluorescent microscopy using a green fluorescent protein (GFP) tagged Bok construct. H1299 cells were co-transfected with GFP-Bok and either Bok d-siRNA or siControl using lipofectamine reagent and observed under the fluorescent microscope after 24 hrs. Figure 18 demonstrates that GFP-Bok localizes primarily to the cytoplasm. Thus, in our hands nuclear Bok appears minimal.

In order to determine if Bok's apoptotic activity is enhanced in the nucleus we developed H1299 cells that stably express an HA tagged- estrogen receptor (ER) tagged-fusion of Bok (Fig 19A). Western blot against HA reveal that all three cell lines express HA-ER-Bok fusion protein, however cell line number 2 less strong. Figure 19B demonstrate that HA-ER-Bok fusion protein is express in the cytoplasm and after treatment with the ER ligand, OHT, the fusion protein is forced into the nucleus, however some stays in the cytoplasm. Using this experimental approach H1299 HA-ER-Bok cell lines were treated with or without 300nM OHT and analyzed by FACS for subG1-DNA content as a measure of apoptosis. In contrast to reports this did not induce any spontaneous apoptosis at 48 or 72 hrs (Fig 20 lanes 2, 6 and 10). We also performed a similar experiment where H1299 HA-ER-Bok cell line was treated with and without OHT and also 200nM Flavopiridol, since H1299 cells undergo apoptosis after FP treatment (86). Consistent with our previews result this did not enhance Bok's apoptotic activity. If observed carefully, shuttling Bok into the nucleus slightly decreased sub-G1 DNA content (Fig 20, compare lanes 3 and 4, 11 and 12) suggesting that Bok's apoptotic role is in the cytoplasm. Similar results were seen in an additional experiment; however,

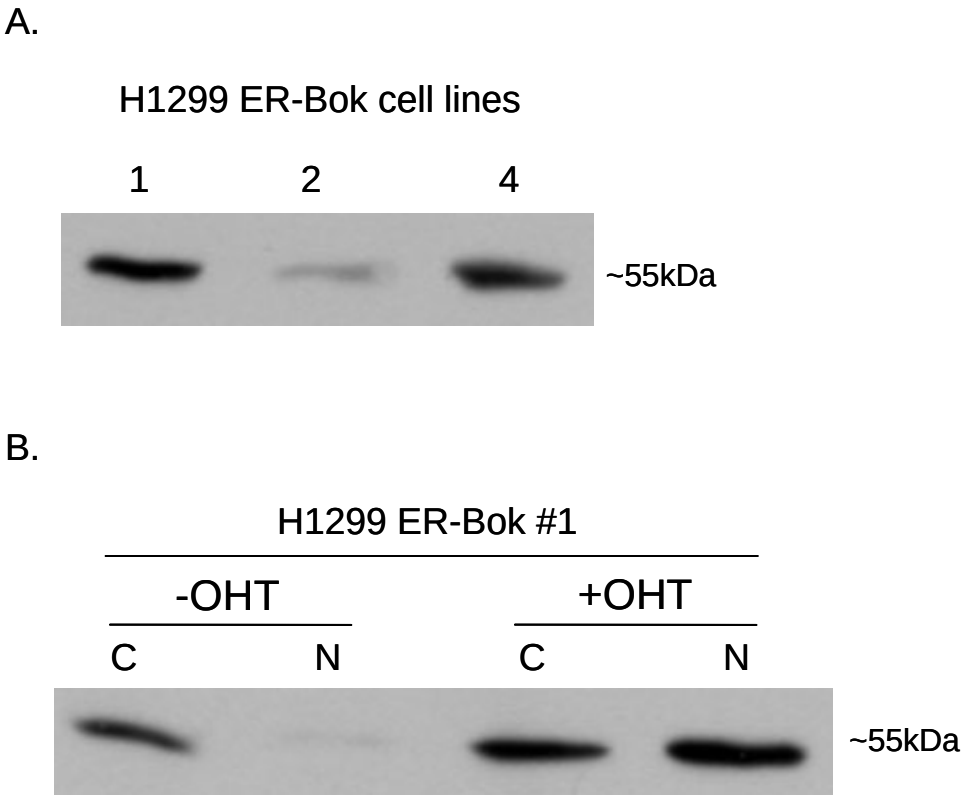


Figure 19. Generation of responsive H1299 HA-ER-Bok cell line. HA-ER-Bok expressing H1299 cell lines were generated by transfection with pcDNA3-HA-ER-Bok followed by selection with G418 (see Methods). (A) At least three cell lines emerging from this screen expressed HA-ER-Bok as measured by anti-HA Western blot. (B) Fractionatio experiment reveal that ER-Bok is expressed in the cytoplasm and after treatment with 300 nM OHT more than half of ER-Bok is shuttled into the nucleus.

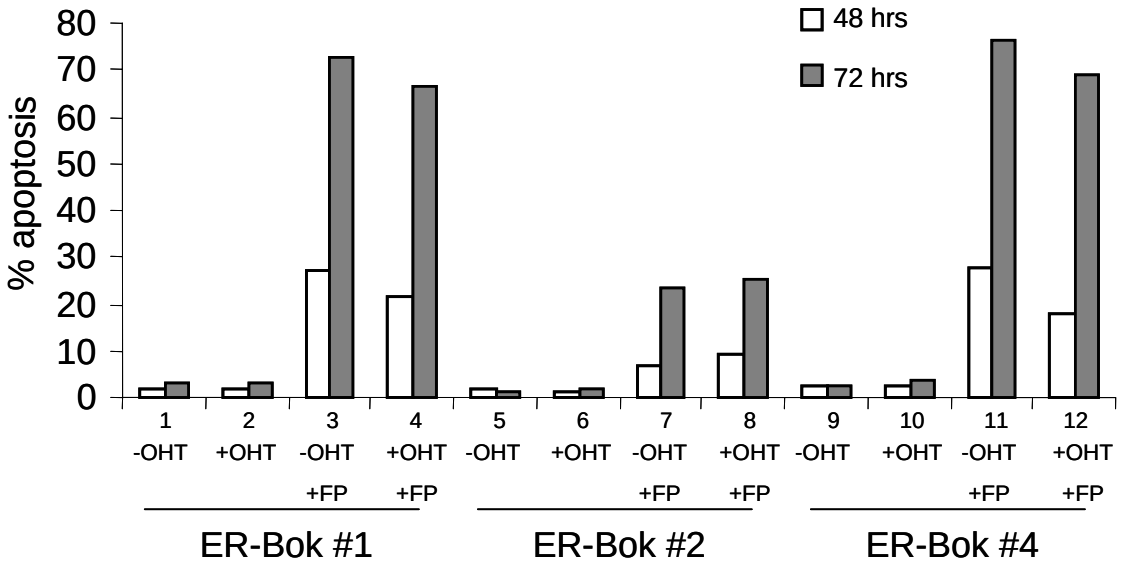


Figure 20. Bok's apoptotic role is not enhanced in the nucleus. H1299 HA-ER-Bok cell lines1, 2 and 4 were treated with or without 300 nM OHT in order to shuttle Bok into the nucleus. In contrast to reports this did not induce any spontaneous apoptosis as measured by FACS for sub-G1 DNA content, after 48 and 72 hrs of OHT treatment (compare lane 1 and 2, 5 and 6, 9 and 10). We also combined OHT and flavopiridol treatment (to induce apoptosis) and tested if Bok in the nucleus had an enhance apoptotic activity, however we saw a slightly opposite effect (compare lanes 3 and 4, 7 and 8, 11and 12). Experiment was performed twice in singles each. Representative result is shown.

further experimentation in duplicates or triplicates is needed to solidify this observation. Additionally this experiment is limited by the lack of good antibody against endogenous Bok, since tagging Bok with GFP or HA-ER might affect the ability of Bok to move to different compartments in the cell.

Bok Expression Sensitizes Cells to Stress-Induced Apoptosis

The Flag-Bok expressing cells generated in Figure 13 grew at the same rate as parental H1299s (Fig 21). In light of the observation that Bok over-expression alone is not sufficient for apoptosis induction, we sought to determine whether over-expression of Bok sensitizes cells to stress-induced apoptosis. To this end, the H1299-Flag-Bok #8 cell line (as well as parental H1299s) were assayed for viability after treatment with the cyclin-dependent kinase inhibitor flavopiridol, which we have previously shown to induce apoptosis in H1299 cells [86,88]. Fig. 21 reveals that flavopiridol-induced loss of viability is greatly accelerated in Bok expressing cells.

We next sought to verify our viability assay in a more direct measurement of apoptosis induction. The H1299–Flag-Bok cell line and control H1299s were treated with flavopiridol, harvested at 24 hrs intervals, stained with propidium iodide (PI) and assayed for sub-G1 DNA content via flow cytometry. In agreement with low viability, there was a significant increase in sub-G1 content within the flavopiridol treated H1299-Flag-Bok cell lines in comparison to the parental controls (Fig. 21). Similar results were obtained with other genotoxic agents (Fig 22). For further confirmation, we conducted Western blot analysis for the presence of poly-ADP ribose polymerase (PARP) cleavage (a

measurement of apoptosis) within the same experiment. As expected, both H1299-Flag-Bok and parental H1299s displayed cleavage of PARP, however, PARP cleavage began 24 hrs post flavopiridol treatment and was maximal at 48 hrs in the Bok expressing cell line, whereas PARP cleavage was noticeable 48 hrs and maximal at 72 hrs within the H1299 parental controls (Fig. 23). Taken together, these data suggest that expression of Bok sensitizes cells to rapid apoptosis induction.

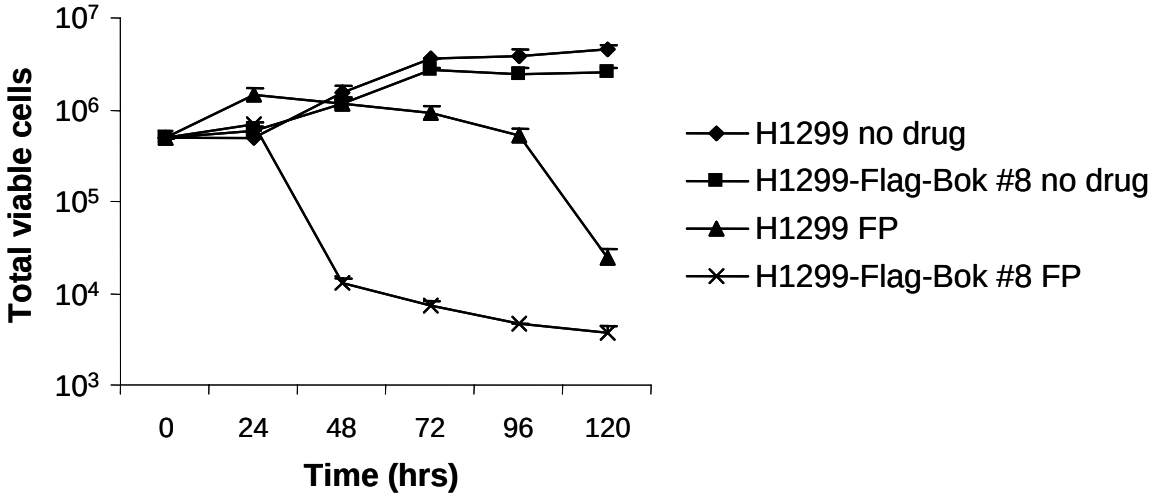


Figure 21. Kinetics of apoptosis induction in response to Flavopiridol treatment. H1299 and H1299 Flag-Bok cell line #8 were plated in 60-mm plates and their growth rate/survival was measured by trypsinization, followed by counting trypan blue excluding cells after treatment with DMSO control or flavopiridol [200 nM]. H1299 and H1299 Flag-Bok cell lines grow at similar rates (circles and squares), however upon treatment with FP, the Flag Bok cell line dies faster. Experiment was performed in triplicates and p values were less than 0.05.

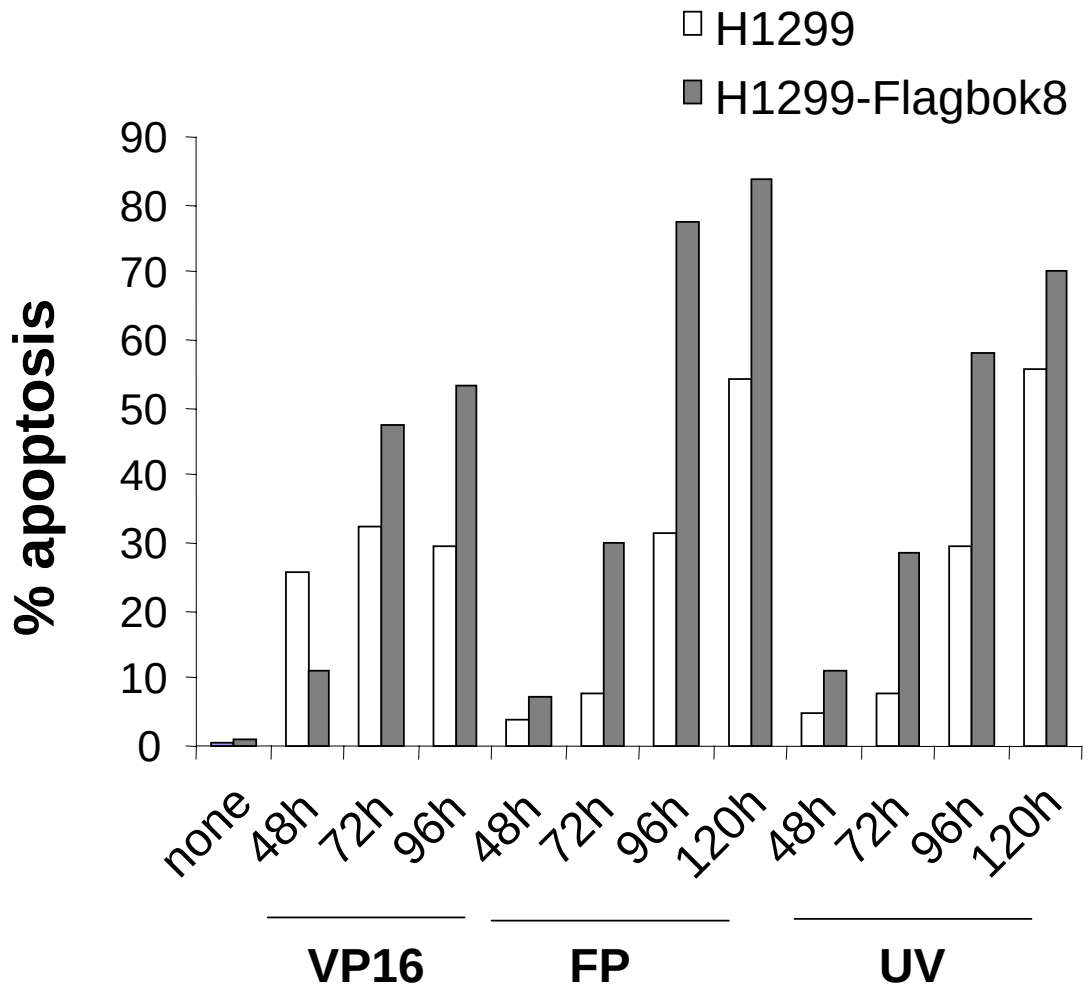


Figure 22. Constitutively Bok expression sensitizes to apoptosis after DNA damage. DNA damage was induce in H1299 or H1299 Flag-Bok by treatment with chemotherapeutic drugs (VP16 or Flavopiridol) or UV irradiation. Cells were then harvested at 48, 72, 96 or 120 hrs, fixed with 70% ethanol-PBS, stained with PI and analyzed for sub-G1 DNA content.

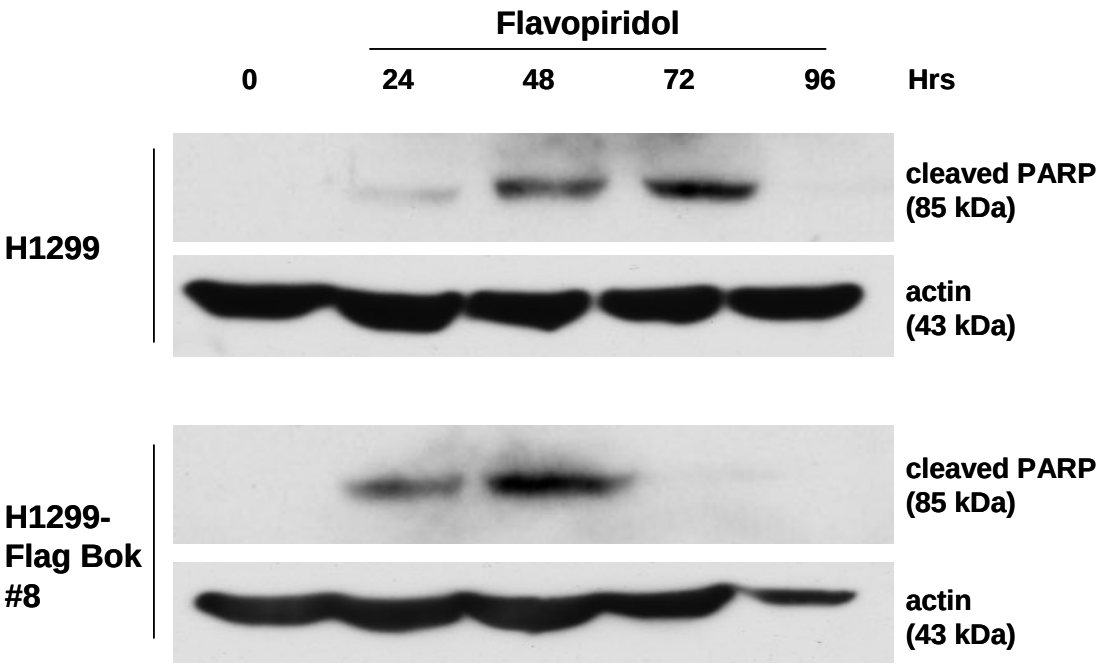


Figure 23. A molecular marker of apoptosis is seen by 24 hr after FP treatment in the Flag-Bok expressing cell line. Protein extracts of H1299 or H1299 Flag-Bok cell lines after treatment of flavopiridol were harvested in 24 hr intervals and Western blotted for PARP as a measure of apoptosis. PARP cleavage was visible by 24 hrs and maximal at 48 hrs on H1299 Flag Bok cell line in contrast to being maximal at 72hrs on parental H1299 cells

Chapter 4: Discussion

In the current work we show that the Bok promoter is activated by serum addition in a manner dependent upon a conserved E2F site in the promoter. The Bok promoter is also activated by over-expression of S phase promoting members of the E2F family. We also show by ChIP assay that E2F1 and E2F3 both bind the Bok promoter region *in vivo.* Finally we find that Bok over-expression sensitizes to flavopiridol-induced apoptosis.

Our understanding of the interactions between the E2F and Bcl-2 families of proteins that modulate survival are growing increasingly complex and interwoven. This is the first example of a pro-apoptotic member of the Bcl-2 family found to have its expression tied to cell cycle progression, although this is no t the first example of regulation of Bcl-2 family by E2F1 in its apoptotic role. It has been known for some time that E2F1 can repress the expression of Bcl-2 [25] and, we have demonstrated that E2F1 can directly repress the Mcl-1 promoter [19,87]. Other laboratories have found that several pro-apoptotic BH3-only members of the Bcl-2 family (PUMA, Noxa, Bim, and Hrk/DP5) are also activated by E2F1 [46]. In the current work, we find that E2F1 can directly activate expression of Bok. Since E2F1 is a well-characterized inducer of apoptosis its effects on Mcl-1, PUMA, Noxa, Bim, Hrk/DP5 and Bok are logical. The net consequence of over-active E2F1 is thus to tip the balancing act within the Bcl-2 family toward apoptosis.

The transcriptional activation of Bok at the G1/S boundary by serum stimulation was not anticipated since Bok is considered a pro-apoptotic member of the Bcl-2 family. Bok might have a number of roles at G1/S. Bok might serve a specific G1/S or S phase function. For example, recent work has shown that BID (a pro-apoptotic Bcl-2 protein) can induce an S phase arrest following its phosphorylation by ATM [35,70,152]. While we cannot formally exclude the possibility that Bok has a specific G1/S function, we have performed extensive siRNA and shRNAi experimentation aimed at depleted proliferating cells of Bok. Though we are confident in our ability to deplete cells of 80-90% of endogenous Bok mRNA or exogenous protein, we obtained no convincing evidence that Bok deficiency affects cell cycle progression. Of course these studies are hampered by the lack of good quality antibody to Bok, and so, it is possible that future studies will find an additional role for Bok in cell cycle.

An alternative role for Bok induction at the G1/S boundary would be to serve as a checkpoint. G1/S phase cells are known to be highly sensitive to apoptosis induction and it reasonable that expression of Bok might mediate this sensitivity, at least in part. This model would lead to the prediction that cells expressing exogenous Bok would survive and grow normally, but would be sensitive to apoptosis-inducing stresses. Indeed, this appears to be the case since Flag-Bok expressing H1299 cell lines are obtained with high efficiency and they grow normally, yet they are much more readily killed by treatment with flavopiridol, as well as by other death-inducing agents. Taken together the results in

this dissertation demonstrate that Bok is a cell cycle regulated member of the Bcl-2 family that serves as a checkpoint sensitizing replicating cells to stress-induced apoptosis.

Future Studies

Throughout my graduate studies, we have demonstrated that Bok mRNA is upregulated by serum stimulation and by E2F1 over-expression. We have also cloned and characterized the Bok promoter and demonstrated that E2F1 directly binds and transactivates it via a conserved E2F element. In addition we have shown that high expression of Bok sensitizes cells to apoptosis after treatment with chemotherapeutric agents. Furthermore, it is known that appropriate patterns of apoptosis are essential for normal tissue development, and since Bok is an inducer of apoptosis and it is expressed at various levels in diverse tissues [126], it may play a significant role in mouse development.. To test these hypotheses future experiments should use the reagents we have developed to generate Bok-deficient mice using the conventional targeting approach.

Bok mRNA expression is highest in the uterus, ovaries and testes; therefore these tissues are most likely to be dramatically affected by Bok deficiency. However, we may also expect to see tumor suppressor effects in other tissue that express Bok including lung, brain, liver, mammary epithelium and lymphoid tissues. Assuming that Bok heterozygous mice are viable and fertile, we expect them to be developmentally normal, however litter sizes and animal weights will be monitored and recorded to detect any differences from control animals. Mice will be observed carefully for overall health and behavior in aspects such as appearance, viability, growth rate, fertility and longevity. Any

outstanding abnormalities will be noted and affected animals euthanized. Organs from age matched wt and heterozygous animals will be removed, examined for gross abnormalities and weighed to determine if Bok deficiency affects organ size. The organs we are particularly interested include the testes, ovaries, uterus, lung, brain, liver and lymph nodes. Once weighed, organs will be fixed, paraffin-embedded and sectioned for histological examination and immunohistochemistry for BrdU, TUNEL and levels of Bok protein.

Two heterozygous mice will be crossed and by Mendelian genetics we expect that about twenty-five percent of the offspring to be homozygous null. The first question that this analysis will answer is whether we can establish Bok-deficient mice. Since Bok is an apoptotic protein we predict that Bok-deficient mice will display apoptotic defects in multiple tissues. The defects we may observe could be organ enlargement, but perhaps poor organization and differentiation due to impaired apoptosis. Assays such as TUNEL and apo-BrdU would reveal fewer positive cells compared to wild type counter parts. For example, mice over-expressing Bcl-2 in the ovaries results in suppression of follicular cell apoptosis, enhancement of folliculogenesis and increased germ cell tumorigenesis [56]. Interestingly, the Bcl-2 transgenic mice were fertile and their litters were on average 2 pups larger than litters of wild-type females, due to enhanced folliculogenesis. We think that it is likely that the effects of Bok deficiency, at least within the ovary, will be similar to the effects of Bcl-2 over-expression, and thus, Bok deficient animals will probably be more fertile than control mice. Again, health and behavior will be monitored for up to twenty-four months to determine these animals develop spontaneous tumors.

In the case of successfully developing Bok-deficient mice, we could cross these animals with commercially available Bax- and Bak-deficient animals in order to identify unique versus redundant or cooperative functions within this family of proteins. If viable, we anticipate that pathological effects of either Bax or Bak deficiency may be significantly aggravated by deficiency of Bok, particularly in lymphoid or reproductive tissue where the Bcl-2 family members are co-expressed at high levels. In addition, combined deficiencies may result in a stronger predisposition toward tumor formation.

In summary, future studies with a Bok-deficient animal will seek to determine the role of Bok *in vivo* and in mouse tumorgenesis. We anticipate that Bok will be found to be a tumor suppressor and that Bok-deficient animals will be predisposed to hyperplasia and neoplasia. We also anticipate that Bok may play a role in reproductive development or physiology.

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