

Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy

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Summary

Defective apoptosis not only promotes tumorigenesis, but also can confound chemotherapeutic response. Here we demonstrate that the proapoptotic BH3-only protein BIM is a tumor suppressor in epithelial solid tumors and also is a determinant in paclitaxel sensitivity in vivo. Furthermore, the H-ras/mitogen-activated protein kinase (MAPK) pathway conferred resistance to paclitaxel that was dependent on functional inactivation of BIM. Whereas paclitaxel induced BIM accumulation and BIM-dependent apoptosis in vitro and in tumors in vivo, the H-ras/MAPK pathway suppressed this BIM induction by phosphorylating BIM and targeting BIM for degradation in proteasomes. The proteasome inhibitor Velcade (P-341, Bortezomib) restored BIM induction, abrogated H-ras-dependent paclitaxel resistance, and promoted BIM-dependent tumor regression, suggesting the potential benefits of combinatorial chemotherapy of Velcade and paclitaxel.

Introduction

Programmed cell death or apoptosis occurs in response to developmental cues and disease states, and serves to sculpt tissues, eradicate abnormal or infected cells, and maintain homeostasis. Acquired resistance toward apoptosis is considered to be a universal feature of cancer (Cory et al., 2003; Hanahan and Weinberg, 2000). Defects in apoptosis not only provide cancer cells with an intrinsic survival advantage, but may also confer inherent resistance to chemotherapeutic drugs (Johnstone et al., 2002). BCL-2 family proteins are central players in the regulation of apoptosis, and modulate death signaling through the intrinsic or mitochondrial pathway (Cory and Adams, 2002). Whereas BCL-2 and several close relatives promote cell survival, other family members that share several domains with BCL-2, such as BAX and BAK, instead favor apoptosis. Members of the more distantly related BH3-only subfamily (BIM, PUMA, NOXA, BID, BAD, and BIK, for example) have the critical role of initiating the apoptotic program. They act by antagonizing the function of antiapoptotic BCL-2 and its homologs,

and perhaps also by directly activating the function of BAX and BAK (Adams, 2003b). Either BAX or BAK is required for apoptosis, which is associated with their oligomerization in the mitochondrial outer membrane and its permeabilization to proapoptotic mitochondrial proteins such as cytochrome c and SMAC/Diablo. Loss of both BAX and BAK therefore results in profound resistance to many apoptotic stimuli, including death receptor signaling and genotoxic agents (Degenhardt et al., 2002a; Wei et al., 2001; Zong et al., 2001).

The strong impact of the BCL-2 family on tumor development is well illustrated by findings with baby mouse kidney epithelial (BMK) cells transformed by E1A and dominant negative p53 (p53DD). Transformed BMK cells from mice deficient for both BAX and BAK, or that overexpress BCL-2, readily form tumors in nude mice, whereas those retaining functional BAX and/or BAK do not (Degenhardt et al., 2002a; Nelson et al., 2004). In epithelial cells, ischemic conditions in the tumor microenvironment cause induction of the BH3-only protein PUMA, and BAX- and BAK-mediated apoptosis that suppresses tumorigenesis independent of the RB and p53 pathways (Nelson

SIGNIFICANCE

Tumorigenesis results in the acquisition of mutations that promote tumor growth and chemoresistance, and relating tumor genotype to prognostic indications and to effective treatment regimens is essential for successful therapeutic outcome. Determining the mechanism of apoptosis induction by the chemotherapeutic drug paclitaxel revealed that BIM suppressed tumorigenesis and was required for paclitaxel responsiveness. The targeting of BIM for degradation in proteasomes by the H-ras/MAPK pathway was the molecular basis for paclitaxel resistance in tumors with activating mutations in RAS, and paclitaxel responsiveness was restored by joint administration of the proteasome inhibitor Velcade. Thus, rational combinatorial chemotherapy using proteasome inhibitors to enhance chemosensitivity to paclitaxel in tumors where the H-ras/MAPK pathway is activated may be therapeutically beneficial.

et al., 2004). Blockade of this p53-independent apoptotic pathway by either loss-of-function of BAX and BAK or gain-of-function of BCL-2 in vivo does not merely extend viability, but also allows survival of genetically unstable cells which may further promote tumor development (Nelson et al., 2004). However, how the RB, p53, and BCL-2 pathways impact chemotherapeutic response and functionally interact with other known pathways, such as those of receptor tyrosine kinases, is still not clear.

The frequent activation of RAS signaling pathways during tumorigenesis is also likely to reflect in part their ability to inhibit apoptosis (Downward, 2003). Downstream of RAS, AKT/PKB signaling has been shown to desensitize cells to apoptosis by phosphorylating various targets, whereas the mechanisms by which the RAF/MAPK pathway blocks apoptosis are less well defined. In the MAPK pathway, a survival signal is transduced from RAS through serine/threonine kinase RAF, which in turn phosphorylates and activates MAPK kinases MEK1/2 and thereby the p42/44 MAPK (ERK1/2) (Downward, 2003). The MEK/ERK cascade can inhibit apoptosis by upregulating the antiapoptotic proteins BCL-2, BCL-X_L, and MCL-1 (Jost et al., 2001; Leu et al., 2000; Liu et al., 1999). It may also promote survival both by phosphorylating BCL-2 and blocking its degradation in proteasomes (Dimmeler et al., 1999) and by phosphorylating BIM and accelerating its proteasomal degradation (Akiyama et al., 2003; Ley et al., 2003; Luciano et al., 2003).

In this study, we have investigated the role of BIM and its regulation by the RAS/RAF/MAPK pathway in control of apoptosis during both tumorigenesis and chemotherapeutic response. There are two major BIM isoforms: the longer BIM-EL predominating over BIM-L, and a shorter form (BIM-S) which is sometimes detectable (O'Connor et al., 1998). A central role for BIM-mediated apoptosis in maintenance of hematopoietic homeostasis was indicated by the aberrant accumulation of lymphoid and myeloid cells in BIM-deficient mice (Bouillet et al., 1999; Bouillet et al., 2002). BIM functions through protein-protein interactions, and its proapoptotic activity can be inhibited by association with BCL-2, BCL-X_L, BCL-w, and MCL-1 (Opferman et al., 2003; Wilson-Annan et al., 2003; Yamaguchi and Wang, 2002; Zhu et al., 2004). Genetic evidence for the mutually antagonistic roles of BIM and BCL-2 came with the demonstration that the apoptosis of kidney cells, melanocytes, and lymphocytes provoked by BCL-2 deficiency was precluded by concomitant deficiency for BIM (Bouillet et al., 2001). Finally, BIM also interacts with microtubule-associated dynein complex by binding to dynein light chain 1 (DLC1) LC8, which may relate BIM function to regulation of the cytoskeleton (Puthalakath et al., 1999).

BIM has been reported to be a tumor suppressor for mature B lymphocytes in Eμ-*myc*-transgenic mice, and is haploinsufficient (Egle et al., 2004). However, due to the multiplicity of BH3-only proteins, and the complexity of their expression and regulation, it is not possible to predict whether BIM could serve that role in other cell types, such as the epithelial cells that produce all the common solid tumors. Interestingly, in vitro studies (Bouillet et al., 1999; Sunters et al., 2003) have also implicated BIM in the apoptosis induced by paclitaxel, an anti-cancer agent that interferes with microtubule dynamics. Paclitaxel is a frontline antineoplastic agent that is efficacious in the treatment of a number of malignancies, including ovarian, breast, lung, and prostate cancers. Although it is known to sta-

bilize microtubules, causing mitotic arrest and activating the spindle assembly checkpoint, the signal transduction pathways by which paclitaxel induces apoptosis are still poorly understood (Bhalla, 2003).

We have developed and utilized isogenic transformed epithelial cells with defined combinations of gain- and loss-of-function mutations to evaluate the impact on both tumor growth and treatment response. We report here that paclitaxel induces BIM accumulation that is required for paclitaxel-induced apoptosis both in vitro and in vivo. However, constitutive activation of the H-ras/MAPK pathway suppresses BIM induction by phosphorylating BIM and targeting BIM for degradation in proteasomes, thereby blocking response to paclitaxel. Nevertheless, in cells with the RAS pathway activated, combined treatment with a proteasome inhibitor and paclitaxel restores BIM accumulation and apoptosis. Finally, we show that BIM is indeed a tumor suppressor in epithelial cells, and that BIM deficiency results in paclitaxel-resistant tumors. This provides a mechanistic explanation for chemotherapeutic-mediated apoptosis and suggests that combining a proteasome inhibitor with paclitaxel would provide therapeutic benefit among tumors with H-ras/MAPK pathway activation.

Results

Expression of H-ras confers resistance to paclitaxel

Since mutations that activate a *ras* gene are among the most common found in tumors, we sought to determine how constitutive RAS activity alters the apoptotic response during chemotherapy. Hence, we have investigated how imposed H-ras expression affects the paclitaxel sensitivity of transformed BMK epithelial cells with genetically defined defects in apoptosis. Transformed BMK cells that express BAX and BAK (W2 cells) (Degenhardt et al., 2002a) were transfected with plasmids directing constitutive expression of H-ras or BCL-2, or with the vector. Isogenic BAX- and BAK-deficient transformed BMK cells (D3 cells) (Degenhardt et al., 2002a) were also transfected with plasmids directing constitutive expression of H-ras or with the vector to test if H-ras enhanced the survival in the background of BAX/BAK deficiency. Three independent clones of each genotype that stably express H-ras (data not shown) were treated with paclitaxel and viability was determined. The viability of one representative clone of each genotype is shown in Figure 1A.

Importantly, the vector control cell line derived from W2 cells (W2.3.1-5) was readily killed by paclitaxel, and this cell death was blocked by the expression of H-ras (W2.Hras-3). Expression of antiapoptotic BCL-2 (W2.Bcl2-3) or deficiency of proapoptotic BAX and BAK (D3.zeo-2) (Nelson et al., 2004) conferred slightly more resistance to apoptosis than H-ras (W2.Hras-3) (Figure 1A). Furthermore, the expression of H-ras in BAX/BAK-deficient cells (D3.Hras-1) did not further enhance survival (Figure 1A), suggesting that H-ras inhibited paclitaxel-mediated apoptosis by blocking the BAX/BAK apoptotic pathway.

Paclitaxel induces BIM accumulation that is blocked by H-ras

As BH3-only proteins are essential triggers of apoptosis upstream of BAX and BAK (Adams, 2003b; Cheng et al., 2001), H-ras might act by affecting the function of BH3-only proteins that mediate paclitaxel-induced apoptosis. The resistance of

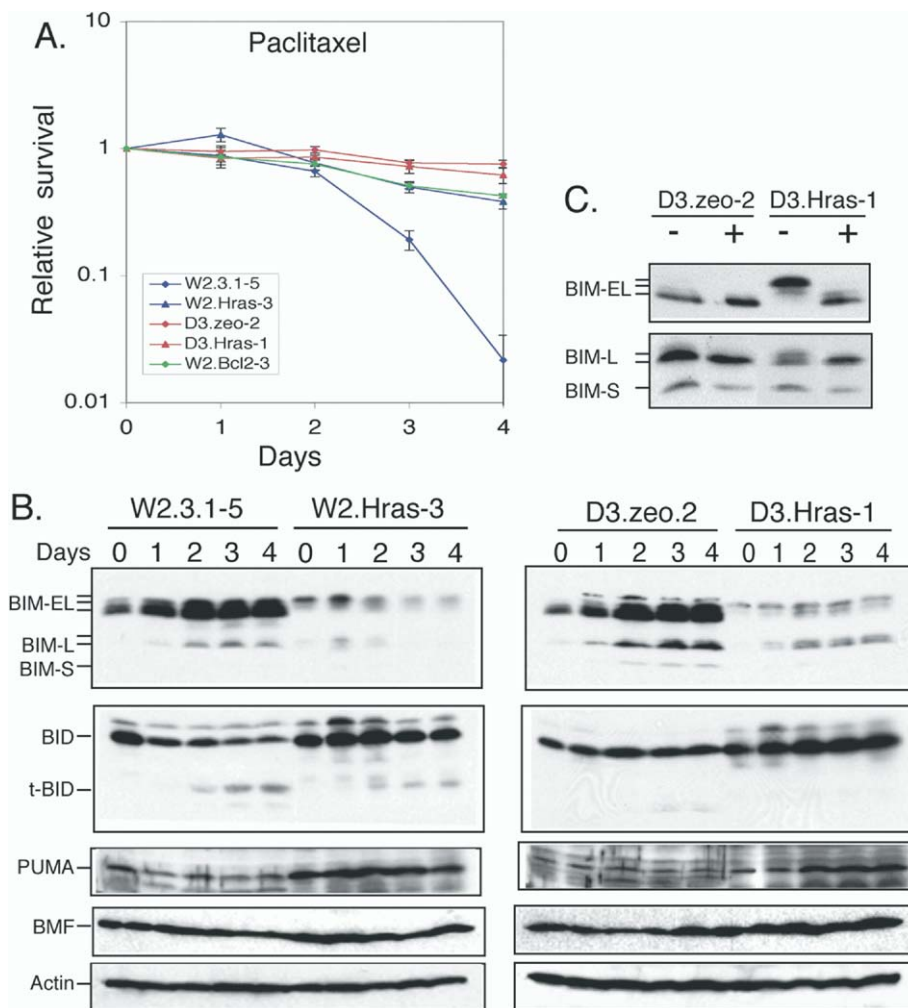


Figure 1. H-ras phosphorylates BIM, blocks BIM induction by paclitaxel, and confers resistance to paclitaxel

A: Viability of transformed BMK cells in response to paclitaxel. W2.3.1-5, W2.Bcl2-3, W2.Hras-3, D3.Hras-1, and D3.zeo-2 were treated with 300 nM paclitaxel for four days. The viable cell number was counted before (day 0) and each day after treatment by trypan blue exclusion. Results are presented as relative survival with error bars representing standard deviation.

B: Paclitaxel induces BIM accumulation, which is blocked by H-ras. W2.3.1-5, W2.Hras-3, D3.zeo-2, and D3.Hras-1 were untreated or treated with 300 nM paclitaxel for four days, and the expression of BH3-only proteins BIM, BID, tBID, PUMA, and BMF was analyzed by Western blotting. Actin is shown as a control for protein loading.

C: H-ras causes BIM-EL and BIM-L phosphorylation. D3.zeo-2 and D3.Hras-1 whole cell lysates were incubated with lambda protein phosphatase, resolved by SDS-PAGE, and Western blotted for BIM to establish that the slower migrating forms of BIM are the result of phosphorylation.

BIM-deficient lymphocytes in vitro to paclitaxel (Bouillet et al., 1999) led us to investigate the role of BIM in H-ras-dependent paclitaxel resistance in epithelial cells. Western blotting revealed that the levels of BIM-L and particularly the more predominant BIM-EL isoform were upregulated dramatically in W2.3.1-5 and D3.zeo-2 cells following paclitaxel treatment (Figure 1B). As D3.zeo-2 cells did not undergo apoptosis in response to paclitaxel (Figure 1A), BIM induction was not a consequence of cell death. Notably, BIM induction by paclitaxel was largely abrogated by the H-ras expression in both W2.Hras-3 and D3.Hras-1 cells (Figure 1B). Thus, H-ras regulates both BIM accumulation and apoptosis by paclitaxel. Because the levels of other BH3-only proteins, namely BID, tBID, PUMA, and BMF, were unchanged by paclitaxel treatment (Figure 1B), BIM appears to have a specific role in paclitaxel-induced cell death. BIM induction is not merely a general response to cellular damage, because another cytotoxic drug, the topoisomerase II inhibitor etoposide, did not induce BIM accumulation (see Supplemental Figure S1). Interestingly, BID was only cleaved to tBID in W2.3.1-5 cells that underwent apoptosis in response to paclitaxel, and not in apoptosis-resistant D3 cells, suggesting that BID cleavage resulted from caspase activation (Figure 1B).

BIM-EL and BIM-L were present as multiple distinctively migrating bands, the slowest of which were preferentially observed in H-ras-expressing cells (Figure 1B), and BIM-EL is a substrate for phosphorylation by the RAF/MAPK pathways downstream of H-ras (Marani et al., 2004). In contrast, W2.3.1-5 and D3.zeo-2 cells predominantly displayed the faster migrating BIM-EL and BIM-L, suggesting that they were unphosphorylated. To confirm that the slower migrating forms of BIM-EL and BIM-L resulted from phosphorylation by H-ras, lysates of D3.zeo-2 and D3.Hras-1 cells were incubated with lambda protein phosphatase. As expected, phosphatase treatment converted both BIM-EL and BIM-L to monomeric forms with enhanced mobility (Figure 1C). Thus, H-ras expression caused BIM phosphorylation and specifically prevented BIM induction by paclitaxel, suggesting that H-ras may confer resistance to the drug by modulating BIM levels.

The MAPK pathway regulates BIM and blocks the apoptotic response to paclitaxel

To define the downstream effector pathway through which H-ras interferes with paclitaxel-induced apoptosis, W2 cells were transfected with the vector, or vectors that express constitutively activated forms of RAF (RAF-CAAX) or AKT (Myr-

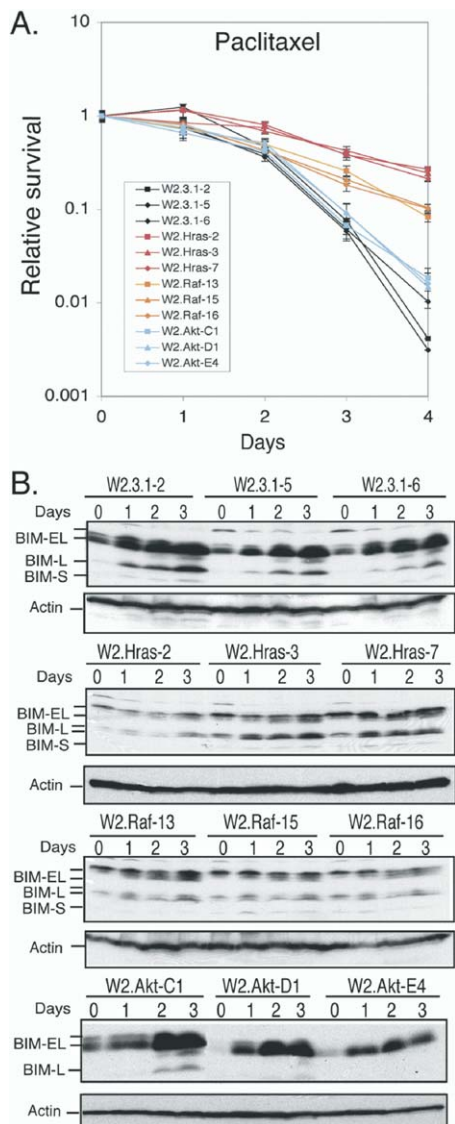


Figure 2. Activation of MAPK pathway blocks BIM induction and confers paclitaxel resistance

A: Viability of transformed BMK cells expressing constitutively activated RAF or AKT in response to paclitaxel. Three independent W2-derived stable BMK cell lines expressing constitutively activated H-ras, RAF, AKT, or vector controls were treated with 300 nM paclitaxel for four days, and viability was evaluated as described above. Results are presented as relative survival with error bars representing standard deviation.

B: H-ras phosphorylates BIM and blocks BIM induction through the RAF/MAPK pathway. The above cell lines were untreated or treated with 300 nM paclitaxel for three days, and cell extracts were subjected to Western blotting for BIM. Actin is shown as a loading control.

AKT). Three independent cell lines of each genotype were treated with paclitaxel, and viability was determined. BMK cells expressing RAF-CAAX were resistant to paclitaxel to a similar extent as cells expressing H-ras (Figure 2A). In contrast, cells expressing Myr-AKT were as sensitive to paclitaxel as vector control cell lines (Figure 2A). These data suggest that H-ras provides resistance to paclitaxel in BMK cells through RAF/MAPK pathway.

To test if phosphorylation of BIM by H-ras was also a function of H-ras signaling through RAF/MAPK pathway, the cell lines were treated with paclitaxel, and BIM protein levels were measured by Western blotting. As expected, BIM isoforms were phosphorylated in H-ras- or RAF-CAAX-expressing cells, neither of which accumulated BIM in response to paclitaxel (Figure 2B). In contrast, BIM was unphosphorylated and highly induced in response to paclitaxel in both W2 vector control cells and W2 cells expressing Myr-AKT (Figure 2B). Hence, H-ras may suppress paclitaxel-induced apoptosis through MAPK pathway by phosphorylating BIM and blocking BIM accumulation. To confirm that the MEK/ERK pathway downstream of H-ras is responsible for phosphorylation of BIM by H-ras, the specific MEK1/2 inhibitor U0126 was used to block MEK1/2 activity, which as expected prevented BIM phosphorylation in H-ras expressing cells (see Supplemental Figure S2).

H-ras provides resistance to paclitaxel by preventing BIM accumulation

To determine if paclitaxel-induced apoptosis was mediated by BIM, multiple independent E1A and p53DD transformed BMK cell lines that were deficient for BIM (*bim*^{-/-}) or expressed BIM (*bim*^{+/+}) were derived from primary BMK cells from kidneys of *bim*^{-/-} and *bim*^{+/+} mice, littermates from a *bim*^{+/-} cross. As expected, BIM was expressed only in the three independent *bim*^{+/+} cell lines, whereas E1A, p53DD, BAX, and BAK were expressed in all cell lines (Figure 3A). Significantly, all three BIM-deficient BMK cell lines (BIM^{-/-}A, B, and C) remained largely viable following three days of paclitaxel treatment, whereas all three BIM wild-type BMK cell lines (BIM^{+/+}A, B, and C) were readily killed, indicating that BIM was required for paclitaxel-induced apoptosis (Figure 3B).

To determine if H-ras and RAF-CAAX blocked paclitaxel-induced apoptosis by inhibiting BIM accumulation, the BIM-deficient BMK cell line BIM^{-/-}A was engineered to express H-ras (BIM^{-/-}A.Hras-A, B, and C) or RAF-CAAX (BIM^{-/-}A.Raf-A, B, and C) (Figure 3C). Only H-ras expressing BIM^{-/-}A cells showed overexpression of RAS, and both H-ras- and RAF-CAAX-expressing BIM^{-/-}A cells displayed phosphorylated ERK, a measure of MAPK activation, which was not present in vector control BIM^{-/-}A and BIM^{+/+}A cells (Figure 3C). These cell lines were then tested for paclitaxel sensitivity to determine if H-ras and RAF-CAAX-mediated apoptosis-resistance was BIM-dependent. Three independent BIM deficient cell lines expressing H-ras or RAF-CAAX showed paclitaxel resistance similar to the BIM-deficient control cells (Figures 3D and 3E). Note that BAX/BAK-deficient D3 cells (with or without H-ras) were more resistant to paclitaxel than W2.Hras-3 cells (Figure 1A), presumably because of their extreme defect in apoptosis. Thus, a gain-of-function of H-ras or RAF in the background of BIM deficiency should have been apparent. The absence of a gain-of-function of H-ras or RAF in BIM-deficient cells indicates that they block paclitaxel-induced apoptosis by inhibiting BIM.

BIM, but not PUMA, suppresses tumorigenesis

Since tumorigenesis and chemoresistance often concur in apoptotic defective cells, we investigated the impact of BIM deficiency on tumorigenesis, by comparing tumor development with three independent BIM-deficient (BIM^{-/-}A, B, and C) and BIM wild-type (BIM^{+/+}A, B, and C) transformed BMK cell

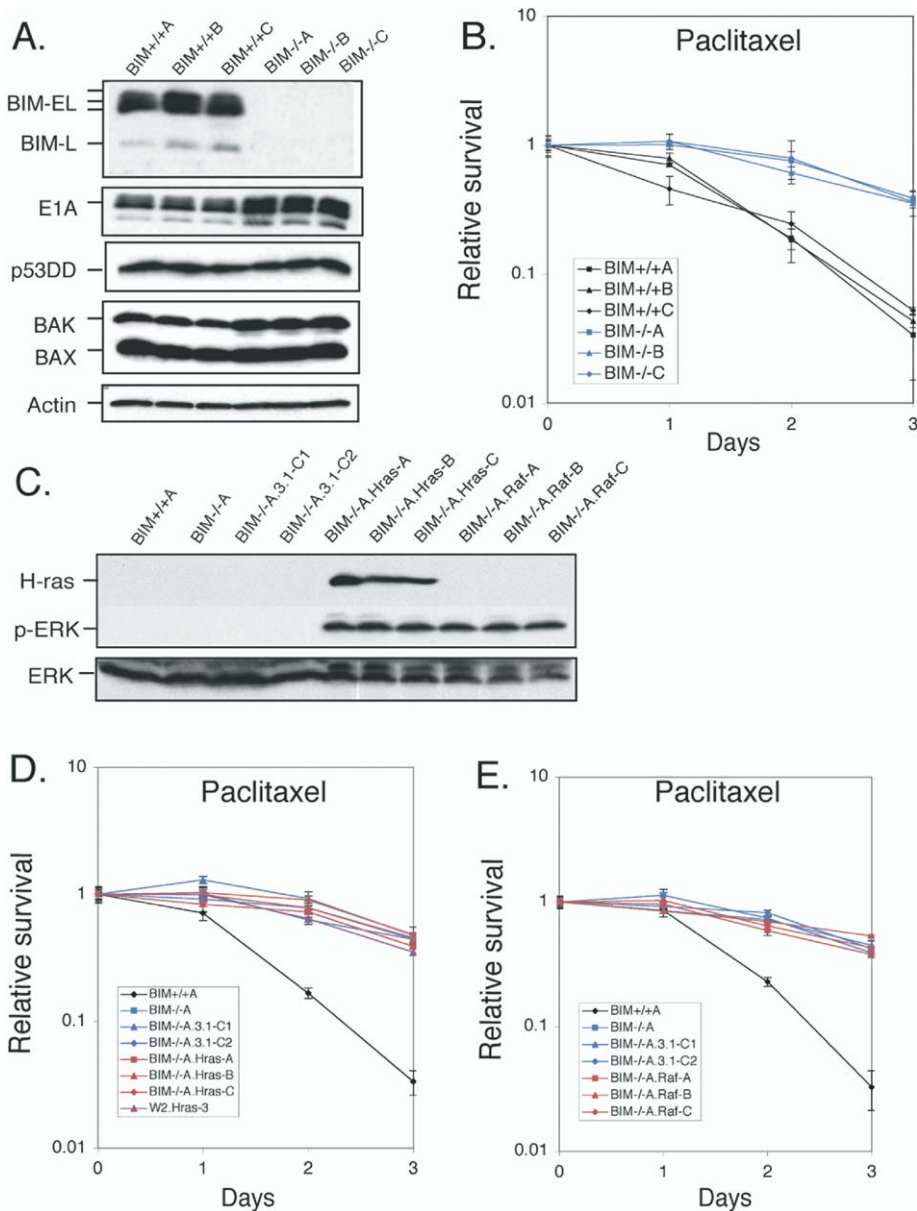


Figure 3. H-ras provides resistance to paclitaxel by blocking BIM induction

A: Generation of wild-type and BIM-deficient BMK cell lines. Cell extracts generated from three independent BMK cell lines that express BIM ($BIM^{+/+A}$, $BIM^{+/+B}$, and $BIM^{+/+C}$), and that are deficient for BIM ($BIM^{-/-A}$, $BIM^{-/-B}$, and $BIM^{-/-C}$) were subjected to Western blotting with antibodies specific for BIM, E1A, p53, BAK, BAX, and actin.

B: BIM deficiency confers resistance to paclitaxel. Three independent BMK cell lines that express BIM or that are deficient for BIM were treated with 100 nM paclitaxel for three days, and viability was evaluated as described above. Results are presented as relative survival with error bars representing standard deviation.

C: Generation of BIM-deficient BMK cell lines expressing H-ras or RAF. Cell extracts generated from a BMK cell line that expresses BIM ($BIM^{+/+A}$), a cell line that is deficient for BIM ($BIM^{-/-A}$), and three independent cell lines derived from $BIM^{-/-A}$ that express H-ras, or RAF-CAAX, along with vector controls, were subjected to Western blotting with antibodies specific for H-ras and phosphorylated ERK. The level of total ERK is shown as a loading control.

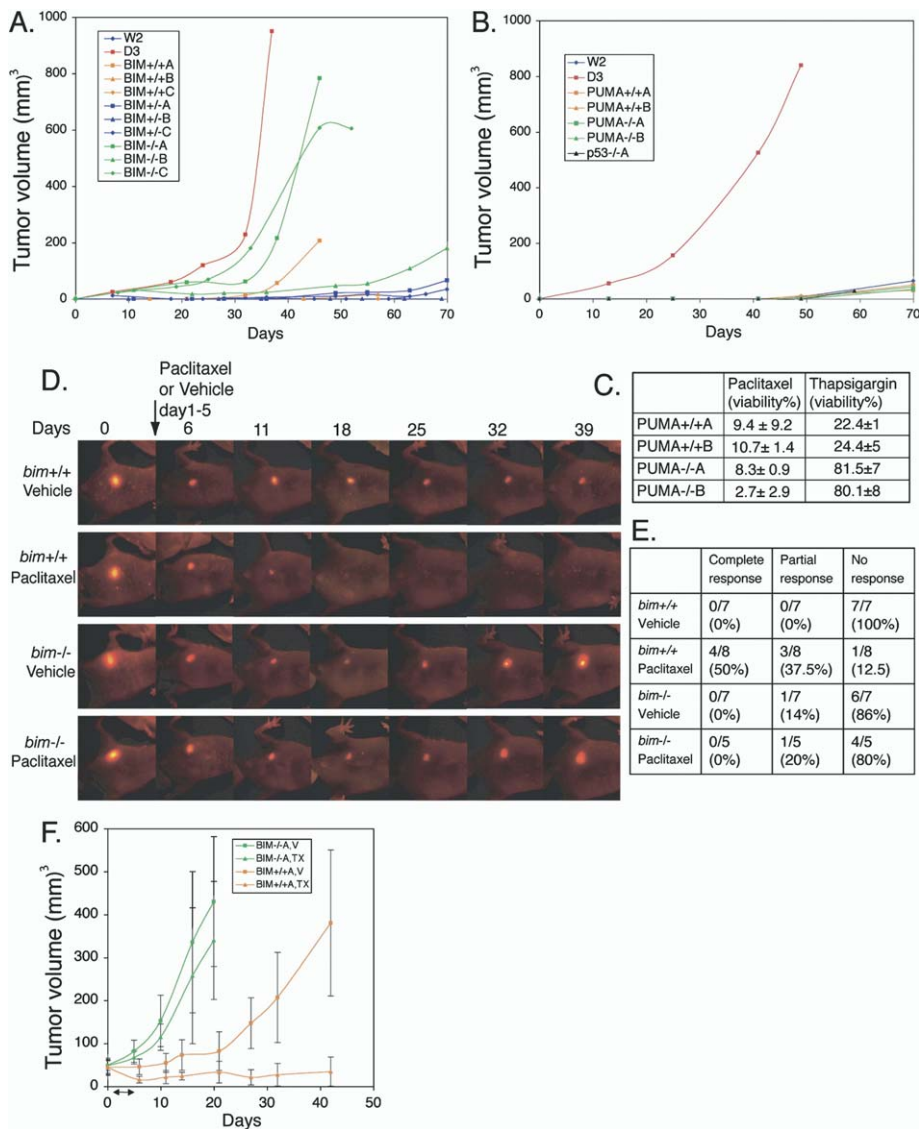
D and E: Overexpression of H-ras or RAF-CAAX did not further enhance paclitaxel resistance over BIM deficiency. Three independent BMK cell lines expressing H-ras (**D**) or RAF-CAAX (**E**) derived from $BIM^{-/-A}$ cells were treated with 100 nM paclitaxel for three days, along with $BIM^{+/+A}$ cells, $BIM^{-/-A}$ cells, and two independent cell lines with vector control derived from $BIM^{-/-A}$, and viability was evaluated by trypan blue exclusion. Results are presented as relative survival with error bars representing standard deviation. Note that H-ras or RAF-CAAX had no additional capacity to inhibit paclitaxel-mediated apoptosis over BIM deficiency.

lines. Moreover, to evaluate if BIM is haploinsufficient in epithelial tumorigenesis, as it was recently shown to be in B lymphocytes (Egle et al., 2004), three independent BIM heterozygous transformed BMK cell lines ($BIM^{+/-A}$, B, and C) were also tested. Cells from all the lines were injected subcutaneously into nude mice, and tumor growth was monitored.

As previously reported (Degenhardt et al., 2002a; Nelson et al., 2004), the BAX/BAK-deficient control (D3) formed large tumors (carcinomas) within 30 days, whereas a transformed cell line expressing BAX and BAK (W2) formed no measurable tumors (Figure 4A). Significantly, two of the three BIM-deficient transformed BMK cell lines formed tumors at a rate comparable to that of the BAX/BAK deficient D3 cells, and the third cell line also eventually formed tumors (Figure 4A). In contrast, all three BMK cell lines heterozygous for BIM and two of the three BMK cell lines wild-type for BIM were as nontumorigenic as

the W2 control cells, while a single BMK cell line that expressed BIM ($BIM^{+/+A}$) displayed modest tumorigenic activity (Figure 4A). Thus, BIM deficiency promotes epithelial tumorigenesis nearly as well as loss of BAX and BAK ($p < 0.05$), although unlike the $E\mu$ -*myc* B cell lymphomas (Egle et al., 2004), we did not observe haploinsufficiency. As BAX and BAK are absolutely required for apoptosis, their deficiency may be more effective at blocking apoptosis than the loss-of-function of one of the upstream BH3-only proteins such as BIM.

To address the possibility that BH3-only protein deficiency may shift the balance toward the antiapoptotic state and that deletion of any BH3-only protein would nonspecifically promote tumorigenesis, we tested the tumorigenic potential of E1A plus p53DD-transformed *puma*^{-/-} BMK cells (Figure 4B) derived from PUMA-deficient mice (Villunger et al., 2003). The observation that PUMA-deficient transformed BMK cells were



response is defined as progressive loss of RFP signal beyond three weeks following treatment, but still detectable, and no response is defined as no detectable loss of RFP signal. The number of mice showing a response in each category is shown over the total number of mice in each group, and the percent responding is shown in parenthesis.

F: BIM deficiency abrogates paclitaxel-mediated inhibition of tumor progression of established tumors. Six mice bearing subcutaneous tumors of either BIM^{+/+}A or BIM^{-/-}A were divided into vehicle and paclitaxel groups, with three mice in each group. When the tumors reached 0.5 cm in diameter (day 0), treatment with paclitaxel at 16 mg/kg or vehicle was given intraperitoneally for five consecutive days (day 1–5), and the average tumor volume of each group, with standard deviation, is shown as a function of time. Double arrow indicates the days of drug treatment.

as nontumorigenic as the W2 control cells indicates that in contrast to BIM deficiency, PUMA deficiency was not sufficient to promote tumorigenesis of epithelial cells (Figure 4B). In addition, PUMA-deficient cells were sensitive to paclitaxel, but displayed resistance to thapsigargin, an inducer of ER-stress-mediated apoptosis (Figure 4C), consistent with a previous study (Reimertz et al., 2003). Thus, although BIM and PUMA are both proapoptotic BH3-only BCL-2 antagonists, their functions are apparently pathway-specific.

BIM confers paclitaxel sensitivity in vivo

We investigated the role of BIM in the apoptotic response to paclitaxel in vivo, by testing the ability of the drug to prevent

tumorigenesis. To visualize injected cells noninvasively, transformed BMK cell lines BIM^{+/+}A and BIM^{-/-}A were engineered to express red fluorescent protein (RFP), and the mice were monitored by whole-animal optical imaging. Figure 4D shows representative mice from each treatment group (vehicle or paclitaxel) prior to and after paclitaxel administration. BIM^{+/+}A cells persisted in the vehicle control-treated animals and eventually formed tumors of 0.5 cm in diameter by eight weeks, whereas the RFP signal progressively regressed and completely disappeared after paclitaxel treatment, and no tumors formed during the total 14 weeks of monitoring. In contrast, BIM^{-/-}A cells persisted in both the paclitaxel and vehicle control groups for over three weeks, and tumors formed within

Figure 4. BIM suppresses tumorigenesis and confers paclitaxel sensitivity in vivo

A: BIM deficiency facilitates tumor growth. Three independent transformed BMK cell lines of each genotype (*bim*^{+/+}, *bim*^{+/-}, or *bim*^{-/-}) were injected subcutaneously into nude mice, and tumor growth was monitored over time. Each point represents the average tumor volume for five animals.

B: PUMA deficiency is not sufficient to promote tumorigenesis. Two independent transformed wild-type (PUMA^{+/+}A and B) and PUMA-deficient (PUMA^{-/-}A and B) BMK cell lines were derived from primary BMK cells from *puma*^{-/-} and *puma*^{+/+} mice, littermates from a *puma*^{+/-} cross. The E1A-alone transformed BMK cell line p53^{-/-}A similarly derived from p53^{-/-} mice is shown as a control. Tumor formation was performed as described above.

C: PUMA-deficient cells are sensitive to paclitaxel but resistant to thapsigargin. PUMA^{+/+}A and B and PUMA^{-/-}A and B were treated with 100 nM paclitaxel or 1 μM thapsigargin, and viability was evaluated by trypan blue exclusion after 72 hr or 24 hr of treatment, respectively, as described above. Results are presented as relative survival with standard deviation.

D: BIM deficiency abrogates paclitaxel-mediated inhibition of tumorigenesis. BIM^{+/+}A and BIM^{-/-}A cells expressing RFP were injected subcutaneously into nude mice. Three days after implantation, treatment with paclitaxel at 16 mg/kg dissolved in normal saline or vehicle (84.3 mg/ml cremophor and 8% [v/v] ethanol dissolved in normal saline) was given intraperitoneally for five consecutive days. Day 0 represents the day before treatment. Mice were ear-tagged and individual mice were monitored over time using a whole-animal fluorescence imaging system to monitor tumor growth. One representative animal of each treatment group is shown.

E: Quantitation of BIM-dependent paclitaxel responsiveness. The response of BIM^{+/+}A and BIM^{-/-}A implanted into nude mice to vehicle or paclitaxel was quantitated and categorized. Complete response is defined as the complete disappearance of the RFP signal, partial response is defined as progressive loss of RFP signal beyond three weeks following treatment, but still detectable, and no response is defined as no detectable loss of RFP signal.

six weeks, irrespective of paclitaxel treatment. Whereas only one of five mice bearing BIM^{-/-}A cells responded partially to paclitaxel, 7 of 8 mice bearing BIM^{+/+}A cells responded completely or partially (Figure 4E). Thus, BIM is required for paclitaxel responsiveness in vivo as well as in vitro.

To test the efficacy of paclitaxel on established tumors, BIM^{+/+}A and BIM^{-/-}A cells were implanted subcutaneously into nude mice, and paclitaxel or vehicle treatment was initiated when tumors reached 0.5 cm in diameter. Subsequent monitoring of tumor volume (Figure 4F) revealed that paclitaxel did not affect the growth rate of BIM-deficient tumors (BIM^{-/-}A) but induced striking regression of tumors bearing BIM (BIM^{+/+}A) that persisted during the six weeks of observation. Thus, BIM is a major determinant in the response of tumors to paclitaxel, both during tumor development and in established tumors.

H-ras/MAPK signaling promotes BIM degradation in proteasomes

It has been reported recently that phosphorylation of BIM by the ERK1/2 pathway accelerates BIM-EL degradation in proteasomes (Ley et al., 2004; Luciano et al., 2003). Therefore, we explored whether RAS prevented BIM induction by paclitaxel by inducing its phosphorylation and turnover. To obviate any contribution of cell death to BIM turnover, the effect of H-ras on BIM stability was assessed in the BAX/BAK-deficient D3 cells. D3.zeo-2 and D3.Hras-1 cells were incubated with or without paclitaxel for 24 hr, the protein synthesis inhibitor cycloheximide (CHX) was added, and the subsequent decay in BIM levels was monitored by Western blotting. In the absence of paclitaxel, BIM-EL levels fell slowly in D3.zeo-2 cells, with a half-life in excess of four hours, but H-ras expression accelerated BIM turnover to a half-life of less than two hours (Figure 5A). Though paclitaxel enhanced BIM stability in the D3.zeo-2 cells, BIM turnover was greatly accelerated in the D3.Hras-1 cells (Figure 5A). Thus, paclitaxel enhances BIM stability, but H-ras expression facilitates BIM turnover even in the presence of paclitaxel.

To determine if the degradation of BIM-EL proceeded via a proteasome-dependent pathway, we examined BIM stability in the presence of the proteasome inhibitor epoxomicin. Significantly, epoxomicin completely blocked BIM-EL degradation in the absence (Figure 5B) or presence (Figure 5C) of paclitaxel and restored BIM accumulation to H-ras expressing cells (Figures 5B and 5C). Moreover, in D3.zeo-2 cells, the slower migrating forms of BIM-EL representing phosphorylated BIM-EL accumulated over time following epoxomicin treatment, suggesting that BIM-EL phosphorylation occurred prior to its degradation in proteasomes (Figure 5B and 5C). Thus, the enhanced turnover of BIM in H-ras expressing cells could be attributed to stimulation of BIM phosphorylation and degradation via the ubiquitin proteasome pathway. Since paclitaxel induces BIM accumulation, and BIM function is required for apoptosis induction by paclitaxel, this suggests that H-ras expression suppresses BIM accumulation by targeting BIM for degradation in proteasomes, thereby blocking apoptosis.

Since BIM protein levels reportedly can be regulated at the transcription level through the MAPK, AKT/Forkhead, or JNK pathways in certain cell types (Marani et al., 2004; Putcha et al., 2003), we tested whether RAS also blocks BIM induction in response to paclitaxel at the mRNA level. The basal BIM mRNA levels in BMK cells with (D3.Hras-1) or without (D3.zeo-2)

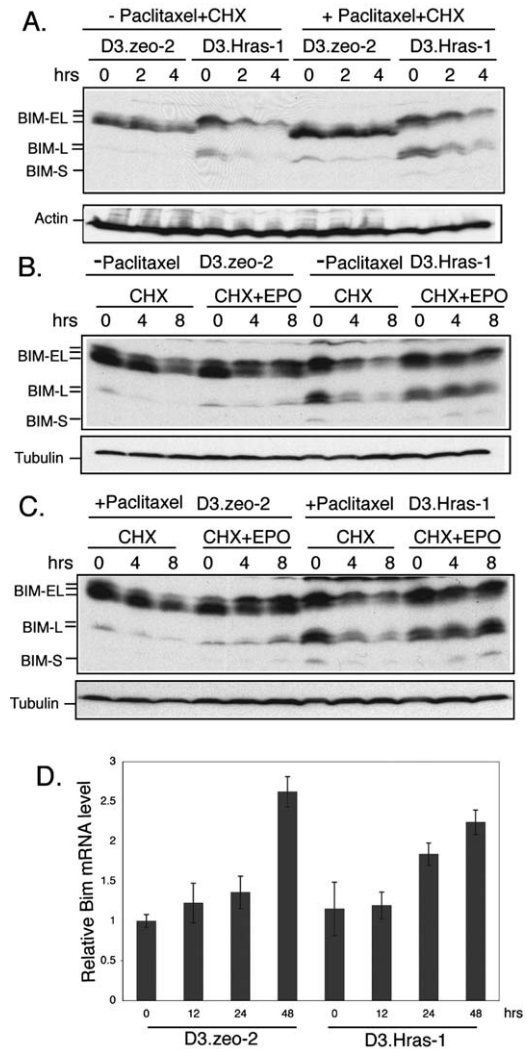


Figure 5. H-ras promotes BIM degradation in proteasomes

A: Phosphorylation by H-ras promotes BIM turnover. D3.zeo-2 and D3.Hras-1 were untreated or treated with 300 nM paclitaxel for 24 hr and then treated with 0.4 μ g/ml of CHX or 0.4 μ g/ml of CHX and paclitaxel, respectively. Cell lysates were analyzed by Western blotting at the indicated times for BIM. Actin is shown as a loading control.

B and C: Proteasome inhibition rescues BIM turnover. D3.zeo-2 and D3.Hras-1 cells were untreated (**B**) or treated with 300 nM paclitaxel (**C**) for 24 hr and then 0.4 μ g/ml of CHX or the combination of CHX and 200 nM of epoxomicin was added to the cells. Cell lysates were analyzed by Western blotting at the indicated times for BIM. α -tubulin is shown as a loading control. **D:** BIM is transcriptionally upregulated in response to paclitaxel that is not significantly affected by H-ras. Total RNA was prepared from D3.zeo-2 and D3.Hras-1 cells following 300 nM paclitaxel treatment at the times indicated, and level of BIM mRNA was analyzed by RT-PCR. Standard deviation was calculated from the triplicate samples.

H-ras expression were comparable (Figure 5D). After 48 hr of paclitaxel treatment, the mRNA level increased modestly in both D3.zeo-2 cells and D3.Hras-1 cells, suggesting that upregulation of BIM mRNA by paclitaxel may contribute to accumulation of the protein independently of H-ras. We conclude that the predominant mechanism by which H-ras prevents BIM induction by paclitaxel is stimulation of BIM turnover.

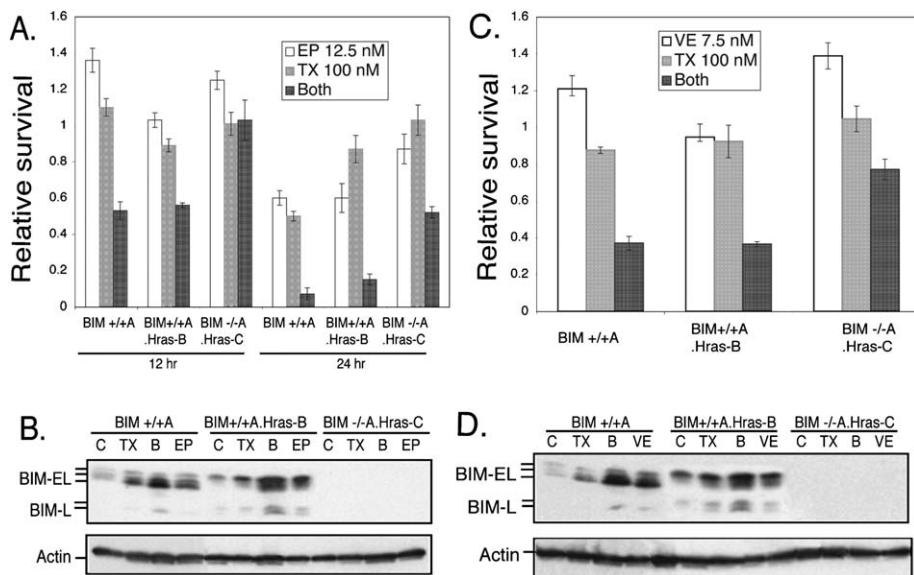


Figure 6. Proteasome inhibition restores BIM function to H-ras expressing cells

A: Epoxomicin abrogates H-ras-dependent resistance to paclitaxel in BIM wild-type but not in BIM-deficient cells in vitro. BIM^{+/+}A, BIM^{+/+}A.Hras-B, and BIM^{-/-}A.Hras-C were treated with 12.5 nM epoxomicin alone or 24 hr. Viability was evaluated by trypan blue exclusion. Results are presented as relative survival with error bars representing standard deviation.

B: Epoxomicin rescues BIM induction by paclitaxel in the presence of H-ras. Cell lysates from 24 hr treatment were then subjected to Western blotting for BIM. Control lysates without treatment (C), paclitaxel alone (TX), the combination of epoxomicin and paclitaxel (B), and epoxomicin alone (EP) are indicated.

C: Velcade abrogates H-ras-dependent paclitaxel resistance in BIM wild-type but not in BIM-deficient cells in vitro. BIM^{+/+}A, BIM^{+/+}A.Hras-B, and BIM^{-/-}A.Hras-C were treated with 7.5 nM Velcade alone or 100 nM paclitaxel alone, or both, for 24 hr. Viability was evaluated by trypan blue exclusion. Results are presented as relative survival with error bars representing standard deviation.

D: Velcade rescues BIM induction by paclitaxel in the presence of H-ras. Cell lysates of the above were subjected to Western blotting for BIM. Control lysates without treatment (C), paclitaxel alone (TX), the combination of Velcade and paclitaxel (B), and Velcade alone (VE) are indicated.

Proteasome inhibition restores BIM-dependent apoptosis by paclitaxel in the presence of H-ras

If H-ras renders cells refractory to paclitaxel predominantly by promoting BIM degradation, proteasome inhibitors might restore cell death in H-ras-expressing cells by blocking BIM turnover, and only BIM-expressing cells should be sensitized to paclitaxel. To test this hypothesis, BIM^{+/+}A, BIM^{+/+}A.Hras-B, and BIM^{-/-}A.Hras-C were treated with paclitaxel, epoxomicin, or both. At time points and drug concentrations where the BIM wild-type cells were refractory to paclitaxel or epoxomicin alone, the combined treatment was highly cytotoxic, even when H-ras was expressed (Figure 6A). In striking contrast, BIM-deficient cells expressing H-ras were markedly resistant to the combination of paclitaxel and epoxomicin (Figure 6A). The small epoxomicin and paclitaxel synergy observable at 24 hr in BIM-deficient cells might indicate the existence of proteasome targets other than BIM. Restoration of paclitaxel sensitivity by epoxomicin in BIM^{+/+}A.Hras-B cells correlated with restoration of BIM accumulation (Figure 6B). This is not a specific effect of epoxomicin, because the proteasome inhibitor Velcade, which is currently in clinical trials as an anticancer agent and has been approved by the FDA for use in multiple myeloma, also restored BIM induction by paclitaxel and abrogated H-ras-dependent resistance in BIM wild-type but not BIM-deficient cells (Figures 6C and 6D). Thus, H-ras inhibits paclitaxel-mediated apoptosis by phosphorylating BIM via the MAPK pathway and promoting its degradation in proteasomes.

Proteasome inhibition abrogates H-ras-dependent paclitaxel resistance in vivo

The ability of proteasome inhibitors to abrogate the paclitaxel resistance conveyed by H-ras encouraged us to test whether proteasome inhibition could sensitize H-ras-expressing cells to paclitaxel in vivo. The efficacy of cotreatment with Velcade

and paclitaxel was compared to that with each drug alone or to the vehicle control in BIM^{+/+}A.Hras-B and BIM^{-/-}A.Hras-C BMK cells implanted subcutaneously into nude mice. BIM^{+/+}A.Hras-B and BIM^{-/-}A.Hras-C were both highly tumorigenic (Figures 7A and 7B). The tumors formed by BIM^{+/+}A.Hras-B responded poorly to either Velcade or paclitaxel alone, but the cotreatment significantly reduced tumor growth ($p < 0.01$) (Figure 7A). In contrast, tumors formed by BIM^{-/-}A.Hras-C BMK cells responded poorly to the combination treatment (Figure 7B), demonstrating that the synergy between Velcade and paclitaxel requires BIM. Thus, proteasome inhibition abrogated the paclitaxel resistance in vivo imposed by H-ras, by rescuing BIM responsiveness to paclitaxel.

Discussion

Many anticancer agents induce apoptosis, and chemoresistance can occur when tumors are unable to efficiently engage apoptotic programs. Since apoptotic defects are selected for during tumorigenesis, the process of tumor evolution may also select for drug resistance. Indeed, BIM has proven to be a tumor suppressor both in epithelial solid tumors (Figure 4A) and lymphomas (Egle et al., 2004), and also is a major determinant in paclitaxel sensitivity (Figures 4D–4F). Similarly, H-ras and the MAPK pathway are commonly mutated in human tumors, and we show that RAS/MAPK activation suppresses BIM accumulation and thereby ablates the proapoptotic response to paclitaxel. Thus, common mutational events that arise during tumor formation can directly or indirectly disable the apoptotic machinery and confound cancer treatment.

Just as BCL-2 is an oncoprotein, evidence is emerging that several of the BH3-only proteins may suppress the development of very specific types of tumors. Mice lacking BID have an increased tendency to develop chronic myelomonocytic

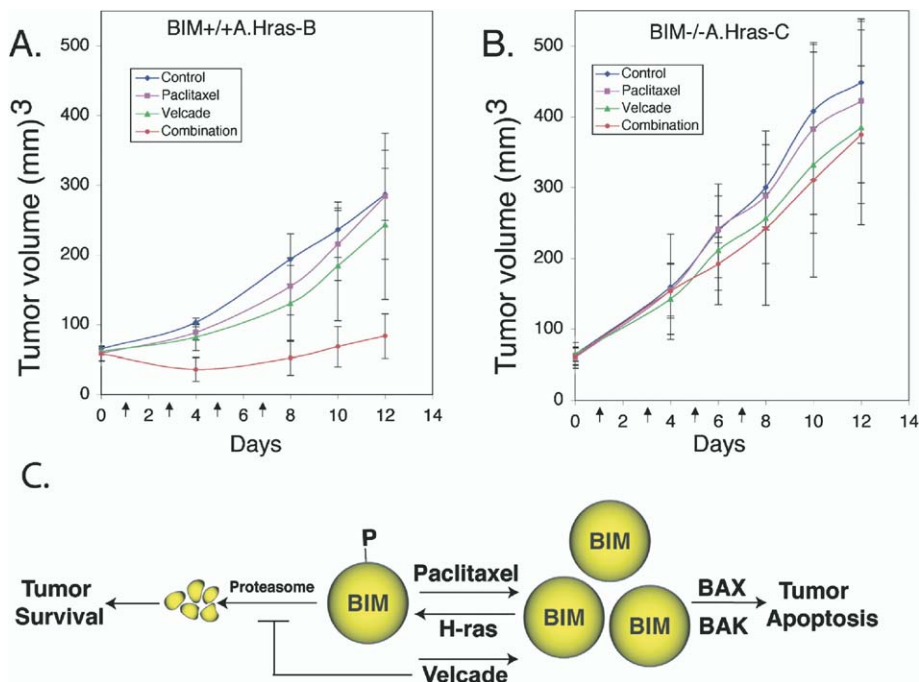


Figure 7. Velcade-enhanced paclitaxel-induced apoptosis in vivo requires BIM

A and B: BIM^{+/+}A.Hras-B or BIM^{-/-}A.Hras-C cells were injected subcutaneously into nude mice. When the tumors reached 0.5 cm in diameter (day 0), the mice were assigned randomly into each of the following groups with 5–9 mice/group: control, Velcade, paclitaxel, or Velcade and paclitaxel. Each mouse received 0.55 mg/kg Velcade and/or 10 mg/kg paclitaxel intraperitoneally on days 1, 3, 5, 7 as arrows indicate. Mannitol dissolved in normal saline was used as vehicle for Velcade; 52.7 mg/ml cremophor and 5% (v/v) ethanol dissolved in normal saline was used as vehicle for paclitaxel. Day 0 represents the day before treatment. Each point represents the average tumor volume of each group, with standard deviation. Note that Velcade sensitized H-ras-expressing BMK cells to paclitaxel in the presence of BIM (**A**) but failed to sensitize H-ras-expressing BMK cells to paclitaxel in the absence of BIM (**B**).

C: A model for the mechanism by which H-ras modulates BIM and confers resistance to paclitaxel. See text for details.

leukemia (Zinkel et al., 2003), and some aged BAD-deficient mice develop diffuse large cell lymphoma (Ranger et al., 2003). Similarly, either PUMA knockdown or BIM deficiency promotes the development of B cell lymphomas in E μ -myc transgenic mice (Egle et al., 2004; Hemann et al., 2004). The contribution of BH3-only proteins to suppression of epithelial solid tumor formation, however, was unknown. We showed previously that BAX- or BAK-dependent apoptosis suppresses tumorigenesis of transformed BMK cells independently from p53 function (Deegenhardt et al., 2002a). As BH3-only proteins function upstream of BAX and BAK, identification of those BH3-only proteins, and the apoptotic pathways that they regulate to control apoptosis during solid tumor formation, is of great interest. Here we demonstrate that BIM, but not PUMA, deficiency promotes epithelial tumorigenesis (Figures 4A and 4B). The human *Bim* gene (official name *BCL2L11*) may well also be a tumor suppressor, because it is located in a region (chromosome 2q13) where alterations, predominantly deletions, have been reported in human malignancies, more than half of the cases being epithelial in origin (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>).

Since the BMK cells studied here had been transformed with genes that disrupt the RB and p53 pathways, BIM clearly controls a checkpoint independent of those major routes to tumorigenesis. BIM deficiency was somewhat less efficient in tumor promotion than combined deficiency in BAX and BAK. As BAX and BAK seem to be required for apoptosis mediated by all BH3-only proteins, whereas the latter are activated by specific stimuli (Cory and Adams, 2002), this result may well indicate that another BH3-only protein can also contribute to epithelial tumorigenesis. Indeed, PUMA and not BIM is induced by ischemic conditions in the tumor microenvironment in vivo during BAX- and BAK-mediated tumor regression (Nelson et al., 2004), and PUMA may synergize with BIM to suppress tumori-

genesis. As PUMA deficiency alone was insufficient to promote epithelial tumorigenesis, PUMA function may only be manifested in the absence of BIM. Alternatively, PUMA may be a tumor suppressor in other situations, as it mediates both p53-dependent and -independent apoptosis, and NOXA or other BH3-only proteins may function redundantly with PUMA.

BIM has been implicated in mediating paclitaxel-induced apoptosis in vitro (Bouillet et al., 1999; Sunters et al., 2003), and we demonstrate here that there is an essential role of BIM in paclitaxel-induced apoptosis in vivo. Paclitaxel not only effectively inhibited epithelial tumor establishment, but also strikingly induced regression of established tumors. However, this chemotherapeutic effect required BIM, because it was completely abrogated by BIM deficiency. Moreover, H-ras expression conferred resistance to paclitaxel-induced apoptosis by promoting BIM degradation in proteasomes, and BIM inhibition promoted both epithelial tumor growth and drug resistance. Pertinently, combined chemotherapy with paclitaxel and the MER/ERK inhibitor U0126 was shown to enhance induction of cell death in human tumors (MacKeigan et al., 2000; McDaid and Horwitz, 2001), but the underlying mechanism is not known.

Here, we correlate RAS-dependent paclitaxel resistance and BIM regulation by the RAS/MAPK pathway (Figure 7C). In transformed BMK epithelial cells, paclitaxel caused upregulation of both BIM mRNA and protein, resulting in BIM accumulation and apoptosis. Although BIM has been shown to be regulated transcriptionally by RAS or its downstream effectors in different scenarios, basal levels of BIM mRNA and its upregulation by paclitaxel were not significantly affected by H-ras. However, activation of the RAS/MAPK pathway prevented BIM induction by paclitaxel by stimulating BIM phosphorylation and degradation via the ubiquitin proteasome pathway, thereby producing resistance to paclitaxel (Figure 7C). As BCL-2 and

MCL-1 bind to and compromise BIM function, we tested whether phosphorylation of BIM by H-ras would modulate its binding to BCL-2 and/or MCL-1, but this was not the case (data not shown). Thus, phosphorylation of BIM by H-ras likely regulates BIM protein levels and not its function per se.

Our studies demonstrate that proteasome inhibitors restore BIM induction and abrogate H-ras-dependent resistance to paclitaxel. Velcade, the first proteasome inhibitor to reach clinical trials, shows *in vitro* and *in vivo* activity as a single agent against multiple myeloma, chronic lymphocytic leukemia, and prostate, pancreatic, and renal cell cancer (Adams and Kauffman, 2004). Preclinical studies have shown that Velcade also produced additive or synergistic antitumor activity in a mouse Lewis lung cancer model, when combined with paclitaxel or a few other cytotoxic agents (Teicher et al., 1999), but the underlying mechanism of the cooperative effect is not known.

A notorious oncogenic pathway activated by proteasome degradation is the NF- κ B pathway (Ravi and Bedi, 2004). Proteasome inhibitors can diminish the ubiquitin-mediated turnover of the NF- κ B inhibitor I κ B, thereby extinguishing NF- κ B survival signaling and other activities to promote tumor progression. NF- κ B is constitutively activated in many tumor types, and it can also be induced by chemotherapy, radiation, and other cellular stresses. Inhibition NF- κ B activation has been proposed as a mechanism of Velcade action (Adams, 2003a), but NF- κ B-independent activity of Velcade has also been reported (An et al., 2004). Our results identify an alternative pathway responsive to Velcade that may be both drug- and tumor genotype-specific. Velcade sensitized BIM wild-type but not BIM-deficient BMK cells expressing H-ras to paclitaxel-induced apoptosis both *in vitro* and *in vivo*, indicating that this paclitaxel/Velcade synergy is BIM-dependent. Thus, combination therapy with paclitaxel and proteasome inhibitors may be particularly useful in treating paclitaxel-resistant tumors with mutations activating MAPK signaling. Interestingly, combination therapy with Velcade and the paclitaxel-related drug docetaxel was not always effective against pancreatic cancer, which frequently harbors *Ras* mutations (Nawrocki et al., 2004), suggesting that the regulation of chemotherapeutic responses by BIM and H-ras may be influenced by yet other factors.

The armamentarium of potential anticancer drugs is expanding; however, single agents may not suffice to achieve complete remission. Velcade is currently being evaluated in combination with paclitaxel, docetaxel, and a few other chemotherapeutic drugs in Phase I and II clinical trials. As it is not likely feasible to clinically test every possible combination of agents, the predictive value of knowing what drugs to use to treat specific cancer genotypes based on their mechanism of interaction can maximize the potential for effective treatment. Ultimately, successful therapy may rely upon relating tumor genotype to its capacity to response to specific agents, singly and in combination.

Experimental procedures

Materials

The materials were obtained as follows: cycloheximide, cremophore, and D-mannitol (Sigma, St. Louis, MO); U0126, etoposide, thapsigargin, and epoxomicin (Calbiochem, La Jolla, CA); paclitaxel (Calbiochem and Bristol-Myers Squibb Co., Princeton, NJ); and Velcade (Millennium Pharmaceuticals, Inc., Cambridge, MA).

Generation of stable cell lines

The generation of E1A plus p53DD transformed BMK cell lines were described previously (Degenhardt et al., 2002b). The BMK cell lines were grown in Dulbecco's modified Eagle media (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 38.5°C. Stable BMK cells expressing human BCL-2, H-ras, RAF-CAAX, Myr-AKT, or the vector controls were derived by electroporation with pcDNA3.1-hBCL-2 (Nelson et al., 2004), pcDNA1.H-rasV12 (Lin et al., 1995), pcDNA3.RAF-CAAX (provided by Dr. Peter Sabbatini, UCSF, San Francisco, CA), pcDNA3.Myr-AKT (Plas et al., 2001), or vector (W2, pcDNA3.1; D3, pcDNA3.1zeo, Invitrogen, Carlsbad, CA), followed by selection (W2, geneticin; D3, zeocin). For RFP expression, cells were transfected with pDsRed-C1 (Clontech, Palo Alto, CA) and selected with geneticin, and the pool of transfected cells was used for subcutaneous injection in nude mice. Tumor formation in nude mice by subcutaneous injection was performed essentially as described previously (Degenhardt et al., 2002a). Briefly, 10⁷ cells were injected in each of 5 or more mice for each cell line (NCR nu/nu athymic nude mice, 5–6 weeks of age; Taconic, Germantown, NY), and tumor growth was monitored over time.

Western blotting

The following antibodies were used: rabbit anti-BIM (Axxora, LLC, San Diego, CA); rabbit anti-mouse BMF (Abcam, Cambridgeshire, UK); rabbit anti-PUMA (Nelson et al., 2004); goat anti-mouse BID and tBID (R&D Systems, Minneapolis, MN); rabbit anti-BAX, and anti-BAK (Upstate Biotechnology, Lake Placid, NY); mouse anti-p53 (Ab-1), mouse anti-E1A, mouse anti-pan-ras, mouse anti-actin, and anti- α -tubulin (Oncogene, Cambridge, MA); and rabbit anti-MAPK p42/p44 and phosphorylated MAPK p42/p44 (Cell Signaling Technology, Beverly, MA). Cell extracts were generated in Laemmli buffer, and Western blotting was performed as previously described (Nelson et al., 2004).

Phosphatase digestion of BIM

D3.zeo-2 and D3.Hras-1 cells were harvested in 2% CHAPS lysis buffer with protease inhibitors, as previously described (Cuconati et al., 2003). Whole cell lysates were incubated with lambda protein phosphatase (NEB, Beverly, MA) at 30°C for two hours and then analyzed by Western blotting.

Real-time PCR

RT-PCR was carried out as previously described (Cuconati et al., 2003). Gene-specific primer pairs and probe sequences for *bim*: forward (5'-TGC GCC CGG AGA TAC G-3'), reverse (5'-CCT CCT TGT GTA AGT TTC GTT GAA C-3'), and probe (5'-/56-FAM/CGA TCC GCC GCA GCT CCT GT/36-TAMTph/-3'). Primers and probe for mouse *gapdh* PCR were obtained from Applied Biosystems, which served as an internal control and were used to normalize for variances in input cDNA.

Supplemental data

Supplemental data for this article can be found at <http://www.cancercell.org/cgi/content/full/7/3/227/DC1/>.

Acknowledgments

We thank Thomasina Sharkey for assistance with preparation of the manuscript. This work was supported by the Howard Hughes Medical Institute and a National Institutes of Health grant (R37 CA53370) to E.W. We thank Drs. David R. Plas, Peter Sabbatini, Eric Rubin, and Vassiliki Karantza-Wadsworth for providing reagents, and Dr. Andreas Strasser and members of the White laboratory for helpful advice.

Received: November 2, 2004

Revised: January 19, 2005

Accepted: February 18, 2005

Published: March 14, 2005

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