Active N-Ras and B-Raf Inhibit Anoikis by Downregulating Bim Expression in Melanocytic Cells

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B-Raf and N-Ras proteins are often activated in melanoma, yet their roles in producing inherent survival signals are not fully understood. In this study, we investigated how *N-RAS*^{Q61K} and *B-RAF*^{V600E} contribute to melanoma's resistance to apoptosis induced by detachment from the extracellular matrix (anoikis). We found that expression of constitutively active *N-RAS*^{Q61K} and *B-RAF*^{V600E} downregulated the proapoptotic Bim protein in an immortalized melanocyte cell line. Bim is one of the main proapoptotic mediators of anoikis. Western blot analysis showed that detachment increased Bim expression in melanocytes, and Annexin V staining indicated that detachment induced cell death significantly in melanocytes. Blocking Bim expression by using RNAi vectors or by expressing *N-RAS*^{Q61K} significantly inhibited anoikis in melanocytes. In summary, this report indicates that *N-RAS*^{Q61K} and *B-RAF*^{V600E} contribute to melanoma's resistance to apoptosis in part by downregulating Bim expression, suggesting that Bim is a possible treatment target for overriding melanoma's inherent defenses against cell death.

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INTRODUCTION

Malignant melanoma is a devastating disease as it metastasizes early and is highly resistant to all conventional treatments, including chemo-, immuno-, or radiation therapy. Unfortunately, the treatment of this disease has not improved significantly over the past 30 years. Gaining a better understanding of the predominant gene alterations and their downstream signals that occur during melanoma's initiation and progression will help to develop effective molecular targeted strategies for combating the disease.

Activation of Ras or Ras effector pathways is associated with melanoma initiation and progression (see review (Chudnovsky *et al.*, 2005; Miller and Mihm, 2006; Gray-Schopfer *et al.*, 2007)). Two major Ras effector pathways are important for melanoma development and progression: the B-Raf/Mek/Erk and the PI3K/Akt pathways (Chudnovsky *et al.*, 2005; Miller and Mihm, 2006; Gray-Schopfer *et al.*, 2007).

High frequencies of activating mutations in Ras or members of its effector pathways suggest that these signaling cascades are important in melanoma tumorigenesis, progression, and development. The mutation frequency of the Ras family in human melanoma (mainly *N-RAS*) has been estimated at 10–20% (Chudnovsky *et al.*, 2005). Oncogenic Ras activates both the Raf and PI3K cascades.

In addition to Ras alterations, the discovery of activating mutations in the *B-RaF* gene in 80% of short-term melanoma cell cultures and 66% of uncultured melanomas was the first success of the Cancer Genome Project (Davies *et al.*, 2002). A great number of follow-up studies have further investigated the frequency of *B-RAF* mutations in melanocytic lesions, and the resulting estimates have varied from 30 to 70%. Taken together, the published data suggest that approximately 50% of melanomas carry the activating *B-RAF*^{V600E} mutation (see review (Chudnovsky *et al.*, 2005)).

In a review on apoptosis regulation and melanoma resistance to therapies, Soengas and Lowe (2003) indicated that during melanoma progression, malignant cells become ''bullet proof'' against a variety of chemotherapeutic drugs by exploiting their intrinsic apoptotic defenses and by reprogramming their proliferation and survival pathways (Soengas and Lowe, 2003).

Both activating *N-RAS* and *B-RAF* mutations have been demonstrated to be very important for melanoma survival as suppression of either of them by RNA interference induces apoptosis in human melanoma cells (Hingorani *et al.*, 2003; Eskandarpour *et al.*, 2005).

Decreased sensitivity to anoikis, or detachment-induced cell death, has been shown to be a characteristic of oncogenic progression in epithelial cells (Reginato *et al.*, 2005). Adhesion of cells to appropriate extracellular matrix is important for the survival of many normal cells and the tissue organizations of multicellular organisms, and anoikis is considered as the mechanism by which cells use extracellular

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Abbreviations: ANOVA, analysis of variance; HA, hemagglutinin; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; poly-HEMA, poly-(2-hydroxyethylmethacrylate; shControl, Control shRNA

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matrix-derived signals to maintain tissue integrity *in vivo* (see review (Gilmore, 2005)). The insensitivity of cells to anoikis has been suggested to be associated with epithelial to mesenchymal transition, transformation, and immortalization (see review (Gilmore, 2005)).

The Bcl-2 family of proteins is crucial in regulating apoptosis (see review (Thomadaki *et al.*, 2006)), and the proapoptotic BH3-only protein member, Bim, has been shown to be important in detachment-induced apoptosis (anoikis) in mammary epithelial cells, epidermal keratinocytes, and NIH3T3 cells (Reginato *et al.*, 2003; Marani *et al.*, 2004; Wang *et al.*, 2004; Collins *et al.*, 2005; Quadros *et al.*, 2006; Woods *et al.*, 2007).

Here, we investigated the role of Bim in regulating anoikis in human melanocytic cells, and how active N-Ras and B-Raf contribute to their resistance to anoikis by regulating Bim expression. We found that detachment significantly induced Bim-mediated apoptosis in immortalized human melanocytes. In addition, *N-RAS*^{Q61K} or *B-RAF*^{V600E} provides protection from anoikis for the cells by the activation of mitogen-activated protein kinase (MAPK) signaling and the subsequent repression of Bim protein expression.

RESULTS

Overexpressed HA-tagged *B-RAF*^{V600E}, *myr-AKT3*, and *N-RAS*^{Q61K} were functionally active in PIG1 melanocytes

To examine the functions of active B-Raf, Akt, and N-ras in melanoma development, we transiently transfected PIG1 cells, an immortalized human melanocyte cell line (Le Poole *et al.*, 1997), with vectors containing constitutively active hemagglutinin (HA)-tagged *B-RAF*^{V600E}, *myr-AKT3*, and *N-RAS*^{Q61K}. We have achieved 60–90% transfection efficiency routinely estimated by fluorescence-activated cell sorting staining with anti-HA-Alexa488 antibody (data not shown).

Further, western blotting confirmed strong expression of HA-tagged proteins in PIG1 cells (Figure 1a). As active B-Raf and N-Ras should upregulate phospho-p44/42 MAPK (p-p44/42 MAPK or p-Erk) expression, and active N-Ras and Akt3 should upregulate phospho-Akt expression, we performed western Blot analyses of p-p44/42 MAPK (Thr202/Tyr204) and phospho-Akt (Ser473) to determine whether the transfected proteins were functionally active in PIG1 cells. Figure 1a shows that indeed all transfected proteins were functionally active. For example, expression of *B-Raf*^{V600E} and *N-Ras*^{Q61K} but not *myr-AKT3* significantly upregulated p-p44/42 MAPK protein level compared to the control. In addition, expression of *N-Ras*^{Q61K} and *myr-AKT3* but not *B-Raf*^{V600E} drastically upregulated phospho-Akt protein levels compared to the control. The smaller molecular weight of HA-myr-Akt3 compared to wild-type Akt is due to the truncation of the active myr-Akt3 construct.

Overexpression of *B-RAF*^{V600E} and *N-RAS*^{Q61K} but not *myr-AKT3* downregulated Bim expression in PIG1 melanocytes grown in monolayer culture

We used western blotting to examine whether overexpression of *B-Raf*^{V600E}, *myr-AKT3*, and *N-Ras*^{Q61K} regulate Bcl-2 family members in PIG1 cells grown in monolayer culture. We found that overexpression of *B-Raf*^{V600E} or *N-Ras*^{Q61K} but not *myr-AKT3* downregulated Bim protein expression significantly (Figure 1b) compared to other Bcl-2 family members (Figure S1). Figure 1b also shows that the Bim-EL protein band in *B-Raf*^{V600E} or *N-Ras*^{Q61K} transfectants migrated slightly slower, indicating that they are phosphorylated forms.

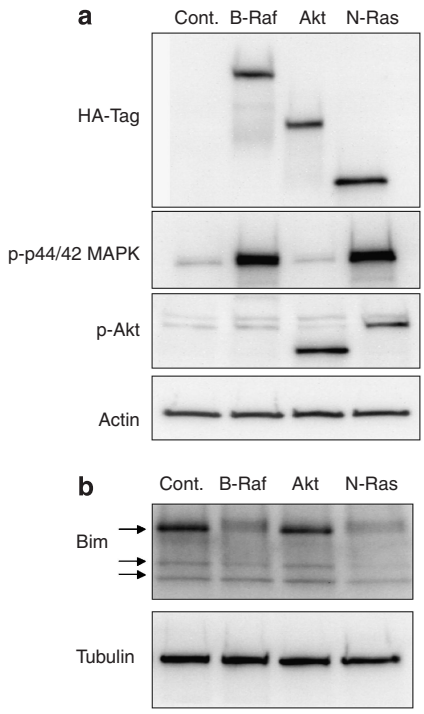

Figure 1. Transfection of PIG1 melanocytes with active BRaf and NRas decreased Bim protein expression in monolayer culture. PIG1 cells were transiently transfected with expression vectors pMEV-2HA(a) (Control), pMEV-2HA-BRAF-V600E (Braf), pMEV-2HA-AKT3-Myr (Akt), or pMEV-2HA-NRAS-Q61K (NRas), and grown in monolayer culture for 24 hours before being subjected to indicated analysis. (**a**) Western Blot analysis for the activation of downstream signaling. (**b**) Western Blot analysis of Bim expression in transfected PIG1 cells grown in monolayer. Three putative isoforms of Bim are indicated by arrows, with Bim-EL at top, Bim-L in the middle, and Bim-S at bottom.

Detachment-induced Bim expression and apoptosis in PIG1 melanocytes, and knocking down Bim expression protected PIG1 cells partially from anoikis

Bim has been shown to be important in detachment-induced apoptosis (anoikis) in mammary epithelial cells, epidermal keratinocytes, and NIH3T3 cells (Reginato *et al.*, 2003; Marani *et al.*, 2004; Wang *et al.*, 2004; Collins *et al.*, 2005; Quadros *et al.*, 2006; Woods *et al.*, 2007). Growing cells on poly-(2-hydroxyethylmethacrylate) (poly-HEMA)-coated dishes prevent attachment and have been shown to induce anoikis (Reginato *et al.*, 2003; Marani *et al.*, 2004; Wang *et al.*, 2004; Collins *et al.*, 2005; Quadros *et al.*, 2006; Woods *et al.*, 2007).

To assess whether Bim expression is involved in anoikis in human melanocytes, we used shRNA retroviral vectors to knock down Bim expression specifically in PIG1 cells. We transduced PIG1 melanocytes with retroviral vectors encoding Bim shRNA or Control shRNA (shControl), and then cultured the stable pools of cells in growth factor-free medium on normal or poly-HEMA-coated dishes to induce

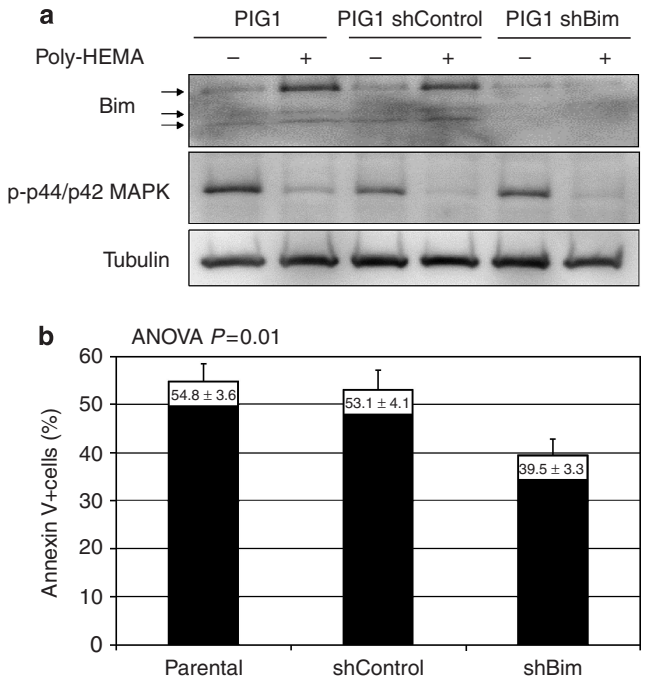

Figure 2. Detachment induced Bim expression and apoptosis and knocking-down Bim expression provided survival signals against anoikis. PIG1 cells were transduced with retroviral vectors encoding Bim shRNA (shBim) or Control shRNA (shControl), and stable pools were then grown in normal or poly-HEMA-coated dishes in medium without growth factors for indicated times. (**a**) Protein expression in PIG1 parental and transduced cell lines grown for 24 hours in monolayer (without poly-HEMA) or detachment (with poly-HEMA) culture conditions. (**b**) Annexin V apoptosis assays for PIG1 parental and transduced cell lines grown for 48 hours in poly-HEMA culture conditions. Averages represent seven independent experiments ($P=0.01$ by ANOVA).

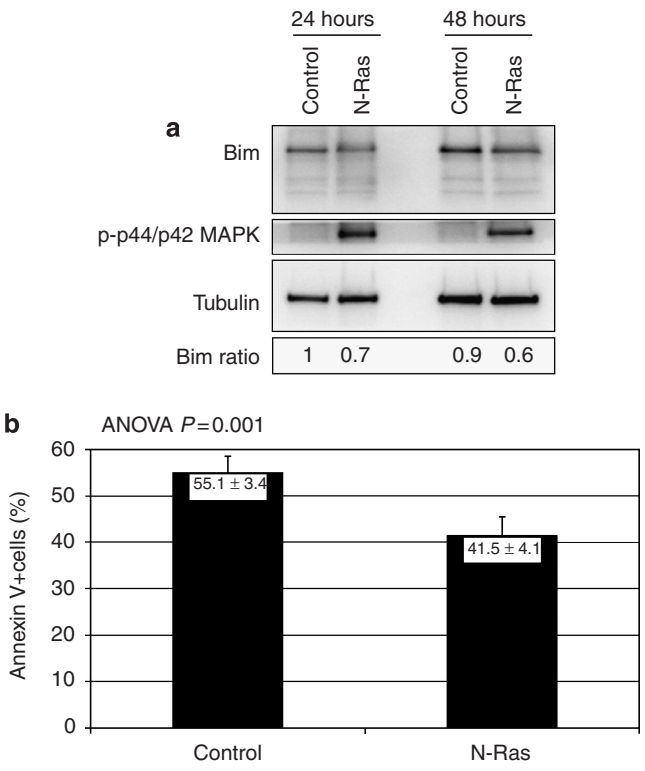

Figure 3. Transfection of PIG1 melanocytes with active NRas decreased Bim expression and apoptosis induced by detachment. PIG1 melanocytes were transiently transfected with pMEV-2HA-NRAS-Q61K (NRas) or pMEV-2HA(a) (Control) vectors, and grown in poly-HEMA-coated dishes using medium without growth factors for indicated times. (**a**) Immunoblots. The Bim ratio refers to Bim protein expression levels as normalized to Tubulin levels. (**b**) Annexin V apoptosis assay for transfected PIG1 melanocytes grown for 48 hours in poly-HEMA-coated dishes. Averages represent five independent experiments ($P=0.001$ by ANOVA).

anoikis. Figure 2a shows that detachment (cultured in poly-HEMA-coated dishes) decreased MAPK signaling activity in all PIG1 cells, as indicated by reduced expression of the phospho-p44/p42 MAPK protein. In addition, detachment induced Bim protein expression in both PIG1 parental and PIG1 shControl cells. However, expression of Bim shRNA in PIG1 cells dramatically reduced Bim protein levels in both monolayer and detachment conditions compared to both parental and shControl PIG1 cells.

We then performed Annexin V apoptosis assays to quantify total apoptotic cells induced by detachment in these cell lines (Figure 2b). Both parental (54.8% ± 3.6) and shControl (53.1% ± 4.1) PIG1 cells exhibited significantly higher cell death, compared to Bim shRNA (39.5% ± 3.3) PIG1 cells ($n=7$; analysis of variance (ANOVA); $P=0.01$). This decrease of 13.6% in the death rate constitutes 26% of relative cell death when the death rate in shControl cells is set as 100%. These results suggest that anoikis in human melanocytes cells is in part mediated by Bim upregulation.

Overexpressing HA-tagged *N-Ras*^{Q61K} in PIG1 melanocytes inhibited detachment-induced Bim protein expression and apoptosis

We transfected PIG1 melanocytes with expression vectors encoding HA-tagged *N-Ras*^{Q61K} (NRas) or the empty vector control (Control) and cultured them in growth factor-free medium on poly-HEMA-coated dishes for indicated times. Figure 3a illustrates that PIG1 cells transfected with *N-Ras*^{Q61K} exhibited significantly higher MAPK signaling activity, as shown by increased expression of the phospho-p44/p42 MAPK protein. Further, Bim protein expression was reduced in active N-Ras transfectants compared to vector control transfectants, and the Bim-EL protein band migrated slightly slower in those samples. We note that the degree of Bim downregulation by active N-Ras was less in poly-HEMA culture than in monolayer culture.

We then performed Annexin V apoptosis assays to quantify apoptotic cell death in transfected PIG1 cells grown for 48 hours in poly-HEMA-coated dishes (Figure 3b). HA-N-Ras^{Q61K}-transfected PIG1 cells (NRas; 41.5% ± 4.1) showed significantly less apoptosis compared to empty vector-transfected PIG1 cells (Control; 55.1% ± 3.4) ($n=5$; ANOVA; $P=0.001$). This is a 25% reduction of relative cell death.

DISCUSSION

Dysregulation of apoptosis is a hallmark of cancer development and the Bcl-2 family proteins are major regulators of apoptosis. Bim is a BH3-only proapoptotic member of the

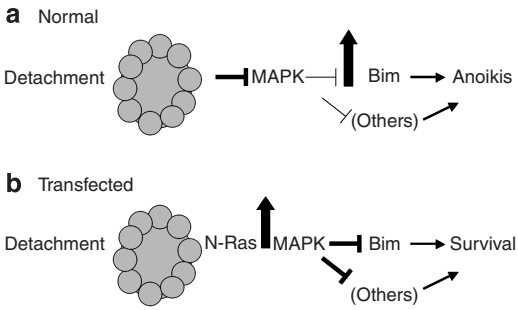

Figure 4. Constitutively active MAPK signaling protects melanocytic cells from Bim-mediated anoikis. (**a**) In melanocytes, detachment reduces MAPK signaling, which allows Bim expression levels to increase that leads to anoikis. (**b**) Expression of *N-RAS*^{Q61K} activates MAPK signaling, which suppresses Bim induction and results in cell survival.

Bcl-2 family, and it can antagonize all the pro-survival Bcl-2 proteins, leading to apoptosis. Bim is one of the main proapoptotic mediators of anoikis in normal cells (Reginato *et al.*, 2003; Marani *et al.*, 2004; Wang *et al.*, 2004; Collins *et al.*, 2005; Quadros *et al.*, 2006; Woods *et al.*, 2007).

In this study, we examined whether *N-RAS*^{Q61K} and *B-RAF*^{V600E} regulate expression levels of Bcl-2 family members and how this might be involved in melanoma's resistance to apoptosis, specifically anoikis. Transient transfection experiments with a human melanocyte cell line PIG1 indicated that *B-RAF*^{V600E} and *N-RAS*^{Q61K} downregulated the proapoptotic Bim protein (Figure 1), and conversely MEK inhibition in melanoma cells upregulated Bim protein expression (data not shown). Our results suggest that constitutively activated MAPK signaling in melanoma cells suppresses expression of the proapoptotic Bim protein.

Figure 2 shows that detachment-induced anoikis in the human melanocyte cell line, PIG1, which was at least partially Bim-mediated. In addition, expression of *N-RAS*^{Q61K} in these cells protected them from this anoikis significantly (Figure 2). Figure 4 illustrates our hypothesis: detachment significantly induced Bim-mediated apoptosis in human melanocytes. In addition, *N-RAS*^{Q61K} or *B-RAF*^{V600E} provides protection from anoikis by the activation of MAPK signaling and the subsequent repression of Bim protein expression.

Normal melanocytes are kept in the correct tissue compartment by proper cell–extracellular matrix interactions for survival. When melanoma progresses to more advanced stages, cells invade into the dermis that does not provide the normal cell–extracellular matrix survival signals required for melanocyte survival. Our results suggest that *N-RAS*^{Q61K} or *B-RAF*^{V600E} in melanoma might be one of the factors that provide these protections by downregulation of the proapoptotic protein Bim when cells invade into the dermis.

Although knocking down Bim in PIG1 cells protected cells from anoikis, it was not sufficient to rescue the cells completely (Figure 2). Our data suggest that Bim is not the only mediator of anoikis in melanocytes. It has been recently shown that Bad is also involved in anoikis in melanoma cells (Boisvert-Adamo and Aplin, 2008). In addition, Bmf upregulation and Mcl-1 degradation have also been implicated in anoikis in certain cells (Puthalakath *et al.*, 2001; Woods *et al.*, 2007). The roles of these proteins will be investigated in future studies.

Figure 1b also shows that the Bim-EL protein band in *N-RAS*^{Q61K} or *B-RAF*^{V600E} transfectants migrated slightly slower, resembling to the phosphorylated form of Bim-EL seen by others (Harada *et al.*, 2004; Tan *et al.*, 2005). Harada *et al.* (2004) have demonstrated that phosphorylation of Bim by MAPK (Erk) can inhibit Bim's proapoptotic activity. It is conceivable that *B-RAF*^{600E} and *N-RAS*^{Q61K} initiate this stream of events by activating Erk, and inducing downstream phosphorylation events leading to inactivation of Bim.

In other cell types, including human mammary epithelial cells and Chinese hamster lung fibroblast cells, it has been reported that activation of MAPK (Erk) signaling pathways promotes phosphorylation and proteasome-dependent degradation of Bim (Ley *et al.*, 2003; Reginato *et al.*, 2003). Oncogenes such as *ERbB2*, *v-Src*, and *H-Ras*, suppress expression of Bim by an MAPK-dependent pathway, contributing to cell transformation and resistance to chemotherapy (Reginato *et al.*, 2005; Tan *et al.*, 2005). However, our report describes the link between Bim and the Ras/B-Raf/Erk signaling pathway in human melanocytic cells.

These results are consistent with a very recent study (Boisvert-Adamo and Aplin, 2008), in which the authors used melanoma cell lines to show that Bim and Bad are major B-RAF responsive proteins that regulate resistance to anoikis. Our results complement theirs, and they indicate that Bim is one of the Ras/B-Raf/Erk responsive proteins that regulates apoptosis in melanocytic cells.

High frequencies of activating mutations in *B-RAF* or *N-RAS* are found in melanoma that probably contributes to constitutive MAPK (Erk) signaling. Our data suggest that low expression of Bim might be quite common in melanomas. Indeed, Dai *et al.*(2008) reported that Bim expression is reduced as melanoma progresses, and decreased Bim expression is also significantly correlated with poor 5-year survival in melanoma patients. Results here and by others suggest that Bim is a possible treatment target for overriding cancer's inherent defenses, including melanomas.

Several studies support the concept of Bim as a treatment target in cancers, including melanoma (Tan *et al.*, 2005; Deng *et al.*, 2007; Kashiwagi *et al.*, 2007). For example, Kashiwagi *et al.* (2007) utilized a synthetic peptide called TAT-Bim (containing the BH-3 domain from Bim) to treat various cancer cells and found that it induced apoptosis in a dose-dependent fashion in many cancer cell lines *in vitro*, and it significantly slowed tumor growth in murine models of pancreatic cancer and melanoma. These studies indicate that reversing the low levels of Bim expression in cancer cells can be exploited to develop new treatments.

In summary, our study indicates that *N-RAS*^{Q61K} and *B-RAF*^{V600E} contribute to melanoma's resistance to apoptosis, such as anoikis, in part by downregulating Bim expression. Because of the high frequency of *B-RAF*^{V600E} and *N-RAS*^{Q61K} mutations in melanomas, more studies are warranted for developing rational therapies that address low expression levels of the proapoptotic protein Bim in these cells.

MATERIALS AND METHODS

Cell lines and culture conditions

An immmortalized human melanocyte cell line, PIG1, was kindly provided by Dr Le Poole (Le Poole *et al.*, 1997). Cells were maintained in Medium 254 with Human Melanocyte Growth Supplement-2 (Cascade Biologics, Portland, OR) and antibiotics, in a 5% CO_2 incubator at 37 °C. The medical ethical committee of University of Colorado Denver approved all the described studies.

Constructs

The following expression plasmids employing the human cytomegalovirus immediate-early promoter were obtained from Biomyx Technology (San Diego, CA): the empty vector (pMEV-2HA, reading frame A), and a vector encoding activated human B-RAF (pMEV-2HA-BRAF-V600E). To facilitate detection of the protein of interest, these vectors use two 9-amino-acid peptides (HA) derived from the hemagglutinin influenza virus as an N-terminal affinity tag. The myristoylated human Akt3 expression vector (pMEV-2HA-AKT3-Myr) was made by PCR subcloning the myristoylated human *Akt3* gene sequence from the vector pcDNA3.1/myc-his-hAKT3myr (kindly provided by Kohsuke Kanekura, KEIO University School of Medicine, Tokyo, Japan) (Kanekura *et al.*, 2005) into pMEV-2HA (reading frame A) at the *Nhe*I and *Xma*I restriction sites. The standard PCR protocol was used for PCR amplifying the myristoylated human *Akt3* gene with the following primers: AKT3myr *Nhe*I, forward: 5′-GCGCTAGCATGGGGAGTAGCAAGAGCAAGC and AKT3myr *Xma*I, backward: 5′-GCCCCGGGTTATTCTCGTCCACTTGCAGAG. The activated human N-RAS expression vector (pMEV-2HA-NRAS-Q61K) was made by PCR subcloning the activated human *N-RAS* gene sequence from the vector pZIPneoSV(x)1-NRas-61K (kindly provided by Channing Der, University of North Carolina at Chapel Hill, Chapel Hill, NC) (Plattner *et al.*, 1996) into pMEV-2HA (reading frame A) at the *Bam*HI and *Eco*RI restriction sites. The standard PCR protocol was used for PCR amplifying the activated *N-RAS* gene using the following primers: NRas *Bam*HI, forward: 5′-CGGGATC CATGACTGAGTACAAACTGGTG and NRas *Eco*RI, backward: 5′-GCGAATTCTTACATCACCACACATGGCAA. All resulting clones were verified by sequencing at UCDHSC cancer center sequencing core. The pMAX-GFP vector was obtained from Amaxa Inc. (Gaithersburg, MD) for optimizing nucleofection conditions.

The pSUPER.Retro.Puro vector containing the human H1 RNA promoter for expressing shRNA was purchased from Oligoengine (Seattle, WA). Validated mRNA target sequences were selected to generate DdRNAi oligonucleotide inserts for all isoforms of human BIM (GenBank AF032457, nucleotides 37–56; GACCGAGAAGG TAGACAATT; (Lu *et al.*, 2006)), and a nontargeting control sequence (AATTCTCCGAACGTGTCACGT; (Lu *et al.*, 2006)) using Oligoengine's software. The annealed inserts were cloned into the *Bgl*II/*Hin*dIII sites of pSUPER.Retro.Puro according to Oligoengine's protocol, and all vectors were confirmed by sequencing in the University of Colorado Cancer Center DNA Sequencing and Analysis Core.

Reagents

poly-HEMA was purchased from Sigma Chemical (St Louis, MO). Poly-HEMA was suspended in 95% ethanol at a final concentration of 12 mg ml^{-1}, and dissolved overnight at 65 °C. The MEK inhibitor U0126 was purchased from Promega (Madison, WI) and was dissolved in dimethyl sulfoxide before use. A final concentration of 10 μM U0126 was used to treat the cells.

Anoikis growth

Culture dishes were coated with 1.5 ml (6-cm dish) and 5 ml (10-cm dish) poly-HEMA (12 mg ml^{-1}) that had been previously dissolved in ethanol at 65 °C. Dishes were allowed to dry at least overnight in an empty 37 °C incubator, to ensure all ethanol had evaporated. PIG1 cells were plated at densities of 6×10^5 cells per 6-cm dish, whereas nucleofected PIG1 cells were plated at densities of one reaction per 6-cm dish and two reactions per 10-cm dish.

Transient transfection

Plasmids were introduced into cells by nucleofection, using an Amaxa Nucleofector II device (Amaxa Inc.). For PIG1, we used two million cells and 20 μg of DNA per reaction, using Amaxa's Reagent V and program T-020. PIG1 cells were cultured in 6-cm dishes after nucleofection.

Generation of stable cell lines with retroviral transduction

Phoenix amphitropic packaging cells were transiently transfected with pSUPER vectors using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol, and allowed to produce virus for 48 hours. Viral supernatants were filtered using 0.45-μM polyethersulfone filters and added to target cells for 24 hours in the presence of 5 μg ml^{-1} Polybrene (hexadimethrine bromide; Sigma Chemical). Target cells were allowed to recover in normal growth medium for 24 hours, and infection was repeated two more times for a total of three rounds. At 24 hours after the third infection, puromycin (Sigma Chemical) at 0.5 μg ml^{-1} was added for at least 5 days to select stably transduced pools of cells. For all experiments, the stable pool of nontargeting control cells was used.

Immunoblot

At 24 hours after nucleofection, adherent cells were washed once with phosphate-buffered saline (PBS) and lyzed directly in the dish with 0.15 ml of 1 × Laemmli Sample Buffer (Bio-Rad, Hercules, CA). Cells from poly-HEMA-coated plates were centrifuged at 1,000 *g*. for 5 minutes, rinsed with PBS, centrifuged again, and lyzed directly in centrifuge tubes using 0.10 ml of 1 × Laemmli Sample Buffer. Lysates were heated at 95 °C for 5–10 minutes and then clarified by centrifugation at 13,200 *g* for 5 minutes. Samples were electrophoresed on SDS–PAGE 4–20% acrylamide gradient gels (Bio-Rad), and transferred to p olyvinylidene difluoride membranes using a Transblot SD Semi-Dry Transfer Cell apparatus (Bio-Rad) at 15 V for 30 minutes. Membranes were probed with primary antibodies in PBS with 5% nonfat milk, washed with PBS for 5 minutes, Tris-buffered saline/Tween for 2 × 10 minutes, then PBS for 5 minutes, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody in PBS with 5% nonfat milk. Blots were developed with HRP substrate (West Pico or Femto developing solutions; Pierce, Rockford, IL) for 5 minutes at room temperature, and analyzed using a Chemi-doc chemiluminescence detector (Bio-Rad). The following antibodies were used at the suggested dilutions from each manufacturer: anti-Bim rabbit polyclonal antibody was from Chemicon/Millipore (Billerica, MA); anti-phospho-Akt (Ser473), anti-HA-Tag, and anti-phospho-p44/42 MAPK (Thr202/Tyr204) mouse monoclonal antibodies were from Cell Signaling Technology Inc. (Beverley, MA); anti-Actin and anti-Noxa mouse monoclonal

antibodies were from EMD Biosciences Inc. (San Diego, CA); HRP-conjugated goat anti-rabbit IgG was from Transduction Laboratories (Lexington, KY); HRP-conjugated goat anti-mouse IgM was from EMD Biosciences Inc.; HRP-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA).

Annexin V apoptosis assay

The Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (San Jose, CA) and cells were stained at indicated times according to the manufacturer's protocol. Cells were analyzed by flow cytometry using a Beckman Coulter FC500 with CXP software (Beckman Coulter, Hialeah, FL) in the University of Colorado Cancer Center Flow Cytometry Core. ANOVAs were performed to compare group means.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Transfection of PIG1 melanocytes with active BRaf, Akt3, and NRas-modulated expression levels of Bcl-2 family members.

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