

# p53-independent NOXA induction overcomes apoptotic resistance of malignant melanomas

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## Abstract

Once melanoma metastasizes, no effective treatment modalities prolong survival in most patients. This notorious refractoriness to therapy challenges investigators to identify agents that overcome melanoma resistance to apoptosis. Whereas many survival pathways contribute to the death-defying phenotype in melanoma, a defect in apoptotic machinery previously highlighted inactivation of Apaf-1, an apoptosome component engaged after mitochondrial damage. During studies involving Notch signaling in melanoma, we observed a  $\gamma$ -secretase tripeptide inhibitor (GSI; z-Leu-Leu-Nle-CHO), selected from a group of compounds originally used in Alzheimer's disease, induced apoptosis in nine of nine melanoma lines. GSI only induced G<sub>2</sub>-M growth arrest (but not killing) in five of five normal melanocyte cultures tested. Effective killing of melanoma cells by GSI involved new protein synthesis and a mitochondrial-based pathway mediated by up-regulation of BH3-only members (Bim and NOXA). p53 activation was not necessary for up-regulation of NOXA in melanoma cells. Blocking GSI-induced NOXA using an antisense (but not control) oligonucleotide significantly reduced the apoptotic response. GSI also killed melanoma cell lines with low Apaf-1 levels. We conclude that GSI is highly effective in killing melanoma cells while sparing normal melanocytes. Direct enhancement of BH3-only

proteins executes an apoptotic program overcoming resistance of this lethal tumor. Identification of a p53-independent apoptotic pathway in melanoma cells, including cells with low Apaf-1, bypasses an impediment to current cytotoxic therapy and provides new targets for future therapeutic trials involving chemoresistant tumors. [Mol Cancer Ther 2004;3(8):895–902]

## Introduction

Treatment failure in metastatic melanoma (average survival period is only 6 to 10 months) reflects not only an impressive array of tumor cell survival pathways but also a resistance to apoptotic stimuli including radiotherapy and chemotherapy (1, 2). Because most genotoxic agents act primarily via p53 to induce apoptosis, it is somewhat surprising the p53 gene is not frequently mutated in melanoma (3–5). However, this lack of p53 mutations may be explained in part by CDKN2A aberrations affecting p14<sup>ARF</sup> and also inactivation of other downstream apoptotic effectors (3). An approach to developing new melanoma treatments is to identify compounds that can "switch on" the apoptotic machinery in resistant cells. Ideally, these compounds could induce apoptosis in a p53-independent manner in melanoma cells but not in normal melanocytes.

To identify a pathway that could effectively kill melanoma cells but not normal melanocytes, we established five different cultures of human melanocytes from normal skin and used nine different melanoma cell lines obtained from metastatic melanomas. Eight melanoma cell lines had been maintained in culture for prolonged periods (>100 passages), whereas one melanoma cell line (RJ002L) was examined relatively early after removal from the patient (<20 passages). Preliminary analysis comparing normal melanocytes with melanoma cells revealed that all melanoma cells had greater levels of activated Notch receptors (Notch-1 and Notch-4; refs. 6, 7). Notch signaling mediates cell fate decisions in mammalian cells during embryogenesis and has become a therapeutic target for investigators interested in treating cancer and Alzheimer's disease (6–10). Notch receptors are activated by intramembrane proteolysis catalyzed by  $\gamma$ -secretase (6). Because all four Notch receptors use  $\gamma$ -secretase for their activation, by targeting this enzyme, it is possible to simultaneously interfere with all Notch receptor-mediated signaling events.

To further investigate a potential survival role for Notch signaling in melanoma, we selected a synthetic tripeptide aldehyde containing  $\gamma$ -secretase tripeptide inhibitor (GSI; ref. 8) because of its ability to block Notch receptor processing and activation (9). This identical peptide aldehyde compound was used previously to reduce intracellular production of amyloid peptides in neurons (10). In this

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report, we show that GSI induces apoptosis in malignant melanoma cells in a NOXA-dependent fashion. Furthermore, GSI only induces a G<sub>2</sub>-M growth arrest but not apoptosis in melanocytes.

## Materials and Methods

### Cells

Cutaneous (C8161, SK-Mel-28, SK-Mel-100, SK-Mel-5, and C81-61), uveal (MUM2B, MUM2C, and OCM-1A), and pulmonary metastases (RJ002L) melanoma cell lines were maintained in RPMI supplemented with 10% fetal bovine serum. Normal human melanocytes were isolated from neonatal foreskins and cultured with medium 154 containing growth supplements (Cascade Biologics, Portland, OR). A malignant prostate cell line (PC-3) and an osteogenic cell line (SAOS-2) were obtained from American Type Culture Collection (Rockville, MD) and grown using DMEM plus 10% FCS. Assessment of tumor formation in nude mice was done as described (11). Briefly, aggressive melanoma cells (C8161) were injected ( $1 \times 10^6$ ) s.c. in nude mice on day 0, and tumors were allowed to grow for 1 week. Subsequently, the control group received intraleisional injections (100  $\mu$ L) of carrier only (DMSO) versus experimental group that received GSI (1 mmol/L in DMSO) every other day for 1 week (total amount of GSI administered was 150  $\mu$ g). Average tumor size (mm<sup>2</sup>) was measured at weeks 1 and 2. Average tumor size for both groups was normalized, and size of tumors at 2 weeks was averaged. Each exon of p53 was amplified and PCR reactions were purified after thermocycling using solid-phase reversible immobilization-based technology (AMPure, Agencourt Biosciences Corp., Beverly, MA). Sequencing reactions were done using BigDye Terminator v3.1 premix on GeneAmp 9700 PCR machines (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using CleanSEQ (Agencourt Biosciences) and analyzed on 3730xl DNA analyzers (Applied Biosystems). We assessed sensitivity of melanoma cells to TRAIL by using trimeric recombinant human Apo2L/TRAIL (Genentech, Inc., San Francisco, CA) with or without pretreatment using cycloheximide (Sigma Chemical Co., St. Louis, MO).

### Cell Viability Assays

Apo Target Annexin V-FITC staining kits (Biosource, Camarillo, CA) were used to measure apoptosis according to the manufacturer's instructions followed by flow cytometric analysis using FACSCalibur (Becton Dickinson, Palo Alto, CA; ref. 12). In some cases, cell cycle and apoptosis analysis was measured using propidium iodide staining and flow cytometry as described. Cells with DNA content less than the G<sub>0</sub> amount of untreated cells were considered apoptotic. For cell analysis, DNA histograms were analyzed using MultiCycle for Windows (Phoenix Flow Systems, San Diego, CA) as described (13). Detection of apoptosis in tissue sections was done using terminal deoxynucleotidyl transferase-mediated nick end labeling staining. In some experiments, cells were exposed to UV

light (30 mJ/cm<sup>2</sup>) using a Panelite Unit (Ultralite Enterprise, Inc., Lawrenceville, GA) as described previously (13). The output wavelengths of the bulbs are 65% UVB, 34% UVA, and 1% UVC. The UV dose was monitored with a radiometer (International Light, Inc., Newburyport, MA) fitted with a UVB detector. Mycoplasma detection using representative early passage (RJ002L) and late passage (C8161 and MUM2B) melanoma cells was negative (MycoAlert, Cambrex BioScience, Inc., Rockland, ME).

### Immunoblot Analysis and Subcellular Fractionation

Whole cell extracts were prepared and analyzed as described (13). The enriched mitochondria pellet and mitochondria-free cytosol of melanoma cells were prepared with the Apo Alert cell fraction kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions.

### Antisense Treatment

NOXA antisense oligonucleotide (ASO; ISIS156882: TCAGTCTACTGATTACTGG) and control oligonucleotide (CO: ISIS141923: CCTTCCCTGAAGGTTCTCC) were provided by ISIS Pharmaceuticals, Inc. (Carlsbad, CA). Cells ( $20 \times 10^4$ ) were seeded in six-well plates 1 day before transfection. Opti-MEM was preincubated for 30 minutes at room temperature using a ratio of 3  $\mu$ L/mL Lipofectin per 100 nmol/L to produce a final oligonucleotide concentration of 50 nmol/L. Cells were washed with PBS, and transfection mix (1 mL) was added. After 4 hours of incubation, RPMI 1640 (1 mL) containing 20% fetal bovine serum and GSI was added.

### Chemical Reagents and Antibodies

GSI was obtained from Calbiochem (La Jolla, CA) and dissolved in DMSO. Cycloheximide was purchased from Sigma Chemical. Genotoxic agents included Adriamycin and etoposide (both from Bedford Labs, Bedford, OH). Antibodies used were obtained as follows: Bcl-2 (SC-7382), Bcl-x<sub>L</sub> (SC-634), Mcl-1 (SC-819), survivin (SC-10811), Bax (SC-493), Bak (SC-832), Bim (SC-11425), PUMA (SC-19187), p53 (SC-126), and GADD45 (SC-796) from Santa Cruz Biotechnology (Santa Cruz, CA); Bid (2002) and cleaved caspase-9 (9501) from Cell Signaling (Beverly, MA); NOXA (OP180) from Oncogene Research Products (La Jolla, CA); Apaf-1 (559683) from BD Biosciences (San Diego, CA); SMAC/DIABLO (IMG-248) from Imgenex (San Diego, CA); and cytochrome *c* (Apo Alert cell fraction kit).

### Small Interference RNA Synthesis and Transfection

Smart pools of p53 small interference RNA duplexes were purchased from Upstate Biotechnology (Charlottesville, VA). Scramble control duplex was obtained from Dharmacon (Lafayette, CO). Melanoma cells were plated in six-well plates at a density of  $1.5 \times 10^5$  cells per well. Small interference RNA duplexes were transfected with Oligofectamine in Opti-MEM medium using the manufacturer's protocol. After 48 hours, transfected cells were treated with GSI for another 24 hours.

### Statistical Analysis

The mean and SD were derived from at least three independent experiments. Results were considered significant when  $P < 0.05$ .

## Results

Addition of GSI (1–10  $\mu\text{mol/L}$ ) to all cultures of proliferating normal human melanocytes did not significantly induce apoptosis (Fig. 1A), but GSI (10  $\mu\text{mol/L}$ ) did induce a G<sub>2</sub>-M growth arrest (Fig. 1C). In contrast, GSI triggered a dose-dependent increase in apoptosis of all nine different melanoma cell lines tested, which was preceded by a G<sub>2</sub>-M growth arrest (Fig. 1A and C). Representative apoptotic results are portrayed for two different melanocyte cultures (MC004 and MC007) and five different melanoma cell lines (RJ002L and SK-Mel-100 carrying wild-type p53; C8161, MUM2B, and SK-Mel-28 carrying mutated p53) before and 24 hours after increasing concentrations of GSI (Fig. 1A). Epigenetic inactivation of Apaf-1 was reported previously to occur in ~50% of melanoma cell lines (4). Therefore, we investigated Apaf-1 levels in our panel of melanoma cell lines (Fig. 1B). Higher Apaf-1 levels were observed in most melanoma lines compared with melanocytes, with only two melanoma lines (C8161 and SK-Mel-100) having low Apaf-1 levels. Importantly, GSI effectively killed melanoma cells but not melanocytes regardless of Apaf-1 expression levels.

To evaluate the relative effectiveness of killing melanoma cells, an initial dosing study was done comparing GSI against three different chemotherapeutic agents (i.e., Adriamycin, etoposide, and cisplatin). In agreement with a previous report (4), SK-Mel-28 cells, carrying a mutated p53, were relatively resistant to killing by Adriamycin as well as other chemotherapeutic agents. The maximal apoptotic response for Adriamycin, etoposide, cisplatin, and GSI were 12%, 2.6%, 4.0%, and 29.4%, respectively, for SK-Mel-28 melanoma cells and 23.9%, 12.7%, 3.4%, and 45.7%, respectively, for RJ002L melanoma cells, indicating that GSI was superior to these chemotherapeutic agents.

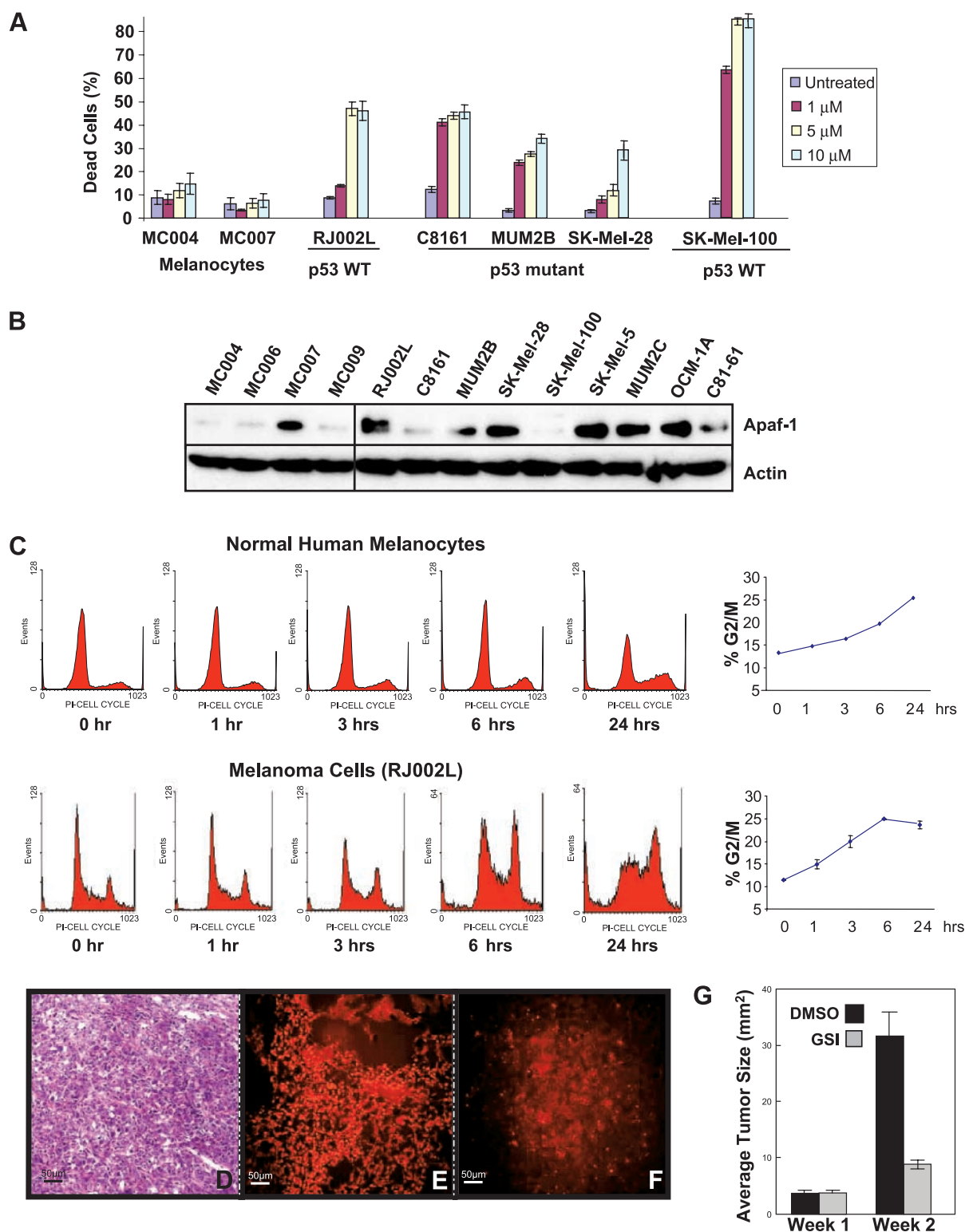
To confirm and extend these *in vitro* findings, the effects of GSI were investigated by injecting the cutaneous melanoma cell line C8161 s.c. into nude mice. Figure 1D to G reveals light microscopic appearance and terminal deoxynucleotidyl transferase-mediated nick end labeling staining, demonstrating induction of apoptosis of these melanoma cells by GSI using this orthotopic *in vivo* model. Reduction in average tumor size after 2 weeks following GSI administration was significant ( $P < 0.05$ ).

Kinetic analysis using GSI (10  $\mu\text{mol/L}$ ) revealed induction of apoptosis beginning during the initial 6 to 8 hours of exposure, but the most striking killing of all melanoma cell lines was apparent at the 18- and 24-hour time points (Fig. 2A). This delay in induction of apoptosis suggested a requirement for new protein synthesis. To dissect the mechanism of apoptosis induction by GSI, a series of biochemical studies was done. RJ002L melanoma cells were pretreated with or without cycloheximide (1  $\mu\text{g/mL}$ ; 1 hour; Fig. 2B) followed by exposure to either GSI or TRAIL. Whereas cycloheximide reduced the GSI-induced apoptotic response of melanoma cells in a concentration-dependent fashion (0.1, 0.5, and 1.0  $\mu\text{g/mL}$ ), it enhanced the number of melanoma cells undergoing apoptosis induced by TRAIL (Fig. 2C; ref. 14). These results suggested

a requirement for GSI-mediated induction of protein(s) that triggered apoptosis in melanoma cell lines. Thus, a search began to define relevant apoptotic mediators following GSI treatment. Because forced overexpression of a FADD dominant-negative did not reduce the susceptibility to GSI in any of the melanoma cell lines (data not shown), we turned our attention away from death receptor pathways and focused on the mitochondrial-based pathways.

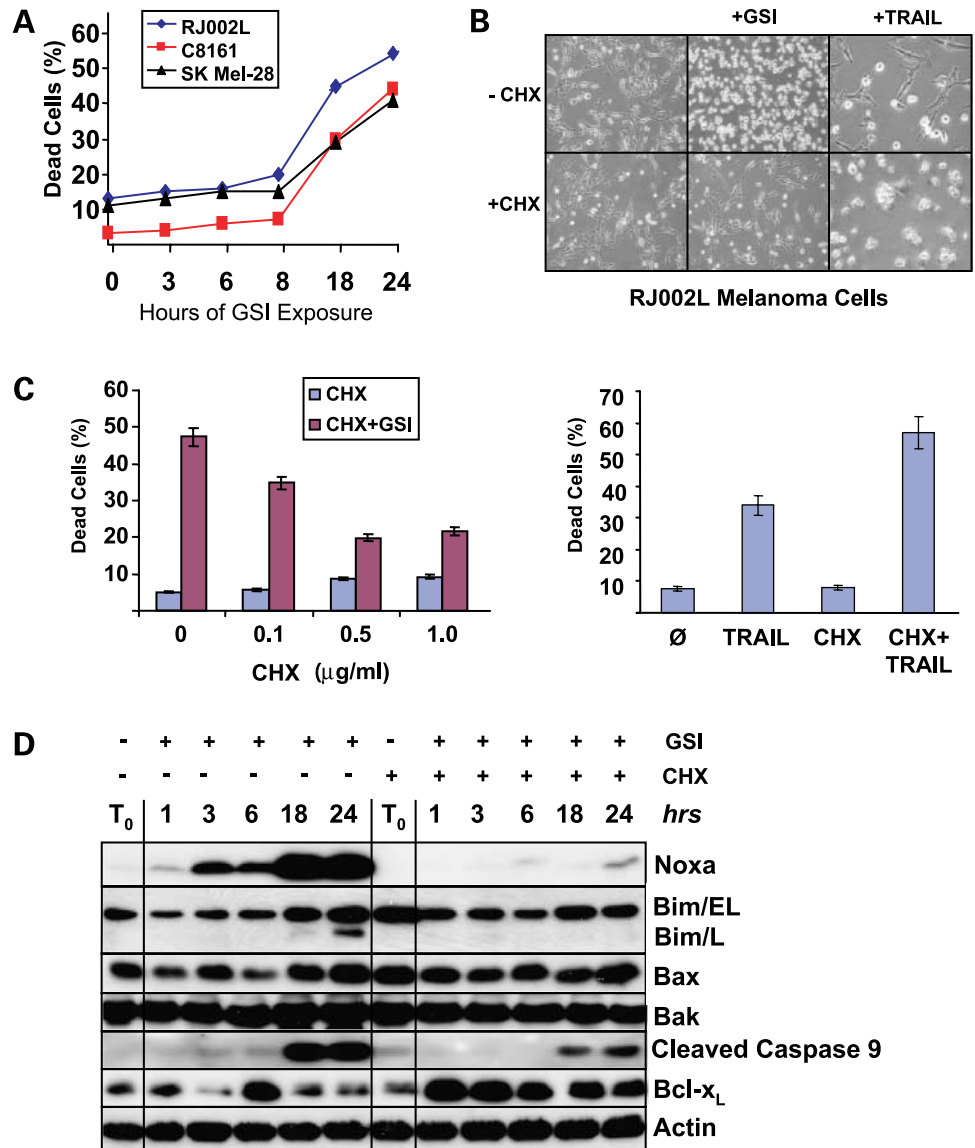
Western blot analysis of survival and proapoptotic proteins following GSI treatment of RJ002L melanoma cells revealed an induction of Bak, Bim, and NOXA, with PUMA and Bax levels unaffected (Fig. 3A). NOXA induction was detectable as early as 1 hour and consistently elevated between 3 and 6 hours followed by massive expression at 18 to 24 hours (Figs. 2D and 3A). Induction of these proapoptotic proteins in melanoma cell lines coincided with onset of apoptosis, Bid degradation, and appearance of cleaved caspase-9 and poly(ADP-ribose) polymerase. There was little to no decrease at 6 hours in the levels of the survival proteins Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, and survivin. However, at longer time intervals, such as 18 and 24 hours, decreases in Bcl-2 and survivin levels were observed in the melanoma cells. Figure 3B reveals the lack of NOXA induction by GSI (10  $\mu\text{mol/L}$ ) in normal melanocytes and the inability of DNA-damaging agents Adriamycin, etoposide, or UV light exposure to induce NOXA in RJ002L melanoma cells. Note that both melanocytes and RJ002L melanoma cells responded to GSI and genotoxic agents by increasing p53 levels (Fig. 3B). We assessed mitochondrial integrity before and after exposure to GSI and observed the presence of cytochrome *c* and SMAC/DIABLO released from the mitochondria into the cytoplasm initially detectable at 6 hours and increasing over time (18 and 24 hours) following GSI exposure (Fig. 3C).

Because NOXA was prominently induced by GSI in melanoma cells but not in melanocytes (Fig. 3A and B), a role for NOXA in GSI (10  $\mu\text{mol/L}$ )-induced apoptosis was further pursued. To determine if p53 status influenced induction of NOXA, three different melanoma cell lines were examined that contained p53 mutations (i.e., C8161, SK-Mel-28, and MUM2B) and compared with two melanoma cell lines with wild-type p53 (RJ002L and SK-Mel-100) as shown in Fig. 3D. Both MUM2B and SK-Mel-28 cells carry homozygous inactivating mutations (R196Stop and L145R, respectively), and little to no p53 was detected before or following GSI exposure. In contrast, C8161 carried a R196Stop mutation in only one allele, and p53 expression, similar to that observed in wild-type cell lines, was evident in response to NOXA. Because SK-Mel-100 cells with wild-type p53 and low Apaf-1 levels (Fig. 1B) were highly sensitive to GSI-mediated killing (Fig. 1A), Apaf-1 and NOXA levels were examined before and after GSI treatment. NOXA induction occurred earlier (1 hour) with further increases after 3 hours (Fig. 3C); however, this increased sensitivity to GSI was not due to any increase in Apaf-1 levels induced by GSI (data not shown). In summary, NOXA was induced in all cell lines regardless of p53 mutation status, albeit with a delayed onset in lines



**Figure 1.** GSI induces apoptosis in melanoma cells *in vitro* and *in vivo*. **A** and **B**, GSI induces apoptosis in melanoma cells carrying both wild-type and mutated p53 but not melanocytes regardless of Apaf-1 expression levels. **C**, GSI (10  $\mu$ mol/L) induces apoptosis in melanoma cells, but only a G<sub>2</sub>-M growth arrest in melanocytes, most notable at 24 hours. **D** to **G**, GSI induces apoptosis in melanoma xenografts. **D**, H&E-stained frozen section of highly aggressive human cutaneous melanoma tumor xenograft (C8161 cells). High level of apoptosis observed by immunofluorescent terminal deoxynucleotidyl transferase-mediated nick end labeling assay following 1 week treatment with GSI (**E**) versus DMSO carrier (**F**). **G**, GSI treatment for 1 week significantly reduced tumor size ( $P = 0.046$ ) compared with DMSO ( $n = 11$ ).

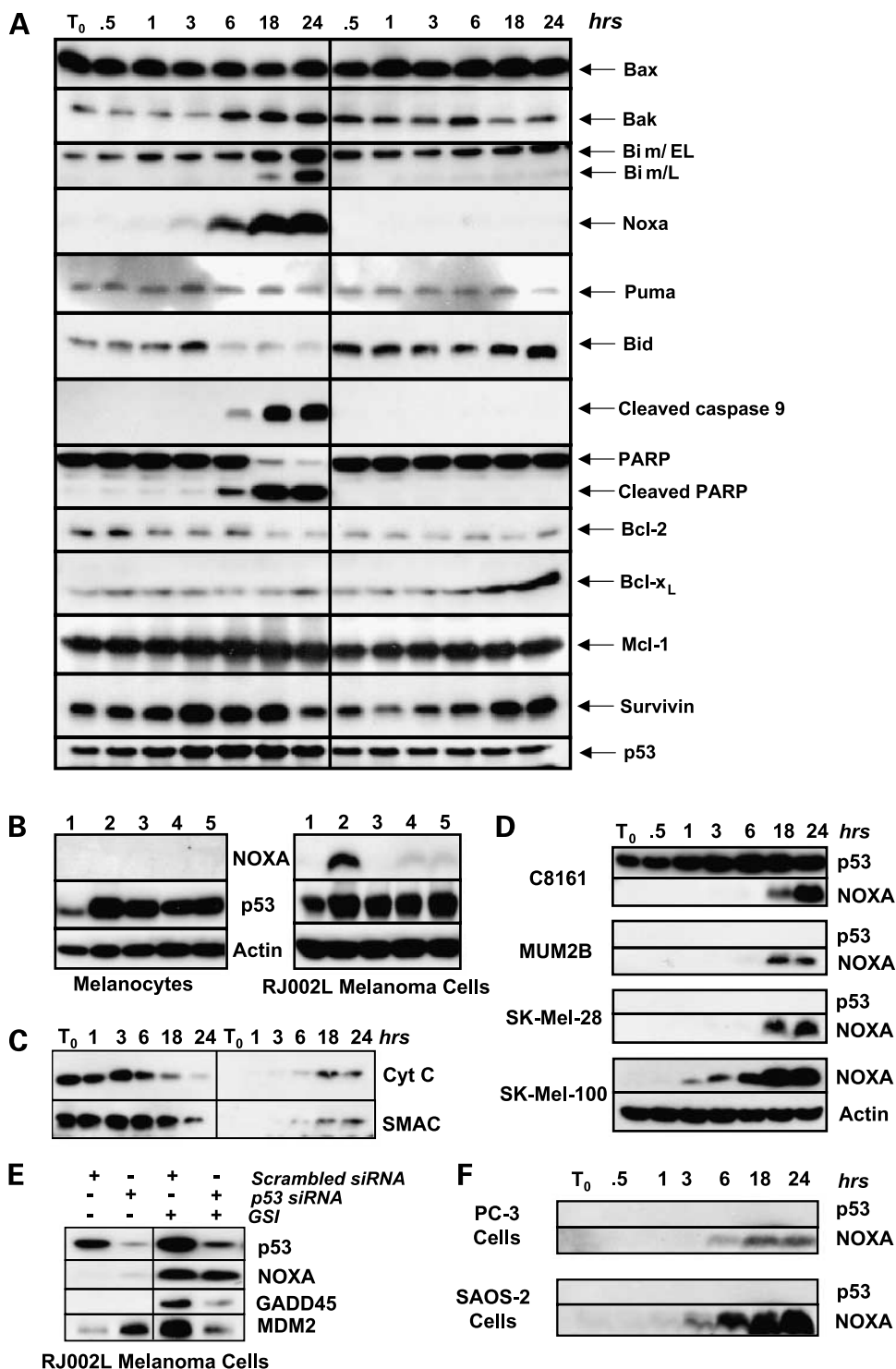
**Figure 2.** GSI-induced apoptosis requires new protein synthesis. **A**, induction of apoptosis occurred at 6 hours but was most apparent at 24 hours in three melanoma cell lines (SE < 20%). **B**, cycloheximide pretreatment reduces GSI-induced apoptosis while enhancing TRAIL-induced apoptosis. Phase-contrast appearance of RJ002L melanoma cells 24 hours after addition of either GSI (10  $\mu$ mol/L) or TRAIL (100 ng/mL). *Lower panels*, cells were pretreated with cycloheximide (1  $\mu$ g/mL, 1 hour) prior to addition of either GSI or TRAIL. **C**, cycloheximide pretreatment reduces GSI-induced apoptosis in a dose-dependent manner while enhancing TRAIL-induced apoptosis. **D**, cycloheximide pretreatment reduces GSI-mediated induction of NOXA and cleaved caspase-9 but not Bim or Bak (actin is loading control).



carrying mutated p53 (Fig. 3A and D). In addition, whereas all normal melanocytes contained abundant p53 levels (data not shown), none of the normal melanocyte responses to GSI included induction of NOXA (Fig. 3B). Thus, within these two different cell types (i.e., melanocytes versus melanoma cells), p53 does not seem to be either necessary or sufficient for induction of NOXA. Further studies were done in which small interference RNA against p53 was used in the RJ002L cell line, and despite significant reduction in p53 levels (>80%), NOXA was still induced by GSI (Fig. 3E). Confirmation of the p53 small interference RNA activity included diminished induction of levels for other p53-inducible proteins such as GADD45 and MDM2 (Fig. 3E). Two different cell lines known to be p53 null (PC-3 prostate cells and SAOS-2 osteosarcoma cells) were

also found to induce NOXA following GSI exposure with prominent apoptotic responses (>50% dead cells; 24 hours; Fig. 3F), suggesting that this mechanism may not be limited to melanoma cell lines.

To more definitively establish a role for NOXA in the GSI-mediated apoptosis of melanoma cells, both cycloheximide-pretreated melanoma cells and ASO-targeting NOXA in melanoma cell lines were used. GSI treatment (10  $\mu$ mol/L) results in an induction of apoptosis accompanied by induction of Bak, Bim, and NOXA (Figs. 2 and 3). Pretreatment with cycloheximide reduced apoptosis induction (Fig. 2B and C) and reduced levels of cleaved caspase-9, with minimal effects on Bim, Bax, or Bak (Fig. 2D). However, a marked reduction in NOXA induction was observed, accompanied by enhanced Bcl-x<sub>L</sub> levels (Fig. 2D).



**Figure 3.** GSI induces proapoptotic proteins including p53-independent induction of NOXA in melanoma cells but not melanocytes. **A**, profile of pro-survival and proapoptotic proteins in RJ002L melanoma cells following GSI exposure (10  $\mu\text{mol/L}$ ; left) compared with medium alone (right). Top, time of maturation beginning at 30 minutes and extending up to 24 hours. **B**, NOXA and p53 levels in normal melanocytes (MC009) and RJ002L melanoma cells before (lane 1) and 18 hours after exposure to GSI (lane 2; 10  $\mu\text{mol/L}$ ) and genotoxic agents: Adriamycin (lane 3; 1  $\mu\text{g/mL}$ ), etoposide (lane 4; 10  $\mu\text{g/mL}$ ), and UV light (lane 5; 30  $\text{mJ/cm}^2$ ). Actin serves as a loading control. **C**, GSI (10  $\mu\text{mol/L}$ ) triggers release of cytochrome *c* and SMAC from the mitochondria (left panels) into the cytoplasm (right panels) of RJ002L cells first detected at 6 hours. **D**, p53-independent induction of NOXA by GSI. Induction of NOXA by GSI in melanoma cell lines with either mutant p53 (C8161, MUM2B, and SK-Mel-28) or wild-type p53 (SK-Mel-100). **E**, reduction of p53 levels in RJ002L cells using p53 small interference RNA leads to a reduction of GSI-induced (18 hours) GADD45 and MDM2 levels but not NOXA levels. **F**, induction of NOXA by GSI in two different nonmelanoma cell lines with null p53 (PC-3 and SAOS-2).

Preincubation of RJ002L melanoma cells with the anti-NOXA ASO but not CO blocked induction of NOXA but not Bim or Bak (Fig. 4A). The ability of the anti-NOXA ASO to block NOXA induction was accompanied by a significant reduction in the apoptotic response (Fig. 4B and C). Interestingly, GSI treatment in the absence of NOXA induction

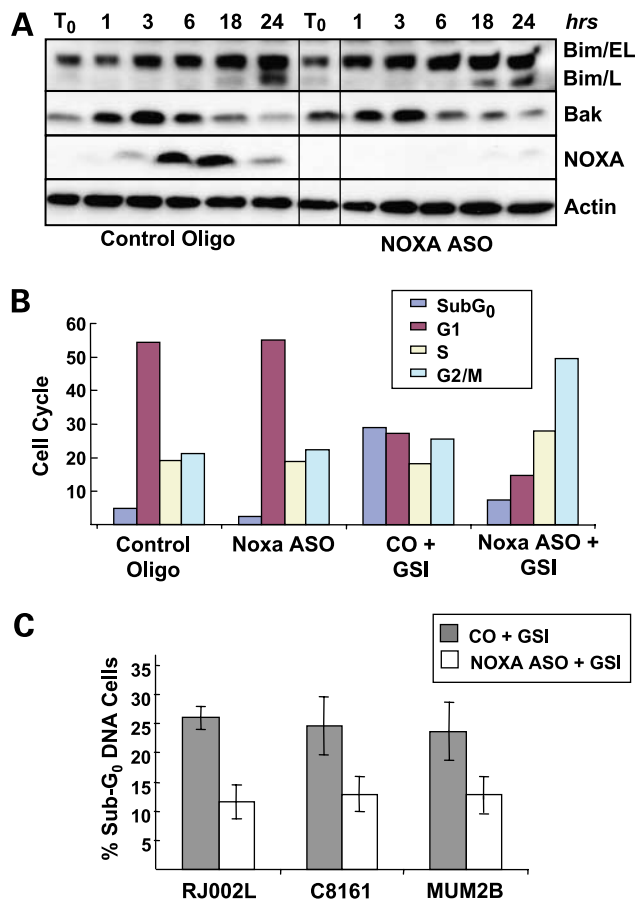
enhanced the relative percentage of melanoma cells in G<sub>2</sub>-M, thereby resembling the melanocyte response to GSI (compare Fig. 1C with the representative result portrayed in Fig. 4B). Whereas neither the NOXA ASO nor the CO alone influenced the cell cycle or spontaneous apoptosis for RJ002L, C8161, or MUM2B cells, the GSI-mediated apoptosis

(determined using sub-G<sub>0</sub> DNA content) observed in three to four independent experiments for each of these melanoma cell lines was significantly reduced ( $P < 0.01$  for all cell lines comparing CO + GSI versus NOXA ASO + GSI; Fig. 4C).

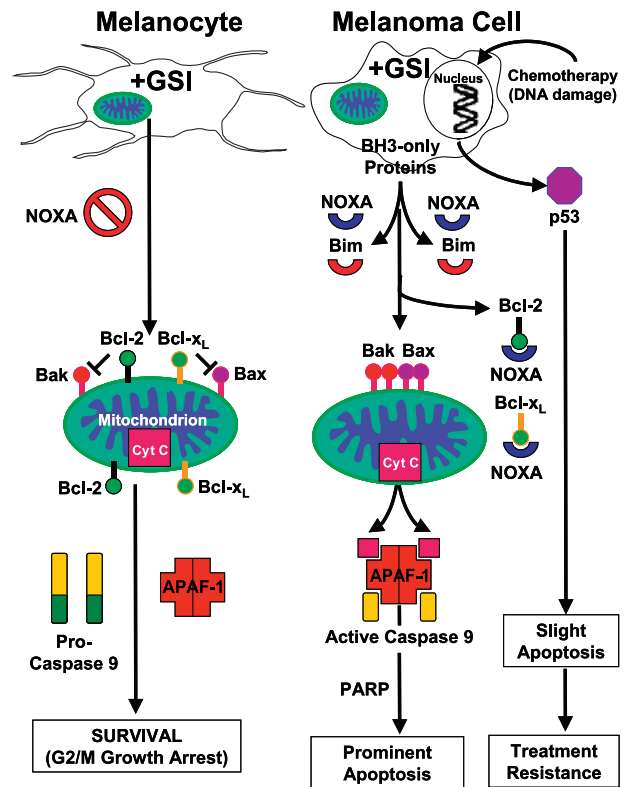
A role for caspase activation in GSI-mediated apoptosis was confirmed by pretreating RJ002L cells with various membrane permeable inhibitors against a broad group of caspases (i.e., ZVAD-CHO) as well as more selective inhibitors targeting caspase-9 (LETD-CHO) and caspase-3 (DEVD-CHO). Each of these caspase inhibitors reduced the GSI-mediated apoptotic response in melanoma cells by ~30% to 50% (data not shown).

## Discussion

Taken together, these results show the ability of GSI to induce significant apoptosis in all human melanoma cell lines tested, both *in vitro* and *in vivo*, without triggering



**Figure 4.** Knockdown of NOXA with ASO reduces induction of apoptosis. **A**, NOXA ASO but not CO reduced GSI (10  $\mu$ mol/L)-induced NOXA levels but not Bim or Bak (actin is loading control) in RJ002L cells. **B**, NOXA ASO but not CO reduced GSI (10  $\mu$ mol/L)-induced apoptosis (sub-G<sub>0</sub> DNA content) accompanied by increased G<sub>2</sub>-M growth arrest in RJ002L cells. **C**, NOXA ASO but not CO reduced GSI-mediated induction of apoptosis (sub-G<sub>0</sub> DNA content) in three different melanoma cells (RJ002L, C8161, and MUM2B;  $P < 0.01$ ).



**Figure 5.** Summary of distinctive responses of melanocytes versus melanoma cells to GSI highlighting key roles for BH3-only proteins (i.e., NOXA and Bim), which are induced in a p53-independent fashion. By contrast to chemotherapeutics mediated via p53, which trigger only slight apoptosis leading to treatment resistance, GSI triggers prominent apoptosis in melanoma cells.

the killing of normal human melanocytes (summarized in Fig. 5). In contrast to conventional cytotoxic agents that are not clinically effective and only indirectly induce apoptosis (i.e., via p53), treatment of melanoma cells with GSI can activate apoptotic machinery via the action of BH3-only proteins, including a key role for NOXA. Not only can GSI induce NOXA in the absence of p53, but it can also kill melanoma cell lines with mutant p53 and low Apaf-1 levels as well as abundant levels of antiapoptotic proteins such as Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, and survivin (15).

The proximal mechanism of action of GSI in these cells is still unclear but most likely will involve more than a single molecular target.  $\gamma$ -Secretase has numerous substrates, including all four Notch receptors, several Notch ligands, ErbB4, syndecan, CD44, and several other molecules (6). Moreover, we cannot rule out that GSI may have additional targets besides  $\gamma$ -secretase (10). Whereas we will continue to dissect the proximal mechanisms of action of GSI, we have determined that the downstream apoptotic pathway activated by this molecule is novel and unique. Because Notch signaling has been implicated in stem cell biology, by targeting Notch, we may be targeting cancer stem cells. Further studies are warranted to determine the role for GSI and Notch signaling in the survival and apoptotic

responses involving melanoma cells. As shown in Fig. 3, GSI was able to also trigger NOXA induction in malignant cell lines besides melanoma cells including PC-3 and SAOS-2 cells, indicating that the apoptotic response primarily characterized in melanoma cells may also be relevant to other tumor cell types.

The current findings reveal several new insights into the biology of melanoma cells. (1) Melanoma cells are not as consistently deficient in Apaf-1 as originally suggested (4). Specifically, Apaf-1, a component of the apoptosome engaged after mitochondrial damage, is epigenetically silenced in a subset (10 of 19) of melanoma cell lines (4). In our current series of melanoma cell lines, only three of nine were characterized by relatively low Apaf-1 levels and none of these melanoma cells had completely silenced Apaf-1 expression. (2) We observed that the mitochondrial pathway could be engaged to kill melanoma cells by the use of GSI. (3) Even in the absence of p53 and barely detectable levels of Apaf-1, biochemical mediators can execute an apoptotic program in melanoma cells that does not become activated in proliferating normal melanocytes. (4) Whereas several reports have linked the p53-mediated apoptotic response to NOXA, we have uncovered a p53-independent mechanism for induction of NOXA and another BH3-only (Bim) family member (16–18). (5) This newly discovered, directly activated pathway to NOXA and other proapoptotic proteins does not involve enhanced PUMA levels (19, 20). Moreover, GSI-initiated apoptosis in tumor cells with high constitutive levels of both Bcl-2 and Bcl-x<sub>L</sub> highlights the importance of the BH3-only proteins (21). As NOXA and Bim bind to antiapoptotic Bcl-2 family members, this promotes subsequent binding of multidomain proapoptotic proteins Bax and Bak to the mitochondrial outer membrane (22–24), and we confirmed release of cytochrome *c* and SMAC/DIABLO into the cytoplasm with activation of caspase-9 and caspase-3 (25, 26). (6) The *in vivo* data using a xenograft animal model lay the foundation for additional preclinical testing and future clinical trials in melanoma patients.

As a variety of compounds related to GSI are already in the clinic for the treatment of Alzheimer's disease, such bench to bedside translational efforts for melanoma patients may be facilitated by the ongoing use of such agents in nonneoplastic settings. Finally, GSI may be indicated for the treatment of other highly chemoresistant tumor cell types, including those with frequent p53 mutations.

## References

- Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin* 2002;52:23–47.
- Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. *Oncogene* 2003;22:3138–51.
- Chin L. The genetics of malignant melanoma: lessons from mouse and man. *Nat Rev Cancer* 2003;3:559–70.
- Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 2001;409:207–11.
- Chipuk JE, Kuwana T, Bouchier-Hayes L, et al. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 2004;303:1010–4.
- Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* 2003;22:6598–608.
- Hendrix MJ, Seftor RE, Seftor EA, Gruman LM, Lee LM, Nickoloff BJ, et al. Transendothelial function of human metastatic melanoma cells: role of the microenvironment in cell-fate determination. *Cancer Res* 2002;62:665–8.
- Wolfe MS. Secretase targets for Alzheimer's disease: identification and therapeutic potential. *J Med Chem* 2001;44:2039–60.
- Josien H. Recent advances in the development of  $\gamma$ -secretase inhibitors. *Curr Opin Drug Discov Dev* 2002;5:513–25.
- Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, et al. Distinct sites of intracellular production for Alzheimer's disease A $\beta$ 40/42 amyloid peptides. *Nat Med* 1997;3:1016–20.
- Hendrix MJ, Seftor EA, Seftor RE, Trevor KT. Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior. *Am J Pathol* 1997;150:483–95.
- Qin J, Chaturvedi V, Bonish B, Nickoloff BJ. Avoiding premature apoptosis of normal epidermal cells. *Nat Med* 2001;7:385–6.
- Denning MF, Wang Y, Nickoloff BJ, Wrone-Smith T. Protein kinase C $\delta$  is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. *J Biol Chem* 1998;273:29995–30002.
- Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol* 1998;161:2833–40.
- Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001; 292:727–30.
- Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000;288:1053–8.
- Bouillet P, Cory S, Zhang LC, Strasser A, Adams JM. Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. *Dev Cell* 2001;1:645–53.
- Shibue T, Takeda K, Oda E, Tanaka H, Murasawa H, Takaoka A, et al. Integral role of Noxa in p53-mediated apoptotic response. *Genes Dev* 2003;17:2233–8.
- Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 2001;7:673–82.
- Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001;7:683–94.
- Villunger A, Michalak EM, Coultas L, Mullaer F, Bock G, Ausserlechner MJ, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003;302:1036–8.
- Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* 1999;399:483–7.
- Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 2001;8:705–11.
- Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires anti-apoptotic MCL-1. *Nature* 2003;426:671–6.
- Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;102:33–42.
- Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000;102: 43–53.



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