SUPPLEMENTAL INFORMATION

Protein Immunoblotting. Primary antibodies used in this study include: Casp-9, Casp-3 and Survivin from Novus Biologicals (Littleton, CO); Casp-8 (Ab-3) from Oncogene Research Products (San Diego, CA); Casp-7, Bid, Bak, Bad, phospho-IκB-α (Ser32) and IκB-α from Cell Signaling Technology (Beverly, MA); FLIP (ab8421) from Abcam (Cambridge, MA); TRAF-2 and Bim/Bod from Imgenex Innovations in Functional Genomics (San Diego, CA); XIAP and Bcl-x from BD Transduction Laboratories (Franklin Lakes, NJ); Bax (B-9), CHOP (GADD-153; R-20), Mcl-1 (S-19), AIF (E-1), and Ubiquitin (P4D1) from Santa Cruz Biotechnology (Santa Cruz, CA); Bax and Bak from Upstate Cell Signalling Solutions (Lake Placid, NY); Bcl-2 from Dako Diagnostics (Glostrup, Denmark); Hsp-70 (Hsp-72) and Grp-78 from Stressgen Biotechnologies (San Diego, CA); Cyt *c* (clone 7H8.2C12) from BD PharMingen (Franklin Lakes, NJ); Smac (222-237) and Noxa from Calbiochem (San Diego, CA); HrtA2 from Alexis Biochemicals (San Diego, CA); Cyt *c* Oxidase Subunit IV (COX IV) from Molecular Probes (Eugene, OR); p53 from Novocastra Laboratories (Newcastle upon Tyne, UK); and Tubulin and β-Actin (clone AC-74) from Sigma Chemical (St Louis, MO). Protein levels were estimated by densitometry and normalized with respect to Tubulin or Actin, used as a loading controls.

Visualization of Chromatin Condensation (DAPI Staining). Melanoma cells (4×10^4) were treated with Adriamycin (0.5 μg/ml), Bortezomib (50 nM) or Cisplatin (30 μg/ml) for 36 h. Floating cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed with PBS, and stained with 2 μg/ml DAPI (Sigma) for 5 min at room temperature. Apoptotic cells were identified by condensation and fragmentation of nuclei using an Olympus IMT-2 inverted light microscope. A minimum of 400 cells were counted for each treatment. Percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted. Images were captured using a SPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Scanning Electron Microscopy. Cells (4×10^4), growing on 13 mm Thermanox coverslips, were treated with Adriamycin (0.5 μg/ml), Bortezomib (50 nM) or Cisplatin (30 μg/ml) for 30 h. Coverslips were rinsed briefly with 0.1 M Sorensen's buffer, pH 7.4, and immediately fixed in 2.5% glutaraldehyde in the same buffer for 1.5 h. After a Sorensen's buffer wash, they were postfixed with 1% osmium tetroxide for 15 min. The cells were again rinsed with Sorensen's buffer and dehydrated in a graded series of ethanol. At that time, cells were treated with hexamethyldisilazane and allowed to dry. The samples were sputter coated with gold and the cellular blebbing was analyzed using an

AMRAY 1000-B scanning electron microscope at 10 kV. Digital images were collected with a Semicaps 2000-A Imaging System.

Luciferase Assays. Melanoma cell lines were seeded into 6-well plates (1-1.5×10^5 cells per well). After incubating at 37°C for 18 h, cells were transiently transfected with 250-500 ng of pGL2-2κB-*luciferase* reporter plasmid containing two canonical κB sites or an empty vector control (generous gifts from Dr. Colin Duckett laboratory at the University of Michigan). The transfections were performed in DMEM medium without FBS using the LIPOFECTAMINE™ Plus reagent (Life Technologies) or Fugene 6 (Roche) according to the manufacturer's instruction. Cells were incubated for 3 h at 37°C, and then fresh DMEM-10% FBS medium was added. After 18 h, cells were treated with either Bortezomib (50 nM) or human Tumor Necrosis Factor-α (hTNF-α) (1,000 U/ml) alone, or in combination, for 6, 8 and 12 h, at which time luciferase activity was measured using the Promega Luciferase Assay kit (Promega, Madison, WI). Briefly, cells were washed with PBS and lysed with 150-250 μl of Reporter Lysis Buffer for 15 min with gentle shacking. Cells were subsequently harvested into a 1.5 ml microcentrifuge tube, vortex-mixed for 15 s; centrifuged at 12,000 × g for 30 s, and the supernatant (cell lysate) was collected. Luciferase activity was measured with 20 μl of cell lysate and 100 μl of substrate using a TR 717 Microplate Luminometer after normalizing for internal controls and protein concentration. Results are expressed as the mean of relative light units (RLU) ± SEM. All transfection experiments were carried out in triplicate wells and repeated separately at least three times. The significance of differences between treated and control groups was determined using the One-Way analysis of variance, and the comparison of means was conducted using post hoc Tukey multiple comparison test.

FIGURE LEGENDS OF SUPPLEMENTAL INFORMATION

Supplemental Table 1. Genetic background of the metastatic melanoma lines used in this study. SK-Mel melanoma lines were coded for consistency following a previously reported nomenclature (1). p53, as well $p14^{ARF}$, $p16^{INK4a}$ and Apaf-1 were analyzed as described in Soengas *et al.* (1). Samples with polymorphism p53 P72R are indicated as wt^{R}. *Cell line #3 and #10 have been reported as bearing $p16^{INK4a}$ mutations R58Stop and P114L respectively; cell line #10 has in addition a homozygous deletion of exon 1β of $p14^{ARF}$; cell line #4 and #5 have a R24C mutation in CDK4. [d, e]Mutational status of BRAF and N-Ras at exons 15 and 3, respectively, determined by direct sequencing of PCR-amplified genomic fragments. [f]Apaf-1, Casp-9, Casp-8, Bcl-2, Bcl-x_L, Mcl-1, XIAP and Survivin protein levels were estimated by immunoblotting followed by densitometry and normalized to melanocyte control. Line expressing protein levels ≤ 25%, melanoma cells are labeled as (-), and those expressing intermediate levels of protein are indicated as (-/+ and +), defined as having an amount of protein >25-50% and >50-75%, respectively with respect to normal melanocytes. Positive cells (++ and +++), defined as having an amount of protein >75- ≤ 150% and >150%, respectively. ND: not determined.

Supplemental Fig. 1. Apoptotic features of Bortezomib-induced cell death. Adriamycin, Bortezomib and Cisplatin were compared in their ability to induce chromatin condensation and nuclear fragmentation (blue fluorescence corresponding to nuclear DAPI staining; *a-d, i-l*), and membrane blebbing (determined by scanning electron microscopy; *e-h, m-p*). Note the drastic effects of Bortezomib on cell line #9, otherwise resistant to Adriamycin.

Supplemental Fig. 2. Caspase-dependent and -independent cell death induced by Bortezomib. The indicated cell lines were treated with 50 nM in the presence or absence of the pan-caspase inhibitor z-VAD-fmk (50 μM). Cell viability was estimated at each time point by trypan blue exclusion. Note that zVAD-fmk cannot completely block Bortezomib-induced killing suggesting the implication of alternative death mechanisms to classical (caspase-dependent) apoptotic pathways.

Supplemental Fig. 3. Bortezomib does not significantly downmodulate intrinsic NF-κB dependent transcriptional activity. Relative luciferase units (RLU) driven by a reporter plasmid containing canonical κB sites transfected into melanoma lines #3 or #9. Non Treated (NT) and Borterzomib-treated cells (Bor) had similar luciferase activities. Bortezomib was able, however, to

reduce NF-κB dependent transcriptional activation by hTNF-α. The data shown are representative of three independent experiments performed in triplicate, and represent the mean ± SEM.

Supplemental Fig. 4. Accumulation of Bim and Mcl-1 in normal melanocytes and melanoma cells. Protein immunoblots corresponding to total cell extracts of normal melanocytes (Normal) and melanoma cell lines #3 and #9 left untreated (NT) or treated with 50 nM Bortezomib (Bor) for the indicated times. Shown are protein immunoblots probed simultaneously with specific antibodies against Bim or Mcl-1. Both normal and tumor cells responded to Bortezomib by increasing the levels of Bim and Mcl-1, and there was no correlation between the levels of the two proteins and the final extent of cell death. Total ubiquitinated proteins (Ub) are shown as a surrogate for proteasome inhibition and Tubulin as a loading control.

Supplemental Fig 5. **Generalized upregulation of Noxa by Bortezomib in metastatic melanoma cells**. Basal and Bortezomib-induced levels of Noxa were determined in a pool of normal melanocytes isolated from foreskins of 3 donors of caucasian ethnic origin (C) and in an independent population of normal foreskin melanocytes from highly pigmented African-American donors (D). The indicated 16 metastatic melanoma lines were also treated and analyzed in parallel. Cells were incubated for 18 h in media with solvent (0.05% DMSO) or 50 nM Bortezomib for 6 or 18 h. Total cell extracts were prepared and processed as indicated in *Material and Methods* to visualize the amount of Noxa by protein immunoblotting. Cell line #3 treated with Bortezomib for 18 h (*) was included in each membrane as internal reference to ensure equivalent immunostaining among samples loaded in different polyacrylamide gels. Tubulin is shown as a loading control. (see *Supplemental* Table 1 for the full nomenclature of the lines referred with a #n code number).

Supplemental Fig 6. **Impact of Bortezomib of tumor growth (mouse xenografts).** Comparative analysis of the localized growth of melanoma cells (lines #9 and #10) implanted s.c.. *(Left)* Plots show tumor growth (± SEM) in animals treated systemically with placebo control (white squares) or Bortezomib (black diamonds). *p* values correspond to day 17 post-tumor implantation. *(Right)* Representative examples of animals corresponding to each treatment group at day 17 after implantation (see additional details on *Materials and Methods* about dose and treatment schedule).

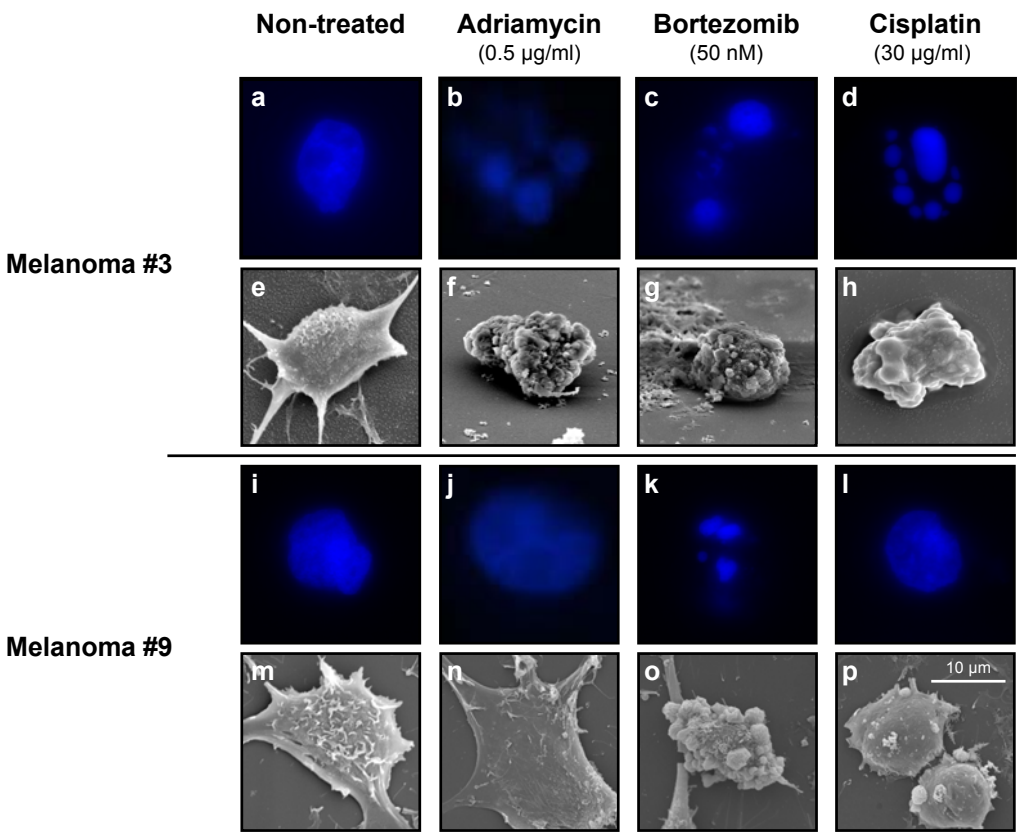
REFERENCES

1. Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., Lazebnik, Y. A., Cordon-Cardo, C., and Lowe, S. W. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001; *409:* 207-211.

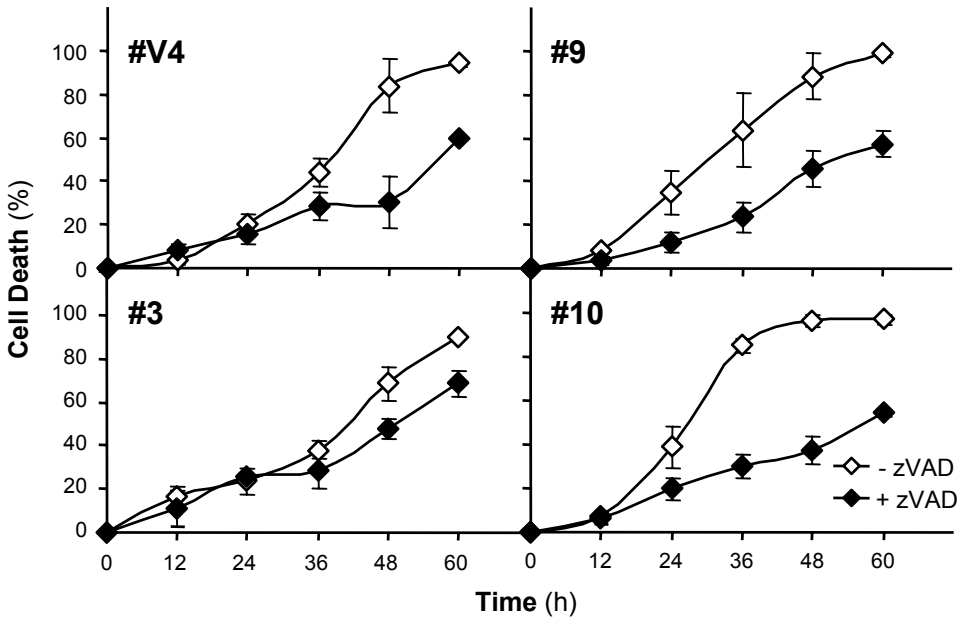
Fernández *et al.*, **Supplemental Information Table 1**

Invasive Features	Cell line	Code	p53[a]	p14[b] (mRNA)	p16[c] (mRNA)	B-Raf[d] (V599)	N-Ras[e] (exon 3)	Apaf-1[f] (protein)	Casp-9[f] (protein)	Casp-8[f] (protein)	Bcl-2[f] (protein)	Bcl-x_L[f] (protein)	Mcl-1[f] (protein)	XIAP[f] (protein)	Survivin[f] (protein)
Melanocytes	**NHEM**	**Normal**	ND	ND	ND	wt	wt	++	++	++	++	++	++	++	++
VGP	**WM-1366**	**V4**	ND	ND	ND	wt	Q61R	++	++	+++	-/+	+++	+++	+++	++
Metastatic	**SK-Mel-19**	**3**	wt	+	+'*	mutant	wt	+++	++	+++	++	++	++	+++	+++
	SK-Mel-28	**4**	$R273H^R$	ND	+'*	mutant	wt	+++	++	+++	++	+	++	++	++
	SK-Mel-29	**5**	wt	+	+'*	mutant	ND	+++	+	+++	++	++	+++	+++	+++
	SK-Mel-94	**7**	wt	+	+	mutant	wt	-/+	++	-/+	++	++	+++	++	+++
	SK-Mel-103	**9**	wt^R	+	+	wt	Q61R	-/+	++	-/+	+	+++	++	+++	++
	SK-Mel-147	**10**	wt^R	-	+'*	wt	Q61R	-/+	++	++	-/+	+++	++	++	++
	SK-Mel-173	**11**	wt^R	ND	-	wt	wt	++	-/+	++	++	+++	++	++	+++
	Malme-3M	**15**	wt^R	-	-	wt/mutant	wt	++	++	+++	++	+++	++	+++	+
	UACC-62	**17**	wt	-	-	mutant	wt	++	+	+++	++	++	++	+++	++

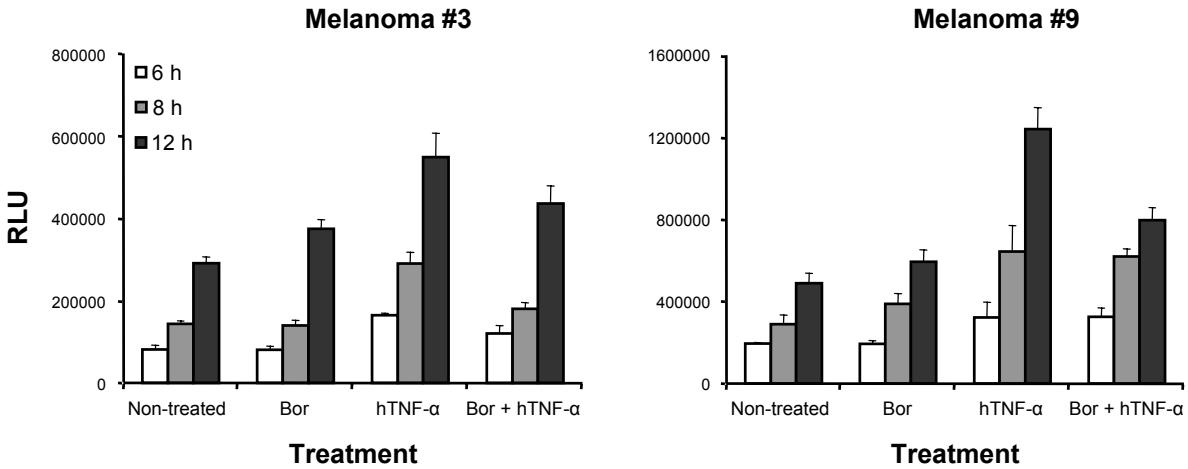
Fernández *et al.*, **Supplemental Information Figure 1**



Fernández *et al.*, **Supplemental Information Figure 2**



Fernández *et al.*, **Supplementary Information Figure 3**



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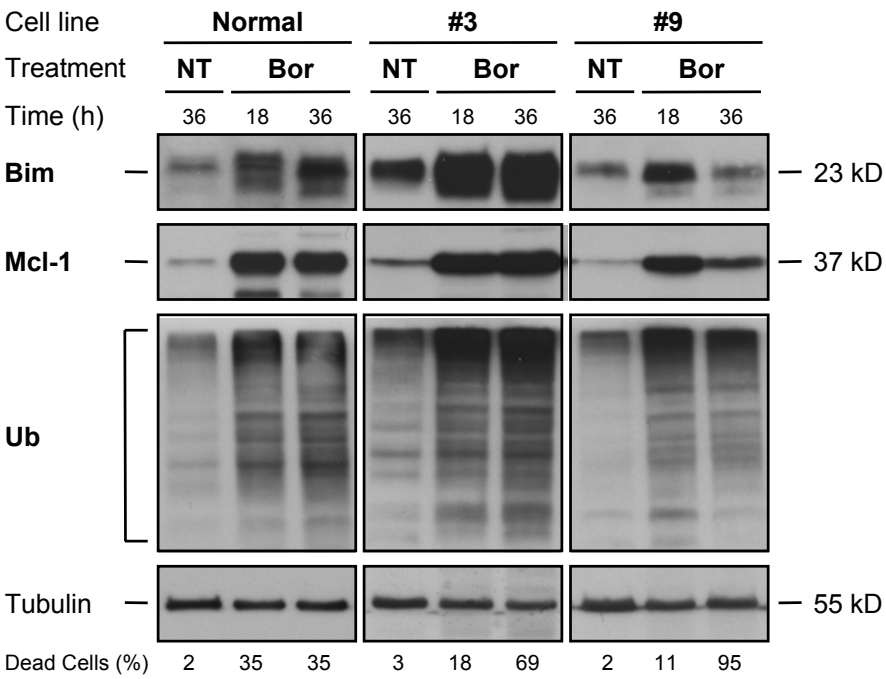

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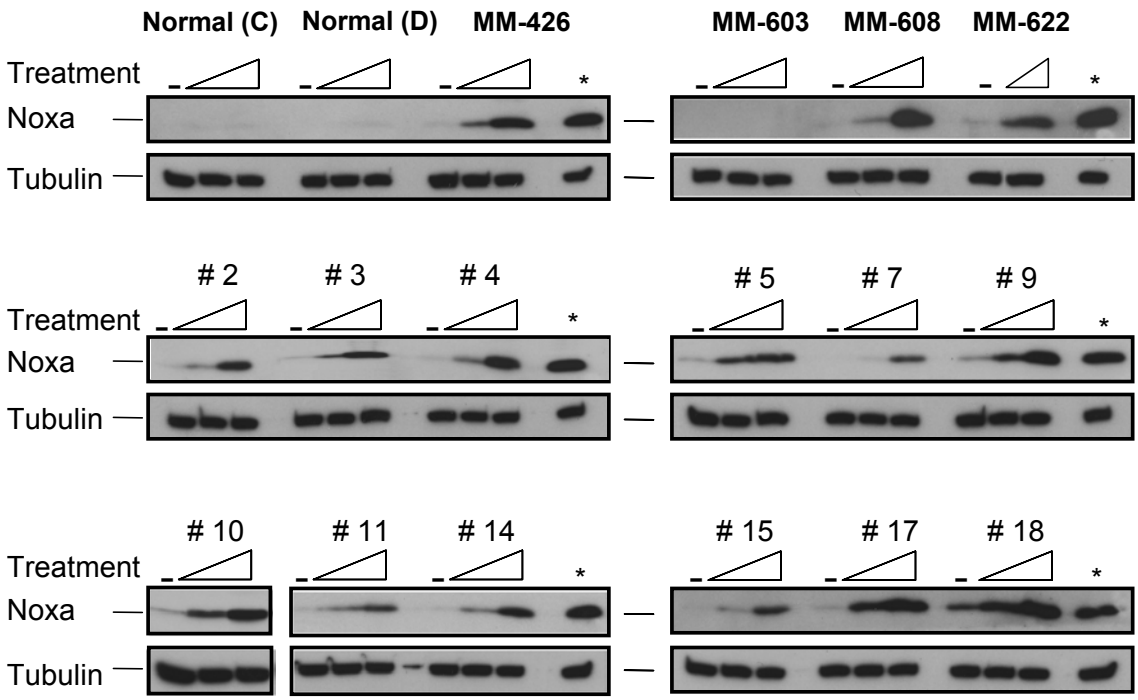

Fernández *et al.*, **Supplementary Information Figure 6**