



Ubiquitin B: an essential mediator of trichostatin A-induced tumor-selective killing in human cancer cells

P Wu^{1,2}, Y Tian^{1,2}, G Chen¹, B Wang¹, L Gui¹, L Xi¹, X Ma¹, Y Fang¹, T Zhu¹, D Wang¹, L Meng¹, G Xu¹, S Wang¹, D Ma^{*1} and J Zhou^{*1}

Although histone deacetylase inhibitors (HDACis) are emerging as a new class of anticancer agents, the mechanism of tumor-selective killing by HDACi is not well understood. We used suppression of mortality by antisense rescue technique (SMART) to screen the key genes responsible for the tumor-selective killing by trichostatin A (TSA). Twenty-four genes were identified, the most significant of which was ubiquitin B (UbB). The expression of UbB was selectively upregulated by TSA in tumor cells, but not non-malignant cells. Further observation indicated that TSA induced a substantial dissipation of mitochondrial transmembrane potential, release of cytochrome *c* into the cytosol, and proteolytic cleavage of caspases-3/9 in HeLa cells, which was apparently mediated by ubiquitylation and the subsequent degradation of mitochondrial membrane proteins including BCL-2 and MCL-1. In contrast, knockdown of UbB expression inhibited the TSA-induced apoptotic cascade by abolishing TSA-induced ubiquitylation and the subsequent degradation of mitochondrial membrane proteins. Furthermore, apicidine, another HDACi, exhibited activity similar to that of TSA. Interestingly, TSA induced UbB-dependent proteasomal degradation of BCR–ABL fusion protein in K562 leukemic cells. Thus, our findings highlight the essential role of UbB and UbB-dependent proteasomal protein degradation in HDACi-induced tumor selectivity. The mechanism provides a novel starting point for dissecting the molecular mechanism underlying the tumor selectivity of HDACi.

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Histone deacetylase inhibitors (HDACis) are now emerging as a new class of anticancer agents with potent activity in the inhibition of proliferation and induction of apoptosis and differentiation in a wide spectrum of tumors. At least 12 HDACis are under evaluation in over 100 clinical trials and have produced encouraging therapeutic responses with surprisingly good safety profiles.^{1–3} The clinical potential of HDACis has been well exemplified by the successful development of Vorinostat, which was recently approved by the US Food and Drug Administration for treatment of cutaneous T-cell lymphoma.⁴ Despite the rapid clinical progress achieved, the mechanism of action of HDACis is not yet well understood. One of the central questions is how these agents selectively kill tumor cells while sparing normal cells. Identification of the critical intracellular targets responsible for the tumor selectivity of HDACis will further improve the design of optimized clinical protocols. More attractively, unraveling the potential ‘death programs’ selectively activated in tumor but not in normal cells will have broader implications for the understanding of tumorigenesis and the design of targeted therapies.

Identification of the target genes essential for the selective induction of apoptosis in tumor cells has proven to be very

difficult. It is well established that HDACis could affect up to 10% of all known genes at the transcriptional level.⁵ In addition to HDACs, many non-histone proteins are also regulated by HDACis by influencing the molecular events of acetylation, protein–protein interactions and stability, and so on. Previously, a significant number of apoptotic and cell-cycle regulatory genes have been identified by different groups and proposed to be effectors responsible for the tumor-selective action of HDACis.⁶ In acute promyelocytic leukemia, however, preferential induction of tumor-necrosis factor-related apoptosis inducing ligand or FAS by HDACis was proposed to serve as the target responsible for the tumor-selective action.⁷ Although these findings have greatly extended our understanding of the action of HDACis, some basic questions still remain to be addressed.^{8,9} One of the obviously unanswered questions is whether a universal, essential target responsible for HDACi-mediated apoptosis exists. To address this, new analytic approaches are needed to identify the essential genes from the numerous proposed effectors of HDACis.

Suppression of mortality by antisense rescue technique (SMART) is a powerful technique to hunt for functionally relevant genes involved in cell death induced by certain

¹Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, People's Republic of China

*Corresponding authors: D Ma, Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Ave., Wuhan, Hubei 430030, People's Republic of China. Tel: +86 27 8366 3351; Fax: +86 27 8366 2681; E-mail: dma@tjh.tjmu.edu.cn or J Zhou. Tel: +86 27 8366 2680; Fax: +86 27 8366 2681; E-mail: jfzhou@tjh.tjmu.edu.cn

²These authors contributed equally to this work.

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Abbreviations: HDACi, histone deacetylase inhibitor; SMART, suppression of mortality by antisense rescue technique; UbB, ubiquitin B; REC, respiratory endothelial cells; APL, acute promyelocytic leukemia; TRAIL, tumor-necrosis factor-related apoptosis inducing ligand; $\Delta\Psi_m$, mitochondrial transmembrane potential

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agents. The SMART screen used an antisense cDNA expression library knocking out the global gene expression of tumor cells in a random and unbiased manner. The transfected tumor cells are then continuously exposed to treatment with the agent. The rationale is that the tumor cells will survive treatment of the agent providing silencing genes required for the agent-induced apoptosis are inactivated.¹⁰ A number of investigators have successfully applied this technique in the isolation of death-associated genes involved in various agent-induced apoptosis.¹¹ In this study, we performed SMART to identify the key genes responsible for the tumor-selective action of trichostatin A (TSA), one of most extensively studied HDACis. We identified 24 genes including Liv-1 and ubiquitin B (UbB), which were potentially essential for the TSA-induced apoptosis. Of these, loss of function of UbB gene conferred the strongest resistance to TSA treatment. We further underscore the potential role of UbB in HDACi-induced apoptosis. Our findings highlight a previously unrecognized mechanism whereby UbB contributes to the tumor-selective killing of HDACis.

Results

Preferential induction of apoptosis of tumor cells by TSA.

To determine the kinetics and dosage range within which TSA would preferentially induce apoptosis in tumor cells, we treated MCF-7 and a non-malignant cell line of same tissue origin, MCF-10A, with various doses of TSA. Although TSA caused only slight growth inhibition in MCF-10A at a dose range between 250 and 1000 nM, it led to profound growth inhibition of MCF-7 in a dose-dependent manner. The growth inhibition became evident at 24 h and reached a maximum at 72 h (Figure 1a). We then chose the lowest concentration of TSA (250 nM) to compare the induction of apoptosis in MCF-7 *versus* MCF-10A. Exposure of MCF-7 or MCF-10A to TSA displayed a similar kinetics of apoptosis, which was detectable within 48 h and peaked at 72 h. At every time point examined, TSA exhibited preferential induction of apoptosis in MCF-7 (Figure 1b). To test whether TSA at a dose of 250 nM also preferentially induced apoptosis in other tumor cell types, tumor (HeLa, C13K, OV2008) and primary normal respiratory endothelial cells (RECs) were treated with TSA for 72 h and subjected to apoptosis analysis. Again, 250 nM TSA preferentially induced apoptosis in tumor (HeLa, C13K, or OV2008) but not in REC (Figure 1c). Thus, we concluded that 250 nM TSA would preferentially induce apoptosis in tumor cells.

Identification of genes essential for TSA-induced apoptosis using SMART screen.

To identify the genes essential for the preferential induction of apoptosis in tumor cells, we conducted SMART (Supplementary Figure 1). Theoretically, only those cells in which 'death genes' were randomly inactivated by the antisense cDNA library could survive and continue to grow in the presence of HDACi. An antisense cDNA library was initially generated from TSA-treated MCF-7 cells, and electroporated into HeLa cells. After selection with TSA plus hygromycin-B for 4 weeks, the cells yielded some drug-resistant colonies. Under the same

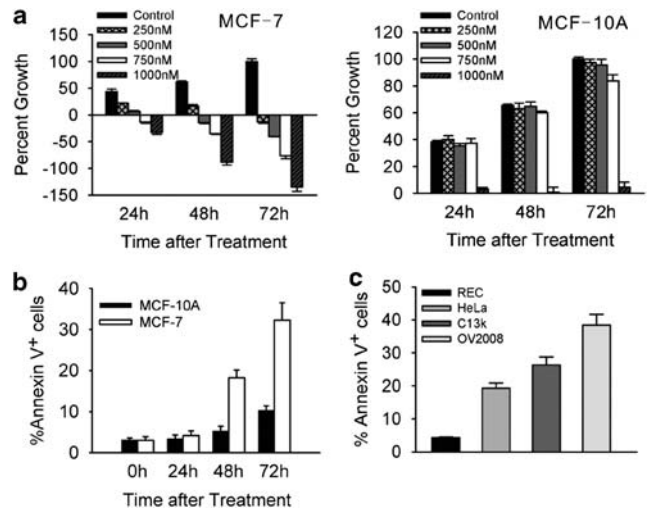


Figure 1 Preferential induction of apoptosis of tumor cells by TSA. (a) MCF-7 and MCF-10A cells were incubated with TSA at a dose ranging between 250 and 1000 nM for the indicated times. At the end of the experiment, the cells were fixed and stained with sulforhodamine B. The absorbance at 570 nm of bound dye was quantified and expressed as a percentage of untreated controls. Each data point is the mean \pm standard error (S.E.) of six replicates. (b) MCF-7 and MCF-10A cells were exposed to 250 nM TSA for the indicated times and then analyzed for apoptosis using FACS analysis. Each data point represents the mean \pm S.E. apoptosis percentage of six replicates. (c) Different tumor cell lines (HeLa, C13k, and OV2008) and non-malignant respiratory endothelial cells (REC) were treated with 250 nM TSA for 72 h, and the apoptosis percentage was determined using FACS analysis

conditions, cells transfected with the control vector yielded no surviving colonies. The episomes were recovered for sequencing. A total 24 cDNA inserts were identified, 6 of which were selected for in a second round of screening to confirm the resistance to TSA (Table 1). Of the six vectors examined, plasmids containing antisense cDNA for UbB displayed the strongest resistance to TSA. The UbB antisense plasmid-contained library HeLa cells that survived the screen were confirmed to be with suppressed expression of UbB mRNA (Supplementary Figure 2A) and profound reduction of free cellular ubiquitin (Supplementary Figure 2B). Transfection of UbB antisense plasmid into HeLa or MCF-7 cells displayed significant resistance to TSA in the presence of hygromycin-B (Figure 2a) and provided long-term survival as defined by clonogenic assays (Supplementary Table 1). To rule out the possible interference of hygromycin-B, HeLa or MCF-7 cells were stably transfected with UbB antisense plasmid and treated with TSA for 4 days, then examined for growth inhibition. Again, the cells displayed significant resistance to TSA treatment compared with HeLa or MCF-7 cells stably transfected with empty vector (Figure 2b). Furthermore, stable transfection of HeLa or MCF-7 with UbB antisense plasmid also made these cells resistant to treatment with apicidine, a structurally diverse HDACi (Figure 2c). To address whether the cells expressing UbB antisense plasmid are also resistant to other cell death triggers, HeLa cells stably transfected with UbB antisense construct were exposed to commonly used chemotherapeutic drugs

Table 1 Positive clones identified from the anti-sense library sorted by their known cellular functions

Clone number	Gene name	Symbol	Activities	Processes involved, known	Confirmation
<i>Gene expression regulation</i>					
2	Thyroid hormone receptor-associated protein 1	THRAP1	Transcriptional co-activator	Induce gene expression	N/A
15	High-mobility group nucleosome-binding domain 1	HMG1	Altering the interaction between the DNA and the histone octamer	Maintains transcribable genes in a unique chromatin conformation	Confirmed
112	Peptidylprolyl isomerase A (cyclophilin A)	PPIA	Encodes a member of the peptidyl-prolyl <i>cis-trans</i> isomerase (PPIase) family.	Folding of proteins	N/A
335	Cold shock domain containing E1, RNA binding	CSDE1	RNA chaperones	IRES-mediated translation	N/A
<i>Protein kinase</i>					
23	Phosphohistidine phosphatase 1	PHPT1	Protein histidine phosphorylation	Protein histidine phosphorylation	N/A
66	Ubiquinol-cytochrome <i>c</i> reductase core protein II	UQCRC2	Protein binding	Oxidative phosphorylation	N/A
130	Serine carboxypeptidase 1	SCPEP1	Serine carboxypeptidase activity	Proteolysis	N/A
<i>Cell signaling related</i>					
38	Homo sapiens gene for SMAD4	SAMD 4	Transcriptional cofactor activity	Regulate cell death and proliferation	Confirmed
42	Mitogen-activated protein kinase kinase kinase 4	MAP4K4	Member of the serine/threonine protein kinase family	Activate MAPK8/JNK and mediate the TNF- α signaling pathway	Confirmed
<i>Cell death/stress related</i>					
46	Prothymosin, α	PTMA	Formation, maintenance, or functioning of the mitotic spindle and chromatin decondensation	Differentiation and progression of human cancers	N/A
54	Heat shock 90 kDa protein 1, β	HSPCB	Molecular chaperones	Cell stress	N/A
58	Tumor rejection antigen (gp96) 1	TRA1	Unknown	Cell death	N/A
64	Cytochrome <i>c</i> oxidase subunit VIa polypeptide 1	COX6A1	Catalyzes the electron transfer from reduced cytochrome <i>c</i> to oxygen	Mitochondria-mediated cell apoptosis	N/A
265	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, γ polypeptide 1	ATP5C1	Encodes a subunit of mitochondrial ATP synthase	Mitochondria-mediated cell apoptosis	N/A
<i>Others</i>					
281	Homo sapiens clone CDABP0036 mRNA sequence	Unknown	Unknown	Unknown	N/A
297	Metastasis-associated lung adenocarcinoma transcript 1	MALAT1	Unknown	Unknown	N/A
301	Transmembrane protein 30A	TMEM30A	Unknown	Unknown	N/A
B1	Zinc-finger protein 28 (KOX 24)	ZNF28	Unknown	Unknown	N/A
27	Solute carrier family 39 (zinc transporter), member 6	LIV-1	An integral plasma membrane protein that transports zinc into cells	Protein, nucleic acid, carbohydrate, and lipid metabolism	Confirmed
16	Homo sapiens chromosome 8, clone RP11-44N12	Unknown	Unknown	Unknown	N/A
21	RAB14, member RAS oncogene family	RAB14	Member RAS oncogene family	Unknown	Confirmed
81	Homo sapiens BAC clone RP11-143I21 from 7, complete sequence	Unknown	Unknown	Unknown	N/A
242	Ubiquitin B	UBB	Encodes ubiquitin protein	Ubiquitin-mediated proteolysis	Confirmed
65	Human DNA sequence from clone RP11-65J3 on chromosome 9	Unknown	Unknown	Unknown	N/A

The positive colonies that have been confirmed describing in 'Materials and Methods' N/A, not available (has not been tested)

(cisplatin, paclitaxel, or etoposide). UbB antisense construct, however, did not reduce the sensitivity of HeLa cells to undergo apoptosis in response to treatment of chemotherapeutic drugs (Supplementary Figure 2C). These data indicated that UbB specifically modulated the tumor cell sensitivity to HDACis but not other cell death triggers.

Next, we examined the influence of TSA on UbB expression in several tumor and non-malignant cell lines. As shown in Figure 2d, transcriptional expression of UbB was highly induced in tumor cells, but not non-malignant cells. The induction of UbB seems to be gene specific, as expression of UbC, another member of ubiquitin gene family, was not

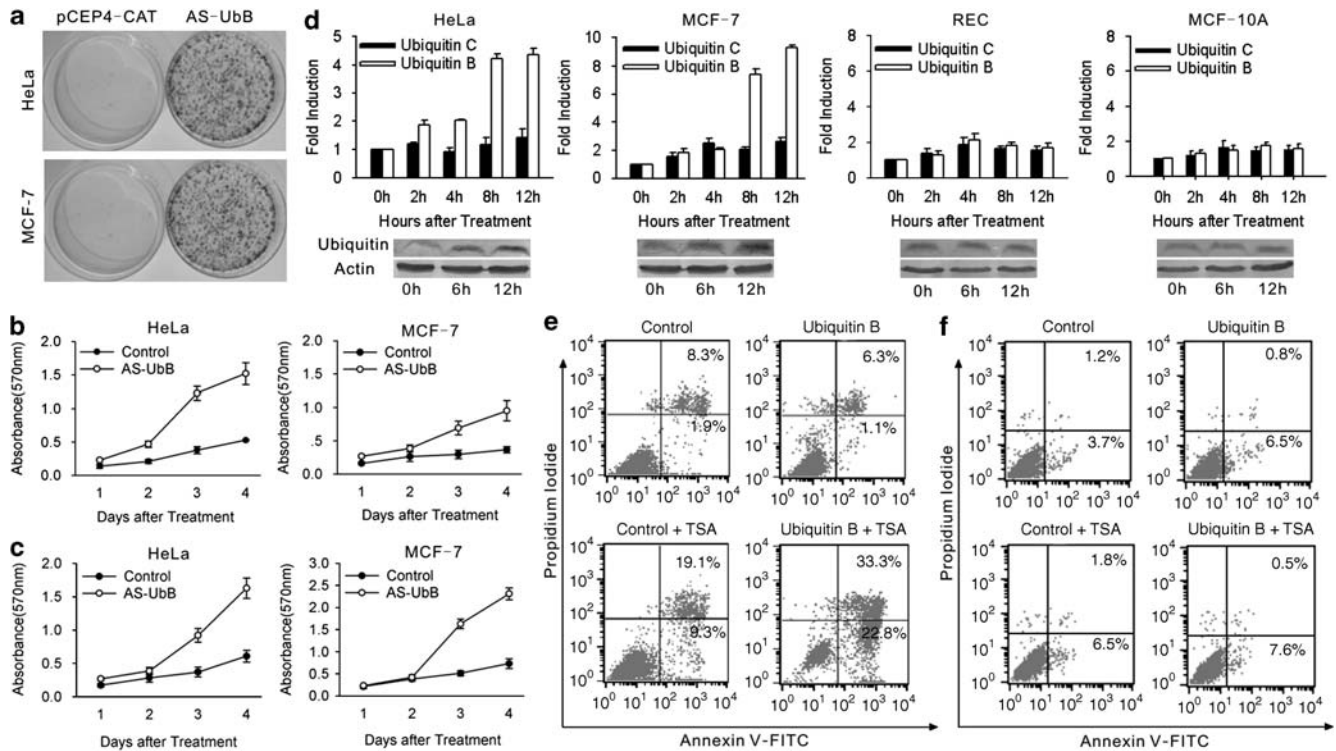


Figure 2 UbB is an authentic mediator in resistance to TSA-induced apoptosis. (a) HeLa or MCF-7 cells were treated as SMART protocol described, and the surviving colonies were fixed and stained with SRB. AS-UbB, pCEP4 carrying antisense UbB cDNA. (b) HeLa or MCF-7 cells were stably transfected with pCEP4-CAT (Control) or AS-UbB and selected with hygromycin-B and reseeded in 96 wells. The cells were then exposed to TSA at a concentration of 500 nM for HeLa or 250 nM for MCF-7. Cell growth was analyzed at the time points indicated on the x axis. Each data point represents the mean \pm S.E. of five replicates. (c) HeLa or MCF-7 cells were treated as described in b, exposed to apicidine at a concentration of 500 nM for HeLa and 250 nM for MCF-7, and analyzed for cell growth. (d) HeLa, MCF-7, REC, or MCF-10A cells were treated with 500 nM TSA for the indicated times and subjected to analysis by real-time PCR for the mRNA levels of UbB and UbC or by western blot for the levels of free cellular ubiquitin protein. Upper panel: results are expressed as fold induction compared with an untreated or 0 h group, and normalized to that of 18s RNA (the untreated or 0 h group being set as 1). Lower panel: the levels of free cellular ubiquitin were detected by western blot. HeLa (e) and REC (f) stably transfected with pcDNA3.1-UbB (ubiquitin) or pcDNA3.1(control) were treated with 500 nM TSA for 48 h, cell apoptosis was analyzed by flow cytometry, values represent the means \pm S.D. for four experiments. The graphs represent typical results of cell apoptosis

induced. Accordingly, elevated level of free cellular ubiquitin protein was induced in tumor cell, but not non-malignant cells. The similar induction of UbB and free cellular ubiquitin was further confirmed by the treatment of apicidine (Supplementary Figure 2D). Interestingly, the induction of UbB mRNA and elevation of free cellular ubiquitin seems to be HDACi specific, with the only exception of UbC mRNA induction by cisplatin, a number of chemotherapeutic drugs failed to induce expression of UbB/UbC mRNA and increase free cellular ubiquitin in HeLa cells (Supplementary Figure 3A). To test whether knockdown of UbC also prevent apoptosis, HeLa cells transfected with UbC siRNA were confirmed to be with reduced expression level of UbC mRNA (data not shown) and subsequently exposed to TSA or chemotherapeutic drugs. UbC siRNA, however, did not reduce the sensitivity of HeLa cells to undergo apoptosis in response to treatment of TSA or chemotherapeutic drugs (Supplementary Figure 3B). To determine whether overexpression of UbB directly induced apoptosis, HeLa, MCF-7, and REC were transiently transfected with UbB expression plasmid and subjected to apoptosis analysis. Although a significant increase in the level of the UbB mRNA and free ubiquitin protein was detected

in these cells (Supplementary Figure 4A and B), no obvious increase in apoptosis was observed (Supplementary Figure 4C). Thus, UbB *per se* is not sufficient to induce apoptosis. We then tested whether UbB sensitized tumor cells to TSA-induced apoptosis. HeLa cells stably transfected with UbB expression plasmid were much more sensitive to TSA-induced apoptosis compared with cells stably transfected with empty vector ($54.9 \pm 5.4\%$ versus $31.8 \pm 3.5\%$, $P < 0.01$, Figure 2e), and the similar effects were also observed in MCF-7 cells ($63.3 \pm 6.2\%$ versus $32.6 \pm 4.7\%$, $P < 0.01$; Supplementary Figure 4D). On the other hand, transfection of UbB expression plasmid did not sensitize REC to TSA treatment ($9.1 \pm 1.6\%$ versus $8.9 \pm 0.9\%$, $P > 0.05$, Figure 2f). Therefore, overexpression of UbB sensitized tumor cells to TSA. More importantly, it seems that induced expression of UbB and consequently elevated level of free cellular ubiquitin protein are indispensable for TSA-induced apoptosis.

RNAi knockdown of UbB gene suppressed TSA-induced loss of $\Delta\Psi_m$ and apoptosis in HeLa cells. Next, we examined the effects of UbB depletion on the apoptotic cascade induced by TSA. UbB siRNA (Supplementary

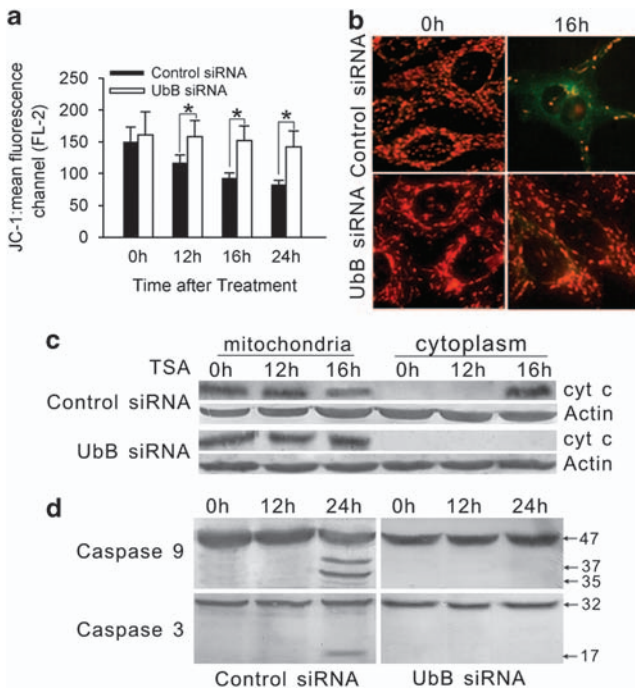


Figure 3 Knockdown of UbB expression suppresses TSA-induced loss of $\Delta\Psi_m$ and the apoptotic cascade. (a) HeLa cells transfected with UbB siRNA or control siRNA were treated with 500 nM TSA and subjected to determination of $\Delta\Psi_m$ as defined by fluorescence of J-aggregates emitting at 590 nm. Data represent the mean of triplicates at each time point. * $P < 0.01$, HeLa transfected with UbB siRNA versus those transfected with control siRNA. (b) HeLa cells were treated as described in a; the panel shows representative images of JC-1-stained cells 16 h after treatment with 500 nM TSA. (c) HeLa cells were transfected with UbB siRNA or control siRNA and treated with 500 nM TSA for the indicated times. The cytosolic and mitochondrial fractions of pretreated cells were then analyzed for levels of cytochrome c. (d) HeLa cells were treated as described in c and analyzed for cleavage of caspases-3 and -9

Figure 4E) was confirmed to significantly knockdown the basal and TSA-induced level of UbB mRNA in HeLa cells (Supplementary Figure 4F) and knockdown effects of UbB siRNA was confirmed in REC (Supplementary Figure 4G and H). Accordingly, levels of free cellular ubiquitin were also significantly reduced by UbB siRNA in HeLa and REC cells (Supplementary Figure 4I). Considering the mitochondrial outer membrane permeabilization (MOMP) centers in the apoptotic process, we examined the effects of UbB depletion on the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) induced by TSA. Although TSA induced a substantial dissipation of $\Delta\Psi_m$, knockdown of UbB expression significantly blocked this event (Figure 3a and b). Consistent with this finding, significant release of cytochrome c into the cytosol was detected in HeLa cells 16 h post TSA treatment. After 24 h, TSA treatment caused a subsequent cleavage of caspases-3 and -9. However, knockdown of UbB transcription appeared to block the TSA-induced release of cytochrome c into the cytosol (Figure 3c) and the subsequent proteolytic cleavage of caspases-3 and 9 (Figure 3d). These data show that the induction of UbB is essential for TSA-induced MOMP and the subsequent apoptotic cascade.

TSA-induced apoptotic cascade is mediated by ubiquitylation of mitochondrial proteins, which is inhibited by knockdown of UbB. In light of the finding indicating that UbB has an essential role in TSA-induced MOMP, we tested whether TSA and UbB affect ubiquitylation and the subsequent degradation of mitochondrial proteins. Treatment of REC with TSA did not induce increase in polyubiquitylation of total mitochondrial protein (Figure 4a). In contrast, TSA induced significant polyubiquitylation of total mitochondrial protein in HeLa cells. Strikingly, TSA-induced polyubiquitylation was abolished by knocking down UbB expression, indicating the level of UbB gene expression is required for the process (Figure 4b). Next, we chose several mitochondrial proteins, which are pivotal in the control of MOMP, for further study. Treatment with TSA reduced endogenous levels of BCL-2 and MCL-1, but not apoptosis inducing factor (AIF), Bcl-X_L, voltage-dependent anion channels (VDAC), or adenine nucleotide translocator (ANT). On the other hand, the expression of pro-apoptosis gene BimEL (the most abundant isoform of Bim) and Bax was increased moderately on TSA treatment. The MCL-1 and BCL-2 reduction could be abolished by the proteasome inhibitor MG132 or UbB siRNA, implying proteasomal degradation is responsible for the action of TSA, and the induction of UbB gene expression is required for the process (Figure 4c). In contrast, no obvious change in the expression levels of the above mitochondria proteins was observed in REC treated by either TSA, MG132, or UbB siRNA (Supplementary Figure 5A). To further clarify this, HeLa cells were transfected with either UbB or control siRNA, and treated with TSA, total mitochondria proteins from the cells were then extracted for subsequent polyubiquitylation analysis. BCL-2 or MCL-1 protein was immunoprecipitated with antibody against BCL-2 or MCL-1 and examined for polyubiquitylation with the anti-ubiquitin antibody. Significant polyubiquitylation of MCL-1 protein was detected in TSA-treated HeLa cells. Obviously, the TSA-induced polyubiquitylation of MCL-1 protein was abolished by transfection of UbB siRNA (Figure 4d). Similarly, on TSA treatment, BCL-2 polyubiquitylation was also detected in HeLa cells (Supplementary Figure 5B). In the same experiment, polyubiquitylation of MCL-1 was not induced in REC by TSA treatment (Supplementary Figure 5C). On the other hand, treatment of TSA induced enhanced transcription of MCL-1 and Bim, the levels of BCL-2 and Bcl-X_L mRNA were not affected by TSA treatment in HeLa cells (Supplementary Figure 5D). Furthermore, in the presence of cycloheximide (CHX), a protein synthesis inhibitor, the half-life of MCL-1 protein was significantly shortened by treatment of TSA (Figure 4e). MCL-1 protein exhibited a half-life of 48 min in HeLa cells in the absence of TSA. Addition of TSA accelerated the half-life of MCL-1 protein to 26 min (Figure 4f). However, the accelerated half-life of MCL-1 protein induced by TSA was reverted by UbB siRNA to 46 min (Figure 4g), indicating the degradation of MCL-1 protein occurs through an ubiquitin/proteasomal mechanism and the process requires the participation of UbB. To address whether degradation of MCL-1 is critical for the TSA-induced apoptotic cascade, we examined the effects of depletion or overexpression of MCL-1 on the action of TSA.

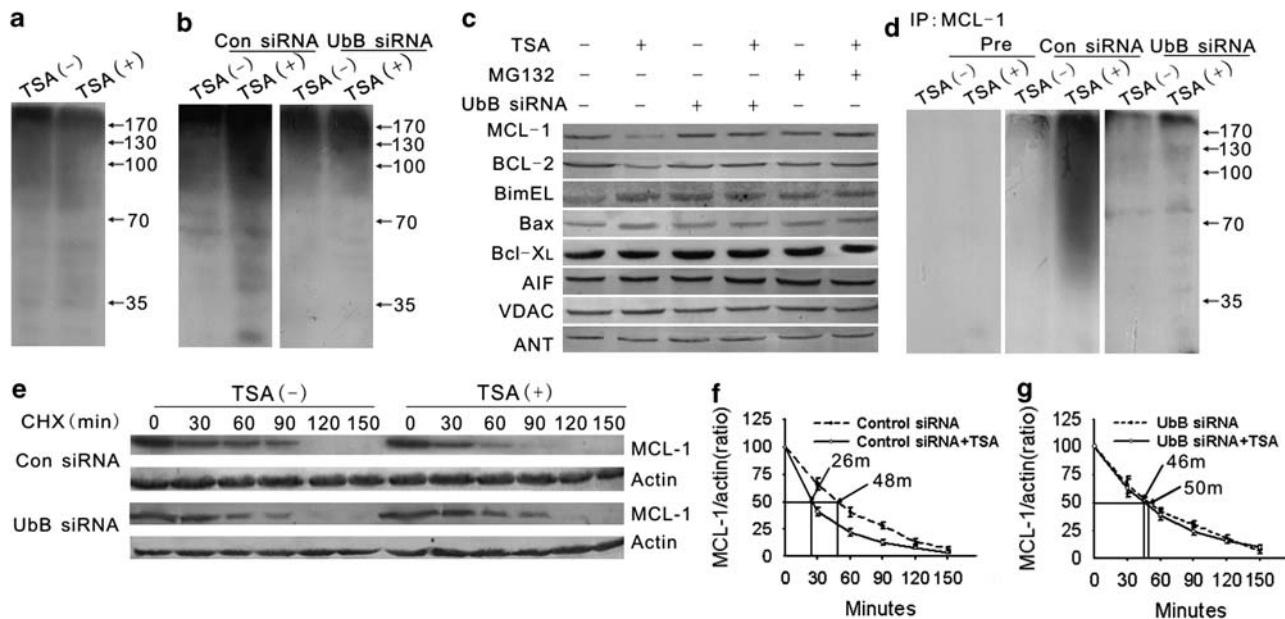


Figure 4 TSA induced UbB-dependent degradation of mitochondrial proteins through an ubiquitin/proteasomal mechanism. (a) REC cells were treated with 500 nM TSA or PBS for 12 h and the proteasome inhibitor MG132 (20 μ M) was added 6 h before cell collection. Total 80 μ g mitochondrial proteins were isolated and analyzed for ubiquitylation using anti-ubiquitin antibody. (b) HeLa cells transfected with UbB siRNA or control siRNA were treated as described in a and analyzed for ubiquitylation of mitochondrial proteins. Con siRNA, control siRNA. (c) HeLa cells were treated as described in a, collected for extraction of mitochondrial proteins, and analyzed for MCL-1, BCL-2, BimEL, Bax, Bcl-X_L, AIF, VDAC, and ANT protein levels. (d) HeLa cells were treated as described in a, 200 μ g of total mitochondria protein was subjected to immunoprecipitation using anti-MCL-1 antibody. The immunoprecipitates were subjected to western blot analysis with anti-ubiquitin antibody. Con siRNA, control siRNA. (e) HeLa cells transfected with UbB siRNA or control siRNA were preincubated for 1 h with 500 nM TSA or PBS. The cells were then incubated with 200 μ g/ml of the protein synthesis inhibitor cycloheximide (CHX) for the indicated times. The cells were collected and subjected to western blot analysis for protein levels of MCL-1. MCL-1 protein half-life of control siRNA transfected cells (f) and UbB siRNA transfected cells (g) was determined, the graph represents MCL-1/actin ratio levels as measured by densitometry, MCL-1 levels at time 0 h were calculated as 100%, and data averaged from three experiments are shown. Error bars represent standard deviation

RNA interference dramatically knocked down the endogenous MCL-1 (Figure 5a). Furthermore, depletion of MCL-1 sensitized HeLa cells to TSA-induced apoptosis (Figure 5b) and loss of $\Delta\Psi$ m (Figure 5c and d). On the other hand, overexpression of MCL-1 could effectively rescue cells from TSA-induced apoptosis ($23.2 \pm 4.5\%$ versus $42.4 \pm 3.1\%$, $P < 0.01$, Figure 5e and f).

As overexpression of UbB alone is not sufficient to induce apoptosis (Supplementary Figure 4C) and degrade MCL-1 (Supplementary Figure 5E), extra modulators must be required for the process of cell death. Considering the chaperone functions of heat shock protein 90 (HSP90) is affected by acetylation and critical for the stability of many client proteins, we sought to determine whether HSP90 and UbB cooperated to modulate the degradation of MCL-1 and sensitivity of apoptosis. Interestingly, 17-allylamino 17-demethoxygeldanamycin (17-AAG), a HSP90 inhibitor, selectively degraded MCL-1 in HeLa but not in non-malignant cells. Although overexpression/knockdown of UbB alone did not affect the protein levels of MCL-1, the 17-AAG-induced degradation of MCL-1 could be abolished by UbB siRNA (Supplementary Figure 5E). Furthermore, 17-AAG-induced G2/M cell-cycle arrest and apoptosis in HeLa cells could be profoundly reverted by UbB siRNA (Supplementary Figure 5F–H). These data strongly imply that HSP90 and UbB cooperate to mediate the degradation of MCL-1 and apoptosis.

The action of TSA could be mimicked by other HDAC inhibitors and TSA-induced degradation of other oncogenic proteins is dependent on expression of UbB. We have shown that TSA induces apoptotic cascade through an unknown UbB-dependent proteasomal degradation of mitochondria proteins. Next, we tested whether the action is TSA specific, or is shared with other HDAC inhibitors, and whether TSA induces an UbB-dependent proteasomal degradation of oncogenic proteins other than mitochondrial proteins. Similarly, apicidine reduced the endogenous levels of BCL-2 and MCL-1 but not Bim, Bax, Bcl-X_L, AIF, VDAC, and ANT in HeLa cells (Figure 6a). Again, the reduction could be abolished by MG132 and UbB siRNA, indicating UbB-dependent proteasomal degradation of mitochondrial protein might be a common mechanism of action shared by other HDAC inhibitors. Previously, some investigators had shown the proteasomal degradation of BCR–ABL fusion protein after HDACi treatment.¹² To address whether expression of UbB is required for TSA-induced proteasomal degradation of BCR–ABL, the leukemia cell line, K562 was treated with TSA and examined for the reduction of BCR–ABL fusion protein. TSA reduced the level of BCR–ABL but not MCL-1 protein in a time- and dose-dependent manner in K562 cells (Figure 6b). Apparently, reduction of BCR–ABL was not because of inhibition of BCR–ABL at transcriptional level (Figure 6c). Although expression of UbB mRNA was not

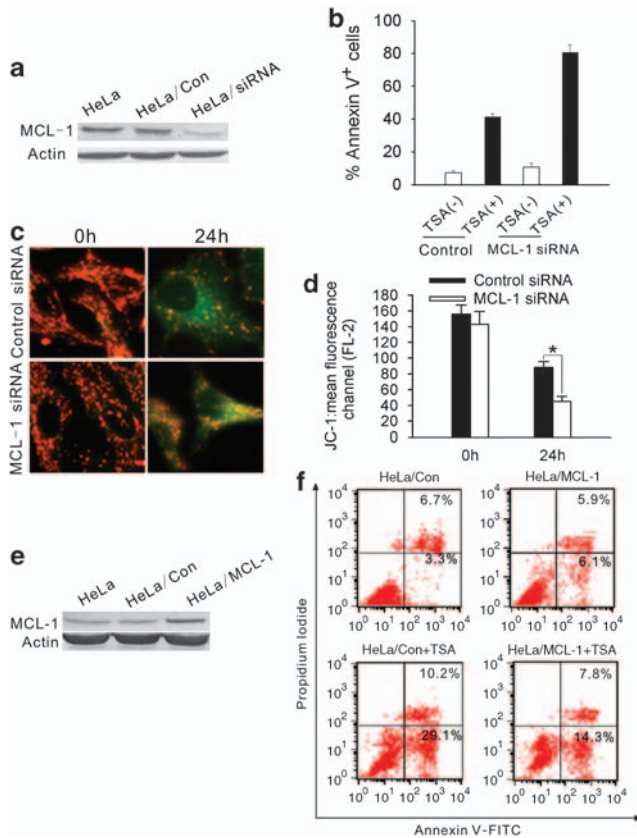


Figure 5 MCL-1 is a critical regulator of TSA-induced loss of $\Delta\Psi_m$ and apoptosis. (a) HeLa cells were transfected with MCL-1 or control siRNA. The levels of MCL-1 in whole-cell lysates were determined by western blotting. HeLa/Con, HeLa cells transfected with control siRNA; HeLa siRNA, HeLa cells transfected with MCL-1 siRNA. (b) HeLa cells were transfected with MCL-1 siRNA and exposed to 500 nM TSA for 48 h. The cells were then stained with propidium iodide and annexin V-FITC, and analyzed by flow cytometry. Results are presented as the means \pm S.D. of triplicates. (c) HeLa cells transfected with control or MCL-1 siRNA were treated with 500 nM TSA for 24 h, and then stained with JC-1; the panel shows representative photomicrographs of JC-1-stained cells. (d) Quantitation of the percentage of cells staining positive for J-aggregates was analyzed by flow cytometry. * $P < 0.05$, HeLa cells transfected with MCL-1 siRNA versus with control siRNA. (e) V5-tagged MCL-1 or empty control vector was introduced into HeLa cells. Three days after transfection, MCL-1 was determined by western blot. HeLa/Con, HeLa cells transfected with pcDNA3.1/V5-His-TOPO; HeLa/MCL-1, HeLa cells transfected with pcDNA3.1-V5tagged MCL-1. (f) HeLa cells were treated as described in e and were exposed to 500 nM TSA for 48 h, cell apoptosis was analyzed by flow cytometry, and values represent the means \pm S.D. of four experiments. The graphs represent typical results of cell apoptosis

induced, level of free cellular ubiquitin significantly increased after TSA treatment (Figure 6d). Again, the reduction of BCR-ABL protein was blocked by UbB siRNA or MG132 (Figure 6e), indicating the proteasomal degradation of oncogenic protein other than mitochondrial proteins also requires the participation of UbB. Notably, K562 cells were resistant to TSA-induced apoptosis in the experiment (Figure 6f), and in contrast to previous TSA-sensitive cell lines, upregulation of free cellular ubiquitin in K562 failed to degrade MCL-1 protein, indicating these two events could be dissociated.

Discussion

Significant advances in clinical trials involving the use of HDACis have been achieved; nevertheless, the mechanism of action of these agents is not yet well understood. One of the key questions is whether there are universal, essential targets responsible for the tumor-selective action of HDACis.¹³ Identification of these putative tumor-associated 'death programs' selectively activated by HDACis is fundamentally important.¹⁴ To address this, we conducted a SMART analysis on a genome-wide scale in this study. The obvious advantage of the current approach is that it allows potential effectors pivotal to TSA-induced apoptosis to be identified in an unbiased and random manner. Twenty-four positive hits were identified by virtue of the induced resistance to TSA-induced apoptosis. These genes provide interesting primary data for a deeper understanding of the mechanism of action of HDACi and the critical molecular events leading to tumor cell survival.

One of the most significant hits is UbB, one of the ubiquitin gene family members. Ubiquitin is a small, highly conserved protein expressed in all eukaryotic cells, and can be covalently linked to certain target proteins to mark them for degradation by the ubiquitin-proteasome system (UPS). The abundance of cellular ubiquitin is in a dynamic balance, which is ultimately maintained by *de novo* synthesis of ubiquitin from ubiquitin gene transcripts. Human ubiquitin is encoded by a family of multiple genes, composed of UbA₅₂, UbA₈₀, UbB, and UbC, of which UbB and UbC are inducible by various cell stresses.¹⁵⁻¹⁸ Up to now, the role of UPS in apoptosis has been extensively investigated, and ubiquitin-activating enzyme (E1), ubiquitin conjugating enzymes (E2s), ubiquitin ligases (E3s), and proteasomes have been well characterized.¹⁹ On the other hand, knowledge of the role of the ubiquitin gene family in regulating various cellular functions has been generally poor and overlooked owing to the assumption that these genes would not determine the specificity of ubiquitylation. One of the first reports linking the process of ubiquitylation with apoptosis emerged from studies of intersegmental muscle programmed cell death in the tobacco hawkmoth, *Manduca Sexta*.²⁰ Further evidence for the involvement of ubiquitin genes in apoptosis came from studies of murine lymphocytes treated with teniposide.²¹ However, the finding that HDACi activates UbB and favors apoptosis by elevating the UbB transcription and level of free cellular ubiquitin selectively in tumor cells has not been recognized earlier. Indeed, identification of UbB as the essential regulator in selective apoptosis of tumor cells is somehow unanticipated in that UbB was believed to be ubiquitously abundant in both normal and tumor cells. Subsequent to the selective induction of UbB, several mitochondrial proteins pivotal to the control of MOMP were degraded through activation of the UPS in tumor cells. Obviously, TSA-induced proteasomal degradation of mitochondrial proteins is gene specific, rather than a global effect because not all mitochondrial proteins examined were affected. Notably, the action of UbB could be duplicated in various tumor cell types using structurally diverse HDAC inhibitors but not commonly used chemotherapeutic drugs, indicating UbB probably represents one of the universal key targets responsible for HDACi-induced selective apoptosis in various tumors.

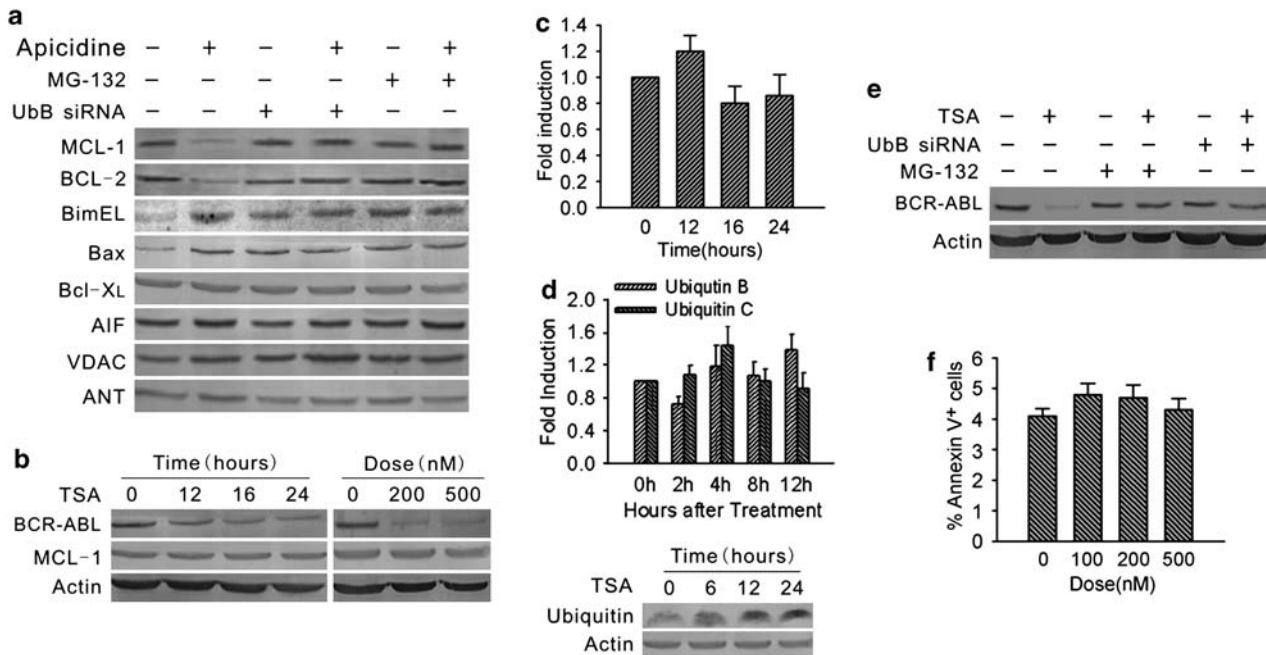


Figure 6 HDAC inhibitor apicidine mimics the action of TSA, and TSA induces degradation of BCR-ABL fusion protein is dependent on expression of UbB. (a) HeLa cells were transfected with UbB or control siRNA, treated with 500 nM apicidine for 12 h, and MG 132 (20 μ M) was added 6 h before cell collection. Mitochondrial protein was extracted and examined for the protein levels of genes as indicated. (b) K562 cells were exposed to 200 nM TSA for the indicated times (left panel), or the indicated dose of TSA for 16 h (right panel). Protein levels of BCR-ABL and MCL-1 in K562 cells were determined by western blot analysis. (c) K562 cells were incubated with 500 nM TSA for the indicated times and subjected to analysis of BCR-ABL mRNA by real-time PCR. Values were expressed as fold induction compared with the untreated or 0 h group. Error bars represent the S.E.M. of five replicates. (d) K562 cells were treated with 500 nM TSA for the indicated times. Levels of UbB/UbC mRNA and free cellular ubiquitin protein were examined. Upper panel: levels of UbB/UbC mRNA are expressed as fold induction compared with the untreated or 0 h group. Lower panel: the levels of free cellular ubiquitin were detected by western blot. (e) K562 cells transfected with UbB or control siRNA were treated with 500 nM TSA for 16 h, and MG 132 (20 μ M) was added 6 h before cell collection. Protein levels of BCR-ABL in K562 were determined by western blot. (f) K562 cells were exposed to TSA for 16 h then subjected to apoptosis analysis. Error bars represent the S.E.M. of five replicates

Recently, accumulating evidence suggested that HDACs may regulate apoptosis by decreasing the half-life of several oncogenic proteins through activating the proteasomal degradation pathway.^{22,23} However, whether the proteasomal degradation of oncogenes represents a general mechanism central to HDACi-induced apoptosis was not systematically evaluated on a genome-wide scale. Thus, our present finding that UbB works as a universal key target responsible for HDACi-induced selective apoptosis in tumor cells is a novel and important concept. Up to now, it is not known that the exact mechanism whereby HDACi could selectively activate expression of UbB, and subsequently target only key anti-apoptotic mitochondrial proteins for proteasomal degradation in tumor but not in non-malignant cells. In our hands, HSP90 and UbB cooperate to mediate the degradation of MCL-1 and apoptosis in HeLa cells, indicating HSP90 and acetylation-dependent release of client proteins might have functions in the process.^{24–26} In contrast, our data in K562 clearly highlight that in the leukemia cell line TSA instead induces the UbB-dependent degradation of BCR-ABL but not MCL-1. The lack of MCL-1 degradation seems to correlate with the resistance of these cells to HDACi-induced apoptosis. These findings might suggest BCR-ABL is a more abundant/critical HSP90 client protein than MCL-1 in K562 cells. Most recently, an independent group had used a genome-wide loss-of-function screen to identify HR23B, which shuttles ubiquitinated cargo

proteins to the proteasome, as a sensitivity determinant for HDAC inhibitor-induced apoptosis.²⁷ These findings strongly imply aberrant profiles of components mediating proteasomal degradation pathway including HSP90, HDAC6, HR23B, and UbB proposed by us would be cooperated to form the molecular basis responsible for the HDACi selectivity. Our study is now underway to test the hypothesis.

Materials and Methods

Cells and constructs. MCF-7 cells were cultured in phenol red-free Eagle's minimal essential medium supplemented with 5% charcoal-stripped fetal bovine serum and 10^{-11} M estradiol while being treated with TSA. Cells were grown in phenol red-free medium 24 h before treatments were initiated. The breast epithelial cell line, MCF-10A, was obtained from ATCC (Manassas, VA, USA) and cultured in DMEM/F12 supplemented with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% horse serum. A cisplatin-sensitive ovarian cancer cell line (OV2008) and its resistant variant (C13K) were gifts from Dr. Rakesh Goel from the Ottawa Regional Cancer Center, Ottawa, Canada and cultured as described earlier.²⁸ Primary normal RECs were isolated from freshly excised human nasal polyp tissue using the protease method.²⁹ The proteasome inhibitor, MG132, was from Calbiochem (San Diego, CA, USA); CHX, TSA, and apicidine were from Sigma (St. Louis, MO, USA). Constructs encoding full-length MCL-1 were kindly provided by Ulrich Maurer (Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität, Freiburg, Germany). The pOTB7-UbB was from Proteintech Group, Inc. (Chicago, IL, USA) and subcloned into pcDNA3.1 (+). Antibodies to MCL-1, AIF, ANT, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); to Bax and cytochrome c were purchased from Neomarker (Neomarker, Fremont, CA, USA); to VDAC were

purchased from Biovision (Biovision, Mountain View, CA, USA); to Bim, BCL-2, Bcl-X_L, BCR-ABL, caspases-3 and -9 were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA).

Cell growth assay. Pretreated cells were plated in 96-well plates and cell growth was monitored following our previous description.³⁰ Briefly, the cells were fixed with 10% trichloroacetic acid (TCA) and were stained with 0.4% sulforhodamine B (SRB, Sigma) in 1% acetic acid for 1 h at the end of the experiment. One control plate was fixed with TCA 8 h after plating to determine the absorbance representing the starting cell number. When expressed as a percentage of the value for untreated controls, an increase in cell number falls on the positive scale and a decrease in cell number appears on the negative scale. Annexin-V binding assays were performed using a commercially available kit (PharMingen, San Diego, CA, USA) according to the manufacturer's recommendation.

Suppression of mortality by antisense rescue technique. Construction of an antisense cDNA library and subsequent analysis using SMART were performed following protocols described elsewhere with some modifications.¹⁰ Initially, at 0, 8, 12, 24, 36, and 48 h post treatment with 250 nM TSA, MCF-7 cells were collected and total mRNA was extracted for construction of antisense cDNA libraries by random insertion of cDNA into the mammalian replication permissive plasmid, pCEP4. Forty micrograms of library DNA containing approximately 2×10^6 cDNA inserts of various sizes was electroporated into 1×10^7 HeLa cells. The HeLa cells transfected with the cDNA library were then selected by incubation with 150 nM TSA in the presence of 200 μ g/ml hygromycin-B for 4 weeks. Parallel plates of cells transfected with pCEP4-CAT were similarly selected. The culture medium was changed, and TSA was re-added daily for the first week, and then every other day for the remaining weeks. At the end of 4 weeks of selection, the surviving colonies were pooled and amplified in the presence of hygromycin-B (200 μ g/ml) for isolation of plasmids using the Hirt DNA extraction method.³¹ The isolated DNA was digested with Dpn I and electroporated into *Escherichia coli* DH10B. The resultant colonies were picked up and screened by restriction enzyme digestion to detect the presence of inserts. The individual plasmids with inserted antisense cDNA were subjected to a second round of selection to confirm the resistance to TSA treatment. The cells were then selected with TSA (150 nM) and hygromycin-B (200 μ g/ml) for 20 days. Episomes that were confirmed to be TSA resistant were chosen for DNA sequencing and further characterization.

Determination of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Pretreated cells were stained with JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide; Molecular Probes, Eugene, OR, USA), analyzed by flow cytometry, and visualized as described earlier.³²

Subcellular fractionation. Cells were lysed in mitochondria lysis buffer [210 mmol/l mannitol, 70 mmol/l sucrose, 10 mmol/l HEPES (pH 7.4), 1 mmol/l EDTA, and protease inhibitor cocktail] with a Dounce homogenizer and subjected to centrifugation at $1000 \times g$ to pellet nuclei. Postnuclear supernatant was centrifuged at $10\,000 \times g$ to pellet the mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged to obtain the cytosolic fraction.

Half-life assay. HeLa cells were plated in 100 mm plates, the cells were preincubated for 1 h with 500 nM TSA, then treated with 120 μ g/ml CHX for 0, 15, 30, 60, 90, 120, and 150 min. The cells were collected, and 50 μ g of protein lysate was separated on a 12% SDS-PAGE, transferred to membranes, and probed with anti-MCL-1 polyclonal antibodies. The protein levels were calculated as mentioned above, and the values of MCL-1 were plotted against the values of actin. The values of MCL-1/actin at time 0 were taken as 100%.

RNA interference. Annealed, purified, and desalted double-stranded siRNA MCL-1 (AAG AAA CGC GGU AAU CGG ACU) and Ubb (UGG GCA CUG CGA AUG CCA UGA CUG A; AAU GGC UAU AGU GCA GAG UAA UGC C; UUU GAA CAG GUU CAG CUA UUA CUG A) and UbC (GGU GAA CGC CGA UGA UUA U) and control siRNA were ordered from Invitrogen Corporation (Carlsbad, CA, USA). Then, 1.5×10^5 cells were plated per well in a 6-well dish on day 0. On day 1, the cells were transfected with 200 nM siRNA in Opti-MEM medium (Invitrogen) without FBS using Lipofectamine 2000 reagent (Invitrogen).

Real-Time PCR. Quantitative PCR was performed using the SYBR Green Real Time PCR method. Each sample was tested in triplicate, and relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method,³³ and the results expressed as fold induction compared with the untreated group. PCR was performed with a 5' sense primer (5'-GGT GAG CTT GTT TGT GTC CCT GT-3') and a 3' antisense primer (5'-TCC ACC TCA AGG GTG ATG GTC-3') for Ubb; a 5' sense primer (5'-TGC ACC TGG TAC TCC GTC TCA-3') and a 3' antisense primer (5'-CAG TGA GTG TCT TCA CGA AGA TTT G-3') for UbC; a 5' sense primer (5'-GTG GAG TTC TTC CAC GTA CAG GA-3') and a 3' antisense primer (5'-AGC AAC ACC CGC AAA AGC-3') for MCL-1; a 5' sense primer (5'-CGT GTG TGA AAC TCC AGA CTG TCA-3') and a 3' antisense primer (5'-CTT CAG CGG CCA GTA TCT-3') for BCR-ABL. A 5' sense primer (5'-AGC ACC CAT GAG TTG TGA CAA ATC-3') and a 3' antisense primer (5'-CGT TAA ACT CGT CTC CAA TAC GC-3') for Bim; a 5' sense primer (5'-GTA AAC TGG GGT CGC ATT GT-3') and a 3' antisense primer (5'-TGC TGC ATT GTT CCC ATA GA-3') for Bcl-X_L; a 5' sense primer (5'-TCG CCC TGT GGA TGA CTG AG-3') and a 3' antisense primer (5'-CAG AGT CTT CAG AGA CAG CCA GGA-3') for BCL-2; To amplify 18s RNA internal control, a 5' sense primer (5'-AGT CCC TGC CCT TTG ACA CA-3') and a 3' antisense primer (5'-GAT CCG AGG GCC TCA CTA AAC-3') were used.

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Conflict of Interest

The authors declare no conflict of interest.

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