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CREB-Binding Protein Is a Mediator of Neuroblastoma Cell Death Induced By the Histone Deacetylase Inhibitor Trichostatin A[1]

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Abstract

The cytotoxic mechanism of the histone deacetylase inhibitor (HDACI) Trichostatin A (TSA) was explored in a neuroblastoma (NB) model. TSA induces cell death in neuroblastic-type NB cells by increasing the acetylation of Ku70, a Bax-binding protein. Ku70 acetylation causes Bax release and activation, triggering cell death. This response to TSA depends on the CREB-binding protein (CBP) acetylating Ku70. TSA-induced cell death response correlates with CBP expression. In stromal-type NB cell lines with low levels of CBP and relative resistance to TSA, increasing CBP expression disrupts Bax–Ku70 binding and sensitizes them to TSA. Reducing CBP expression in neuroblastic cell types causes resistance. Cytotoxic response to TSA is Bax-dependent. Interestingly, depleting NB cells of Ku70 also triggers Bax-dependent cell death, suggesting that conditions that leave Bax unbound to Ku70 result in cell death. We also show that CBP, Ku70, and Bax are expressed in human NB tumors and that CBP expression varies across cell types comprising these tumors, with the highest expression observed in neuroblastic elements. Together, these results demonstrate that CBP, Bax, and Ku70 contribute to a therapeutic response to TSA against NB and identify the possibility of using these proteins to predict clinical responsiveness to HDACI treatment.

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Introduction

Protein function can be regulated by posttranslational modifications, including phosphorylation, sumoylation, ubiquitination, and acetylation. The acetylation status of proteins is regulated by two groups of enzymes: histone acetyltransferases (HATs), including CREB-binding protein (CBP) and p300, and histone deacetylases (HDACs). Despite the nomenclature, both HATs and HDACs act on nonhistone proteins as well as on histones [1], and protein acetylation is involved in the regulation of nearly every major cellular process. As such, activators and inhibitors of HATs and HDACs are important to understanding molecular regulation and are of increasing interest as therapeutic agents. For example, inhibitors of HDACs have been approved for clinical use as antitumor agents [2]. Despite evidence supporting their efficacy in several types of cancer, molecular mechanisms linking HDAC inhibition to effects on cell survival and growth are not understood. Consequently, the HDAC sensitivity or resistance of tumors cannot be rationally predicted.

Because histones and transcription factors were the first proteins to be identified as HDAC substrates, gene transcription and chromatin remodeling were the initial foci of experiments studying the antitumor mechanisms of histone deacetylase inhibitors (HDACIs) [3]. However, we have provided strong evidence for a non–chromatin-based mechanism, involving Ku70 protein, that accounts for antitumor proapoptotic response in neuroblastoma (NB) tumor cells [4]. In neuroblastic NB cell lines (N-type) analogous to the predominant tumor cell type in NB tumors, about 50% of Ku70 protein is localized in the cytosol where it is bound to Bax, a multidomain proapoptotic member of the Bcl-2 family of proteins [5]. Ku70 was originally characterized as an autoantigen protein [6,7], which is essential for the repair of nonhomologous DNA double-strand breaks as part of the DNA-binding component of DNA-dependent protein kinase [8,9]. Distinct from this biochemical activity, Ku70 regulates tumor cell survival through its association with Bax [10]. The carboxy-terminal region of Ku70, which binds Bax, contains a domain that interacts with HATs (CBP and p300/

Abbreviations: CBP, CREB-binding protein; HAT, histone acetyltransferase; HDACI, histone deacetylase inhibitor; NB, neuroblastoma; TSA, Trichostatin A

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CBP–associated factor) [10]. Specific residues in this domain can be acetylated (lysine-539 and lysine-542), preventing or disrupting Bax binding. Treating cells with Trichostatin A (TSA; an inhibitor of class I/II HDACs) or nicotinamide (a class III HDACI) increases the acetylation of these residues [10], disrupts the Ku70–Bax complex, and sends Bax to the mitochondria to trigger apoptotic cell death. Thus, it appears that the balance of acetylation on these key regulatory sites is, at least partially, determined by HDACs [11]. Moreover, high levels of Ku70 expression are observed in N-type cell lines. Although this may be a factor that limits cellular sensitivity to radiation and certain types of chemotherapy, the cellular sensitivity of N-type cell lines to HDACIs appears instead to be directly proportional to Ku70 expression.

In addition to neuroblastic cells, NB tumors contain a second tumor-derived or tumor-associated cell population, stromal-type (S-type) cells. Whereas N-type cell lines express N-myc protein and neuronal markers, represent a larger fraction of total tumor cells in high-risk tumors, and are tumorigenic in xenografts, S-type cell lines do not express N-myc, express stromal cell markers, are not tumorigenic in mice, and are associated with less malignant tumor behavior. We conducted experiments to determine if the response of S-type NB tumor cell lines to TSA was similar to that of N-type cell lines. Interestingly, S-type NB cells are significantly less sensitive to this HDACI. This lack of responsiveness is seen both with overall cell survival and at the mechanistic level of disruption of the Bax–Ku70 complex. Comparing S-type cell lines with N-type cell lines showed that CBP is differentially expressed (higher in N-type than S-type) so as to potentially account for differential responsiveness. When CBP expression was increased in S-type cell lines, TSA treatment results in cell death mediated by Bax release that depends on acetylation-sensitive sites in Ku70. When CBP expression in N-type cell lines was reduced, cells became relatively resistant to TSA treatment. Reducing Bax expression in these cells, like decreasing CBP, similarly reduced their sensitivity to treatment with this HDACI. Conversely, reducing Ku70 levels in these cells caused cell death even without HDACI treatment. This was likewise dependent on Bax expression. Together with data presented on the expression patterns of CBP, Ku70, and Bax in a collection of human NB tumor specimens, our results showing that these proteins together mediate cell death in response to TSA in NB cells provide evidence that these proteins are potential biomarkers for predicting and monitoring treatment responsiveness to HDACIs in NB tumors.

Materials and Methods

Cell Culture and Cell Transfection

Human NB cell lines SH-SY5Y, IMR32, SH-EP1, SK-N-AS, GOTO, SMS-KCN-69n, LA1-55n LA1-5s, SH-310, and WSN were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified 5% CO_2 incubator. For transient transfection, SH-EP1 cells were transfected with the vector pcDNA3-CBP-FLAG or pcDNA3-p300-FLAG, with or without pCMV2B-Ku70 wild-type FLAG or pCMV2B-Ku70 K539/542R-FLAG, and a green fluorescence protein (GFP) expression vector (Amaxa, Gaithersburg, MD) using Nucleofector kit V (Amaxa). Briefly, immediately before transfection, 2×10^6 cells were trypsinized, washed with phosphate-buffered saline, and then resuspended in Nucleofector V solution containing 5 μg of indicated plasmids along with 0.5 μg of the GFP vector to a final concentration of 3×10^6 to 4×10^6 cells/100 μl. Samples were subjected to nucleofection using the Nucleofector device at settings recommended by the manufacturer for SH-SY5Y cells (Amaxa). After transfection, cells were resuspended in prewarmed MEM and plated. Twenty-four hours after transfection, the efficiency of transfection was determined by GFP positivity.

Cell Viability Assays

Two S-type (SH-EP1 and SK-N-AS) and two N-type (IMR32 and SH-SY5Y) human NB cell lines were treated with different concentrations of TSA, and viability was determined after 24 and 48 hours by MTT assay, as previously described [4]. The viabilities of CBP-transfected and CBP knockdown cells were similarly measured. All experiments were performed at least three times, with triplicates in each experiment. Data from a representative experiment are shown as mean values and standard deviations.

siRNA-Mediated Silencing

Briefly, 24 hours before transfection, SH-SY5Y cells were plated at a density of 2×10^6 cells per 10-cm plate. On the following day, cells were transfected with SMART-pool CBP siRNA (Dharmacon, Inc., Lafayette, CO) using a Nucleofector transfection reagent (Amaxa), as per the manufacturer's protocol. As a measure of transfection efficiency, we used siGlo (Dharmacon). Mock transfection and transfection with scrambled siRNA served as controls. siRNA-induced depletion of CBP expression was measured 48 to 72 hours after transfection by Western blot analysis using CBP antibodies (06-893; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Western Blot Analysis and Coimmunoprecipitation

Whole-cell extracts of NB cell lines SH-SY5Y, IMR32, SH-EP1, SK-N-AS, GOTO, SMS-KCN-69n, LA1-55n LA1-5s, SH-310, and WSN were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PDVF membrane, and then immunoblotted for CBP, p300, Bax, or Ku70. Tubulin was used as a loading control. The following antibodies were used for Western blot analyses: tubulin (T-4026) and FLAG (F-1804) antibodies were from Sigma (St. Louis, MO); Ku70 (A-9), CBP (A-22), and p300 (N-15) antibodies were from Santa Cruz Biochemicals; and Bax-NT (06-499) antibody was from Upstate (Billerica, MA). Western blot analyses were visualized by using Enhanced Chemiluminescence Plus (Amersham Pharmacia Biotech, Piscataway, NJ). Coimmunoprecipitation of endogenous and FLAG-tagged Ku70 before and after TSA treatment

was performed in CHAPS buffer, according to the procedure described by Sawada et al. [5].

NB Tissue Array

Tissue arrays were constructed using triplicate 1.0-mm cores taken from 45 paraffin-embedded formalin-fixed neuroblastic tumors (30 NBs, 7 ganglioneuroblastomas, 7 ganglioneuromas, and 5 composite/mixed-histology tumors), 5 normal adrenal glands, and 20 other neural crest–derived and unrelated neoplasms. After constructing and curing array cores in a paraffin block, 10-μm sections on poly-L-lysine slides were taken for routine hematoxylin–eosin staining and immunohistochemistry. Staining was performed according to the manufacturer's recommended Immunohistochemistry (IHC) protocols using the same antibodies described above. A semiquantitative system was used to measure staining intensity, with a score from 0 to 3 assigned to each component (neuroblastic and stromal) of each tumor core. Mean scores and component ratios (neuroblast staining intensity divided by stromal cell staining intensity) were then derived for each tumor from its triplicate cores. Correlations between immunohistochemical staining and histologic cell type were performed using bivariate analysis with Pearson coefficients and two-tailed significance tests.

Results

S-Type NB Cells Are Less Sensitive to TSA and Have Lower Levels of HATs Than N-Type Cells Growing in Culture

To determine the relative responsiveness of N-type and S-type NB cell lines to TSA-induced cell death, we treated two N-type (IMR32 and SH-SY5Y) and two S-type (SH-EP1 and SK-N-AS) NB cell lines with TSA for 24 or 48 hours, and then measured cell viability with MTT assay. After 48 hours, 1 μM TSA reduced the viability of N-type cells to less than 10% to 20% (Figure 1*A*). In contrast, S-type cells were significantly less sensitive to TSA: at 1 μM, viability remained above 50% (Figure 1*B*). Consistent with our previous report, TSA disrupts the molecular interaction between Ku70 and Bax in N-type SH-SY5Y (Figure 2*A*, *lanes 5* and *6*) and IMR32 cells (data not shown), which we proposed is due to increased acetylation on Ku70 lysine residues K539 and K542 [4]. In contrast, although SH-EP1 cells have similar levels of Ku70–Bax complex detected in cytosolic extracts (Figure 2*A*, *lanes 1–4*), TSA does not affect Bax and Ku70 binding in these cells (Figure 2*A*, *lanes 7* and *8*). These data are generally consistent with findings recently reported by Furchert et al. [12] comparing growth inhibition in response to TSA and other HDACIs in a panel of cells, including five

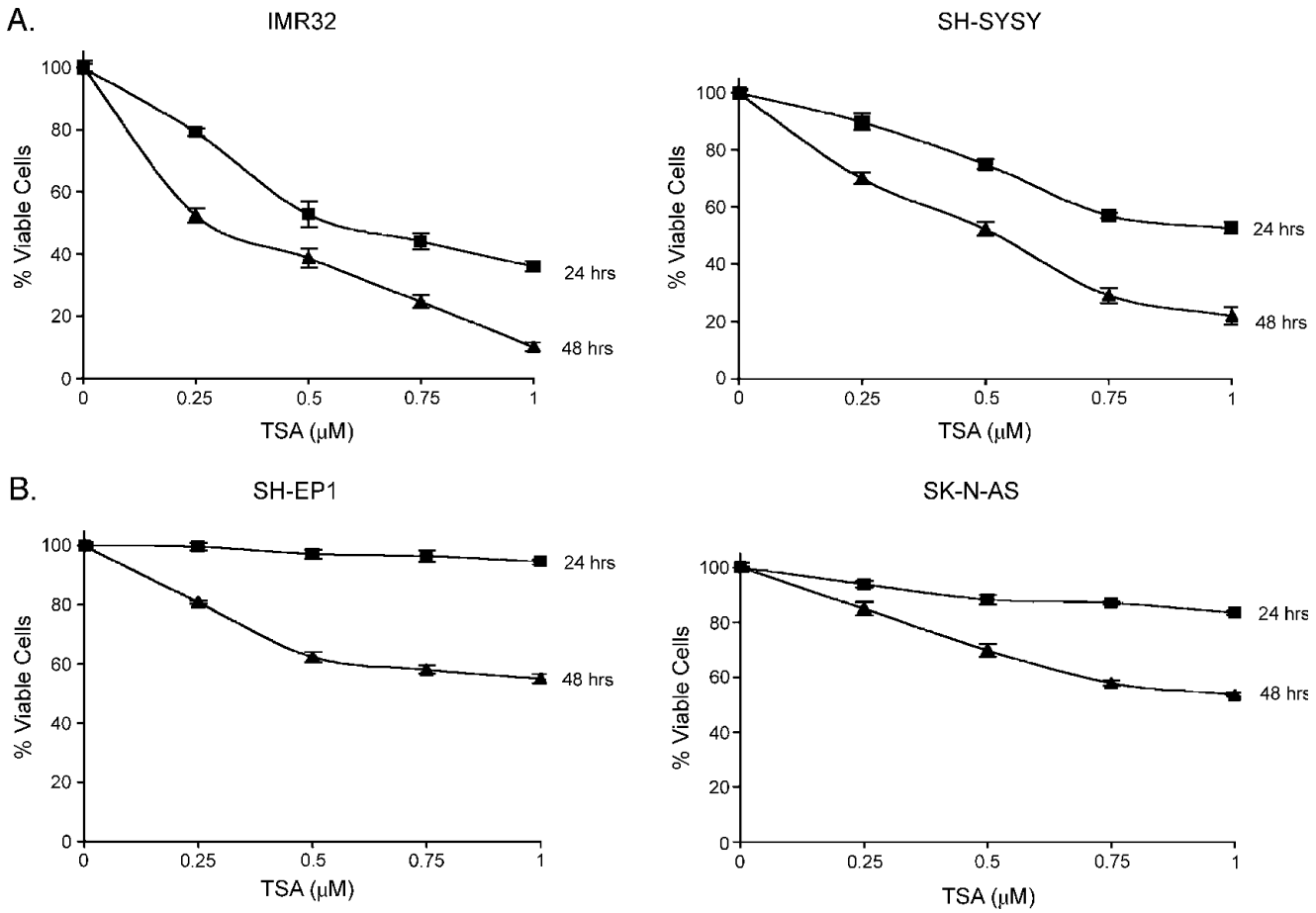

Figure 1. *TSA induces cell death in N-type, but not S-type, NB cells. N-type cell lines (IMR32 and SH-SY5Y) (A) and S-type cell lines (SH-EP1 and SK-N-AS) (B) were treated with various concentrations of TSA for 24 or 48 hours, as indicated. Cell viability was determined by MTT assay. Results are expressed as the percentage of viable cells compared with vehicle-treated controls (mean ± SD; n = 3).*

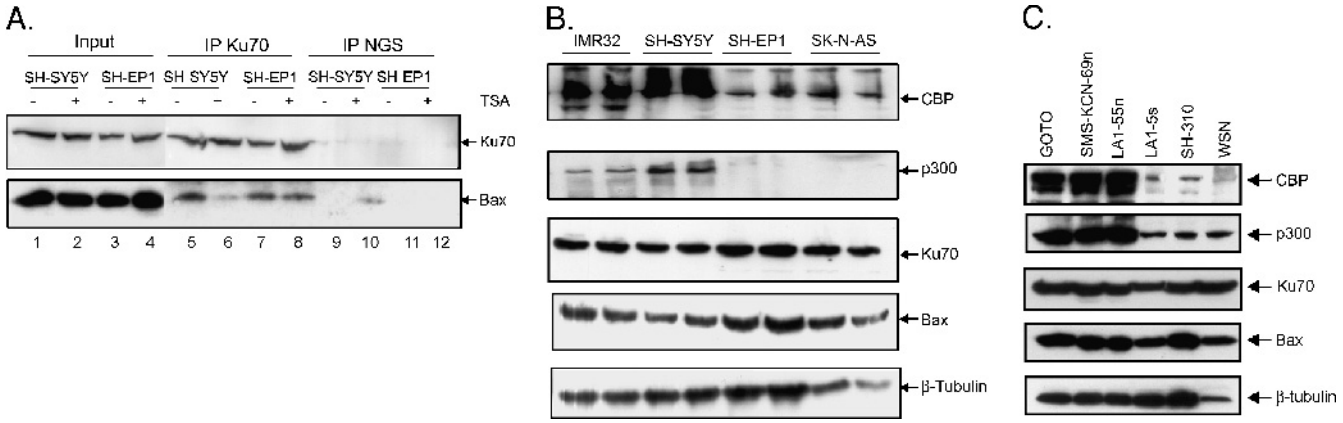

Figure 2. *N-type NB cells have higher CBP and p300 levels, and Bax is released from Ku70 in response to TSA. (A) Both N-type (SH-SY5Y) and S-type (SH-EP1) cells were treated with 1 μM TSA or vehicle for 4 hours. Endogenous Ku70 was immunoprecipitated using goat anti-Ku70 antibodies or normal goat serum (NGS). Immunoprecipitates were separated by SDS-PAGE and probed with anti-Bax or anti-Ku70 antibodies. Input represents 20% of the sample used in immunoprecipitation. (B and C) Whole-cell extracts of N-type (IMR32 and SH-SY5Y in B; GOTO, SMS-KCN-69n, and LA1-55n in C) or S-type (SH-EP1 and SK-N-AS in B; LA1-5s, SH-310, and WSN in C) NB cells were separated by SDS-PAGE and probed with anti-CBP, anti-p300, anti-Ku70, and anti-Bax antibodies. β-Tubulin was probed for equal loading.*

NB cell lines. With respect to sensitivity to HDACIs (TSA, MS-275, and SAHA), they report that SHEP-SF cells, an S-type line, are less responsive than N-type cells (CH-LA 90, IMR-5, and SHSY-5Y), with a single exception in that SH-SY5Y cells were relatively resistant to SAHA.

HDAC inhibition can only increase Ku70 acetylation when HAT activity capable of acetylating the corresponding sensitive sites is present. Hence, these results showing that TSA fails to disrupt Ku70–Bax binding in S-type cells could be explained by the possibility that the cells lack adequate HAT expression or activity. To test this possibility, we determined the relative expression of Ku70, Bax, p300, and CBP, the last of which is known to acetylate Ku70 [10]. As shown in Figure 2, *B* and *C*, the N-type (IMR32 and SH-SY5Y in Figure 2*B*; GOTO, SMS-KCN-69n, and LA1-55n in Figure 2*C*) and S-type (SH-EP1 and SK-N-AS in Figure 2*B*; LA1-5s, SH-310, and WSN in Figure 2*C*) cell lines express nearly equal amounts of Ku70 and Bax; however, the levels of CBP and p300 are significantly lower in S-type cell lines than in N-type cell lines.

Low-Level CBP Expression Limits the Responsiveness of S-Type Cells to TSA Treatment

Next, we designed a series of experiments to determine whether the level of expression of CBP or p300 governs responsiveness to HDACIs in NB cell types. First, we tested the hypothesis that increasing the level of CBP or p300 in S-type cells would result in a more TSA-responsive phenotype. FLAG-tagged CBP or p300 expression vectors were transiently transfected into SH-EP1 cells by an electroporation method that results in a transfection efficiency of $\geq$ 95% (data not shown). Twenty-four hours after transfection, SH-EP1 cell viability was assessed. Remarkably, although the expression levels of FLAG-tagged CBP and p300 were similar (Figure 3*B*), the transfection of CBP, but not p300, had toxic effects; CBP reduced SH-EP1 viability to 30% of vector-transfected control cells (Figure 3*A*). Mechanistically, CBP transfection, but not p300 transfection, disrupted the interaction between Bax and Ku70, as evidenced by the failure to detect Bax in immune complexes precipitated with anti-Ku70 antibody from CBP-transfected cells (Figure 3*C*). Furthermore, when CBP or p300 transfection was combined with TSA treatment, only CBP-transfected cells had increased sensitivity to TSA-induced death (Figure 3*D*, *left panel*). These experiments were performed by harvesting cells that remained viable after CBP transfection; these cells were then treated with vehicle control, TSA, or doxorubicin (Dox). Unlike the increased sensitivity to TSA seen in cells transfected with CBP, there was no change in the sensitivity of cells to Dox, which represents a cytotoxic agent that does not act by blocking HDAC activity (Figure 3*D*, *right panel*). Indeed, the TSA dose response of CBP-transfected S-type cells was comparable to that of N-type cells (Figure 1*A*). Together, these results show that the level of expression of CBP in S-type cell lines can independently affect Ku70–Bax binding and that increased CBP expression enhances cell death in response to TSA.

To directly link Ku70 acetylation with CBP-induced Bax release, activation, and cell death, we took advantage of a *Ku70* mutant protein in which two acetylation-sensitive lysine residues (539 and 542) were replaced by arginines. This mutant has been previously shown to block TSA-induced apoptosis in N-type cell lines [4]. As shown in Figure 4*A*, a single cotransfection using the CBP and Ku70 plasmids resulted in the expression of target proteins. It was noted that the level of CBP expression was lower when Ku70 plasmid was also present, potentially due to transcription-level interference. As expected, transfection to overexpress wild-type Ku70 or Ku70 lysine mutant did not significantly affect viability (84% and 95% of controls; Figure 4*B*, *lanes 5* and *6*, respectively), whereas CBP was toxic (52% viable; Figure 4*B*, *lane 2*). Cotransfecting wild-type Ku70 along with CBP resulted in slightly higher cell deaths (42% viability; Figure 4*B*, *lane 3*) compared with transfecting CBP alone, whereas cotransfection of *Ku70* mutant completely blocked the toxic effects of CBP overexpression (81% viability;

Figure 4*B*, *lane 4*). It is worth noting that the levels of CBP expression were nearly equal in cotransfections with wild-type Ku70 and with *Ku70* mutant (Figure 4*A*); therefore, differences in CBP expression do not account for differences in cell survival. We monitored the Bax–Ku70 complex following each transfection. Consistent with effects on cell survival, transfection of CBP alone or CBP with wild-type Ku70 resulted in dissociation of Ku70–Bax (Figure 4*C*, *lanes 6* and *7*, respectively), whereas the complex remained intact in cells cotransfected with CBP and *Ku70* mutant (Figure 4*C*, *lane 8*). These results provide evidence that CBP depends on critical acetylation-sensitive lysine residues in Ku70 for its ability to disrupt the interaction between Ku70 and Bax in NB cells. However, these interpretations may be limited by the unphysiologically high level of CBP expression introduced by transient transfection in these cells.

High-Level CBP Expression Is Required for HDACI-Induced Cell Death in N-Type Cells

Our data suggest that CBP may be required to acetylate Ku70 in NB cells. If so, we reasoned that depletion of CBP in N-type cell lines should render cells less sensitive to TSA-induced cell death. Using siRNA targeting CBP, we reduced CBP protein expression in SH-SY5Y cells (up to 90%; Figure 5*A*). Depleting CBP in these cells significantly blocks the cytotoxic response observed after 24 or 48 hours of TSA treatment (Figure 5*B*). These results indicate that CBP is a critical mediator of TSA-induced cell death.

Bax Is Required for TSA-Induced Cell Death in NB Cells

We used siRNA knockdown of Bax and Ku70 in SH-SY5Y cells (Figure 6*A*) to determine whether Bax is required for TSA-induced killing and to establish whether the balance in the expression of Ku70 and Bax can affect viability. Consistent with the model developed above, reducing Bax expression in SH-SY5Y cells protects cells from TSA-induced death (Figure 6*C*). Knocking down Bax expression, by itself, does not affect NB cell viability, whereas Ku70 knockdown alone led to cell death, even without TSA treatment (Figure 6*B*). SH-SY5Y cells survived Ku70 knockdown, however, when Bax was simultaneously knocked down and, as expected, death response to TSA was significantly reduced in double-knockdown cells (Figure 6*C*). These results show both that the Ku70–Bax complex is a relevant target for therapeutic

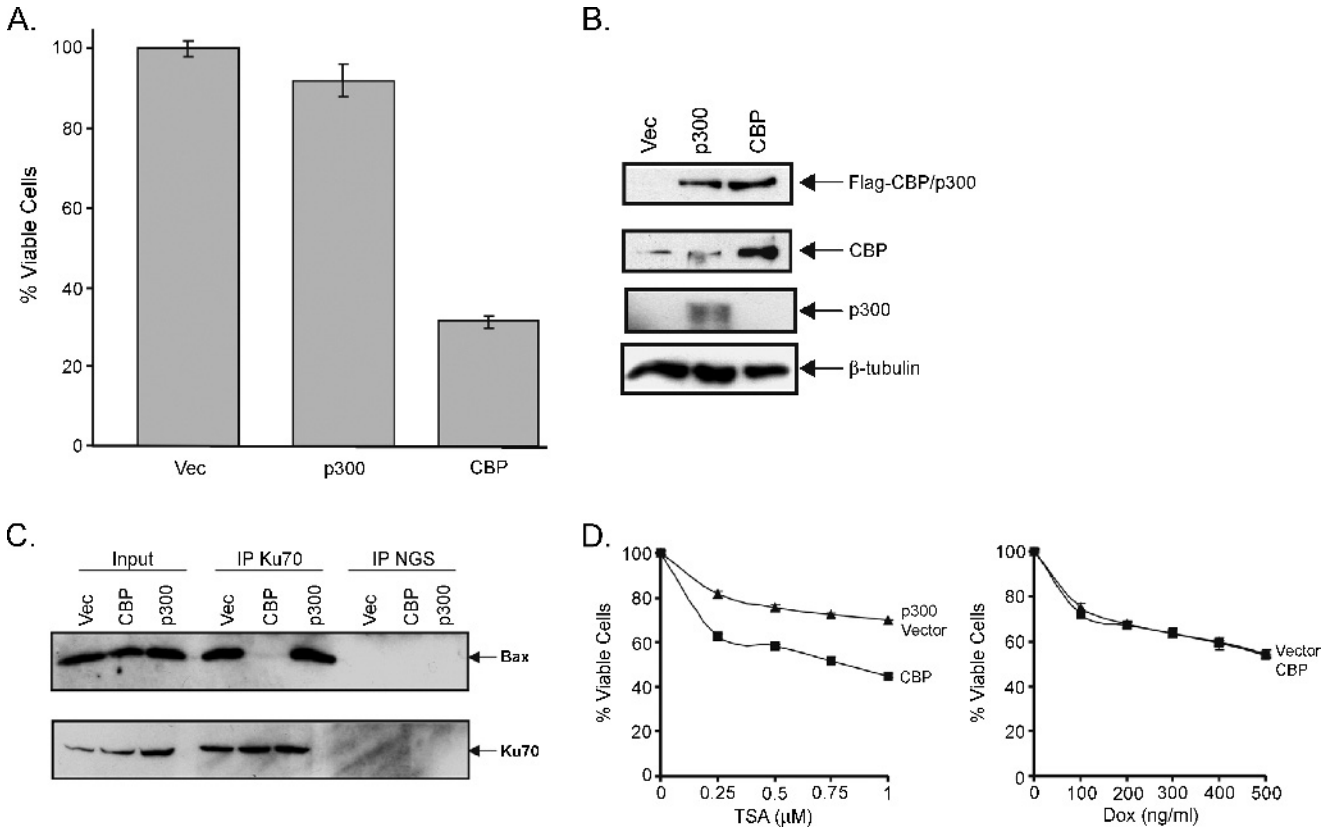

Figure 3. *Increasing CBP in S-type NB cells reduces cell viability and Bax binding to Ku70. SH-EP1 (S-type) cells were transfected with FLAG-tagged CBP, FLAG-tagged p300 expression vector, or empty vector. (A) Cell viability was determined by MTT assay 24 hours after transfection. Results are expressed as the percentage of viable cells compared with vehicle-treated controls (mean ± SD; n = 3). (B) The levels of CBP and p300 after transfection, as described above, are detected by immunoblotting with anti-FLAG, anti-CBP, and anti-p300 antibodies, as shown. β-Tubulin was probed for equal loading. (C) SH-EP1 cells were transfected with FLAG-tagged CBP, FLAG-tagged p300 expression vectors, or empty vector. Twenty-four hours after transfection, Ku70 was immunoprecipitated using goat anti-Ku70 antibodies or NGS. Immunoprecipitates were separated by SDS-PAGE and probed with anti-Ku70 or anti-Bax antibodies. Input represents 20% of the sample used for immunoprecipitation. (D) SH-EP1 cells were transfected with FLAG-tagged CBP, FLAG-tagged p300 expression vector, or empty vector. Twenty-four hours after transfection, cells remaining viable were treated with various concentrations of TSA or Dox, as indicated, for an additional 24 hours, after which viability was determined by MTT assay. Results are expressed as the percentage of viable cells compared with vehicle-treated controls (mean ± SD; n = 3).*

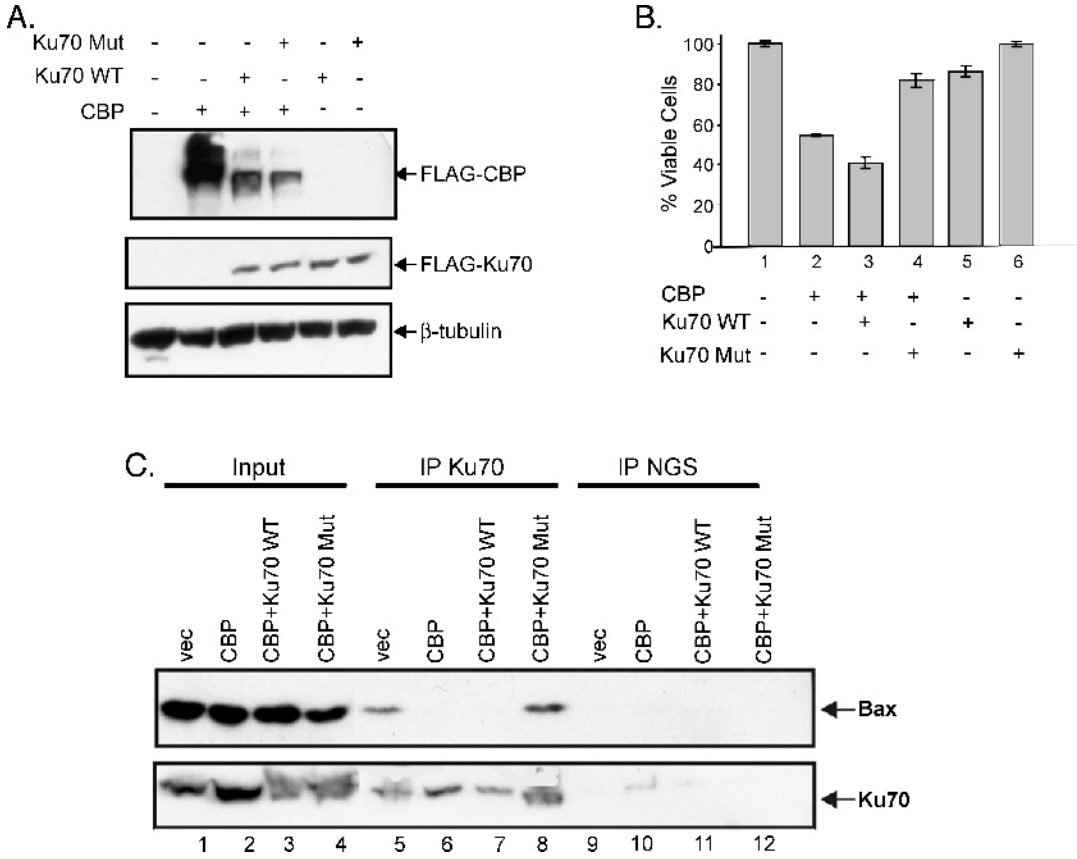

Figure 4. *Coexpression of a Ku70 acetylation mutant rescues CBP-induced reduction of cell viability in S-type NB cells. SH-EP1 (S-type) cells were transfected with FLAG-tagged CBP, FLAG-tagged wild-type Ku70, or FLAG-tagged Ku70 mutant (K539R/K542R) expression vectors, as indicated. The expression of transfected proteins is shown in (A) by immunoblotting using anti-FLAG antibodies. β-Tubulin was probed for equal loading. (B) Twenty-four hours after TSA treatment, cell viability was determined by MTT assay. Results are expressed as the percentage of viable cells compared with vector alone (mean ± SD; n = 3). (C) Twenty-four hours after transfection, Ku70 was immunoprecipitated using goat anti-Ku70 antibodies or NGS. Immunoprecipitates were separated by SDS-PAGE and probed with anti-Ku70 or anti-Bax antibodies. Input represents 20% of the sample used in immunoprecipitation.*

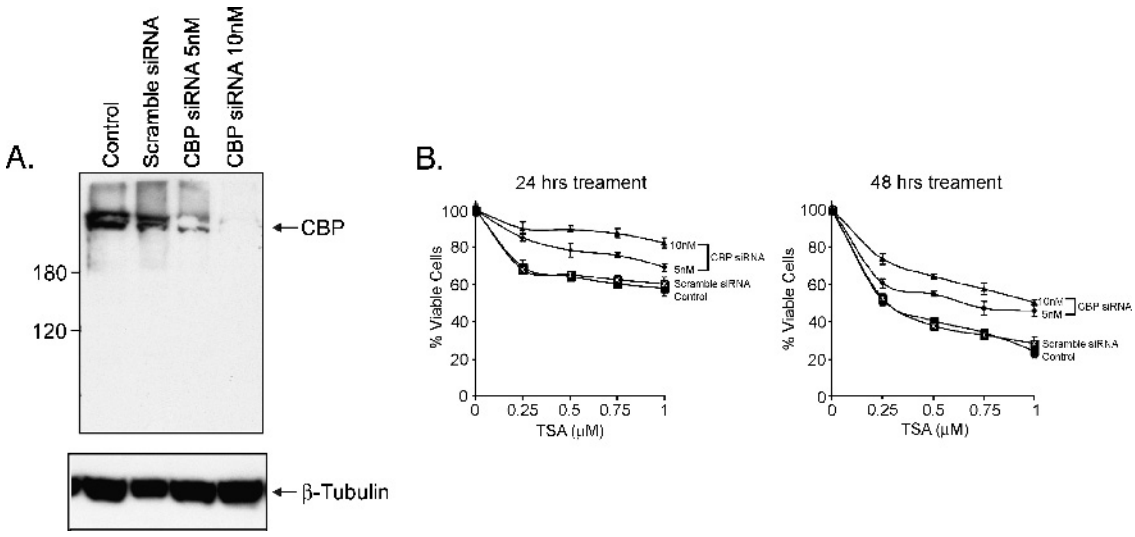

Figure 5. *Reduction of CBP in N-type NB cells reduces TSA-induced cell death. (A) SH-SY5Y (N-type NB) cells were transfected with CBP-specific siRNA (5 or 10 nM) or scrambled siRNA. The control received no siRNA. Forty-eight hours after transfection, the level of CBP was determined by immunoblotting using anti-CBP antibodies. β-Tubulin was probed for equal loading. (B) Forty-eight hours after siRNA transfection, the cells were treated with various concentrations of TSA for 24 or 48 hours, as indicated. Cell viability was then determined by MTT assay. Results are expressed as the percentage of viable cells compared with vector alone (mean ± SD; n = 3).*

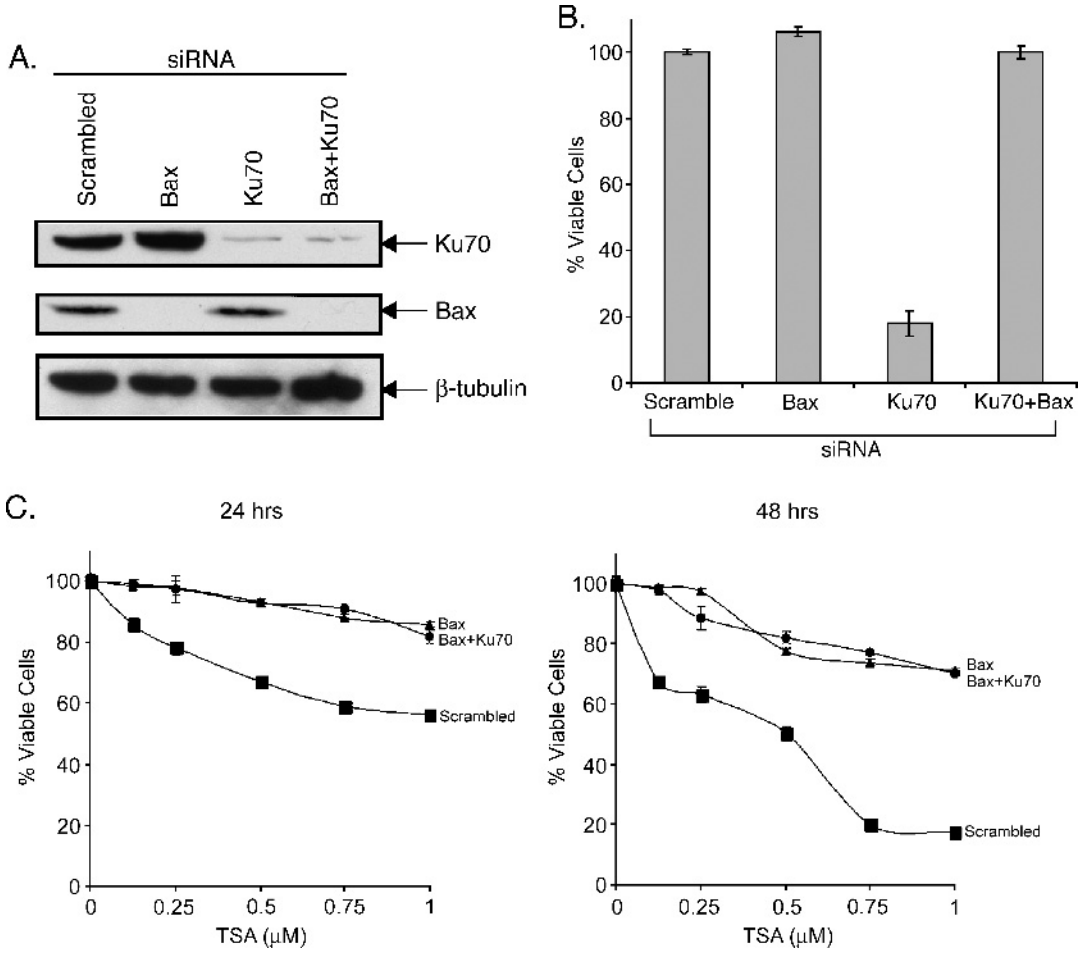

Figure 6. *Ku70 and Bax are critical for TSA-induced death of N-type NB cells. (A) SH-SY5Y cells were transfected with scrambled, Bax (10 nM), Ku70 (10 nM), or both Bax/Ku70–targeted siRNA. The levels of Bax or Ku70 protein at 48 hours after transfection are shown in immunoblots using Bax-specific or Ku70-specific antibodies. β-Tubulin was probed for equal loading. (B) Forty-eight hours after siRNA transfection, the viability of Bax, Ku70, or Ku70/Bax knockdown in SH-SY5Y cells was determined by trypan blue exclusion assay. The results are expressed as percentages of viable cells relative to cells transfected with scrambled siRNA (mean ± SD; n = 3). (C) Forty-eight hours after siRNA transfection, Bax or Ku70/Bax knockdown in SH-SY5Y cells was treated with various concentrations of TSA, as shown. The viability of cells was determined by MTT assay 24 or 48 hours after TSA treatment by comparison to untreated SH-SY5Y cells. The results are expressed as mean ± SD (n = 3).*

strategies to decrease NB survival and that Bax expression is required for TSA-induced cell death.

Proteins Required for HDACI-Induced Cell Death Are Expressed in Human NB Tumors

Next, we determined the protein-level expression of Bax, Ku70, and CBP in human NB tumors to assess the potential of these proteins to be developed as biomarkers to select tumors that are most likely to respond to HDACIs. To do this, we constructed NB tumor tissue microarrays and used immunohistochemical detection methods to semiquantitatively determine the expression of Bax, Ku70, CBP, and p300 (Figure 7*A*).

We found that every NB tumor had detectable levels of these proteins. This suggests that the critical components of the TSA response mechanism defined *in vitro* are present in tumors. It is interesting to note that CBP and p300 are expressed at higher levels in neuroblastic regions of tumors compared to the stroma (the CBP neuroblast/stroma ratio is 3.65), although there is a smaller difference in the expression of Ku70 or Bax between neuroblastic and stromal regions (neuroblastic/stromal ratios are 2.21 and 1.36, respectively). Semiquantitative analyses of CBP and p300 expression levels in neuroblastic foci reveal that > 90% of tumors demonstrate expression that reaches the upper half of the range (levels 2 and 3) (Figure 7*B*). In contrast, CBP and p300 expression in stromal regions reaches the upper range in only 20% to 35% of tumors and is barely detectable in > 35% of stromal regions. Thus, CBP and p300 are differentially expressed between neuroblastic and stromal cell regions in tumors, mirroring the differential expression observed between N-type and S-type NB cell lines. In addition to demonstrating the presence of these proteins in all NB tumor specimens, these data show a range for the expression for each protein across tumors that varies according to neuroblastic and stromal histology. The differences in expression observed across this small set of NB tumors support the possibility of detecting correlations between the expression of

levels of CBP, Ku70, or Bax and clinical variables such as responses to HDACI treatment in a future translational study.

Discussion

Our results support a model in which the association of Bax with Ku70 in NB cells defines a target for TSA and in which response to TSA treatment is regulated by CBP. This model further predicts that the sensitivity of NB tumor cells to the apoptotic effects of certain HDACIs depends on the three components: Ku70, Bax, and CBP. We have tested this model in S-type NB cell lines in which the level of CBP is low. Overexpression of CBP in S-type cell lines favors the dissociation of Bax from Ku70, increasing the sensitivity of these cells to TSA. A Ku70 acetylation site mutant, which we have previously shown, blocks the apoptotic effects of HDACIs in N-type NB cell lines and also blocks CBP-induced cell death in S-type NB cells. Conversely, reducing CBP expression in N-type NB cell lines significantly reduces the cytotoxic effects of TSA, further establishing that CBP is a factor determining HDACI responsiveness. The results of experiments in which Bax and Ku70 expression was modulated not only support the importance of the interaction between Bax and Ku70 in maintaining NB cell viability but also demonstrate that TSA-induced cell death in NB cells depends on these proteins because cells lacking both Ku70 and Bax are insensitive to HDACI-induced killing.

CBP Mediates Cell Death By Affecting Ku70–Bax Binding in the Cytoplasm

Our results suggest that CBP acetylates Ku70 as part of a mechanism leading to the disruption of cytoplasmic Ku70–Bax complexes. These data are consistent with a model in which CBP, typically considered a nuclear protein, may be active in the cytoplasm where it acetylates Ku70. CBP and the related molecule p300 have previously been detected in the cytoplasm of other cells. For example, CPB and p300 are cytoplasmic in mouse oocytes isolated from primordial follicles, but as follicles develop, oocyte CBP and p300 become progressively localized in the nucleus [13]. Cohen et al. [10] have shown that, after HDACI treatment, CBP translocates from the nucleus to the cytoplasm in 293T cells. In NB, HeLa, and HEK 293 cells, we have recently demonstrated that CBP and p300 are detected in both the cytoplasm and the nucleus (manuscript in preparation). These results provide for the possibility that cytoplasmic CBP acetylates Ku70. It is also possible that the interaction between CBP and Ku70 occurs in the nucleus and that shuttling of Ku70 between the nucleus and the cytoplasm allows the acetylation that occurs in the nucleus to ultimately affect the binding equilibrium of cytoplasmic Ku70 and Bax. Ongoing work is directed at answering this question.

CBP, But Not p300, Reduces the Viability of S-Type NB Cells

Because CBP and p300 are highly homologous (61% identical at the amino acid level overall, and 95% similar in functional domains), it is surprising that only the expression of CBP, and not p300, is cytotoxic to S-type NB cells (Figure 3). CBP and p300 are functionally equivalent in terms of their interactions with many cellular proto-oncogenes and a wide variety of transcription factors [14]. Mutations of CBP and p300 in humans are both associated with the Rubinstein-Taybi syndrome, a developmental disorder characterized by mental retardation, digital abnormalities, and an increased incidence of certain types of tumors, including NB [15,16]. Nevertheless,

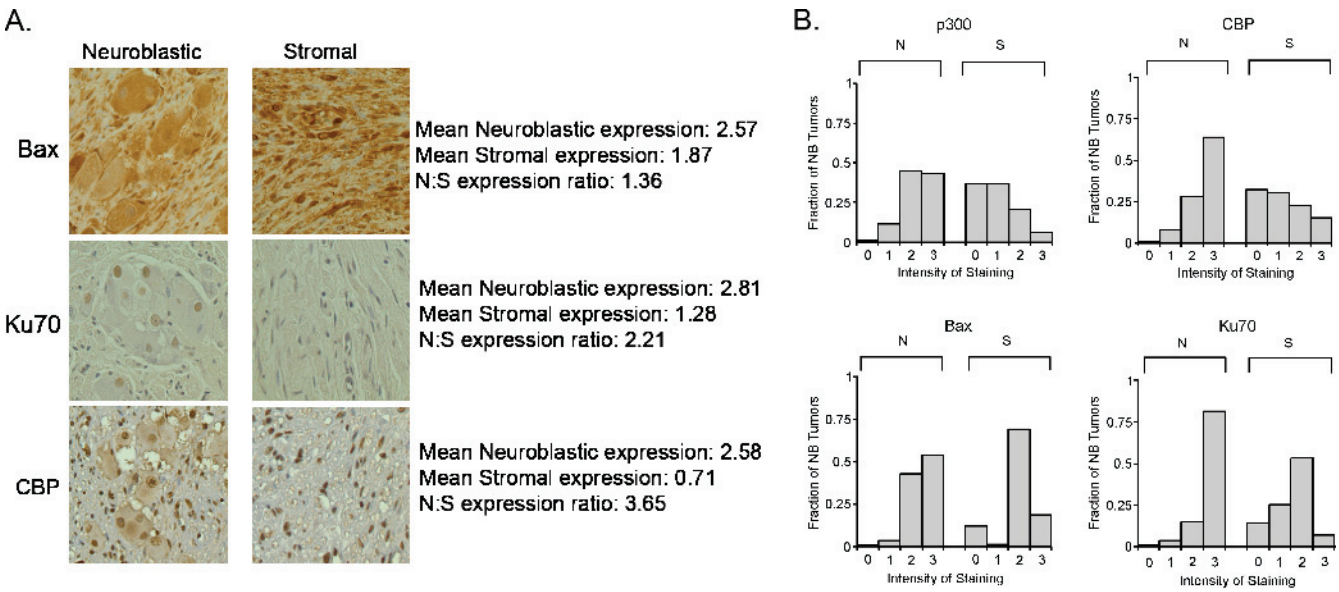

Figure 7. *Expression of Bax, Ku70, and CBP in a tissue microarray of neuroblastic tumors. (A) Cores of formalin-fixed paraffin-embedded neuroblastic tumors were stained using peroxidase-conjugated anti-Bax, anti-Ku70, or anti-CBP antibodies (brown signals). The slides were then counterstained with hematoxylin (blue background). (B) Fraction of N-type or S-type cells that express different staining intensities (levels, 0–3+) of CBP, p300, Ku70, or Bax in our NB tissue microarray studies.*

several examples provide evidence that the functions of CBP and p300 do not completely overlap. In F9 cells, retinoic acid–induced differentiation is dependent on p300, but not CBP, because expressing a p300-specific hammerhead ribozyme capable of cleaving p300 messenger RNA, but not CBP, makes these cells resistant to retinoic acid–induced differentiation [17]. Although p300, and not CBP, is crucial for proper hematopoietic differentiation [18], only CBP is essential for hematopoietic stem cell self-renewal. The results in the current study show that CBP and p300 are not functionally equivalent in NB cells and suggest that this may result from differences in substrate specificity for Ku70 acetylation. The precise mechanism that determines CBP and p300 substrate specificity remains an area of intense research interest.

Clinical Relevance

NB tumors are comprised of neuroblastic and stromal cells [19,20]. Differences in the proportion of neuroblastic and schwannian stromal cells present in neuroblastic tumors, which include NB, ganglioneuroblastoma, and ganglioneuroma, are one of the important criteria used to establish clinical prognosis. Tumors that are composed primarily of neuroblasts and have low numbers of schwannian stromal cells are more aggressive and are usually the most difficult to cure [21,22]. Our analysis of a panel of neuroblastic tumors identifies a difference in the level of CBP expression between neuroblastic and schwannian stromal cells. Specifically, neuroblastic cells express three-fold more of this protein. Because TSA is more active against N-type cells compared to S-type cells in culture and because its killing depends on adequate CBP expression, we predict that HDACIs will preferentially kill neuroblastic cells within these tumors, which may mean that tumors with higher neuroblastic content (NBs) will respond better to these agents than stromal-predominant ones (ganglioneuromas). Further experiments and clinical trials that validate the safety and efficacy of HDACIs in patients with neuroblastic tumors may consider evaluating whether clinical variables, including responses to this therapeutic class, correlate with the expression of Bax, Ku70, and CBP.

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