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Targeting histone deacetylase activity to arrest cell growth, reduce pluripotency, and promote differentiation in Ewing sarcoma

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Abstract

There is an urgent need for advances in the treatment of Ewing sarcoma (EWS), an aggressive childhood tumor with possible neuroectodermal or mesenchymal origin. Inhibition of histone deacetylases (HDAC) can revert aberrant epigenetic states and reduce growth in different

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Author Contributions

B.K.S., P.L.C.L. and R.R. designed experiments. B.K.S. and R.R. drafted the manuscript. B.K.S. performed all experiments, analysis and interpretation of data, and figure generation. P.L.C.L. provided technical assistance for flow cytometry, immunofluorescence experiments and interpretation of data. P.R.M. provided technical assistance for cell culture drug treatments and tumorsphere induction experiments. I.A.V. and N.K. provided technical assistance for cell culture experiments, protein extraction and western blotting. A.L.A., A.T.A., A.L.B., L.G., C.B.F. and R.R. acquired materials. C.B.F. and R.R. supervised the project. A.L.A., A.T.A., A.L.B., L.G., C.B.F., C.J.T. and R.R. critically edited the manuscript. All authors reviewed the manuscript.

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experimental cancer types. Here, we investigated whether the potent HDAC inhibitor, sodium butyrate (NaB) has the ability to reprogram EWS cells towards a more differentiated state and affect their growth and survival. Exposure of two EWS cell lines to NaB resulted in rapid and potent inhibition of HDAC activity (1 h, IC₅₀ 1.5 mM) and a significant arrest of cell cycle progression (72 h, IC₅₀ 0.68–0.76 mM), marked by G0/G1 accumulation. Delayed cell proliferation and reduced colony formation ability were observed in EWS cells after long-term culture. NaB-mediated effects involved suppression of cell proliferation accompanied by decreased expression of key survival and pluripotency-associated genes, and the re-expression of the differentiation neuronal marker β III-tubulin. Finally, we observed a marked activity of NaB in impairing survival of putative EWS cancer stem cells. Our findings support the use of HDAC inhibition as a therapeutic approach to impair cell growth and survival and to reprogram EWS tumors towards differentiation.

Introduction

Ewing sarcoma (EWS), a highly aggressive bone and soft tissue cancer, is the second most common primary solid bone malignancy in children and young adults¹. Despite advances in multimodal therapy, patients with the disease have a poor prognosis, with a survival rate of 50 – 65% at 5 years and less than 30% for metastatic or refractory tumors². EWS tumors typically harbor a specific genetic alteration characterized by a chromosomal translocation resulting in fusions between the EWS RNA Binding Protein 1 (*EWSR1*) gene and one of the several *ETS* family genes (most frequently *FLI-1*) which is frequent in 85% of cases^{1,3}. EWS tumors are poorly differentiated and its cell of origin remains elusive and highly debated. Evidence indicates that EWS may arise from either developing neural crest cells^{4–7} or mesenchymal stem cells^{8–11}.

Overall, EWS tumors are relatively genetically stable, since they harbor few somatic mutations compared with most other cancer types¹². However, many epigenetic alterations are likely crucial for EWS tumorigenesis. *EWSR1-FLI-1* acts as an aberrant transcription factor that induces chromatin remodeling to repress tumor suppressors while activating oncogenes¹³. The chromatin state in EWS is strikingly similar to that found in bone-marrow-derived mesenchymal stem cells. The increased chromatin accessibility in stem cells may lead to a state that facilitates oncogenic alterations induced by *EWSR1-FLI-1*, suggesting a stem cell origin for EWS¹⁴. Epigenetic alterations in EWS include DNA methylation or post-translational modifications to histones, including acetylation, which are key in regulating gene expression^{15,16}.

Epigenetic changes provide potentially druggable targets¹⁷. A balance between the opposing activities of histone acetyltransferases (HATs) and deacetylases (HDACs) is key in regulating gene expression. Histone acetyltransferases (HATs) control histone acetylation activity through the transfer of acetyl groups to the amino-terminal lysine residues of histones, thus increasing transcriptional activity. In contrast, histone deacetylases (HDACs) remove acetyl groups, favoring chromatin condensation and repression of gene expression¹⁸. HDAC inhibitors (HDi), represent a class of experimental antineoplastic agents to target aberrant epigenetic alterations found in cancer. The anti-tumor effects caused by HDi

commonly involve alterations in cell cycle, differentiation and apoptosis. Several HDIs are being developed and extensively evaluated *in vitro* and *in vivo*¹⁹. However, to date only three HDAC inhibitors have been approved by the Food and Drug Administration, namely vorinostat (SAHA), romidepsin (Istodax) and belinostat (Beleodaq) for treatment of peripheral T-cell and cutaneous T-cell lymphomas²⁰. Sodium butyrate (NaB) is a short fatty acid that acts as a potent class I and IIa HDAC inhibitor inducing arrest in proliferation of cancer cells by increasing histone acetylation. In mammalian cells, NaB promotes hyperacetylation of the histones H3 and H4, resulting in chromatin decondensation and increased transcriptional activity²¹. In EWS cells, we have previously shown a synergistic antitumor effect of NaB combined with commonly chemotherapeutic drugs²². Moreover, SAHA showed a synergistically enhanced antitumor activity upon combination with etoposide²³, and NaB, SAHA or entinostat or MS-275 as monotherapies or combined with TRAIL display an additive cytotoxic effect in EWS cells²⁴.

Here, we show that inhibition of global HDAC activity by NaB suppresses multiple pathways involved in cell proliferation, survival and pluripotency. In addition, NaB exposure stimulates morphologic and biochemical aspects of neural differentiation in EWS cells.

Results

Inhibition of HDAC activity induces growth arrest in EWS cells

To examine the effect of NaB on HDAC activity in EWS cells, we exposed SK-ES1 and RD-ES cells to NaB for 1 and 3 h. HDAC activity was significantly reduced after 1 h when NaB doses higher than 1 mM were used, resulting in a target IC₅₀ of approximately 1.5 mM (Fig. 1A, C). HDAC activity in cells exposed to NaB for 3 h was not as effectively reduced in comparison to 1 h treatments, and a significant decrease was observed only in RD-ES cells exposed to 2 mM of NaB (see Supplementary Fig. S1 online), suggesting that NaB optimally inhibits HDAC activity in EWS cells at a kinetic rate of 1 h.

In order to evaluate the biological effect of HDAC activity inhibition, we exposed EWS cells to varying concentrations of NaB (0.5 – 5 mM) for 72 h. HDAC activity inhibition potently affected cell viability of both cell lines (Fig. 1B). At 72 h, the biological IC₅₀ of NaB was 0.76 and 0.68 mM for SK-ES 1 and RD-ES EWS cell lines, respectively (Fig. 1C). Interestingly, cells exposed to NaB showed a change in morphology accompanied by the appearance of short neurite-like extensions (Fig. 1D).

Next, we verified whether inhibition of HDAC activity by NaB would change cell cycle distribution. HDAC inhibition resulted in a significant alteration in EWS cell cycle featuring an accumulation of cells in the G0/G1 phase 35 h after NaB exposure. In the SK-ES1 EWS cell line, we also observed a significant decrease in the S and G2/M phases of the cell cycle while in the RD-ES cell line there was a significant reduction in polyploidy (Fig. 2A). To determine whether HDAC activity inhibition disrupts histone 3 phos-S10 (chromosome condensation marker during mitosis²⁵), we immunostained EWS cell lines exposed to NaB for 72 h against anti-H3 phos-S10 plus anti-Alexa488, measured by laser confocal microscopy. As expected, H3 phos-S10 immunolocalized to the nucleus in both EWS cell lines (Fig. 2B). In addition, we observed that both EWS cell lines exposed to higher doses of

NaB (2 and 5 mM) showed a reduced H3 phos-S10 level, whereas there was no significant change at lower doses (0.5 and 1 mM) compared to untreated cells (Fig. 2C). Given that Cyclin D1 is a G1-phase regulator protein overexpressed in EWS, we investigated whether the HDAC activity function regulate Cyclin D1 expression after 72 h of NaB exposure. SK-ES 1 cells exposed to 0.5 or 1 mM of NaB showed an approximately 1.5-fold change decrease in Cyclin D1 levels, and 1 mM NaB also decreased Cyclin D1 levels also in RD-ES cells (Fig. 2D). These results suggest that HDAC activity inhibition induces growth arrest, which inhibits EWS cell proliferation potential.

HDAC inhibition abrogates cell proliferation and survival pathways in EWS cells

To investigate molecular mechanisms associated with the NaB-induced decreases in EWS cell growth, we examined the expression of key proteins that control cell survival and proliferation. After exposure to either 0.5 or 1.0 mM NaB, a decrease in the levels of survival-promoting neurotrophins, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were observed (Fig. 3A). We also immunostained tropomyosin receptor kinase B (TrkB) in EWS cell lines against anti-TrkB, plus anti-Rhodamine. As expected, TrkB immunoreactivity was detected in the cytoplasm. In addition, we observed that TrkB levels were reduced in a dose-dependent manner in both cell lines after NaB exposure (Fig. 2B, C). Consistently with this finding, we also observed a decrease in protein levels of downstream targets phosphorylated extracellular-regulated kinase (pERK), but not total ERK1 levels, and pAKTS473 as well as total AKT. Essentially similar effects were observed in RD-ES cells, however only at the 1.0 mM dose of NaB (Fig. 3D). The analysis of relative densitometric units related to the immunoblots is depicted in Fig. 3E. These results support the possibility that signaling mediated by neurotrophins, ERK and AKT are involved in mediating the inhibitory effects of NaB in EWS cells.

Persistence of the anti-proliferative effect induced by HDAC inhibition in EWS cells

We performed a cumulative population doubling (CPD) of EWS cells to examine the persistence of the anti-proliferative effect of NaB. After 72 h of treatment, cells were maintained for 16 days in drug free media, as shown in Fig. 4A. Significant delays in cell proliferation rate were found with 2 and 5 mM of NaB (Fig. 3B). Because the number of cells was too small, we modeled the growth from day 19 to 35 using a mathematical prediction described by Silva et al.²⁶. We were able to predict whether EWS cells exposed to the higher doses of NaB over 35 days would have a persistent reduction in the ability to proliferate. Lower doses (0.5 and 1 mM) of NaB resulted in a gain in cell proliferation by day 20 which by 35 days was even higher than our prediction (Fig. 4B). It is possible that only a resistant subset of EWS cells could restore cell proliferative ability to an equal or higher degree compared to controls.

We then went on to perform a CPD profile analysis that examines long-term changes in cell population size. On the basis of CPD graphs from cell counting we calculated four different parameters: relative end CPD (RendCPD) to quantitatively assess the end point of cell proliferation analysis; relative area under curve (rAUC) to determine a global effect after treatment intervention; relative time to cross a threshold (RTCT) to measure the delay of the cell population that recovers growth after treatment; and relative proliferation rate (RPR) to

quantify the relative regrowth velocity of the cells which survived after treatment intervention. The average of all parameters described above is termed the long-term survival index (LSI). HDAC activity inhibition in both cell lines, particularly after treatment with 5 mM NaB, resulted in a robust decrease in RPR and RTCT parameters (Fig. 4C), except the RPR parameter in which there was no significant change in SK-ES 1 cells (data not shown). These data show that the effects of NaB can persist in the long-term to delay the regrowth of the EWS cell population.

Finally, we performed a colony formation assay for 10 days in drug free media, after a 72-h exposure to NaB. There was a significant reduction in the number of colonies in both cell lines after exposure to NaB. Moreover, there was a significant decrease in the size of the colonies in SK-ES 1 cells after exposure to 5 mM NaB (Fig. 4D). These results indicate that the decrease in cell growth is also associated with the cells' colony forming capability.

HDAC inhibition suppresses pluripotency-associated genes

Previous studies have shown that the EWSR1/FLI-1 oncoprotein stimulates the pluripotency gene BMI-1, thus influencing EWS cell self-renewal⁷. We sought to evaluate with immunoblot analysis whether the expression of key pluripotency transcriptional regulators NANOG, c-MYC, OCT3/4, KLF4, BMI-1 and ALDH1A1 were altered because of HDAC inhibition by NaB. Both SK-ES 1 and RD-ES cells showed a 2–3-fold reduction in the levels of pluripotency-associated genes OCT3/4, c-MYC and BMI-1 compared to untreated control cells. The levels of KLF4, NANOG and ALDH1A1 decreased in SK-ES 1 cells but were relatively unchanged or increased in RD-ES cells (Fig. 5A, B). Thus, SK-ES 1 cells show higher sensitivity to NaB regarding suppression of pluripotency-associated genes.

Interestingly, both EWS cell lines exposed to NaB showed an approximately 2-fold increase in complexity or granularity measured by the SSC-A mean parameter in flow cytometry (Fig. 6A, B). This alteration was accompanied by cell morphology changes with formation of neurite extensions (Fig. 6C). Furthermore, we assessed the expression level of the neural differentiation marker β -III Tubulin (TUBB3) (Fig. 6D). We observed an increase in TUBB3 in cells exposed to HDAC activity inhibition by NaB, with a 2-fold enhancement in protein levels compared to controls. These findings suggest that HDAC activity inhibition may act to suppress a set of genes related to pluripotency, promoting neural differentiation of EWS cells.

HDAC inhibition impairs EWS tumorsphere formation

Cancer stem cells displaying tumor-initiating properties and treatment resistance ability have been identified and characterized in EWS²⁷. Tumorsphere (TS)-forming assays have been widely used as models to study cancer stem cell-enriched cultures²⁸. To verify whether HDAC inhibition altered TS-forming ability of EWS *in vitro*, we measured TS-formation efficiency (TFE) and size through optical microscopy after exposure of cells to NaB in a serum-free TS-inducing medium for 7 days. HDAC inhibition by NaB significantly reduced TFE in both EWS cell lines. In addition, NaB reduced the size of EWS tumorspheres (Fig. 7). It is noteworthy that the higher dose of NaB (5 mM) fully impaired the cells' ability to produce tumorspheres. These findings suggest that HDIs can decrease the ability of EWS

cancer stem cells to expand and survive, thus revealing HDAC as a potential novel EWS cancer stem cell target.

Discussion

Inhibition of HDAC activity has become an increasingly promising therapeutic strategy given its potential to ameliorate the aberrant epigenetic state that underlies a variety of cancers and allows the expression of silenced genes that can promote cell death or differentiation. We found that NaB efficiently inhibits HDAC global activity in EWS cells, along with cell growth, long-term proliferation, and colony formation. Similar IC₅₀ values for HDAC inhibition and cell growth impairment were observed in both cell lines, leading to G1-phase accumulation. These findings support previous reports of NaB arresting cell cycle, particularly at G1-phase, and inducing cell differentiation and apoptosis in multiple cell lines^{29–31}. The overexpression of cyclins D (cyclin D1, D2, and D3) can drive aberrant cell cycle regulation and signaling in many human malignant tumors, including EWS tumors and RD-ES cells^{32,33}. Inhibition of HDAC by NaB resulted in a decrease of cyclin D1 protein level in both cell lines, similarly to what was previously reported in neuroblastoma³⁴. The expression level of cyclin D1, a G1- phase regulator protein, may represent an important component that controls growth arrest prior to cell differentiation in EWS cells.

Polyploidy induction is a proposed mechanism by which HDIs control tumor cell growth. For instance, SAHA induced polyploidy in human colon cancer and breast cancer cells, leading to senescence, particularly in cells harboring defective p21WAF1 or p53³⁵. In contrast, we observed a significant reduction in the proportion of cells showing polyploidy after NaB exposure. HDAC inhibition induced changes in pathways related to cell proliferation and survival in EWS cells, including components of neurotrophin signaling. In other types of pediatric and neuroendocrine cancers, differential neurotrophin and Trk expression is associated with patient prognosis and tumor properties including angiogenesis, metastasis, and chemosensitivity³⁶. In EWS, we have recently shown the expression of Trk in tumors, and that Trk inhibition reduced cell growth and increased chemosensitivity³⁷. Here we show that NaB exposure in EWS cells resulted in a reduced NGF and BDNF protein levels, which is accompanied by reductions in components of the ERK and AKT pathways.

Given that neurotrophins are known to mediate neuronal survival via activation of ERK, phosphatidylinositol 3-kinase (PI3K) and phospholipase C γ (PLC- γ)³⁸, which supports our hypothesis that the inhibition of global HDAC activity suppresses Trk-mediated survival pathways in EWS cells.

We also observed a NaB-induced decrease in the levels of the mitotic marker H3 phos-S10. During mitosis in late G2 and M phase of cell cycle, the post-translational modification phosphorylation on serine 10 of histone 3 (H3 phos-S10) is taken as a hallmark of condensed chromatin³⁹. High levels of H3 phos-S10 in oncogene-transformed cells and human cancer cells are associated with amplified activation of the Ras-Mitogen-activated protein kinase (MAPK)-MSK1 pathway⁴⁰. Moreover, H3 phos-S10 overexpression is associated with chromosome instability that plays a role in the maintenance of ploidy⁴¹ and

carcinogenesis⁴². There is a crucial role of phosphorylation on serine 10 of histone 3 at the p21 promoter to activate p21 gene through HDAC inhibition by trichostatin A⁴³.

Carcinogenesis mediated by cancer stem cells may be considered a sort of epigenetic reprogramming where there is loss of expression of specific genes that control differentiation and reactivation of genes specifying stemness⁴⁴. Consistent with this rationale, our results showed that HDAC inhibition potently suppresses pluripotency-associated genes and this is associated with neurite outgrowth and expression of the neuronal differentiation marker β -III tubulin. A microarray analysis report revealed that HDAC inhibition by TSA suppresses pluripotency genes including as Nanog, Klf4, Sox2 and Sall4 in embryonic stem cells while activating differentiation-related genes⁴⁵. β -III tubulin is a neuron-specific marker and is expressed in early postmitotic and differentiated neurons⁴⁶, hence this differentiation neuronal marker has been used to evaluate neural differentiation in EWS^{47,48}. Our findings support the possibility that NaB can reprogram EWS cells towards neural differentiation.

Sphere formation in cancer cell cultures is a widely used platform for cancer stem cell expansion. Cells from tumorspheres had higher levels of pluripotency-related genes such as Oct3/4 and BMI-1, supporting a stem cell phenotype. In addition, tumorspheres showed higher levels of TrkB compared to cells grown in monolayer, suggesting a possible increase in neurotrophin signaling in EWS stem cells⁴⁹. Importantly, NaB reduced sphere formation, indicating that it is capable of targeting EWS stem cells.

Mechanisms other than histone acetylation may be involved in the effects of NaB in cancer cells. Histone lysine butyrylation is another type of epigenetic post-translational mark that can cooperate or compete with acetylation to promote gene expression programs^{50–52}. Increases in butyrylation in neuroblastoma cells after exposure to SAHA have been reported⁵³. It should also be noted that NaB has been shown to enhance cAMP levels and stimulate the activation of multiple protein kinase pathways through mechanisms at least partially mediated by effects independent of epigenetic influences, through direct interactions with cAMP/PKA signaling in the cytoplasm^{54–56}. Thus, we cannot exclude the possibility that NaB-induced effects on cell machinery components other than HDACs are involved in our findings.

A schematic model of the biological actions of NaB in EWS is depicted in Fig. 8. This model proposes a mechanism by which NaB acts through HDAC inhibition on transcriptional regulators to change the differentiation status of EWS cells. Together our results indicate that HDIs can reduce EWS growth and survival and lead to a differentiated state, thus representing a potentially useful strategy to treat EWS tumors.

Materials and Methods

Cell lines and cell culture

Human Ewing sarcoma cell lines SK-ES-1 and RD-ES-1 obtained from American Type Culture Collection (Rockville, MD, USA) were grown in RPMI- 1640 medium (Gibco-BTL, Carlsbad, CA, USA), containing 0.1% fungizone® (250 mg/kg; Invitrogen Life Technologies, São Paulo, Brazil), 4 mg/ml gentamicin and 10% fetal bovine serum (FBS,

Gibco® by Thermo Fisher Scientific, Life Technologies, Brazil). Cells were cultured at 37 °C in a humidified incubator under 5% CO₂.

Drug treatment

Sodium butyrate (NaB) (Sigma Aldrich - St. Louis, MO, USA) was diluted in sterile ultrapure water in a stock solution of 100 mM. EWS cells (2×10⁴) were plated in 24-well plate and exposed to NaB in concentrations ranging from 0.5 to 5 µM for 72 h⁵⁷. For calculation of IC₅₀, data were fitted in a dose response curve (Graphpad Prism v. 6.0) using the equation: $y = min + (max - min)/(1 + 10^{((LogIC50 - x)*Hillslope + Log((max - min)/(50 - min) - 1))})$.

Cell viability

Cells were exposed to NaB as described above. Both cells in the supernatant and adhered cells were detached, collected, centrifuged and washed with PBS twice. To assess viability, cells were incubated with 1 µg/ml propidium iodide (PI) (Sigma Aldrich) in PBS at 4 °C in the dark. PI uptake was assessed by flow cytometry analysis using an Attune Acoustic focusing cytometer (Applied Biosystems, Thermo Fisher Scientific, USA). Data was analyzed using Attune Cytometric Software version 1.2.5. Four individual replicates were performed.

Cell cycle

To assess cell cycle, the EWS cells were cultured in 24-well plates under the same conditions of described above, and then detached, centrifuged and washed with PBS twice. After, the cells were resuspended in 50 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, Mo., USA) in 0.1% Triton X-100 in 0.1% sodium citrate solution containing 5 µg/ml RNase A. One million cells were stained in 1.0 ml of the PI cell cycle solution for 5 min. followed by assessment on an Attune Acoustic focusing cytometer by Applied Biosystems (Thermo Fisher Scientific, USA) analysis. In each sample 20,000 cells were analysed. Data was analyzed using FlowJo Cytometric Software version 10.1. Three individual experiments were performed.

Cumulative population doubling

Cells were plated [2×10⁴ cell/mL] and exposed to NaB, in the same conditions described above, in quadruplicates. Every 4 days, supernatant and adhered cells were collected, quantified by PI uptake and re-plated. The Population Doubling (PD) level was calculated to determine the proliferation potential of the cell lines SK-ES 1 and RD-ES unexposed versus NaB exposed cells. The PD at each passage was calculated using the equation $PD = (\log Xe - \log Xb)/\log 2$ and doubling time was calculated using $DT = T * \ln 2 / \ln(Xe/Xb)$. In both cases 'Xe' is the cell number at the end of the incubation time, 'Xb' is the cell number at the beginning of the incubation time and T is the number of days between time points, as described in the ATCC® Animal Cell Culture Guide. Data is displayed as cumulative PD (CPD), calculated as the sum of all previous PDLs.

All the CPD parameters were analyzed according to Silva and colleagues²⁶. Briefly, Relative end CPD (RendCPD) quantifies the end point of cell proliferation analysis, and is obtained

by the ratio between the final CPD of cells exposed to drug and control cells. To determine the global effect after the treatment intervention, the relative Area Under Curve (rAUC) was calculated (Graphpad Prism v. 6.0 software) using the lower CPD of the initial population to obtain the minimal threshold. RPR (Relative Proliferation Rate) and RTCT (Relative Time to Cross a Threshold), linear regression for both exposed and control groups was calculated using the higher angular coefficients of the last two or more CPD intervals. RPR is the ratio of the angular coefficients of exposed and control groups. RTCT was determined by the ratio of between the control and exposed cells Time to Cross the Threshold (TCT), *i.e.*, a threshold set on a CPD value where both treatment and control conditions present the highest velocity of proliferation. Long-term survival index (LSI) is calculated by the average of the four parameters described above.

Colony formation

Cells were exposed to NaB for 72h and 1×10^2 cells per well were re-plated in 6-well plates and cultured for 10 days with the RPMI medium being changed every 3 days. Cells were then fixed with methanol, followed by staining with 0.5% crystal violet. The stained colonies images were scanned and analyzed using the cell counter plugin on Image J software (NIH, Bethesda, MD, USA).

Tumorsphere culture

Cells were dissociated with trypsin-EDTA into single cell suspension and seeded at 2×10^3 cells/well in 6-well plates. The cells were cultured in a serum-free tumorsphere (TS)-inducing medium, containing DMEM-F12 (1:1) supplemented with 2% B27 supplement (Gibco, Invitrogen, CA, USA), 20 ng/mL recombinant human EGF (Sigma-Aldrich, MO, USA), 20 ng/mL human leukemia inhibitor factor (Thermo Fisher Scientific, Life Technologies), 10 IU/mL (5 mg/mL) heparin (Roche, Mannheim, Germany) and antibiotics as described by Leuchte and colleagues⁵⁸. The media was changed every 4 days. After 8 – 9 days, tumorspheres were dissociated with a non-enzymatic solution containing 1 mM EDTA, 40 mM Tris-HCl and 150 mM NaCl, and re-plated in a 96-well plate to evaluate their capacity to self-renew through secondary EWS tumorsphere formation. Tumorspheres with at least 80µm were analyzed and quantified by inverted phase microscopy (Leica Microsystems, Mannheim Germany). The effect of NaB on tumorsphere formation ability during 7 days was examined.

HDAC activity

Cells (1×10^4) were seeded in a 96 black well plate/clear flat-bottom (Greiner-bioone, Frickenhausen, DE) and exposed to NaB, at the same concentrations described above, for 1 or 3 h. HDAC enzymatic activity was measured by *In Situ* HDAC Activity Fluorometric Assay kit (EPI003, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Three individual experiments were performed. HDAC enzyme activity was calculated across the groups as pmol/min and the percent of HDAC activity was corrected by control (unexposed cells).

Immunofluorescence

For immunofluorescent staining, cells were plated on the glass coverslips, pre-coated with 2 µg/ml of fibronectin. After 72 h of exposure to NaB, cells were fixed in 4% PFA for 15 minutes at room temperature. After two additional washes, the fixed cells were incubated with blocking buffer (1X PBS containing 3% BSA and 0.3% Triton X-100) for 1 h at room temperature, followed by an overnight incubation at 4°C with the following primary antibodies: Histone 3 phos-S10 (1:200, Cell Signaling), Trkb (1:100, Santa Cruz). After washing with PBS twice, cells were incubated with secondary antibodies conjugated with either Alexa fluor-488 (1:800; Invitrogen) or Rhodamine (1:300, Invitrogen) at room temperature for two h. After three additional washes, DNA was stained with 4 µg/ml of Hoechst 33342 (Invitrogen) for 15 minutes at room temperature. After washing, slides were mounted with Prolong Gold anti-fade reagent. Slides were viewed with Leica TCS SP5 Confocal Laser Scanning Microscope, using Leica LAS AF Lite 2.0.2 software to acquire images. For quantitative analysis, was calculated by the percent of mean intensity corrected by the control (unexposed cells). For each group, at least five random images were taken, and the results were determined from at least three independent experiments.

Real time qPCR

Total RNA was extracted from EWS cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Prior to RT-PCR, the samples were treated with DNase I (Promega Corporation, WI, USA). Total RNA was quantified with NanoDrop (Thermo Fisher Scientific, DE, USA). SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) enabled first strand synthesis. Expression of target genes (TrkB and Oct3/4) were quantified using KiCqStart™ SYBR Green qPCR ReadyMix™, with ROX™ (Sigma-Aldrich, St. Louis, MO, USA) and normalized to the expression of Gapdh (housekeeping gene). The primer sequences used are shown in Supplementary Table S1 online.

Western blot

Proteins were separated using 8 – 14% SDS Tris-glycine gels and transferred onto a polyvinylidene difluoride membranes. Membranes were blocked with 5% fat-free milk and incubated with antibodies against BDNF (H117, SC-20981, Santa Cruz, CA, USA), NGF (H-20, sc-548, Santa Cruz, CA, USA), pERK (E-4 sc-7383, Santa Cruz, CA, USA), ERK1 (K-23, sc-94, Santa Cruz, CA, USA), pAKT (S473) (SAB4300042, Sigma Aldrich, MO, USA), AKT (pan) (C67E7, #4691, Cell Signaling Technology, MA, USA), OCT3/4 (sc-5279, Santa Cruz, CA, USA), NANOG (M-149, sc-33760, Santa Cruz, CA, USA), c-MYC (D84C12 - Cell Signaling Technology, MA, USA), KLF4 (H-180, SC-20691, Santa Cruz, CA, USA), ALDH1A1 (ab23375, Abcam, Cambridge, UK), BMI-1 (D20B7, #6964 - Cell Signaling Technology, MA, USA), Cyclin D1 (D71G9 - Cell Signaling Technology, MA, USA), βIII-Tubulin (D71G9 - Cell Signaling Technology, MA, USA) and ACTB (A2228, Sigma Aldrich, MO, USA) used as loading control. Incubation with appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 1 h at RT was performed. Chemiluminescence was detected using ECL Western Blotting substrate (EMD Millipore, DE) and analyzed by ImageQuant LAS500 (GE Healthcare Life Sciences, UK). Densitometric analyses were performed using Image J (NIH, MD, USA). Relative

densitometric unit (RDU) was calculated by the normalization of interest protein level to the housekeeping genes β -actin (ACTB) or Histone 3 (H3) and corrected by control (unexposed cells). Three individual replicates were performed.

Statistical analysis

For statistical analysis, GraphPad Prism version 6.0 was used. Each assay was performed at least three times in biologically independent assays. The significance of differences in mean $\pm$ standard error values was analyzed by one-way ANOVA and Tukey's multiple comparison test or Student's t-test; p values of less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HDAC Inhibition by NaB hinders the growth of EWS cells.

(a) HDAC activity (%) in EWS cell lines SK-ES 1 and RD-ES after 1 h of exposure to NaB; $n = 4$ independent experiments (b) Percent of viable SK-ES 1 and RD-ES cells after 72 h of exposure to NaB; $n = 4$ independent experiments (c) Heat map showing the target IC_{50} calculated by the percentage of HDAC activity in cells exposed to NaB for 1 h, and biological IC_{50} calculated by the percent of viable cells exposed to NaB for 72 h. (d) Morphology of EWS SK-ES 1 (upper panel) and RD-ES (lower panel) cells after 72 h of NaB exposure; black arrows indicates neurite-like extensions (NE). Scale bar: 50 μ m. Data in the graphs are shown as mean $\pm$ s.e.m.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. controls.

Figure 2. Cell cycle changes induced by HDAC inhibition in EWS cells.

(a) Cell cycle distribution of EWS SK-ES 1 (upper panel) and RD-ES (lower panel) cells after 35 h of NaB exposure; $n = 3$ independent experiments (b) Representative images of H3 phos-S10 level (immunofluorescence) from SK-ES 1 (upper panel) and RD-ES (lower panel) cells exposed to NaB for 72 h. (c) Percent of H3 phos-S10 mean intensity (immunofluorescence) from EWS cell lines exposed to NaB for 72 h; $n = 3$ independent experiments. (d) Western blot (upper panel) of Cyclin D1 protein levels in EWS cells exposed to NaB for 72 h, and Relative Densitometric Unit (RDU) analysis (lower panel)

normalized by ACTB, and corrected by control. Data in the graphs are shown as mean $\pm$ s.e.m.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. controls.

Figure 3. HDAC activity inhibition decreases components of signaling pathways related to proliferation and survival in EWS cells.

(a) Western Blot of BDNF and NGF from SK-ES 1 (left) and RD-ES (right) cells exposed to NaB for 72 h. (b) Percent of TrkB mean intensity (immunofluorescence). (c) Representative images of TrkB level (immunofluorescence) from SK-ES 1 (upper panel) and RD-ES (lower panel) cells exposed to NaB for 72 h. (d) Western Blot analysis of pERK, ERK1, pAKT(Ser473), AKT in SK-ES 1 (left panel) and RD-ES (right panel) cells. (e) Radar graph showing Relative Densitometric Unit (RDU) analysis were normalized by ACTB, and

corrected relative to control cells. Data in the graph are shown as mean $\pm$ s.e.m. from three independent experiments; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. controls.

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Figure 4. Persistent inhibition of cell proliferation rate and colony formation ability in EWS cells treated with NaB.

(a) Cumulative Population Doubling (CPD) *in vitro* assay schematic and timeline. (b) Cumulative CPD from SK-ES1 (upper panel) and RD-ES (lower panel) EWS cells after 72 h of NaB treatment plus 16 days in a drug free media, plus 19 days of a mathematical prediction (total 35 days); $n = 3$ independent experiments. (c) CPD profile from SK-ES1 (upper) and RD-ES (lower) EWS cells after 72 h of NaB (5 mM). (Green square - RendCPD, Blue diamond - rAUC, yellow circle - RPR, red triangle - RTCT, black line - LSI). (d) Colony formation in SK-ES1 (left panel) and RD-ES (right panel) cells after 72h

of NaB exposure plus 10 days in a drug free media, measured by percent of colony number and size; $n = 3$ independent experiments. Data in the graphs are shown as mean $\pm$ S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. controls.

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Figure 5. Changes in the expression of pluripotency-associated genes induced by HDAC inhibition in EWS cells.

(a) Western Blot analysis of pluripotency-associated genes in SK-ES 1 (left panel) and RD-ES (right panel) cells after 72 h of exposure to NaB. (b) Radar graph showing Relative Densitometric Unit (RDU) analyses normalized by ACTB level, and corrected by control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls.

Figure 6. Cell complexity and differentiation of EWS cells after HDAC inhibition.

(a) Representative scatter plot from SK-ES 1 (upper) and RD-ES (lower) exposed to NaB for 72 h, measured by cytometric analysis. (b) Percent of SSC-A mean from EWS cells exposed with the indicated concentrations of NaB for 72 h; $n = 4$ independent experiments. (c) Morphology of SK-ES 1 (upper panel) and RD-ES cells (lower panel) after 72 h of NaB exposure; black arrows indicate neurite-like extensions (NE). Scale bar: 50 μ m. (d) Western Blot analysis of the differentiation neural marker TUBB3 (upper panel); $n = 3$ independent experiments, and Relative Densitometric Unit (RDU) analyses (lower panel) normalized by

ACTB, and corrected by control. Data in the graph are shown as mean $\pm$ S.E.M; * $p < 0.05$, ** $p < 0.01$ vs. controls.

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Figure 7. Reduced tumorsphere formation in EWS cells after HDAC inhibition.

(a) Percent of number and size of EWS tumorspheres from SK-ES 1 induced with a serum-free tumorspheres medium compared with 10% FBS medium for 9 days; $n = 3$ independent experiments. (b) Oct3/4 and TrkB mRNA levels in SK-ES 1 cells grown in monolayer (10% FBS medium) and tumorspheres (serum-free tumorspheres medium) induced for 9 days. Gene expression levels were normalized by gapdh level and corrected by monolayer; $n = 3$ independent experiments. (c) Representative images from EWS tumorspheres from SK-ES1 (upper left panel) and RD-ES (upper right panel) cells after 7 days of NaB exposure. The percentage of EWS tumorspheres formation efficiency (TFE) (number of tumorspheres normalized by the number of plated single cells and corrected by control (lower panel) and the size of EWS tumorspheres is shown. Only tumorspheres $\geq 80\mu\text{m}$ were analyzed. Scale bars: $200\mu\text{m}$ (upper), $100\mu\text{m}$ (lower); $n = 3$ independent experiments. (d) Western blot of c-MYC, BMI-1 and TrkB in SK-ES 1 cells in monolayer (ML), Tumospheres (TS) induced for 7 days, and tumorspheres exposed to 1 mM of NaB for 7 days (TS + N); $n = 3$ independent

experiments. (e) Relative Densitometric Unit (RDU) analyses were normalized by coomassie loading control, and corrected by monolayer. Data in the graphs are shown as mean $\pm$ s.e.m.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. controls.

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Figure 8. Schematic model depicting biological actions HDAC inhibition by NaB in EWS.

(a) Unbalance between the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) results in an aberrant epigenetic state that occurs often in EWS cells. HDAC inhibitors such as NaB have been employed to revert this setting by increasing histone acetylation and gene transcription. (b) As a result of epigenetic dysregulation, EWS cells present an aberrant expression of genes associated cell cycle, proliferation, survival, and pluripotency pathways. HDAC inhibition by NaB leads to transcriptional change by suppressing cell cycle, proliferation, survival, and pluripotency-associated genes, whereas de-repressing expression of the neural differentiation protein TUBB3. (c) A poor differentiated phenotype is a feature of EWS. HDAC inhibition change cell fate towards increased cellular complexity, arrested cell growth by G1-phase accumulation, decreased long-term clonal repopulation and reduced tumorsphere formation. Inhibiting HDAC might epigenetically reprogram EWS cells from an undifferentiated to a more differentiated state associated with a better prognosis (d).