Supporting Information

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SI Materials and Methods

Cell Culture. The human neuroblastoma cell lines BE(2)-C [European Collection of Cell Cultures (ECACC)], Kelly (DSMZ), and IMR32 (DSMZ) were cultured under standard conditions in DMEM with L-glutamine and 4.5 g/L glucose containing 10% FCS (Sigma) and 1% nonessential amino acids (NEAAs) (Invitrogen). Astrocyte culture medium also contained 1% astrocyte growth supplement. Primary human fibroblasts (ECACC) were cultured in DMEM/HAM's F12 (Invitrogen) supplemented with 10% FCS (Sigma) and 1% NEAAs (Invitrogen). All neuroblastoma cell lines were verified using DNA fingerprinting by the DSMZ. Human infant astrocytes were obtained from S. Pfister (German Cancer Research Center, Heidelberg, Germany) (1).

To demonstrate the tumor cell-selective effects of histone deacetylase (HDAC)10, JoMa1 cells were used (2, 3). Murine JoMa1 cells were derived from the neural crest of mouse embryos harboring ubiquitous cMycER^{T} expression (ROSA^{cMycERT}). Supplementing JoMa1 cell culture medium with (*Z*)-4-hydroxytamoxifen (4-OHT) (Sigma Aldrich; no. H7904) maintains conditional low-level cMycER^{T} activation and retains JoMa1 cells in an immortalized and undifferentiated state. JoMa1 cells express early neural crest stem cell markers and can be instructed to differentiate into neurons, glia, smooth muscle cells, melanocytes, and also chondrocytes upon 4-OHT removal.

Transient Transfections. Commercially available siRNAs were used to silence *HDAC10*: siRNA #1 (ID 33581; Exon14/15; Ambion); siRNA #2 (ID 120681; Exon5; Ambion). For *HDAC6* knockdown, siRNA #120451 (Ambion) was used. Heat shock cognate 70 (*HSC70*) (*HSPA8*) was targeted by an siRNA with the sequence: sense, 5′-GAU CGA UUC UCU CUA UGA ATT-3′; antisense, 5′-UUC AUA GAG AGA AUC GAU CTT-3′ (Ambion). Lysosome-associated membrane protein type 2A (*LAMP2A*) was targeted by an siRNA with the sequence: sense, 5′-CCA UCA GAA UUC CAU UGA ATT-3′; antisense, 5′-UUC AAU GGA AUU CUG AUG GTT-3′ (Ambion). The siRNA against *BECN1* was described previously (4). Qiagen FlexiTube Hs_APG7L_5 was used to silence autophagy-related gene 7 (*ATG7*). The following siRNAs were used as negative controls: RISC-Free (siCONTROL RF siRNA #1; Dharmacon); NC #1 (Silencer Negative Control #1; Ambion); NC #5 (Silencer Negative Control #5; Ambion). Transfection efficacy in BE(2)-C cells was 90.3 ± 2.3% as determined by fluorescently labeled siRNA siGLO Lamin A/C (Dharmacon). The reverse-transfection protocol was used in a 96-well plate format for siRNAs according to the manufacturer's instructions (Qiagen). Cells were seeded into six-well plates at 2×10^5 cells per well for transfection with expression constructs. Transient transfection was carried out 24 h after seeding using Effectene (Qiagen), according to the manufacturer's instructions. HDAC10 was overexpressed using the C-terminal FLAG-tagged HDAC10 pCMX construct, and the C-terminal FLAG-tagged HDAC10 H135A mutant construct (5) were provided by T.-P. Yao (Duke University Medical Center, Durham, NC). The negative control vector, pCMX-FLAG, was provided by R. Schuele (University of Freiburg Medical Center, Freiburg, Germany). Microtubule-associated protein 1 light chain 3 (LC3) was overexpressed using the EGFP-LC3 construct (6) and mCherry-EGFP-LC3B construct (no. 22418; Jayanta Debnath) from Addgene.

Generation of Stable Cell Lines. For lentiviral gene knockdown, pLKO.1 shRNA sequences against human HDAC10 [SHCLNG Mission shRNAs; Sigma; shR-1, the RNAi consortium number (TRCN) 0000195664; shR-2, TRCN 0000004859; shR-3, TRCN 0000004863; shR-4, TRCN 0000004861] and shRNA negative control (5′-CCG GAA TTG CCA GCT GGT TCC ATC ACT CGA GTG ATG GAA CCA GCT GGC AAT TTT TTT G-3′ and 5′-CCG GAA TTG CCA GCT GGT TCC ATC ACT CGA GTG ATG GAA CCA GCT GGC AAT TTT TTT G-3′) were used. pLKO lentiviral vectors containing shRNA were transfected into 293T cells together with psPAX2 (packaging vector) and pMD2.G (vesicular stomatitis virus G glycoprotein envelope protein expression vector) using calcium-phosphate transfection (Sigma). Virus-containing supernatants were collected and filtered after 24, 40, and 48 h. BE(2)-C cells were infected in six-well plates at 60,000 cells per well, and transgene expressing cells selected and maintained with puromycin (Sigma; 2–5 μg/mL). Once plated for experiments, cells were kept in puromycin-free medium.

Human IMR32 cell lines stably expressing HDAC10 were established by transfection using Effectene (Qiagen) with pCMV/hygro-FLAG HDAC10 cDNA (Sino Biological) or hygromycin-resistant empty vector control. Transfected cells were selected with hygromycin (200 μg/mL) for 3 wk. A mixed population of hygromycin-resistant cells was used for experimental analysis.

Real-Time Reverse Transcription–PCR. The following specific primer pairs were used: *HDAC10* (forward: 5′-ATC TCT TTG AGG ATG ACC CCA G-3′; reverse: 5′-ACT GCG TCT GCA TCT GAC TCTC-3′), *HDAC6* (forward: 5′-CAA GGA ACA CAG TTC ACC TTC G-3′; reverse: 5′-GTT CCA AGG CAC ATT GAT GGT A-3′), *HSC70* (*HSPA8*) (forward: 5′-TGC TGC TGC TAT TGC TTA CG-3′; reverse: 5′-TCA ATA GTG AGG ATT GAC ACA TCA-3′), *LAMP2A* (QT00077063; Qiagen), *BECN1* (4), *ATG7* (forward: 5′-ATG CCT GGG CAT CCA GTG AAC TTC-3′; reverse: 5′-CAT CAT TGC AGA AGT AGC AGC CA-3′), *SDHA* (succinate dehydrogenase complex, subunit A; forward: 5′-TGG GAA CAA GAG GGC ATC TG-3′; reverse: 5′-CCA CCA CTG CAT CAA ATT CAT G-3′) and *HPRT* (hypoxanthine phosphoribosyltransferase 1; forward: 5′-TGA CAC TGG CAA AAC AAT GCA-3′; reverse: 5′-GGT CCT TTT CAC CAG CAA GCT-3′).

Immunoblotting. The following antibodies were used: anti-HDAC10 (Sigma), anti-LC3 (Sigma), anti-HDAC6 (Cell Signaling), anti-protein (p)62/sequestosome 1 (5F2; MLB), anti-Hsp70/Hsc70 (H-300; Santa Cruz Biotechnology), anti-Hsc70 (13D3; Abcam), anti-FLAG-M2 (Sigma), anti-GAPDH (Millipore), anti-Ac-Lysine (rabbit, Cell Signaling), anti-LAMP2 (H4B4; Santa Cruz Biotechnology), anti-Beclin1 (D-18; Santa Cruz Biotechnology), anti-ATG7 (Cell Signaling), anti–acetyl-H4 (Millipore), anti–acetyl-tubulin (6-11B-1; Sigma), and anti-β-actin (AC-15; Sigma). Ratios were calculated with the Bio-1D Version 12.10a software (Peqlab).

Determination of K_d Values for Tubastatin. HDACs were enriched from stable isotope labeling by amino acids in cell culture (SILAC)-encoded MV4-11 (DSMZ) cell lysates using an affinity matrix for HDACs. The concentration of the immobilized compound to obtain 50% binding of each target protein was determined in quantitative binding experiments. In a second step, tubastatin was used within a concentration range of 3 nM to 30 μM to compete with target binding to the affinity matrix. IC_{50} values were determined for each target protein. K_d values for tubastatin were calculated for each target protein using the Cheng–Prusoff equation (7). Binding and competition experiments were performed for 90 min at 4 °C.

1. Eisenhardt AE, et al. (2011) Functional characterization of a BRAF insertion mutant associated with pilocytic astrocytoma. *Int J Cancer* 129(9):2297–2303.
2. Maurer BJ, et al. (2007) Improved oral delivery of N-(4-hydroxyphenyl)retinamide with a novel LYM-X-SORB organized lipid complex. *Clin Cancer Res* 13(10):3079–3086.
3. Molenaar JJ, et al. (2012) LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. *Nat Genet* 44(11):1199–1206.
4. Böck BC, et al. (2010) The PEA-15 protein regulates autophagy via activation of JNK. *J Biol Chem* 285(28):21644–21654.
5. Guardiola AR, Yao TP (2002) Molecular cloning and characterization of a novel histone deacetylase HDAC10. *J Biol Chem* 277(5):3350–3356.
6. Jackson WT, et al. (2005) Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol* 3(5):e156.
7. Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22(23):3099–3108.

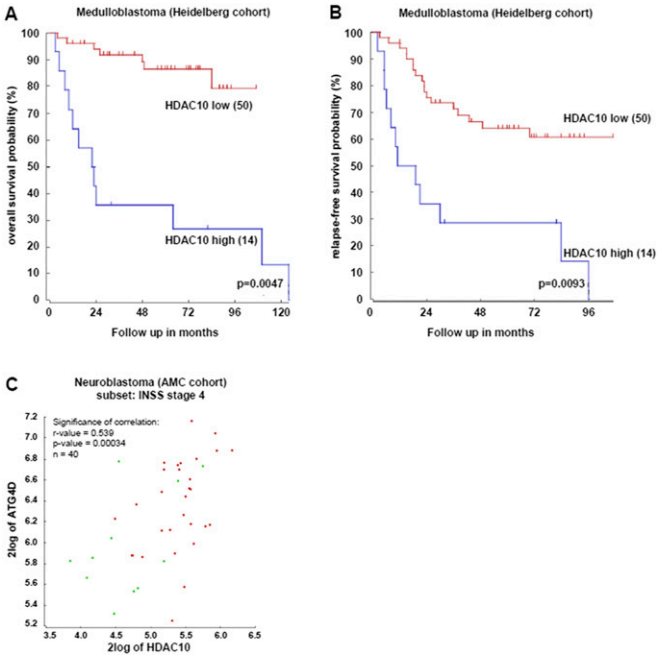


Fig. S1. Kaplan–Meier curves are shown for overall (*A*) and relapse-free (*B*) survival in medulloblastoma patients whose tumors expressed low ($n = 14$) or high ($n = 50$) levels of *HDAC10*. Scan modus was used for cutoff determination and *P* values were corrected for multiple testing (Bonferroni). (*C*) A correlation graph is shown for the expression of *HDAC10* and *ATG4D* in stage 4 tumors [Academic Medical Center (AMC) cohort]. Green dots, alive; red dots, dead. The R2 microarray analysis and visualization platform (http://r2.amc.nl) was used for all calculations and is the source of the data.

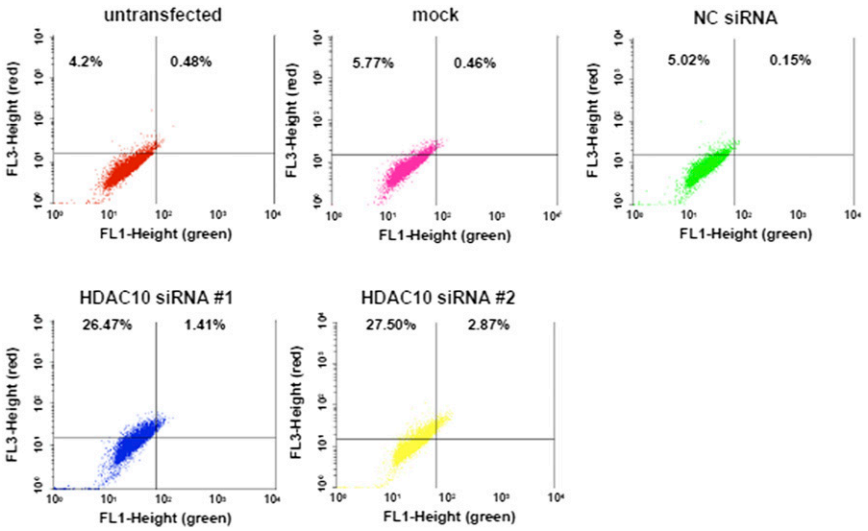


Fig. S2. Scatter plots showing fluorescence 1 (FL1) (green) and FL3 fluorescence (red) of acridine orange-stained cells 72 h after transfection. A shift from green to red fluorescence indicates an increase of acidic vesicular organelles. HDAC10 siRNA #1 and #2, cells transfected with siRNAs against two different regions on *HDAC10* mRNA; mock, cells treated with transfection reagent only; NC siRNA, cells transfected with negative control siRNA #1; untransfected, BE(2)-C cells cultured in medium only.

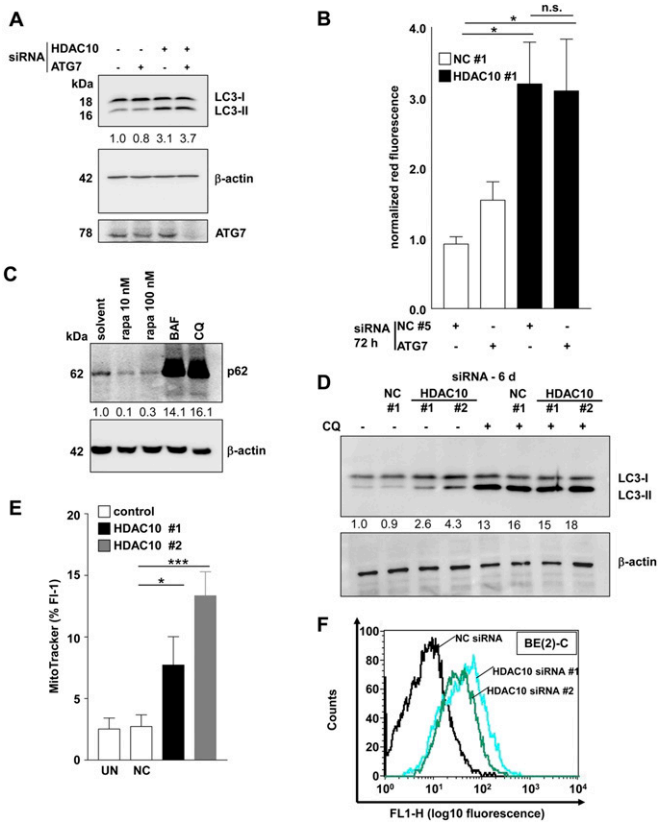
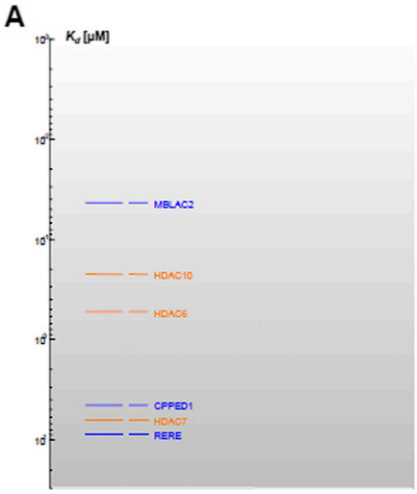


Fig. S3. (*A*) Western blot showing LC3-I and LC3-II expression in whole-cell lysates of BE(2)-C cells 6 d after transfection with *HDAC10* siRNA #1 or *ATG7* siRNA. β-actin was used as a loading control. Ratios of LC3-II to LC3-I are shown below the Western blot. (*B*) Accumulation of acidic organelles (red fluorescence) detected by acridine orange staining in FACS analysis after knockdown of HDAC10 with and without *ATG7* knockdown compared with the negative controls, as indicated. Means of at least three independent experiments are shown. Error bars represent SEM. Significant differences between groups were detected by unpaired two-tailed *t* test. $*P < 0.05$; n.s., not significant. (*C*) Expression of p62 protein levels after treatment with 10 or 100 nM rapamycin (rapa) to induce autophagy or 10 nM bafilomycin A1 (BAF) or 25 μM chloroquine (CQ), both lysosomal inhibitors. β-Actin served as a loading control. Numbers indicate p62 expression normalized to β-actin expression. (*D*) Western blot showing LC3-I and LC3-II expression in whole-cell lysates of BE(2)-C cells 6 d after transfection with *HDAC10* siRNA #1, #2, or negative control siRNA #1 (NC#1), as well as in combination with 25 μM chloroquine (CQ) treatment. β-Actin was used as a loading control. Ratios of LC3-II to LC3-I are shown below the Western blot. (*E*) Flow-cytometric mitochondrial detection using MitoTracker Green dye (FL1-H) in HDAC10-depleted BE(2)-C cells (6 d after transfection). Means (±SEM) of at least three independent experiments are shown. Significant differences between groups were detected by unpaired two-tailed *t* test. HDAC10 siRNA #1 and #2, cells transfected with siRNAs against two different regions on *HDAC10* mRNA; NC, transfected with negative control siRNA #1; UN, untransfected cells. $*P < 0.05$; $***P < 0.001$. (*F*) Flow-cytometric detection of reactive oxygen species in BE(2)-C cells 72 h after transfection with *HDAC10* siRNAs #1 or #2 or negative control siRNA (NC #1) using the oxidant-sensitive probe 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester. Fluorescence was detected in Fl-1 channel.



	Tubastatin
HDAC1	no target
HDAC2	no target
HDAC3	no target
HDAC4	no target
HDAC5	no target
HDAC6	0.53
HDAC7	>6.33
HDAC8	no target
HDAC10	0.22

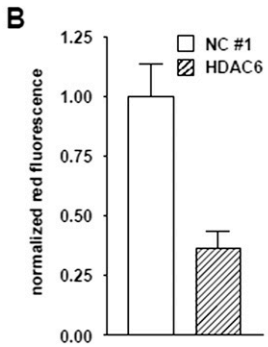


Fig. S4. (*A*) Epigenetics target profiling showing target profiles for tubastatin and K_d values of HDACs determined for tubastatin. (*B*) Flow-cytometric detection of acidic organelles after acridine orange staining (red fluorescence) 6 d after transfection of BE(2)-C with siRNAs specific for *HDAC6* or the negative control siRNA (NC #1). Means from at least three independent experiments are shown, and error bars represent SEM.

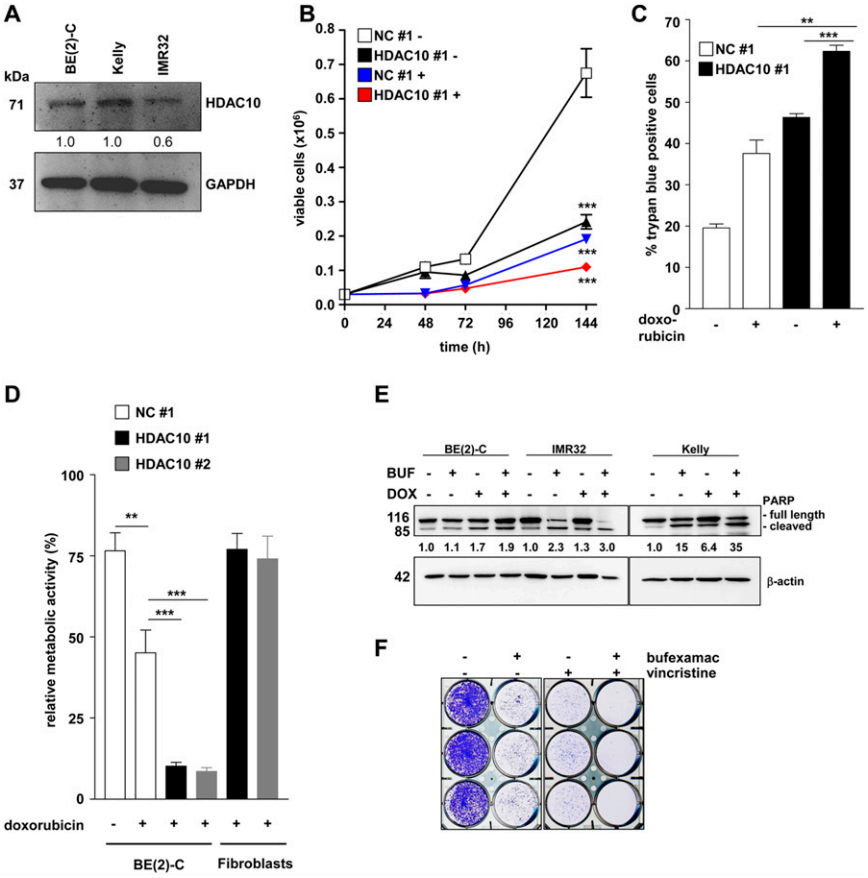


Fig. S5. (*A*) Endogenous HDAC10 protein levels in three neuroblastoma cell lines detected via Western blotting. GAPDH served as a loading control. Numbers indicate HDAC10 expression normalized to GAPDH expression. (*B*) Population growth over time of BE(2)-C cells transfected with *HDAC10* siRNA #1, or negative control siRNA #1 (NC#1). +, transfected cells were cotreated with 0.1 μg/mL doxorubicin. Viable cells were counted (trypan blue exclusion) and are displayed as means (±SEM) of at least three independent experiments. Significant differences between groups were detected by unpaired two-tailed *t* test. ***$P < 0.001$. No significant differences in growth were detected between doxorubicin-treated and HDAC10-depleted and doxorubicin-treated and control-transfected cells 48 or 72 h after transfection, but differences in growth were significant 144 h after transfection ($P < 0.001$). (*C*) BE(2)-C cells transfected with *HDAC10* siRNA #1, or negative control siRNA #1 (NC#1) were treated with 0.1 μg/mL doxorubicin and then monitored 6 d after transfection and 72 h after doxorubicin treatment for cell death using trypan blue staining (dead cells, trypan blue-staining cells). (*D*) The BE(2)-C cell line and fibroblasts were transfected with *HDAC10* siRNAs (siRNA #1 or #2) or negative control siRNA (NC #1) and then treated 72 h after transfection with 0.1 μg/mL doxorubicin for 72 h. Metabolic activity was measured by WST-1 assay and is shown relative to untransfected, untreated cells. (*E*) Poly(ADP-ribose) polymerase (PARP) cleavage in three neuroblastoma cell lines detected via Western blotting. Cells were treated with the HDAC6/10 inhibitor, bufexamac (BUF), alone or in combination with the cytotoxic agent, doxorubicin (DOX). Where treatment is indicated, all cell types were treated with 30 μM bufexamac for 6 d. Doxorubicin treatment, where indicated, started 72 h before lysate generation and was at varied concentrations: neuroblastoma cell lines, BE(2)-C (0.1 μg/mL), Kelly (0.05 μg/mL), and IMR32 (0.01 μg/mL). β-Actin served as a loading control. Numbers indicate cleaved PARP expression normalized to β-actin expression. (*F*) BE(2)-C cells were treated with bufexamac alone or in combination with vincristine, and colonies were stained after 10 d. All bars represent means (±SEM) of at least three independent experiments. Significant differences between groups were tested using an unpaired, two-tailed *t* test. **$P < 0.01$; ***$P < 0.001$.

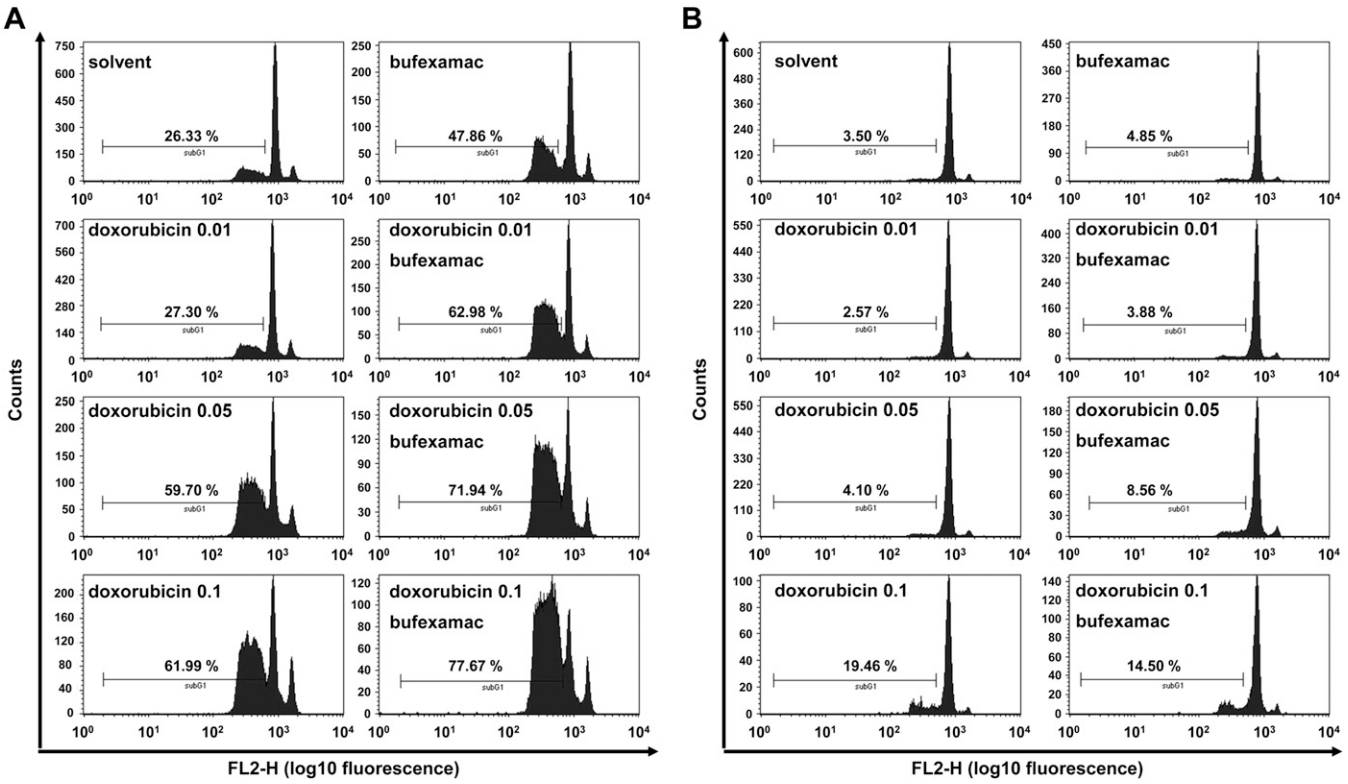


Fig. S6. Evaluation of tumor cell selectivity with JoMa1 cells. JoMa1 cells were seeded with (*A*) or without (*B*) 4-OHT. Supplementing JoMa1 cell culture medium with 4-OHT retains JoMa1 cells in an immortalized and undifferentiated state attributable to induction of MYC expression, whereas withdrawal of 4-OHT results in more differentiated, nonmalignant neuroblasts. After 3 d, medium was changed and bufexamac (30 μM), or DMSO was added. After another 3 d, doxorubicin (0.01, 0.05, and 0.1 μg/mL) was added to the media. Three days later, the cells and supernatants were collected for the detection of apoptotic cell death by propidium iodide (PI) staining of ethanol-fixed cells.

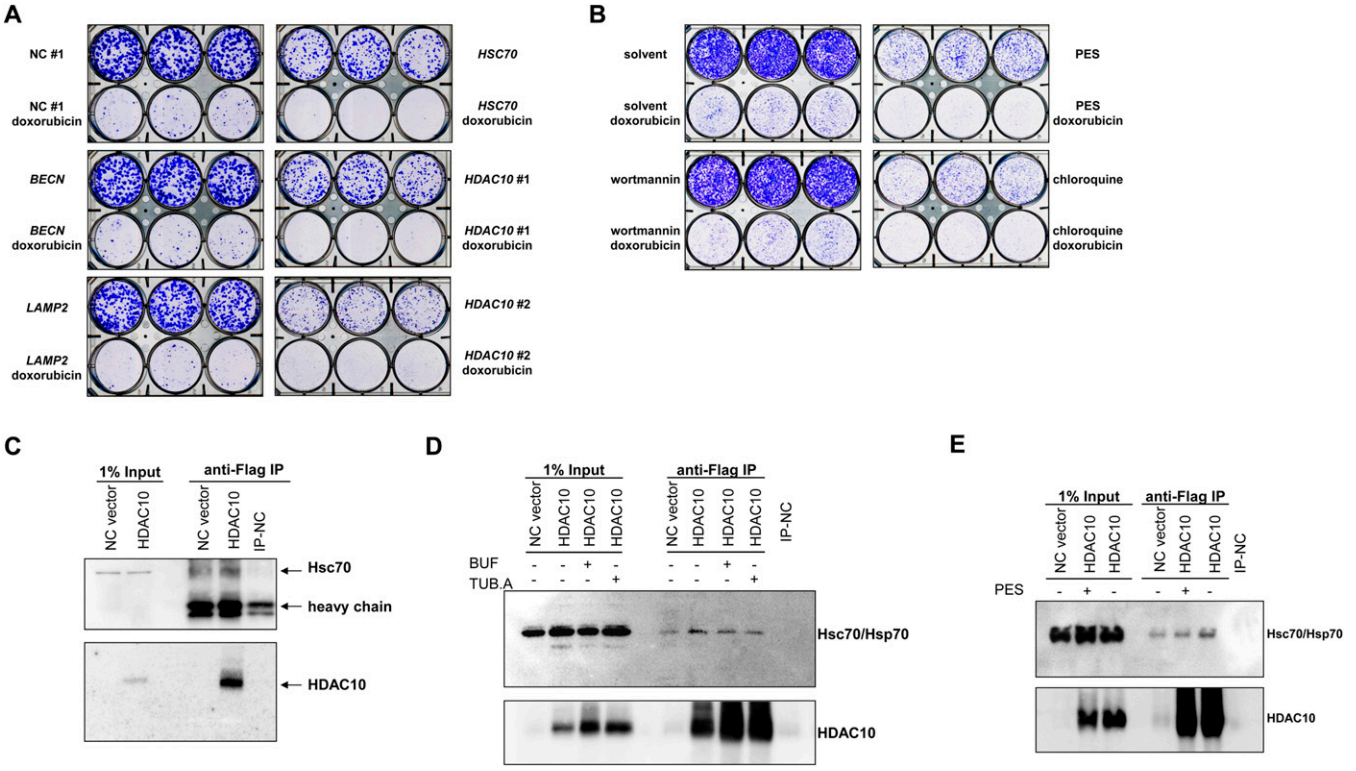


Fig. S7. (*A*) BE(2)-C cells were transfected with siRNAs targeting *BECN1*, *LAMP2*, *HSC70*, *HDAC10* (siRNA #1 and #2), or the negative control siRNA (NC #1). Cells were treated with 0.1 μg/mL doxorubicin or normal culture medium, and colonies were stained after 10 d. (*B*) BE(2)-C cells were treated with wortmannin (100 nM), PES (10 μM), chloroquine (15 μM), or the solvent control. Where indicated, cells were treated with 0.1 μg/mL doxorubicin. Colonies were stained after 10 d. (*C*) Whole-cell extracts (input) of BE(2)-C cells transfected with either a HDAC10 expression construct (HDAC10) or empty vector (NC vector) were immunoprecipitated with an antibody directed against the FLAG tag. HDAC10 and Hsc70 proteins in the extracts and immunoprecipitated complexes were detected on Western blots. The specific Hsc70 (13D3) antibody was used for the detection of Hsc70. (*D*) Whole-cell extracts (input) of BE(2)-C cells transfected with either a HDAC10 expression construct (HDAC10) or empty vector (NC vector) and treated with bufexamac (BUF) or tubastatin A (TUB.A), as indicated, were immunoprecipitated with an antibody directed against the FLAG tag. HDAC10 and Hsc70/Hsp70 proteins in the extracts and immunoprecipitated complexes were detected on Western blots. IP-NC indicates the immunoprecipitation negative control. (*E*) Whole-cell extracts (input) of BE(2)-C cells transfected with either a HDAC10 expression construct (HDAC10) or empty vector (NC vector) and treated with PES or solvent control, as indicated, were immunoprecipitated with an antibody directed against the FLAG tag. HDAC10 and Hsc70/Hsp70 proteins in the extracts and immunoprecipitated complexes were detected on Western blots. IP-NC indicates the immunoprecipitation negative control.

Table S1. Impact of HDAC expression in INSS stage 4 neuroblastomas on the patients overall survival

HDAC family member	HDAC-specific probe set	Overall survival* (high vs. low HDAC tumor† expression)
HDAC1	201209_at	NS
HDAC2	201833_at	NS
	242141_at	NS
HDAC3	216326_s_at	NS
HDAC4	204225_at	NS
	228813_at	NS
	1554322_a_at	NS
HDAC5	202455_at	NS
	229408_at	NS
HDAC6	206846_s_at	NS
	216224_s_at	NS
HDAC7	217937_s_at	NS
HDAC8	223345_at	NS
	223909_s_at	NS
	223908_at	NS
HDAC9	205659_at	NS
	1552760_at	NS
	1552758_at	NS
	234393_at	NS
HDAC10	226672_s_at	$P = 0.020$
HDAC11	227679_at	NS
	219847_at	NS

INSS, International Staging System; NS, not significant.

*Scan modus for cutoff determination with Bonferroni correction of *P* values for multiple testing was used. $P < 0.05$ was considered significant.

†$n = 40$ INSS stage 4 neuroblastomas.

Table S2. Genes whose expression correlates with *HDAC10* expression in primary neuroblastomas

Positively correlated*
HDAC10
EGFL7
AXIN1
TRIM10
FBXL6
ADAMTS10
URM1
ADRBK1
C19orf61
PCDHGB5
ZDHHC8
ATG4D
XAB2
RAVER1
GRWD1
BRD2
CORO1B
FLT4
CLDN5
SLC26A6
TAF6L
PPIL2
Negatively correlated*
ARMCX3
RNF103
ENSA
AVL9
Overrepresented gene set in gene-set analysis†
Regulation of autophagy *(ATG4D)*

*R $P < 0.01$.

†Gene sets for which the number of *HDAC10*-correlated genes are overrepresented ($P < 0.05$ from 2×2 contingency table analysis with continuity correction).