HOKKAIDO UNIVERSITY

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Supporting information for

Manipulating Histone Acetylation Leads to Antitumor Effects in Hemangiosarcoma Cells

Case Number	Breed	Age	Sex	Location
1	Miniature Dachshund	10y	F Spay	Spleen
2	Jack Russell Terrier	9y	M Cast	Spleen
3	Jack Russell Terrier	7y	F Spay	Spleen
4	Miniature Dachshund	14y	M Cast	Spleen
5	French Bulldog	7y	M Cast	Spleen
6	Miniature Dachshund	9y	F	Spleen
7	Miniature Schnauzer	11y	M	Spleen
8	Miniature Dachshund	10y	F	Spleen
9	Flat-coated retriever	11y	F	Spleen
10	Golden Retriever	9y	M	Liver

*F: female, M: male, Spay: spayed, Cast: castrated

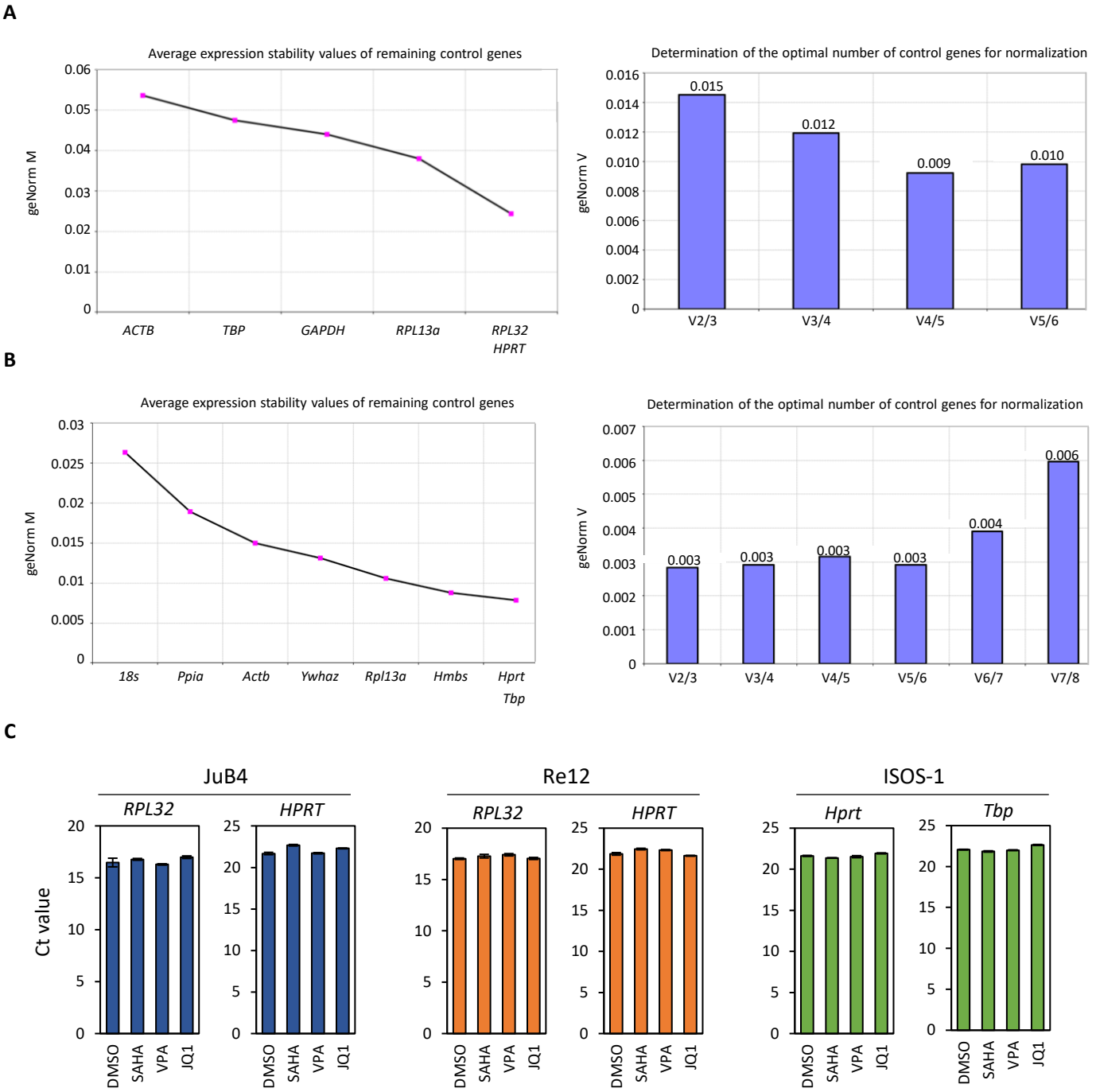
Supplementary Table 1. Case information

Protein Name	Maker	Host	Dilution	Catalog Number
Acetylated Histone H2B	Santa Cruz Biotechnology, Inc.	Mouse	1:500	sc-515937
Acetylated Histone H3	Active Motif	Rabbit	1:5000	39040
Acetylated Histone H4	Santa Cruz Biotechnology, Inc.	Mouse	1:500	sc-377520
Cleaved-caspase 3	Cell Signaling Technology	Rabbit	1:1000	9661S
pAKT	Cell Signaling Technology	Rabbit	1:1000	4060
tAKT	Cell Signaling Technology	Rabbit	1:1000	4691
pERK1/2	Cell Signaling Technology	Rabbit	1:1000	4370S
tERK1/2	Cell Signaling Technology	Rabbit	1:1000	4695S
LC3	MBL	Rabbit	1:1000	PM036
Actin	Sigma-Aldrich	Mouse	1:10000	MAB1501
H3	MAB Institute	Mouse	1:10000	MABI0001-20
Goat anti-Mouse IgG (H+L)	Thermo Fisher Scientific	Goat	1:10000	G21040
Goat anti-Rabbit IgG (H+L)	Thermo Fisher Scientific	Goat	1:10000	G21234

Supplementary Table 2. Antibody list

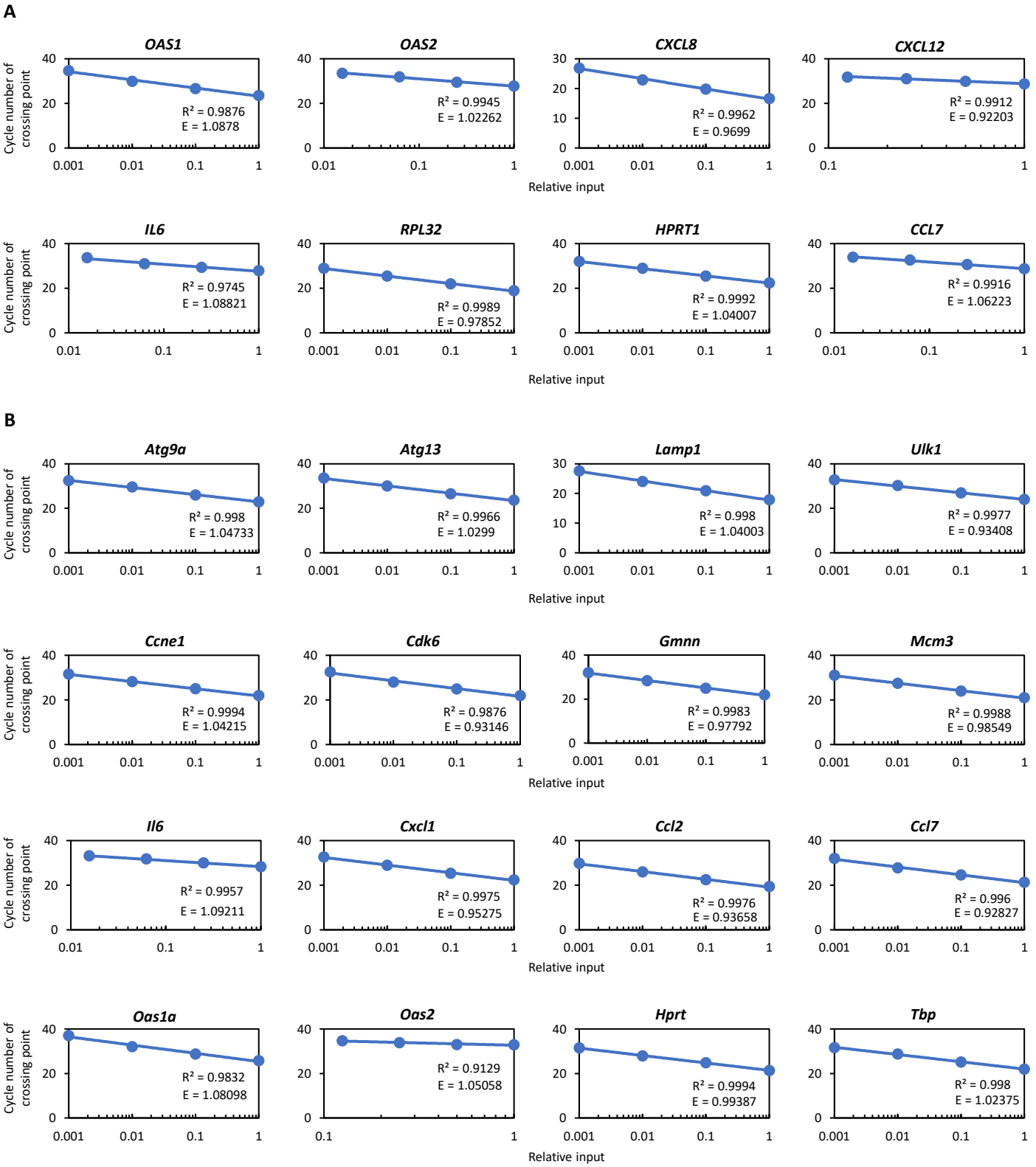
Species	Target	Sequence (Forward)	Sequence (Forward)	Gene ID
Canine	*OAS1*	TGTGCGGGTGTCTAAAGTTG	TGAACTGTCCTCGTTTCTCG	ENSCAFG00000023556
	OAS2	TGACCCAGATCCAGAAAACC	CCATTCGGTAGCGTCTTTTG	ENSCAFG00000023107
	CXCL8	GGCAGCTTTTGTCCTTTCTG	ACACTGGCATCGAAGTTCTG	ENSCAFG00000003029
	CXCL12	AGCCAACGTCAAGCATCTCA	TCAATGCACACCTGTCTGCT	ENSCAFG00000007026
	IL6	TCGGCAAAATCTCTGCACTG	TTTCTGCCAGTGCCTCTTTG	ENSCAFG00000002733
	RPL32	TGGTTACAGGAGCAACAAGAAA	GCACATCAGCAGCACTTCA	ENSCAFG00000004871
	HPRT1	CACTGGGAAAACAATGCAGA	ACAAAGTCSGGTTTATAGCCAACA	ENSCAFG00000018870
	GAPDH	ATTCCACGGCACAGTCAAG	TACTCAGCACCAGCATCACC	ENSCAFG00000015077
	TBP	ATAAGAGAGCCCCGAACCAC	TTCACATCACAGCTCCCCAC	ENSCAFG00000004119
	RPL13A	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAATTC	ENSCAFG00000029892
Mouse	*Atg9a*	ATCACCTTGGCACCACATTG	TGGTGAAGGCAACCACAAAG	ENSMUSG00000033124
	Atg13	ATTTGCACCCGCTCATCATC	AGGGCCTTCTTTGCTTCATG	ENSMUSG00000027244
	Lamp1	AGTCTTGTGTTGGCGTTCAG	AGGCAATGCATTACGTGAGC	ENSMUSG00000031447
	Ulk1	AAACATCGTGGCGCTGTATG	TTCACTCAGTGTGCGCATAG	ENSMUSG00000029512
	Ccne1	AAGCCCAAGCAAAGAAAGCC	TGGCAGGTTTGGTCATTCTG	ENSMUSG00000002068
	Cdk6	AAGCTGCTGACCAATTGTGC	ATACGCATGCACACACACTC	ENSMUSG00000040274
	Gmnn	AGGAGAACGCTGAAGATGATCC	TGCTAGCTGGTCATCCCAAAG	ENSMUSG00000006715
	Mcm3	CGTTCCAAGGATGTCTTTGAGC	ATGTGGCTGCCGTTTTCAAG	ENSMUSG00000041859
	Il6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC	ENSMUSG00000025746
	Cxcl1	CAAACCGAAGTCATAGCCACAC	TTTCTCCGTTACTTGGGGACAC	ENSMUSG00000029380
	Ccl2	TTTCCACAACCACCTCAAGC	TTAAGGCATCACAGTCCGAGTC	ENSMUSG00000035385
	Ccl7	AAAACCCCAACTCCAAAGCC	ACAGCGGTGAGGAATTTTGC	ENSMUSG00000035373
	Oas1a	AAGCACTGGTACCAACTGTG	AGGCAAAGACAGTGAGCAAC	ENSMUSG00000052776
	Oas2	TAGACCAGGCCGTGGATG	GTTTCCCGGCCATAGGAG	ENSMUSG00000032690
	Hprt	GCTTGCTGGTGAAAAGGACCTCTCGAAG	CCCTGAAGTACTCATTATAGTCAAGGGCAT	ENSMUSG00000025630
	Tbp	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA	ENSMUSG00000014767
	Ywhaz	GAAAAGTTCTTGATCCCCAATGC	TGTGACTGGTCCACAATTCCTT	ENSMUSG00000022285
	Actb	AGGCCAACCGTGAAAAGATG	TGGATGGCTACGTACATGGC	ENSMUSG00000029580
	Hmbs	ATGAGGGTGATTCGAGTGGG	TTGTCTCCCGTGGTGGACATA	ENSMUSG00000032126
	Rpl13a	AGGGGCAGGTTCTGGTATTG	TGTTGATGCCTTCACAGCGT	ENSMUSG00000074129
	Ppia	CGCGTCTCCTTCGAGCTGTTTG	TGTAAAGTCACCACCCTGGCACAT	ENSMUSG00000071866
	18s	CGGCTACCACATCCAAGGAA	AGCTGGAATTACCGCGGC	ENSMUSG00000119584

Supplementary Table 3. Primer list



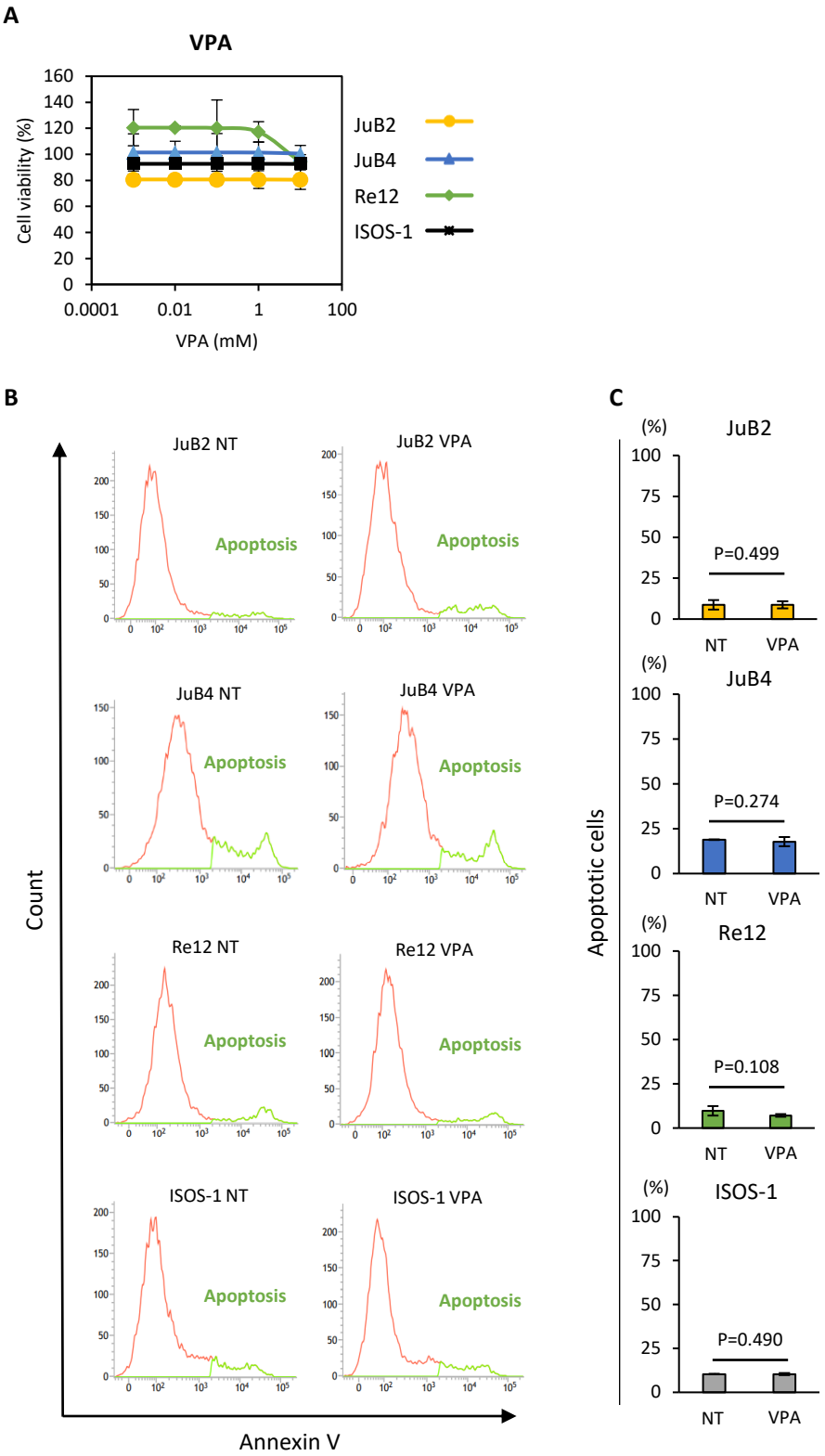
Supplementary Figure 1. geNorm analysis to determine appropriate reference gene sets

(A, B) (Left) Average expression stability values (geNorm M) of candidate genes. The gene with the lowest M value has the most stable expression in dogs (A) and mice (B). (Right) Determination of optimal number of control genes for geometric normalization for dogs (A) and mice (B). To determine the optimal number of reference genes, 0.15 V value was used as the cut-off value as Vandesompele J et al. (2002. *Genome Biol.*) recommended. **(C)** Ct values of reference genes in canine cell lines (JuB4 and Re12) and the mouse cell line (ISOS-1) treated with/without HDACi (SAHA and VPA) or BETi (JQ1). Data are plotted as average percentages ± SD.



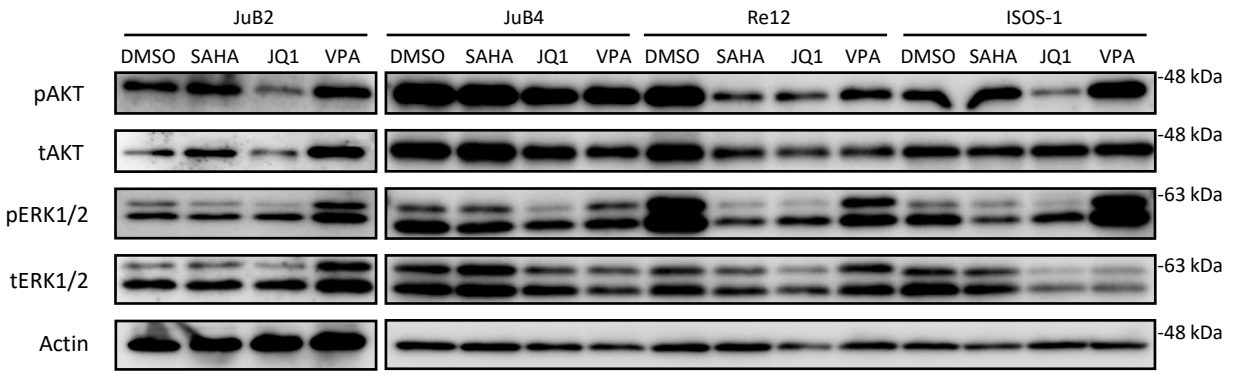
Supplementary Figure 2. Standard curves of each primer set.

(A, B) Standard curves of the primers for canine (A) and murine (B) genes. The slopes were used to calculate primer efficiencies. R^2 = coefficient of determination. E = efficiency.



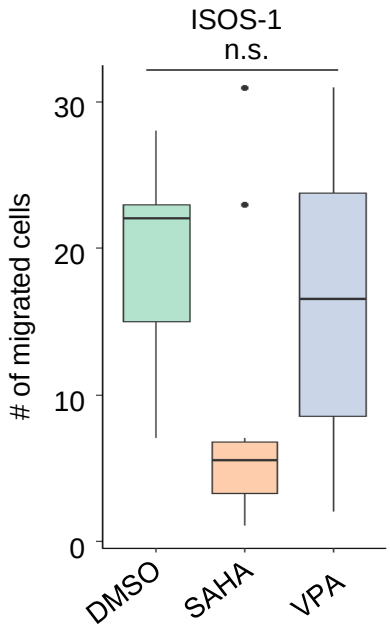
Supplementary Figure 3. VPA did not affect cell viability in HSA cells.

(A) Survival curves of canine and murine HSA cell lines treated with VPA (10 mM, 1 mM, 0.1 mM, 0.01 mM, 0.001 mM) for 60 h. **(B)** Representative results of annexin V staining in canine and murine HSA cells treated with/without VPA. **(C)** Percentages of apoptotic cells (annexin V-positive) in canine and murine HSA cells treated with/without VPA. NT = non treated. All samples were analyzed in triplicate. Data are plotted as average percentages ± SD.



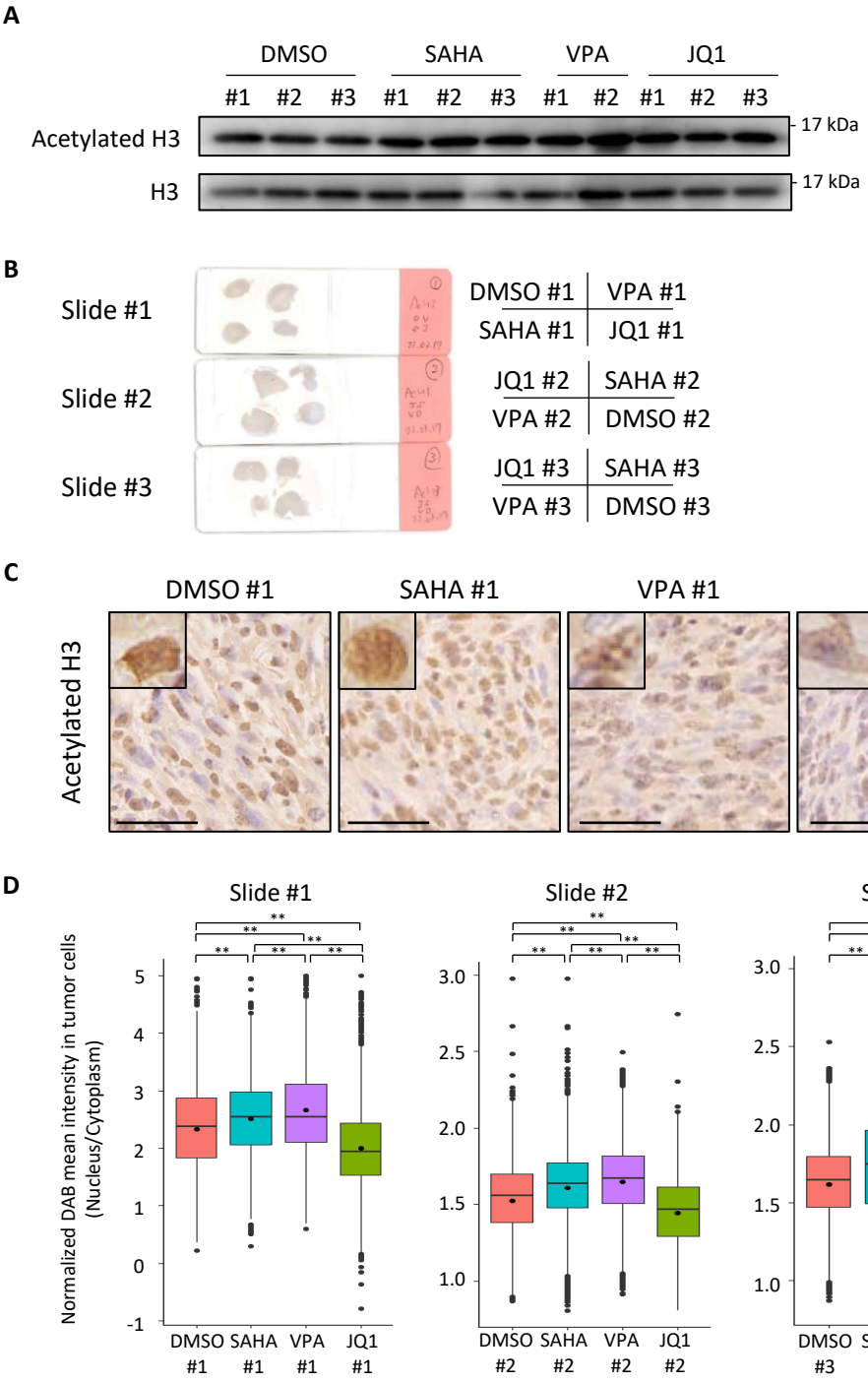
Supplementary Figure 4. AKT and ERK expressions in HSA cell lines.

Western blot analysis for phosphorylated and total AKT and ERK expressions in canine and murine HSA cell lines treated with DMSO, SAHA, JQ1 or VPA.



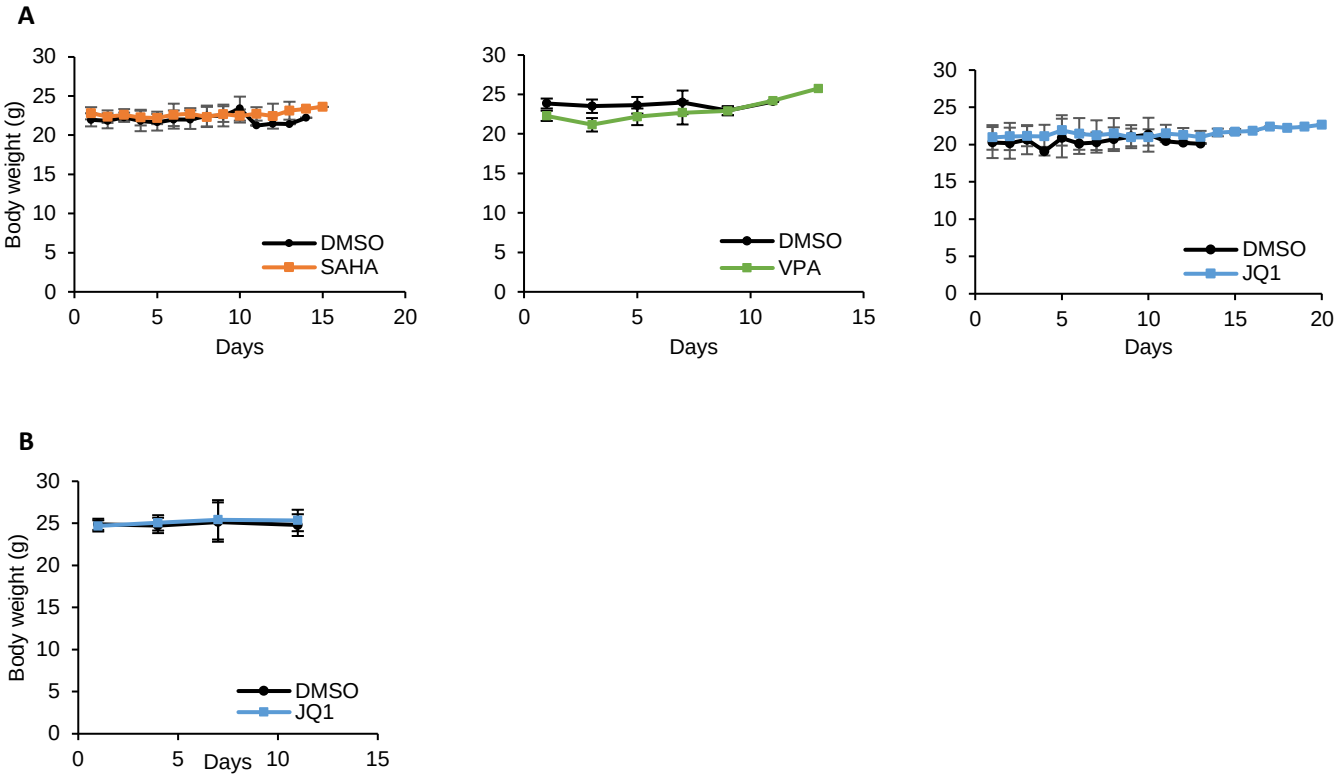
Supplementary Figure 5. RAW264 migration assay in ISOS-1

Quantitative analysis of migrated RAW264 cells co-cultured with SAHA or VPA-treated ISOS-1. Data are plotted as average percentages ± SD. n.s. means not significant.



Supplementary Figure 6. Histone acetylation levels in *in vivo* study

(A - C) Western blot images (A), tissue layout (B), and representative IHC images (C) of acetylated H3 in ISOS-1 tumor tissues obtained from ISOS-1 inoculated mice after three days treatment of DMSO, SAHA, VPA and JQ1. For B, D = DSMO, S = SAHA, V = VPA, J = JQ1. **(D)** Box plots for nucleus DAB OD mean values of tumor cells normalized by cytoplasm DAB OD mean values in the same cells. One tumor tissue sample from each treatment group was placed on the same slide and analyzed in the same digital image. Scale bars = 50 μm.



Supplementary Figure 7. Average body weight of mice in *in vivo* study

(A) Average body weight of Balb/c mice treated with DMSO, SAHA, VPA or JQ1 after starting treatments. **(B)** Average body weight of KSN/Slc mice treated with DMSO or JQ1 after starting treatments.

FULL MATERIALS AND METHODS

1. Histopathology and immunohistochemistry (IHC)

Tumor samples were obtained from patients presenting to a veterinary hospital with written informed consent (Table S1). The samples were fixed in 10% neutral buffered formalin, dehydrated through an ethanol series, cleared with xylene and infiltrated with paraffin wax in Tissue-Tek VIP5 Jr (Sakura Finetek Japan Co.,Ltd, Tokyo, Japan). Then, the samples were embedded in paraffin wax and sliced into 2 μm thickness. For hematoxylin and eosin staining, tissues were deparaffinized with xylene and placed in 99%, 95%, 90%, 80%, and 70% ethanol for 2 min each in this order. After washing ethanol out with tap water and distilled water (DW), the tissues were stained with hematoxylin for 1 min and then washed with tap water for 5 min. Then, they were stained with eosin for 1.5 min after being placed in 95% ethanol for 2 min. Remaining eosin was washed with 95% ethanol. Afterwards, the tissues were dehydrated with absolute ethanol and cleared with xylene. Finally, the tissues were mounted with Eukitt (#6.00.01.0001.06.01.EN, ORSAtec, Bobingen, Germany,) and covered with cover glasses for histopathological analysis. For IHC, after deparaffinization, the tissues were washed with phosphate buffered saline (PBS) three times, and then antigens were retrieved in Tris-EDTA buffer (pH 9.0) while being heated in a microwave for 10 min. Endogenous peroxidases were inactivated with 0.3% H_2O_2 in methanol for 25 min at room temperature (RT) before blocking the tissue sections with 10% normal goat serum (#426042, Nichirei biosciences, Tokyo, Japan) for 1 hour at RT. Tissues were stained with anti-acetylated histone H3 antibody (1:250; #39140, Active Motif, CA, USA) overnight at 4°C. Afterwards, the tissues were washed with PBS three times and stained with biotinylated goat anti-rabbit IgG (#426012, Nichirei biosciences) for 30 min at RT. After washing the tissues with PBS three times, they were treated with peroxidase conjugated streptavidin for 10 min at

RT. The slides were washed with PBS three times again, and then signals were developed by reaction with 3.3'-diaminobenzidine (349-00903, Dojindo, Kumamoto). For quantification of IHC results, the stained slides were scanned with NanoZoomer 2.0-RS (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with QuPath ver.0.2.1.[1] Three visual fields per slide in each case were randomly selected for quantitative analysis. Areas within 500 μm from the tissue edge was not used for analysis to avoid the edge effect. At least total 1,000 tumor cells and 100 normal endothelial cells were analyzed for each case. Nucleus DAB OD mean values in tumor cells were normalized against those in normal endothelial cells in adjacent tissue on the same slides, and the normalized values were used for quantitative analysis. To assess inhibitor efficacy in ISOS-1 tumors, nucleus DAB OD mean values were normalized with cytoplasm DAB OD mean values because there were not enough normal endothelial cells in the adjacent tissues of the isolated tumors.

2. Cell culture and Cell Line Validation Statement

Canine aortic endothelial cells (CnAOEC: #Cn304-05, Cell Applications, CA, USA), seven canine HSA cell lines (JuA1, JuB2, JuB4, Re12, Re21, Ud2, and Ud6),[2] ISOS-1 obtained from the Cell Resource Center for Biomedical Research Cell Bank (Tohoku University),[3] and RAW264 obtained from RIKEN Bioresource Center, were cultured with Dulbecco's Modified Eagle Medium with High Glucose (DMEM; #044-29765, Fujifilm Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; #S1580-500, Biowest, UT, USA) and penicillin–streptomycin solution (#168-23191, Fujifilm Wako) at 37℃ with 5% CO_2. CnAOEC, ISOS-1 and RAW264 were purchased from the company and cell banks and used for this study. We did not conduct validation tests for these cell lines in our lab. Canine HSA cell lines were given from the researcher who

established the cell lines, and we performed PCR using animal specific primers to validate that they were canine cells.

3. Drug preparation

SAHA (#10009929, Cayman Chemical, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 150 mg/ml. For *in vivo* studies, the stock solution of SAHA was diluted with 30% polyethylene glycol 400 and 5% Tween 80. VPA (#227-01071, Fujifilm Wako) was dissolved in PBS. JQ1 (#T117580, Toronto Research Chemicals, Toronto, Canada) was initially dissolved in DMSO to a stock solution of 50 mg/ml. For *in vivo* studies, the stock solution of JQ1 was diluted with 5% Tween 80. Bafilomycin A1 (#554-29211, Fujifilm Wako) was dissolved in DMSO to a stock solution of 1 mM.

4. Cell viability assay

Two thousand cells were seeded in 96 well cell culture plates (#655180, Greiner Bio-One, Kremsmünster, Austria), and they were cultured with 100 µl culture medium. On the next day, they were treated with DMSO or five different concentrations (10 nM, 100 nM, 1µM, 10 µM, and 100 µM) of SAHA or JQ1. Survival rates were analyzed using Cell Counting Kit-8 (CCK-8; #343-07623, Dojindo) according to the manufacturer's instructions with slight modifications. Briefly, 10 µl of CCK-8 solution was added to each well 60 hours after adding DMSO or inhibitors. After 2 hours incubation, 10 µl of 0.1% SDS solution was added to stop the reaction and the absorbance at 450 nm was measured with NanoDrop 2000 (Thermo Fisher Science, MA, USA). Survival rates were calculated by setting that of DMSO-treated samples as 100%. KyPlot 5.0 software (KyensLab, Inc., Tokyo, Japan) was used to

draw survival curves[4]. For growth inhibition curves of JQ1 treatment in ISOS-1, the absorbance at the time of treatment (Tz) was measured 24 hours after seeding, and the absorbance at the end of treatment (Ti) was measured 60 hours after JQ1 treatment. DMSO treated cells were used as the control (C). Growth inhibition rates were calculated as: [(Ti-Tz)/(C-Tz)] × 100 for JQ1 concentrations in which Ti>/=Tz, [(Ti-Tz)/Tz] × 100 for JQ1 concentrations in which Ti < Tz. [5]

5. Apoptosis assay

Apoptosis assay was performed using the FITC Annexin V Apoptosis Detection Kit I (#556547, BD Biosciences, NJ, USA) according to the manufacturer's instructions. Briefly, JuB4 and Re12 cells were treated with 10 μM SAHA or JQ1 for 24 h and detached from dishes using TrypLE Express Enzyme (#12604013; Thermo Fisher Scientific). One million cells were suspended in the binding buffer and stained with annexin V. Cells were analyzed with a BD FACSVerse flow cytometer (BD Biosciences, NJ, USA), and the results were analyzed with FCS Express 4 (De Novo Software, CA, USA) or FACSuite ver 1.0.5.3841 (BD Biosciences).

6. RNA-Sequencing

ISOS-1 were treated with 1 μM SAHA for 24 h, 1 μM JQ1 for 36 h, or 2 mM VPA for 36 h in triplicate. Total RNA was extracted with a NucleoSpin RNA isolation kit (#740955.50, Macherey-Nagel GmbH & Co. Düren, Germany) according to the manufacturer's instructions. RNA samples were submitted to Kazusa DNA Research Institution (Chiba, Japan) for further analyses. RNA-seq libraries were constructed using a

QuantSeq 3'mRNA-Seq Library Prep Kit (LEXOGEN, Vienna, Austria) and sequenced with the NextSeq500 (Illumina, CA, USA) to generate a minimum of two million single-end 75-bp reads. Sequencing reads were mapped to the mm39 mouse reference genome using STAR, and expression levels were estimated using RSEM.[6,7] Differential expression and gene expression profiles were analyzed by edgeR and GSEA v4.1.0, respectively.[8–10]

7. SA-β-gal staining

SA-β-gal staining was performed using a Senescence β-Galactosidase Staining Kit (#9860, Cell Signaling Technology, MA, USA) according to the manufacturer's instructions. Briefly, ISOS-1 was treated with DMSO or 10 μM JQ1 for 24 h and fixed with 4% paraformaldehyde (PFA) for 10 min at RT. Cells were stained with β-Galactosidase Staining Solution containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal: #16495, Cayman Chemical) for 13 h at 37℃. Positive cells were counted manually using an inverted microscope (BIOREVO BZ-9000; KEYENCE, Tokyo, Japan).

8. Cell cycle analysis

ISOS-1, JuB2, JuB4, and Re12 cells were treated with DMSO or JQ1 for 24 hours. ISOS-1 was treated with 10 μM JQ1, and other cell lines were treated with 5 μM JQ1. The cells were stained with 30 μM bromodeoxyuridine (BrdU) for 45 min at 37℃ on 70% confluency. Cells were washed with PBS and detached from dishes with 0.25% Trypsin-EDTA (#201-16945, Fujifilm Wako). Afterwards, cells were fixed in 70% ethanol overnight and washed with 0.5% Triton X-100 in PBS (PBST). The cells were resuspended in 500 μL of 2N HCl-0.5% Triton X-100 for 30 min at RT and neutralized with 500 μL of 0.1 M

sodium borate buffer (pH 8.5) for 30 min at RT. Then, cells were blocked with 1% bovine serum albumin (BSA, #017-23294, Wako) in PBST for 1 hour. 1.2×10^6 cells were incubated with/without anti-BrdU monoclonal antibody (1:100; MOBU-1 clone, #B35128, Thermo Fisher Scientific) in PBST with 1% BSA for 1 hour at RT. Cells were washed with PBST and stained with donkey anti-mouse IgG (H+L) conjugated with AlexaFluor 488 (1:1,000; #A32766, Thermo Fisher Scientific). DNA was stained with 25 μg/ml propidium iodide (PI, #P378, Dojindo). Cell cycle and proliferation were analyzed with BD FACSVerse flow cytometer. Results were analyzed with FCS Express version 4 software.

9. Migration assay

JuB4 and Re12 were treated with DMSO, 2 μM SAHA, or 2 mM VPA for 48 h in 6-well plates. Then, after replacing the medium to the one without inhibitors, the cells were co-cultured with RAW264 seeded on ThinCert Cell Culture Inserts (#657630, Greiner Bio-One) for 24 h. RAW264 on the culture surface of the inserts were removed with a cotton swab. Migrated RAW264 on the bottom side of the inserts were fixed with 4% PFA for 30 min at RT and then stained with 0.01% crystal violet for 30 min at RT. The number of cells was counted manually in 10 fields at 200× under a light microscope (BH-2; Olympus, Tokyo, Japan).

10. Western blotting

SDS lysis buffer {2% SDS, 50 mM Tris-HCl (pH6.8), 1 mM EDTA (pH 8.0)} was added to cultured cells or minced tumor tissues after washing them with PBS twice. Cell lysates and the supernatant of the minced tumor tissues were then sonicated using

BRANSON Sonifier 450 (Branson Ultrasonics Corporation, CT, USA) for 2 seconds at power 2. Protein concentrations were measured with TaKaRa BCA Protein Assay Kit (#T9300A, Takara Bio, Kusatsu, Japan) before adding 4× sample loading buffer {200 mM Tris-HCl buffer (pH 6.8), 8% SDS, 40% glycerol, 1% bromophenol blue, 20% 2-mercaptoethanol} and denaturing at 98°C for 5 min. 2 μg protein was separated on 12, 15 and 18% SDS polyacrylamide gels by electrophoresis and transferred to Immobilon-P transfer membranes (#IPVH00010, Merck Millipore, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline with 5% Tween 20 (TBST), or 5% BSA in TBST for 1 hour at RT and incubated with primary antibody in Can Get Signal Solution 1 (#NKB-101, TOYOBO, Osaka, Japan) overnight at 4°C. The membranes were washed with TBST three times before incubating with the corresponding secondary anti-mouse (#G21040) or anti-rabbit (#G21234) IgG antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific) in Can Get Signal Solution 2 (TOYOBO). Signals were developed with Immobilon Western Chemiluminescent HRP substrate (#WBKLS0500, Merck Millipore) and visualized in Image Quant LAS 4000 mini luminescent image analyzer (Cytiva, MA, USA). Captured data were processed using ImageJ.[11] Antibodies used in this study are listed in Table S2.

11. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted with TriPure Isolation Reagent (#11667157001, Roche, Basel, Switzerland) according to the manufacturer's instructions. Reverse transcription was performed using Primescript II 1st strand cDNA Synthesis Kit (#6210, Takara Bio, Kusatsu, Japan) for 1 μg total RNA of samples according to the manufacturer's instructions. qPCR samples were prepared using KAPA SYBR FAST qPCR Kit Master Mix (2×) ABI Prism

(KK4605, KAPA Biosystems, MA, USA). Reaction solution contains 1× KAPA SYBR FAST qPCR Master Mix, 200 nM forward and reverse primers, 1 μl cDNA and UltraPure DNase/RNase-free distilled water (UPDW, #10977-015, Thermo Fisher Scientific). The samples were applied in triplicate and analyzed by StepOne Real-time PCR system (Thermo Fisher Scientific). Samples were denatured at 95 °C for 10 min followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. RT-qPCR was performed using the primers listed in Table S3. Results were normalized using the geometric mean of reference genes (*RPL32* and *HPRT* for canine genes, and *Hprt* and *Tbp* for murine genes), which were selected from potential internal controls by geNorm (Fig. S1).[12] UPDW (no template) and no RT samples were used as negative controls and confirmed that no signal was detected for all primer sets. Gene sequences were obtained Ensembl. Primer3 (ver 3.2.4) was used to design primers targeting 80-150 bp products. The primers bind all splice variants and exon-exon junctions. The BLAST database was used to confirm that each primer set does not detect other genes. Primer efficiency was calculated based on the slope obtained from the standard curves and was confirmed to be between 90% and 110% for all primer sets (Fig. S2). Primer set specificity was evaluated by checking that each primer set has identical and singular peak in the melting curve. Relative expression levels were calculated by setting the expression levels in the DMSO-treated samples as 1.

12. Animal studies

All mouse experiments were performed under the guidelines of an institute, which follows the ARRIVE guidelines. Five-week-old female Balb/c and KSN/Slc mice (Japan SLC, Inc. Shizuoka, Japan) were used. A day before tumor cell inoculation, KSN/Slc mice were treated with 100 μL 2.5 mg/ml anti-asialo GM1 (#014-09801, Fujifilm Wako) to

increase the success rate of JuB2 transplantation.[13] Five million cells were inoculated subcutaneously in both flanks of Balb/c and KSN/Slc mice, which were anesthetized with 0.3 mg/kg medetomidine (Domitor, ZENOAQ, Tokyo, Japan), 4 mg/kg midazolam (Dormicum, Maruishi Pharmaceutical Co., Ltd. Osaka, Japan) and 5 mg/kg butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd. Tokyo, Japan). After tumor cell inoculation, mice were awoken by intraperitoneal injection of 3 mg/kg atipamezole (Atipame, Kyoritsu Seiyaku Corporation, Tokyo, Japan). Tumor volumes were calculated using the formula: Volume = (Length × $Width^2$)/2. When the tumor volume reached 100 mm^3, mice were treated with the vehicle control or inhibitors. Mice were intraperitoneally injected with SAHA (150 mg/kg) daily,[14] VPA (200 mg/kg) five times weekly,[15] or JQ1 (50 mg/kg) daily.[16,17] For preliminary experiments to confirm whether these inhibitors function *in vivo*, tumor tissues were collected from three mice for each inhibitor three days after starting treatments and were subject to western blotting or IHC. Comparison in IHC was done among the samples placed on the same slides. For treatment experiments, mice were examined at least twice weekly to check their health status and to measure tumor sizes. Balb/c mice were euthanized with CO_2 when tumors reached 1,000 mm^3 in volume. KSN/Slc mice were euthanized with CO_2 11 or 12 days after treatment initiation. Survival times were defined as the period from beginning of drug administration to euthanasia.

13. Statistical analysis

Statistical analyses were performed with R (version 4.1.0). Fisher's exact test was used to analyze the ratio of acetylated H3 staining levels. Student's *t*-test or Mann-Whitney test were used to analyze the differences between two groups, whereas Tukey's test was used to analyze differences among multiple groups. Survival curves were analyzed using the log-

rank test. Overall tumor growth was analyzed with two-way ANOVA.

References

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