Supporting Information

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SI Materials and Methods

Marker Gene Selection. RNA was extracted with TRIzol per the manufacturer's protocol (Invitrogen) from BE (2)-C and SHSY5Y neuroblastoma cells treated in triplicate with vehicle (ethanol) versus 5 μM *all-trans*-retinoic acid (ATRA) for 5 days and in SHSY5Y treated with 16 nM phorbol 12-myristate 13-acetate (PMA) for 5 days. Gene expression was evaluated with Affymetrix U133A DNA microarrays. GeneChip MAS5 Software (Affymetrix) was used for preprocessing the raw data, and all scans within the experiment were scaled to the array with the median overall microarray intensity. Thresholds were set to a minimum value of 20 and a maximum value of 16,000, and a variation filter with a 3-fold minimum variation and a minimum absolute difference of 50 was applied. We next identified genes meeting the following criteria: (*i*) at least a 2-fold variation between one of the classes (BE (2)-C vehicle vs. ATRA, SHSY5Y vehicle vs. ATRA, or SHSY5Y vehicle vs. PMA) with $P < 0.1$ by *t* test and (*ii*) the appropriate direction of change in the other cell line. Raw microarray data are available at http://www.broad.mit.edu/cancer/pub/Neuroblastoma_GE-HTS; mapping of data file sample names is presented in supporting information (SI) Table S1 and probe sequence data are in Table S2.

Small-Molecule Library Screen Methods. ***Cell and compound addition.*** BE (2)-C cells were plated in 384-well tissue culture plates in 50 μl of medium at 2,000 cells per well with 1 mM VPA. Compounds were added at a final approximate concentration of 20 μM in DMSO and incubated for 3 days at 37°C with 5% CO_2. We screened in triplicate the National Institute of Neurological Disorders and Stroke (NINDS) small-molecule collection containing 1,040 compounds (http://www.broad.harvard.edu/chembio/platform/screening/compound_libraries/ninds.htm). Three-quarters of the compounds in this collection of characterized bioactive molecules are FDA-approved. The following controls were also included: medium, undifferentiated cells treated with VPA alone and 5 μM ATRA differentiated controls.

RNA extraction and reverse transcription. After 3 days of chemical incubation, cells were then lysed with 25 μl of lysis buffer for 30 min at room temperature. Lysate was transferred to a 384-well oligo(dT)-coated plate and incubated for 1 h at room temperature. Reverse transcription was carried out in a 5-μl MMLV reaction (Promega) for 1.5 h at 37°C. Lysis buffers and 384-well oligo(dT) plates were originally purchased from RNAture, Hitachi Chemical, and now supplied by Qiagen.

Ligation-mediated amplification (LMA). After 1.5 h, solutions were removed by centrifugation into an absorbent towel. Signature gene-specific oligonucleotide probes were hybridized to the cDNA by using 2 nM each probe and Taq ligase buffer (New England Biolabs) in a 5-μl reaction. Upstream probes contained the T7 primer site, Luminex-designed FlexMap barcode tag (24 nt), and gene-specific sequence (20nt). Downstream probes were phosphorylated at the 5′ end and contained gene-specific sequence (20 nt) followed by the T3 primer site. Gene-specific sequence was chosen such that the 20-bp sequences of the upstream and downstream probes contain similar base composition, minimal repeats, and C-G or G-C juxtaposing nucleotides (Table S2). Hybridization was performed at 95°C for 2 min followed by 50°C for 1 h. Excess probe was then spun out. Probes were ligated in a 5-μl reaction by using TaqDNA ligase (New England Biolabs) at 45°C for 1 h followed by incubation at 65°C for 10 min. Excess ligation mix was spun out. The ligated products were amplified with the universal T3 primer (5′-ATT AAC CCT CAC TAA AGG GA-3′) and universal biotinylated T7 primer (5′-TAA TAC GAC TCA CTA TAG GG-3′) (Integrated DNA Technologies) by using HotStarTaq DNA Polymerase in a 5-μl reaction system. PCR was performed at 92°C for 9 min, followed by 34 cycles of denaturation at 92°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Amplicon detection. xMAP MultiAnalyte COOH microspheres (Luminex) (2.5 million) were coupled to complementary FlexMap barcode sequence (4 μM) with 2.5 μl of 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Pierce) in 25 μl of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 4.5 (Mes) buffer (Sigma). This 30-min coupling reaction was then repeated. Microspheres were then washed sequentially with 0.02% Tween-20, 0.1% SDS, and TE (pH 8) and resuspended in 50 μl of TE (pH 8). Next, the LMA sample was hybridized to microspheres by incubating 2,500 of each microsphere in 18 μl of 1.5× TMAC [4.5 M tetramethylammonium chloride, 0.15% *N*-lauryl sarcosine, 75 mM Tris·HCl (pH 8), and 6 mM EDTA (pH 8)] and 5 μl of TE (pH 8) at 95°C for 2 min and then 45°C for 1 h. For detection, the sample was incubated with 10 μl of streptavidin-phycoerythrin (SA-PE) {1% SA-PE in 1× TMAC [3 M tetramethylammonium chloride, 0.1% *N*-lauryl sarcosine, 50 mM Tris·HCl (pH 8), and 4 mM EDTA (pH 8)]} for 5 min at 45°C and then washed once and resuspended with 1× TMAC. Dual-color fluorescence was detected with a Luminex high-throughput detection instrument. A minimum of 100 events was recorded for each microsphere and median fluorescent intensities (MFI) computed.

Hit identification. The median fluorescence level for each gene (where each gene was represented by a particular bead color) from the Luminex detector was used as a measure of the gene's expression. To maximize consistency between and within plates, we normalized genes by using the expression ratio between each gene and the average of the four reference genes *GTF*, *GAPDH*, *TUB*, and *HNRPAB*. Next, filtering was performed to eliminate poorly performing wells from further analysis where *GTF* expression levels were used as a proxy for cell viability. Because ratios with a denominator of the reference gene average were used for subsequent analysis, we needed to eliminate wells where reference gene expression was nominally zero. Forming a ratio with such a low denominator could potentially falsely flag a well where the cells were merely dead or dying or where the well failed for technical reasons. For our filtering level, we used the mean of the medium only control wells. For this screen, 91 of 3,840 compound wells were filtered. After filtering, each measurement was scaled by using the robust *Z* score to further reduce plate-to-plate variation of measurements within the screen. The robust *Z* score normalizes the raw ratios for each gene in each well by assuming that the majority of compounds are inactive and can supplement any negative controls.

$$Z_i = \frac{x_i - m_p}{\mathrm{MAD}_p} \qquad [1]$$

where m_p is the median of the compounds and negative controls (VPA-only wells) for plate p and MAD_p is the median absolute deviation of the compounds and negative controls from the median. The robust *Z* score has the advantage over the traditional *Z* score in that it is relatively insensitive to statistical outliers. After normalization, we used a combination of the summed score, weighted summed score, naïve Bayes, and *k*-

nearest neighbor metrics to identify candidate neuroblastoma differentiation agents.

The summed-score metric combined expression ratios by summing them with a sign determined by the expected direction of regulation as determined from the differentiated positive controls. The weighted summed-score metric is a variant of the summed-score metric that combines expression ratios by summing them with a weight and sign determined by the signal-to-noise ratio determined from the differentiated positive controls and the undifferentiated negative controls. Signal-to-noise ratio is defined by:

$$W_i = \frac{\mu_{i1} - \mu_{i2}}{\sigma_{i1} + \sigma_{i2}} \quad [2]$$

μ_{i1} represents the mean expression of samples from class 1 for feature i and σ_{i1} represents the standard deviation of class 1 for feature i (1). Composite scores for both the summed score and weighted summed score were formed by finding the average of the scores from the three replicates. Each compound's summed score and weighted summed score was assigned a probability that the compound caused the cells to have an expression signature similar to the differentiated positive-control wells. The calculation of the probability was based on finding the Bayesian probability density of the score by using normal distributions to model each of two classes of controls:

$$p(C = c|X = x) = \frac{p(C = c)p(X = x|C = c)}{p(X = x)}, \quad [3]$$

where

$$p(X = x|C = c) = N(x;\ \mu_c,\ \sigma_c). \quad [4]$$

$N(x;\mu_c,\sigma_c)$ is the probability density function for a normal (or Gaussian) distribution with mean μ_c and standard deviation σ_c (2). The parameters for the Gaussian distribution were trained on the positive and negative controls, and $p(C = c)$ was the *a priori* probability of class c controls (in this case, we assumed that the positive and negative controls have equal *a priori* probabilities). Composite probabilities were found by taking the product of the probabilities for the three replicates (but leaving out filtered replicates) and renormalizing the probabilities to ensure that the probability that the compound is a positive control and the probability that the compound is a negative control sum to one. Compounds were ranked for follow-up according to the probability that they looked like the differentiated positive control.

A naïve Bayes classifier was also used to evaluate the expression signatures for the compounds. The naïve Bayes classifier is based on the Bayes probability rule and naïvely assumes that the features are independent within each class. The independence assumption greatly simplifies the calculation of the class probabilities and has been shown to work well even in cases where the features have significant dependencies. The probabilities are calculated as follows:

$$p(C = c|\mathbf{X} = \mathbf{x}) = \frac{p(C = c)p(\mathbf{X} = \mathbf{x}|C = c)}{p(\mathbf{X} = \mathbf{x})}, \quad [5]$$

where

$$p(\mathbf{X} = \mathbf{x}|C = c) = \prod_i p(X_i = x_i|C = c), \quad [6]$$

where, for continuous values like the gene expression ratios, $p(X_i = x_i|C = c)$ were a kernel distribution formed out of a mixture of Gaussians (3).The parameters for the distribution for each class c and each feature i are trained by using the controls for the screen. The overall probability for each compound is found by multiplying the probabilities for the individual replicates (leaving out filtered replicates) and renormalizing the probabilities so the two classes sum to one. Compounds were ranked for follow-up according to the probability that they looked like a differentiated positive control.

The k-nearest-neighbor (KNN) classifier was also used to identify possible hits. It assigns samples to the class most frequently represented among the k nearest control samples (2). A KNN predictor was trained by using the undifferentiated and differentiated control samples and the compound-treated wells were tested by using $k = 5$ with a Pearson correlation for the distance metric and weights for the neighbors based on the Pearson distance. A modified version of the KNN method was used here where the genes were weighted based on their signal-to-noise ratio in the control samples. Compounds were ranked for follow-up according to how well they performed in the four metrics.

Bliss Independence Model. The Bliss independence model predicts the combined response C for two single compounds with A and B according to the relationship C = A + B − A * B, where A is the fractional inhibition of compound A at the particular concentration and B is the fractional inhibition of compound B at the particular concentration. According to this model, the excess above the predicted Bliss independence represents the synergistic effect of the combination treatment.

Western Blot Analysis. Cells were lysed in Cell Signaling Lysis Buffer with protease inhibitor (Complete Mini EDTA free protease inhibitor tablets, Roche Diagnostics), resolved by electrophoresis on 4–20% gradient Tris·HCl precast Ready Gel (BioRad Laboratories) and transferred to nitrocellulose membranes (Protran, Schleicher and Schuell). All proteins were detected by using chemiluminescence and antibodies to NF-M (SC-20013, Santa Cruz), GAPD (Ab 22556, Abcam), anti-acetyl-histone H3 (06-599, Upstate), and anti-acetyl-Histone H4 (06-866, Upstate). Control histones were untreated HeLa cell acid extract (13-112, Upstate) and sodium butyrate-treated HeLa cell acid extract (13-113, Upstate).

Histone Extraction. Ten million cells were lysed in ice-cold lysis buffer [10 mM Tris, 50 mM sodium bisulfite, 20 mM magnesium chloride, 0.25 M sucrose, 1% Triton X-100, pH 6.5] and thoroughly homogenized. Nuclei were collected by centrifugation, washed three times with lysis buffer, and resuspended in 100 μl of 10 mM Tris (pH 7.4) and 13 mM EDTA. Concentrated sulfuric acid (1.1 μl) was added and the samples were incubated at 4°C for 3 h to extract acid-soluble protein. The supernatant was retained and incubated overnight at 4°C in 1 ml of acetone. The next day, the precipitate containing histones was collected by centrifugation, washed with acetone, and dissolved in water.

Xenograft Studies. Male NCr nude mice were injected with 10^6 BE (2)-C cells in 0.1 ml of 50% Matrigel (BD Biosciences) in the right flank via s.c. injection. Tumors were established for 10 days at which time mice with palpable tumors between 50 and 150 mm^3 were divided into four groups with statistically equivalent average tumor volumes. The four treatment groups were vehicle, ATRA alone (2.5 mg/kg), SAHA alone (25 mg/kg), or a combination of ATRA and SAHA. All drugs were prepared fresh daily and were delivered by IP injection. Animals were weighed daily to adequately adjust drug dosage during the 21 days of treatment. Caliper measurements were used to calculate tumor volume by using the formula: Volume = 0.5 × length × $(\text{width})^2$. Mice with signs of distress or tumor size with a single linear dimension of 2 cm were killed. In a second xenograft experiment,

BE (2)-C xenografts were established in NCr nude mice until tumor volume reached 100 mm^3. They were divided into treatment cohorts and received either vehicle, ATRA 2.5 mg/kg IP daily, SAHA 25 mg/kg IP daily, or a combination of ATRA and SAHA for 4 days. Tumors were then harvested, formalin-fixed, and stained with hematoxylin and eosin or snap-frozen and then RNA-extracted with TRIzol per the manufacturer's protocol and the neuroblastoma differentiation signature measured. All animal experiments were performed following approval from the Institutional Animal Care and Use Committee.

1. Golub TR, *et al.* (1999) Molecular classification of cancer: Class discovery and class prediction by gene expression monitoring. *Science* 286(5439):531–537.
2. Duda RO, Hart PE (1973) *Pattern Classification and Scene Analysis* (Wiley, New York).
3. John GH, P. L. (1995) Estimating continuous distributions in Bayesian classifiers. *Proceedings of the 11th Conference on Uncertainty in Artificial Intelligence, Montreal, Canada*, eds Besnard P, Hanks S (Morgan Kaufman, San Francisco), pp 338–345.

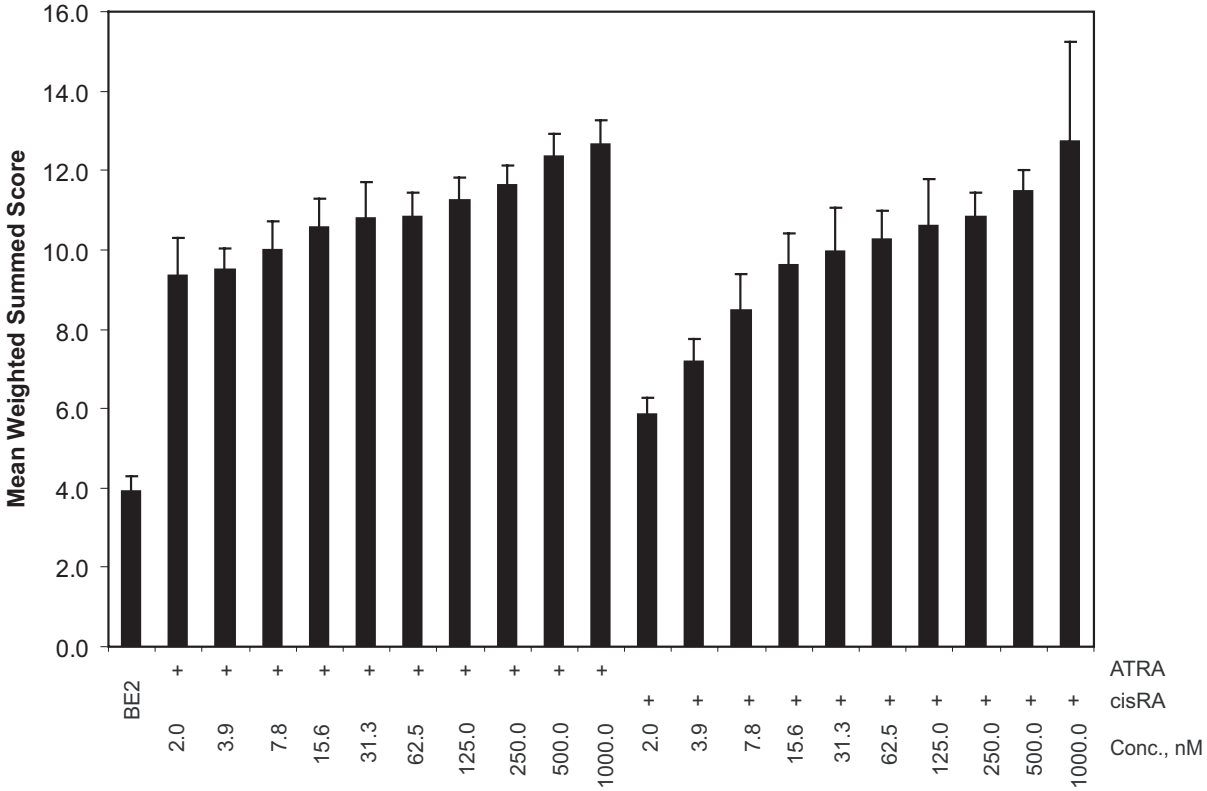

Fig. S1. GE-HTS is quantitative. BE (2)-C cells were treated in replicate of 16 for 3 days in a 2-fold dilution series with ATRA or 13-*cis*-RA and the mean weighted summed score was determined for the neuroblastoma differentiation signature. A dose–response is seen for both drugs.

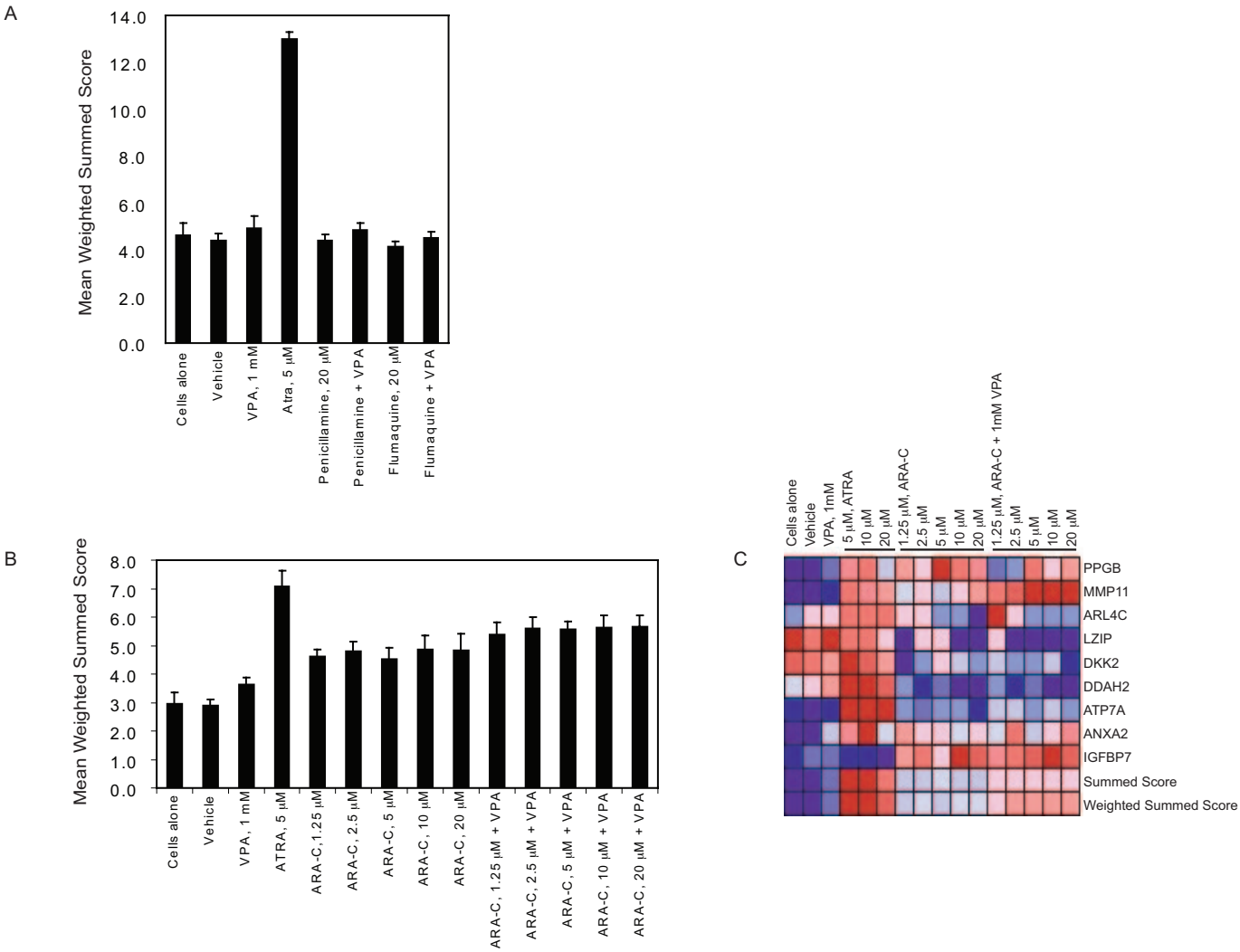

Fig. S2. Evaluation of candidate hits. (*A*) BE (2)-C cells were treated in replicate of 16 with either vehicle, 1 mM VPA, 5 μM ATRA, 20 μM penicillamine, 20 μM penicillamine + 1 mM VPA, 20 μM flumaquine, or 20 μM flumaquine + 1 mM VPA. After 3 days, differentiation was assessed with the GE-HTS assay by using the 14-gene neuroblastoma differentiation signature with a mean weighted summed score. Only ATRA induced the differentiation signature. (*B*) BE (2)-C cells were treated in replicate of 16 with either vehicle, 1 mM VPA, 5 μM ATRA, or cytarabine (ARA-C) in a 2-fold dilution series from 20 μM to 1.25 μM ± 1 mM VPA. After 3 days, differentiation was assessed with the GE-HTS assay by using the 14-gene neuroblastoma differentiation signature with a mean weighted summed score. ARA-C induced a slight induction in the overall weighted summed score. (*C*) The performance of the individual genes for each condition in *B* are depicted in a row-normalized heat map where blue represents relative poor expression and red represents relative high expression. Although there is a slight increase in the overall score induced by ARA-C, there is poor individual gene performance with the expression of several of the genes changing in the wrong direction.

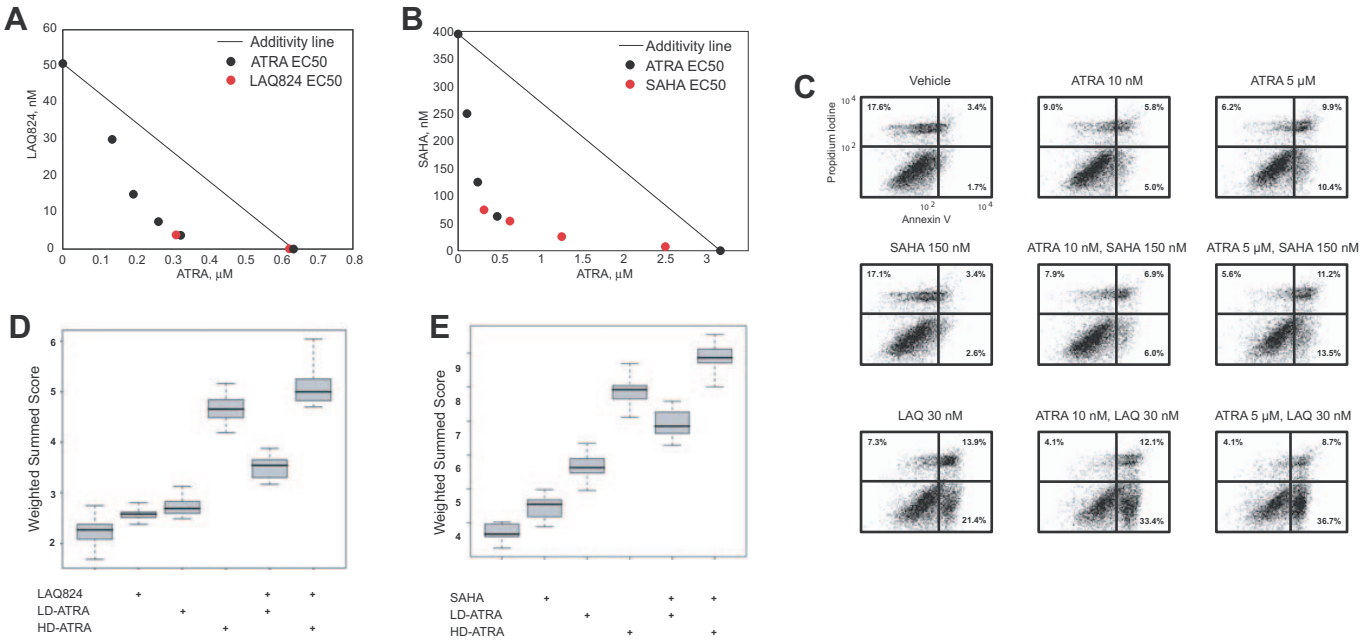

Fig. S3. Multiple HDAC inhibitors act synergistically with ATRA to inhibit cell viability and increase differentiation. (*A*) LAQ-824 shows synergistic growth inhibition with ATRA. The combined effect of LAQ824 and ATRA on BE (2)-C viability at 5 days, as determined by ATP level, is shown by isobologram. Synergy appears as points below the line of additivity. (*B*) Similarly, the combined effect of SAHA and ATRA on BE (2)-C viability at 5 days is shown by isobologram. (*C*) BE (2)-C cells were treated as shown for 3 days and apoptosis evaluated. Combination treatment induced early apoptosis (annexin V positive, PI negative) with an additive interaction at the low-dose ATRA + SAHA combination and synergistic interaction with low-dose ATRA + LAQ824 and high-dose ATRA + SAHA or LAQ824 as evaluated by excess above Bliss independence. (*D*) BE (2)-C cells were treated with combinations of LAQ824 (30 nM), LD-ATRA (10 nM), and HD-ATRA (5 μM) and the neuroblastoma differentiation signature evaluated with LMA/Luminex fluorescent bead-based detection. A box-and-whisker plot demonstrated the distribution of weighted summed scores for each sample type where the heavy lines inside the box show the median, the boxes show the quartiles, and the whiskers show the extremes of the observed distribution of scores. LAQ824 in combination with either low-dose or high-dose ATRA induced a higher score than did either agent alone. (*E*) BE (2)-C cells were treated with combinations of SAHA (150 nM), LD-ATRA (10 nM), and HD-ATRA (5 μM) and the neuroblastoma differentiation signature weighted summed score was evaluated as described above.

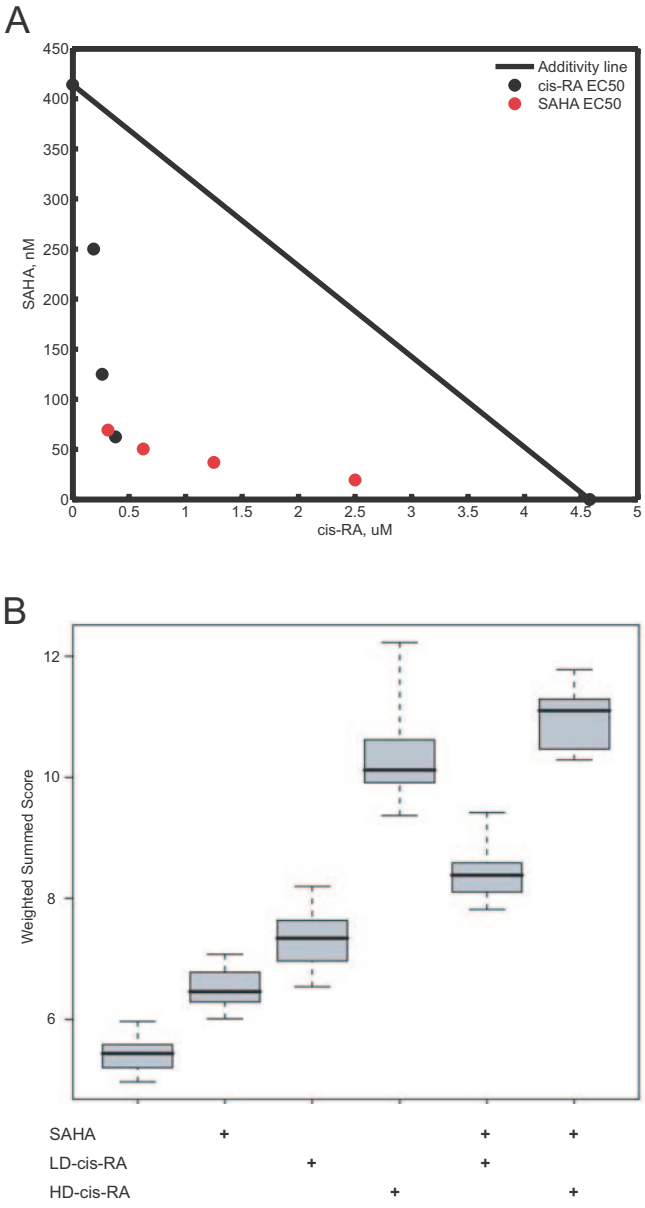

Fig. S4. 13-*cis*-RA and SAHA induce synergistic effects on BE (2)-C. (*A*) The combined effect of SAHA and 13-*cis*-RA on BE (2)-C cell viability at 5 days, as determined by ATP level, is shown by isobologram. Synergy appears as points below the line of additivity. (*B*) The combination of SAHA and 13-*cis*-RA induced increased differentiation compared with either agent alone. BE (2)-C cells were treated with combinations of SAHA at 150 nM, LD-13-*cis*-RA (10 nM), and HD-13-*cis*-RA (5 μM) and the neuroblastoma differentiation signature weighted summed score was evaluated with the LMA/Luminex bead-based detection. A box-and-whisker plot demonstrated the distribution of weighted summed scores for each sample type where the heavy lines inside the box show the median, the boxes show the quartiles, and the whiskers show the extremes of the observed distribution of scores. SAHA in combination with either low-dose or high-dose 13-*cis*-RA induced a higher weighted summed score than did either alone.

Table S1. Mapping from data file sample names (used in Broad Institute formatted res files) to scan names (Affymetrix CEL files)

Sample name	Cell line	Treatment	CEL file name
SH_Etoh_1	SHSY5Y	Vehicle	CL2004071613AA
SH_Etoh_2	SHSY5Y	Vehicle	CL2004071616AA
SH_Etoh_3	SHSY5Y	Vehicle	CL2004071619AA
SH_ATRA_1	SHSY5Y	5 μM ATRA for 5 days	CL2004071614AA
SH_ATRA_2	SHSY5Y	5 μM ATRA for 5 days	CL2004071617AA
SH_ATRA_3	SHSY5Y	5 μM ATRA for 5 days	CL2004071620AA
SH_PMA_1	SHSY5Y	16 nM PMA for 5 days	CL2004071615AA
SH_PMA_2	SHSY5Y	16 nM PMA for 5 days	CL2004071618AA
SH_PMA_3	SHSY5Y	16 nM PMA for 5 days	CL2004071621AA
BE_Etoh_1	BE(2)-C	Vehicle	CL2004071622AA
BE_Etoh_2	BE(2)-C	Vehicle	CL2004071624AA
BE_Etoh_3	BE(2)-C	Vehicle	CL2004071626AA
BE_ATRA_1	BE(2)-C	5 μM ATRA for 5 days	CL2004071625AA
BE_ATRA_2	BE(2)-C	5 μM ATRA for 5 days	CL2004071627AA

Table S2. Neuroblastoma differentiation marker genes

Gene name	Lua tag	RefSeq	Probe sequence
IGFBP7	33	NM_001553	5′-TAATACGACTCACTATAGGGTCAATTACTTCACTTTAATCCTTTCATGGTTAAAAGTAGTCATG-3′ 5′-PO_4-GATAACTACATTACCTGTTCTCCCTTTAGTGAGGGTTAAT-3′
PPGB	38	NM_000308	5′-TAATACGACTCACTATAGGGCAATCATTACACTTTTCAACAATCAAGCAGCCATACTGATGAC-3′ 5′-PO_4-CACAGCAACCAGCTCCACGGTCCCTTTAGTGAGGGTTAAT-3′
MMP11	40	NM_005940	5′-TAATACGACTCACTATAGGGCTTTCTACATTATTCACAACATTACCCTGGAGGCTGCAACATAC-3′ 5′-PO_4-CTCAATCCTGTCCCAGGCCGTCCCTTTAGTGAGGGTTAAT-3′
ARL4C	42	BC001051	5′-TAATACGACTCACTATAGGGCTATCTTCATATTTCACTATAAACCGGTTCAGCGGGCACGGAGC-3′ 5′-PO_4-GCTGTGCTGGCCTGGCTGGGTCCCTTTAGTGAGGGTTAAT-3′
LZIP	43	AF029674	5′-TAATACGACTCACTATAGGGCTTTCAATTACAATACTCATTACACTGCCTGCTGCATTACATGC-3′ 5′-PO_4-CTCAGGCTCCCAGTGCAGAGTCCCTTTAGTGAGGGTTAAT-3′
DKK2	44	NM_014421	5′-TAATACGACTCACTATAGGGTCATTTACCAATCTTTCTTTATACTTCTTCCCAAACCTCTGCAG-3′ 5′-PO_4-CATCTGCTTTATTGCCAAAGTCCCTTTAGTGAGGGTTAAT-3′
DDAH2	45	NM_013974	5′-TAATACGACTCACTATAGGGTCATTTCACAATTCAATTACTCAACTCTGATGTCACCCTGGTAC-3′ 5′-PO4-CTCAGCACACGCCCCCACAGTCCCTTTAGTGAGGGTTAAT-3′
ASCL1	46	BC001638	5′-TAATACGACTCACTATAGGGTACATCAACAATTCATTCAATACAGAAGAACAAAGTTTATGCAG-3′ 5′-PO4-CTGTGTCCTGCTCAGAACTGTCCCTTTAGTGAGGGTTAAT-3′
ATP7A	51	NM_00052	5′-TAATACGACTCACTATAGGGTCATTTCAATCAATCATCAACAATGGACGATGTTGTGCAATATC-3′ 5′-PO4-CATCTACTGTAGTTAAGATATCCCTTTAGTGAGGGTTAAT-3′
ANXA2	53	BC001388	5′-TAATACGACTCACTATAGGGTAATTATACATCTCATCTTCTACACCCTGTACTATTATATCCAG-3′ 5′-PO4-CAAGACACTAAGGGCGACTATCCCTTTAGTGAGGGTTAAT-3′
GAPD	34	M33197	5′-TAATACGACTCACTATAGGGTCATTCATATACATACCAATTCATATCTCCCCTCCTCACAGTTG −3′ 5′-PO4-CCATGTAGACCCCTTGAAGATCCCTTTAGTGAGGGTTAAT-3′
TUBG1	47	NM_001070	5′-TAATACGACTCACTATAGGGCTTCTCATTAACTTACTTCATAATTCAGAGCACAGATCAGGGAC-3′ 5′-PO4-CTCACGCATCTCTTTCTCATTCCCTTTAGTGAGGGTTAAT-3′
GTF3A	48	NM_002097	5′-TAATACGACTCACTATAGGGAAACAAACTTCACATCTCAATAATGAGAGTCACCCAACTGTGTG-3′ 5′-PO4-GAAGACAAGATGCTCTCGACTCCCTTTAGTGAGGGTTAAT-3′
HNRPAB	52	NM_004499	5′-TAATACGACTCACTATAGGGTCAATCATCTTTATACTTCACAATGCCTGGACCTGTGGACCCTG-3′ 5′-PO4-GTTGTAAAGAGTAAATTGTATCCCTTTAGTGAGGGTTAAT-3′

Table S3. Summary of top hits

Compound name	Wells filtered, *n*	Prob diff SS	Prob diff WSS	Prob diff naïve Bayes	Diff calls KNN, *n*
ATRA	0	1	1	1	3 of 3
Flumequine	0	1	1	1	0 of 3
Cytarabine	1	0.01	1	1	0 of 3
Penicillamine	0	0	1	0	0 of 3

The probability (Prob) of each compound inducing a differentiated (diff) signature is shown for the summed score (SS), weighted summed score (WSS), and naïve Bayes metrics. The last column indicates the number of replicates of the triplicate samples called "differentiated" in a KNN analysis ($K = 5$).