Pharmacologic inhibition of histone demethylation as a therapy for pediatric brainstem glioma

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Pediatric brainstem gliomas often harbor oncogenic K27M mutation of histone H3.3. Here we show that GSKJ4 pharmaco logically inhibits K27 demethylation. JMJD3 increases cellular H3K27 methylation in K27M tumor cells and demonstrate potent antitumor activity both in vitro against K27M cells and in vivo against K27M xenografts. Our results demonstrate that increasing H3K27 methylation by inhibiting K27 demethylase is a valid therapeutic strategy for treating K27M-expressing brainstem glioma.

Oncogenic mutations of the H3F3A gene, which encodes histone variant H3.3, are present in the majority of pediatric brainstem gliomas. The most common H3F3A mutation causes a substitution of lysine 27 with methionine (i.e., K27M), abolishing a crucial site of regulatory post-translational methylation. We, and others, have demonstrated that these oncogenic mutations have a dominant effect, sequestering polycomb repressive complex 2 (PRC2) and reducing cellular H3K27 methylation. We hypothesized that pharmacologic reversal of brainstem glioma H3K27 demethylation could serve as a therapeutic strategy for this uniformly lethal malignancy.

To test this hypothesis, we studied two K27M-mutant brainstem glioma cell lines (SF7761 (ref. 8) and SF8628 (ref. 9)), four glioma cell lines with wild-type H3.3 (SF9012, SP9402, SP9427 and GBM43), a glioma cell line expressing G34V-mutant H3.3 (KNS42) and isogenic human astrocyte lines (HAs) with and without transgene expression of K27M H3F3A (refs. 3-6). As such, tumor-derived histone gene mutations are thought to drive tumorigenesis by causing reduced histone K27 methylation and thereby altering gene expression in cells of the developing pons. We hypothesized that pharmacologic reversal of brainstem glioma H3K27 demethylation could serve as a therapeutic strategy for this uniformly lethal malignancy.

To confirm that the effect of GSKJ4 on tumor cell growth was due to activity against JMJD3, we studied the effects of depleting JMJD3 and UTX on tumor cell growth and the response of these depleted cells to GSKJ4. siRNA depletion of JMJD3 and UTX in glioma cells depleted K27M cells revealed a dose-dependent inhibition of cellular viability, with 50% growth inhibition reached at concentrations of 1.3-3.0 µM (Fig. 1d). In contrast, GSKJ4 concentrations as high as 8 µM did not achieve 50% growth inhibition for any of the cells expressing wild-type and G34V H3.3. GSKJ4 treatment of HAs revealed that HAs expressing K27M were more sensitive to GSKJ4 than HAs without the mutation (Fig. 1d). Analysis of cell cycle distributions revealed S phase as being the most diminished cell cycle stage in tumor cells expressing K27M (Supplementary Fig. 4). GSKJ4 also led to more apoptosis of K27M-expressing cells, as indicated by annexin V staining (Supplementary Fig. 5) and by cell counts of GSKJ4-treated samples (Supplementary Fig. 6). Finally, GSKJ4 completely inhibited the clonal growth of all K27M-expressing cells but had no effect on the clonal growth of the cells expressing wild-type and G34V H3.3 (Fig. 1e).

To determine whether GSKJ4 is active against K27M brainstem gliomas in vivo, we administered GSKJ4 by intraperitoneal injection, at 100 mg per kg body weight per day for 10 consecutive days, to athymic (nu/nu genotype, BALB/c background) mice harboring subcutaneous SF8628 K27M xenografts. The drug showed significant toxicity in these mice. GSKJ4 treatment of wild-type xenografts reduced tumor growth, but GSKJ4 treatment of K27M-expressing xenografts did not achieve 50% growth inhibition (Fig. 1f).

In summary, GSKJ4 shows antitumor activity against pediatric brainstem gliomas expressing the K27M mutation. GSKJ4 treatment is associated with potent antiproliferative, proapoptotic, and anticolonial effects, and it is well tolerated in a rodent xenograft model. GSKJ4 is an orally bioavailable agent and is being developed for the treatment of pediatric brainstem gliomas expressing K27M mutation.

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growth-inhibitory activity against SF8628 subcutaneous tumors (Fig. 2a). The same cells, when transfected with JMJD3 (KDM6B) siRNA and then injected subcutaneously, showed substantial growth delay relative to the growth of SF8628 cells transfected with scrambled siRNA (Fig. 2b).

We next tested for GSKJ4 activity against orthotopic K27M brainstem glioma xenografts (SF7761 and SF8628). Administration of GSKJ4 for 10 consecutive days significantly reduced the growth of K27M tumors engrafted in mouse brainstem and significantly extended animal survival (Fig. 2c). Analysis of K27M tumors obtained from mice at the end of therapy showed significantly less Ki-67 staining—a measure of cell proliferation—as well as significantly higher TUNEL positivity—a measure of apoptotic activity—after GSKJ4 treatment (Fig. 2d). In addition, GSKJ4 treatment led to significantly higher tumor cell K27me3 positivity (P = 0.0016, two-tailed unpaired t-test; Supplementary Fig. 10). In contrast to K27M tumors, xenografts established from GBM43 cells expressing wild-type H3.3 showed no response to GSKJ4 treatment (Fig. 2c).

To address GSKJ4 brain access, we administered GSKJ4 to three non-tumor-bearing mice that were euthanized 3 h after the third GSKJ4 treatment. Their brains were immediately resected, and each brainstem was dissected from the surrounding brain. HPLC analysis of tissue extracts revealed detectable GSKJ1, the hydrolysis product and activated derivative of GSKJ4, in all three brainstem samples (Supplementary Table 2). This finding indicated that the drug entered the brain: specifically, the site of brainstem tumor development.

Drawing on known associations between K27 methylation and gene regulation, we applied expression array and K27me3 chromatin immunoprecipitation–sequencing (ChIP-seq) analysis to SF8628 K27M cells. Results for sequences showing the most significant inverse correlation between GSKJ4-associated gene expression changes and K27me3 sequence association are indicated in Supplementary Figure 11 and Supplementary Table 3 (see also Gene Expression Omnibus data set GSE59497).

The recent discovery of histone K27M mutation in pediatric gliomas and the identification of the altered epigenetic program resulting from these mutations has prompted the investigation of small-molecule inhibitors targeting H3 demethylases as new cancer therapies. Here we have demonstrated that pharmacologic modulation of histone K27 methylation, by inhibiting H3 demethylation, is an approach warranting further investigation for treating K27M pediatric gliomas.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** NCBI Gene Expression Omnibus: transcriptional profiling and ChIP-seq results available at accession number GSE59497.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Figure 2 GSKJ4 treatment shows K27M antitumor activity in vivo. (a) Mice with SF8628 subcutaneous tumors were treated with either vehicle (DMSO) or GSKJ4 (100 mg per kg body weight per day) daily for 10 d. Left, tumor bioluminescence in mice 47 d after tumor cell injection. Horizontal bars indicate the mean from 3 mice for each treatment group. A two-tailed unpaired t-test was used to compare vehicle and GSKJ4 treatment. Right, tumor bioluminescence overlay images showing relative bioluminescence intensities from representative vehicle-treated versus GSKJ4-treated mice. (b) Left, growth plots for subcutaneous tumors, established by injection of SF8628 cells transfected with scrambled (Control) or JMJD3 (K6M6B) siRNA. Tumor bioluminescence values were normalized against bioluminescence values obtained 3 d after tumor cell injection. Values are mean ± s.e.m. from 3 mice at each time after implantation. Right, corresponding tumor bioluminescence intensity overlay images for representative scrambled and JMJD3 siRNA–treated mice 29 d after tumor cell implantation. (c) Left, bioluminescence distributions of SF7761 (top; 106 d after tumor cell implantation), SF8628 (66 d after tumor cell implantation) and GBM43 (12 d after tumor cell implantation) brainstem xenografts, following 10 d (SF7761 and SF8628) or 7 d (GBM43) of treatment with GSKJ4 or vehicle. Horizontal bars indicate the mean from 4 mice for each treatment group in SF7761, 5 for each group in SF8628, and 6 for Control and 7 for GSKJ4 treatment in GBM43. A two-tailed unpaired t-test was used to compare vehicle and GSKJ4 treatment. Center, corresponding tumor bioluminescence intensity overlay images for representative vehicle- and GSKJ4-treated mice. Right, survival plots for each experiment. Statistical analysis was performed using a log-rank test. Pink shaded areas indicate the duration of treatment. (d) Left, representative Ki-67 and TUNEL staining for vehicle- versus GSKJ4-treated SF8628 tumors from mice euthanized at the end of treatment. Right, average percentage of positive cells in four high-powered fields for each tumor. Error bars, s.e.m. Two mice were analyzed in each treatment. A two-tailed unpaired t-test was used to compare vehicle and GSKJ4 treatment.

AUTHOR CONTRIBUTIONS
R.H. designed and conducted all of the experiments and wrote the manuscript. N.A. performed bioinformatics analysis for the data from gene expression and ChIP-seq. Y.I. performed the in vitro studies and provided valuable discussion. R.L. performed the cell cycle analysis, flow cytometry analysis and quantitative RT-PCR. H.G., X.C. and D.F. performed the ChIP-seq experiments. X.H. provided valuable material and intellectual input. M.W.T. performed the in vitro studies. Y.N. performed microarray analysis. D.S. provided valuable material and intellectual input. S.M. provided clinical information and conceptual advice. P.L.P. supervised microarray analysis and provided valuable discussion and intellectual input. Z.Z. supervised ChIP-seq experiments and provided valuable discussion. C.P. supervised the in vitro experiments and provided valuable discussion and intellectual input. N.G. provided clinical information and supervised the projects. T.A.W. provided valuable discussion and intellectual input and edited the manuscript. C.D.J. supervised the projects, provided valuable discussion and conceptual advice and edited the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Cell sources and propagation. Primary pediatric human glioma cells, designated "SF" were obtained from surgical biopsy of tumor from patients admitted to UCSF Medical Center and in accord with an institutional approved protocol by the UCSF Committee for Human Research (IRB# 10-01318). Subjects, or their legal guardians if the subject was a minor, provided informed consent. Human astrocytes (HAS) were obtained from Z. Zhang (Mayo Clinic); consent was not required for the use of this established line. These cells were modified to express wild-type (WT) H3F3A or K27M H3F3A transgene as previously described. Establishment of glioma cell cultures, from surgical specimens, and tumor cell modification for expression of firefly luciferase, for in vivo bioluminescence imaging, have been described. Glioblastoma multiforme cells were purchased from Simonsen Laboratories and housed under nongenetic conditions.

Cell line KN542, with H3F3A G34V mutation (substitution of glycine 34 with valine), was obtained from the Japanese Collection of Bioresources and was established from a 16-year-old male.

Nuclear protein extraction and immunoblotting. Nuclear proteins were extracted from proliferating cells in acid extraction buffer (0.2 N HCl), following the extraction of cytoplasmic proteins with TNE buffer: 150 mM NaCl, 10 mM Tris pH 7.4, 1% Triton X-100, 5 mM EDTA, 1% NP40, 1 μM DTT, and proteinase (Roche) plus phosphatase (Sigma) inhibitor cocktails. Protein extracts were resolved by SDSPAGE and transferred to poly(vinylidene difluoride) (PVDF) membranes. After probing with primary antibodies, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody, and signals visualized by ECL (GE Healthcare). Antibodies specific for total histone H3 (H662C10, 1:1,000), H3K27me3 (C36B11, 1:1,000), H3K27me2 (D18C8, 1:1,000), H3K27me1 (#7693 1:1,000) and EZH2 (D2C9, 1:1,000) were obtained from Cell Signaling Technologies. Histone H3.3 antibody (ab97968, 1:1,000) was obtained from Abcam, and histone K27M mutant antibody (ABE419, 1:1,000) was from EMD Millipore.

Immunocytochemistry. Tumor cells were fixed on coverslips in 4% paraformaldehyde (PFA), rinsed in PBS, and blocked in PBS containing 0.3% Triton X-100 and 5% FBS for 1 h at room temperature. Coverslips were incubated with H3K27me3 antibody (C36B11, Cell Signaling, 1:800) or JMJD3 antibody (ab154985, Abcam, 1:800) in PBS containing 0.3% Triton X-100 and 5% FBS overnight at 4 °C. Overnight incubations were followed by incubations using goat anti-rabbit Alexa Fluor 568 secondary antibody (Invitrogen, 1:800) in PBS containing 5% FBS for 50 min at room temperature and in the absence of light. Coverslips were rinsed in PBS four times, and the nuclei were stained with DAPI in PBS at room temperature. Coverslips were then subjected to successive rinses in PBS and sterile water and then mounted on glass slides using Vectashield (Vector Laboratories) and analyzed with a Zeiss LSM 510 NLO Meta microscope at 1,000x magnification.

Cell viability, cell proliferation and clonogenic assays. For determination of cell viability effects of GSKJ4, tumor cells were seeded in 96-well plates, with 5,000 cells per well, and cultured in the presence of 0–8 μM GSKJ4 (R&D Systems) or GSK126 (Xcess Biosciences) for 72 h, with quadruplicate samples for each incubation condition. Relative numbers of viable cells were determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). IC50 values were calculated by nonlinear least-squares curve-fitting. Inhibitor proliferation effects were determined by cell counting from samples obtained at 0, 1, 2, 3 and 6 d in the presence of vehicle (DMSO) or with IC50 concentrations of GSKJ4 (SF7761, SF8628, HA KM) or with 3 μM GSKJ4 (SF9402, SF9427, HA WT, GBM43, KN542). Clonogenic assays were performed by plating at 300–1,000 cells/ml in 60-mm plates, with cells treated with vehicle, with IC50 concentrations of GSKJ4 (SF7761, SF8628, HA KM), or with 3 μM GSKJ4 (SF9402, SF9427, HA WT, GBM43, KN542) for 72 h, beginning 3 d after plating. Cells were incubated for 3 weeks at 37 °C and then stained with 0.05% crystal violet.

Xenograft studies. Six-week-old female athymic mice (nu/nu genotype, BALB/c background) were purchased from Simonsen Laboratories and housed under aseptic conditions. All protocols, described below, were approved by the UCSF Institutional Animal Care and Use Committee. Tumor cells were implanted into the pontine tegmentum of athymic mice as previously described. Briefly, mice were anesthetized by intraperitoneal (ip) injection of a mixture containing ketamine (100 mg per kg body weight (mg per kg)) and xylazine (10 mg per kg) in 0.9% saline. A 1-cm sagittal incision was made along the scalp, and the skull suture lines were exposed. A small hole was created by puncture with a 25-gauge needle at 1.5 mm to the right of the bregma and posterior to the lambda bone suture. With use of a sterile Hamilton syringe (Stoelting), 1 × 106 cells/ml in Hank’s balanced salt solution without Ca2+ and Mg2+ were slowly injected into the pontine tegmentum at 5-mm depth from the inner base of the skull. Mice were monitored daily and euthanized at induction of progressive neurologic deficit or if found in a moribund condition. For subcutaneous tumor cell implantation, 4 × 106 cells, in 0.4 ml of cell culture medium with Matrigel (BD Biosciences), were injected in the right flank of mice. For testing the effect of JMJ3 siRNA in vivo, 2 × 105 tumor cells transplanted with JMJ3 siRNA (Ambion oligo ID: #23110) or scrambled control siRNAs for 72 h were injected into the right flank of mice. Tumor growth and response to therapy were determined twice weekly by bioluminescence imaging. For imaging, mice were anesthetized as described above and examined for tumor bioluminescence 10 min following ip injection of α-luciferin (potassium salt, 150 mg per kg, Gold Biotechnology). Signal intensities were quantified within regions of interest, as defined by the LivingImage software. Bioluminescence measurements for each animal at each time point were normalized against corresponding readings obtained at the beginning of therapy.

In vivo therapy-response analysis, ten mice were randomly assigned to vehicle (DMSO, n = 5) and GSKJ4 (n = 5) treatment groups, with GSKJ4 administration by ip injection at 100 mg per kg, with once-daily administration for 10 consecutive days. An additional two mice, not included in the survival analysis, were sacrificed 6 h after administration of final treatment, with the brains of these mice resected and placed in 4% PFA.

Immunohistochemical analysis. PFA-fixed mouse brains were paraffin embedded and sectioned (10 μm) for immunohistochemical analysis of effect of therapy on cell proliferation, as indicated by positivity for Ki-67 staining (2 μg/ml, Ventana Inc.), and on K27 methylation, as indicated by positivity for H3K27me3 staining (C36B11, Cell Signaling, 1:500). To assay for apoptotic response to treatment, TUNEL staining was performed using the ApopTag Peroxidase In situ Apoptosis Detection Kit (S7100, Millipore) according to the manufacturer's protocol.

Statistical analyses. The Kaplan-Meier estimator and Prism software were used to generate and analyze survival plots. Differences between survival plots were calculated using a log-rank test. Two-way ANOVA with Bonferroni post test was used for comparison of annexin V staining. For all other comparisons, a two-tailed unpaired t-test was used (GraphPad software). No statistical method was used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment. The experiments were not randomized. In the in vivo therapy-response experiments, intracranial tumor bioluminescence signals were ranked from highest to lowest on the day of initiating therapy, and animals were assigned to treatment groups on an alternating basis, as determined by rank order, such that the mean tumor bioluminescence signals for each treatment group were approximately equal at the time of therapy initiation.

Cell cycle analysis and apoptosis assay. GSKJ4 cell cycle effects were determined by treating cells with 3 μM GSKJ4 for 72 h, pulsing cells with 10 μmol/l 5-ethyl-2'-deoxyuridine (EdU) for 30 min, and then collecting and staining cells with Alexa Fluor 647–azide using a Click-IT assay kit (Life Technologies). DNA was stained using 1 μg/ml DAPI. Cells were then subjected to flow cytometric analysis, using a BD FACSCalibur instrument, with data analyzed using FlowJo 8.1 software. Cell apoptosis was assessed with the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Life Technologies).

siRNA oligonucleotide treatments. JMJ3 (KDM6B), UTX (KDM6A), and scrambled control siRNAs (Ambion oligo IDs: JMJ3 siRNA A: s23109, B: s23110; and UTX siRNA A: s14737, B: s14736) were used to transfect tumor cells at a concentration of 30 nM using Oligofectamine reagent (Invitrogen).
Quantitative PCR for determination of JMJD3 (KDM6B) and UTX (KDM6A) gene expression. Total RNA was extracted from cell lines following treatment of JMJD3 and UTX siRNA for 72 h, and cDNA was synthesized by reverse transcription (RT) using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed in a 15-µl reaction mixture using Maxima SYBR master mix (#K0221, Thermo Scientific). Fluorescence data were collected at annealing stages, and real-time analysis performed with SDS v2.3 software (Applied Biosystems). Ct values were determined using automated baseline and fluorescence thresholds. Fold changes were calculated using the ΔΔCt method and normalized against GAPDH and ACTB expression. Forward and reverse primers, respectively, used for PCR were 5′-CCTCGAAATCCCATACAGT-3′ and 5′-CAGGGTCTTGGTGGAGAAGA-3′ for KDM6B (81-bp fragment), and 5′-ATTCAATGCGAAGGACAGCC-3′ and 5′-CTGGACAGCCGCTCTT-3′ for KDM6A (91-bp fragment).

Immunoblotting for JMJD3 and UTX proteins. Cell lysates were collected from asynchronously proliferating cells in buffer (Cell Signaling) supplemented with proteasein (Roche) and phosphatase (Sigma) inhibitor cocktails. Lysates were resolved by SDS-PAGE, and detected protein expression as described above. Antibody specific for JMJD3 (AP1022a, 1:1,000) was obtained from Abgent, and UTX antibody (A302-374A, 1:1,000) was from Bethyl.

Analysis of brain-tissue drug concentration. Athymic mice were administered GSKJ4 at 100 mg per kg per day for 3 d, with brains resected following mouse euthanasia 3 h after the third administration. Brainstem was dissected from surrounding brain, with tissues snap frozen and stored at −80 °C. GSKJ4 was extracted from homogenized tissues using a Bullet Blender (Next Advance, Inc.). Homogenates were extracted with organic solvent and further processed before transfer to an autosampler for high-performance liquid chromatography (HPLC) analysis (Shimadzu VP Series 10 System), and determination of GSKJ4 and GSKJ1 content (Integrated Analytical Systems).

Microarray analyses. RNAs from SF8628 K27M DIPG cells, either untreated or treated with GSKJ4 for 24 and 72 h, were extracted and then labeled (Cy3 for treated and Cy5 for untreated). 100-ng quantities of corresponding treated-untreated pairs were hybridized to SurePrint G3 Human Gene Expression 8 × 60K Microarrays (Agilent Technologies), in duplicate, for each length of treatment 17 h at 65 °C. Subsequent to post-hybridization rinsing, arrays were scanned using an Agilent microarray scanner (model G2505C), and signal intensities were extracted using Agilent’s Feature Extraction 10.5.1 software. Agilent’s two-color technology option was used for thresholding of signal values to 5, ratio computing (Cy3/Cy5) and log transformation. No baseline transformation was performed. Comparison of log signal intensities distributions among all eight samples indicated concordance. The distribution of Cy3/Cy5 intensity ratios plotted against the average intensity indicated the absence of bias, i.e., no normalization was necessary.

ChIP-seq. The ChIP-seq experiments were performed as described1. Briefly, 1 × 10^6 cells, treated with vehicle or 6 µM GSKJ4 for 24 and 72 h, were harvested, washed with PBS and fixed with 1% formaldehyde for 5 min at room temperature. Cells were then quenched with 125 mM glycine for 5 min. After washing, cell extracts were prepared using lysis buffer (50 mM HEPES/KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM PMSF, 1 mM Pellobloc, 1 mM benzamidine, 1 mg/ml bacitracin) on ice for 10 min. Chromatin was sheared by sonication (Bioruptor, high power, 15 × 2 cycles) to average lengths of 500 bp and then immunoprecipitated using an antibody against H3K27me3 (#9733, Cell Signaling, 1:50). After repeated washings, the DNA was recovered from the beads by incubating the beads in elution buffer (10 mM Tris at pH 8.0, 10 mM EDTA at pH 8.0, 1% SDS, 150 mM NaCl, 5 mM DTT) at 65 °C. Both input DNA and eluted DNA were subsequently purified using Qiagen Mini Elute PCR purification kit. ChIP DNA libraries were prepared with the Ovation Ultralow DR Multiplex system (NuGEN). The DNA libraries were sequenced using paired-end method by an Illumina Hi-seq 2000. Reads were aligned to the human genome (hg19) using Bowtie2 (ref. 18) software and preset parameters. Only uniquely mapping reads were used for further analysis.

Informative tags were selected from the aligned ChIP-seq data of each sequenced sample on the basis of the cross-correlation profile of positive- and negative-strand tag densities. Background anomalies resulting from extremely high-density peaks at single chromosome positions were removed. After these filtering steps, the remaining tag list from each K27me3-immunoprecipitated sample was normalized against tag lists from sample-matched input DNA (treated with only DMSO). Broad regions of binding enrichment (i.e., ChIP signal over input) for each immunoprecipitated sample were obtained by comparing scaled ChIP and input tag counts to see whether their ratio exceeds that expected from a Poisson process. The R library SPAI19 was used to perform all aforementioned steps. Distance to the nearest transcription start site (TSS) was obtained for each enriched binding region using Bioconductor package ChIPpeakAnno20 (function annotatePeakInBatch, AnnotationData: TSS_human_GRCh37).

Combined ChIP-seq and gene expression analysis. To identify both differentially expressed and differentially immunoprecipitated genes, we derived P values and fold changes separately for differential expression and K27me3 immunoprecipitation as described below.

To identify differential gene expression associated with GSKJ4 treatment, we performed a t-test to verify the null hypothesis that the Cy3/Cy5 intensity log ratios derived from GSKJ4-treated cells (24 and 72 h; two replicates per time point) are distinct from 0. The R package DBCHIP was used to identify sequences for which differential K27me3 immunoprecipitation is associated with GSKJ4 treatment. Enrichment sites estimated from vehicle-treated SF8628 cells and SF8628 cells incubated with 6 µM GSKJ4 (24 and 72 h) were merged into consensus binding sites (function site.merge), and fold changes of non-differential binding at each consensus site were calculated (function test.diff.binding). P values for each gene were obtained by performing a two-sided Wilcoxon signed-rank test of the hypothesis that fold changes associated with a gene come from a distribution whose median is 0. The resulting P values reflect both the differential length of K27me3-immunoprecipitated regions between GSKJ4-treated and untreated samples and the differential sequencing depth within those regions. The 34 differentially expressed and differentially immunoprecipitated genes (P ≤ 0.1 and absolute fold change ≥ 1.5), and those for which there was an inverse correlation between GSKJ4-associated gene expression change and corresponding change in K27me3 sequence association, are shown in Supplementary Figure 11 and Supplementary Table 3.