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Targeted inhibition of histone H3K27 demethylation is effective in high-risk neuroblastoma

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Abstract

High-risk neuroblastoma is often distinguished by amplification of *MYCN* and loss of differentiation potential. We performed high-throughput drug screening of epigenetic-targeted therapies across a large and diverse tumor cell line panel and uncovered the hypersensitivity of neuroblastoma cells to GSK-J4, a small-molecule dual inhibitor of lysine 27 of histone 3 (H3K27) demethylases ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX), and histone demethylase Jumonji D3 (JMJD3). Mechanistically, GSK-J4 induced neuroblastoma differentiation and endoplasmic reticulum (ER) stress, with accompanying up-regulation of p53 up-regulated modulator of apoptosis (PUMA) and induction of cell death. Retinoic acid (RA)-resistant neuroblastoma cells were sensitive to GSK-J4. In addition, GSK-J4 was effective at

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Data and materials availability: The RNA-seq data from this study are available in the GEO database (accession no. GSE110709). COG cell lines are available from C.P.R. via the Childhood Cancer Repository (www.cccells.org) under a material transfer agreement with the COG. Venetoclax (ABT-199) is available from A. Souers (AbbVie) under a material transfer agreement with VCU.

blocking the growth of chemorefractory and patient-derived xenograft models of high-risk neuroblastoma in vivo. Furthermore, GSK-J4 and RA combination increased differentiation and ER stress over GSK-J4 effects and limited the growth of neuroblastomas resistant to either drug alone. In *MYCN*-amplified neuroblastoma, PUMA induction by GSK-J4 sensitized tumors to the B cell lymphoma 2 (BCL-2) inhibitor venetoclax, demonstrating that epigenetic-targeted therapies and BCL-2 homology domain 3 mimetics can be rationally combined to treat this high-risk subset of neuroblastoma. Therefore, H3K27 demethylation inhibition is a promising therapeutic target to treat high-risk neuroblastoma, and H3K27 demethylation can be part of rational combination therapies to induce robust antineuroblastoma activity.

Introduction

High-risk neuroblastoma remains a lethal cancer, despite comprehensive therapy (1). The failure of neural crest cell precursors to differentiate is at the root of the formation of neuroblastic tumors (2, 3). Retinoic acid (RA), including all-*trans* retinoic acid (ATRA) and other vitamin A derivatives that can induce the differentiation of neuroblastoma tumors have reduced the risk of neuroblastoma recurrence after induction therapy and stem cell transplant (1). However, a substantial number of patients do not benefit from RA therapies.

High-throughput drug screening (HTS) using the Genomics of Drug Sensitivity in Cancer (GDSC) platform has revealed unanticipated sensitivities of subsets of cancers (4–7). We have recently expanded these large-scale screening efforts to include a greater number of epigenetic-targeted therapies. Changes in the epigenome have come into focus as important mediators of tumorigenesis, particularly in pediatric cancers such as neuroblastoma (8–10). In pediatric cancers, the overall mutation burden is relatively low (10, 11), suggesting that epigenetic-driven wholesale changes in the genome landscape may play large and still unappreciated roles in tumorigenesis (12). For instance, in diffuse intrinsic pontine glioma (DIPG), mutations in the genes encoding H3.1 and H3.3 histones are rampant, resulting in a lack of methylation at lysine 27 of histone 3 (H3K27) (13). Focused studies (14, 15) have demonstrated that the histone demethylase inhibitor GSK-J4 (16), through on-target inhibition of H3K27 demethylation activity, had activity in DIPG mouse models. Neuroblastomas also have a number of alterations to key genes in the epigenome (12, 17). Thus, understanding how multiple epigenetic mechanisms affect neuroblastoma growth is essential for more successful therapies against this pediatric cancer.

Here, we demonstrate that a large subset of neuroblastomas, including high-risk neuroblastomas, is exquisitely sensitive to inhibition of histone H3K27 demethylase activity. Furthermore, our studies establish the mechanistic activity of demethylase inhibitor GSK-J4 to include the induction of differentiation, implicating aberrant H3K27 trimethylation (H3K27me3) in the differentiation block involved in *MYCN*-amplified neuroblastoma formation. Finally, GSK-J4 potentiated the activity of the B cell lymphoma 2 (BCL-2) inhibitor venetoclax in neuroblastomas in vivo, including chemoresistant and high-risk models. Together, these data reveal a drug target and potential treatment strategy for high-risk neuroblastoma.

Results

Demethylase inhibition has antineuroblastoma activity

As part of the continuing effort of the GDSC HTS (7, 18), we identified the histone H3K27 demethylase inhibitor GSK-J4 (16, 19) as having potent activity in neuroblastoma cell lines, among ~800 solid tumor cancer cell lines tested ($P = 1.7 \times 10^{-10}$; Fig. 1A). We observed a range of sensitivity across the 31 neuroblastoma models included in our initial screen, with 8 models among the top 3% most sensitive models and 28 of 31 found in the top 50% of sensitivity (773 solid tumor cell lines screened; table S1).

Sensitivity was not differentiated by *MYCN* status, p53 functionality, chromosomal changes, or expression of *KDM6A* [ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX)], *KDM6B* [histone demethylase Jumonji D3 (JMJD3)], enhancer of zeste homolog 2 (*EZH2*), or other H3-lysine modifiers [Fig. 1B, figs. S1 and S2 (A and B), and table S2]. GSK-J4 treatment also led to near-complete loss of cell viability in sensitive neuroblastomas in 6-day viability assays (fig. S2C). Two additional histone demethylase inhibitors, IOX1 (20) and JIB-04 (21), albeit with less narrow specificity, demonstrated a pattern of sensitivity similar to GSK-J4 in neuroblastoma lines (fig. S3, A and B). In addition, knockdown of JMJD3 but not of UTX led to a decrease in cell viability (fig. S3, C and D), suggesting that JMJD3 is the key target of GSK-J4 in neuroblastoma.

High-risk and chemorefractory neuroblastomas are sensitive to GSK-J4 in vivo

We next assessed the activity of GSK-J4 in several in vivo mouse models, including the *MYCN* wild-type and chemorefractory model, CHLA20 (22), and the *MYCN*-amplified, high-risk model, IMR32. We also studied the effects of GSK-J4 on the *MYCN* wild-type (FELIX) and high-risk *MYCN*-amplified (COG-N-561) patient-derived xenograft (PDX) models. GSK-J4 delivered daily was sufficient to induce tumor regressions in the IMR32 model, completely block growth in the COG-N-561 PDX, and inhibit tumor growth in the FELIX PDX and CHLA20 models (Fig. 1, C to F). The global amount of H3K27me3 in individual tumors increased with GSK-J4 treatment (fig. S4, A and B). These effects were seen without behavioral changes in the mice and with only minor weight loss of the GSK-J4-treated animals throughout the experiments (fig. S4C). These data further support H3K27 demethylation as a therapeutic target for the treatment of neuroblastoma.

GSK-J4 induces broad expression changes in sensitive neuroblastomas

To gain insight into the mechanism of GSK-J4 efficacy in neuroblastoma, we performed high-throughput RNA sequencing (RNA-seq) in sensitive neuroblastoma cell lines (IMR5, LAN5, and SK-N-F1) and the resistant SK-N-BE(2) neuroblastoma cell line, for comparison. There were far more significant gene expression changes in the sensitive cell lines (>7000 genes altered; $P < 0.05$) compared to the resistant cell line (<1500 genes altered; $P < 0.05$; Fig. 2A and table S3). Consistent with differential sensitivity to H3K27 demethylation inhibition, GSK-J4 sharply increased H3K27 methylation (16) only in the sensitive IMR5 cell line and not the resistant SK-N-BE(2) cell line (fig. S5, A and B). Because GSK-J4 treatment may also inhibit other H3 histone demethylases (19), we assayed for changes at other H3 marks and also found an increase in H3K36me3 (fig. S5C), which is

positively associated with transcriptional elongation (23). We also saw an increase of H3K4me3 (fig. S5C), which is associated with transcriptional activation (24). We did not observe changes in the methylation status of H3K9me3 (fig. S5C). Subsequent RNA-seq analyses indicated that many of the gene changes were similar in the sensitive cell lines (fig. S6 and table S4). Among the most affected functions were differentiation pathways, including neuronal differentiation and neuritogenesis (tables S5 and S6). Some prominent changes among differentiation genes included the critical differentiation genes enolase 2 (neuron-specific enolase, *ENO2*) (25), chromodomain helicase DNA binding protein 5 (*CHD5*) (26), nerve growth factor (*NGF*) (26), and neuregulin 1 (*NRG1*) (27), which were up-regulated, whereas the differentiation blockers, achaete-scute homolog 1 (*ASCL1*) (28, 29) and *MYCN* (2), were reduced (Fig. 2B). Similarly, we also found that protein expression of CHD5 was increased in sensitive neuroblastoma cell lines (Fig. 2C and fig. S7). In addition, MYCN expression decreased in the *MYCN*-amplified cell lines (Fig. 2C). Overall, the transcriptional changes were most prominent in the *MYCN*-amplified cell lines, consistent with MYCN as an important inhibitor of neuroblastoma differentiation (2). Furthermore, the induction of differentiation markers occurred even in ATRA-resistant IMR5 and SK-N-FI cells (Fig. 2B) (26).

In addition to induction of differentiation, GSK-J4 also induced apoptosis and/or necrosis, as observed by annexin V/propidium iodide staining (fig. S8, A to C), whereas no increase in apoptosis/necrosis was observed in the GSK-J4-resistant SK-N-BE(2) model (fig. S8D). Marked cell death was seen at 48 and 72 hours (fig. S8, A to C), similar to the kinetics of ATRA treatment (30). In addition, caspase-3 activity was increased in the GSK-J4-treated-sensitive cell lines IMR5, SMS-SAN, and KELLY, implicating apoptosis as a prominent mode of cell death (fig. S9). Consistent with the hypothesis of apoptosis-driven cell death, pretreatment of GSK-J4-sensitive neuroblastoma cells with the caspase inhibitor Z-VAD-FMK protected against GSK-J4-mediated apoptosis (fig. S10).

Because BCL-2 family members govern intrinsic apoptosis (31), we analyzed key BCL-2 family members in the RNA-seq data for expression changes and found a marked up-regulation of p53 up-regulated modulator of apoptosis (PUMA) (32) in the sensitive cell lines (Fig. 2D). Although p53 is a driver of PUMA up-regulation (33), we did not observe p53 stabilization upon GSK-J4 treatment (Fig. 2E). Endoplasmic reticulum (ER) stress is also known to cause PUMA up-regulation and can accompany differentiation induced by RA (34–36). In addition to an increase in cleaved poly(adenosine diphosphate-ribose) polymerase (cl.PARP), we found that several markers of ER stress, including inositol-requiring protein 1 [IRE1 α ; encoded by ER-to-nucleus signaling 1 (*ERN1*)] and activating transcription factor 4 (*ATF4*), were up-regulated at the mRNA level (Fig. 2D) and confirmed that both IRE1 α and ATF4 were increased at the protein level (Fig. 2E and fig. S7). A significant ($P=0.0063$) decrease in *BCL2* binding component 3 (*BBC3*, the gene encoding PUMA) mRNA expression is observed in relapsed neuroblastoma tumors, compared to tumors at diagnosis (37), suggesting that increasing *BBC3* expression may provide a therapeutic benefit (fig. S11A). Consistent with a role for PUMA in GSK-J4-mediated efficacy, RNA-seq and Western blot analyses indicated induction of *BBC3*/PUMA (Fig. 2, D and E), whereas knockdown of *BBC3* by short hairpin-mediated RNA (shRNA) silencing protected cells from GSK-J4-induced apoptosis/necrosis (fig. S11, B and C). Furthermore,

cell death from GSK-J4 treatment was decreased through the knockdown of IRE1 α (fig. S11, D to F), further supporting the hypothesis that ER stress directs GSK-J4-mediated toxicity.

GSK-J4 combines with RA to overcome drug resistance

ATRA and 13-*cis*-RA are vitamin A-derived retinoids that can induce differentiation in neuroblastoma (2, 26), and 13-*cis*-RA is used clinically in high-risk neuroblastoma (38). Given that we found several differentiation-related genes up-regulated after GSK-J4 treatment, we asked whether there was an overlap between GSK-J4 and RA treatment effects. We found that a large subset of genes affected by GSK-J4 treatment was similarly affected by RA (Fig. 3A and table S7) (39). We confirmed that this gene overlap was statistically significant both through Fisher's exact test and gene set enrichment analysis (GSEA) for both the RA up- and down-regulated gene sets (fig. S12 and table S7). From the GSEA, we found that the LAN5, IMR5, and SK-N-FI cells had a positive normalized enrichment score (NES), indicating that a large subset of RA-increased genes were also up-regulated with GSK-J4 treatment (fig. S12A). Conversely, we found negative NESs in the RA-decreased gene set, suggesting a similar effect with GSK-J4 treatment (fig. S12B). In contrast to the large set of affected genes we found in common with GSK-J4 and RA treatment, only nine genes were similarly affected between GSK-J4 and the H3K27 methyltransferase inhibitor EPZ-6438 (table S8) (40).

In addition, subtoxic amounts of GSK-J4 induced axonal outgrowth in LAN5 cells (Fig. 3B and fig. S13), further indicating induction of differentiation. We then asked whether cotreatment with RA and GSK-J4 could block neuroblastoma growth in vitro. We found that cotreatment of the GSK-J4-resistant neuroblastoma cell lines CHLA90, SK-N-AS, and SK-N-BE(2) with GSK-J4 and RA was highly effective at reducing cell viability (Fig. 3, C and D, and fig. S14). Furthermore, the combination of RA and GSK-J4 induced both the differentiation marker CHD5 and several ER stress markers, including PUMA, after 6 days of treatment (Fig. 3E). These data reveal that, in GSK-J4-insensitive neuroblastoma cells, the combination of GSK-J4 and RA induces differentiation and ER stress and that these compounds synergize to induce cell death (fig. S14).

Venetoclax and GSK-J4 combine to induce tumor regression

PUMA interacts with the main antiapoptotic proteins, BCL-2, B cell lymphoma extra large (BCL-XL), and myeloid cell leukemia 1 (MCL-1) (31) to sensitize cells to BCL-2 interacting mediator of cell death (BIM)-mediated apoptosis (36, 41), and we had previously identified BCL-2 homology domain 3 mimetics as promising therapeutics in *MYCN*-amplified neuroblastoma (5). Therefore, we asked whether PUMA induction by GSK-J4 may sensitize *MYCN*-amplified neuroblastomas to the BCL-2 inhibitor venetoclax. The venetoclax/GSK-J4 combination suppressed the growth of several *MYCN*-amplified neuroblastoma cell lines (Fig. 4A and fig. S15, A and B), which was due to an increase in the induction of apoptosis by the combination compared to GSK-J4 or venetoclax single-agent therapy (Fig. 4B). Furthermore, immunoprecipitation of the proapoptotic protein BIM in *MYCN*-amplified LAN5 cells treated with venetoclax or the venetoclax/GSK-J4 combination demonstrated the expected disruption of BIM/BCL-2 complexes (fig. S15C).

However, only the venetoclax/GSK-J4 combination treatment led to marked reduction of BIM/MCL-1 complexes (fig. S15C). Overall, these findings are consistent with the combination therapy stimulating cell death. In vivo, although both GSK-J4 and venetoclax had some single-agent tumor growth inhibition activity, the combination had marked antineuroblastoma activity (Fig. 4, C and D), with no behavior changes or marked weight loss in the mice (fig. S16).

Discussion

High-risk neuroblastoma remains fatal in more than half the cases (42). Recent genomic analysis of recurrent neuroblastomas has indicated a low rate of mutations in druggable targets; with the exception of anaplastic lymphoma receptor tyrosine kinase (*ALK*), there are no kinases mutated at a rate greater than 5% (17). Furthermore, amplified *MYCN*, which is currently undruggable, remains by far the most prevalent genetic driving alteration in the refractory population (17). The second most commonly mutated cancer gene in the refractory population is the chromatin remodeler alpha thalassemia/mental retardation syndrome X-linked (*ATRX*), with the mutation occurring in about 25% of non-*MYCN*-amplified neuroblastoma cases (43). *ATRX* is part of a complex responsible for deposition of H3.3 variants at naked DNA and, hence, widely affects the expression of different genes (44). In addition, mutations in other epigenetic components, including *EZH2* (45), stromal antigen 2 (*STAG2*) (46), and AT-rich interaction domain 1AB (*ARID1A/B*) (47), are found in neuroblastoma.

Therefore, successful development of targeted therapies in neuroblastoma may best be accomplished by engaging nonkinase targets, particularly those that cause key changes in the epigenome and that present as particular vulnerabilities in this cancer. To this point, Veschi *et al.* (8) recently reported that the lysine methyltransferase Su(var)3-9 enhancer-of-zeste and trithorax domain containing 8 (SETD8) is a promising therapeutic target in high-risk neuroblastomas, and disruptor of telomeric silencing-1-like histone lysine methyltransferase (DOT1L) was recently reported to act as a key cofactor in *MYCN*-induced transcripts in neuroblastoma (48).

Here, we demonstrate that pharmacologically inhibiting the histone H3K27 demethylases UTX and JMJD3 with GSK-J4 has profound activity on key differentiation genes and pathways in neuroblastoma, including reduction of *MYCN* expression. *MYCN* remains the most important target in neuroblastoma not only because the gene is amplified in ~25% of cases but also because it portends poor outcomes (49). Although GSK-J4 can inhibit other H3 demethylases (19), a comprehensive analysis of chromatin changes after GSK-J4 treatment in sensitive neuroblastoma cells only revealed marked changes in H3K27, with increases at H3K36me3 and H3K4me3, consistent with on-target activity (15).

A recent report demonstrated that inhibition of PRC2 with the *EZH2* inhibitor EPZ-6438 and DNA methylation inhibitor decitabine can together induce differentiation markers and control neuroblastoma growth in vitro (40). Earlier work also showed that *EZH2* inhibition could induce neurite outgrowth in neuroblastoma cells (50). In addition, a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 screen

demonstrated sensitivity of *MYCN*-amplified neuroblastomas to EZH2 disruption (51). Whereas these studies involved inhibiting H3K27 methylation, our study involves inhibiting H3K27 demethylation; however, epigenetic control of gene expression tends to be a balance between a repressed and an activated state, with imbalances in either direction possibly producing adverse effects to cells (52).

Relatively few genes were similarly up-regulated in both GSK-J4-treated cells and in the EZH2 inhibitor EPZ-6438-treated cells from the study of Henrich *et al.* (40). Although there was some overlap of differentially expressed genes with H3K27 methylation inhibition and demethylation inhibition, GSK-J4 induced a much more robust gene expression response, suggesting that blocking either H3K27 methylation or demethylation is a valuable strategy in neuroblastoma treatment. Although ER stress was not specifically assessed in their study, we demonstrated that histone demethylation induces ER stress-mediated PUMA up-regulation and that ER stress and PUMA up-regulation are important for GSK-J4 efficacy. There may be a causal link between ER stress and differentiation in neuroblastoma, as has been found in other normal and cancerous cells (53, 54). Specifically, it has been shown that ER stress induction causes an induction of differentiation, offering a potential mechanism for the induction of differentiation of neuroblastoma cells with GSK-J4 treatment. However, what specific role ER stress may have in neuroblastoma differentiation will require further exploration. In addition, we also reported a strong decrease in *MYCN* expression upon treatment with GSK-J4, which was not reported (40) with EPZ-6438 treatment. This result may be due to an increase in H3K27me3 at the enhancer or promoter of *MYCN* (55). In addition, both UTX and JMJD3 have regulatory functions independent of their demethylase activity and may act as transcription factors themselves or affect chromatin remodeling (56).

We also found that GSK-J4 could sensitize neuroblastomas to RA therapy. These data suggest a role of histone demethylation in differentiation failure, which is at the core of neuroblastoma tumorigenesis. It is therefore tempting to speculate that a critical misstep resulting in neuroblastoma formation is at the level of histone demethylation, creating a differentiation block, in effect preventing cells from maturation into differentiated cells while making them vulnerable to tumor-forming mutations such as amplification of *MYCN*. However, this model is highly speculative, and further studies will be required to test the hypothesis. In addition, in the cell lines tested here, engagement of the RA receptor seems ineffective. However, cotreatment with GSK-J4 lifts the differentiation block, allowing RA to induce differentiation.

GSK-J4 induced tumor growth inhibition in models of high-risk and chemorefractory neuroblastoma, and the combination of GSK-J4 with the BCL-2 inhibitor venetoclax induced marked antineuroblastoma activity superior to single-agent therapy in multiple xenograft models. GSK-J4 sensitized these tumors to venetoclax through induction of ER stress and PUMA, which caused a marked decrease in MCL-1/BIM complexes after venetoclax treatment. We propose a model where GSK-J4 first increases ER stress and PUMA, followed by PUMA-driven displacement of BIM from the antiapoptotic BCL-2 proteins. This allows for BIM to activate BAK and BAX (41) directly, exacerbating venetoclax-mediated apoptosis, which occurs via BIM in *MYCN*-amplified neuroblastoma

(5). Thus, together, these data provide a strong rationale for targeting H3K27 demethylation in combination with venetoclax in *MYCN*-amplified neuroblastoma.

Overall, we provide evidence that targeting the H3K27 demethylases induces differentiation and triggers ER stress-mediated apoptosis and cell death in a large subset of neuroblastomas, including those characterized by high-risk features. We also demonstrate two rational GSK-J4-based combinations: with RA or with venetoclax. Furthermore, both RA and venetoclax are U.S. Food and Drug Administration-approved drugs. These data demonstrate the promise of rational epigenetic-targeted therapy combination in neuroblastoma.

Materials and Methods

Study design

The objective of this study was to test the hypothesis that GSK-J4 is a potential therapeutic for neuroblastoma. For the in vivo studies, GSK-J4 efficacy as a single agent was determined in both cell line-based xenograft and PDX neuroblastoma models. We also determined the efficacy of GSK-J4 in combination with venetoclax in neuroblastoma xenograft models. Sample sizes were determined on the basis of previous experimental experience and on the growth profiles of the individual models ($n = 3$ to 7). With the exception of the LAN5 model, all mice were randomized. The LAN5 model was separated into cohorts as described below. For all experiments, tumors were measured every 2 to 3 days in two dimensions via calipers, and tumor volume was calculated as height $\times$ width $\times$ width $\times$ 0.52, where height was the larger of the two measurements. Investigators were not blinded during experiments. Animals were euthanized if they lost 20% of their weight or exhibited signs of impaired health. Mice were euthanized when any tumor volume within their cohort exceeded ~ 1500 mm³. For the combination of GSK-J4 with venetoclax, remaining mice within each cohort were monitored until two or fewer mice remained. Primary data are presented in table S9.

Drug screening

Authenticated cell lines from the GDSC (7) were screened against GSK-J4 at the Center for Molecular Therapeutics at the Massachusetts General Hospital. Area under the dose-response curve and median inhibitory concentration were determined as previously described (7).

Expression analysis

Basal RNA expression data for the GDSC cell lines screened were obtained through the R2: Genomics Analysis and Visualization Platform (<http://hgserver1.amc.nl>). Expression data came from ArrayExpress (accession no. E-MTAB-3610).

Cell lines, GSK-J4, and venetoclax

The neuroblastoma cell lines IMR32, SMS-SAN, LAN5, IMR5, KELLY, LAN6, NB1643, NB12, SK-N-AS, SK-N-BE(2), SK-N-SH, and SK-N-DZ were from the Molecular Center Therapeutics Laboratory at Massachusetts General Hospital, which performs routine testing of cell lines by single-nucleotide polymorphism and short tandem repeat analysis. The NB-

EBC1 and SK-N-F1 cell lines were provided by the Children's Hospital of Pennsylvania (Y. Mossé) and have been previously published (5). FELIX, COG-N-415, COG-N-440, COG-N-452, COG-N-561, CHLA20, CHLA90, CHLA140, and CHLA172 were obtained from the Children's Oncology Group (COG) Cell Culture and Xenograft Repository. COG-N-561 and FELIX were obtained as both cell lines and PDX single-cell suspensions. The NB-EBC1 cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The SK-N-AS, SK-N-BE(2), SK-N-SH, SK-N-DZ, IMR32, IMR5, and SK-N-FI cell lines were cultured in DMEM/F12 (50:50) supplemented with 10% FBS. The KELLY, NB1643, LAN6, LAN5, SMS-SAN, and NB12 cell lines were cultured in RPMI 1640 supplemented with 10% FBS. The COG-N-440, CHLA20, CHLA90, CHLA140, and CHLA172 cell lines were cultured in DMEM supplemented with 20% FBS and 1× insulin-transferrin-selenium (ITS; Thermo Fisher Scientific, catalog #41400045). The COG-N-415, COG-N-452, COG-N-561, and FELIX cell lines were cultured in Iscove's modified Dulbecco's medium supplemented with 20% FBS and 1× ITS. GSK-J4 was purchased from AbMole BioScience. Venetoclax was provided by AbbVie.

Cell viability assays

Neuroblastoma cells were seeded in quadruplicate in 96-well microtiter plates at a concentration of 2×10^4 cells per well in 180 μ l of growth medium as described above. Twenty-four hours after seeding, cells were treated with 1 μ M GSK-J4 for 72 hours. Cell viability was measured by the CellTiter-Glo protocol per the manufacturer (Promega).

Crystal violet assays

Cells were seeded at 1×10^5 cells per well in a six-well dish and treated the following day with 1 μ M GSK-J4. Five or six days later, when untreated cells reached confluency, cells were stained with 0.1% crystal violet (Sigma-Aldrich).

Western blotting

Cells were lysed with NP-40 lysis buffer as previously described (5), followed by acid extraction of the nuclear pellet by incubation with 0.2 M HCl overnight to obtain histones. Alternatively, radioimmunoprecipitation assay (RIPA) buffer was used to extract both total protein and histones in a single fraction. All in vitro data shown in the manuscript are from the NP-40 lysis buffer and acid extraction method. In vivo tumor lysates were prepared with RIPA buffer. Western blots were performed as previously described (5). Differentiation and ER stress protein observation experiments were performed a minimum of three times. H3K27 methylation blotting was performed three times. Other histone marks were measured a single time. Antibodies were used as follows: from Cell Signaling Technologies, H3K27me3 (catalog #9733S), H3K4me3 (catalog #9751S), H3K27me2 (catalog #9727S), H3K36me3 (catalog #4909S), H3 (catalog # 3638S), cl.PARP (catalog #5625S), EZH2 (catalog #5246S), UTX (catalog #33510S), N-MYC (catalog #9405S), JMJD3 (catalog #3457S), BIM (catalog # 2933S), MCL1 (catalog #5453S), BCL-2 (catalog #2933S), BCL-XL (catalog #2764S), PUMA (catalog #4976S), IRE1 α (catalog #3294S), ATF4 (catalog #11815S), NOXA (catalog #14766S), p53 (catalog #2527S); from Santa Cruz Biotechnology, GAPDH (catalog #sc-32233), CHD5 (catalog #sc-68390); from Active Motif, H3K27me1 (catalog #61015), H3K9me3 (catalog #39161); and from Abcam, PUMA

(catalog #ab9643). Densitometry was performed using the GeneTools software suite (Syngene).

Immunoprecipitation

A confluent 15-cm dish of LAN5 cells was treated with 1 μ M GSK-J4, 1 μ M venetoclax, or a combination of both for 16 hours. Cells were lysed as above, and 500 μ g of each lysate were incubated with BIM antibody (500 ng; Cell Signaling Technology, catalog #2933S), or rabbit immunoglobulin G (500 ng; Santa Cruz Biotechnology, catalog #sc-2027). Twenty-five microliters of 1:1 phosphate-buffered saline/prewashed protein A beads was added to the antibody/lysate mix, and then, the samples were incubated with rotating motion overnight at 4°C. Equal amounts of extracts (5% of the immunoprecipitated protein lysates) were prepared in parallel. Experiments were performed in biological duplicate, and a representative assay is presented.

RNA sequencing

Cells were seeded in triplicate at 15×10^4 cells (untreated) or 25×10^4 cells (GSK-J4-treated) in six-well plates. Twenty-four hours after seeding, cells were treated with 1 μ M GSK-J4 for 72 hours. The medium was removed, and RNA was extracted using the TRIzol reagent as per the manufacturer's instructions (Thermo Fisher Scientific). RNA quantity and quality were determined using the Agilent Bioanalyzer, and only samples above an RNA integrity number of 8.6 were processed for sequencing. RNA-seq libraries were created with the KAPA Stranded mRNA-Seq Kit (Kapa Biosystems), and sequencing was performed on the Illumina HiSeq 2500 using paired-end sequencing (2×125). RNA-seq data are available at the Gene Expression Omnibus (GEO) Database under the accession no. GSE110709.

RNA-seq analysis

RNA-seq data quality was confirmed using FastQC, and reads were mapped to the Ensembl hg38 release 85 assembly RNA annotation. Reads were mapped using the Spliced Transcripts Alignment to a Reference aligner under default settings. Mapped reads were quantitated in R using the SummarizedExperiment library, and differential expression analysis was performed using DESeq2 with default settings. Bonferroni-Hochberg correction (false discovery rate of 10%) was applied to determine the P_{adj} used to define significance. Pathway analysis was performed using QIAGEN Ingenuity Pathway Analysis (IPA) on significantly up-regulated genes ($P_{\text{adj}} < 0.05$; absolute log fold change, > 0.8).

RNA interference assays

The plasmid for sh*BBC3* (clone ID: NM_014417.2-785s1c1) was obtained from the MISSION shRNA repository (Sigma-Aldrich) and used to knock down PUMA. A nonspecific shRNA (MISSION pLKO.1-shRNA control) served as a negative control. The pLKO1.1 plasmid contains a puromycin resistance gene, which allowed for cell selection (2 μ g/ml). Viral particles were collected from virus generated in 293T cells. Knockdown was confirmed by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR). Briefly, RNA was extracted from puromycin-selected cells using the Zymo Quick-RNA Miniprep Plus. Generation of complementary DNA (cDNA) was performed on 0.5 μ g

of RNA using iScript Advanced (Bio-Rad) as per the manufacturer's instructions. Quantitation of cDNA was performed on six separate samples on two separate days ($n = 3$ per day) by qPCR using the PowerUp SYBR Green Master Mix (Applied Biosciences) using primers for *BBC3* (forward primer, ACTCACCAAACCAGAGCAGG; reverse primer, CTCCTGGGGCCACAAATC). The primers for the endogenous control *ACTB* have been previously described (5). Quantitation of RNA was performed using the delta delta cycle threshold (C_t) method (57). On each day, each biological replicate was performed in technical triplicate, and the average C_t values from all control siRNA replicates were used to normalize expression for all samples. For the small interfering RNA (siRNA) experiments, siControl (Ambion, catalog #4390843), si*KDM6A* (Ambion, catalog #S14736), and si*KDM6B* (Ambion, catalog #S23110) were transfected in with Lipofectamine 2000 (Invitrogen), using the manufacturer's reverse transfection protocol. After reverse transfection, cells were incubated with the siRNA for 96 hours, followed by Western blotting or the CellTiter-Glo cell viability assay as described above. Alternatively, siControl (GE Healthcare Life Sciences, catalog #D-001810-10-20) or si*ERN1* (GE Healthcare Life Sciences, catalog #L-004951-02-0005) was reverse transfected as above for 24 hours, followed by 48 hours of 1 μ M GSK-J4 treatment. Cells were processed for whole-cell lysates or fluorescence-activated cell sorting (FACS) analysis of apoptosis. All siRNA experiments were performed at a final siRNA concentration of 50 nM.

FACS apoptosis assays

Cells were seeded at 1×10^5 cells per well in a six-well plate. Twenty-four hours after seeding, 1 μ M GSK-J4 was added for the 72-hour time point. Forty-eight hours after seeding, 1 μ M GSK-J4 was added for the 48-hour time point. Both the 72- and 48-hour time points were collected and assayed at the same time. For the combination therapy, cells were seeded at 2×10^5 cells per well in a six-well plate and were treated for 48 hours with 1 μ M GSK-J4, 1 μ M venetoclax, or a combination of both drugs. Untreated cells served as a negative control. For apoptosis assays, cells were stained with propidium iodide and annexin V–Cy5 (BD Biosciences) and assayed on a Guava easyCyte flow cytometer (Millipore Sigma). Analysis was performed using FlowJo by separating the measured cells into four quadrants, and cells within annexin V–positive and annexin V/propidium iodide–double positive quadrants were counted as apoptotic.

Cleaved caspase-3 activity assays

Cells were seeded in duplicate (SMS-SAN) or triplicate (IMR5 and KELLY) at 2×10^4 cells in a 96-well plate. After 24 hours, the medium was changed to 1 μ M GSK-J4 and incubated for 36 hours. Cells were assayed for caspase-3 activity as per the manufacturer's protocol (Cell Signaling Technology, catalog #5723S), with a 2-hour incubation period before reading the plate. Untreated cells were used as a negative control.

Caspase inhibition assays

Cells were seeded as above and cotreated with 50 μ M Z-VAD-FMK (Selleckchem, catalog #S7023) and 1 μ M GSK-J4 or 1 μ M venetoclax for 48 hours. After treatment, cells were assayed for apoptosis as above.

Xenograft and patient-derived xenograft models

The CHLA20 and IMR32 cell lines were injected into the right flank of female Nu/Nu mice at a concentration of 5×10^6 cells per mouse using a 1:5 ratio of cells/Matrigel (Corning, catalog #354248). When the tumors reached an average size of 150 mm^3 , they were enrolled in the study. For the CHLA20 model, mice were randomized into four untreated and four GSK-J4-treated mice. For the IMR32 model, mice were randomized into five untreated and four GSK-J4-treated mice. Mice were dosed with GSK-J4 (100 mg/kg) in dimethyl sulfoxide by intraperitoneal injection 5 days/week. FELIX and COG-N-561 PDX models were injected into female NSG mice at 5×10^5 cells per mouse using a 1:1 ratio of cells/Matrigel. These mice were randomized when they reached an average tumor size of 120 mm^3 , with cohorts being seven controls and six GSK-J4-treated (COG-N-561) or three controls and three GSK-J4-treated (FELIX). Mice were treated with GSK-J4 (50 mg/kg) 4 days/week. The LAN5 cell line was injected into the right flank of female Nu/Nu mice at a concentration of 5×10^6 cells per mouse as above. After their tumors reached an average size of 200 mm^3 , animals were enrolled as follows: The three largest and three smallest tumors were enrolled in combination therapy, the next three largest and smallest were enrolled as GSK-J4, the next three largest and two smallest were enrolled as venetoclax, and the remaining mice were enrolled as control. Animals were treated with GSK-J4 (100 mg/kg) by intraperitoneal injection, venetoclax (100 mg/kg) by oral gavage (200 μl ; 60% PHOSAL 50PG, 30% PEG-400, 10% ethanol), or a combination of both drugs 5 days/week. SK-N-DZ and IMR5 xenograft models were injected into NSG mice as described above. After tumors reached an average size of 120 mm^3 , mice were randomized and enrolled in control (SK-N-DZ and IMR5, $n = 5$), GSK-J4 (50 mg/kg, 4 days/week; SK-N-DZ and IMR5, $n = 5$), venetoclax (100 mg/kg, 5 days/week; SK-N-DZ, $n = 4$; IMR5, $n = 5$), or combination cohorts (SK-N-DZ and IMR5, $n = 5$). All animal experiments were approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee (protocol #AD10001048).

RA and axonal length assays

SK-N-BE(2), CHLA90, and SK-N-AS cell lines were seeded at 1×10^5 cells per well in a six-well dish. The day after seeding, cells were treated with $1 \mu\text{M}$ GSK-J4, $20 \mu\text{M}$ 13-*cis*-RA (Sigma-Aldrich), or a combination of both drugs for 6 days. Cells were then prepared for crystal violet staining as above. For Western blotting, 2×10^5 cells were seeded in a 10-cm dish and treated as above. For LAN5 axon growth assays, cells were seeded as above, and cells were treated with $0.1 \mu\text{M}$ GSK-J4 or $10 \mu\text{M}$ RA. Cells were imaged on an Olympus microscope by both differential interference contrast (DIC) and phase-contrast (PC) imaging. DIC images are displayed in fig. S13. Measurement of axonal length was performed on the PC images in Fiji (<https://fiji.sc/>) using the simple neuronal tracer tool.

Synergy assay

Cells were seeded in quadruplicate at 1×10^3 cells in a 96-well plate. Twenty-four hours after seeding, cells were treated with varied concentrations of GSK-J4 (0 to $2 \mu\text{M}$) and 13-*cis*-RA (0 to $100 \mu\text{M}$) for 6 days, followed by measurement of cell viability by CellTiter-Glo. Alternately, cells were seeded in quadruplicate at 2×10^3 cells per well in a 96-well

plate. Twenty-four hours later, cells were treated with GSK-J4 (0 to 1 μ M) and venetoclax (0.1 to 10 μ M) for 72 hours, followed by cell viability measurements as above. Percent viability was constrained to a maximum of 100. Percent over the bliss score was calculated as previously described (58).

PUMA (*BBC3*) expression analysis from patient data

The Schramm data set (37) was downloaded from the R2: Genomic Analysis and Visualization Platform. Primary and relapse tumor expression data came from the GEO database (accession no. GSE65303).

Statistical analyses

Biological replicates were used unless explicitly stated otherwise. Statistical analyses were performed using the Mann-Whitney *U* test for Fig. 1A and figs. S1 and S2A. For fig. S12, gene overlap analysis (Fisher's exact test) was performed using the GeneOverlap package within R. The total number of genes possible within each analysis was set as the total genes reported in the RNA-seq described above: LAN5 (33,561), IMR5 (30,271), and SK-N-FI (32,760). GSEA was also performed using the GSEA software (<http://software.broadinstitute.org/gsea>) (59, 60). Briefly, the regularized log normalized counts from the RNA-seq were compared against the up- or down-regulated gene sets as described in the study of Duffy *et al.* (39). The Illumina_HumRef8_v2.chip was used as the reference annotation, and the maximum gene set size was set as 750. All other settings were left as default. All other statistical analyses were performed using Student's *t* test. All analyses were two-tailed. Results were considered significant when $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Neuroblastomas are sensitive to the H3K27 demethylase inhibitor GSK-J4.

(A) High-throughput drug screen of 773 solid tumor cell lines. Models are split into neuroblastomas (N; $n = 31$) and all other solid tumors (O; $n = 742$). The Mann-Whitney U test was used to assess statistical significance. LN, natural log; IC_{50} , median inhibitory concentration. (B) Neuroblastoma cell lines were treated with $1 \mu M$ GSK-J4 for 72 hours, and cell viability was determined by CellTiter-Glo. Cell lines with less than 50% viable cells (red dashed line) under these conditions are termed “sensitive” (blue line), and those with more than 50% viable cells are termed “resistant” (red line). *MYCN*-amplified (Amp) cell

lines are indicated with red bars. (C to F) Mice treated with GSK-J4 in CHLA20 (C) or IMR32 (D) xenograft models or FELIX (E) or COG-N-561 (F) PDX models. Black lines correspond to control mice, and red lines correspond to GSK-J4-treated mice. Data are shown as means $\pm$ SEM. Asterisks indicate a significant separation between control and GSK-J4 cohorts using Student's *t* test ($P < 0.05$). For the experiments in (B), $n = 4$. For (C), $n = 4$ for both cohorts. For (D), cohorts are control ($n = 5$) and GSK-J4 ($n = 4$). For (E), $n = 3$ for both cohorts. For (F), cohorts are control ($n = 7$) and GSK-J4 ($n = 6$).

Fig. 2. RNA-seq of GSK-J4-sensitive neuroblastoma cell lines demonstrates gene expression changes.

(A) Mean of normalized counts versus mean $\log_2$ ratio plots of differential gene expression analysis for IMR5, LAN5, SK-N-FI, and SK-N-BE(2) treated with GSK-J4 for 72 hours compared to untreated cells. Red dots indicate significantly altered genes [adjusted $P(P_{adj}) < 0.05$]. (B) $\log_2$ ratio for a panel of genes involved in neuroblastoma differentiation from the RNA-seq data in neuroblastoma cell lines treated with GSK-J4 for 72 hours. Asterisks indicate significance ($P_{adj} < 0.05$). (C) Western blot of IMR5 and KELLY neuroblastoma cell lines treated with GSK-J4 for 24, 48, or 72 hours. Blot was probed with the indicated antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. No Rx, no treatment. (D) $\log_2$ ratio for expression of apoptotic and ER stress-related genes from the RNA-seq data in neuroblastoma treated with GSK-J4 for 72 hours.

Asterisks indicate significance ($P_{\text{adj}} < 0.05$). Data are shown as the means $\pm$ SEM. (E) Western blot of IMR5 and KELLY lysates, probed for ER stress-related apoptosis proteins. Asterisks denote bands at about 23 kDa (top; PUMA- α) and 18 kDa (bottom; PUMA- β) (32). GAPDH was used as a loading control.

Fig. 3. GSK-J4 induces axonal growth and combines with RA to control the growth of GSK-J4-resistant neuroblastomas.

(A) Venn diagram showing the overlap of significant gene expression changes in sensitive neuroblastomas compared to ATRA-treated SH-SY5Y cells from the study of Duffy *et al.* (39). (B) Axonal length was measured in the images similar to those shown in fig. S13 using ImageJ and graphed for no treatment ($n = 215$), GSK-J4 ($n = 189$), and RA ($n = 177$). Statistical significance was determined by Student's *t* test. AU, arbitrary units. (C) CHLA90, SK-N-AS, and SK-N-BE(2) cell lines were treated with 1 μ M GSK-J4, 20 μ M RA, or a

combination of both drugs and stained with crystal violet 6 days later. **(D)** Cell viability assays for CHLA90, SK-N-AS, and SK-N-BE(2) cell lines treated with 0 or 1 μ M GSK-J4 and the indicated concentrations of RA for 6 days. **(E)** Western blot of CHLA90, SK-N-AS, and SK-N-BE(2) cell lines treated with 1 μ M GSK-J4, 20 μ M RA, or a combination of both drugs for 6 days. Blots were probed with the indicated antibodies. All bar graphs are shown as the means $\pm$ SEM. For the experiments in (D), $n = 4$. Asterisks denote bands at about 23 kDa (top) and 18 kDa (bottom), matching PUMA isoforms.

Fig. 4. GSK-J4 combines with venetoclax and induces tumor regression.

(A) Seventy-two-hour CellTiter-Glo assay of the indicated cell lines with GSK-J4 alone or combined with 1 μ M venetoclax. Data are graphed as the fraction of viable cells relative to no treatment. (B) Apoptosis assay in IMR5, LAN5, SMS-SAN, and KELLY cells treated with 1 μ M GSK-J4, 1 μ M venetoclax, or the combination for 48 hours. Bars plotted represent only apoptotic annexin V-positive cells. (C) Nu/Nu mice injected with LAN5 cells (left) were treated daily with GSK-J4 (100mg/kg), venetoclax (100mg/kg), or the combination of both drugs. Nonobese diabetic severe combined immunodeficient gamma

(NSG) mice injected with SK-N-DZ (center) or IMR5 (right) cells were treated with GSK-J4 (50mg/kg) for 4 days a week, venetoclax (100 mg/kg) for 5 days a week, or the combination of both drugs. **(D)** Tumors shown in (C) were followed until the end of the experiment or until the humane limit of tumor volume was reached. The percentage change from initial tumor size is plotted for individual tumors. Student's *t* test was used to determine significance of differences between groups within the waterfall cohorts. Data are presented as means $\pm$ SEM. For (A), $n = 4$. For (B), $n = 3$. For (C) and (D), LAN5 data are presented as control ($n = 4$), GSK-J4 ($n = 6$), venetoclax ($n = 5$), and combination ($n = 6$); SK-N-DZ data are presented as control ($n = 5$), GSK-J4 ($n = 5$), venetoclax ($n = 4$), and combination ($n = 5$). For IMR5, $n = 5$ for all cohorts.