EWS/ETS-Driven Ewing Sarcoma Requires BET Bromodomain Proteins


Abstract

The EWS/ETS fusion transcription factors drive Ewing sarcoma (EWS) by orchestrating an oncogenic transcription program. Therapeutic targeting of EWS/ETS has been unsuccessful; however, identifying mediators of the EWS/ETS function could offer new therapeutic options. Here, we describe the dependency of EWS/ETS-driven transcription upon chromatin reader BET bromodomain proteins and investigate the potential of BET inhibitors in treating EWS. EWS/FLI1 and EWS/ERG were found in a transcriptional complex with BRD4, and knockdown of BRD2/3/4 significantly impaired the oncogenic phenotype of EWS cells. RNA-seq analysis following BRD4 knockdown or inhibition with JQ1 revealed an attenuated EWS/ETS transcriptional signature. In contrast to previous reports, JQ1 reduced proliferation and induced apoptosis through MYC-independent mechanisms without affecting EWS/ETS protein levels; this was confirmed by depleting BET proteins using PROTAC-BET degrader (BETd). Polycomb repressive complex 2 (PRC2)-associated factor PHF19 was downregulated by JQ1/BETd or BRD4 knockdown in multiple EWS lines. EWS/FLI1 bound a distal regulatory element of PHF19, and EWS/FLI1 knockdown resulted in downregulation of PHF19 expression. Deletion of PHF19 via CRISPR-Cas9 resulted in a decreased tumorigenic phenotype, a transcriptional signature that overlapped with JQ1 treatment, and increased sensitivity to JQ1. PHF19 expression was also associated with worse prognosis in patients with EWS. In vivo, JQ1 demonstrated antitumor efficacy in multiple mouse xenograft models of EWS. Together these results indicate that EWS/ETS requires BET epigenetic reader proteins for its transcriptional program and can be mitigated by BET inhibitors. This study provides a clear rationale for the clinical utility of BET inhibitors in treating EWS.

Significance: These findings reveal the dependency of EWS/ETS transcription factors on BET epigenetic reader proteins and demonstrate the potential of BET inhibitors for the treatment of EWS.

Introduction

Ewing sarcoma (EWS) represents the second-most common primary bone malignancy in children and young adults after osteosarcoma with an annual incidence of 2.9 cases per million population (1). Although, the 5-year survival rate for primary EWS has improved following the introduction of systemic chemotherapy in the adjuvant and neoadjuvant setting, several clinical studies indicate a plateau phase for conventional therapies (2). Further, the survival rate for patients with high-risk recurrent disease (<2 years) is extremely poor with <10% survival at 5 years, therefore, novel therapies are urgently needed to improve outcomes. EWS is driven by characteristic chromosomal translocations that generate fusions between the EWS breakpoint region 1 (EWSR1) gene with members of the ETS family of transcription factors, most commonly to FLI1 (3) and with less frequency to ERG (4). Unlike adult cancers, the EWS/ETS (EWS/FLI1 and EWS/ERG) oncogenic fusion proteins are often the only genetic alteration in these pediatric tumors functioning as an aberrant transcription factor (5, 6). Importantly, EWS tumors are dependent on these fusion products and require their persistent expression/function to maintain a transformed phenotype (7). Similar to other transcription factors, direct pharmacological targeting of the EWS/ETS fusion proteins remains a formidable challenge, and proteins in this class are often considered undruggable due to a lack of small molecule recognizable pockets (8). Thus, identification and targeting of functionally relevant EWS/ETS-associated factors should be considered to mitigate its oncogenic function.

ETS-family gene fusions are also prominent drivers in prostate cancer (9), where our group recently identified BET bromodomain inhibition as a promising new therapeutic strategy (10). The bromodomain and extraterminal (BET) family of proteins includes ubiquitously-expressed BRD2, BRD3, and BRD4—and
BRD4, which is specific to the testis. Each protein contains two tandem bromodomain motifs (BD1 and BD2)—deep hydrophobic pockets that have been described chiefly as chromatin readers that bind to acetylated lysine on histones, recruit chromatin modifying enzymes, and function as transcriptional coactivators (11). Of the BET family proteins, BRD4 is well studied and known to recruit mediator complex and positive transcription elongation factor b (P-TEFb) thereby helping in the release of promoter proximal pausing of RNA polymerase II (12, 13). BRD4 also binds to acetylated non-histone proteins, such as acetylated transcription factors including FLI1 and ERG, and mediates their transcriptional activity (14, 15). Potent and selective small-molecule inhibitors that reversibly bind the BD1 and BD2 domains of BET proteins and prevent their interaction with acetylated histones and transcription factors have been developed and are being evaluated in the clinic for treatment of a variety of cancers, including NUT-midline carcinoma, acute leukemia, multiple myeloma and castration-resistant prostate cancer (16–18).

Here we investigated the dependency of EWS/ETS-driven transcription on BET bromodomain proteins in EWS. Using multiple EWS cell lines, we found BET proteins including BRD4, are essential for their proliferation and invasion. Through gel exclusion chromatography and pulldown assays, EWS/FLI1 and EWS/ERG protein were found to be in a transcriptional complex containing BRD4, and knockdown or pharmacologic inhibition of BRD4 resulted in downregulation of EWS/ETS target genes. Further, we discovered PHF19—a PRC2-associated protein as a novel and essential EWS/ETS transcriptional target that requires BRD4 function. Moreover, in EWS mouse xenograft models, treatment with BET inhibitor JQ1 showed antitumor activity that was associated with PHF19 downregulation. These findings illustrate an important functional role for BET proteins in EWS/ETS-mediated oncogenic transcription program and demonstrate the therapeutic potential of BET bromodomain inhibitors in treating EWS.

Materials and Methods
Cell culture and compounds
RD-ES (obtained from CLS), CADO-ES1 (obtained from DSMZ), and SK-N-MC cell lines (obtained from ATCC) maintained in RPMI1640 media (Life Technologies) supplemented with 10% FBS. CHLA-10 cells (obtained from Children’s Oncology Group) were maintained in DMEM (Life Tech) supplemented with 10% FBS. Cell lines were STR pro
tested negative for mycoplasma by MycoAlert Kit (Lonza). All cells included in this study were tested and certified free of mycoplasma by MycoAlert Kit (Lonza). All cells were grown at 37°C in a 5% CO2 incubator. JQ1 was acquired from Cayman Chemical for in vitro experiments and from Dieckmann (HK) Chemical Ind. for in vivo experiments. PRTOAC BET degrader (BETd) compound ZBC260 was a gift from Dr. Shaoeng Wang from University of Michigan.

Lenti virus preparation
siRNAs against BRD2/3/4, FLI1, and ERG (Penn Core Facilities) were packaged using second-generation lentiviral packaging systems. Briefly, 1 x 10^6 HEK-293T cells were seeded in 10 cm plates. siRNA plasmids (4 µg) were cotransfected with pMD2.G (VSVG; 1 µg) and pSPAX2 (GAG-POL; 3 µg) using 25 µL Lipofectamine 2000 (Life Tech). Media was collected after 48 hours of transfection, centrifuged, and clarified using 0.45 µm filter before infection.

RNA extraction and quantitative RT-PCR
Total RNA was isolated from cells using the miRNeasy Kit (QIAGEN) and cDNA was synthesized from 1,000 ng total RNA using SuperScript IV (Life Tech). qPCR was performed using SYBR Green PCR Master Mix (Life Tech) on QuantStudio3 (Applied Biosystems). Relative expression was calculated using ΔΔCT values after being normalized against GAPDH expression. All primers were designed using primer 3 (http://frodo.wi.mit.edu/primer3/) and synthesized by Integrated DNA Technologies. The primer sequences used is provided in the Supplementary Table S1.

siRNA knockdown
For siRNA mediated PHF19 knockdown experiments, cells were seeded in 10 cm plates and transfected with 50 nmol/L ON-TARGETPlus SMARTpool siRNA nontargeting control or siRNA pools targeting PHF19 (Dharmacon) using Oligofectamine (Invitrogen 12252011). Two successive transfections were carried out at an interval of 24 hours. Cells were harvested 48 hours after first transfection for Western blot analysis, qRT-PCR validation and cell proliferation assay.

Cell viability assay
Cells were seeded onto a 96-well cell culture plate at a density of 2,000 cells/well in 100 µL of normal cell culture medium. One hundred microliters of serially diluted compound was added to each well 12 hours later. After 96 hours of exposure to the compound, cell viability was assessed using the CellTiter-GLO assay (Promega). IC50 values were calculated using GraphPad Prism software.

For knockdown studies, cells were infected with respective lentiviruses and selected for 3 days. After antibiotic selection, cells were seeded in 24-well plates at a density of 10,000 cells/well and counted on day 2, 4, and 6 post seeding. Growth curves were plotted as percent growth compared with cells at Day 0. For the long-term growth, assay cells were seeded in six-well plates at 5,000 cells/well and grown in media containing desired concentrations of drugs. Media was changed every 4 days and after 12 days cells were fixed with 10% methanol and stained with 0.1% crystal violet. Absorbance was taken at 590 nm on BioTek Synergy Multimode reader.

Antibodies and immunoblot analysis
For immunoblot analysis, cells were lysed in RIPA buffer (Boston BioProducts) supplemented with Halt protease and phosphatase inhibitor and Pierce Protease Inhibitor (LifeTech). Quantified protein lysates (Life Tech; 23250) were boiled in Laemmli buffer (BioRad) and 10 to 20 µg of protein was resolved using SDS-PAGE and transferred to PVDF membrane followed by blocking for 1 hour with blocking buffer [Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk], and incubated overnight at 4°C in primary antibody. Blots were washed with TBS-T and incubated with HRP-conjugated secondary antibody for 1 hour at room temperature. Blots were washed again with TBS-T and visualized after incubation with Supersignal West Femto (Life Tech). Primary antibodies included: FLI1 (Abcam; ab15289), BRD4 (Bethyl Laboratories A301-985A50), BRD3 (Santa Cruz; sc-81202), BRD2 (Cell Signaling Technology; 5848), ERG (Abcam; ab92513), MHC (Abcam; ab32072), cPARP (Cell Signaling Technology; 9541), PHF19 (Cell Signaling Technology; 77271), and GAPDH-HRP (Cell Signaling Technology; 3683).
Gel-filtration chromatography

Nuclear extracts were obtained using the NE-PER Nuclear Extraction Kit (Thermo Scientific), and dialyzed against FPLC buffer [20 mmol/L Tris-HCl, 0.2 mmol/L EDTA, 5 mmol/L MgCl2, 0.1 M KCl, 10% (v/v) glycerol, 0.5 mmol/L DTT, 1 mmol/L benzamidine, 0.2 m MPMSF, pH 7.9]. 2.5 mg of nuclear protein was concentrated to 500 μL using a Microcon centrifugal filter (Millipore) and then applied to a Superose 6 size-exclusion column (10/300 GL GE Healthcare) precalibrated using the Gel Filtration HMW Calibration Kit (GE Healthcare). Five hundred microliters of eluate was collected for each fraction at a flow rate of 0.5 mL/min, and eluted fractions were subjected to SDS-PAGE and immunoblotting.

Immunoprecipitation

Nuclear pellet was lysed in IP buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Triton-X 100, Protease Inhibitor) by sonication. Nuclear lysates (0.5–1.0 mg) were preclenched with protein G Dynabeads (Life Technologies) for 1 hour on a rotator at 4°C. Five micrograms of antibody was added to the preclenched lysates and incubated on a rotator at 4°C overnight prior to the addition of protein G Dynabeads for 1 hour. Beads were washed thrice in IP buffer and resuspended in 40 μL of 2× loading buffer and boiled at 90°C for 10 minutes for separation of the protein and beads. Samples were then analyzed by immunoblotting as described above.

Cellular thermal shift assay

Cells were seeded in 10-cm cell culture plates at a density of 7 × 10⁶ cells. Cells were exposed to DMSO or IQ1 for 3 hours. The cells were washed once with PBS to remove excess compound and trypsinized. Cells were resuspended in PBS containing protease and phosphatase inhibitors (Thermo Fisher) and divided in 50 μL aliquots. The aliquots were heated at different temperatures for 3 minutes (Veriti thermal cycler; Applied Biosystems), followed by cooling for 3 minutes at room temperature. Cells were lysed using two cycles of freeze-thawing in liquid nitrogen. Lysates were centrifuged at 20,000 × g for 20 minutes at 4°C to separate soluble fractions from precipitates. The supernatants were transferred to new microtubes and analyzed by SDS-PAGE followed by immunoblot analysis.

Chromatin immunoprecipitation

Posttreatment with the compound, cells were trypsinized, washed, and cross-linked with 1% formaldehyde solution in PBS for 10 minutes. Freshly prepared 0.125 mol/L glycine was added followed by gently shaking the cells at room temperature to stop the cross-linking process. chromatin immunoprecipitation (ChIP) was performed using Diagenode Kit following manufacturer’s instructions. Briefly, cells were collected by centrifugation and resuspended in ChIP buffer (100 mmol/L NaCl; 50 mmol/L Tris-HCL, pH 8.1; 5 mmol/L EDTA, pH 8.0; 0.5% SDS; 5% triton X-100, 1× protease inhibitor). Cells were then subjected to sonication in a Bioruptor pico machine on 30 seconds on/off cycle for 5 minutes. Sonication efficiency was confirmed by observing 200bp DNA fragments. Chromatin equivalent to 10 × 10⁶ cells were used for ChIP using various antibodies (anti-Fli1 Abcam, Catalog No. ab152289; anti-BRD4 Bethyl Laboratories, Catalog No. A301-985AS0; IgG Diagenode, Catalog No. C01010061). ChIP DNA was isolated (IPure Kit, Diagenode) from samples by incubation with the antibody at 4°C overnight followed by washing and reversal of cross-linking. The eluted DNA was used for SYBRgreen qPCR. The primer sequences used for ChIP-qPCR are provided in Supplementary Table S1.

Matrigel invasion assays

After 3 days of infection with respective shRNA containing lentiviruses, cells were selected for another 3 days with puromycin. After selection and expansion, cells were trypsinized, and 100,000 cells were resuspended in serum-free medium and added into the invasion chamber (BD Biosciences). The bottom of the chamber was filled with media containing 10% serum. Cells that had degraded the matrix and migrated through the porous membrane (8 μm pore size) to the other end after a period of 48 hours were fixed and stained with crystal violet (0.5%), and images were captured using phase contrast microscopy.

Chicken chorioallantoic membrane assay

Fertilized chicken eggs were incubated in a rotary humidified incubator at 38°C for 10 days. A small hole was drilled through the eggshell into the air sac and another hole was drilled near the allantoic vein that penetrates the eggshell, keeping the chick chorioallantoic membrane (CAM) intact. The CAM was dropped by applying mild vacuum to the hole over the air sac. Subsequently, a cutoff wheel (Dremel) was used to cut a 1 cm² window encompassing the second hole near the allantoic vein to expose the underlying CAM. Cells were prepared for implantation by trypsinizing and resuspending in media (without FBS) at the density of 1 × 10⁶ cells/50 μL. The CAM was gently abraded with a sterile cotton swab to provide access to the mesenchyme and 50 μL cell suspension was implanted on top of it. The windows were sealed and the eggs were returned to a stationary incubator. The eggs were incubated for seven more days, after which the extra-embryonic tumor were isolated and weighed to determine the difference in tumor growth between control and test group. For metastatic assay, embryonic liver was harvested analyzed for the presence of tumor cells by isolating genomic DNA by Puregene DNA purification system (Qiagen) and quantitative Human Alu PCR.

RNA-seq library preparation for next-generation sequencing

Total RNA was isolated using miRNeasy Kit (QIAGEN) and the quality of the RNA was analyzed on Bio-analyzer (Agilent). After confirming that all of the RNA samples have RNA integrity number greater than 8, RNA-seq libraries were constructed using the TruSeq sample Prep Kit V2 (Illumina) according to the manufacturer’s instructions. Briefly, 2 μg of purified RNA was poly-A selected and fragmented with fragmentation enzyme. After first- and second-strand synthesis from a template of poly-A selected/fragmented RNA, other procedures from end-repair to PCR amplification were done according to library construction steps. Libraries were purified and validated for appropriate size (~300 bp) on a 2100 Bioanalyzer High Sensitivity DNA chip (Agilent Technologies, Inc.). The DNA library was quantified using Qubit and normalized to 4 nmol/L concentration before pooling. Libraries were pooled in an equimolar fashion and diluted to 1.8 pmol/L concentration. Library pools were clustered and sequenced on Nextseq500 platform with paired-end reads of 151 or 75 bases, according to the manufacturer’s recommended protocol (Illumina Inc.). Raw reads passing the Illumina RTA quality filter were preprocessed using FASTQC for sequencing base quality control.
Mapping and expression quantification

The raw fastq data were adaptor-trimmed and mapped to hg19 human reference genome using the STAR Alignment Tool within the Illumina BaseSpace app suite (www.basespace.illumina.com) to generate the BAM files. Cufflinks Assembly & DE (BaseSpace Workflow) with default setting was used to generate expression matrix.

Differential expression and GSEA gene enrichment analysis

Pairwise comparisons between treatment groups (shBRD4, shFLI1, JQ1 treatment) and control group (DMSO treatment) were performed to detect differentially expressed (DE) genes using the Bioconductor package DESeq2 (version 1.10.1; ref. 19). DE genes were selected with a threshold of 10−2 on adjusted P-value. Heatmap of DE genes was generated using the R package pheatmap (version 1.0.8).

GSEA (version v2.2.3) was used to perform enrichment analysis (20). GSEA Preranked function was used to perform enrichment analysis for RNA-seq data, following the official user guide of GSEA. Ranked list is constructed with the log2-fold change values from DE analysis. GeneSet/signature is constructed with the name of selected gene (e.g., common downregulated genes between shBRD4 and shFLI1).

Deletion of PHF19 using CRISPR-Cas9 system

CRISPR-Cas9–mediated genome editing was used to delete PHF19 in SK-N-MC cells. The genomic region in the human PHF19 exon 10 was targeted using gRNA (CACCGTGTACGTC-CAAACTGCGGA) designed using CRISPR Design Tool (http://tools.genome-engineering.org). The gRNA was ligated into the one vector lentCRISPRv2 plasmid system (Addgene #52961) as previously described (21). The resulting plasmid was sequence verified before packaging into pMD2.G (VSVg) along with pSPAX2 (GAG-Pol) into a lentiviral system. Cells were infected with the guide RNA containing lentivirus along with a control virus (without guide RNA) for 48 hours and selected using Puromycin for 4 to 7 days. PHF19 deletion was verified using immunoblot analysis.

Tumor xenograft studies

All procedures involving mice were approved by the University Committee on Use and Care of Animals (UCLCA) at the University of Michigan and conform to their relevant regulatory standards. Four- to six-week-old male CB17 SCID mice were anesthetized using 2% Isofluorane (inhalation) and 1 to 2 × 106 EWS cells (SK-N-MC, CHLA-10, or CADO-ES1) suspended in 100 μL of PBS with 50% Matrigel (BD Biosciences) were implanted subcutaneously into the dorsal flank on both sides of the mice. Once the tumors reached an average size of 80 to 100 mm3 the animals were randomized and treated once per day with IQ1 (50 mg/kg) or vehicle (10% hydroxypropyl-β-cyclodextrin) via intraperitoneal injection 5 days per week. Growth in tumor volume was recorded using digital calipers in 100 mm3 the animals were randomized and treated once per day with IQ1 (50 mg/kg) or vehicle (10% hydroxypropyl-β-cyclodextrin) via intraperitoneal injection 5 days per week. Growth in tumor volume was recorded using digital calipers in

Physical association of BRD4 with other transcription factors

BRD4 exists in a large protein complex along with EWS-FLI1/ERG, RNA Pol II, and mediator complex 1

Physical association of BRD4 with other transcription factors has been described in other cancers (18, 19). As BRD4 knockdown displayed a phenotype similar to EWS/FLI1 or EWS/ERG knockdown, we hypothesized that EWS/ETS fusions might exist in a transcriptional complex consisting of BRD4, playing a critical role in regulating genes essential for EWS growth and survival. To test this, we performed gel filtration chromatography using nuclear lysates from CHLA-10 and CADO-ES1 cell lines. A majority of EWS/FLI1 and EWS/ERG existed in complexes devoid of BRD4 supporting the previous observations of self-aggregation of these fusion proteins due to intrinsically disordered low-complexity prion-like EWS domains (23, 24). However, a fraction of EWS/FLI1 and EWS/ERG fusion proteins and BRD4 eluted together in a
Knockdown of BET proteins or EWS/FLI1/ERG fusion inhibits EWS cell proliferation, invasion, and tumor growth. 

**A.** Heat map showing RT-qPCR validation of BRD2, BRD3, BRD4, FLI1, and ERG mRNA expression in the indicated EWS cells stably expressing NT-shRNA or shBRD2/shBRD3/shBRD4/shFLI1/shERG. Target gene expression was normalized using GAPDH with values in NT-shRNA control set to 1.

**B.** Representative immunoblot showing the knockdown of the indicated target protein in EWS cells as in A. GAPDH was used as a loading control. sh-targets: BRD2, BRD3, BRD4, FLI1, or ERG.

**C.** Control or knockdown cells were seeded at a density of 10,000 cells/well in a 24-well plate and counted after 2, 4, and 6 days. Cell growth was plotted as percentage of growth compared with Day 0. Data show mean ± SEM with *P < 0.05.*

**D.** Colony formation assays. Indicated cells were seeded at a density of 5,000/well in a six-well plate and grown for 12 to 14 days, followed by staining (top) and quantification (bottom; mean ± SEM, n = 3). *P < 0.05.*

**E.** Matrigel invasion assay with the indicated cells. Forty-eight hours postincubation, invaded cells were fixed in methanol and stained with 0.5% crystal violet. Percentage cell invasion is shown with mean ± SEM; n = 3. *P < 0.05.*

**F.** BRD4 or EWS/FLI1 knockdown reduces tumor growth and attenuates distant metastasis in a chicken CAM model. Control, BRD4, or EWS/FLI1 knockdown CHLA-10 cells were inoculated atop CAM of 10-day-old chick embryos. Tumors growing on top of the CAM and fetal liver were extracted after 7 days. Tumor weights were measured (left). Genomic DNA from lungs was extracted, and metastasized cells were detected by human Alu-qPCR. Graphs plotted as percentage metastasized cells with mean ± SEM (right). Statistical significance was calculated by two-tailed Student t test.
high-molecular weight complex (Fig. 2A and B). In addition, we found evidence for the presence of RNA polymerase II and the mediator complex (Med1)—known members of the BRD4 transcriptional coregulatory network, in the same complex (25, 26). To further confirm the endogenous association between EWS/ETS and BRD4, we immunoprecipitated ERG in EWS/ERG positive CADO-ES1 nuclear extracts and probed for BRD4. Immunoblot with BRD4 antibody confirmed the endogenous interaction with EWS/ERG (Fig. 2C). PARP and DNA-PK that is known to interact with EWS/ERG were used as positive control (27). Reciprocal immunoprecipitation experiments using antibody specific to BRD4 confirmed these interactions (Fig. 2D). However, treatment with the BET inhibitor JQ1, that binds the acetyl-binding pockets of BRD4 (Supplementary Fig. S1A), did not disrupt the interaction between EWS/ERG and BRD4 (Fig. 2E), indicating an indirect interaction between these two proteins in EWS cells. A physical interaction between ERG and BRD4 has been reported in AML and prostate cancer cells, which was shown to be dependent on the N-terminal (K96/99) acetylation of ERG (14, 15). However, the ERG portion of EWS/ERG fusion lacks K96/99, which might explain the inability of the BET inhibitor to disrupt the ERG/BRD4 interaction. Consistent with this, treatment with an HDAC inhibitor did not result in an increased EWS/ERG pulldown by BRD4 further implicating an acetylation-independent indirect interaction (Supplementary Fig. S1B). In addition, endogenous EWS/FLI1 and BRD4 interaction were observed in EWS/FLI1 positive
CHLA-10 cells (Supplementary Fig. S1C). Collectively, these data demonstrate that EWS/ETS associates indirectly with BRD4 in a complex that includes Med1/RNAPoII in an acetylation independent manner in EWS cells.

Genetic or pharmacologic targeting of BRD4 inhibits EWS/ETS transcriptional activity

Our data support a model in which BRD4 is involved in EWS/ETS-driven transcriptional programs. To directly test this, we conducted RNA-seq experiments after EWS/FLI1 and BRD4 knockdown or JQ1 treatment of CHLA-10 cells (Supplementary Fig. S2A). As expected, knockdown of EWS/FLI1 had profound effects on transcription, with 3,900 genes significantly altered, of which 1,057 genes overlapped with genes modulated upon BRD4 knockdown (Fig. 3A). Importantly, integrating the EWS/FLI1 ChIP-seq data (21) showed greater than 50% (573/1,057) of these overlapping genes associated with EWS/FLI1 peaks (P < 0.0001) in their regulatory regions. Interestingly, JQ1 treatment led to transcriptional changes in genes that showed significant overlap with genes associated with EWS/FLI1 and BRD4 knockdown, including known EWS/FLI1 targets such as NGFR, FOXM1, STEAP1, and SYT4 (Fig. 3B and C). Subsequent gene ontology analysis of the downregulated genes revealed enrichment of processes such as RNA splicing, cell cycle, and DNA replication, all of which are known to be associated with EWS/ETS function (Fig. 3D). Next, RNA-seq analysis of RD-ES, SK-N-MC, and CADO-ES1 cells treated with JQ1 also showed overlapping expression profiles of genes that included EWS/ETS targets (Fig. 3E). Interestingly, gene set enrichment analysis (GSEA) with a EWS/ETS targets (Fig. 3F). Although MYC has been shown to be a major downstream target of BET inhibitor in multiple cancers including EWS (28, 29), GSEA analysis did not reveal modulation of any MYC-associated concepts in all four JQ1-treated cells and this was associated with lack of MYC downregulation (Fig. 3G). Interestingly, JQ1-treated cells showed slightly increased MYC expression compared with vehicle-treated controls. Moreover, knockdown of EWS/FLI1 or BRD2/3/4 had no effect on MYC transcript levels in all three tested cells (Fig. 3G–3I; Supplementary Fig. S2B). In contrast to previous reports, these observations rules out MYC as a direct transcriptional target of EWS/ETS or BET inhibitors. A commonly altered gene was PHF19-PHD finger protein 19, a polycomb-like protein that forms a subcomplex with the polycomb repressive complex 2 (PRC2) core complex. PHF19 was one of the top downregulated transcripts in all treatment conditions, suggesting it may be a novel EWS/ETS target that requires BET proteins for its transcription (Fig. 3H; Supplementary Fig. S2C). These results imply that knockdown of BRD4 or its inhibition by JQ1 selectively targets a subset of EWS/ETS-dependent transcripts.

BET inhibitor downregulates PHF19 and reduces proliferation of EWS cells

Recent reports of BET inhibitor efficiency in cancer cells including EWS led us to examine the effect of JQ1 on a panel of EWS cell lines. We treated CHLA-10, CADO-ES1, SK-N-MC, and RD-ES cells with JQ1 and found them to be sensitive with IC50 values ranging from 250 to 500 nmol/L (Fig. 4A). Similar results were obtained in a long-term cell viability assay where cell lines displayed a concentration-dependent decrease in colony formation in response to JQ1 treatment (Fig. 4B). Interestingly, unlike previous reports (28, 29), treatment with JQ1 had no effect on the expression of EWS/FLI1 mRNA or protein in CHLA-10, SK-N-MC, and RD-ES cells or EWS/ERG in CADO-ES1 cells (Fig. 4C; Supplementary Fig. S2D) but apoptosis was apparent as increased level of cleaved-PARP was observed in all four cell lines. Furthermore, no change in MYC protein levels was found in accordance with our transcript data, suggesting no correlation between MYC and sensitivity to BET inhibitors in the EWS cells. Consistent with our transcript data, PHF19 protein levels were decreased in JQ1-treated cells compared with vehicle-treated controls. Importantly, treatment with a pan-BETi compound- ZBC260, led to a dose-dependent decrease in the PHF19 levels without affecting EWS/FLI1 or MYC (Fig. 4D), further validating our observation that BET inhibition does not affect EWS/ETS fusion or MYC levels, whereas PHF19 expression seems to be dependent on BET proteins in EWS cells.

PHF19 is a direct EWS/ETS target and is required for EWS tumorigenesis

PHF19, also known as PCL3 is a substoichiometric component of PRC2 (30). PHF19 is involved in the recruitment of the PRC2 complex to gene bodies to silence its expression through histone H3 lysine 27 trimethylation (H3K27me3; ref. 31). As our data strongly indicate a role for EWS/ETS and BRD4 in regulating the expression of PHF19, we investigated whether it is a direct transcriptional target of EWS/ETS fusion. Analysis of publicly available EWS/FLI1 ChIP-seq data (GSE61953; ref. 21) from A675 and SK-N-MC cells showed strong binding of EWS/FLI1 to the downstream regulatory region approximately 20kb from the PHF19 gene (Fig. 5A). Further, RNA-seq from the same data set showed reduced expression of PHF19 upon EWS/FLI1 knockdown, supporting the notion that PHF19 is a direct transcriptional target of the fusion protein (Fig. 5B). Interestingly, the immediate neighboring genes located on 5' and 3' ends of PHF19 such as PSMD5, FBXW2, MEG9, and TRAF1 did not show change in the expression upon EWS/FLI1 knockdown, BRD4 knockdown, or JQ1 treatment, further implicating PHF19 as the sole transcriptional target of EWS/FLI1 in that locus (Supplementary Fig. S3A, S3B, and S3C). To validate this further, we examined PHF19 expression levels after knockdown of EWS/FLI1 or EWS/ERG and found significant downregulation in all the EWS cell lines (Fig. 5C and D). As expected, knockdown of BRD4 led to the loss of PHF19 expression, indicating the dependency of EWS/ETS protein on BRD4 to regulate PHF19 gene expression. Furthermore, treatment with JQ1 led to significant reduction in the recruitment of BRD4 to the PHF19 regulatory region, whereas EWS/FLI1 binding was unchanged compared with vehicle control (Supplementary Fig. S4A), suggesting that fusion protein binding to its target region on the chromatin is independent of BRD4. Next, to investigate the role of PHF19 in EWS cells, we performed CRISPR-Cas9–mediated knockout (ko) of PHF19 gene and validated its loss of expression by Western blotting (Fig. 5E). Deletion of PHF19 led to a significant reduction in proliferation, colony forming ability, and invasive potential of SK-N-MC cells (Fig. 5F–H). We then performed RNA-seq analysis in SK-N-MC cells with PHF19 ko or JQ1 treatment (500 nmol/L for 48 hours) and found differential expression of 861 transcripts in PHF19 ko, of which 47% (n = 401) overlapped with transcripts affected by
Figure 3.
Genetic or pharmacologic targeting of BRD4 disrupts EWS/ETS transcriptional activity. A, Venn diagram showing significantly altered genes (>two-fold; \( P < 0.001 \)) between BRD4 or EWS/FLI1 knockdown (shFLI1) in CHLA-10 cells. The 573/1057 overlapping genes are bound by EWS/FLI1 (GSE61953). The \( \chi^2 \) determined \( P \) value is indicated. B, Venn diagram, as in A, including genes altered by JQ1 treatment. C, Heatmap displaying common up- and downregulated genes between BRD4 knockdown, EWS/FLI1 knockdown, and JQ1 treatment in CHLA-10 cells. D, DAVID Gene Ontology analysis of common genes downregulated by BRD4 or FLI1 knockdown or JQ1 treatment in CHLA-10 cells. E, Heatmap showing commonly altered genes in control versus JQ1-treated EWS cells. Log2-fold change compared with control is plotted. F, GSEA plot with shBRD4/shFLI1 common downregulated gene signature in JQ1-treated CHLA-10, RD-ES, SK-N-MC, and CADO-ES1 cells. G, MYC is not a transcriptional target of EWS/FLI1 or BRD4. RNA-seq FPKM values of MYC in the indicated cells. Mean ± SE from two independent RNA-seq is plotted. H, RNA-seq FPKM values as in G for the PHF19 transcript.
Figure 4.
BET bromodomain inhibitor downregulates PHF19 and reduces proliferation of EWS cells independent of MYC. A, Cell viability curves for CHLA-10, CADO-ESI, SK-N-MC, and RD-ES cells on treatment with JQ1. Bottom, table showing IC50 values for each cell line along with the EWS/ETS fusion status. B, Colony formation assay; cells were cultured in the presence or absence of JQ1 as indicated for 12 days, followed by staining. Representative images from multiple experiments are shown. C, Immunoblot analysis for EWS/FLI1, EWS/ERG, MYC, PHF19, cleaved-PARP (cl.PARP), and GAPDH in CHLA-10, CADO-ESI, SK-N-MC, and RD-ES cell lines treated with DMSO, 0.5 or 2.5 μmol/L JQ1 for 48 hours. D, BET protein depletion results in reduced PHF19 levels but does not affect EWS/FLI1 or MYC protein levels. Immunoblot for the indicated target protein with total lysates from SK-N-MC cells treated with DMSO or PROTAC BETd compound ZBC260 at increasing concentration for 24 hours.
PHF19 is a direct EWS/ETS target and is required for EWS cell proliferation and invasion. 

A, Genome browser tracks views showing enrichment of H3K27ac and EWS/FLI1 at PHF19 loci in SK-N-M and A675 cells. The black line below the enrichment peaks is the region used to amplify for ChIP-qPCR, with BRD4 and FLI1 antibody validation shown in Supplementary Fig. S4A. The ChIP-seq data were downloaded from NCBI GEO with accession number GSE619534. 

B and C, RNA-seq and qRT-PCR showing downregulation of PHF19 transcript upon EWS/FLI1, EWS/ERG, or BRD4 knockdown in EWS cells.

D, Immunoblot for PHF19 in EWS/FLI1, EWS/ERG, or BRD4 knockdown cells.

E, CRISPR knockout of PHF19 leads to reduced proliferation, invasion, and increased sensitivity to JQ1 in SK-N-Mc cells.

F, Immunoblot for indicated targets in control gRNA or PHF19 gRNA CRISPR-Cas9 lentivirus–infected cells.

G, Control or PHF19 knockout cells were seeded at a density of 10,000/well in a 24-well plate and counted after 2, 4, and 6 days. Cell growth was plotted as percentage of growth compared with Day 0.

H, Matrigel-invasion assay with control and PHF19 knockout cells. Top, invaded cells were fixed with methanol and stained with 0.5% crystal violet. Bottom, percentage cell invasion is shown with mean ± SEM from n = 3.

I, Venn diagram showing significantly altered genes between JQ1 or PHF19 knockout in SK-N-Mc cells. Heatmap displaying common up- and downregulated genes.

J, GSEA plot with PHF19 knockout signature in JQ1-treated CHLA-10, RD-ES, SK-N-Mc, and CADO-ES1 cells.
BET bromodomain inhibition blocks EWS growth in vivo. **A** and **B**, SK-N-MC, CHLA-10, and CADO-ES1 cells were implanted subcutaneously in mice and grown until tumors reached a size of approximately 80 to 100 mm³. Xenografted mice were randomized (n = 8–12 mice/arm) and then received vehicle or JQ1 (50 mg/kg) as indicated 5 days/week. Mean tumor volume ± SEM is shown. *P* values for the final tumor volumes were determined by two-way ANOVA comparing the treatment to the vehicle group using GraphPad Prism. **B**, Kaplan–Meier plots are shown for progression-free survival (progression defined by tumor volume tripling); *P* values were determined by the log-rank test using GraphPad Prism. **C**, qRT-PCR for PHF19 and MYC in the SK-N-MC tumor xenografts from control and JQ1-treated mice. *N* = 5 tumors from each group. **D**, Immunoblot analysis of PHF19 and MYC in SK-N-MC xenograft tumors as in **C**. GAPDH was used as loading control. **E**, Quantification of PHF19 and MYC protein levels from **D**. *P*-values determined by Student *t* test. **F**, Schematic illustrating the dependency of EWS/ETS on BRD4. Inhibition or knockdown of BRD4 disrupts the transcription complex consisting of EWS/ETS, Med1, and RNA PolII, thereby impairing the ability of EWS/ETS fusion proteins to alter gene expression and cell death.

**Figure 6.**

1- Downregulation of EWS/ETS target gene expression e.g., PHF19, FOXM1, NKX2-2, STEAP1 etc.  
2- Reduced proliferation and death
Identify PHF19 as an Essential EWS/ETS Target

JQ1 (Fig. 5I). Interestingly, GSEA using PHF19 ko signature (downregulated genes) revealed negative enrichment upon JQ1 treatment in all four cell lines (Fig. 5I). These data suggest that the PHF19 modulation of the regulation of a significant subset of genes including FOXM1 that gets downstream regulated by JQ1. Interestingly, FOXM1 is overexpressed in EWS and identified as an indirect target of EWS/ETS (32). These observations strongly indicate the role of PHF19 in partially mediating the JQ1 phenotype. To further support this, we performed transient knockdown of PHF19 and tested whether it synergizes with BET inhibition. Interestingly, PHF19 knockdown, which resulted in decreased proliferation of SK-N-MC cells, was further affected by JQ1 treatment compared with nontarget control (Supplementary Fig. S4B and S4C). In addition, the PHF19 knockout cells displayed increased sensitivity to JQ1, suggesting a synthetic lethal relationship between these two epigenetic regulators (Supplementary Fig. S4D). To further validate the potential prognostic significance of PHF19, we explored SurvExpress—a publicly available platform of gene expression and associated survival data (33). The prognostic value of high PHF19 mRNA expression was significantly correlated with overall survival rates in 88 patients with EWS (Supplementary Fig. S3). Furthermore, the PHF19 levels in the high-risk cohort were significantly upregulated compared to the low-risk cohort. These results indicate that PHF19 is a bona fide EWS/ETS target gene requiring BRD4 for its expression, and plays a significant role in the EWS/ETS-mediated pathogenesis of EWS.

BET bromodomain inhibition blocks EWS growth in vivo

Collectively, our data indicate BRD4 function is critical for the EWS/ETS transcriptional program in EWS. We treated xenograft models of SK-N-MC, CHLA-10, and CADO-ES1 daily with the BET inhibitor JQ1 and examined tumor growth. Our studies show that daily treatment with JQ1 alone was sufficient to significantly attenuate tumor growth as compared to vehicle control (Fig. 6A; Supplementary Fig. S6A) with no toxicity to mice (Supplementary Fig. S6B). Further, to determine the effect of drug treatment on progression free survival, we assessed the tripling of tumor volume for all three xenografts (time interval between initiation of treatment to tumor progression) by generating Kaplan–Meier survival curves and compared the treatment groups using log rank test. Tumor progression for all three xenografts was delayed for the JQ1-treated group with a median progression free survival of 13.5 days versus 8 days for vehicle (\(P = 0.0007\)), for CHLA-10 xenograft 10 days versus 6 days for vehicle (\(P = 0.0003\)), and for CADO-ES1 xenograft 10 days versus 7 days for vehicle treated control group (\(P = 0.0009\); Fig. 6B). SK-N-MC and CHLA-10 tumors from the JQ1-treated group displayed downregulation of PHF19 at the transcript and protein levels in addition to increased cleaved PARP levels (Fig. 6C–E; Supplementary Fig. S6C–S6E). As expected, MYC expression was unaffected both at the transcript and protein level in tumors from JQ1-treated animals. These observations strongly suggest that BET inhibitor affects the transcriptional program of EWS/ETS fusion and in particular PHF19 to reduce tumor growth in vivo.

Discussion

As a master transcription factor EWS/ETS activates an oncopgenic transcriptional program by modulating the chromatin landscape in EWS. Unfortunately, targeting this oncogenic fusion protein represent a drug discovery challenge, not only because of the difficulty of targeting transcription factors but also contributed by the unstructured prion-like domains in the EWSR1 portion of the fusions (34). In this study, our findings reveal a critical role for chromatin reader BET bromodomain proteins, mainly BRD4, in regulating the oncogenic transcriptional program of the two main EWS/ETS fusions namely EWS/FLI1 and EWS/ERG. Genetic knockdown or small-molecule blockade of BRD4 with the potent inhibitor, JQ1, comprehensively disrupted the transcriptional signature of EWS/FLI1 and EWS/ERG as well as the consequent downstream malignant features of EWS cells (Fig. 6F). The anti-tumorigenic effect of JQ1 was found to be MYC-independent. MYC is a pleiotropic transcription factor that contributes to proliferation, metabolic adaptation, tumorigenesis, and resistance to apoptosis in cancer. Indeed, amplification of MYC is one of the most common genetic alterations in cancer genomes (35). MYC has been suggested as a major oncogenic target for the BET inhibitors in many different malignancies including EWS (18, 28, 29, 36, 37). Although we observed exquisite sensitivity to JQ1 in EWS cells, MYC expression was unaffected upon treatment with JQ1 or BETd. It would be worth exploring whether MYC knockdown or inhibitors targeting MYC transcription such as CDK7 inhibitors (38) will synergize with the BET inhibitors to elicit a more robust antitumorigenic effect in EWS cells. Furthermore, unlike previously reported studies (28, 29), we did not observe downregulation of EWS/FLI1 or EWS/ERG at both mRNA and protein levels upon JQ1 or BETd treatments. This discrepancy in the result we believe is most likely due to the secondary effects and toxicity caused by high concentrations of JQ1 used in those studies. In addition, a recent report also showed a lack of effect of JQ1 on the EWS/ETS mRNA and proteins (39). However, PHF19 appears to be one of the essential EWS/ETS targets dependent on BRD4 function that were downregulated by JQ1 or the PROTAC BETd.

Mechanistically, EWS/FLI1 preferentially binds to chromatin regions enriched for microsatellite GGAA repeats, induces the formation of de novo enhancers and super-enhancers marked by H3K27ac, and activates transcription of target genes (21, 40, 41). BET proteins as chromatin readers bind to H3K27ac in addition to other acetylated lysine residues on histone H4, and regulate the enhancer activity and gene expression. Recently, proteins involved in chromatin remodeling have been shown to interact with EWS/FLI1 in this process. In EWS cells, the BRG1/BRM-associated factor (BAF) chromatin remodeling complex was found to be recruited by the EWS–FLI1 fusion protein to tumor-specific enhancers contributing to target gene activation (24). Unlike the BAF complex, in our study no direct interaction of BRD4 with EWS/ETS fusions was observed. However, their assembly into a transcriptional complex comprising MED1 and RNPAP1 further indicates a central role for BET proteins in EWS/ETS transcriptional program. The ChIP-PCR data showing lack of recruitment of the EWS/FLI1 from the target site upon JQ1 treatment highlights an indirect interaction between EWS/FLI1 and BRD4; nonetheless, the transcriptional downregulation of PHF19 suggests its dependency on BRD4 for target transcription. The mechanisms mediating the indirect interactions between EWS/FLI1 or EWS/ERG and BRD4 need further investigation. Furthermore, a more comprehensive ChIP-seq study with EWS/ETS and all the three BET proteins along with histone modifications will provide
additional mechanistic insights into the role of BET proteins in maintaining the EWS/ETS transcriptional network.

Polycomb group proteins such as PRC2, which is composed of EZH2, EED, and SUZ12 core complex, mediate transcriptional repression through heterochromatin formation by trimethylating the K27 of histone H3 tails (H3K27me3). Interestingly, deregulation of EZH2—the catalytic SET domain containing subunit—is associated with EWS, and is known to be a downstream mediator of EWS/FLI1-induced transcription of human mesenchymal stem cells into EWS phenotype (5, 42, 43). PRC2 also associates with PHF19 and forms subcomplexes that bind H3K36 tri-methyl (H3K36me3) marks through its N-terminal Tudor domain and induces a switch to the H3K27me3 mark on the gene bodies, thereby switching from active transcription to repressed transcription state (30, 44). Although the EWS/FLI1-bound PHF19 regulatory element lacked the canonical GGAA repeat, our transcriptional and phenotypic data does suggest PHF19 is an essential BRD4-dependent EWS/ETS target, and represents a plausible mechanism by which EWS/ETS induces transcriptional silencing of differentiation genes in mesenchymal stem cells (42). Future investigations should include a comprehensive study of PHF19 role in the transcriptional control of direct and indirect EWS/ETS targets and the potential involvement of PHF19 in PRC2-mediated oncosgenesis in EWS. PHF19 was shown to induce proliferation, migration, and invasion in hepatocellular carcinoma cells (45) and was also found to be overexpressed in glioblastoma (46), supporting its involvement in protumorigenic activities.

In summary, our findings identify BET proteins especially BRD4 as an essential dependency factor in EWS/ETS transcriptional program and EWS carcinogenesis. Further, we show that the therapeutic inhibition of BET proteins using a pan-BET inhibitor JQ1 in multiple EWS xenograft models has single agent antitumor activity, which was associated with decreased PHF19 expression and increased apoptosis. Although BET inhibition was not curative in our xenograft studies, we anticipate our findings to have far-reaching implications for developing future combinatorial thera-


EWS/ETS-Driven Ewing Sarcoma Requires BET Bromodomain Proteins
