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Characterizing the Therapeutic Potential of a Potent BET Degrader in Merkel Cell Carcinoma[1]

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Abstract

Studies on the efficacy of small molecule inhibitors in Merkel cell carcinoma (MCC) have been limited and largely inconclusive. In this study, we investigated the therapeutic potential of a potent BET degrader, BETd-246, in the treatment of MCC. We found that MCC cell lines were significantly more sensitive to BETd-246 than to BET inhibitor treatment. Therapeutic targeting of BET proteins resulted in a loss of "MCC signature" genes but not *MYC* expression as previously described irrespective of Merkel cell polyomavirus (MCPyV) status. In MCPyV+ MCC cells, BETd-246 alone suppressed downstream targets in the MCPyV-LT Ag axis. We also found enrichment of HOX and cell cycle genes in MCPyV− MCC cell lines that were intrinsically resistant to BETd-246. Our findings uncover a requirement for BET proteins in maintaining MCC lineage identity and point to the potential utility of BET degraders for treating MCC.

Neoplasia (2019) 21, 322–330

Abbreviations: MCPyV, Merkel cell polyomavirus; BET, bromodomain and extra terminal domain.

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Introduction

Patients presenting with metastatic Merkel cell carcinoma (MCC), an invasive cutaneous neuroendocrine cancer, have an overall 5-year survival rate of 13.5%-18% [1,2]. Despite FDA approval for immunotherapy in the treatment of metastatic MCC, 44%-68% of patients do not demonstrate durable response to treatment in clinical trials [3,4]. Since these patients are not candidates for surgical intervention, their options are limited to standard chemotherapy regimens which have only palliative benefit. Furthermore, targeted therapy, such as those against Bcl-2, PI3K/AKT, and tyrosine kinases, has failed in clinical trials [5] despite success in preclinical studies and individual clinical case reports [6–9].

Therapies inhibiting the bromodomain and extra terminal (BET) family of proteins, Brd2, Brd3, and Brd4, have shown promise in preclinical and early phase clinical studies [10–12]. The BET proteins have been demonstrated to drive transcription of oncogenes, such as *MYC*, by binding to acetylated lysine residues on histones present in super enhancer regions of chromatin [10,11,13]. A previous study identified *MYC* as a target of the BET inhibitor JQ1 in Merkel cell polyomavirus (MCPyV) negative MCC cell lines, nominating it as a clinical candidate drug [14]. More recently, compounds with the ability to degrade BET proteins have shown greater efficacy and a potentially distinct mechanism of action from BET inhibitors [15–17].

Here, we investigate the potential of BETd-246, a potent BET degrader, for the treatment of MCC [16,18]. We show that MCC cell lines undergo apoptosis at markedly lower concentrations of BET degrader when compared to BET inhibitors. Using microarray analysis, we found early downregulation of genes involved in MCC lineage specification [19–21]. Furthermore, apoptosis induced by BETd-246 was not coupled to *MYC* regulation in MCPyV+ or MCPyV– cell lines. Finally, we explored possible mechanisms of efficacy and resistance to BETd-246 by MCPyV status.

Materials and Methods

Cell Lines

The MCC cell lines used in this study, with the exception of the MKL-1 cell line, were established at the University of Michigan and cultured as previously described [6]. Briefly, University of Michigan MCC cell lines were cultured in a modified neural crest stem cell self-renewal medium supplemented with 15% chick embryo extract, while the MKL-1 MCC cell line was grown in RPMI medium with 10% FBS [6]. All cell lines were used within 6 months after thawing from liquid nitrogen stocks. They were tested biweekly for mycoplasma contamination and were confirmed by genotyping every 2-6 months.

Reagents

OTX-015, an *in vivo* grade BET inhibitor, was purchased from Active Biochem. BETi-211, BETd-246, and BETd-260 were developed and provided by Dr. Shaomeng Wang at the University of Michigan [16,18]. BETi-211 is a BET inhibitor. BETd-246 is a BET degrader synthesized from the conjugation of BETi-211 to thalidomide, which targets BET proteins for proteasomal degradation [16,18]. Dr. Wang then optimized BETd-246 for *in vivo* efficacy, which resulted in the new BET degrader BETd-260 [18].

Dose-Response Curves

Ninety-six–well plates were seeded (in triplicate) with 5×10^3 MCC suspension cells per well. IC_{50} curves were generated following treatment with serial dilutions of OTX-015, BETi-211, BETd-246, and thalidomide. DMSO-treated cells were used as a negative control. Cell viability was assessed on day five by a CellTiter-Glo luminescence assay (Promega Corporation).

Immunoblot Analysis

Cell lysates were collected in RIPA lysis buffer with 1% Halt Protease Inhibitor Cocktail (Thermo Fischer Scientific). Western blot was performed by standard protocols using NuPAGE 4%-12% Bis-Tris Protein Gels (Thermo Fischer Scientific). Protein signals were identified by enhanced chemiluminescence (Pierce ECL substrate, Thermo Scientific) using x-ray film.

Anti-ATOH1 antibody (1:1000-5000) was generously provided by Dr. Tom Coates and Dr. Matthew Kelley at NIDCD/NIH [22]. We purchased the following antibodies: Bethyl Laboratories: Brd4 (A700–004, 1:1000), Brd4 (A302-368A, 1:1000), and Brd2 (A700–008, 1:1000); Cell Signaling Technologies: cMyc (5605, 1:1000), cMyb (12,319, 1:1000), and GAPDH (2118, 1:1000).

RNA Interference

SiRNA knockdown experiments were performed using standard protocols for Lipofectamine RNAiMAX transfection reagent (Thermo Fischer Scientific). Cells were seeded at 1×10^6 and 5×10^3 cells in 6- and 96-well plates, respectively, followed by transfection with 25 nM of siRNA at 0 and 24 hours in complete media. Cells were collected for analysis 96 hours postseeding. The following siRNAs (Silencer Select, Thermo Fischer Scientific) were used: BRD4 (s23901, s23902), ATOH1 (s1714, s194299), MYB (s9108, 9110), and Negative Control #1 (AM6411).

RNA Isolation and RT-qPCR

Cell lysates were collected in QIAzol lysis reagent. RNA isolation was performed using the miRNAeasy Mini Kit (Qiagen). cDNA was synthesized using Superscript III reverse transcriptase, and RT-qPCR was performed using SYBR Green dye (Thermo Fischer Scientific). The following primer pair sequences were used (Forward = F, Reverse = R): *GAPDH*-F: GTCTCCTCTGACTTCAACAGCG, GAPDH-R: ACCACCCTGTTGCTGTAGCCAA, *BRD4*-F: CGCTATGTCACCTCCTGTTTGC, *BRD4*-R: ACTCTGAGGAC-GAGAAGCCCTT, *MYC*-F: CGTCCTCGGATTCTCTGCTC, *MYC*-R: GCTGGTGCATTTTCGGTTGT, *ATOH1*-F: CCTTCCAGCAAACAGGTGAATGG, *ATOH1*-R: GAACGACGG-GATAACATTGCGC. *MYB*-F: GGGAACAGATGGGCA-GAAATCG, *MYB*-R: GCTGGCTTTTGAAGACTCCTGC.

Microarray

Cells were treated with DMSO, thalidomide, BETd-246, or BET-211 for 3 and 24 hours, and RNA was isolated as described above. Expression data were captured using the Agilent Whole Human Genome Oligo Microarray (Santa Clara, CA) and were analyzed using the Bioconductor *limma* package in R as previously described [23–25]. Data are available on NCBI GEO database (19550104). All samples were run in duplicate with dye swap. Significantly differentially expressed genes between DMSO and each of the three treatments were identified as ≥0.6-fold change expression with a Bonferroni adjusted P value < .05.

RNA Sequencing

Untreated cells lysates were collected and processed as described previously. Expression data were captured using the Illumina Tru-Seq Stranded mRNA Library Prep Kit (San Diego, CA). Reads per kilobase of transcript per million mapped reads values were generated

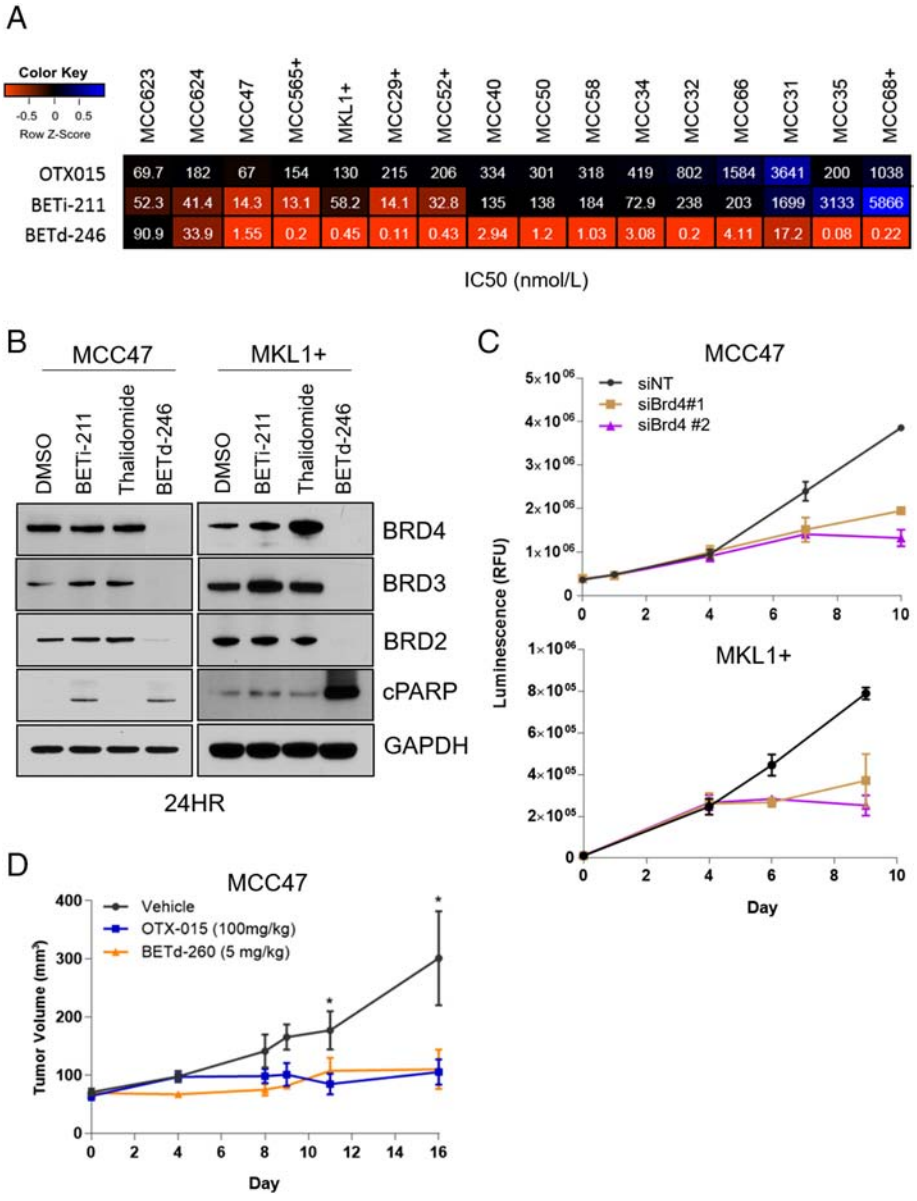

Figure 1. Human MCC cell lines are sensitive to BET degrader BETd-246. (A) Heat map of IC50 values for OTX-015, BETi-211, and BETd-246 (nM). (B) MCC47 and MKL1+ cells were treated with DMSO, BETi-211 (250 nM), thalidomide (10 nM), or BETd-246 (10 nM) for 24 hours. Western blot shows on-target effect of BETd-246 by degradation of Brd4 and Brd2 protein and appearance of cleaved PARP with BETd-246 and BETi-211 treatment. (C) Cell viability following *BRD4* siRNA transfection in MCC47 and MKL1+ cells. *NT*, nontargeting. Error bars represent mean ± SD. (D) Tumor volume in mm^3 and weight in grams between treatment groups following 16 days of treatment. Mice were treated with OTX-015 (100 mg/kg, PO), BETd-260 (5 mg/kg, IV), or vehicle. Error bars represent mean ± SEM.

using the Bioconductor *edgeR* package in R as previously described [26]. Gene set enrichment analysis (GSEA) was performed to identify significantly enriched gene sets (FDR <0.20). Data are available on NCBI SRA database (PRJNA503609).

In Vivo Xenograft Studies

All experimental studies utilizing mice were approved by the University Committee on Use and Care of Animals at the University of Michigan (PRO00006645) and conformed to all regulatory standards. BETd-260 was prepared in 10% PEG400: 3%Cremophor: 87% PBS [16]. OTX-015 was prepared in 40% PEG300 + 5% Tween 80 + ddH2O per manufacturer's instructions. Five million MCC47cells were injected subcutaneously into SCID mice (Charles River Laboratories) in 50% Matrigel solution. When tumors reached 100-150 mm^3, mice were randomized and treated with BETd-260 (5 mg/kg, administered 3 times per week IV), OTX-015 (100 mg/kg administered 5 times per week PO), or vehicle for 16 days.

Statistical Analysis

All experiments included in this publication were performed in duplicate or triplicate. Two-sided Student's *t* tests were used for comparison of continuous data. Significance was designated as follows: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

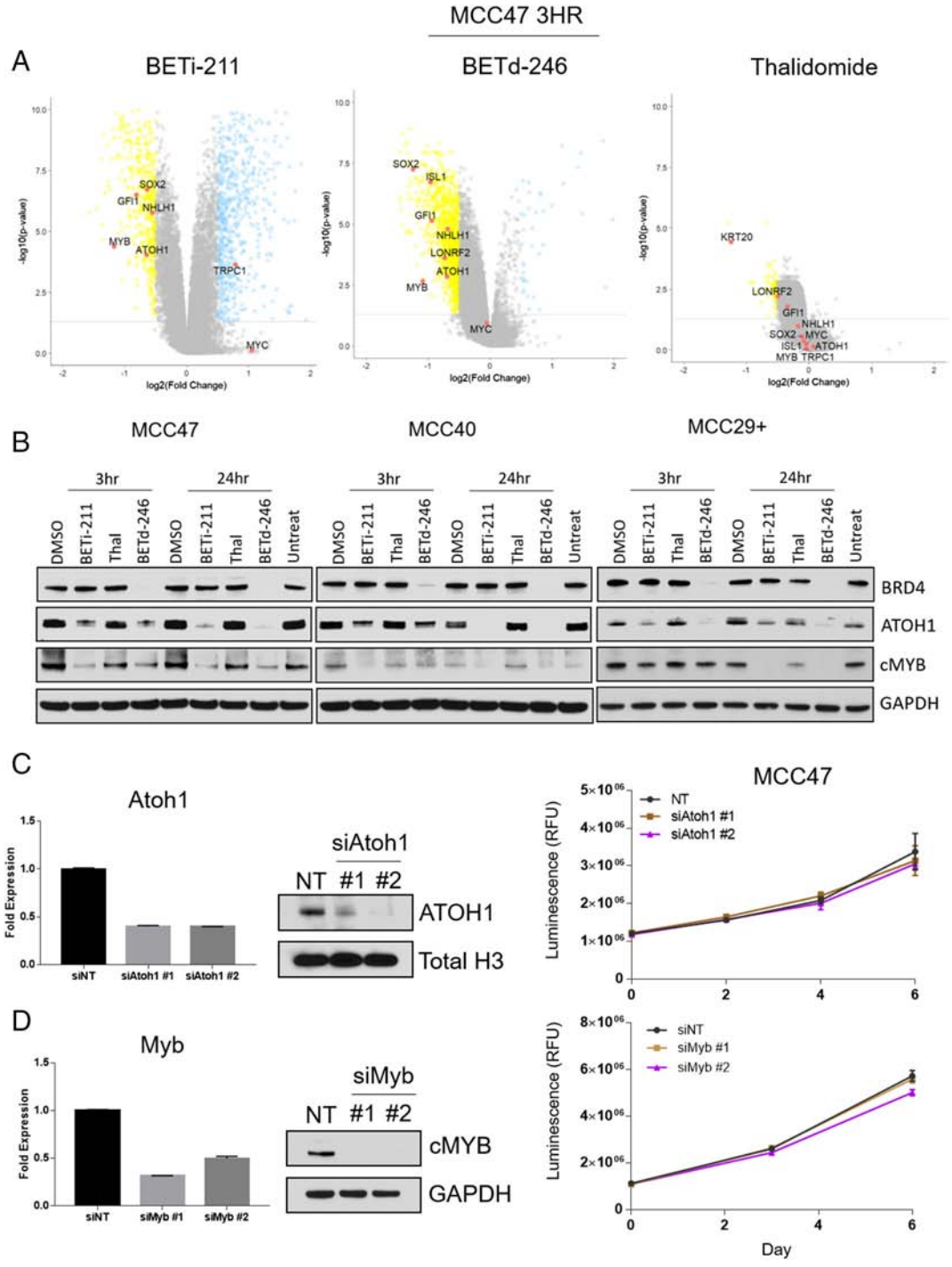

Figure 2. BET inhibitor and degrader treatment downregulates genes required for MCC lineage specification. (A) Volcano plot of top differentially expressed genes compared to DMSO (lfc > 0.6, $P < .05$). Microarray analysis was performed following 3 hours of treatment with DMSO, BETi-211 (250 nM), thalidomide (10 nM), or BETd-246 (10 nM). (B) MCC47 and MCC29+ cells were treated for 3 and 24 hours. Untreated cells were included as an additional control. Western blot shows Atoh1 protein with on-target degradation of Brd4 by BETd-246. MCC47 cells were transfected with siRNA for 96 hours. (c) Left: RT-qPCR for *ATOH1* mRNA and corresponding Western blot for Atoh1 protein show efficacy of siRNA knockdown in MCC47 cells. Right: Cell viability following *ATOH1* siRNA transfection in MCC47 cells. (D) Left: RT-qPCR for *MYB* mRNA and corresponding Western blot for cMyb protein show efficacy of siRNA knockdown in MCC47 cells. Right: Cell viability following *MYB* siRNA transfection in MCC47 cells. All *P* values were determined using paired *t* tests. All error bars represent mean ± SD.

Results

Human MCC Cell Lines are Sensitive to a BET Degrader

To investigate the therapeutic potential of BET degraders in MCC, we screened 16 human MCC cell lines (5 MCPyV+ and 11 MCPyV–) for sensitivity to BET degrader, BETd-246, and BET inhibitors, BETi-211 and OTX-015. All cell lines were sensitive to BETd-246 (IC_{50} [80 pM-91 nM]) (Figure 1*A*, Supp. Figure 1). Additionally, most cell lines were more sensitive to BETd-246 than to either BETi-211 (IC_{50} [13 nM-5.9 μM]) or OTX-015 (IC_{50} [67 nM-3.6 μM]).

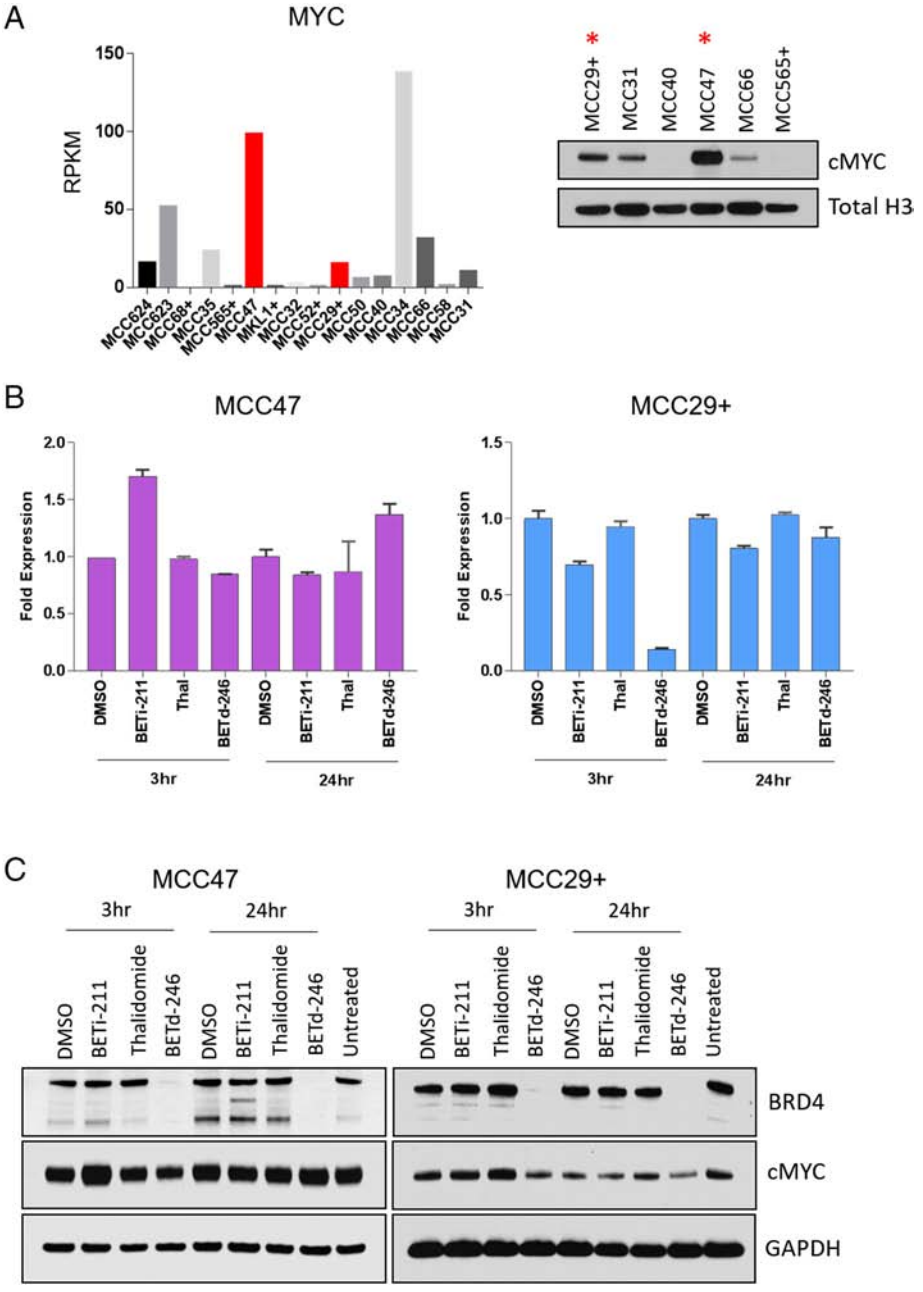

Figure 3. Efficacy of BET inhibitor and degrader in MCC is *MYC*-independent. (A) Left: Comparison of pretreatment *MYC* reads per kilobase of transcript per million mapped reads values across all MCC cell lines. Red bars indicate cell lines with high *MYC* expression nominated for further studies. Right: Western blot shows cMyc protein levels in a subset of MCyV+ and MCyV− cell lines. Asterisks indicate cell lines chosen nominated for further studies. (B-C) MCC47 and MCC29+ cells were treated with DMSO, BETi-211 (250 nM), thalidomide (10 nM), and BETd-246 (10 nM) for 3 and 24 hours. Untreated cells were included as an additional control. (B) RT-qPCR shows expression of *MYC* mRNA. (C) Western blot shows levels of cMyc protein with degradation of Brd4 by BETd-246. All error bars represent mean ± SD.

Interestingly, MCPyV+ cell lines were more sensitive the BETd-246 when compared to MCPyV– cell lines. Complete degradation of Brd4, Brd3, and Brd2 proteins confirmed the on-target effect of BETd-246 at 24 hours posttreatment with BETi-211 and BETd-246 in MCC47 (MCPyV–) and MKL1 (MCPyV+) cell lines (Figure1*B*). Furthermore, induction of apoptosis was indicated by the appearance of cleaved PARP at this time point. As expected, siRNA knockdown of Brd4 resulted in decreased cell viability (Figure 1*C*, Supp. Figure 1). To evaluate the *in vivo* efficacy of a BET inhibitor and degrader, we treated mice with MCC47 subcutaneous xenografts with OTX-015 (100 mg/kg) or BETd-260 (5 mg/kg), respectively. Despite being administered at a significantly lower dose, BETd-260 demonstrated degradation of Brd4 protein and comparable efficacy to OTX-015 (Figure1*D*, Supp. Figure 1), resulting in significantly decreased tumor volume compared to vehicle-treated tumors after 16 days (P = .03). Additionally, changes in weight did not exceed 10% from pretreatment values and were comparable between OTX-015 and BETd-260 groups (Supp. Figure 1).

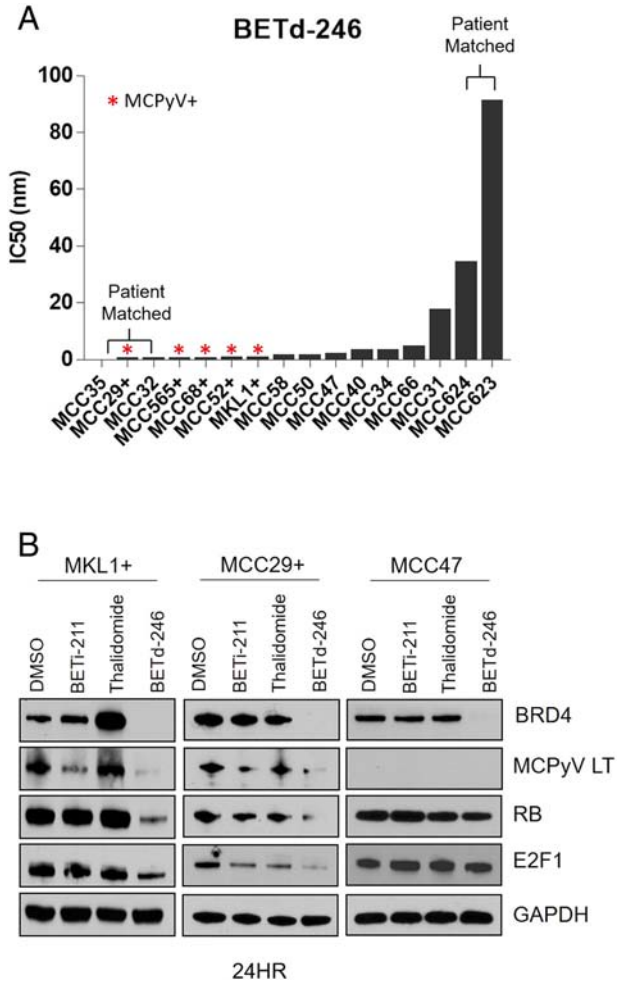

Figure 4. BETd-246 alone downregulates the MCPyV-LT axis. (A) Bar graph of BETd-246 IC50 values across MCC cell lines. MCPyV+ cells are noted with a red asterisk. Cells were treated with DMSO, BETi-211 (250 nM), thalidomide (10 nM), or BETd-246 (10 nM) for 24 hours. (B) Western blot shows MCPyV LT, Rb, and E2f1 expression with BETd-246 and BETi-211 treatment in MKL1+, MCC29+, and MCC47 cells.

BET Inhibitor and Degrader Downregulate Genes Required for MCC Lineage Specification

To identify novel targets of BET inhibition and degradation in MCC cell lines, we performed microarray analysis in MCC47 cells treated with BETi-211, BETd-246, or thalidomide for 3 and 24 hours compared to DMSO (Supp. Tables 1-4). As expected, thalidomide treatment resulted in few changes. There were an increased number of differentially expressed genes at the 24-hour time point in the drug treatment groups (Supp. Figure 2) [16]. At both time points, BET protein modulation led to significant downregulation of genes previously found to be overexpressed in MCC tumors and hypothesized to be important for defining cells to the MCC lineage, including *ATOH1, SOX2, ISL1, NHLH1*, and *MYB* (Figure 2*A*, Supp. Figure 2). In particular, the *ATOH1-SOX2-ISL1 axis* has been shown to be essential for specifying cells to the Merkel cell lineage during normal development in mouse studies [22,27,28]. A recent study found that co-expression of *ATOH1* and MCPyV small T antigen induced lesions expressing markers of MCC in mice [21]. We confirmed loss of Atoh1 and cMyb, a known oncogene and target of BET inhibition, by immunoblotting (Figure 2*B*). However, we found that siRNA knockdown of either *ATOH1* or *MYB* alone did not result in reduction of cell viability in the MCC47 cell line (Figure 2*C-D*).

Efficacy of BET Inhibitor and Degrader in MCC Is MYC-Independent

We were surprised to find no significant change in *MYC* expression in our microarray data. A previous study had reported downregulation of *MYC* upon treatment with the BET inhibitor JQ1 in MCPyV– cell lines with *MYC* overexpression [14]. Although MCC47 is a MCPyV– cell line with significant overexpression of MYC, we found that treatment with high concentrations of JQ1 and OTX-015 to 1 μM did not significantly downregulate *MYC* expression (Supp. Figure 3). To further confirm this finding, we selected MCC29+, a MCPyV+ cell line with overexpression of *MYC,* for comparison (Figure 3*A*). *In vitro* treatment with apoptosis-inducing concentrations of BETi-211 or BETd-246 did not result in significant loss of *MYC* mRNA or cMyc protein in the MCC47 cell line at 3 or 24 hours (Figure 3, *B-C*). Despite a transient downregulation of *MYC* gene expression by BETd-246 at 3 hours in MCC29+ cells, this was rescued by 24 hours. Additionally, there was no significant loss of protein at either time point, indicating that MYC is not likely a direct target of BET inhibition or degradation.

BET Degrader Alone Downregulates the MCPyV-LT Axis

MCPyV+ cell lines were exceptionally sensitive to BETd-246 treatment, with IC_{50} values <500 pM (Figure 4*A*). MKL1+ and MCC29+ showed downregulation of MCPyV Large T antigen (LT) following 24 hours of BETd-246 and BETi-211 treatment, indicating that it may be a common downstream target (Figure 4*B*). However, only BET protein degradation by BETd-246 downregulated additional downstream targets of MCPyV-mediated oncogenic transformation, such as Rb and E2f1. No changes were seen in the MCC47 cell line.

Overexpression of HOX and Cell Cycle Genes is Associated with Reduced Sensitivity to BET Degrader

Unlike the MCPyV+ cell lines, the MCPyV– cell lines exhibited a large range in sensitivity to BETd-246 treatment. Therefore, we conducted an exploratory analysis of possible intrinsic mechanisms of resistance to BETd-246. MCC32/MCC35 and MCC623/MCC624 are paired MCPyV– cell lines that were derived from matched primary and metastatic tumors from the same patient and were the most sensitive and resistant to BETd-246 treatment, respectively (Figure 4*A*). RNA-seq analysis between these matched pairs revealed that the family of Antp homeobox genes (*HOXB2, HOXB3, HOXB4, HOBB5, HOXB6, HOXB8, HOB9*) was the most highly upregulated genes in the resistant cell lines (Figure 5*A*). GSEA also demonstrated enrichment in G2M checkpoint pathway and E2F target genes (Figure 5*B*, Supp. Table 5).

Discussion

This is the first study to investigate the potential of a BET degrader for treatment of MCC. We found that MCC cell lines were more sensitive to the BET degrader BETd-246 when compared to the BET inhibitor BETi-211 or the widely used, commercially available BET inhibitor OTX-015.

We found both BET degrader and inhibitor induced early downregulation of MCC "signature" genes. Our results suggest

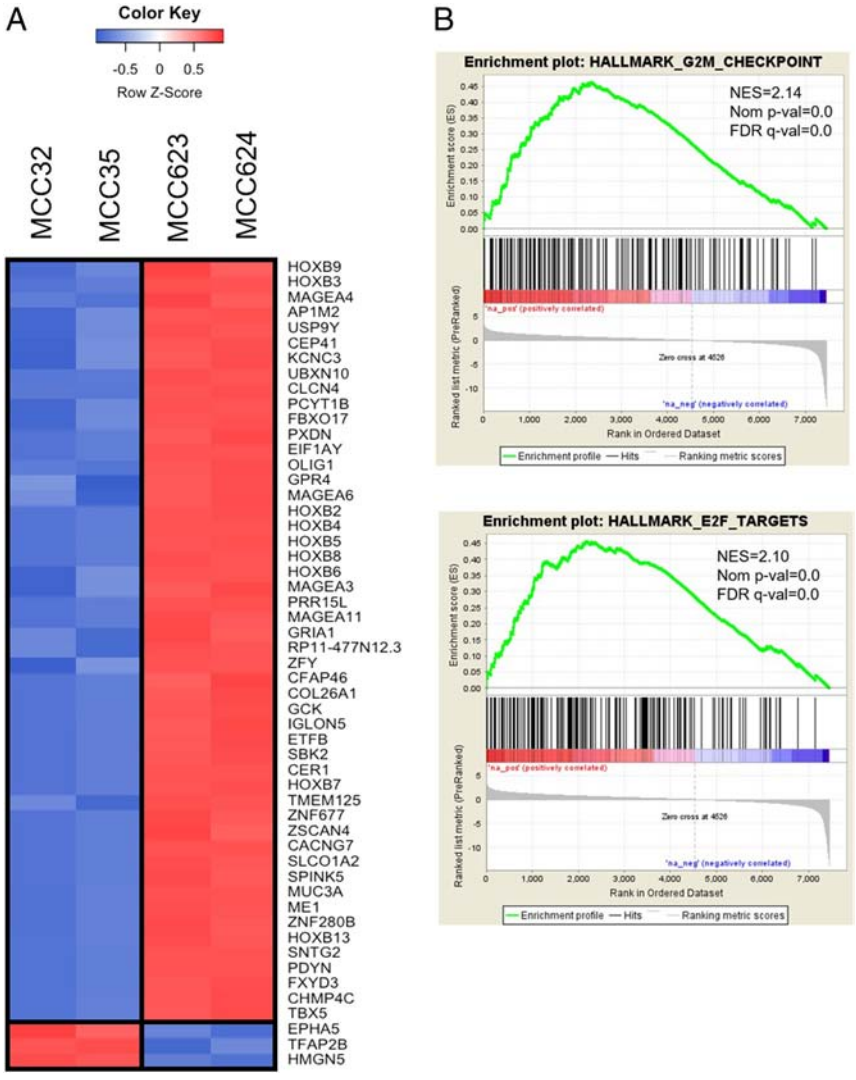

Figure 5. Overexpression of HOX and cell cycle genes attenuates sensitivity to BETd-246. RNA-seq analysis was formed between matched primary and metastatic cell lines MCC32/MCC35 and MCC623/MCC624. (A) Heat map of top differentially expressed genes (lfc > 5, $P < .0005$). (B) Enrichment plots of GSEA analysis of the MSigDB hallmark gene set collection.

that, despite their potential role in lineage and cell fate determination in normal and cancerous cells, *ATOH1* and *MYB*, individually, are not essential for MCC cell survival. Individual loss of gene candidates ATOH1 and MYB did not recapitulate the decrease in cell viability seen with Brd4 knockdown or death phenotype seen with treatment. Additionally, further studies are needed to determine whether simultaneous knockdown of multiple "signature" genes is required for loss of cell viability. Interestingly, previous studies have implicated BET proteins in the initiation, but not maintenance, of lineage-specific gene expression during adipogenesis and myogenesis [31,32], suggesting that any effects on cell fate will be highly dependent on cellular context.

Contrary to previous results, our data suggest that *MYC* is not a target of BET inhibitor or degrader in the MCC cell lines used in this study. *MYC* expression was not downregulated following high concentrations of BET inhibitor treatment with JQ1, OTX-15, and BETi-211 and was sustained following degradation of Brd4 protein in MCPyV+ and MCPyV− cell lines. Despite evidence of Brd4-dependent regulation of *MYC* transcription in MCPyV− MCC and other solid tumors, additional studies have shown that this regulation may be specific to individual cell lines even within a single cancer type [29–32].

We further characterized the response of MCPyV+ cells to BETd-246 as they were all exceptionally sensitive to treatment. Even though there was loss of MCPyV-LT with both BET inhibitor and degrader treatment, only BETd-246 showed loss of downstream targets. As oncogenic transformation and "hijacking" of the Rb/E2F pathway by MCPyV antigens is thought to be a driver of MCPyV+ MCC, complete "shutdown" of this pathway could explain the superior potency of BETd246. However, it remains to be seen if this difference is due to a direct mechanistic interaction between bromodomain proteins and LT antigen or if this a downstream effect of larger effect such as DNA damage. Further studies are required to determine the mechanism by which MCPyV-LT expression is modulated by BET proteins.

In contrast to MCPyV+ cell lines, MCPyV− cell lines had varying sensitivity to BETd-246, suggesting that the mechanism of action may differ by virus status. In addition to enrichment in genes involved

in the cell cycle, we found upregulation of the Antp homeobox family of genes in MCPyV– cell lines most resistant to treatment. These genes are known to be involved in maintaining stem cells and have been associated with various malignancies, including pancreatic cancer and leukemia.

When combined with our observations of the loss of MCC lineage genes with drugs targeting the BET proteins, this suggests that factors influencing cell identity, such as stemness, may be an important marker of intrinsic resistance. Ultimately, our data demonstrate the potential of epigenetic modification through degradation of BET proteins as a promising strategy for treatment of MCC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.01.003.

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Potential Conflict of Interests

The University of Michigan has filed a number of patent applications on BET degraders, including the BET degrader used in this research for which S. W. and B. Z. are co-inventors. These patents have been licensed by Oncopia Therapeutics LLC for clinical development. S. W. and A. M. C. are cofounders of Oncopia Therapeutics LLC. S. W. serves on the board of directors and A. M. C. serves on the SAB of Oncopia. Both S. W. and A. M. C. are paid consultants of Oncopia. S. W. receives a research contract from Oncopia, which did not support this research. Oncopia was not involved in the design, funding, or approval of this study.

References

[1] Lemos BD, Storer BE, Iyer JG, Phillips JL, Bichakjian CK, Fang LC, Johnson TM, Liegeois-Kwon NJ, Otley CC, and Paulson KG, et al (2010). Pathologic nodal evaluation improves prognostic accuracy in Merkel cell carcinoma: analysis of 5823 cases as the basis of the first consensus staging system. *J Am Acad Dermatol* **63**, 751–761. https://doi.org/10.1016/j.jaad.2010.02.056.

[2] Harms KL, Healy MA, Nghiem P, Sober AJ, Johnson TM, Bichakjian CK, and Wong SL (2016). Analysis of prognostic factors from 9387 Merkel cell carcinoma cases forms the basis for the new 8th edition AJCC staging system. *Ann Surg Oncol* . https://doi.org/10.1245/s10434-016-5266-4.

[3] Nghiem PT, Shailender B, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, Berry S, Chartash EK, Daud A, and Fling SP, et al (2016). PD-1 blockade with pembrolizumab in advanced Merkel-cell carcinoma. *N Engl J Med* . https://doi.org/10.1056/NEJMoa1603702.

[4] Kaufman HL, Russell J, Hamid O, Chatia S, Terheyden P, D'Angelo SP, Shih KC, Lebbe C, Linette GP, and Milella M, et al (2016). Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. *Lancet Oncol* **17**, 1374–1385. https://doi.org/10.1016/S1470-2045(16)30364-3.

[5] Samlowski WE, Moon J, Tuthill RJ, Heinrich MC, Balzer-Haas NS, Merl SA, DeConti RC, Thompson JA, Witter MT, and Flaherty LE, et al (2010). A phase II trial of imatinib mesylate in merkel cell carcinoma (neuroendocrine carcinoma of the skin): a Southwest Oncology Group study (S0331). *Am J Clin Oncol* **33**, 495–499. https://doi.org/10.1097/COC.0b013e3181b9cf04.

[6] Verhaegen ME, Mangelberger D, Weick JW, Vozheiko TD, Harms PW, Nash KT, Quintana E, Baciu P, Johnson TM, and Bichakjian CK, et al (2014). Merkel cell carcinoma dependence on bcl-2 family members for survival. *J Invest Dermatol* **134**, 2241–2250. https://doi.org/10.1038/jid.2014.138.

[7] Shiver MB, Mahmoud F, and Gao L (2015). Response to idelalisib in a patient with stage IV Merkel-cell carcinoma. *N Engl J Med* **373**, 1580–1582. https://doi.org/10.1056/NEJMc1507446.

[8] Loader DE, Feldmann R, Baumgartner M, Breier F, Schrama D, Becker JC, and Steiner A (2013). Clinical remission of Merkel cell carcinoma after treatment with imatinib. *J Am Acad Dermatol* **69**, e181–e183. https://doi.org/10.1016/j.jaad.2013.03.042.

[9] Davids M, Charlton A, Ng S, Chong M, Laubscher K, Dar M, Hodge J, Soong R, and Goh BC (2009). Response to a novel multitargeted tyrosine kinase inhibitor pazopanib in metastatic Merkel cell carcinoma. *J Clin Oncol* **27**, e97–e100. https://doi.org/10.1200/JCO.2009.21.8149.

[10] Asangani IA, Dommeti VL, Wang X, Malik R, Cieslik M, Yang R, Escara-Wilke J, Wilder-Romans K, Dhanireddy S, and Engelke C, et al (2014). Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. *Nature* **510**, 278–282. https://doi.org/10.1038/nature13229.

[11] Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, and Felletar I, et al (2010). Selective inhibition of BET bromodomains. *Nature* **468**, 1067–1073. https://doi.org/10.1038/nature09504.

[12] Dombret H, Preudhomme C, Berthon C, Raffoux E, Thomas X, Vey N, Gomez-Roca C, Ethell M, Yee K, and Bourdel F, et al (2014). A phase 1 study of the BET-bromodomain inhibitor OTX015 in patients with advanced acute leukemia. *Blood* **124**, 117.

[13] Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastritis E, Gilpatrick T, Paranal RM, and Qi J, et al (2011). BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146**, 904–917. https://doi.org/10.1016/j.cell.2011.08.017.

[14] Shao Q, Kannan A, Lin Z, Stack Jr BC, Suen JY, and Gao L (2014). BET protein inhibitor JQ1 attenuates Myc-amplified MCC tumor growth in vivo. *Cancer Res* **74**, 7090–7102. https://doi.org/10.1158/0008-5472.CAN-14-0305.

[15] Raina K, Lu J, Qian Y, Altieri M, Gordon D, Rossi AM, Wang J, Chen X, Dong H, and Siu K, et al (2016). PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. *Proc Natl Acad Sci U S A* **113**, 7124–7129. https://doi.org/10.1073/pnas.1521738113.

[16] Bai L, Zhou B, Yang CY, Ji J, McEachern D, Przybranowski S, Jiang H, Hu J, Xu F, and Zhao Y, et al (2017). Targeted degradation of BET proteins in triple-negative breast cancer. *Cancer Res* **77**, 2476–2487. https://doi.org/10.1158/0008-5472.CAN-16-2622.

[17] Winter GE, Mayer A, Buckley DL, Erb MA, Roderick JW, Vittori S, Reyes JM, di Lulio J, Souza A, and Ott CJ, et al (2017). BET bromodomain proteins function as master transcription elongation factors independent of CDK9 recruitment. *Mol Cell* **67**, 5–18. https://doi.org/10.1016/j.molcel.2017.06.004 e19.

[18] Zhou B, Hu J, Xu F, Chen Z, Bai L, Fernandez-Salas E, Lin M, Liu L, Yang CY, and Zhao Y, et al (2017). Discovery of a small-molecule degrader of bromodomain and extra-terminal (BET) proteins with picomolar cellular potencies and capable of achieving tumor regression. *J Med Chem* . https://doi.org/10.1021/acs.jmedchem.6b01816.

[19] Harms PW, Patel RM, Verhaegen ME, Giordano TJ, Nash KT, Johnson CN, Daignault S, Thomas DG, Gudjonsson JE, and Elder JT, et al (2013). Distinct gene expression profiles of viral- and nonviral-associated merkel cell carcinoma revealed by transcriptome analysis. *J Invest Dermatol* **133**, 936–945. https://doi.org/10.1038/jid.2012.445.

[20] Verhaegen ME, Mangelberger D, Harms PW, Vozheiko TD, Weick JW, Wilbert DM, Saunders TL, Ermilov AN, Bichakjian CK, and Johnson TM, et al (2015). Merkel cell polyomavirus small T antigen is oncogenic in transgenic mice. *J Invest Dermatol* **135**, 1415–1424. https://doi.org/10.1038/jid.2014.446.

[21] Verhaegen ME, Mangelberger D, Harms PW, Eberl M, Wilbert DM, Meireles J, Bichakjian CK, Saunders TL, Wong SY, and Dlugosz AA, et al (2017). Merkel cell polyomavirus small T antigen initiates merkel cell carcinoma-like tumor development in mice. *Cancer Res* **77**, 3151–3157. https://doi.org/10.1158/0008-5472.CAN-17-0035.

[22] Ostrowski SM, Wright MC, Bolock AM, Geng X, and Maricich SM (2015). Ectopic Atoh1 expression drives Merkel cell production in embryonic, postnatal and adult mouse epidermis. *Development* **142**, 2533–2544. https://doi.org/10.1242/dev.123141.

[23] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, and Smyth GK (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47. https://doi.org/10.1093/nar/gkv007.

[24] Law CW, Chen Y, Shi W, and Smyth GK (2014). voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* **15**R29. https://doi.org/10.1186/gb-2014-15-2-r29.

[25] Smyth GK and Altman NS (2013). Separate-channel analysis of two-channel microarrays: recovering inter-spot information. *BMC Bioinformatics* **14**, 165. https://doi.org/10.1186/1471-2105-14-165.

[26] Robinson MD, McCarthy DJ, and Smyth GK (2010). edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140. https://doi.org/10.1093/bioinformatics/btp616.

[27] Perdigoto CN, Bardot ES, Valdes VJ, Santoriello FJ, and Ezhkova E (2014). Embryonic maturation of epidermal Merkel cells is controlled by a redundant transcription factor network. *Development* **141**, 4690–4696. https://doi.org/10.1242/dev.112169.

[28] Wright MC, Reed-Geaghan EG, Bolock AM, Fujiyama T, Hoshino M, and Maricichet SM (2015). Unipotent, Atoh1+ progenitors maintain the Merkel cell population in embryonic and adult mice. *J Cell Biol* **208**, 367–379. https://doi.org/10.1083/jcb.201407101.

[29] Baker EK, Taylor S, Gupte A, Sharp PP, Walia M, Walsh NC, Zannettino AC, Chalk AM, Burns CJ, and Walkley CR (2015). BET inhibitors induce apoptosis through a MYC independent mechanism and synergise with CDK inhibitors to kill osteosarcoma cells. *Sci Rep* **5**, 10120. https://doi.org/10.1038/srep10120.

[30] Lenhart R, Kirov S, Desilva H, Cao J, Lei M, Johnston K, Peterson R, Schweizer L, Purandare A, and Ross-Macdonald P, et al (2015). Sensitivity of small cell lung cancer to BET inhibition is mediated by regulation of ASCL1 gene expression. *Mol Cancer Ther* **14**, 2167–2174. https://doi.org/10.1158/1535-7163.MCT-15-0037.

[31] Lockwood WW, Zejnullahu K, Bradner JE, and Varmus H (2012). Sensitivity of human lung adenocarcinoma cell lines to targeted inhibition of BET epigenetic signaling proteins. *Proc Natl Acad Sci U S A* **109**, 19408–19413. https://doi.org/10.1073/pnas.1216363109.

[32] Hong SH, Eun JW, Choi SK, Shen Q, Choi WS, Han JW, Nam SW, and You JS (2016). Epigenetic reader BRD4 inhibition as a therapeutic strategy to suppress E2F2-cell cycle regulation circuit in liver cancer. *Oncotarget* **7**, 32628–32640. https://doi.org/10.18632/oncotarget.8701.