Molecular Therapy
Nucleic Acids
Original Article

# Streamlined DNA-encoded small molecule library screening and validation for the discovery of novel chemotypes targeting BET proteins

Seoyeon Jeong,[1,3] Hwa-Ryeon Kim,[1,3] June-Ha Shin,[1] Min-Hee Son,[2] In-Hyun Lee,[2] and Jae-Seok Roe[1]

[1]Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Korea; [2]Benobio Co., Ltd., Seoul, Korea

**Targeting aberrant epigenetic programs that drive tumorigenesis is a promising approach to cancer therapy. DNA-encoded library (DEL) screening is a core platform technology increasingly used to identify drugs that bind to protein targets. Here, we use DEL screening against bromodomain and extra-terminal motif (BET) proteins to identify inhibitors with new chemotypes, and successfully identified BBC1115 as a selective BET inhibitor. While BBC1115 does not structurally resemble OTX-015, a clinically active pan-BET inhibitor, our intensive biological characterization revealed that BBC1115 binds to BET proteins, including BRD4, and suppresses aberrant cell fate programs. Phenotypically, BBC1115-mediated BET inhibition impaired proliferation in acute myeloid leukemia, pancreatic, colorectal, and ovarian cancer cells *in vitro*. Moreover, intravenous administration of BBC1115 inhibited subcutaneous tumor xenograft growth with minimal toxicity and favorable pharmacokinetic properties *in vivo*. Since epigenetic regulations are ubiquitously distributed across normal and malignant cells, it will be critical to evaluate if BBC1115 affects normal cell function. Nonetheless, our study shows integrating DEL-based small-molecule compound screening and multistep biological validation represents a reliable strategy to discover new chemotypes with selectivity, efficacy, and safety profiles for targeting proteins involved in epigenetic regulation in human malignancies.**

## INTRODUCTION

The onset and progression of cancer is often promoted by the accumulation of epigenetic alterations, including changes in patterns of DNA methylation, histone modifications, and nucleosome positioning.[1] Therefore, the proteins involved in regulating epigenetic processes have long been considered as promising targets for cancer drug discovery and development. In general, epigenetic regulators have been classified into three broad categories: "writers," that is, enzymes involved in installing a specific epigenetic mark on the substrate (e.g., DNA methyltransferases [DNMTs], histone acetyltransferases, histone methyltransferases [HMTs]); "readers," or proteins that bind to the specific marks to modulate function (e.g., bromodomain [BRD] and extra-terminal motif [BET] proteins); and "erasers," or enzymes that remove epigenetic marks from substrates (e.g., histone deacetylases [HDACs], histone demethylases).[2,3] Historically, two major classes of drugs targeting epigenetic regulation, DNMT inhibitors (azacitidine and decitabine), and HDAC inhibitors (vorinostat) were discovered through phenotypic observations and took years to identify specific targets responsible for their mechanism of action.[4,5] Furthermore, although DNMT inhibitors are widely used as chemotherapy agents, their systemic activity causes toxicity, thus resulting in major interest in developing more targeted strategies. Therefore, recent efforts in the field of epigenetic drug discovery and development have been made on specific targets, resulting in the 2020 U.S/ Food and Drug Administration approval of tazemetostat, the first-in-class inhibitor of an HMT, enhancer of zeste homolog 2. Tazemetostat has been reported to be effective for the treatment of metastatic or locally advanced epithelioid sarcoma.[6] In addition, numerous compounds are in different stages of clinical development, including those targeting BRD and BET proteins.[7,8]

The BET proteins (BRD2, BRD3, BRD4, and BRDT) are a family of epigenetic readers that regulate transcription. Attention to these proteins started increasing in 2003, when the BRD4-NUT fusion oncoprotein was found to drive a rare and aggressive form of squamous carcinoma called NUT midline carcinoma.[9] This interest rose further when it was found that unmutated BET proteins play essential roles in the maintenance of various tumors, including breast, prostate, and pancreatic cancer as well as various hematological malignancies.[10] The oncogenic function of BETs is mediated by the two N-terminal bromodomains (BDs) called BD1 and BD2. These domains are evolutionarily conserved, similar in terms of sequence and structure, and preferentially bind to the acetylated lysine residues of either histone (BD1) or non-histone (BD2) proteins.[11,12] This binding drives the acetylation-dependent assembly of transcriptional-regulator complexes.[13,14] In particular, BRD binding leads to high and sustained expression of critical oncogenes such as *MYC* and *CDK6*.[15] The link between the BRDs in BETs and cancer has led to the development of several anti-cancer BET inhibitors, starting with the prototype

Received 21 October 2022; accepted 20 April 2023;
https://doi.org/10.1016/j.omtn.2023.04.023.

[3]These authors contributed equally

**Correspondence:** Jae-Seok Roe, PhD, Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Korea.
**E-mail:** jroe@yonsei.ac.kr

compounds I-BET762 and JQ1.[16,17] Further optimization of prototype scaffolds has led to several new drugs, including a pan-BET inhibitor birabresib (OTX-015 or MK-8628), and apabetalone (RVX-208), which inhibits the BD2s of BRD2 and BRD3.[18,19] Unfortunately, clinical trials on these inhibitors have revealed diverse adverse effects, namely, thrombocytopenia, neutropenia, and anemia,[20] highlighting the need for more effective and safer BET inhibitors with clinical efficacy.

A major challenge in target-based drug discovery has been finding unique chemical matter (chemotypes) that have desired potency and selectivity, as well as suitable pharmacological properties given the vast size of chemical space.[21] DNA-encoded libraries (DELs) used in combination with high-throughput screening platforms have emerged as one of the most promising strategies for profiling large sections of chemical space. These libraries consist of millions of small organic molecules that are conjugated to short DNA fragments that serve as barcodes and can be quickly screened to determine their binding to an immobilized protein target. Thus, this procedure involves multiple rounds of affinity selection followed by next-generation sequencing (NGS) of the amplified barcoded DNA.[22] To date, DEL screening has been used to discover inhibitors and binders of many different enzymes such as kinases, $NAD^+$-dependent enzymes, and epigenetic regulators, including BET proteins.[23–28] However, many BET inhibitors that emerged from these efforts have neither been extensively validated, especially in *in vivo* tumorigenesis models, nor exhibited major improvement of activity over the established inhibitors.

To address this problem, we used DEL technology to screen for chemical compounds that bind to BD1s and BD2s of BRD2, BRD3, and BRD4. We readily identified BBC1115, a new class of pan-BET inhibitor that is structurally distinct from known BET inhibitors. We showed that BBC1115 treatment resulted in a typical response to BET inhibitors, represented by suppression of MYC programs and dissociation of BRD4 from chromatin. Moreover, our cell culture and mouse xenograft models for leukemia, pancreatic cancer, colon cancer, and ovarian cancer showed that BBC1115 suppresses BET-dependent gene expression programs and exhibits anti-proliferative activity *in vitro* and *in vivo*. To summarize, our work highlights the promise of DEL screen in discovering epigenetic drugs with new chemotypes. Furthermore, we suggest that BBC1115 represents a chemically distinct starting point for further hit optimization and lead development of both pan-BET and selective inhibitors of different BET proteins.

## RESULTS

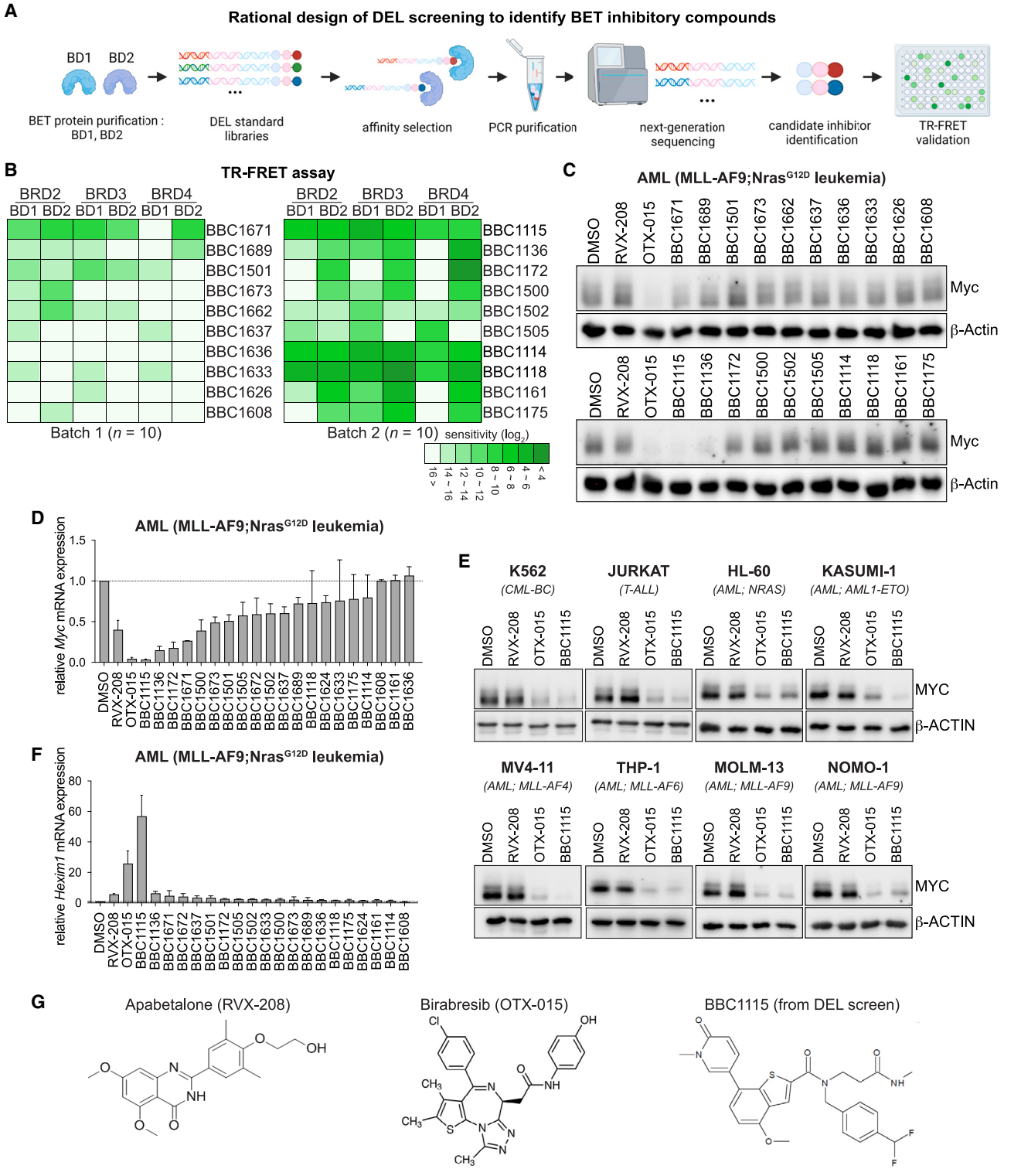
### Experimental platform design to screen BET BRD inhibitors from DNA-encoded small molecule library

DEL technology is an efficient drug screening approach because it employs the "split and pool" strategy to generate large libraries of chemicals tagged with unique DNA barcodes.[22] We screened a commercially available DEL provided by WuXi AppTec,[29] with the BRDs of three BET proteins, including BRD2, BRD3, and BRD4. We chose these targets because the assays used to characterize BET protein inhibitors are well standardized and the potency and selectivity of those BET inhibitors are well established.[30] In particular, we focused on cancer-relevant functions in transcription regulation of the BRDs of BET proteins.[31]

First, we purified His-tagged BD1s and BD2s of BRD2, BRD3, and BRD4, and generated a pool of the BD1s, BD2s, or a mixture of the BD1s and BD2s that are required for affinity selection with the DNA-barcoded chemical compound library (Figure 1A). We then conducted NGS of the DNA tags of small compounds that bound to any of the purified BRDs. As a result, we selected a total of 20 candidates from BET BRD inhibitor screening. To validate our screening results, we used the time-resolved fluorescence resonance energy transfer (TR-FRET) assay and found that these candidates exhibited different binding affinities for the purified BD1s and BD2s (Figure 1B; Table S1). Of note, BBC1115, BBC1114, and BBC1118 were the only candidates that displayed broad binding across BDs of all three BET proteins.

An initial study demonstrating BET proteins as therapeutic targets of cancer revealed that BRD4 maintains proto-oncogene MYC expression in acute myeloid leukemia (AML) cells, blocks terminal myeloid differentiation, and potentiates proliferation of leukemia stem cells.[32] To test whether the newly identified chemical compounds were functionally similar to the known BET inhibitors, we first examined whether the candidates affected MYC expression in cultured murine MLL-AF9; $Nras^{G12D}$ AML cells. As expected, the pan-BET inhibitor OTX-015 downregulated MYC protein levels in AML cells (Figure 1C; Figure S1A). Consistent with previous observations, treatment of RVX-208, a BD2-selective BET inhibitor, only modestly affected MYC protein expression,[19] emphasizing that simultaneous BD1 and BD2 inhibition is needed to abrogate BET-dependent transcription. Notably, BBC1115 suppressed MYC expression to a degree similar to that of OTX-015 (Figure 1D). Moreover, BBC1115 also downregulates MYC protein and mRNA levels in multiple human leukemia cell lines with different genetic backgrounds, such as K562, JURKAT, HL-60, KASUMI-1, MV4-11, THP-1, MOLM13, and NOMO-1 (Figure 1E), suggesting that BBC1115-mediated MYC downregulation is not restricted to MLL-rearranged leukemia cells.

Nonetheless, we wondered if BBC1115-induced MYC downregulation could be an indirect consequence of BBC1115 cytotoxicity. To exclude this possibility, we examined whether BBC1115 increases the transcription of hexamethylene bisacetamide-inducible protein 1 (*HEXIM1*), a well-established marker whose expression is upregulated by BET inhibition.[33] BBC1115 increased *Hexim1* mRNA expression by >50-fold; this effect was even stronger than that of OTX-015 (increase of >20-fold) (Figure 1F). By contrast, BBC1136, another hit from the DEL screening that decreased MYC expression, failed to upregulate *Hexim1* mRNA. To further confirm if BBC1115 is a potent inhibitor of BET BRDs, we checked if other hematopoietic genes, whose expressions depend on BRD4 chromatin occupancy,




*(legend on next page)*

were affected.[34] Consistent with MYC downregulation, BBC1115 interferes with mRNA expression of known BRD4-dependent genes, including *Pu1*, *Fli1*, and *Erg* (Figure S1B).

The chemical composition of BBC1115 is different from that of known BET inhibitors, OTX-015 and RVX-208. Specifically, BBC1115 has a benzothiophene core, while OTX-015 and RVX-208 have diazepine and quinazoline cores, respectively.[35–37] Despite these differences, the key biological consequence of BET inhibition, represented by MYC suppression, is conserved in leukemia cell lines. This suggests that BBC1115 may have unique conformational characteristics that allow it to bind BET proteins and demonstrates the potential of DEL screening to identify novel chemotypes against well validated targets. This opens up new opportunities for the development of targeted therapies.

### BBC1115 abrogates BET-dependent transcriptional programs in association with BRD4 dissociation from chromatin

To further validate BBC1115 biochemically, we conducted BRD profiling with BROMOscan, a robust and sensitive assay measuring binding affinities of chemical compounds to specific BRDs. We focused on a total of 13 BRDs of 4 BET BRD-containing proteins including BRD2, BRD3, BRD4, and BRDT. Although dissociation constant ($K_d$) values varied among tested BRDs, BBC1115 bound to the BD1s and BD2s of all BET proteins with a $K_d$ of <1,000 nM (Figure 2A). Interestingly, BRD4 was the only case in which we identified a significant difference in binding affinities between BD1 and BD2 ($K_d$ [BD1-BBC1115] = 320 nM, and $K_d$ [BD2-BBC1115] = 950 nM) (Figure 2B). Collectively, the results of the BROMOscan analysis indicate that BBC1115 is a sub-micrometer pan-BET inhibitor that engages all BRDs in BRD2-4, with BD2 in BRD4 exhibiting the lowest affinity.

Next, to examine if BBC1115 suppresses BET protein-dependent transcription by displacing BET proteins from chromatin, we tested whether BBC1115 binding causes BRD4 to dissociate from the chromatin where acetylated histones are enriched, such as a distal region of the enhancer cluster that is located >1.8 Mb 3′ of the *Myc* locus (namely, e1-e5).[38] To this end, we treated AML cells with either BBC1115, OTX-015, or RVX-208 and conducted chromatin immunoprecipitation quantitative real-time PCR (ChIP-qPCR) with BRD4-specific antibodies. Similar to OTX-015, BBC1115 impaired the occupancy of BRD4 at the e1-e5 *Myc* enhancers (Figure S2; Table S2). In contrast, in line with its inability to downregulate MYC expression, RVX-208 treatment showed a negligible effect on the BRD4 occupancy at e1-e5.

To evaluate the impact of BBC1115 treatment on BRD4-chromatin binding throughout the genome, we conducted ChIP-sequencing (ChIP-seq). From four different BRD4 ChIP-seq data of AML cells each treated with DMSO, RVX-208, OTX-015, or BBC1115, we isolated a total of 4,145 BRD4 ChIP-seq peaks and achieved high confidence by the disregarding BRD4-peak if the normalized BRD4 tag count was <10. To further define BBC1115-sensitive BRD4 peaks, we considered BRD4-occupied sites where relative changes (BBC1115 to DMSO) of BRD4 are smaller than −1.5 in the log2 scale. To this end, we characterized 330 BRD4 peaks in AML cells that exhibited the greatest sensitivity to BBC1115 (Table S3).

Comparison of the ChIP-seq profiles of BRD4 and acetylated H3 Lys-27 (H3K27ac) demonstrated that BBC1115 strongly reduced the BRD4 occupancy in regions that were enriched with H3K27ac modification (Figure 2C). While the fold changes of BRD4 dissociation in a total of 4,145 BRD4-occupied sites upon BBC1115 treatment showed marginal differences, BBC1115-sensitive BRD4-bound sites were associated with a severe loss of BRD4 signals ($n$ = 330) (Figures 2D and 2E). In addition, we evaluated the impact of BBC1115 on the genome-wide binding of RNA polymerase II (Pol II) as BRD4 regulates Pol II activity at its co-regulated target genes.[39] Consistent with the effects on BRD4, we found that BBC1115 led to a loss of Pol II binding from BRD4-occupied sites to a greater degree at BBC1115-sensitive sites than all BRD4 peaks (Figure 2F; Figure S3). However, compared with BBC1115 and OTX-015, the impact of RVX-208 on chromatin bindings of BRD4 or Pol II was negligible.

Next, we conducted an RNA-sequencing (RNA-seq) analysis to determine if BBC1115-mediated BRD4 loss accompanies transcriptional changes (Table S4). Following ChIP-seq analysis, gene set enrichment analysis (GSEA) revealed that genes located close to the BBC1115-sensitive 330 regions, including MYC, that had lost BRD4 were significantly downregulated by both BBC1115 and OTX-015 (Figure 2G; Table S5). Selective loss of BRD4 occupancy and preferential changes in oncogenic expression upon BET inhibition are a key to therapeutic activity, although the underlying molecular mechanism of such asymmetric changes remains incompletely understood.[34] Nevertheless, our findings indicate that BBC1115, although chemically distinct from OTX-015, exhibits comparable activity, further illustrating the power of DEL screening to discover new chemotypes.

### BBC1115-mediated suppression of BET proteins leads to anti-leukemogenic phenotypes

Together with our results that BBC1115 is a small molecule inhibitor of BET BRDs, we were prompted to compare the therapeutic activity

**Figure 1. DEL screening identifies BBC1115 as a candidate BET inhibitor**

(A) Schematic diagram for rational design of DEL screening for BET-inhibitor compounds. (B) TR-FRET assay to determine the binding affinity of 20 candidate BET inhibitors of the BD1s and BD2s from BRD2, BRD3, and BRD4. (C) Western blot analysis of whole-cell lysates from murine AML (MLL-AF9; $Nras^{G12D}$) cells treated for 6 h with DMSO, RVX-208 (1 μM), OTX-015 (1 μM), or candidate BET inhibitors (10 μM). (D) Real-time quantitative reverse transcription PCR (RT-qPCR) of *Myc* mRNA in murine AML after 6 h treatment with the indicated BET inhibitors or DMSO. The results were normalized against *Tbp*. Mean ± SEM are shown. (E) Western blot analysis of whole-cell lysates from various human leukemia-derived cell lines treated for 48 h with DMSO, RVX-208, OTX-015, or BBC1115. (F) RT-qPCR analysis of *Hexim1* in murine AML after 6 h treatment with indicated drugs. (G) RVX-208, OTX-015, and BBC1115 chemical structures. See also Figure S1.

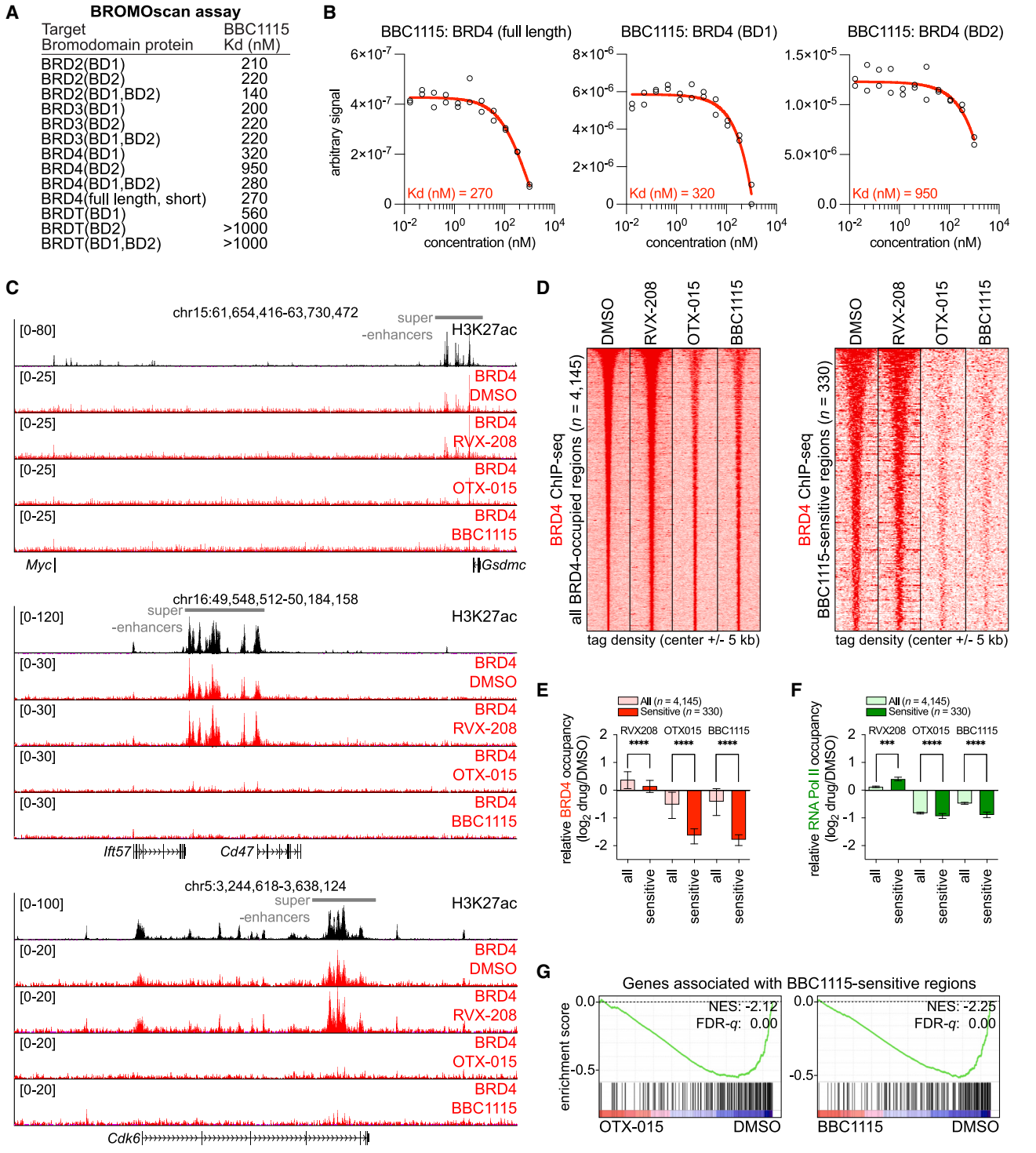



**Figure 2. BBC1115 selectively abrogates BRD4-mediated gene expression programs**

(A and B) BROMOscan assay. The binding affinity of BBC1115 for 13 BRDs are indicated by the $K_d$ values (A), represented by binding curves of BBC1115 to BRD4-BD1 and to BRD4-BD2 (B). (C) Representative ChIP-seq browser tracks of BRD4 and H3K27ac in murine AML cells treated with indicated drugs. Super enhancer regions are indicated

*(legend continued on next page)*

of BBC1115 to JQ1, a first-in-class BET inhibitor.[37] We obtained RNA-seq profiles of RVX-208-, OTX-015-, and BBC1115-treated murine AML cells and compared the effect of these BET inhibitors with JQ1 by focusing on JQ1-rapid targets. A total of 50 genes have been reported to decrease rapidly after a brief exposure to JQ1 in murine AML cells.[34] Consistent with its inability to suppress MYC expression, RVX-208 treatment downregulated some but not all JQ1-rapid targets. In contrast, OTX-015 and BBC1115 strongly downregulated all JQ1-rapid targets (Figure 3A).

Since BET proteins maintain a transcriptional program for self-renewal in AML cells, inhibiting BET proteins decreases the surface expression of Kit, a protein marker for leukemia stem cells (LSCs).[32] To examine if BBC1115 treatment affects LSCs, we performed a GSEA analysis and found that both OTX-015 and BBC1115 significantly suppressed the LSC signature genes, whereas changes driven by RVX-208 were insignificant (Figure 3B). Consistent with our observation, flow cytometric analyses showed that both OTX-015 and BBC1115 downregulated c-Kit in murine AML cells (Figure 3C). Furthermore, OTX-015 and BBC1115 treatment inhibited the proliferation of leukemia cells, while the ability to form colonies on methylcellulose was substantially suppressed (Figures 3D and 3E).

Motivated by these results, we compared the sensitivity of human leukemia cells with OTX-015 or BBC1115 by examining anti-proliferative activity in cell culture. As a result, we observed a broad growth-suppressive effect of OTX-015 in four of eight leukemia cell lines (MV4-11, KASUMI-1, MOLM-13, and THP-1) representing diverse disease subtypes (Figure 3F). On the contrary, the remaining four of eight leukemia cell lines (K562, NOMO-1, JURKAT, and HL-60) showed minimal sensitivity to the compound (Figure 3F). Similar to these results, we observed significant growth-inhibitory effects of BBC1115 on MV4-11, KASUMI-1, MOLM-13, and THP-1 cells (Figure 3G). Notably, we found that BBC1115 showed anti-proliferative activity in the four leukemia cell lines insensitive to OTX-015 with a moderate but more severe degree than OTX-015.

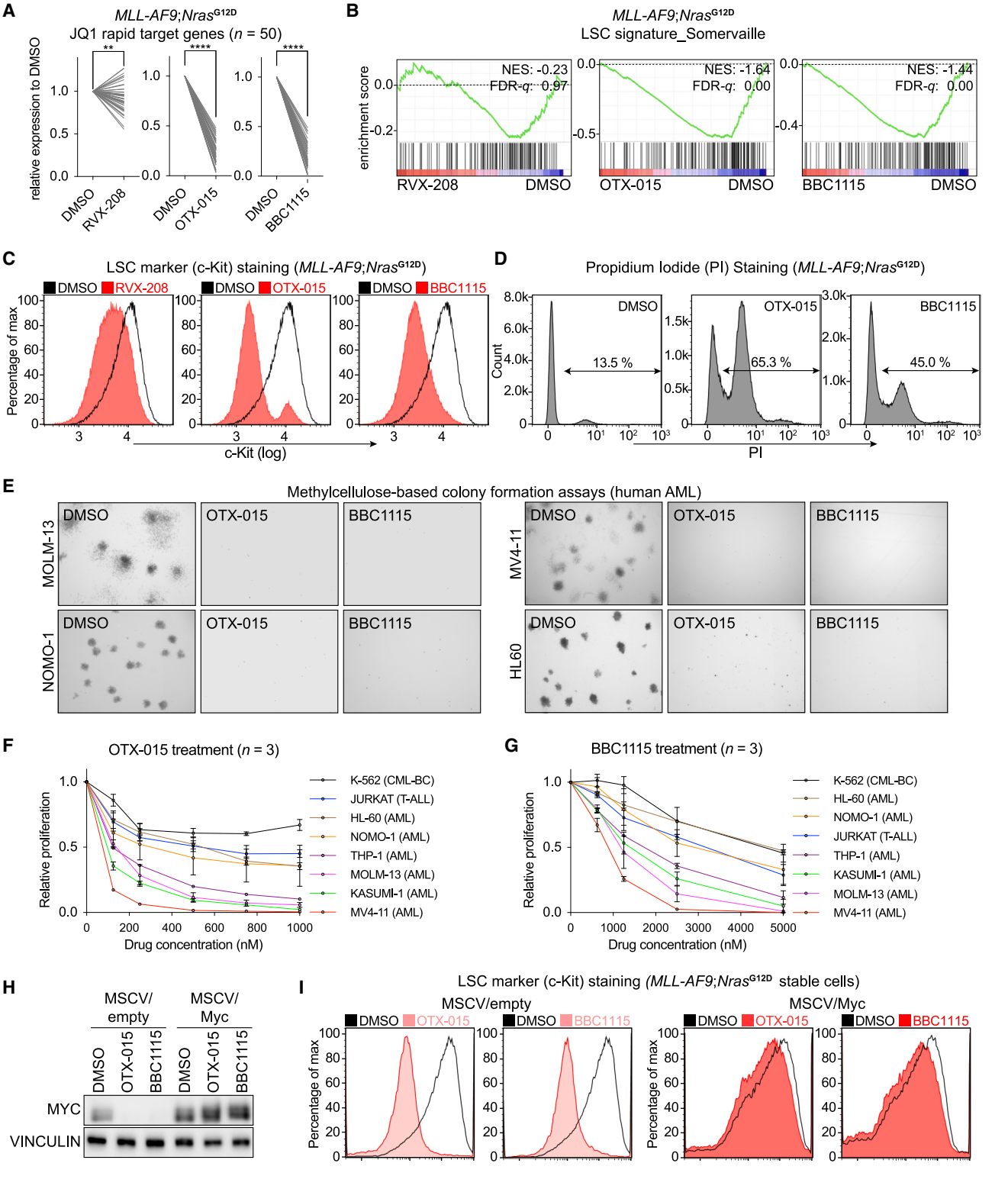
Next, to evaluate the on-target activity of BBC1115, we exploited the cDNA retroviral overexpression system. Since MYC is a well recognized downstream target of BRD4 and is responsible for the anti-leukemic activity of BET inhibition, ectopic expression of Myc cDNA is sufficient to prevent the growth suppression or differentiation phenotype of BET inhibitor.[32] Based on this, we established a murine AML cell culture ectopically expressing Myc cDNA from an MSCV retroviral promoter. As anticipated, we found that ectopic Myc expression conferred resistance to BBC1115, as for OTX-015 (Figure 3H). Furthermore, Myc restoration was sufficient to suppress phenotypic characteristics led by BBC1115 treatment, including reduced Myc protein expression, and, more important, decrease in the cell surface LSC marker, c-Kit (Figure 3I). These findings collectively support that OTX-015 and BBC1115 target Myc as a key downstream effector to maintain cancerous phenotypes. Furthermore, these results implicate that BBC1115 could potentially be a viable hit for further optimization for treating AML and other hematological malignancies.

### BBC1115 treatment suppresses proliferation of pancreatic, colorectal, and ovarian cancer cells *in vitro* and *in vivo*

Next, we investigated whether the anti-tumorigenic activity of BBC1115 is applicable to solid tumors since BET inhibitors are also effective for cancers other than hematologic malignancies. For example, there are several ongoing clinical trials evaluating the ability of BET inhibition to treat solid tumors.[40] To this end, we tested the anti-proliferative effect of BBC1115 in a pancreatic ductal adenocarcinoma (PDA) cell line AsPC-1 and a colorectal cancer cell line SW-480. As a result, OTX-015 and BBC1115 markedly inhibit clonogenic growth of AsPC-1 and SW-480 cells in a dose-dependent manner (Figures 4A and 4B). Similar to the effects on AML cells, BBC1115 decreased MYC and increased HEXIM1 expressions in these cell lines (Figure 4C; Figure S4). We next explored clinically relevant therapeutic activities of BBC1115. Recently, the organoid model of PDA has been established and offered a reliable platform for drug discovery and evaluation.[41,42] Thus, we asked whether BBC1115 shows comparable anti-proliferative effects in PDA organoids. In line with their ability to inhibit proliferation of monolayer cultures, microscopic analysis showed that treatment with OTX-015 and BBC1115, but not RVX-208, attenuated PDA organoid growth (Figure 4D), and these changes were statistically significant according to CellTiter Glo-3D assays (Figure 4E).

Our findings motivated us to examine if BBC1115 is also active in *in vivo* tumor models. Therefore, we first analyzed pharmacokinetic profiles in mice and rats injected with BBC1115 intravenously. We confirmed that the time needed to metabolize and eliminate BBC1115 from the bloodstream increased in a dose-dependent manner in both mice and rats (Figures 4F and 4G). To test if BBC1115 treatment inhibits tumor growth *in vivo*, we generated subcutaneous tumors by injecting AsPC-1 or SW-480 cells. When the tumors were 100–150 $mm^3$ in volume, the mice were intravenously injected with BBC1115 (12 times over 4 weeks). Of note, BBC1115 at doses of 25 and 35 mg/kg did not significantly affect body weight over the next 40–60 days (Figures 4H and 4I). In addition, we also included a xenograft model of the ovarian cancer cell line, SNU-251 since BRD4 amplification occurs most frequently in high-grade serous ovarian cancer patients.[43] Despite the tolerability of BBC1115

with gray above the genome browser tracks at *Myc*, *Cd47*, and *Cdk6* loci. (D) BRD4 ChIP-seq density plot analysis of DMSO or BET inhibitors treated murine AML cells at indicated genomic regions. (E and F) Relative BRD4 occupancy (E) and RNA Pol II occupancy (F) in murine AML cells treated with indicated drugs. ***$p < 0.001$; ****$p < 0.0001$. (G) GSEA of DMSO vs. OTX-015 or BBC1115 RNA-seq data using a custom gene set that was associated with 330 BBC1115-sensitive regions. The GSEA provided the normalized enrichment score (NES) and false discovery rate (FDR) *q*-value. See also Figure S2 and S3.




(legend on next page)

administration, the growth of pancreatic, colorectal, and ovarian tumors was significantly decreased by BBC1115 compared with its vehicle-treated counterpart (Figures 4J and 4K; Figure S5), suggesting the potential use of BBC1115 as a therapeutic reagent in cancer treatment.

In conclusion, our collective findings indicate that DEL screening may be useful as a strategy for expanding available chemical space and opening opportunities for overcoming limitations of the existing chemical matter by discovering new chemotypes with potent and selective activity against established targets.

## DISCUSSION

This study showed that the DEL screening platform can identify novel and unique chemotypes against well established targets in a robust, straightforward, efficient, unbiased, and relatively quick manner. This is especially important for those targets, such as BET proteins, for which inhibitors currently in clinical testing have shown clinical benefit at the cost of diverse adverse effects.[20] Here, the deployment of a DEL platform selected to include a broad range of chemotypes and enable access to thousands of diverse compounds led us to BBC1115, a pan inhibitor of BET proteins that is chemically distinct from the existing inhibitors. Our analyses with this agent showed that it was as effective as the previously identified pan-BET inhibitor OTX-015 in terms of suppressing MYC, JQ1-rapid target genes, and LSC-like signature genes in AML cells. BBC1115 also inhibited the *in vitro* growth of AsPC-1 pancreatic, SW-480 colorectal, and SNU-251 ovarian carcinoma cells, the *ex vivo* growth of pancreatic organoids, and the *in vivo* xenograft tumor growth of pancreatic, colorectal, and ovarian carcinoma cells. Thus, BBC1115 represents a useful addition to the currently available arsenal of compounds targeting BET proteins. Importantly, BBC1115 has anti-tumor effects on cancers of varying lineages and against both models of hematological malignancies and solid tumors.

Our study highlights the usefulness of DEL screening for the discovery of inhibitors that are structurally distinct from those identified through structure-guided approaches, which tend to limit chemical features to the same and/or closely related set of derivatives. For instance, BBC1115 and OTX-015 do not resemble each other structurally, yet BBC1115 exerts epigenome-regulating activities like OTX-015.[44] Furthermore, both compounds have a significantly higher affinity for BRD4 BD1 than BD2, and their effect on BRD4 is likely to be primarily driven by BD1 binding. We also examined binding to other BET proteins and our TR-FRET and BROMOscan analyses showed that BBC1115 also binds to BD1 and BD2 of BRD2 and BRD3 and represents a pan-BET inhibitor. This pan-BET activity is considered to be beneficial for clinical applications, as it is believed to contribute to enhanced efficacy *in vivo*.[45]

In terms of mechanisms of action, we proposed that BBC1115 competes with acetylated lysine for binding to BRD4. This was confirmed by our observation that BBC1115 binding to BRD4 resulted in dissociation of BRD4 from chromatin regions enriched with acetylated lysine. Furthermore, treatment with BBC1115 suppressed aberrant self-renewal and cell fate programs in AML and attenuated the proliferation of pancreatic, colorectal, and ovarian cancer cells. Our study also indicated that these effects of BBC1115 (and OTX-015) are mediated by displacing BRD4 from chromatin in only a subset of sites (330 of 4,145 BRD4-occupied sites in AML cells), thus shutting down the epigenetic reading of and aberrant transcription from these sites.

In agreement with our results from cell-based assays, *in vitro* binding assay (BROMOscan) also confirmed that BBC1115 phenocopies OTX-015 as indicated by tight but permissive binding affinity toward BD1 and BD2. While OTX-015 and BBC1115 do not show significantly different binding affinities between BD1 and BD2 of BRD2/3/4, RVX-208, a quinazolone compound, had shown an approximately 10- to 100-fold stronger binding affinity toward BD2 than BD1.[19,37,46–48] Despite differences in the core chemical structure, our results suggested that BBC1115 harbors pharmacological activities in cells similar to OTX-015, not to RVX-208.

However, several limitations remain to making DEL screening based on the identification of chemical compounds a promising and reliable strategy to develop epigenome-targeted therapeutics for cancer treatment. First, given the role of epigenetic modulation in cellular homeostasis, determining the specificity toward epigenetic addiction in cancer cells, not in normal cells, must be the highest priority. Extended from the first problem, the second issue to be addressed is that it is necessary to better understand the molecular mechanism of epigenetic addictions in the different cancer cells. For example, it is reasonable to think that major epigenetic alterations in hematological malignancies and solid tumors are independent. Therefore, to determine the use of epigenome-targeting compounds, such as BBC1115, in distinct types of cancer and to consider epigenome-targeted therapy as an effective adjunctive therapy (i.e., the combination of immunotherapy and epigenetic therapy), further efforts need to be made to

**Figure 3. BBC1115 treatment exerts anti-leukemogenic phenotypes**

(A) Relative mRNA levels of JQ1-rapid target genes (n = 50) in murine AML cells that were treated with RVX-208, OTX-015, or BBC1115. Relative expression was calculated by dividing normalized expression level of drug-treated to DMSO-treated RNA-seq data. (B) GSEA of LSC signature genes in murine AML cells that were treated with RVX-208, OTX-015, or BBC1115. (C and D) Flow cytometric analysis of c-Kit (C) and propidium iodide (PI)-positive AML cells treated for 48 h with DMSO, RVX-208, OTX-015, or BBC1115. (E) Semi-solid methylcellulose-based colony formation of DMSO, RVX-208, OTX-015, or BBC1115 in human AML cells. (F and G) Proliferation rate of human leukemia cells treated with OTX-015 (F) or BBC1115 (G), and results are normalized to the proliferation rate of DMSO-treated cells (n = 3, mean ± SEM). (H) Western blotting of whole-cell lysates prepared from murine leukemia cells transduced with empty vector or a Myc-cDNA-containing MSCV retrovirus. Cells were treated with DMSO, OTX-015, or BBC1115 for 6 h. (I) Flow cytometry analysis of c-Kit surface expression after 2 days of OTX-015 or BBC1115 treatment in indicated cells.

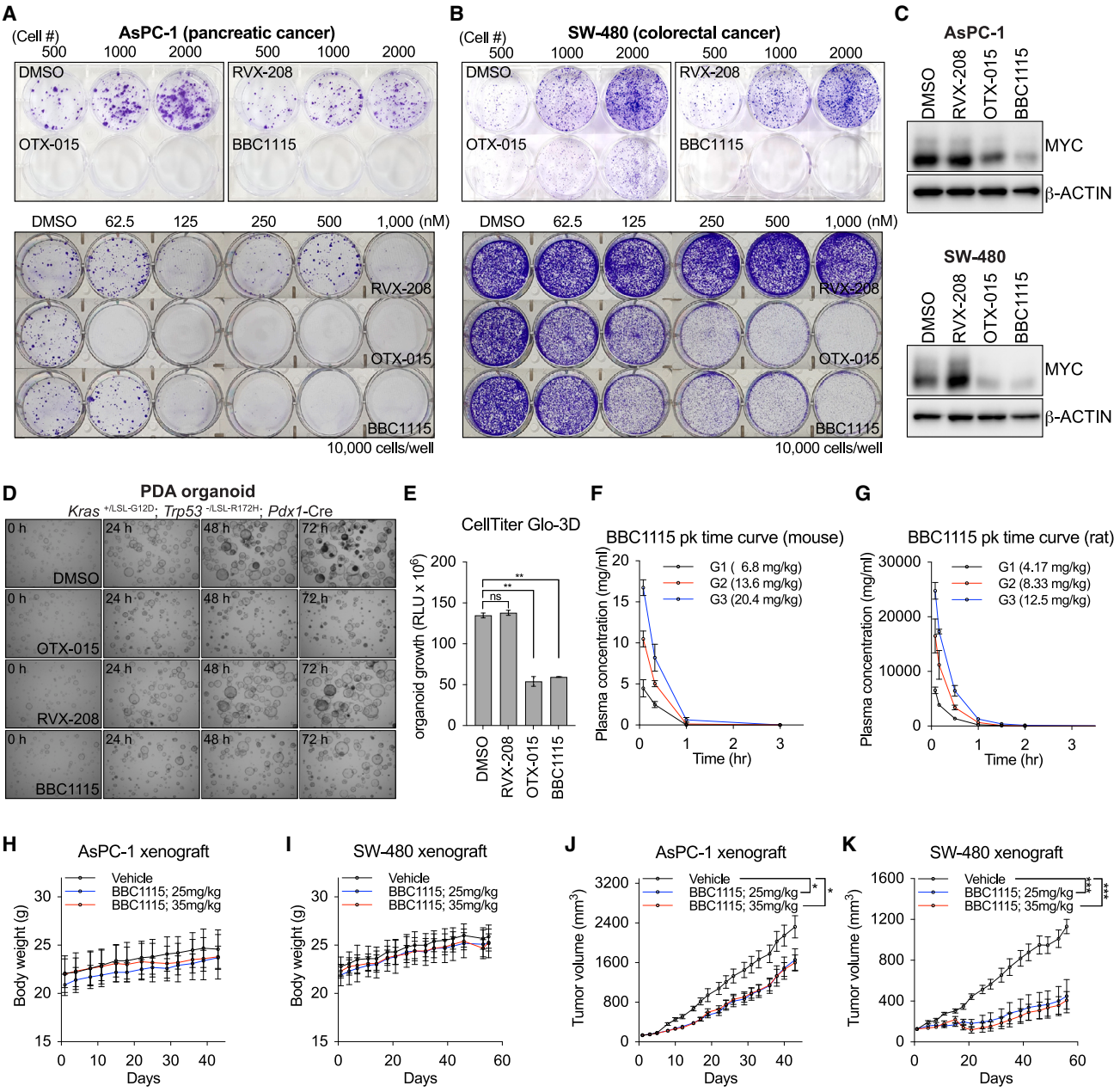



**Figure 4. BBC1115 administration impairs pancreatic and colorectal tumor growth *in vivo***

(A and B) Colony formation of AsPC-1 (A) and SW-480 (B) cells after 5 days of treatment with DMSO, RVX-208, OTX-015, or BBC1115. (C) Western blot analysis of AsPC-1 and SW-480 cells after treatment with the indicated drugs for 3 days. (D) Growth of PDA organoids 24, 48, and 72 h after treatment with DMSO, RVX-208, OTX-015, or BBC1115, as indicated by light microscopy. (E) CellTiter Glo-3D-based quantification of drug-treated PDA organoids. Mean ± SEM are shown. p-values were determined by Student's t test. **$p < 0.005$. (F and G) Pharmacokinetic profile of BBC1115. The mean plasma concentration-time curves are shown for BBC1115 after intravenous injection in mice (F) and rats (G). (H and I) Effect of intravenous BBC1115 injections on the body weight of AsPC-1 (H) and SW-480 (I) tumor xenografts. (J and K) Effect of intravenous BBC1115 injections on the tumor volumes of AsPC-1 (J) and SW-480 (K) xenografts. Mean ± SEM are shown. p Values were determined by Student's t test. *$p < 0.05$; ***$p < 0.0001$. See also Figure S4 and S5.

explore the specific mechanisms underlying epigenetic alterations in different cancers. Finally, both experimental and computational approaches must be used to determine ligand-protein interactions following DEL screening. This will enable an understanding of the distinct binding modality and provide information for hit-to-lead optimization.

Our *in vivo* studies demonstrated activity of BBC1115 in three solid tumor models, pancreatic, colorectal, and ovarian; however, it should be noted that the anti-cancer effects of BBC1115 were only obtained at high doses (25–35 mg/kg). This suggests that structural optimization of BBC1115 would be necessary to maximize its performance. Nonetheless, our study portrays that combining DEL screening platform with thorough biological and functional assessment could accelerate the discovery of novel chemotypes with potentially improved properties for established, high-value cancer drug targets.

## MATERIALS AND METHODS

### DEL screening with BRDs from BRD2, BRD3, and BRD4

Novel BET-inhibitor chemicals were identified by screening a DEL (WuXi AppTec, Shanghai, China) with the BD1s and BD2s from the BRD2, BRD3, and BRD4 proteins. These proteins were generated by amplifying the relevant sections of the *BRD2–4*-encoding genes, placing the sections into pET28a vectors (Novagen, Northumberland, UK), transforming *Escherichia coli* BL21(DE3) (Novagen) with each plasmid, and inducing protein expression by treatment with 0.2 mM isopropyl β-D-thiogalactopyranoside (Sigma, St. Louis, MO) for 16 h at 18°C. The bacteria were then collected, resuspended in lysis buffer (50 mM Tris, pH 8.2, with 300 mM NaCl and 20 mM imidazole), and sonicated. The lysates were centrifuged at 1,550×*g* for 1 h at 4°C, after which the supernatant was loaded onto a nickel affinity HisTrap HP column (GE Healthcare, Chicago, IL). After washing the column, BRD2, BRD3, or BRD4 was eluted with 50 mM Tris (pH 8.2) containing 300 mM NaCl and 500 mM imidazole.

### TR-FRET assay

TR-FRET assay kits from BPS Bioscience (San Diego, CA) that were specific for BRD2 BD1 (Cat# 31022), BRD2 BD2 (Cat# 32522), BRD3 BD1 (Cat# 32513), BRD3 BD2 (Cat# 32523), BRD4 BD1 (Cat# 32613), and BRD4 BD2 (Cat# 32617) were used. The BRD ligands were diluted with water. A master mix consisting of 1× BRD Homogeneous Assay Buffer and diluted BRD ligands was prepared. The BRD proteins were thawed on ice and diluted with 1× BRD Homogeneous Assay Buffer. Subsequently, 1.5 μL master mix was added to each well of a microplate (Cat# LP-0200; Labcyte) and reactions were initiated by adding 5 μL diluted BRD protein to each well. The plate was incubated at room temperature for 30–60 min. GSH Acceptor beads (Cat#AL109C) and Streptavidin-conjugated donor beads (Cat# 6760002S) (both from PerkinElmer, Waltham, MA) were diluted with 1× BRD Homogeneous Detection Buffer 1, after which a 10-μL acceptor bead mixture was added to each well. After incubation at room temperature for 30 min, a 10-μL donor bead mixture was added to each well, followed by incubation at room temperature for 15–30 min. Alpha-counts were read with an EnVision 2105 multimode plate reader (Cat# 2105-0010, PerkinElmer).

### BROMOscan

BBC1115 was dissolved in DMSO at a concentration of 1 mM in 1,000× stock. $K_d$s were measured using an 11-point, 3-fold compound dilution series with 1 DMSO control point. The compounds were then diluted directly into the assays such that the final concentration of DMSO was 0.09%. All reactions were performed in polypropylene 384-well plates in a final volume of 0.02 mL/well. The assay plates were incubated at room temperature with shaking for 1 h, and the affinity beads were washed with wash buffer (1× PBS, 0.05% Tween 20). The beads were then resuspended in elution buffer (1× PBS, 0.05% Tween 20, 2 μM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 min. The BRD concentration in the eluates was measured by qPCR. BROMOscan was then used to determine the $K_d$s to determine the binding affinity of BBC1115 for epigenetic proteins, including BRD2–4. If the initial $K_d$ determined was <0.169 nM (the lowest concentration tested), the measurement was repeated with a serial dilution starting at a lower maximum concentration.

### Mouse studies

ICR mice, SCID mice, athymic mice, and SD-rats were obtained from Orient Bio (Seoul, Korea) and housed under pathogen-free conditions. The animal care was in line with the guidelines of the animal care facility at Biotoxtech Co. Ltd. (Chungcheong Buk-Do, Korea).

### BBC1115 pharmacokinetics

To analyze the pharmacokinetics of BBC1115, ICR mice were intravenously injected with BBC1115 (6.8, 13.6, or 20.4 mg/kg). Alternatively, SD-rats were intravenously injected with BBC1115 (4.17, 8.33, or 12.5 mg/kg). Blood was collected at various time intervals by heart puncture. It was mixed with 3.8% (w/v) sodium citrate and plasma was obtained by centrifuging the samples at 8,000×*g* for 30 min. Mouse or rat plasma samples were then mixed with acetonitrile (33%, v/v), vortexed for 5 min, centrifuged at 13,000×*g* for 10 min. After sample preparation, the concentration of BBC1115 in the plasma was analyzed by UV-absorbance measurement by HPLC-MS. Thus, separation was conducted with the ACQUITYTM UPLC H-Class Plus system (Waters, Milford, MA), which consisted of a quaternary solvent manager, a QDaTM mass detector, and an FTN-H sample manager. Acquity UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm) columns (Waters, Milford, MA) were used. The mobile phase initially consisted of 40% acetonitrile and 60% 10 mM ammonium formate aqueous solution. The mobile phase buffer for ionization was 10 mM ammonium formate. The total analysis time was 5 min, including the 3-min re-equilibration time. The mobile phase was sonicated before use. Separation was conducted at a constant flow rate of 0.3 mL/min with a 2-μL injection volume. The column and autosampler temperatures were set at 40°C and 15°C, respectively. A mass spectrometer was operated in positive electrospray ionization-single ion reaction) mode for all analyses and high-purity nitrogen served as the nebulizing gas. The capillary voltage was 0.8 kV and the source and desolvation temperatures were set at 150°C and 600°C, respectively. Single-ion transition was monitored at 540.2 m/z.

### ChIP assay and ChIP-qPCR

The ChIP assay was performed as described previously.[49] Briefly, 30 million murine AML cells were cross-linked and then incubated for 10 min at 4°C with 600 μL cell lysis buffer containing protease

inhibitor (cOmplete Protease Inhibitor Cocktail, Cat#11873580001; Roche, Basel, Switzerland) and 1 mM DTT (Cat# BP172, Fisher Bioreagents, Waltham, MA). After centrifugation at 7,400 rpm for 30 s, the isolated nuclei were gently resuspended in 600 μL nucleus lysis buffer containing protease inhibitor and 1 mM DTT. The chromatin lysate was then sonicated for 10 cycles (30 s on/30 s off) and centrifuged at 13,000 rpm for 15 min at 4°C. To pre-clear the supernatant, it was incubated for 1 h with 2,400 μL IP dilution buffer, 30 μg rabbit IgG (Cat# I8140, Sigma), and 30 μL Protein A magnetic beads (Cat# 10002D, Invitrogen, Waltham, MA). Immunoprecipitation was then conducted overnight in a 4°C rotator with 3 mL pre-cleared chromatin, 3 μg antibody, and 30 μL Protein A magnetic beads. The antibody was specific for BRD4 (Cat# A301-985A50, Bethyl Laboratories, Montgomery, TX) or H3K27Ac (Cat# ab4729, Abcam, Cambridge, UK) or RNA Pol II phospho-Ser5 (Cat# ab5131, Abcam). The next day, the immunocomplexes were washed six times and eluted with 200 μL elution buffer for 60 min in a 45°C thermomixer at 1,000 rpm. The elute was de-cross-linked with RNase A (1 μg/μL) and 0.25 M NaCl and then incubated overnight at 65°C in a water bath. The next day, 4 μL Proteinase K (Cat# P8107S; New England Biolabs, Ipswich, MA) was added. After 2 h incubation at 42°C, the immunoprecipitated DNA was purified with a QIAquick PCR purification kit (Cat# 28106; Qiagen, Hilden, Germany) in 50 μL elution buffer, after which 2 μL of 1:10 diluted ChIP DNA was subjected to ChIP-qPCR with 1 μL 10 μM primer and SYBR Green Master Mix. All ChIP-qPCR primers are shown in Table S2.

#### ChIP-seq library construction

ChIP-seq libraries were constructed from 40 μL ChIP DNA by using a NEXTflex ChIP-seq kit (Cat# NOVA-5143-02; PerkinElmer) according to the manufacturer's instructions. Briefly, after the end-repair and size selection step, adenylation, adapter ligation, and PCR amplification were performed. The quality and size of the ChIP-seq libraries were confirmed by Bioanalyzer using the High Sensitivity chip (Agilent, Santa Clara, CA). The average size was 250–350 bp. For multiplexing, equal molar quantities of libraries were combined to consider the sequencing depth per sample (20–40 million reads per library). ChIP-seq libraries were then sequenced using an Illumina NextSeq platform with single-end reads of 76 bases.

#### RNA-seq library construction

The RNA-seq libraries were constructed with the NEXTflex Rapid Directional mRNA-seq kit (Cat# NOVA-5138-11; PerkinElmer). Thus, total RNA was purified with QIAzol and the mRNA in 10 μg was isolated by the poly-(A) selection step. After mRNA fragmentation, cDNA was synthesized. After adenylation and adapter ligation, a library was constructed via PCR amplification.

#### Bioinformatic analyses of ChIP-seq and RNA-seq

To align the ChIP-seq, the raw reads were mapped to the reference mouse genome assembly (mm9) by using Bowtie2. SAMtools was used to remove duplicate reads. To permit visualization with the UCSC genome browser, a bigWig file was created by using the makeBigWig tool (in HOMER suite). A density plot was created by using the annotatePeaks tool (in HOMER suite) by centering the BRD4-occupied regions with extension to ±5,000 bp (-size 10000 -hist 25 -ghist). To align the RNA-seq, raw reads were mapped to the reference mouse genome assemblies (mm10) by using STAR tool. Cuffllinks tools were used to analyze differentially expressed genes. To generate a customized set of genes for the BBC1115-sensitive regions, the genes with the closest TSS from the BBC1115-sensitive regions were assigned. GSEA was then performed according to the instructions. The gene set information used in the GSEA analysis is listed in Table S5.

#### Xenograft modeling of pancreatic and colorectal tumors

The human SW-480 colon cancer cell line, the human pancreas adenocarcinoma cell line AsPC-1, and the human SNU-251 ovarian cancer cell line were grown as described above. To generate the colon cancer xenograft model, the dorsal flanks of 6-week-old athymic male BALB/c mice were subcutaneously injected with $0.5 \times 10^7$ SW-480 cells. To generate the pancreas and ovarian cancer xenograft models, the dorsal flanks of 6-week-old male SCID mice were subcutaneously injected with $0.5 \times 10^7$ AsPC-1 and SNU-251 cells. When the tumors were at least 100–150 $mm^3$ in volume, the mice were randomly divided into two groups of three mice (day 0). One group was injected intravenously 12 times over 4 weeks with 100 μL BBC1115 (25 or 35 mg/kg). The other group only received the vehicle. The murine tumors were measured at various time points with vernier calipers. Tumor volume was calculated by using the formula (length × width × height)/2.

## DATA AVAILABILITY

The ChIP-seq and RNA-seq data reported in this study are available in the Gene Expression Omnibus (GEO) database under GSE198843.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.04.023.

## ACKNOWLEDGMENTS

This study was supported by grants from the National Research Foundation of Korea (2021R1A2C4001420) and Samsung Science & Technology Foundation (SSTF-BA2002-12) to J.R.; the Technology development Program funded by the Ministry of SMEs and Startups (S3029615) to J.R. and I.L.; the Brain Korea 21 FOUR Program also supported S.J., H.K., J.S., and J.R.

## AUTHOR CONTRIBUTIONS

S.J., H.K., J.S., M.S., I.L., and K.Y. performed experiments and generated, analyzed, interested data. S.J., H.K., M.S., and J.R. wrote original draft. J.R., S.J., and I.L. conceived of the idea, designed experiments, and provided funding for this study. J.R. supervised throughout this study.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES


1. Flavahan, W.A., Gaskell, E., and Bernstein, B.E. (2017). Epigenetic plasticity and the hallmarks of cancer. Science *357*, eaal2380. https://doi.org/10.1126/science.aal2380.
2. Dawson, M.A., and Kouzarides, T. (2012). Cancer epigenetics: from mechanism to therapy. Cell *150*, 12–27. https://doi.org/10.1016/j.cell.2012.06.013.
3. Cheng, Y., He, C., Wang, M., Ma, X., Mo, F., Yang, S., Han, J., and Wei, X. (2019). Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. Signal Transduct. Target. Ther. *4*, 62. https://doi.org/10.1038/s41392-019-0095-0.
4. Marks, P.A., and Dokmanovic, M. (2005). Histone deacetylase inhibitors: discovery and development as anticancer agents. Expert Opin. Investig. Drugs *14*, 1497–1511. https://doi.org/10.1517/13543784.14.12.1497.
5. Gnyszka, A., Jastrzebski, Z., and Flis, S. (2013). DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. Anticancer Res. *33*, 2989–2996.
6. Rothbart, S.B., and Baylin, S.B. (2020). Epigenetic therapy for epithelioid sarcoma. Cell *181*, 211. https://doi.org/10.1016/j.cell.2020.03.042.
7. Nepali, K., and Liou, J.P. (2021). Recent developments in epigenetic cancer therapeutics: clinical advancement and emerging trends. J. Biomed. Sci. *28*, 27. https://doi.org/10.1186/s12929-021-00721-x.
8. Ganesan, A., Arimondo, P.B., Rots, M.G., Jeronimo, C., and Berdasco, M. (2019). The timeline of epigenetic drug discovery: from reality to dreams. Clin. Epigenetics *11*, 174. https://doi.org/10.1186/s13148-019-0776-0.
9. French, C.A., Miyoshi, I., Kubonishi, I., Grier, H.E., Perez-Atayde, A.R., and Fletcher, J.A. (2003). BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. Cancer Res. *63*, 304–307.
10. Sarnik, J., Popławski, T., and Tokarz, P. (2021). BET proteins as attractive targets for cancer therapeutics. Int. J. Mol. Sci. *22*, 11102. https://doi.org/10.3390/ijms222011102.
11. Slavish, P.J., Chi, L., Yun, M.K., Tsurkan, L., Martinez, N.E., Jonchere, B., Chai, S.C., Connelly, M., Waddell, M.B., Das, S., et al. (2020). Bromodomain-selective BET inhibitors are potent antitumor agents against MYC-driven pediatric cancer. Cancer Res. *80*, 3507–3518. https://doi.org/10.1158/0008-5472.CAN-19-3934.
12. Vollmuth, F., Blankenfeldt, W., and Geyer, M. (2009). Structures of the dual bromodomains of the P-TEFb-activating protein Brd4 at atomic resolution. J. Biol. Chem. *284*, 36547–36556. https://doi.org/10.1074/jbc.M109.033712.
13. Filippakopoulos, P., and Knapp, S. (2014). Targeting bromodomains: epigenetic readers of lysine acetylation. Nat. Rev. Drug Discov. *13*, 337–356. https://doi.org/10.1038/nrd4286.
14. Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K., and Zhou, M.M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature *399*, 491–496. https://doi.org/10.1038/20974.
15. Dawson, M.A., Prinjha, R.K., Dittmann, A., Giotopoulos, G., Bantscheff, M., Chan, W.I., Robson, S.C., Chung, C.W., Hopf, C., Savitski, M.M., et al. (2011). Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature *478*, 529–533. https://doi.org/10.1038/nature10509.
16. Leal, A.S., Williams, C.R., Royce, D.B., Pioli, P.A., Sporn, M.B., and Liby, K.T. (2017). Bromodomain inhibitors, JQ1 and I-BET 762, as potential therapies for pancreatic cancer. Cancer Lett. *394*, 76–87. https://doi.org/10.1016/j.canlet.2017.02.021.
17. Doroshow, D.B., Eder, J.P., and LoRusso, P.M. (2017). BET inhibitors: a novel epigenetic approach. Ann. Oncol. *28*, 1776–1787. https://doi.org/10.1093/annonc/mdx157.
18. Lewin, J., Soria, J.C., Stathis, A., Delord, J.P., Peters, S., Awada, A., Aftimos, P.G., Bekradda, M., Rezai, K., Zeng, Z., et al. (2018). Phase Ib trial with birabresib, a small-molecule inhibitor of bromodomain and extraterminal proteins, in patients with selected advanced solid tumors. J. Clin. Oncol. *36*, 3007–3014. https://doi.org/10.1200/JCO.2018.78.2292.
19. Picaud, S., Wells, C., Felletar, I., Brotherton, D., Martin, S., Savitsky, P., Diez-Dacal, B., Philpott, M., Bountra, C., Lingard, H., et al. (2013). RVX-208, an inhibitor of BET transcriptional regulators with selectivity for the second bromodomain. Proc. Natl. Acad. Sci. USA *110*, 19754–19759. https://doi.org/10.1073/pnas.1310658110.
20. Amorim, S., Stathis, A., Gleeson, M., Iyengar, S., Magarotto, V., Leleu, X., Morschhauser, F., Karlin, L., Broussais, F., Rezai, K., et al. (2016). Bromodomain inhibitor OTX015 in patients with lymphoma or multiple myeloma: a dose-escalation, open-label, pharmacokinetic, phase 1 study. Lancet. Haematol. *3*, e196–e204. https://doi.org/10.1016/S2352-3026(16)00021-1.
21. Bohacek, R.S., McMartin, C., and Guida, W.C. (1996). The art and practice of structure-based drug design: a molecular modeling perspective. Med. Res. Rev. *16*, 3–50.
22. Madsen, D., Azevedo, C., Micco, I., Petersen, L.K., and Hansen, N.J.V. (2020). An overview of DNA-encoded libraries: a versatile tool for drug discovery. Prog. Med. Chem. *59*, 181–249. https://doi.org/10.1016/bs.pmch.2020.03.001.
23. Rianjongdee, F., Atkinson, S.J., Chung, C.W., Grandi, P., Gray, J.R.J., Kaushansky, L.J., Medeiros, P., Messenger, C., Phillipou, A., Preston, A., et al. (2021). Discovery of a highly selective BET BD2 inhibitor from a DNA-encoded library technology screening hit. J. Med. Chem. *64*, 10806–10833. https://doi.org/10.1021/acs.jmedchem.1c00412.
24. Rectenwald, J.M., Guduru, S.K.R., Dang, Z., Collins, L.B., Liao, Y.E., Norris-Drouin, J.L., Cholensky, S.H., Kaufmann, K.W., Hammond, S.M., Kireev, D.B., et al. (2020). Design and construction of a focused DNA-encoded library for multivalent chromatin reader proteins. Molecules *25*, 979. https://doi.org/10.3390/molecules25040979.
25. Modukuri, R.K., Yu, Z., Tan, Z., Ta, H.M., Ucisik, M.N., Jin, Z., Anglin, J.L., Sharma, K.L., Nyshadham, P., Li, F., et al. (2022). Discovery of potent BET bromodomain 1 stereoselective inhibitors using DNA-encoded chemical library selections. Proc. Natl. Acad. Sci. USA *119*. e2122506119. https://doi.org/10.1073/pnas.2122506119.
26. Wang, S., Alpsoy, A., Sood, S., Ordonez-Rubiano, S.C., Dhiman, A., Sun, Y., Jiao, G., Krusemark, C.J., and Dykhuizen, E.C. (2021). A potent, selective CBX2 chromodomain ligand and its cellular activity during prostate cancer neuroendocrine differentiation. Chembiochem *22*, 2335–2344. https://doi.org/10.1002/cbic.202100118.
27. Zhou, Y., Shen, W., Peng, J., Deng, Y., and Li, X. (2021). Identification of isoform/domain-selective fragments from the selection of DNA-encoded dynamic library. Bioorg. Med. Chem. *45*, 116328. https://doi.org/10.1016/j.bmc.2021.116328.
28. Fernández-Montalván, A.E., Berger, M., Kuropka, B., Koo, S.J., Badock, V., Weiske, J., Puetter, V., Holton, S.J., Stöckigt, D., Ter Laak, A., et al. (2017). Isoform-selective ATAD2 chemical probe with novel chemical structure and unusual mode of action. ACS Chem. Biol. *12*, 2730–2736. https://doi.org/10.1021/acschembio.7b00708.
29. Kunig, V., Potowski, M., Gohla, A., and Brunschweiger, A. (2018). DNA-encoded libraries - an efficient small molecule discovery technology for the biomedical sciences. Biol. Chem. *399*, 691–710. https://doi.org/10.1515/hsz-2018-0119.
30. Shorstova, T., Foulkes, W.D., and Witcher, M. (2021). Achieving clinical success with BET inhibitors as anti-cancer agents. Br. J. Cancer *124*, 1478–1490. https://doi.org/10.1038/s41416-021-01321-0.
31. Donati, B., Lorenzini, E., and Ciarrocchi, A. (2018). BRD4 and Cancer: going beyond transcriptional regulation. Mol. Cancer *17*, 164. https://doi.org/10.1186/s12943-018-0915-9.
32. Zuber, J., Shi, J., Wang, E., Rappaport, A.R., Herrmann, H., Sison, E.A., Magoon, D., Qi, J., Blatt, K., Wunderlich, M., et al. (2011). RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature *478*, 524–528. https://doi.org/10.1038/nature10334.
33. Lin, X., Huang, X., Uziel, T., Hessler, P., Albert, D.H., Roberts-Rapp, L.A., McDaniel, K.F., Kati, W.M., and Shen, Y. (2017). HEXIM1 as a robust pharmacodynamic marker for monitoring target engagement of BET family bromodomain inhibitors in tumors and surrogate tissues. Mol. Cancer Ther. *16*, 388–396. https://doi.org/10.1158/1535-7163.MCT-16-0475.
34. Roe, J.S., Mercan, F., Rivera, K., Pappin, D.J., and Vakoc, C.R. (2015). BET bromodomain inhibition suppresses the function of hematopoietic transcription factors in acute myeloid leukemia. Mol. Cell *58*, 1028–1039. https://doi.org/10.1016/j.molcel.2015.04.011.
35. Riveiro, M.E., Astorgues-Xerri, L., Vazquez, R., Frapolli, R., Kwee, I., Rinaldi, A., Odore, E., Rezai, K., Bekradda, M., Inghirami, G., et al. (2016). OTX015 (MK-8628), a novel BET inhibitor, exhibits antitumor activity in non-small cell and small cell lung cancer models harboring different oncogenic mutations. Oncotarget *7*, 84675–84687. https://doi.org/10.18632/oncotarget.13181.
36. Smith, S.G., Sanchez, R., and Zhou, M.M. (2014). Privileged diazepine compounds and their emergence as bromodomain inhibitors. Chem. Biol. *21*, 573–583. https://doi.org/10.1016/j.chembiol.2014.03.004.

37. Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., et al. (2010). Selective inhibition of BET bromodomains. Nature *468*, 1067–1073. https://doi.org/10.1038/nature09504.

38. Zhao, Y., Liu, Q., Acharya, P., Stengel, K.R., Sheng, Q., Zhou, X., Kwak, H., Fischer, M.A., Bradner, J.E., Strickland, S.A., et al. (2016). High-Resolution mapping of RNA polymerases identifies mechanisms of sensitivity and resistance to BET inhibitors in t(8;21) AML. Cell Rep. *16*, 2003–2016. https://doi.org/10.1016/j.celrep.2016.07.032.

39. Jang, M.K., Mochizuki, K., Zhou, M., Jeong, H.S., Brady, J.N., and Ozato, K. (2005). The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. Mol. Cell *19*, 523–534. https://doi.org/10.1016/j.molcel.2005.06.027.

40. Sahai, V., Redig, A.J., Collier, K.A., Eckerdt, F.D., and Munshi, H.G. (2016). Targeting BET bromodomain proteins in solid tumors. Oncotarget *7*, 53997–54009. https://doi.org/10.18632/oncotarget.9804.

41. Ponz-Sarvise, M., Corbo, V., Tiriac, H., Engle, D.D., Frese, K.K., Oni, T.E., Hwang, C.I., Öhlund, D., Chio, I.I.C., Baker, L.A., et al. (2019). Identification of resistance pathways specific to malignancy using organoid models of pancreatic cancer. Clin. Cancer Res. *25*, 6742–6755. https://doi.org/10.1158/1078-0432.CCR-19-1398.

42. Seppälä, T.T., Zimmerman, J.W., Suri, R., Zlomke, H., Ivey, G.D., Szabolcs, A., Shubert, C.R., Cameron, J.L., Burns, W.R., Lafaro, K.J., et al. (2022). Precision medicine in pancreatic cancer: patient-derived organoid pharmacotyping is a predictive biomarker of clinical treatment response. Clin. Cancer Res. *28*, 3296–3307. https://doi.org/10.1158/1078-0432.CCR-21-4165.

43. Rhyasen, G.W., Yao, Y., Zhang, J., Dulak, A., Castriotta, L., Jacques, K., Zhao, W., Gharahdaghi, F., Hattersley, M.M., Lyne, P.D., et al. (2018). BRD4 amplification facilitates an oncogenic gene expression program in high-grade serous ovarian cancer and confers sensitivity to BET inhibitors. PLoS One *13*, e0200826. https://doi.org/10.1371/journal.pone.0200826.

44. Ozer, H.G., El-Gamal, D., Powell, B., Hing, Z.A., Blachly, J.S., Harrington, B., Mitchell, S., Grieselhuber, N.R., Williams, K., Lai, T.H., et al. (2018). BRD4 profiling identifies critical chronic lymphocytic leukemia oncogenic circuits and reveals sensitivity to PLX51107, a novel structurally distinct BET inhibitor. Cancer Discov. *8*, 458–477. https://doi.org/10.1158/2159-8290.CD-17-0902.

45. Xu, Y., and Vakoc, C.R. (2017). Targeting cancer cells with BET bromodomain inhibitors. Cold Spring Harb. Perspect. Med. *7*, a026674. https://doi.org/10.1101/cshperspect.a026674.

46. Olp, M.D., Zhu, N., and Smith, B.C. (2017). Metabolically derived lysine acylations and neighboring modifications tune the binding of the BET bromodomains to histone H4. Biochemistry *56*, 5485–5495. https://doi.org/10.1021/acs.biochem.7b00595.

47. Ran, X., Zhao, Y., Liu, L., Bai, L., Yang, C.Y., Zhou, B., Meagher, J.L., Chinnaswamy, K., Stuckey, J.A., and Wang, S. (2015). Structure-based design of gamma-carboline analogues as potent and specific BET bromodomain inhibitors. J. Med. Chem. *58*, 4927–4939. https://doi.org/10.1021/acs.jmedchem.5b00613.

48. McLure, K.G., Gesner, E.M., Tsujikawa, L., Kharenko, O.A., Attwell, S., Campeau, E., Wasiak, S., Stein, A., White, A., Fontano, E., et al. (2013). RVX-208, an inducer of ApoA-I in humans, is a BET bromodomain antagonist. PLoS One *8*, e83190. https://doi.org/10.1371/journal.pone.0083190.

49. Kim, H.R., Yim, J., Yoo, H.B., Lee, S.E., Oh, S., Jung, S., Hwang, C.I., Shin, D.M., Kim, T., Yoo, K.H., et al. (2021). EVI1 activates tumor-promoting transcriptional enhancers in pancreatic cancer. NAR Cancer *3*, zcab023. https://doi.org/10.1093/narcan/zcab023.