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The Bromodomain protein BRD4 controls HOTAIR, a long noncoding RNA essential for glioblastoma proliferation

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Bromodomain and extraterminal (BET) domain proteins have emerged as promising therapeutic targets in glioblastoma and many other cancers. Small molecule inhibitors of BET bromodomain proteins reduce expression of several oncogenes required for Glioblastoma Multiforme (GBM) progression. However, the mechanism through which BET protein inhibition reduces GBM growth is not completely understood. Long noncoding RNAs (lncRNAs) are important epigenetic regulators with critical roles in cancer initiation and malignant progression, but mechanistic insight into their expression and regulation by BET bromodomain inhibitors remains elusive. In this study, we used Helicos single molecule sequencing to comprehensively profile lncRNAs differentially expressed in GBM, and we identified a subset of GBM-specific lncRNAs whose expression is regulated by BET proteins. Treatment of GBM cells with the BET bromdomain inhibitor I-BET151 reduced levels of the tumor-promoting lncRNA HOX transcript antisense RNA (HOTAIR) and restored the expression of several other GBM down-regulated lncRNAs. Conversely, overexpression of HOTAIR in conjunction with I-BET151 treatment abrogates the antiproliferative activity of the BET bromodomain inhibitor. Moreover, chromatin immunoprecipitation analysis demonstrated binding of Bromodomain Containing 4 (BRD4) to the HOTAIR promoter, suggesting that BET proteins can directly regulate lncRNA expression. Our data unravel a previously unappreciated mechanism through which BET proteins control tumor growth of glioblastoma cells and suggest that modulation of lncRNA networks may, in part, mediate the antiproliferative effects of many epigenetic inhibitors currently in clinical trials for cancer and other diseases.

glioblastoma | long noncoding RNAs | epigenetics | BRD4 | I-BET151

Glioblastoma Multiforme (GBM) is the most common and malignant adult brain tumor. Treatment includes surgical resection followed by radiotherapy and temozolomide (TMZ) chemotherapy. However, tumor recurrence is universal, and patient survival is ∼14 mo (1). Thus, there is an urgent need to identify therapies that increase GBM patient survival. The Cancer Genome Atlas (TCGA) has recently performed DNA and RNA sequencing of over 400 GBM tumors and identified epigenetic modulators as possible GBM therapeutic targets. By contrast to genetic mutations, epigenetic alterations are reversible. Therefore, epigenetic drugs could potentially restore the normal epigenetic landscape in cancer cells. To date, the US Food and Drug Administration (FDA) has approved five epigenetic drugs for use in cancer treatment: two DNA methyltransferase (DNMT) inhibitors and three histone deacetylase (HDAC) inhibitors.

The bromodomain and extraterminal (BET) family of proteins are epigenetic modulators that have emerged as promising drug targets for a number of cancer pathways characterized by changes in the epigenetic cell signature (2, 3). We recently identified upregulation of the BET proteins BRD2, BRD3, and BRD4 (Bromodomain Containing 2, 3, and 4) in GBM (4). These proteins are essential for the recognition of acetylated lysine residues (KAc) of histones during transcriptional activation (5). Among the bromodomain proteins, BRD4 has a well-studied role, which includes initial recognition of acetylated histones followed by recruitment of Mediator, the transcription initiation cofactor, on promoter regions. These events lead to phosphorylation of RNA polymerase II. BRD4 also facilitates the recruitment of elongation cofactor positive transcription elongation factor b (P-TEFb) that results in another step of phosphorylation of Polymerase II necessary for transcription elongation (6–8).

Importantly, highly selective BET bromodomain inhibitors have been recently reported [JQ-1 (3), I-BET151 (9), and I-BET762 (10)]. These inhibitors specifically target the KAc recognition sites of the BET family of proteins (BRD2, BRD3, BRD4, and BRDT), each containing two tandem BRDs (bromodomains BD1 and BD2) (11). BET bromodomain inhibitors exert a broad spectrum of desirable biological effects such as anticancer and anti-inflammatory properties (12). A main transcriptional target of BET proteins is the MYC oncogene (13, 14). The BET bromodomain inhibitors reduce MYC levels and tumor growth in multiple cancers (13, 14), in particular in hematologic malignancies. However, MYC is not always the main transcriptional target of BET proteins (15),

Significance

Glioblastoma Multiforme (GBM) is the most common and deadliest primary brain tumor in adults. As the median survival is approximately 14 mo there is an urgent need for novel therapies. Epigenetic modulators such as bromodomain and extraterminal (BET) proteins are important therapeutic targets in GBM. Bromodomain inhibitors (including I-BET151) suppress proliferation by repressing oncogenes and inducing tumor suppressor genes through unidentified pathways. Here we demonstrate that HOTAIR (HOX transcript antisense RNA) is overexpressed in GBM, where it is crucial to sustain tumor cell proliferation, and that inhibition of HOTAIR by I-BET151 is necessary to induce cell cycle arrest in GBM cells. Our study outlines the mechanism of action underlying the antiproliferative activity of I-BET151, showing for the first time, to our knowledge, that the oncogenic long noncoding RNA HOTAIR is a major target.

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suggesting the existence of additional, unidentified downstream targets of BET proteins in GBM and other cancers.

Recent studies demonstrated that the genome contains many long noncoding transcripts (long noncoding RNAs, lncRNAs), which may be more common than protein-coding genes. The discovery and study of lncRNAs is thus of major relevance to human biology and disease, as they represent an extensive, largely unexplored, and functional component of the genome (16, 17). Moreover, lncRNAs have emerged as players in the cancer paradigm, demonstrating potential roles in both oncogenic and tumor-suppressive pathways (18–21). Increasing evidence indicates that these transcripts are frequently aberrantly expressed in cancer, and some of them have been implicated in diagnosis and prognosis (22, 23). Recent progress suggests that the involvement of lncRNAs in human cancers could be far more prevalent that previously appreciated. To date, lncRNA expression has not been extensively analyzed in GBM samples except in a few studies that used microarrays from TCGA and Gene Expression Omnibus (24, 25). Although informative, such studies likely miss unannotated lncRNAs not represented on arrays and are often biased to polyadenylated fractions that contain fewer lncRNAs (26). We decided to use single molecule sequencing (SMS) to provide a sensitive (27) and accurate (28) signature of lncRNA dysregulated in GBM. Importantly, we discovered that the BET bromodomain inhibitor I-BET151 modulates several GBM-associated lncRNAs. HOTAIR is a transcript necessary for glioblastoma proliferation, and it appears to be the most relevant lncRNA target of this drug. Our findings invoke a previously unidentified important mechanism of action of BET bromodomain inhibitors, which would in part explain the efficacy of this drug in blocking the proliferation of GBM cells in vivo and in vitro (4, 15).

Results

Our prior studies and those from several groups demonstrated that the human genome contains lncRNAs (29–31), which function in diverse biological processes including cell proliferation. Importantly, some of these lncRNAs are deregulated in complex diseases including cancer (32). Thus, we postulated that lncRNAs may be differentially expressed in GBM and mediate the antiproliferative response to BET protein inhibition. To test this directly, we performed SMS on 14 GBM tumor and seven control biospecimens. As it is difficult to obtain fresh brain tissue samples from healthy individuals, we have followed the example of the TCGA Consortium and used epilepsy samples as a control for GBM (33–35). We analyzed the RNA sequencing data by testing expression of each annotated transcript in each individual GBM sample compared against all normal samples, rather than grouping all GBM samples together. The SMS expression analysis achieved significant separation of GBM from control tissues as revealed by principal component analysis (PCA) (Fig. S1*A*). We observed similar separation of control and GBM samples based on either coding or noncoding transcript expression, suggesting that noncoding RNAs may be as important as coding mRNAs in mediating the proliferative and invasive behavior of GBM (Fig. S1*A*). Importantly, our analysis identified the previously characterized glioma-enriched lncRNA H19 (36), suggesting that our SMS pipeline robustly identifies lncRNAs overexpressed in glioma. Fig. 1 illustrates the top 100 most up- and down-regulated lncRNAs that were differentially expressed in GBM (Fig. 1*A* and Fig. S2). We validated several of these, including MEG3 (Maternally Expressed 3), HOTAIRM1 (HOXA transcript antisense RNA, myeloid-specific 1), DGCR5 (DiGeorge Syndrome Critical Region Gene 5), and HOTAIR (Fig. 1*B*). To normalize gene expression across samples, we carefully evaluated multiple housekeeping genes and selected those that had less variability across controls and glioblastoma samples (Fig. S1*B*). Based on this analysis, we decided to normalize qPCR (quantitative PCR) gene expression data

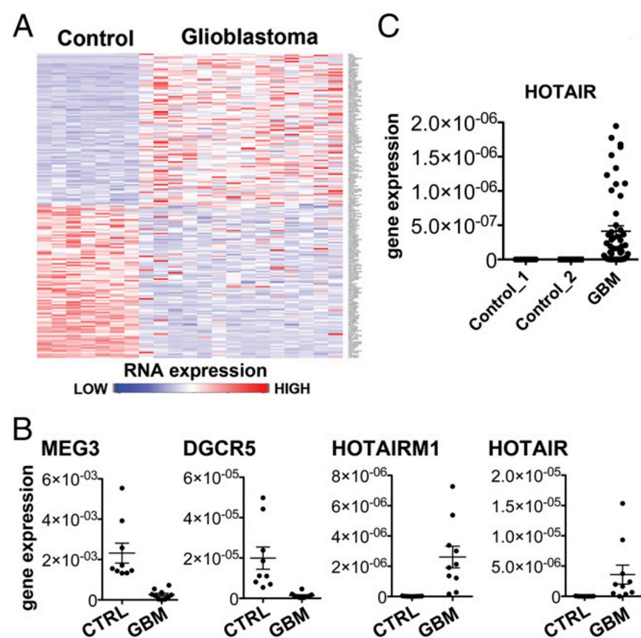

Fig. 1. SMS reveals hundreds of lncRNAs altered in glioblastoma. (*A*) The heat map shows expression of the 200 lncRNAs most up- or down-regulated in GBM compared with normal brain samples. The top 100 lncRNAs up- and down-regulated in GBM are shown in the top and bottom halves, respectively. The heat map was generated with an R package using normalization across rows (tissues). (*B*) A list of the most up-regulated and down-regulated lncRNAs has been selected from *A* for validation by RT-qPCR using the same samples of the RNA sequencing experiment. (*C*) Validation of HOTAIR by RT-qPCR (normalized on 18S) was performed in nine control brain samples from postmortem patients, nine control samples from lobotomy in epileptic patients, and 51 GBM specimens. HOTAIR expression is undetectable in all of the control samples and in nine samples of the glioblastoma cohort.

to*18S* ribosomal RNA (18S; Fig. 1*B*) and glucose-6-phosphate dehydrogenase (*G6PD*) (Fig. S1*C*). HOTAIR has been described as an oncogene in several types of cancers, as it promotes metastasis (37); moreover, the increased HOTAIR expression is a biomarker of poor prognosis (38, 39). HOTAIR is part of a well-established pathway controlling epigenetic gene silencing whereby the Polycomb Repressing Group (PRC2) is targeted to promoters (40–42). Given the importance of HOTAIR in epigenetic regulation, we measured its expression in a larger cohort of 51 GBM and 18 control samples. HOTAIR was expressed in most GBM samples (42 out of 51) and absent in all control brain samples (Fig. 1*C*). In addition, we did not detect HOTAIR in any normal adult brain region tested (insula, pons, frontal lobe, temporal lobe, hippocampus, or cerebellum; Fig. S1*D*). Taken together, these data suggest that HOTAIR is specifically expressed in GBM.

Consistent with its expression in GBM, we show here that HOTAIR is an essential driver of GBM cell proliferation. We transfected the LN18 cell line with two distinct siRNAs targeting HOTAIR (siHOTAIR#1 and siHOTAIR#2) or control siRNA (siControl) (Fig. S3*A*) and measured cell growth via a colony-forming assay (Fig. S3*B*). We observed a dramatic reduction in the number of colonies formed in cells transfected with siRNAs targeting HOTAIR relative to control siRNAs (Fig. S3*B*). HOTAIR knockdown also reduced the number of proliferating LN18 cells in S-phase as measured via an 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay (Fig. 2*A*). Subsequent flow cytometry analysis using an Annexin-V/7-AAD apoptosis assay revealed that 12% of the cells transfected with siHOTAIR#1 were in an early apoptotic stage (Annexin-V+/7AAD–) and 26%

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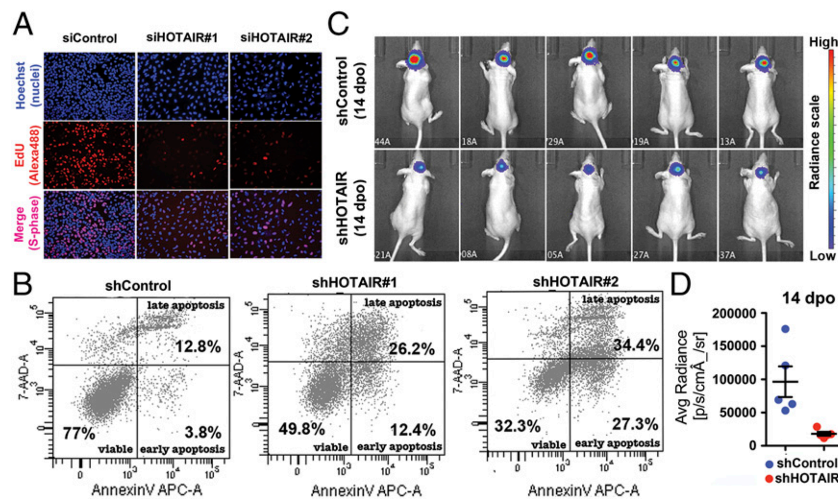

Fig. 2. Knockdown of HOTAIR causes apoptosis and reduces the proliferation of glioblastoma cells in vitro and in vivo. (*A*) LN18 cells were transfected with siRNAs targeting HOTAIR (siHOTAIR#1 and siHOTAIR#2), and after 5 d cells were incubated with EdU, fixed, permeabilyzed, and the EdU (red) was detected by Alexa 488 at the fluorescence microscope. Nuclei were marked by Hoechst (blue) and the cells in S-phase are visible in pink in the merged pictures. (*B*) LN18 transfected with siControl, siHOTAIR#1, and siHOTAIR#2 were harvested 5 d after transfection, washed with binding buffer, and then incubated with Annexin V and 7-AAD to be analyzed by flow cytometry. Early apoptotic cells, Annexin V^{pos}/7-AAD^{neg}; late apoptotis cells, Annexin V^{pos}/7-AAD^{pos}. (*C*) The U87MG GBM cell line stably expressing luciferase was transduced with lentivirus carrying shRNA of control and shRNA targeting HOTAIR. Tumor growth was assessed every week for 2 wk after surgery [14 dpo (day post operation)]. Imaging of the animals was performed with the IVIS. (*D*) Tumor growth of the U87MGLuc GBM cell line implanted in the striatum of nude mice was quantified by IVIS.

were in late apoptosis (Annexin-V+/7AAD+), compared with 3.8% early and 12% late apoptotic cells for siControl (Fig. 2*B*). SiHOTAIR#2 had a stronger effect than siHOTAIR#1, with 27% early and 34% late apoptotic cells (Fig. 2*B*). Importantly, these effects on proliferation and apoptosis were overcome by overexpressing HOTAIR, suggesting that the HOTAIR siRNAs used are specific (Fig. S3*C*). We validated the in vitro effects of depleting HOTAIR in vivo by injecting U87MG glioblastoma cells that stably express luciferase (U87MGLuc) into the brains of immunocompromised mice. Before implantation, U87MGLuc cells were infected with lentiviruses expressing either control or anti-HOTAIR shRNA (shHOTAIR) (Fig. S4 *A* and *B*). As shown in Fig. 2, cells expressing HOTAIR shRNA had significantly reduced tumor growth relative to control cells (Fig. 2 *C* and *D*).

We previously demonstrated that the BET bromodomain inhibitor I-BET151 reduces cell proliferation in both GBM cell lines and patient-derived cells in vitro and in vivo (4). Therefore, we asked whether I-BET151 treatment reduced HOTAIR expression in GBM cells and found that treatment of LN18 cells with I-BET151 for 24 h reduces the expression of HOTAIR by ~80% (Fig. 3*A*). In addition, I-BET151 induced a statistically significant up-regulation of GBM down-regulated lncRNAs including MEG3, NEAT1 (nuclear paraspeckle assembly transcript 1), and DGCR5 (Fig. 3*A*). By contrast, this treatment yielded down-regulation of GBM up-regulated lncRNAs HOTAIRM1, TUG1 (taurine up-regulated 1), and H19 (imprinted maternally expressed transcript) (Fig. 3*A*). Thus, BET proteins modulate the levels of HOTAIR and other lncRNAs potentially involved in cell proliferation and cancer progression. Even at a shorter time point (6 h) HOTAIR is reduced by treating cells with I-BET151 or with the other BET bromodomain inhibitors (I-BET762 and JQ1) (Fig. 3*B*). Inhibition of HOTAIR by I-BET151 was achieved at 6 h and confirmed at 24 h in numerous cell lines such as LN18, U87MG, A172, T98G, and Patient derived xenograft (PDX) (43) cells PDX12 and PDX22 (Fig. 3 *C* and *D*). Interestingly, treatment with TMZ, the DNA alkylating drug administered to GBM patients, at a concentration of 10 μM is able to induce cell cycle arrest [as demonstrated by *CDKN1A* ($p21^{waf1/cip1}$) mRNA induction] (Fig. S4*D*) but does not affect HOTAIR expression (Fig. S4*C*). Therefore, HOTAIR down-regulation after I-BET151 treatment is not merely a consequence of reduced proliferation. Collectively, these data suggest that BET proteins control expression of HOTAIR and other functional lncRNAs.

To demonstrate the importance of I-BET151–mediated down-regulation of HOTAIR, we measured the dose-dependent effect of BET bromodomain inhibition on the proliferation rate (as indicated by EdU incorporation) of U87MG cells overexpressing HOTAIR. We chose to overexpress HOTAIR in U87MG, as this cell line is the one that expresses the lowest levels of HOTAIR compared with LN18, T98G, and A172. We expressed HOTAIR in U87MG cells via a tet-inducible system and observed an increase in HOTAIR expression after doxycycline (DOX) administration (Fig. 4*A*). We demonstrated a shift in the IC_{50} of I-BET151 from~200 nM for control cells (–DOX) to ~700 nM for HOTAIR-overexpressing cells (+DOX) (Fig. S5*B*). Similarly, HOTAIR induction abrogated the antiproliferative effects of I-BET151 treatment of U87MG cells, as determined via an EdU incorporation assay and apoptosis assay (Fig. 4*B* and Fig. S5*A*). However, this was not due to off-target effects of DOX administration, as DOX treatment of U87MG cells without the tet-inducible HOTAIR vector did not increase proliferation (Fig. S4 *E* and *F*). Taken together, these data strongly indicate that I-BET151 inhibits GBM growth in a HOTAIR-dependent manner.

Because I-BET151 inhibits BRD2, BRD3, and BRD4, to identify the bromodomain protein responsible for controlling HOTAIR levels, we systematically depleted BRD2, BRD3, and BRD4 with siRNAs (Fig. 5*A*). We concluded that I-BET151–mediated down-regulation of HOTAIR likely involves BRD4, as only BRD4 depletion reduced HOTAIR levels (Fig. 5*B*). Thus, our findings functionally link BRD4 to HOTAIR. In addition, we discovered that the cell cycle regulator lncRNAs H19 and HOTAIRM1 are also regulated by BRD4 (Fig. 5*C*). To determine whether BRD4 control of HOTAIR is direct, we performed chromatin immunoprecipitation (ChIP) assays to detect an association of BRD4 with the HOTAIR promoter. We saw an enrichment of BRD4 occupancy at the HOTAIR promoter (~1 kb from the transcription start site) with anti-BRD4 antibodies compared with an anti-IgG negative control antibody in ChIP-qPCR

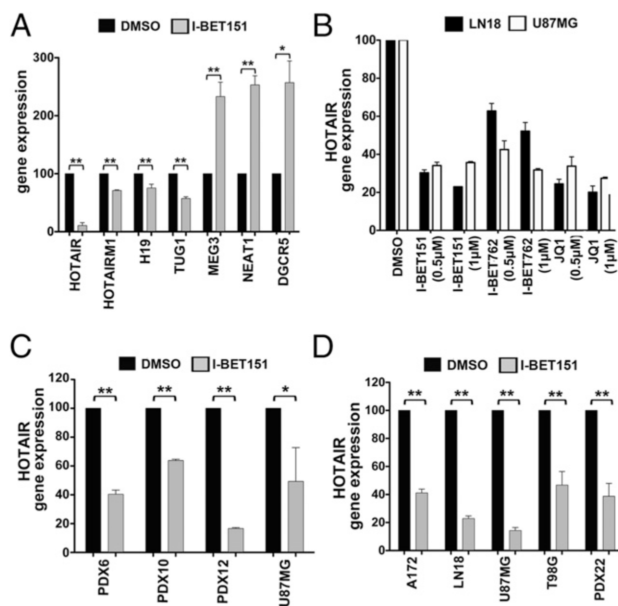

Fig. 3. BET bromodomain inhibitors alter the expression of lncRNAs in glioblastoma cell lines and patient-derived cells. (*A*) LN18 cells have been treated with I-BET151 (1 μM) and DMSO as a control for 24 h, and RNA was extracted. The expression of some lncRNAs present in the GBM-signature was measured by RT-qPCR. (*B*) LN18 and U87MG cell lines were treated with I-BET151, I-BET762, and JQ1 for 6 h. RNA was extracted to measure the expression of HOTAIR by RT-qPCR. (*C* and *D*) PDX cell lines (PDX6, PDX10, PDX12, and PDX22) and GBM cell lines (U87MG, A172, LN18, and T98G) were treated with I-BET151 for 6 h (*C*) and for 24 h (*D*) to collect RNA samples and measure HOTAIR levels by RT-qPCR. Error bars represent the SD calculated over three independent experiments. $*P \leq 0.05$; $**P \leq 0.01$. The *P* value has been calculated using the Student's *t* test (*A*, *C*, and *D*) or one-way ANOVA with Tukey HSD test (*B*). In *B* the ANOVA test gave $P \leq 0.01$ for all of the samples (I-BET151, I-BET762, and JQ1; 500 nM and 1 μM) compared with DMSO, both in LN18 and U87MG experiments.

experiments. Moreover, as shown in Fig. 5*D*, BRD4 occupancy at the HOTAIR promoter is reduced by I-BET151 treatment (1 μM for 24 h). To ensure the specificity of the anti-BRD4 antibody, we show that the binding of BRD4 to HOTAIR's promoter is reduced in LN18 cells when transfected with siRNAs targeting *BRD4*compared with siControl (Fig. S5*C*). Although multiple modes of regulation likely exist, these data suggest that the consequences of I-BET151 treatment may be due to a direct connection between HOTAIR and BRD4.

Discussion

Here we provide, to our knowledge, the first signature of GBM-lncRNAs using Helicos SMS. This technology allows direct measurement of nucleic acid molecules with high accuracy, as it does not require amplification, ligation, cDNA synthesis, or other complex sample manipulations. It avoids biases introduced by other sequencing platforms and sample preparation strategies and generates the most unbiased and quantitative views of genetic material. We validated our signature by quantitative RT-PCR, measuring some of the most up-regulated and down-regulated candidates in GBM and control specimens such as MEG3, HOTAIRM1, HOTAIR, and DGCR5. The lncRNA MEG3 is known to be reduced in several types of cancer and recently has been found to also be down-regulated in GBM (25). MEG3 regulates the cell cycle and apoptosis by increasing TP53 (p53) (44). Here, importantly we report that the BET bromodomain inhibitor I-BET151 can induce MEG3 expression through an as-yet unidentified mechanism. Among the most up-regulated noncoding transcripts in our GBM signature, we found two lncRNAs that originate from the Homeobox gene clusters, HOTAIR and HOTAIRM1. HOTAIRM1 is expressed in the myeloid lineage and is induced during neuronal differentiation, but it has not been extensively studied in cancer. Although its role in GBM is still unclear, we have found that I-BET151 treatment and BRD4 depletion can ameliorate the over-expression of this transcript. Finally, HOTAIR has a well-established role in oncogenic progression in several cancer types due to its interaction with the Polycomb Repressive Complex (PRC2) and LSD1/COREST complex proteins causing genome-wide gene silencing. While interacting with these epigenetic modifiers, HOTAIR increases histone 3 lysine 27 methylation and decreases histone 3 lysine 4 methylation, both of which are events associated with gene silencing. Although LSD1and the PRC2 complex have been demonstrated to be functional partners of HOTAIR, how HOTAIR regulates glioma cell cycle progression remains largely unknown. However, according to a recent publication, EZH2 (the catalytic component of PRC2) inhibition blocked cell cycle progression in glioma cells, consistent with the effects elicited by HOTAIR siRNA (45). As reported in *BrainSpan: Atlas of the Developing Human Brain* [Developmental Transcriptome project (46); brainspan.org/] and confirmed by us, HOTAIR expression is absent or extremely low in the adult brain. The events underlying HOTAIR expression during the process of tumorigenesis in glioblastoma have not yet been investigated. It would be of great interest to identify the transcription factors and/or epigenetic events driving the transcription of HOTAIR in this type of cancer and at what stage of tumorigenesis. It has been recently

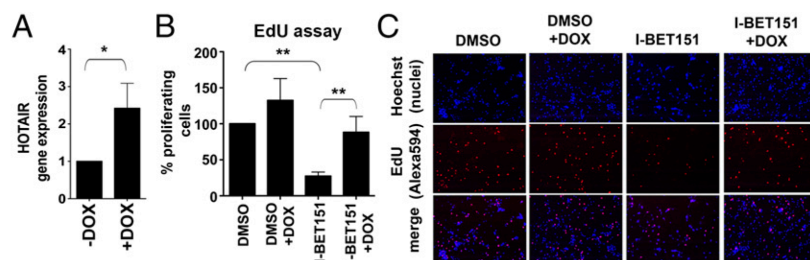

Fig. 4. Overexpression of *HOTAIR* abrogates the antiproliferative effect of I-BET151 in U87MG cells. (*A*) U87MG cells were transduced with HOTAIR-overexpressing lentiviruses and treated with DOX (+/–DOX) for 4 d, harvested, and the RNA was extracted to measure HOTAIR expression by RT-qPCR. (*B*) U87MG cells were transduced with the *HOTAIR*-overexpressing virus, and the infected cells were treated with IBET-151 at a concentration of 0.5 μM for 96 h. HOTAIR expression was induced by DOX for 96 h. In this representative experiment, the proliferating cells were identified with the EdU labeling kit. The automated counting was done using the Termo Scientific imaging platform (Cellomics ArrayScan VTI HCS). (*C*) Fluorescence microscopy images (magnification, 20×) of one representative experiment show EdU-positive cells (in red; in pink in the merged picture) over the total amount of cells (nuclei, Hoechst in blue) in U87MG treated with DMSO or I-BET151 500 nM with +/–DOX for 96 h. Error bars (*B*) represent the SE of six independent experiments. $*P \leq 0.05$; $**P \leq 0.01$. The *P* value was calculated with a *t* test (*A*) or one-way ANOVA Tukey HSD test (*B*).

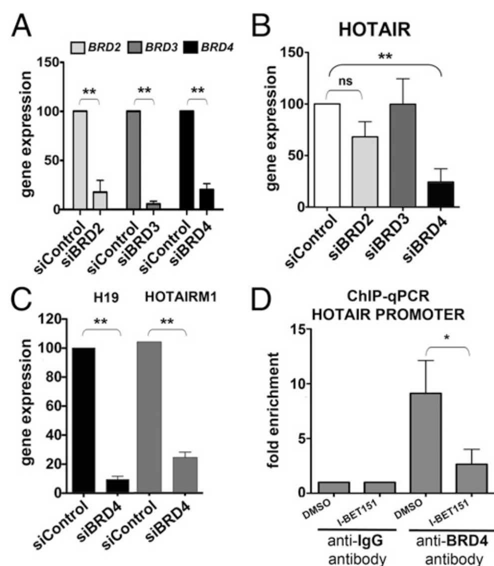

Fig. 5. BRD4 directly regulates the expression of HOTAIR. (*A*) LN18 cells were transfected with siRNAs (20 nM) targeting *BRD2*, *BRD3*, *BRD4*, and control siRNA. The RNA was extracted after 96 h to measure the mRNA levels of *BRD2*, *BRD3*, and *BRD4*. (*B*) HOTAIR expression was measured by RT-qPCR after the *BRD2*, *BRD3*, and *BRD4* knockdown experiments shown in *A*. (*C*) H19 and MEG3 RNA levels were measured by RT-qPCR upon *BRD4* depletion. (*D*) ChIP was performed with anti-BRD4 antibody and anti-IgG as negative control in LN18 cells treated for 24 h with DMSO or I-BET151 (1 μM). The chromatin obtained by the pull-down was used for qPCR to amplify the promoter region of HOTAIR located ∼1 kb upstream of the transcription start site. The graph shows the fold enrichment of BRD4 on HOTAIR's promoter normalized on the IgG signal after normalization on the respective input signal. Error bars (*A–D*) represent the SD calculated over three independent biological replicates. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; ns, not significant. The *P* value has been calculated using the Student's *t* test (*A* and *C*) or one-way ANOVA Tukey HSD test (*B* and *D*). RT-qPCR data (*A–C*) were analyzed with Comparative Cq Method.

proposed that Dicer (a protein canonically involved in the biogenesis of microRNA) and MYC are required for widespread transcriptional initiation and elongation of lncRNAs (47). MYC has potent oncogenic activity in multiple cancers; its regulation of lncRNAs, potentially including HOTAIR, broadens the scope of lncRNA involvement in cancer.

Other lncRNAs such as CRNDE, TUG1, DLEU1, GAS5, TP53TG1, NEAT1, and PAR1 are additional GBM-lncRNAs identified in our signature that can possibly play roles in GBM pathogenesis. Finally, H19 is one of most up-regulated lncRNAs in our RNAseq data, and it has been found to be overexpressed in glioma, where it promotes cell invasion (36). Here, we have shown that I-BET151 and BRD4 knockdown strongly reduce the expression of H19, confirming that functional noncoding RNAs should be taken into consideration when investigating the consequences of drug treatment on the gene expression profile of tumors. In fact, in addition to BET Bromodomain inhibitors, we found that HDAC inhibitors are also potent regulators of HOTAIR expression in GBM cells (Figs. S6 and S7). To date, a multitude of HDAC inhibitors have been tested in clinical trials for a variety of cancers, including GBM (48, 49).

Given the emerging role of lncRNAs such as MEG3 (50), H19 (36), and HOTAIR (39) in regulating the cell cycle of GBM cells, our study uncovers an important connection between these lncRNAs and BET bromodomain proteins. Further, we identify a previously unidentified subset of genes regulated by the BET bromodomain inhibitors. Interestingly, BRD4 may show widespread localization in noncoding regions of the genome. Indeed, a recent report shows that BRD4 occupies vast genomic regions in mouse cells, where it assists the elongation of coding and noncoding transcripts originating from enhancer regions (eRNAs) (51).

Here we have shown that BRD4 localizes to the promoter of HOTAIR, suggesting a direct regulation. As reader of acetylated histones, BRD4 has a central role in transcriptional elongation; therefore, it would be expected to be enriched at all active promoters. Instead it appears that the BET bromodomain inhibitors affect only a small subset of tissue and lineage-specific genes (13, 52, 53). These specific effects mediated by the BET bromodomain inhibitors seem to derive from the removal of BRD4 from enhancer regions (54). In fact, BRD4 is particularly enriched at superenhancer loci, which are genomic regions that stimulate the expression of genes despite being several kilobases away from the transcription start site. Coiling and folding of DNA in the nucleus allows enhancers to physically approach the transcription start site. Enhancer regions can even be on a different chromosome from the genes they regulate (55). This makes the identification of these regulatory regions challenging and requires the use of methods that interrogate the interaction between distant loci such as chIA-PET, Hi-C, and 3C/4C/5C (56, 57). Validated enhancers of HOTAIR have not yet been described, but we propose that transcriptional repression of HOTAIR might be related to the displacement of BRD4 from such regions. However, thus far we have only provided evidence that I-BET151 decreases binding of BRD4 in the promoter region. This suggests that disruption of BRD4's interaction with transcription and elongation factors (e.g., Polymerase II, P-TEFb, Mediator, Activator) may be part of the mechanism by which I-BET151 causes HOTAIR repression. Recently BRD4 has been found to interact with the acetylated transcription factor TWIST1 (diacetylated by Tip60) in the promoter of WNT5A, and pharmacological inhibition of their interaction reduces invasion and tumorigenicity of breast cancer cells (58).

There is currently considerable excitement around BET bromodomain inhibitors, which have shown efficacy in hematological cancers by down-regulating cell proliferation genes such as MYC, CDK4, and CDK6, while inducing proapoptotic genes. However, the mechanism of action of BET bromodomain inhibitors in solid tumors is not as well characterized. We recently demonstrated that BET bromodomain inhibitors can effectively reduce the proliferation of GBM cells in vitro and in vivo in part through the induction of the cyclin-dependent kinase inhibitor $p21/^{Cip1}$ (4). We provide evidence that BET inhibitors may induce $p21^{Cip1}$ expression by reducing HOTAIR, which is known to modulate $p21^{Cip1}$. Our studies are the first, to our knowledge, to demonstrate BRD4 regulation of noncoding RNAs that are involved in cell cycle progression. Further, this is the first indication, to our knowledge, that BET bromodomain inhibitors act on the core of cell cycle machinery through modulating a network of lncRNAs. In sum, lncRNAs, such as HOTAIR, appear to be critically involved in glioblastoma tumor progression and may account for much of the antitumor effects of BET bromodomain inhibitors.

Materials and Methods

A full description of materials and methods can be found in *SI Materials and Methods*. Control and glioblastoma specimen were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank, Florida Center for Brain Tumor Research Bank, and University of Miami Hospital. RNA was extracted from samples and sequenced with Helicos SMS. SiRNAs targeting HOTAIR and the bromodomain proteins were purchased from Ambion. shRNAs targeting HOTAIR were cloned into the vector pLentiLox3.7, and the plasmid overexpressing HOTAIR was purchased from Active Biogene. Proliferation and apoptosis were tested with colony assay, annexinV/7AAD staining, and EdU incorporation. In vivo experiments were conducted implanting U87Luc cells into the brain of Crl:NU-*Foxn1*^{nu} nude mice. Tumor growth was measured by In Vivo Imaging System (IVIS). ChIPs using BRD4 antibody (Bethyl Laboratories) were performed according to the EZ-Magna ChIP protocol according to the manufacturer's instructions.

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