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Bromodomain protein BRD4 is an epigenetic activator of B7-H6 expression in acute myeloid leukemia

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ABSTRACT

B7-H6, a ligand for the NK activating receptor NKp30, has been identified as a biomarker of poor prognosis in several solid cancers. However, little is known about the role of B7-H6 and the mechanisms that control its expression in acute myeloid leukemia (AML). Epigenome modulation, including epigenomic reader dysregulation, is one of the hallmarks of AML. Bromodomain-containing protein 4 (BRD4), the best-known member of the BET family of epigenetic readers, is overexpressed in AML cells and regulates the transcription of genes involved in the pathogenesis of AML, as MYC oncogene. Here, we analyze the role of BRD4 in regulating B7-H6 in AML cells. Results demonstrated that the specific inhibition of BRD4 drastically reduces the expression of B7-H6 in AML cells. Histone acetylation mediated by CBP30/P300 facilitates the binding of BRD4 to the *B7-H6* promoter, which recruits the *P*-TEFb elongation factor that phosphorylates RNA polymerase II, thereby activating *B7-H6* transcription. BRD4 also co-bounded with JMJD6 at the distal enhancer of the *B7-H6* gene. Metabolic modulation with metformin modifies the acetylation pattern in the *B7-H6* promoter, impairing BRD4 binding, thereby inhibiting B7-H6 expression. B7-H6 knockdown induces the apoptosis in HEL-R cell line. Moreover, a high level of *B7-H6* expression in AML patients is related to increased *BRD4* levels, myelodysplastic-derived AML, and del5q, the two latter being associated with poor prognosis. Our data show that BRD4 is a positive regulator of the pro-tumorigenic molecule B7-H6 and that the blockage of the B7-H6 is a potential therapeutic target for the treatment of AML.

Introduction

B7 homolog 6 (B7-H6), also known as NCR3LG1, is a 454-aa-long type I transmembrane protein member of the B7 family that is absent in healthy cells, except under inflammatory conditions.[1–3] However, during malignant transformation B7-H6 expression increases due to the activation of endoplasmic reticulum stress, oncogenes such as MYC, or epigenetic mechanisms.[4–7] B7-H6 was first identified as a ligand of NKp30/NCR3, an activating receptor of NK cells that triggers cytotoxicity and cytokine secretion acting against tumor cells.[8] However, in recent years it has been shown that B7-H6 has a non-immunological role in tumorigenesis because it can inhibit apoptosis and promote the proliferation of tumor cells.[9] B7-H6 is upregulated and associated with poor prognosis (metastasis, worse overall survival) in a variety of solid tumors, including esophageal squamous cell, gastric, small lung, and breast cancers, among others.[10–14] However, little is known about its expression and role in tumorigenesis of hematological malignancies, such as acute myeloid leukemia (AML). One of the few studies being that of Chretien et al., who reported that B7-H6 is expressed on the cell surface of a very small number of leukemic blasts.[15]

Epigenetic mechanisms play an essential role in the differentiation and hematopoiesis of healthy hematopoietic stem cells,[16] but the epigenetic machinery is frequently altered during malignant transformation in AML cells. Using whole-genome and whole-exome sequencing, the Cancer Genome Atlas (TCGA) program identified frequent mutations in genes involved in DNA methylation (*DNMT3A*, *TET2*, *IDH1*, *IDH2*) and chromatin-modifying genes (*ASXL1*, *EZH2*, *KDM6A*).[17] Epigenetic modifications are reversible, making them good candidates for potential anti-tumoral therapies. In recent years, a variety of

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epigenetic drugs have been approved by the US Food and Drug Administration for treating AML, such as hypomethylating drugs (azacytidine, decitabine) and the isocitrate dehydrogenase (IDH1) inhibitor (ivosidenib).[18,19]

The bromodomain and extra-terminal (BET) family proteins (BRD2, BRD3, BRD4, BRDT) contain two conserved N-terminal bromodomains (BD1, BD2) that recognize and bind to acetyl-lysine residues in histones and non-histone proteins.[20] Of these, BRD4 physically interacts with the mediator complex, a ∼30 sub-unit complex, thereby promoting the assembly of transcriptional factors and initiating transcription.[21,22] Moreover, BRD4 recruits the positive transcription elongation factor (P-TEFb) complex (CDK9 and cyclin T1 subunits) that activates RNA pol II elongation.[23] After transcriptional initiation, the expression of several genes may remain paused until a specific stimulus required for their transcription occurs. BRD4 plays an important role in releasing this pause through the interaction with JMJD6 (JmjC-domain-containing protein 6) at super-enhancer regions. This interaction prompts the release of the 7SK snRNA/HEXIM protein inhibitor from RNA pol II, facilitating the transcriptional elongation of paused genes.[24]

The development of diverse BET protein inhibitors, such as JQ1 and I-BETs, had revealed the involvement of BRD4 in tumorigenesis and their great therapeutic potential. BRD4 is deregulated in several human cancers, promoting the progression of cellular cycle, invasion, inflammation, and metastatic phenotype of cancer cells.[25] In fact, the MYC proto-oncogene is one of the most important targets of BRD4.[26] In AML, BRD4 suppression with the JQ1 inhibitor reduces the cellular proliferation and improves the survival in murine models, suggesting that BRD4 has an essential role in the pathogenesis of the disease.[27] Moreover, JQ1 induces apoptosis of leukemic stem and progenitor cells in AML patients so that cellular growth is inhibited.[28] Some phase I clinical trials with BET inhibitors are completed in AML indicating that BET inhibition therapy, alone or in combination with standard therapy in AML, is acceptable although it induces some toxic effects (fatigue, diarrhea, raised bilirubin concentration, etc.) and has antitumor activity.[29–31] Another clinical trial in AML using the BET inhibitor, MK-8628 (NCT02698189), is currently underway. However, the role of BRD4 in regulating molecules involved in the immune recognition is less clear.

In this work, we analyze the potential role of BRD4 in regulating B7-H6 in AML. Our results indicate that BRD4 binds to the distal enhancers and promoter region of *B7-H6* gene, regulating its expression during tumor development. Treatment with BET inhibitors downregulates B7-H6 expression in leukemic cells, suggesting their potential as a therapeutic target.

Methods

Cell lines and treatments

The following cell lines were used: human myeloid leukemia (KG-1a, HL-60, NB-4, and HEL-R), colon cancer (Caco-2), T-lymphoblastic leukemia (Jurkat), and non-Hodgkin lymphoma (Raji). All cell lines were purchased from the American Type Culture Collection and the Leibniz-Institute DSMZ GmbH (Germany), and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and

1% antibiotic-antimycotic solution in a humidified chamber with 5% CO_2 atmosphere at 37°C. For treatments, cells were cultured at a density of 0.2–0.4 × 10^6 cells/ml with a complete medium supplemented with the following inhibitors: BET inhibitor (+) JQ1 or its inactive enantiomer (-) JQ1 (100–500 nM; Cayman Chemical), BET inhibitor I-BET 762 (100–500 nM; Cayman Chemical), CDK9 inhibitor 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside DRB (10–40 μM; Sigma-Aldrich), CBP/P300 inhibitor CBP30 (5–20 μM; Sigma-Aldrich), and metformin (20–60 mM; Sigma-Aldrich).

Patient samples

Bone marrow samples were obtained from 68 AML patients (aged 53.1 ± 3.1 y; 41 males and 27 females) and 21 healthy donors, from Hospital Clinico Universitario Virgen de la Arrixaca (Murcia, Spain). Patients had been diagnosed with AML between 2006 and 2016 according to the morphological and cytochemical criteria of the French-American-British (FAB) classification. Clinical characteristics are listed in Table 1. Peripheral blood samples from two AML patients with a high percentage of blasts (>65%) obtained from Hospital Universitario Central de Asturias (Oviedo, Spain) were used to evaluate the JQ1 treatment in B7-H6 expression. All patients and healthy donors gave their written informed consent according to the Principles of the Declaration of Helsinki, and the study was approved by the local hospitals' ethics committees. To confirm the implication of B7-H6 in the prognosis of AML, a second cohort from UCSC Xena public database (http://xena.ucsc.edu/) was used.

Table 1. Clinical characteristics of AML patients.

Characteristics	AML patients n = 68 (%)
Age (years)	
≤60	31 (45.6%)
>60	37 (54.4%)
Sex	
Male	27 (39.7%)
Female	27 (60.3%)
FAB classification	
M0	2 (3%)
M1	6 (8.8%)
M2	22 (32.3%)
M3	9 (13.2%)
M4	7 (10.3%)
M5	16 (23.5%)
M6	0 (0%)
M7	4 (5.9%)
Unclassified	2 (3%)
Type of AML	
Primary	46 (67.6%)
Secondary to SMD	11 (16.2%)
Secondary to other cancer	11 (16.2%)
Cytogenetic prognosis	
Favorable	20 (29.4%)
Intermediate	16 (23.5%)
Adverse	32 (47.1%)
Therapy	
Chemotherapeutic	28 (41.2%)
Palliative care	13 (19.1%)
Allo-TPH	12 (17.6%)
Auto-TPH	10 (14.7%)
Data missing	5 (7.4%)

Flow cytometry analysis

AML cell lines were stained with a specific human monoclonal antibody (mAb) aimed against B7-H6 protein (FAB7144P, R&D Systems) and analyzed with a Gallios™ flow cytometer and Kaluza for Gallios software (Beckman Coulter). The specific fluorescence index was calculated by dividing the mean fluorescence obtained with the B7-H6 mAb PE-conjugated by that of the isotype control (Biolegend).

Western blot

For protein analysis, AML cells were lysed with RIPA buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Merck) for 30 min on ice. Samples were centrifuged and the supernatant was collected as whole-cell extract. Bradford protein assay (Bio-Rad) was used to measure the total protein concentration. 50 μg of cell extract was run on 10% polyacrylamide-SDS gels, blotted onto nitrocellulose membranes, and blocked in 5% nonfat dry milk TBST buffer for 1 h. Membranes were incubated with the primary antibody anti-B7-H6 (ab121794; Abcam) or anti-β-actin (13E5; Cell Signaling) overnight at 4°C. After that, blots were washed and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Poly4064; Biolegend) for 1 h at room temperature. Immunoreactive bands were visualized using a chemiluminescent substrate (ECL Western Blotting Analysis System; Amersham).

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cultured cells with a GeneMATRIX Universal RNA purification kit (EUR) according to the manufacturer's instructions. Purified RNA (1 μg) was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene expression was measured by quantitative RT-PCR using specific oligonucleotides (**Table S1**) and an MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad). Data were normalized to GAPDH (endogenous control), and all samples were run in triplicate. Transcription levels were calculated by the $2^{-\Delta CT}$ method (ΔCT: CT gene test – CT endogenous control).

Gene silencing and overexpression

In order to overexpress BRD4, its coding sequence was subcloned into the lentiviral pLVX-Puro plasmid. BRD4 was knocked down by transfection with the plasmid containing a pSUPER-shBRD4 (C terminus). B7-H6 knockdown was performed using the lentiviral pLKO.1-Puro plasmid containing an shRNA for B7-H6 (Sigma-Aldrich). pRetrosuper MYC shRNA (#15662, Addgene) was used for MYC knockdown. For experiments, 2×10^4 HEL-R cells were plated in 48-well plates, and plasmids were transfected using XtremeGene HP reagent (Roche), following the manufacturer's instructions. In all assays, transfected clones were selected with 0.5 μg/ml of puromycin (Invitrogen).

For transient silencing assays, JMJD6 and control nonspecific scrambled siRNA were purchased from Qiagen and Dharmacon, respectively. For this purpose, 2×10^4 cells were plated in 48-well plates and transfected with control or specific siRNA (200 nM) using HiPerFect Transfection Reagent (Qiagen).

Chromatin immunoprecipitation analysis

HEL-R cells were previously treated with (+) JQ1 or its enantiomer, SG-CBP30, or metformin. Cells were fixed with 1% formaldehyde in PBS for 30 min at 4°C and the reaction stopped with glycine solution (0.125 M, 10 min at 4°C). Fixed cells were lysed in SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1), and DNA fragments (500–1000 bp) were generated by shearing chromatin on a BioRuptor (Diagenode). The shared chromatin (100 μg) was diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) containing a protease inhibitor cocktail (Merck) and incubated with specific antibodies overnight at 4°C. The antibodies used were against B7-H6 (Abcam), BRD4 (Bethyl Laboratories), H3ac, H4ac and p300 (Millipore), JMJD6 (Biorbyt), and normal IgG as negative control (Millipore). Antibody-chromatin complexes were recovered with Salmon Sperm DNA/Protein A-Agarose beads (Merck) for 1 h at 4°C, washed, and eluted from the beads with elution buffer (1% SDS, 0.1 M NaHCO₃). After reverse crosslinking and proteinase K treatment, DNA was extracted and analyzed by quantitative RT-PCR using specific primers (**Table S1**). Chromatin obtained before immunoprecipitation was used as the input control. Enrichment was calculated as the percentage of input DNA using the formula: % INPUT = 2 exp [Ct (BOUND) – (Ct (UNBOUND) – $\log_2$ (UNBOUND DILUTION FACTOR))] × 100. Data are represented as the magnitude of enrichment of each immunoprecipitation relative to the negative control.

Cytotoxicity assays

KG-1a cells, previously treated with (+) JQ1 or its enantiomer (-) JQ1 (500 nM, 48 h), were used as target cells and primary NKs as effector cells. NKs were obtained from peripheral blood mononuclear cells isolated from healthy donors by Ficoll density gradient separation. Further, NKs were isolated by negative selection using magnetic microbeads (Invitrogen) according to the manufacturer's instructions. The purity of the isolations was always >95%. Target cells were previously cell membrane-labeled with PKH67 Green Fluorescent Cell Linker Kit (Merck) and further co-cultured with effector cells at different effector:target (E:T) ratios (2:1, 3:1, 4:1) for 4 h at 37°C. In blocking experiments, NK cells were incubated with 10 μg/ml anti-human NKp30 blocking mAb (Biolegend) for 1 h at 37°C before co-culture with target cells. After 4 h of co-culture, cells were stained with 5 μl of 7AAD for 20 min at RT and analyzed by flow cytometry. Percentage-specific lysis was calculated as 100 × [(% dead target cells–% spontaneous lysis)/(100–% spontaneous lysis)].

Proliferation and cell apoptosis assays

Cell titer blue assay (Promega) was performed to analyze the effect of B7-H6 knockdown in the cell growth of HEL-R cells. For this, HEL-R control and silenced for B7-H6 (B7-H6 KO) were seeded at a concentration of 5×10^3 cells/well into 96-well plates for 48, 72, and 96 hours. Then, 20 μl of cell titer blue

reagent was added to each well and incubated at 37°C for 4 hours and the fluorescent signal was measured ($560_{Ex}/590_{Em}$).

For apoptosis assays, HEL-R control and B7-H6 KO cells were cultured at a concentration of $2x10^5$/ml during 24 hours. Then, the proportion of live cells, cells undergoing apoptosis, or necrotic cells was evaluated by flow cytometry using the FITC Anexin V Apoptosis Detection Kit with 7-ADD (Biolegend) following the manufacture's instructions.

Statistical analysis

All data are summarized as the mean ± standard deviation. Groups were compared with the non-parametric Wilcoxon rank test (non-normally distributed data) or Student's t-test (normally distributed data). The Pearson correlation coefficient was used to analyze the association between BRD4 and B7-H6 transcription levels in AML patients and Kruskal-Wallis test to compare BRD4 expression among the different AML patient groups. The chi-square test was used to compare the B7-H6 expression level with the clinical characteristics in AML patients. In all cases, a level of $p < .05$ was considered to be statistically significant. Analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY).

Results

Treatment with the BET inhibitor, JQ1, downregulates B7-H6 expression

We determined the effect of the BET bromodomain inhibitor, JQ1, on the expression of B7-H6 in AML cell lines. For this purpose, four AML cell lines (HEL-R, HL-60, KG-1a, and NB-4) were treated with different doses of (+) JQ1 or its inactive enantiomer (-) JQ1, and B7-H6 expression was measured. All AML cell lines under treatment with the enantiomer showed high levels of B7-H6 expression (Figure 1a–c). After (+) JQ1 treatment, B7-H6 transcripts were significantly decreased in a dose-dependent manner (Figure 1a). These results were correlated with reduced protein levels and cell-surface expression in all AML cell lines (Figure 1b and c). Similar results were observed with another BET inhibitor, I-BET 762 (Fig. S1), suggesting the involvement of BET proteins in the regulation of B7-H6 expression.

We also wanted to determine whether B7-H6 downregulation by BET protein inhibitors is a specific mechanism of AML cells or whether it is similarly regulated in other tumor types. For this purpose, tumor cell lines from colorectal cancer (Caco-2), acute lymphoblastic leukemia (Jurkat), and lymphoma (Raji) were analyzed. Results showed that the expression of B7-H6 was again significantly reduced in all cell lines after JQ1 treatment (Fig. S2). Thus, we can conclude that BET protein inhibitors reduced the level of B7-H6 expression in tumor cells independently of their origin.

To evaluate the specific role of BRD4 in B7-H6 regulation, we studied gene silencing using shRNA, targeting the coding region of BRD4 (shBRD4), and BRD4 overexpression with the pLVX-BRD4 lentiviral vector. Results showed that the modulation of BRD4 expression in HEL-R cells is associated with the reduction or increase of B7-H6 molecules (Figure 1d and e). The proto-oncogene MYC was one of the first targets of BRD4

to be identified.[25] In order to determine whether the effect of BET inhibitors is mediated by MYC regulation, B7-H6 expression was analyzed in HEL-R cells after MYC gene silencing using shRNA. No changes in B7-H6 expression levels were observed (Fig. S3), showing that positive regulation of B7-H6 mediated by BRD4 in AML cells is independent of MYC.

BRD4 directly binds to the B7-H6 promoter region, regulating its expression

To determine whether BRD4 directly binds to the B7-H6 promoter region, chromatin immunoprecipitation (ChIP) assays in HEL-R cells were carried out using a specific anti-BRD4 antibody, before or after JQ1 treatment. In the presence of the (-) JQ1 enantiomer, BRD4 is highly enriched in the B7-H6 promoter region. However, the binding of BRD4 was significantly reduced after (+) JQ1 treatment (Figure 2a), indicating that BRD4 binds to the regulatory region of B7-H6 gene modulating its expression.

The initial step in the binding of BRD4 to regulatory regions is the recognition of acetylated residues in histones, a characteristic of active promoters. After that, BRD4 recruits the P-TEFb (CDK9/cyclin T) complex that phosphorylates to the RNA polymerase II (Ser2) promoting the transcriptional elongation.[21] ChIP assays with specific mAb showed enrichment of total acetylation levels in histones 3 (AcH3) and 4 (AcH4), and the presence of RNA pol II (Ser2) in the promoter region of B7-H6 gene (Figure 2a). However, the treatment with (+) JQ1 significantly reduced the presence of both acetylated histones and of the active form of RNA pol II (Figure 2a). Moreover, B7-H6 expression was decreased in a dose-dependent manner in the presence of the CDK9 kinase activity inhibitor, DRB (Figure 2b), confirming that BRD4 acts as a bridge between the B7-H6 promoter and the transcriptional machinery needed for its transcription.

We also investigated whether the CBP/P300 histone acetyltransferase (HAT) could be involved in the histone acetylation in the B7-H6 promoter. First, we observed that treatment with a CBP/P300 inhibitor, SGC-CBP30, significantly reduced the B7-H6 expression (Figure 2c). To corroborate these data, ChIP assays using specific antibodies for BRD4 and CBP/P300 and their opposite inhibitors were performed. Treatment with SGC-CBP30 drastically reduced the binding of BRD4 to the regulatory region of B7-H6, and inversely, the binding of CBP/p300 was abolished in the presence of the BET inhibitor, (+) JQ1 (Figure 2d). Thus, CBP/P300 and BRD4 co-regulates B7-H6 expression.

BRD4 and JMJD6 co-binds in distal enhancers to release the transcriptional pause of B7-H6 in tumor cells

In cancer and other pathologies, many genes remain in a paused state until the presence of a stimulus that promotes their rapid and efficient transcription. In these genes, BRD4 can interact with JMJD6 in the distal regions (also called anti-pause enhancers) to co-regulate the transcriptional pause release located in the proximal promoters.[24] After binding, JMJD6 demethylases the 7SK snRNA methylation group, releasing the 7SK snRNA/HEXIM inhibitory complex from the pTEFb complex and allowing phosphorylation of RNA pol II (Ser2) and transcriptional elongation.
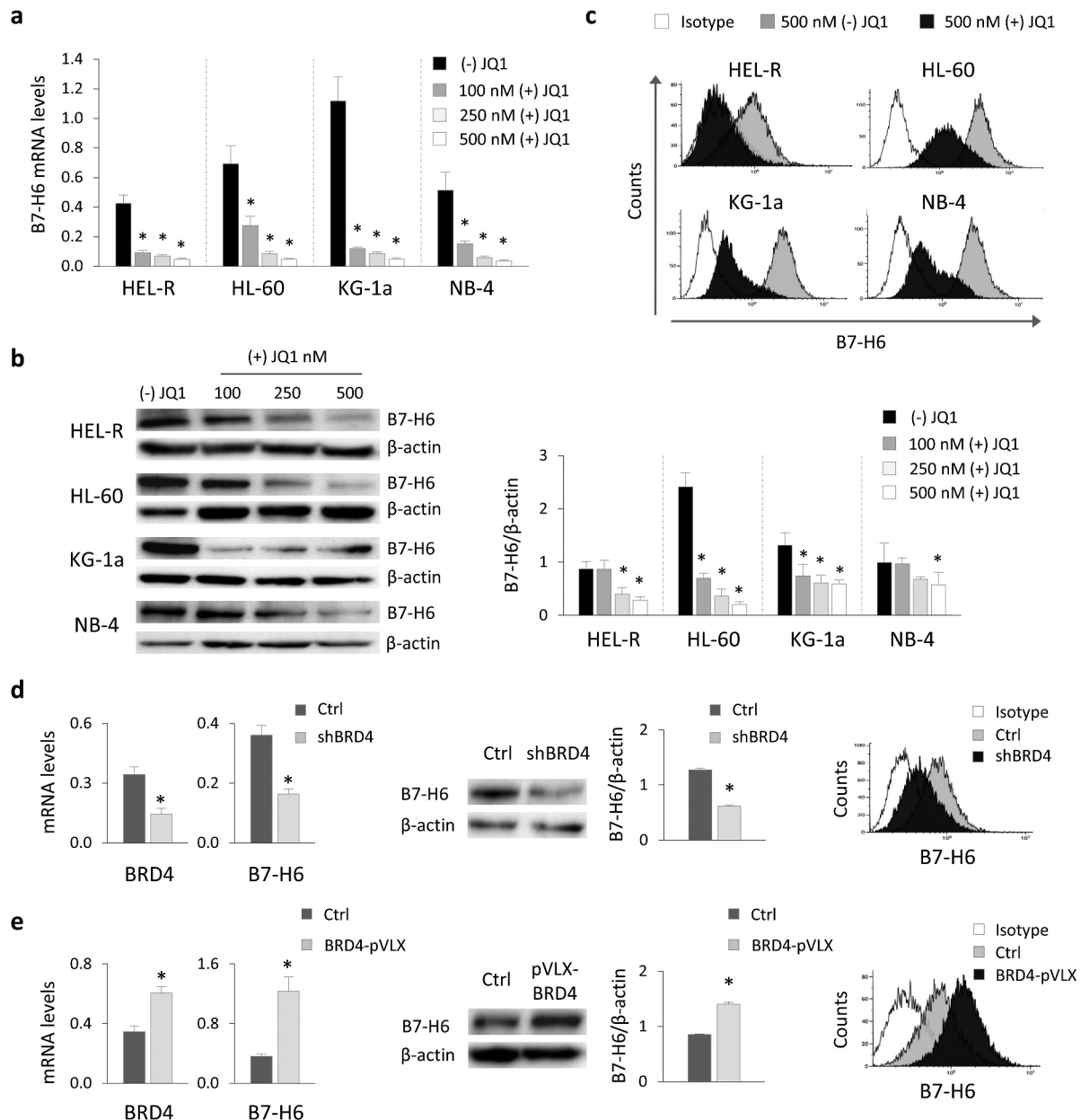
Figure 1. BRD4 inhibition decreases the expression of B7-H6 in AML cell lines. (**a**). AML cell lines (HEL, HL-60, KG-1a, NB4) were treated with (+) JQ1 (100, 200 and 500 nM) or with its negative enantiomer (-) JQ1 (500 nM) for 18 h. mRNA expression of B7-H6 was analyzed by qRT-PCR. Each bar represents the mean ± SEM. *p < .05. (**b, c**). After 48 h of (+) or (-) JQ1 treatment, B7-H6 protein levels were analyzed by western blot (b) and flow cytometry (c) in AML cell lines. (**d, e**) HEL-R cells were transduced with a plasmid containing a specific shRNA for BRD4 (d) or with lentivirus containing the BRD4 coding sequence (BRD4-pVLX)(e). BRD4 inhibition or overexpression was confirmed by qRT-PCR and B7-H6 expression was analyzed for each condition (d, e).

To determine whether JMJD6 collaborates in the regulation of B7-H6, HEL-R cells were transfected with an siRNA specifically targeting JMJD6 or control siRNA. *B7-H6* transcription was significantly reduced in JMJD6-knockdown HEL-R cells, suggesting that JMJD6 may participate in the regulation of this ligand (Figure 3a). To confirm these results, we analyzed the JMJD6 and BRD4 co-binding in a potential enhancer of *B7-H6* located between positions −8440 and −7559 bp, upstream of the translation–initiation site. This region is considered an enhancer-like by Registry of candidate cis-Regulatory Elements (cCREs) of ENCODE, which integrates DNase-seq and H3K4me3, H3K27ac, and CTCF ChIP-

seq data produced by the ENCODE and Roadmap Epigenomics Consortia. A ChIP assay using an anti-JMJD6 mAb was performed in HEL-R cells treated with (+) JQ1 or its enantiomer, and the full enhancer was amplified using adjacent primer pairs. Continuous enrichment of JMJD6 was observed along the complete enhancer, which was significantly reduced after (+) JQ1 treatment (Figure 3b). However, JMJD6 enrichment at promoter region of *B7-H6* was nearly undetectable. Results suggest that BRD4 also interacts with JMJD6 in the distal enhancer of *B7-H6* gene, promoting the release of promoter-proximal pausing and activating its transcription.
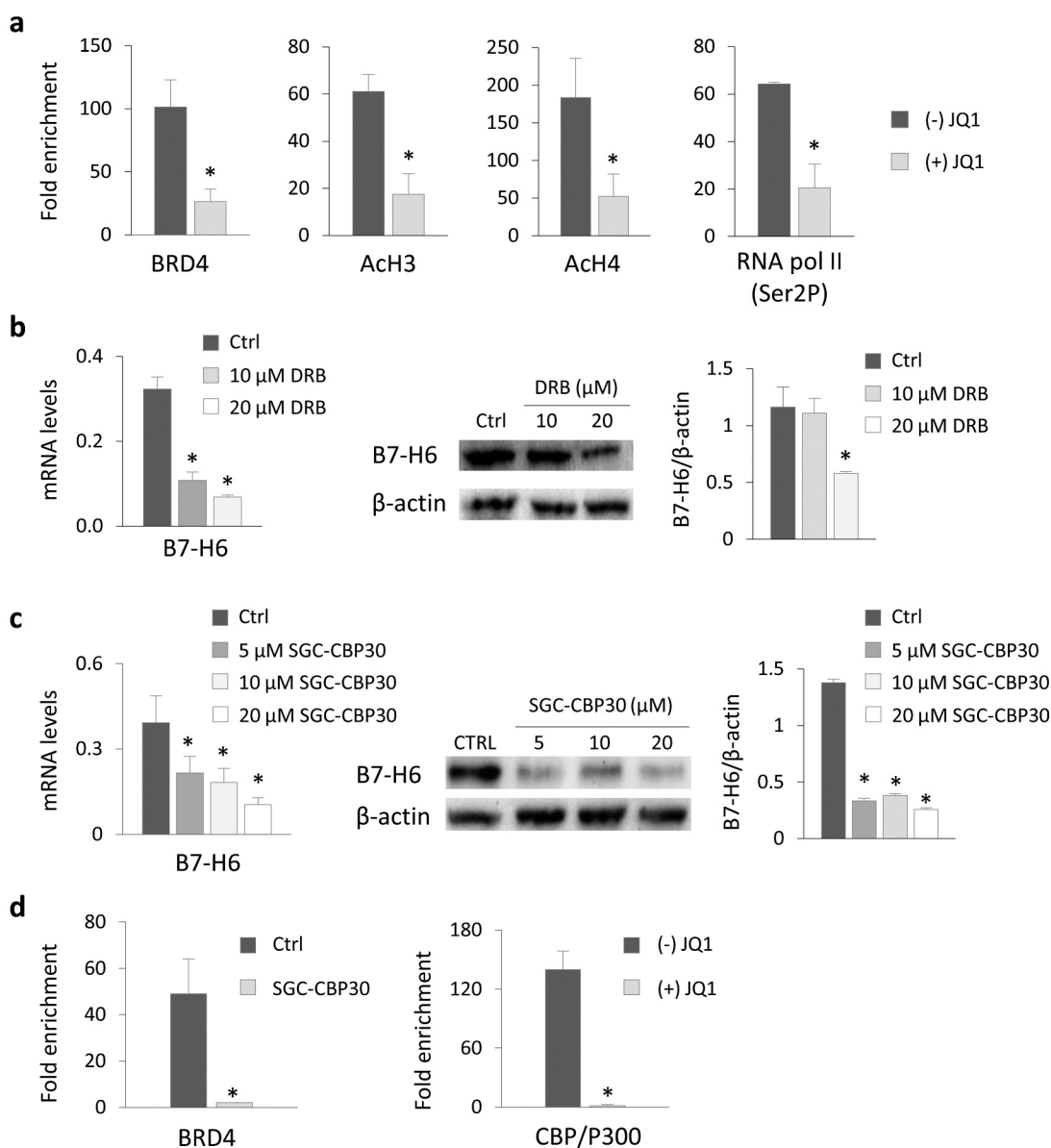
Figure 2. BRD4 binds to *B7-H6* promoter, regulating its expression. (**a**). HEL-R cells were treated with 500 nM of (+) or (-) JQ1 for 48 h. Enrichment of BRD4, acetylation for histones H3 (AcH3) and H4 (AcH4), and phosphorylation of RNA pol II Ser2 were analyzed by ChIP assays. Relative enrichment was determined by the mean ± SEM. Data from three independent experiments are shown and each quantitative PCR was run in triplicate. *p < .05. (**b, c**). HEL-R cells were treated with DRB, an inhibitor of CDK9 kinase (b), or with the inhibitor of CBP/P300, SGC-CBP30 (c). B7-H6 expression was analyzed by qRT-PCR and western blot at 6 h or 48 h post-treatment, respectively. (**d**). Relative enrichment of BRD4 and CBP/P300 after (+) or (-) JQ1 (500 nM) and SGC-CBP30 (20 μM) treatment in HEL-R cells. Values are the mean ± SEM of three independent experiments. Each quantitative PCR was run in triplicate. *p < .05.

Metformin modulates the histone acetylation patterns involved in B7-H6 expression

Epigenetic and metabolic alterations in cancer are closely mechanistically linked to promote the proliferation and survival of tumor cells. Here, we evaluated the effect of metformin on the epigenetic regulation of B7-H6. Metformin is an AMPK (AMP-activated protein kinase) inductor that promotes mTOR-dependent autophagy, but can also modulate the expression of epigenetic enzymes involved in the acetylation of histones and non-histone proteins.[32] In AML cell lines, treatment with metformin reduces B7-H6 expression in a dose-dependent manner (Figure 4a **and b**), lowering the levels of this molecule on the cell surface (Figure 4c). Moreover, the

combined effect of metformin with JQ1 showed an additive effect on the B7-H6 expression (**Figure S4**).

We also analyzed whether the effects mediated by metformin are due to changes in the acetylation patterns in the *B7-H6* gene. To do this, HEL-R cells were treated with metformin (60 mM, 6 h), and the AcH3, AcH4, and BRD4 levels were analyzed by ChIP assay. As shown in Figure 4d, treatment with metformin significantly reduced the histone acetylation levels in the regulatory region of *B7-H6* and, as consequence; the recruitment of BRD4. Therefore, metabolic alterations in AML cells induced during tumor progression might produce important epigenetic changes mediated by BET proteins that lead to overexpression of the B7-H6 molecule.
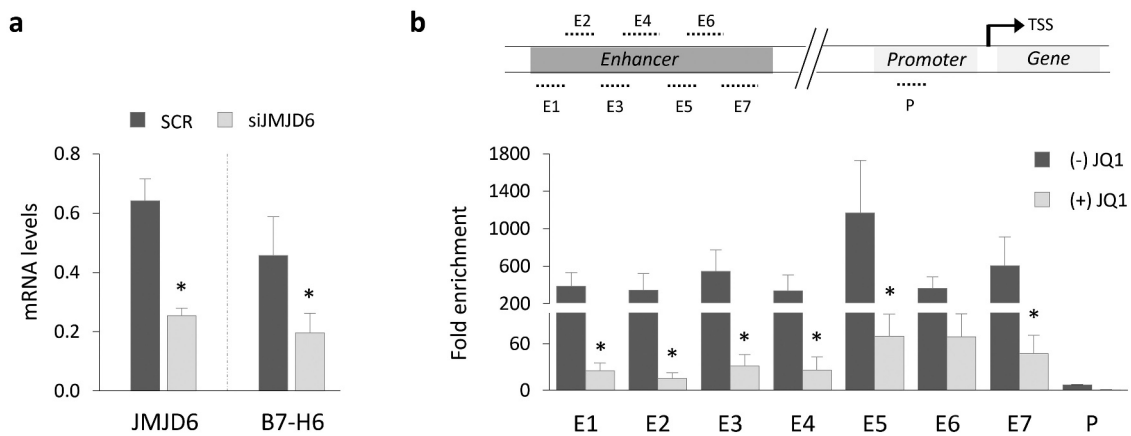
Figure 3. BRD4 and JMJD6 co-bind at anti-pause enhancer regions to promote *B7-H6* transcription. (**a**). *JMJD6* was silenced in HEL-R cells using a specific siRNA. *JMJD6* and *B7-H6* transcription levels were quantified by qRT-PCR. Results are shown as the mean ± SEM of three independent experiments. *p < .05. (**b**). HEL-R cells were treated with (+) or (-) JQ1 (500 nM) for 48 h and relative enrichment of JMJD6 was analyzed in a distal enhancer region (−8440 at −7403 bp) and in the promoter region of *B7-H6* gene by ChIP assay. Enrichment was shown as the mean ± SEM of three independent experiments. Scheme shows the enhancer and promoter regions of *B7-H6* gene amplified by qRT-PCR after chromatin immunoprecipitation, sequence primers and specific binding site are shown in Supplementary Table 1. *p < .05.

Blockage of B7-H6 inhibits the immune recognition mediated by NK cells but increases the cell death of AML cells

As B7-H6 is a ligand of the NKp30 activating NK cell receptor, a functional consequence of B7-H6 overexpression on the tumor cell surface might be NKp30-mediated NK recognition. Given that possibility, we assayed the effect of B7-H6 downregulation by BET inhibition using cytotoxicity assays (Figure 5a-**b**). In this case, the KG-1a AML cell line was used as target cells and primary NK cells as effector. Results showed that pharmacological inhibition of BET proteins with JQ1 reduces the immune recognition mediated by NK cells in a dose-dependent manner compared with the treatment with the inactive enantiomer (18.82 ± 1.31 *vs.* 9.35% ± 1.93 at 3:1 ratio, p < .05) (Figure 5a). Additionally, we observed that previous blockage of NKp30 in the effector cells decreases the lytic ability mediated by NK cells in a similar way to the observed with the JQ1 treatment (Figure 5a). Thus, NKp30/B7-H6 engagement plays an important role in the immune recognition of AML cells, as it has been previously reported, and the JQ1-mediated B7-H6 downregulation might damage that recognition.

On the other hand, it has been shown in other tumors that B7-H6 could have a pro-tumorigenic function, increasing proliferation, invasion, and migration of the tumor cells.[10–14] For this reason, we assayed the effect of B7-H6 knockdown on the cell proliferation and apoptosis of AML cells. B7-H6 was silenced in HEL-R cells (B7-H6 KO cells) using a lentivirus plasmid (plKO.1-puro) containing a specific shRNA B7-H6. Effective B7-H6 inhibition was corroborated by RT-PCR and flow cytometry (Figure 5b). Results showed a moderate and gradual reduction in the proliferation capacity of B7-H6 KO cells compared to the control (Figure 5c), but silencing of B7-H6 induces a significant increase in the number of apoptotic cells (3.1% vs 24%, Figure 5d). All these data suggest that B7-H6 is also involved in the survival of AML cells.

B7-H6 overexpression in AML patients is decreased after BRD4 inhibition

As we had previously shown that BRD4 positively regulates the B7-H6 ligand expression, we wanted to corroborate this association in blasts from AML patients. Accordingly, *BRD4* and *B7-H6* transcription levels were analyzed in bone marrow obtained from AML patients (n8) and healthy donors (n = 21) (Figure 6a). The results showed that whereas *B7-H6* expression was absent from healthy donors, its expression was significantly higher in AML patients (p = .016). No statistically significant difference was observed in the levels of *BRD4* expression between controls and AML patients, although the highest levels were noted in AML patients. However, Pearson's correlation analysis showed a positive association between B7-H6 and BRD4 transcription levels in those patients (r = 0.421, p = .0004) (Figure 6b). Moreover, we wanted to analyze whether JQ1 treatment reduces the expression of B7-H6 in primary AML cells. For this, AML cells isolated from two AML patients were treated with JQ1 and B7-H6 mRNA levels analyzed by RT-PCR. Results showed that JQ1 downregulated the expression of B7-H6 in a dose-dependent manner verifying the role of BRD4 as an essential regulator for B7-H6 expression (Figure 6c).

Next, *B7-H6* expression was analyzed with respect to the clinical characteristics of patients. For this, AML patients were classified into three groups according to the relative B7-H6 mRNA levels as low (≤0.1, n = 36), medium (>0.1-<0.6, n = 23), and high (≥0.6, n = 9). Firstly, we corroborated that the BRD4 levels were significantly different in those three established groups, showing the usefulness of that classification for further analysis (Figure 6d). Clinical association of B7-H6 transcription levels in AML patients was found with the etiology, for which the highest B7-H6 expression levels were observed in patients with secondary AML derived from myelodysplastic syndrome (p = .016) (Figure 6e). Moreover, AML patients with the 5q deletion were significantly increased in the group with B7-H6 high levels (p = .019 in Figure 6f and p = .016 in Table 2). In order to check the clinical impact of B7-H6 in
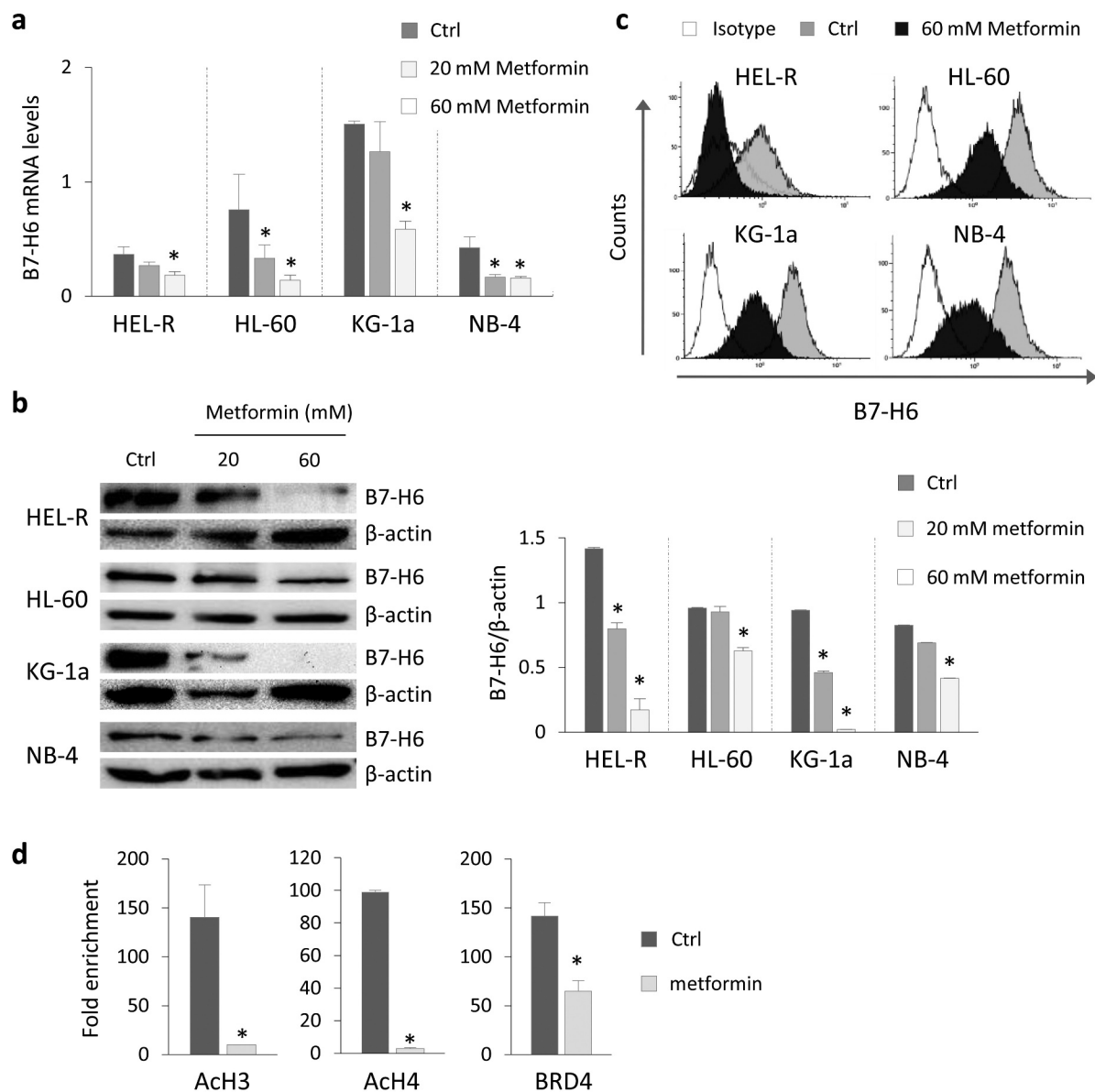
Figure 4. Metformin modulates histone acetylation levels impairing the interaction of BRD4 to the *B7-H6* promoter. (**a**). AML cell lines (HEL-R, HL-60, KG-1a, NB-4) were treated with metformin for 6 h and quantitative *B7-H6* expression was analyzed by RT-PCR. Results are summarized as the mean ± SEM of three independent experiments. *p < .05. (**b, c**). B7-H6 protein levels were analyzed by western blot (b) and flow cytometry (c) after metformin treatment for 24 h in AML cell lines. (**d**). HEL-R cells were treated with 60 mM of metformin for 24 h. The relative enrichment of histones H3 and H4 acetylation (AcH3, AcH4) and BRD4 was analyzed by ChIP assay. Enrichment is shown as the mean ± SEM of three independent experiments. Each quantitative PCR was run in triplicate. *p < .05.

additional AML patient cohorts, we analyzed the clinical data and B7-H6 gene expression in a TARGET AML cohort from UCSC Xena database (https://xena.ucsc.edu/). Data showed that high B7-H6 transcription levels were found in higher risk-group, associated with the presence of cytogenetic characteristics of poor prognosis (t (10;11)(p11.2;q23)) and a worst 4-y survival rate and overall survival (**Fig. S5a-c**). However, low B7-H6 levels were significantly associated with CEBPA and FTL-3 point mutations, both related to a favorable prognosis (**Fig. S5b**).

Discussion

B7-H6 is a ligand for the NKp30-activating receptor that plays an important role in the immune surveillance of cancer, but whose expression has been associated with poor prognosis in several human malignancies.[10–14] However, the role of this ligand in AML is scarcely known. Thus, to elucidate the mechanism controlling its expression could be useful for the development of therapeutic targets. In this study, we found that B7-H6 is highly transcribed in AML cell lines and primary AML cells and it is associated with a worse prognosis. B7-H6 transcription is dependent on the recognition and binding of BRD4 to acetylated histones in the promoter region of this gene. In fact, treatment with JQ1, a BET bromodomain inhibitor, or modification of the acetylation pattern with metformin significantly reduces the B7-H6 expression. Here, we found that the epigenetic reader BRD4 is involved in the regulation of B7-H6 expression, suggesting that the B7-H6 blockage could be useful as a therapeutic target for AML.
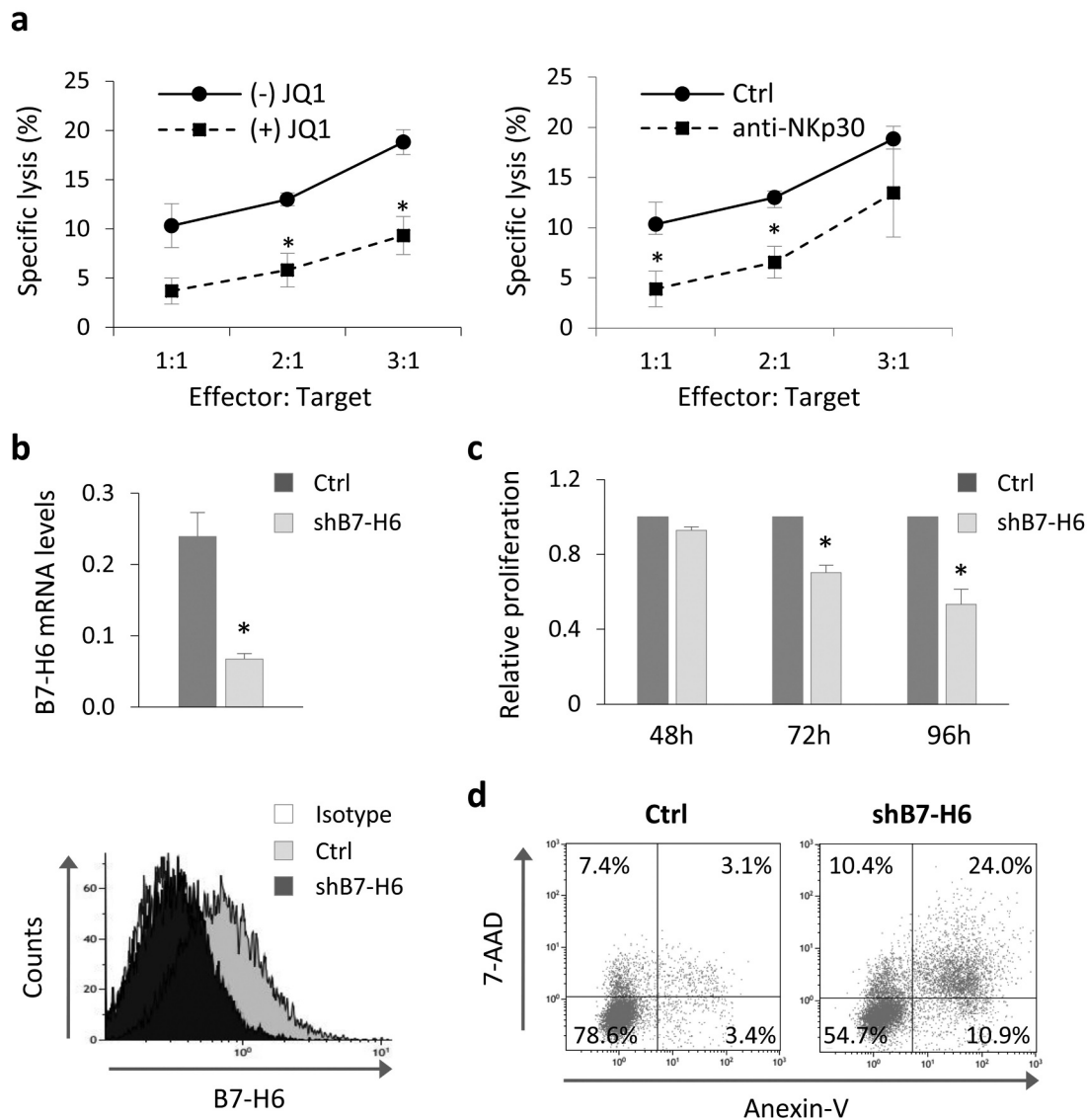
Figure 5. B7-H6 inhibition impair the immune recognition mediated by NK cells but increase the apoptosis of AML cells. (**a**). Isolated NK cells from healthy donors were co-cultured with KG-1a cells previously treated with 500 nM (+) or (-) JQ1 for 48 h, and used as target cells in a cytotoxicity assay. After 4 h of co-culture at different effector:target (E:T) ratios, the percentage of specific lysis was analyzed by flow cytometry. The role of the B7-H6/NKp30 engagement was analyzed pre-cultured the effector cells with an anti-NKp30 blocking mAb previously to the cytotoxicity assay. Data represent the mean ± SEM of three independent experiments. *p < .05. (**b**). HEL-R cells were transduced with a lentivirus plasmid (pLKO.1-puro) containing a specific shRNA for B7-H6 and B7-H6 silencing was confirmed by qRT-PCR and flow cytometry. (**C**). Effect of B7-H6 knockdown on cell proliferation was determined by fluorometric Cell Titer Blue assay. (**d**). Anexin V/7-ADD staining and flow cytometry were used to determine the number of apoptotic cells in control HEL-R cells or after B7-H6 knockdown. Numbers indicate the percentage of positive cells in each quadrant.

The expression of B7-H6 might have a dual effect in tumorigenesis. On the one hand, B7-H6 was first discovered as a ligand of NKp30 which allows the recognition and lysis of tumor cells by NK cells.[2] However, B7-H6 has also an important role in tumorigenesis. So, B7-H6 increases the proliferation and cell migration of tumor cells while reduces their apoptosis via STAT3 pathway.[33–36] As consequence, high expression levels of B7-H6 in many tumors (esophageal, cervical, hepatocellular, and breast cancers or non-Hodgkin lymphoma) were associated with poor prognosis (advanced stages, high level of progression, or worse overall survival).[10–14] It is possible that during tumor development, the role of B7-H6 in the immune recognition and tumor progression coexists over time. In the initial stages, the immune system could be recognizing and lysing the B7-H6-positive tumor cells. However, B7-H6 overexpression or the release in its soluble form might produce a chronic engagement with the NKp30 receptor inducing its downregulation.[37,38] As consequence, B7-H6 positive tumor cells, which have high capacity for proliferation, could escape of the immune system promoting the tumor development. According to that, immunotherapies using bispecific antibodies (anti-CD3 x anti-B7-H6) or B7-H6 specific CAR in polyclonal delta one T cells have been proposed in hematological tumors (lymphoma, AML) due to their ability to decrease the tumor growth improving the survival in murine models.[39–41]

In AML, while it is clearly established that NKp30 expression is reduced and associated with a poor prognosis,[15,42] little
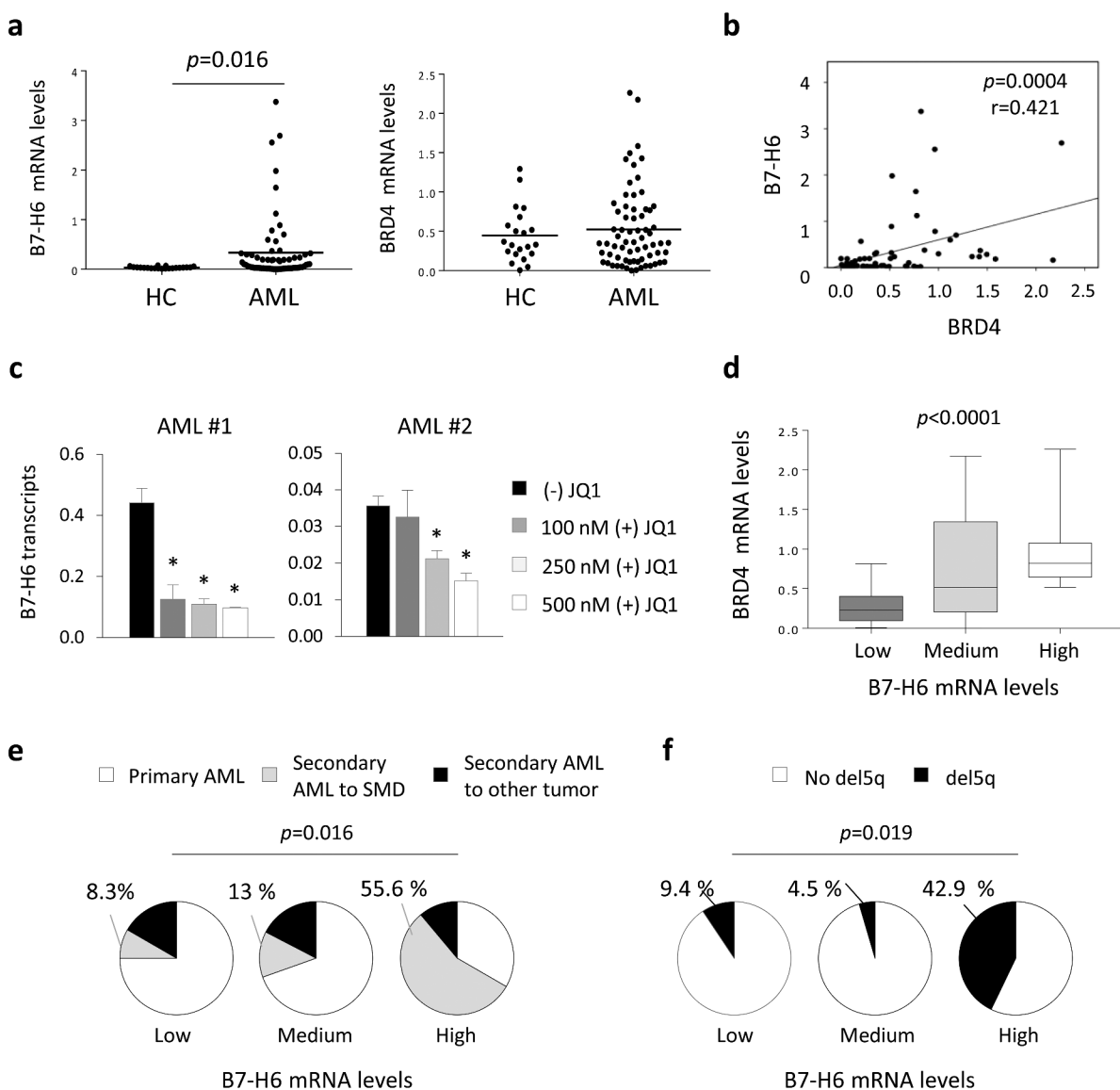
Figure 6. B7-H6 overexpression is correlated with BRD4 levels in AML patients. (**a**). BRD4 and B7-H6 transcripts were analyzed by qRT-PCR in bone marrow samples from AML patients (n = 68) and healthy donors (HC, n = 21). (**b**). Association between B7-H6 and BRD4 mRNA levels was analyzed by the Pearson correlation coefficient (r) and p-value (p) is shown. (**c**). Primary AML cells from two AML patients were treated with JQ1 (+) (100–500 nM) or its negative enantiomer JQ1 (-) during 18 hours and B7-H6 expression was analyzed by qRT-PCR. Statistically significant differences between groups were examined using the Wilcoxon test. * $p < 005$. (**d**). AML patients were classified into three expression groups according to the B7-H6 mRNA levels (low, medium, high). Box plots show the BRD4 mRNA expression among the different groups of AML patients compared by Kruskal–Wallis test. (**e, f**). Pie charts showing the frequency of AML patients with primary or secondary AML (**e**) and the absence or presence of the 5q deletion (**f**) based on B7-H6 expression levels. Statistically significant differences between groups were examined using the chi-square test.

is known about the expression of B7-H6 in this disease. A study by Chretien et al. showed a low level of B7-H6 expression on the cell surface of blasts in a small proportion of AML patients (2.5%).[15] In our study, all analyzed AML cell lines showed high levels of B7-H6 on the cell surface and in a high number of AML patients (19.1%) compared with healthy controls in which B7-H6 expression was undetectable. The highest B7-H6 levels were found in myelodysplastic syndrome-derived AML patients and in patients with deletions in 5q (del5q). Both clinical characteristics are associated with an unfavorable prognosis because they increase treatment resistance and worsen the overall outcome.[43,44] These findings seem to indicate that B7-H6 expression might increase during the tumoral progression associated with poor prognosis in AML. These data were corroborated in a second cohort from the public

UCSC Xena database verifying the relevance of B7-H6 in the pathogenesis of AML. We observed a strong association between high B7-H6 expression and clinical characteristics of poor prognosis, endorsing the essential role of this ligand in the proliferation and survival of AML cells, similar to what was previously observed in other tumor types.[33–36]

One of the hallmarks of cancer is the alterations in DNA–protein interactions that promote abnormal chromatin remodeling.[45] BET proteins are epigenetic readers, proteins that recognize and bind to post-translational histone modifications, involved in the transcriptional regulation of key genes during malignant transformation. BRD4, the best-studied protein of the BET family, has been involved in the pathogenesis of AML. BRD4 was overexpressed in AML patients and associated with shorter overall survival, the presence of specific

Table 2. Genetic alterations of AML patients in association with *B7-H6* expression.

Variant	Low B7-H6 $n = 36$	Medium B7-H6 $n = 23$ (%)	High B7-H6 $n = 9$	p
Cytogenetic characteristics				
t(8;21)	4 (80%)	1 (20%)	0 (0%)	ns
t(15;17)	5 (50%)	4 (40%)	1 (10%)	ns
inv(16) or t (16;16)	7 (77.8%)	1 (11.1%)	1 (11.1%)	ns
t(9;11)	1 (10%)	0 (0%)	0 (0%)	ns
−7/−7q	0 (0%)	1 (50%)	1 (50%)	ns
−21	1 (25%)	3 (75%)	0 (0%)	ns
+4	1 (50%)	1 (50%)	0 (0%)	ns
+8	3 (75%)	1 (25%)	0 (0%)	ns
+11	2 (66.7%)	0 (20%)	1 (33.3%)	ns
+13	0 (0%)	2 (100%)	0 (0%)	ns
+21	2 (50%)	1 (25%)	1 (25%)	ns
del5q	3 (42.8%)	1 (14.4%)	3 (42.8%)	0.016
del7q	2 (33.3%)	4 (67.7%)	0 (0%)	ns
del17p (p53)	5 (83.3%)	1 (16.7%)	0 (0%)	ns
Normal karyotype	9 (56.2%)	6 (37.5%)	1 (6.3%)	ns
Complex karyotype	3 (60%)	2 (40%)	0 (0%)	ns
Gene mutations				
NPM1	5 (71.4%)	1 (14.3%)	1 (14.3%)	ns
FLT3-D835	1 (25%)	3 (75%)	0 (0%)	ns
FLT3-ITD	5 (71.4%)	1 (14.3%)	1 (14.3%)	ns
CEBPA	2 (100%)	0 (0%)	0 (0%)	ns

Notes: ns: not significant. (%): number of positive AML patients for each cytogenetic characteristic or gene mutation with respect to total patients harboring that clinical characteristic.

mutations such as NPM1 or FLT3-ITD, or the expression of various anti-apoptotic proteins (Bcl-2) and oncogenes (CREB, ELK1, ERG).[46] As a consequence, and because of their ability to arrest the cell cycle and promote apoptosis of AML cells in *in vitro* assays and murine models, treatment with BET protein inhibitors has been proposed as a therapeutic strategy.[21,22] Here, we found that treatment with JQ1, a broad-spectrum inhibitor of BET proteins, impairs *B7-H6* transcription and reduces its expression on the cell surface of AML cells. Assays of specific inhibition and overexpression confirm that BRD4 is involved in *B7-H6* transcription. Moreover, the downregulation of B7-H6 transcripts after JQ1 treatment in primary AML cells and the close correlation between BRD4 and B7-H6 expression in AML patients suggests that BRD4 acts as a positive regulator of B7-H6. One of the first and most relevant genes regulated by BRD4 is the MYC proto-oncogene.[26] It has been reported that MYC regulates B7-H6 expression in tumor cell lines and primary samples of hepatocarcinoma, lymphoma, and neuroblastoma.[6] However, we did not find an association between MYC and B7-H6 in AML cell lines.

To regulate gene transcription, BRD4 recognizes and binds to acetylated lysine residues in histones and non-histone proteins, recruiting the *P*-TEFb complex to regulatory regions and allowing the phosphorylation of RNA pol II at the Ser2 residue.[47] Using chromatin immunoprecipitation studies, we demonstrated that the *B7-H6* promoter is highly enriched in acetylated histones (H3 and H4), which are recognized by the bromodomains of BRD4 protein. After that, BRD4 recruits the *P*-TEFb factor, a cyclin-dependent kinase that facilitates the elongation phase of transcription by RNA pol II. We also determined that the histone acetyltransferase (HAT), CBP/P300, is essential for facilitating the interaction of BRD4 to *B7-H6* promoter and for regulating its expression. CBP/P300 promotes the acetylation of lysine residues on histones and non-histone proteins and helps maintain the self-renewal of AML cells, thereby promoting the malignant transformation.[48,49] Pharmacological inhibition of BET (JQ1) and CBP/P300 (CBP30) proteins prevents the recruitment of the transcriptional machinery to the regulatory region of the *B7-H6* gene, thereby blocking its expression.

The RNA pol II pause was first discovered in genome-wide studies of stem cells and *Drosophila*, which revealed that this phenomenon affects 10–40% of all genes.[50] In *Drosophila*, it has been reported that transcriptional pausing controls the rapid antiviral innate immune response.[51] In mammals, multiple genes of rapid response such as heat-shock proteins are exhaustively regulated by RNA pol II pausing.[52] BRD4 plays an important role in the regulation of this phenomenon through its interaction with JMJD6 in distal enhancers.[24] As a consequence of this interaction, JMJD6 demethylates H4R3me2, releasing the binding of the 7SK snRNA/HEXIM inhibitory complex imposed on *P*-TEFb, in turn promoting the phosphorylation of RNA pol II and, thereby, the transcriptional elongation of target genes. We found an enrichment of JMJD6 in a potential enhancer region of *B7-H6* that remains bound to BRD4. Specific blockage of JMJD6 or BRD4 inhibition reduced the level of B7-H6 expression, suggesting that this molecule might be a paused gene in healthy cells waiting for a stimulus, damage signal, or inflammatory condition to be transcribed.

During malignant transformation, tumor cells modulate their metabolism in order to enhance cellular proliferation and development. One of these changes is an increase in TCA cycle metabolism to generate ATP, NADPH, and the substrates needed to synthesize the macromolecules and oncometabolites required for tumoral proliferation.[53] To achieve this, cells need a functional electron transport chain (ETC) to maintain the TCA cycle, which has led to mitochondrial ETC dysfunction being proposed as something that could be exploited in the development of cancer therapies.[54] Metformin, a drug used to treat type 2 diabetes, is a mitochondrial ETC complex I inhibitor.[55] In recent years, many studies have shown that treatment with metformin inhibits the growth of tumor cells by activating the AMPK/mTOR, thereby promoting autophagy.[56,57] Additionally, metformin alters the chromatin structure that regulates the function of enzymes such as CBP/P300, SIRT1, and HAT1, which are involved in histone acetylation.[58–60] We found that metformin reduces B7-H6 expression, to a similar extent to JQ1, by modulating acetylation patterns in the *B7-H6* promoter. Moreover, the treatment of JQ1 plus metformin enhances the inhibition of B7-H6 in AML cells suggesting the potential of the combinatory treatment as a new therapeutic strategy to suppress B7-H6 expression in AML, as occurs in thyroid cancer.[61]

Conclusions

This study shows that the epigenetic reader BRD4 is essential for B7-H6 transcription in AML. We found that the tumor cell surface protein B7-H6 is highly expressed in some AML patients and associated with the clinical characteristics of
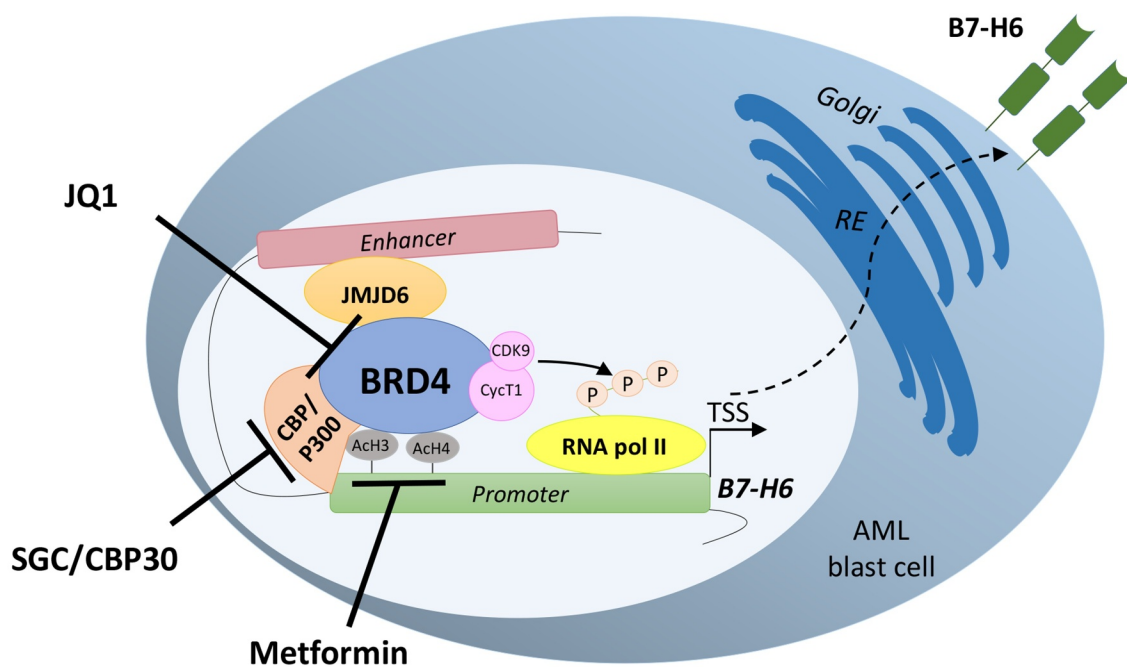
Figure 7. BRD4 maintains expression of the B7-H6 tumorigenic molecule in AML. In AML, BRD4 co-binds with JMD6 in the distal enhancer of *B7-H6* gene, and is recruited to the promoter region after recognition of acetylated histones. As consequence of this binding, BRD4 recruits the *P*-TEFb complex (CDK9, Cyc T1 subunits) releasing the pause of RNA pol II and allowing the transcriptional elongation of the tumorigenic molecule, B7-H6, in AML cells. Treatments with BET inhibitors (such as JQ1), the CBP/P300 HAT inhibitor (SGC-CBP30) and/or metformin can damage the assembly of the transcriptional complexes, preventing *B7-H6* transcription and its expression on the cell surface of AML cells.

poor prognosis. Therefore, the mechanisms involved in its expression could be useful in therapeutic strategies.

In AML cells, BRD4 acts as a bridge to regulate B7-H6 transcription (Figure 7). BRD4 binds to hyperacetylated regions in the *B7-H6* promoter, recruiting the transcriptional machinery that regulates its expression. Moreover, BRD4 and JMJD6 co-bind at the distal enhancer of *B7-H6*, suggesting that this ligand is a paused gene that might be activated during malignant transformation.

Treatment with the BET protein inhibitor, JQ1, or modulation of the acetylation patterns with metformin, a metabolic inhibitor, impairs the assembly of the transcriptional machinery, thereby reducing the B7-H6 expression. Knockdown of B7-H6 decreases the proliferation of AML cells and induces cell death by apoptosis of AML cells showing its essential role in promoting tumorigenesis. Overall, the results obtained here show that JQ1 and metformin, separately or in combination, could be successfully incorporated into an effective therapeutic strategy that targets B7-H6 in AML cells.

Abbreviations

AML: Acute myeloid leukemia
AMPk: *AMP-activated protein kinase*
B7-H6: *B7 homolog 6*
BET proteins: Bromodomain and extra-terminal domain proteins
BRD4: *Bromodomain-containing protein 4*
FAB: French-American-British classification
FLT3: *FMS-like tyrosine kinase-3*
I-BET: BET inhibitors
JMJD6: *JmjC-domain-containing protein 6*
mTOR: *mammalian Target of Rapamycin*
MYC: *Myelocytomatosis viral oncogene*

NKp30/NCR: *Natural cytotoxicity triggering receptor 3*
P-TEFb: *Positive transcription elongation factor*

Ethics approval and consent to participate

All patients and healthy donors gave their written informed consent, in accordance with the Principles of the Declaration of Helsinki. The local hospitals' ethics committees approved the study.

Competing interest

The authors declare no conflicts of interest.

Author contributions

ABR, BSA, and CLL designed the study. ABR, RMR, and ABF did the research. AMP, PP, and EC provided samples. ABR, RMR, and BSA collected, analyzed, and interpreted the results. ABR, BSA, and CLL wrote and revised the manuscript.

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