Targeting BRD4 in acute myeloid leukemia with partial tandem duplication of the *MLL* gene

The lysine methyltransferase 2a (*KMT2A*) (which is also known and hereafter referred to as *mixed-lineage leukemia [MLL], trithorax [Drosophila] homolog gene)* plays a pivotal role in embryogenesis and hematopoiesis. Recurrent, balanced translocations involving the *MLL* gene [t(v;11q23)] are heterogeneous, and more than 75 different fusion partners have been described as important drivers in acute myeloid leukemia (AML) leukemogenesis.[1,2] Beside chromosomal aberrations, a unique gene rearrangement in *MLL* known as partial tandem duplication (PTD) can be found in approximately 5-11% of cytogenetically normal AML (CN-AML) patients. This mutation is associated with poor prognosis.[3-8] Although both t(v;11q23) and the *MLL*-PTD result in increased *HOMEOBOX (HOX)* gene expression in leukemic blasts, t(v;11q23) have been shown to be genetically and functionally distinct from the *MLL*-PTD.[9] While almost all t(v;11q23) lose their C-terminal transactivation and methyltransferase domains, these C-terminal domains are retained in the *MLL*-PTD.[9]

The transcription factor BRD4 is a member of the bromodomain and extra terminal (BET) family of proteins. Aberrant BRD4 binding and gene activation has been shown to be important for t(v;11q23)-mediated leukemogenesis.[10] JQ1 is one of the best-characterized, small molecule bromodomain inhibitors.[11] However, the potential use of JQ1 in *MLL*-PTD AML has not been extensively studied yet. Therefore, we examined whether *MLL*-PTD AML blasts are sensitive to JQ1 treatment and if BRD4 inhibition results in an altered binding of the transcription factor to DNA.[12]

First, we tested whether JQ1 treatment has an impact on cell proliferation and survival in a *MLL*-PTD$^+$ AML cell line (i.e., EOL-1) and in a *MLL* wild-type cell line (i.e., K562).[13] Both cell lines were treated with JQ1 or dimethyl sulfoxide (DMSO) vehicle control for 24 hours (h) at different concentrations and cell growth was assessed by WST-1 assay. We found a significant decrease in cell proliferation in EOL1 cells (IC_{50} = 321 nM) but not in K562 cells (Figure 1A). Concomitantly, we found a significant and dose dependent increase in the number of apoptotic EOL-1 cells (Figure 1B) but not in the K562 cells (*Online Supplementary Figure S1A*). We also analyzed the effect of JQ1 treatment on primary blast cell growth from three AML patients that harbor a *MLL*-PTD compared to normal hematopoietic stem and progenitor cells (HSPC; CD34$^+$ cord blood) controls. We found JQ1 treatment significantly reduced blast cell growth assessed by a decrease in the number of colony-forming cells (CFC) in JQ1-treated *MLL*-PTD AML samples, with no significant decreases of CFC in normal HSPC (Figure 1C) or MLL wild-type (wt) primary patient samples (*Online Supplementary Figure S1B*).

Next, we tested the effect of JQ1 in a murine AML mouse model. For these experiments we used our well established *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ double knockin AML mouse model[14,15] that develops lethal CN-AML with ~100% penetrance. In secondary bone marrow transplantation, it leads to death within 6 to 12 weeks.[14,15] Of note, *MLL*-PTD is predominantly found in CN-AML in humans. First, we wanted to determine whether JQ1 also induced apoptosis in the *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ mouse AML blasts, similar to what we observed in human AML blasts. We found that JQ1 induced a significant increase in apoptosis assessed by Annexin V staining in the *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ blasts (Figure 1D) with essentially no toxicity to normal murine bone marrow cells (*Online Supplementary Figure S1C*). Based on these promising *in vitro* results, we then wanted to test, whether targeting BRD4 *in vivo* would result in prolonged survival of mice with *Mll*-PTD$^+$ leukemia. In order to test the anti-leukemic activity of JQ1 in a murine AML model, we used our previously established *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ mouse model.[14-16] We observed a significant increase in survival of JQ1 treated *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ mice compared to mice treated with vehicle control (Figure 1E). Moreover, the mice that eventually succumbed to disease and were treated with JQ1 had significant lower spleen weight, indicating a lower leukemic burden (Figure 1F). Interestingly, we also found that the leukemic bone marrow cells from JQ1-treated mice had a significant lower engraftment potential after re-transplantation than cells from mice treated with vehicle control (*Online Supplementary Figure S1D*). These data suggested that JQ1 might also have an impact on leukemic cell self-renewal and consequently leukemia stem cells (LSC) in *Mll*-PTD AML, however further experiments are needed to fully address effects on LSC by JQ1 in *Mll*-PTD leukemia.

After identifying the ability of JQ1 to decrease *MLL*-PTD$^+$ AML blast growth *in vitro* and *in vivo*, we wanted to determine whether alterations in BRD4 binding accounts for *MLL*-PTD blast sensitivity to BRD4-inhibition. BRD4 has been shown to be a positive regulator of gene transcription and aberrant BRD4-binding in cancer induces alterations in gene expression. Thus, we hypothesize that *MLL*-PTD leukemogenesis is driven by dysregulation of gene expression patterns resulting from aberrant BRD4 binding. Furthermore, we wanted to determine whether normal BRD4 binding could be restored by treatment with JQ1. In order to address this question, we performed total RNA sequencing (RNA-seq) on primary *MLL*-PTD AML blasts and normal HSPC treated with JQ1 or vehicle control (n=3 for each group, pooled) before and after JQ1 treatment and analyzed as previously described.[18] In addition, we performed chromatin immunoprecipitation sequencing (ChIP-seq) using a BRD4 antibody. Cells from three healthy cord blood donors (CB) and primary leukemic cells from three patients with *MLL*-PTD were treated with either JQ1 (10 nM) or vehicle (DMSO) for 24 hours (h) and ChIP was performed as previously described.[18] The 75-basepair sequence reads were generated using an Illumina sequencing platform (NextSeq 500) and then mapped to the human reference genome (GRCh37/hg19) using the BWA algorithm with default settings.[19] Aligned reads were normalized and genomic regions with local enrichments against corresponding input sample, peaks, were defined using MACS algorithm with a cutoff *P*-value of $1e^{-7}$.[20] Consensus peaks were defined by merging overlapping peak coordinates and peak scores were calculated. The resulting matrix was annotated with gene information by calculating distances from RefSeq gene starts and ends to the center of the consensus peak regions and applying an annotation cutoff of 5 kb. Similar to what has previously been described for t(v;11q23)-AML that BRD4 has an aberrant binding profile, we found three times more and stronger genomic interactions of BRD4 in the *MLL*-PTD patient sample compared to normal HSPC, and only 22% of the BRD4 peaks in *MLL*-PTD overlapped with normal HSPC in ChIP-seq (Figure 2A). When we treated the *MLL*-PTD sample with JQ1, both the number of peaks and their intensity decreased, whereas JQ1 treatment did not affect BRD4 binding in HSPC (Figure 2B). Next, by integrating the RNA-seq and ChIP-

seq data, we wanted to determine which genes had alterations in BRD4 binding, leading to mRNA changes in the AML patient cells compared to normal CD34$^+$ cells. For these analyses, the data obtained from RNA-seq counts, and ChIP-seq using BRD4, were merged by gene id. Our strategy to detect different patterns of changes in peak scores and gene expression consisted in the conversion of the values into quartiles in way to define *MLL*-PTD specific response to JQ1 treatment.

Furthermore, we also wanted to determine the alterations that were reversed by BRD4-inhibiton. First, we identified BRD4 binding sites that were close by to a

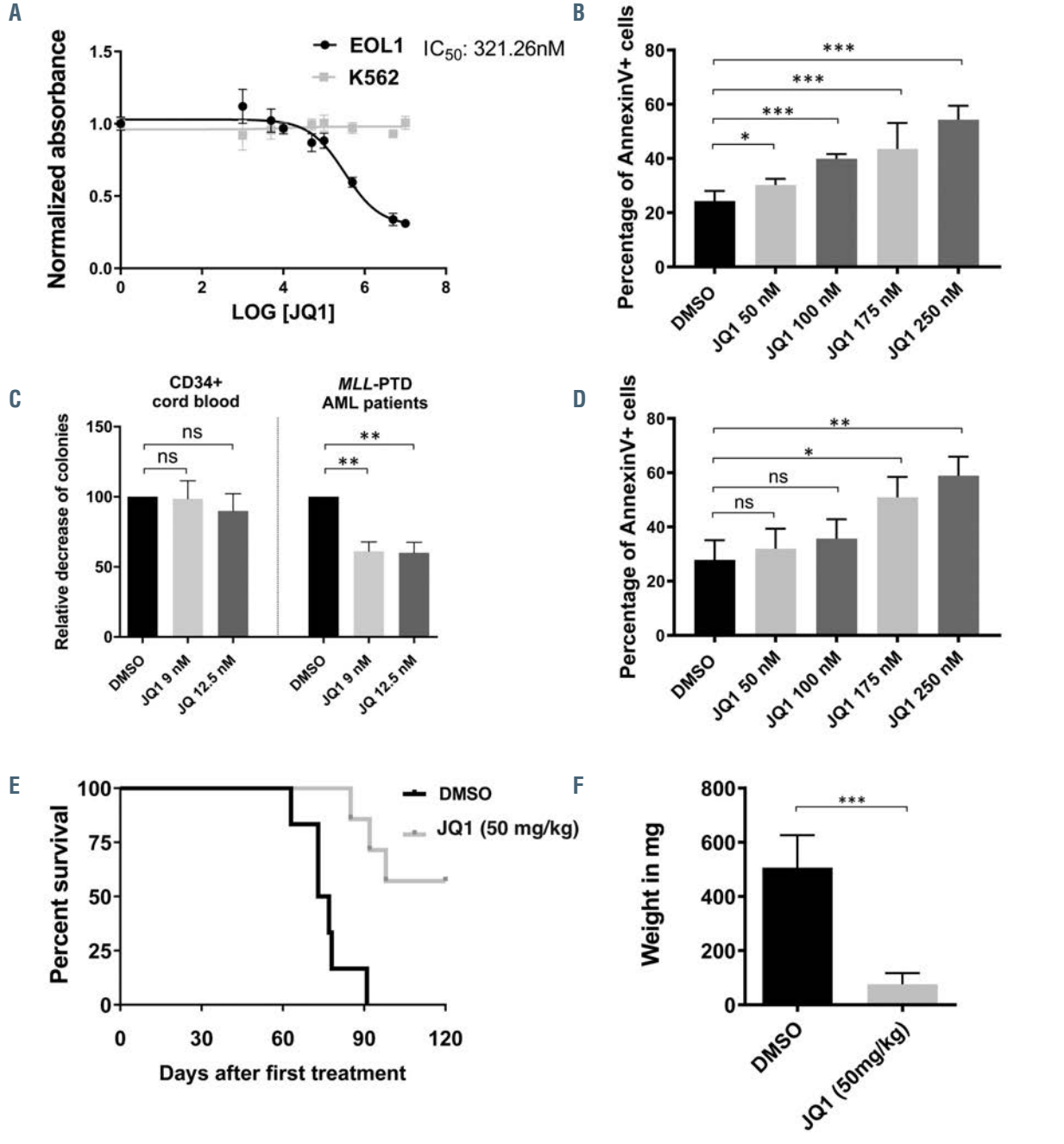

Figure 1. Targeting BRD4/Brd4 using the inhibitor JQ1 has an effect on *MLL*-partial tandem duplication (*MLL*-PTD)/*Mll*-PTD acute myeloid leukemia cells. (A) WST-1 assay on EOL-1 and K562 cells treated with the indicated concentrations of JQ1 for 48 hours (h). (B) EOL-1 cells were treated for 24 h with the indicated concentration of JQ1. Cells were then assessed for apoptosis using Annexin V$^+$ staining and flow cytometry at 24 h post treatment; *P<0.05, ***P<0.001. (C) Colony forming unit assays were performed on three CD34$^+$ cord blood and three *MLL*-partial tandem duplication (*MLL*-PTD) acute myeloid leukemia (AML) patients' samples, cells were plated in triplicates, normalized results are shown.[13] Cells were treated with JQ1 at a concentration of 9 nM or 12.5 nM or with vehicle control (dimethyl sulfoxide [DMSO]); **P<0.01. (D) Primary murine *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ blasts were treated for 24 h with the indicated concentration of JQ1. Cells were then assessed for apoptosis using Annexin V$^+$ staining and flow cytometry at 24 h post treatment; *P<0.05, **P<0.01. (E) *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ blasts were transplanted into sub-lethally irradiated BoyJ mice. Starting at 2 weeks post transplantation, mice were treated with 50 mg/kg body weight of JQ1 or vehicle control for 6 days each week for the entire duration of the study. Mice that died early without any signs of leukemia were excluded. The experiment was stopped after 120 days post treatment initiation. Treatment with JQ1 prolonged survival compared to controls (P=0.001) and (F) also led to a reduced weight of the spleen.

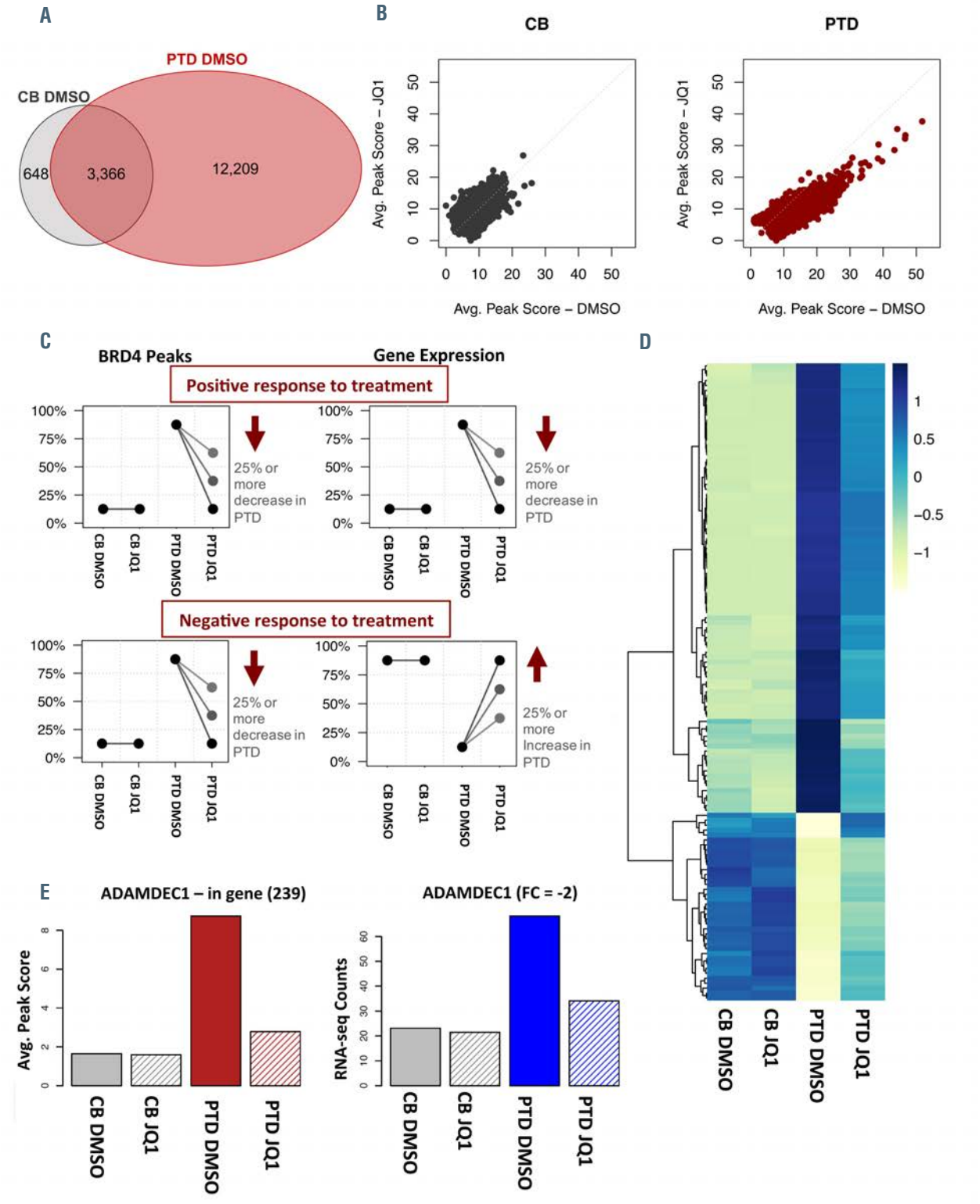

Figure 2. Aberrant BRD4 binding in *MLL*-partial tandem duplication cells drives a distinct gene expression profile that can be restored by JQ1 treatment. (A) Venn diagram showing overlap of BRD4 binding sites between CD34$^+$ selected cord blood (CB) samples primary samples from *MLL*-partial tandem duplication (*MLL*-PTD) acute myeloid leukemia (AML) patients. (B) Scatter plots showing changes in BRD4 binding peak scores upon JQ1 treatment in healthy CB and MLL-PTD AML samples. (C) Schematic overview over the bioinformatic approach used to identify the genes deregulated by BRD4 in *MLL*-PTD cells. A peak and gene pair was classified as *MLL*-PTD specific positive response when peak score and gene expression were in first quartile (0-25%) for CB samples, peak score and gene expression were in the top quartile (75-100%) for MLL-PTD dimethyl sulfoxide (DMSO) sample, and a decreased expression was observed in both peak score and gene expression for *MLL*-PTD sample upon JQ1 treatment (25% or more). Similarly, we identified potential oncosuppressor genes downregulated by MLL-PTD but restored by JQ1 treatment. Genes whose expressions were positively or negatively correlated with changes in BRD4 binding with at least 25% change under JQ1 treatment are shown in the *Online Supplementary Table S1*.(D) Heat map of genes that were found to be deregulated in *MLL*-PTD cells and which also showed a response to JQ1 treatment. (E) Example of one of 130 genes that fulfilled all criteria. ADAMDEC1 expression is increased in *MLL*-PTD AML cells by a binding of BRD4, and is downregulated upon JQ1 treatment.

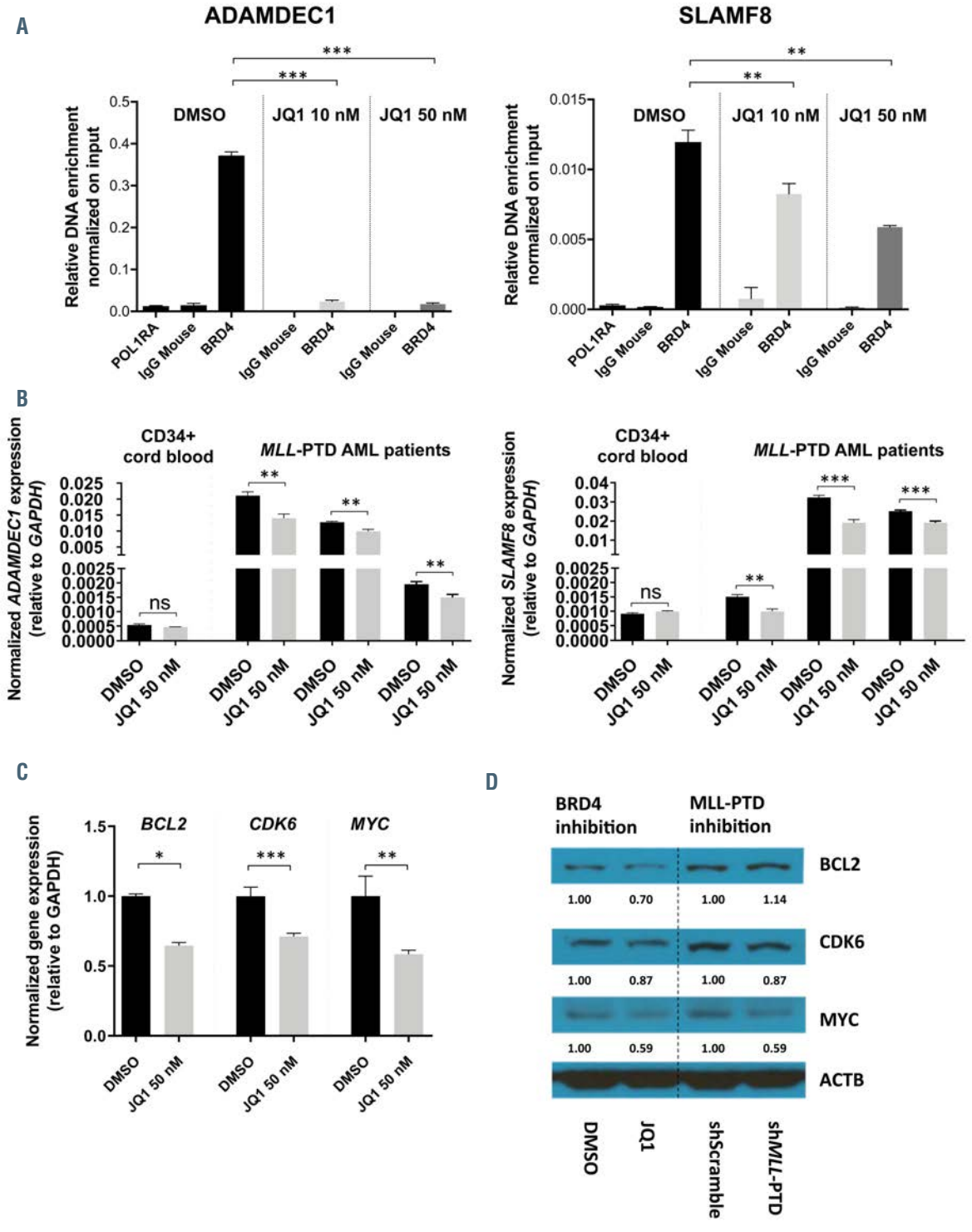

Figure 3. *MLL*-partial tandem duplication drives the aberrant expression profile through BRD4. (A) Cell lysate of EOL-1 cells, either treated with vehicle (dimethyl sulfoxide [DMSO]) or with the indicated concentration of JQ1, were subjected to a chromatin immunoprecipitation (ChIP) assay. Antibodies against BRD4 or POL1RA (control) or an unspecific immunoglobulin G (IgG) were used for the pulldown. DNA of *ADAMDEC1* and *SLAMF8* were quantified using quantitative real-time polymerase chain reaction (qRT-PCR). The enriched binding of BRD4 to both genes, i.e., *ADAMDEC1* and *SLAMF8*, was decreased after treatment of JQ1; $^{**}P<0.01$, $^{***}P<0.001$. (B) Relative expression of *ADAMDEC1* and *SLAMF8* relative to *GAPDH*. qRT-PCR was performed on CD34^{+} selected cord blood (CB) cell samples (n=3, pooled) and three primary cell samples from MLL-partial tandem duplication (*MLL*-PTD) acute myeloid leukemia (AML) patients treated with JQ1 at 50 nM for 24 hours. Treatment with JQ1 leads to significantly lower expression of the genes in *MLL*-PTD patients' cells but not in CB cells; ns = not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (C) Normalized expression of BRD4 downstream targets, *BCL2, CDK6* and *MYC*, relative to *GAPDH* in EOL1 cells. Cells treated with JQ1 for 48 hours showed significantly reduced expression of the genes; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (D) Western Blot analysis of EOL1 cells, which were previously treated with JQ1 or vehicle (DMSO) as control, validated the downregulation of these genes. Similar results were found when cells were transfected with a short hairpin RNA (shRNA) against *MLL*-PTD (shMLL-PTD) but not when transfected with a scramble control. Data of the densitometry are shown. RNA, cDNA, real-time PCR, ChIP and western blots were performed using previously published methods.[13,16-18]

gene's transcription start site (TSS), specifically bound in *MLL*-PTD cells but not in HSPC and could be suppressed by at least 25% through JQ1 treatment. Then, the genes close by such BRD4 binding sites and presenting the same pattern in their expression were classified as positive response to treatment, whereas the genes that present the opposite trend in their expression were classified as negative response to treatment (Figure 2C). As result, we identified genes whose expression was positively or negatively regulated by changes in BRD4 binding specific in the *MLL*-PTD AML sample and could be reversed by JQ1 treatment (Figure 2D). We identified 92 genes which were significantly upregulated in *MLL*-PTD AML cells through BRD4 binding and which were downregulated by BRD4 inhibition (*Online Supplementary Table S1*). As an example, Figure 2E shows the expression of *ADAMDEC1* which is increased in *MLL*-PTD AML cells and correlates with binding of BRD4. On the other hand, 38 genes were downregulated by BRD4 in the *MLL*-PTD AML cells and its inhibition led to a re-expression (*Online Supplementary Table S1*). We also performed ingenuity pathway analysis (IPA) from the ChIP-RNA integration and identified pathways that are affected by BRD4-mediated transcriptional changes (*Online Supplementary Table S2*). These data suggest that the distinct gene expression profile of *MLL*-PTD positive AML, is at least partly driven by the transcription factor BRD4, similar to AML cells which harbor a t(v;11q23). In addition, we showed that this aberrant expression can be restored after JQ1 treatment. Because we compared only cells from one patient (treated *vs.* untreated) with HSPC from three pooled CB samples, next we wanted to validate the direct binding of BRD4 to two genes that were upregulated in *MLL*-PTD AML cells, i.e., ADAMDEC1 and SLAMF8 (Figure 3A). These genes were also shown to have a functional role in *MLL*-PTD cells since knockdown of either of these genes resulted in decreased leukemic cell growth (*Online Supplementary Figure S1E*). Using a chromatin immunoprecipitation followed by quantitative real-time polymerase chain reaction (qRT-PCR), we show that BRD4 binds to *ADAMDEC1* and *SLAMF8*, and this binding can be repressed by JQ1 treatment. Moreover, both genes are upregulated in *MLL*-PTD AML patients cells compared to CB cells (Figure 3B). For both genes, JQ1 treatment led to decreased expression. Similar results were found when *MLL*-PTD was knocked down (*Online Supplementary Figure S1F and G*). We also found similar results in our *in vivo* mouse model. Mice treated with JQ1 had lower expression of several potential oncogenes, including *Adamdec1* and *Slamf8* (*Online Supplementary Figure S1H to J*). Finally, we showed that JQ1 treatment of *MLL*-PTD cells also results in the decreased expression of *BCL2, CDK6,* and *MYC*, well-established downstream targets of BRD4 (Figure 3C).[1,2] We found that knocking down the *MLL*-PTD fusion gene using a short hairpin RNA (shRNA) also resulted in downregulation of these proteins regulated by BRD4, similar to treatment with JQ1 (Figure 3D). Previously, it has been shown that fusion proteins from t(v;11q23) can initiate aberrant gene expression profiles by recruiting BRD4.[1] Our data suggest that in *MLL*-PTD cells utilize a similar mechanism and could account for the distinct gene expression profile.

Taken together, our data shows that targeting BRD4 with the small molecule JQ1 reduces cell proliferation of *MLL*-PTD cells *in vitro* and induces apoptosis. In line, we found that JQ1 treatment decreased leukemic burden *in vivo* and improved survival *in vivo*. We show for the first time to our knowledge, that aberrant BRD4 binding in *MLL*-PTD cells results in a distinct deregulation of genes. By integrating the RNA-seq and ChIP-seq analysis, we identified targets relevant to the *MLL*-PTD subgroup of AML patients and validated in additional samples from *MLL*-PTD$^+$ AML primary patient blasts and our unique AML mouse model. This novel group of genes might be associated with leukemogenesis of *MLL*-PTD$^+$ CN-AML and also with the poor prognosis of this subgroup. Importantly, we were able to reverse the aberrant gene expression patterns by treatment with JQ1. Therefore, targeting BRD4 might be an effective and promising treatment option for patients harboring a *MLL*-PTD to improve their outcome.

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Contributions: AMD designed the study; MB, CG, FP, BM, MK, RK, DP and GF performed the experiments; MB, CG, FP, BM, MK, RK, SK, DP, GF, RG, JB, GM, MAC and AMD contributed to the data interpretation; MB, CG, FP and AMD wrote the manuscript; HGO and XZ performed bioinformatics and statistical analyses. All authors reviewed the manuscript.

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