



BET bromodomain proteins regulate enhancer function during adipogenesis

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Developmental transitions are guided by master regulatory transcription factors. During adipogenesis, a transcriptional cascade culminates in the expression of PPAR γ and C/EBP α , which orchestrate activation of the adipocyte gene expression program. However, the coactivators controlling PPAR γ and C/EBP α expression are less well characterized. Here, we show the bromodomain-containing protein, BRD4, regulates transcription of PPAR γ and C/EBP α . Analysis of BRD4 chromatin occupancy reveals that induction of adipogenesis in 3T3L1 fibroblasts provokes dynamic redistribution of BRD4 to de novo super-enhancers proximal to genes controlling adipocyte differentiation. Inhibition of the bromodomain and extraterminal domain (BET) family of bromodomain-containing proteins impedes BRD4 occupancy at these de novo enhancers and disrupts transcription of *Pparg* and *Cebpa*, thereby blocking adipogenesis. Furthermore, silencing of these BRD4-occupied distal regulatory elements at the *Pparg* locus by CRISPRi demonstrates a critical role for these enhancers in the control of *Pparg* gene expression and adipogenesis in 3T3L1s. Together, these data establish BET bromodomain proteins as time- and context-dependent coactivators of the adipocyte cell state transition.

chromatin | coactivator | BET bromodomain | adipogenesis | transcription

Transcription factors (TFs) guide cell state transitions during development (1). Adipocyte differentiation is controlled by the action of peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer binding protein alpha (C/EBP α), master TFs that control the gene expression program of the developing adipocyte (2). In model systems, proadipogenic stimuli induce PPAR γ and C/EBP α expression by activating an upstream TF cascade comprised of C/EBP β , C/EBP δ , and the glucocorticoid receptor (GR) (3). Disruption of this TF network impairs adipocyte differentiation and adipose tissue formation (4).

These master TF networks signal via chromatin to execute the gene expression programs that drive adipogenesis. Comparative analysis of chromatin states in preadipocytes versus adipocytes has demonstrated massive enhancer reorganization during adipogenesis, as marked by genome-wide changes in the acetylation of histone 3 lysine 27 (H3K27ac) or lysine 9 (H3K9ac) (5, 6). Super-enhancers—cis-regulatory domains comprised of dense clusters of DNA-bound TFs and exceptionally high enrichment of chromatin-associated coactivators [e.g., BRD4, Mediator subunit-1 (MED1)]—have recently been identified in models of adipogenesis (7). Super-enhancers, akin to stretch enhancers or locus control regions, contain a disproportionately large amount of coactivator molecules for any given cell state (8, 9) and drive transcription of genes essential for cell identity (10, 11). During adipogenesis, super-enhancers have been identified near genes important for adipocyte differentiation including *Pparg* (7). However, the specific mechanisms by which dynamically remodeled

enhancers signal to RNA polymerase and regulate adipogenesis are not as well characterized.

The bromodomain and extraterminal domain (BET) family of bromodomain-containing coactivator proteins—BRD2, BRD3, BRD4—associate with chromatin (12–14). BETs coactivate transcription by forming scaffolds with other coregulatory proteins, including Mediator subunits and the positive transcription elongation factor-b (PTEF-b) complex at enhancers. These multi-protein complexes activate RNA polymerase II (RNA Pol II) via long-range chromatin interactions (15). Prior studies establish BRD4 as a dynamic constituent of super-enhancers (10, 16, 17).

Significance

Adipocyte differentiation is necessary for metabolic homeostasis. Transcription factors control adipocyte differentiation by regulating expression of genes required for adipocyte function. Here we show that the bromodomain and extraterminal domain (BET) family of bromodomain-containing proteins are important coregulators of adipogenesis. BRD4 binds to enhancers that drive expression of PPAR γ , a master transcription factor required for normal adipocyte differentiation. Disrupting BRD4 at these enhancers blocks PPAR γ expression and impairs differentiation. Understanding the molecular determinants of PPAR γ expression and adipocyte differentiation may provide insights into the pathogenesis of metabolic diseases related to adipose tissue dysfunction and obesity. Furthermore, these data implicate metabolic pathways and their transcriptional regulators as potential targets of BET bromodomain inhibitors, as currently under investigation in human clinical trials of cancer therapy.

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Conflict of interest statement: J.D.B., J.P., and J.E.B. have a patent filed on Compositions and Methods for Modulating Metabolism (application no. PCT/US2011/036647) in which BET bromodomain inhibition is used to modulate metabolic diseases including obesity and fatty liver. J.E.B. is now an executive and shareholder of Novartis AG.

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Data deposition: ChIP-Seq data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. GSE109940).

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Recently, a distinct role for BETs has been described in transcription control of dynamic, stimulus-coupled cell state transitions in heart failure and inflammation (18, 19). Overall, these data reveal that BETs can transduce dynamic, genome-wide changes in enhancer activity to regulate cell identity.

We hypothesized that BETs are essential coregulators of adipogenesis. Here, we show that BRD4 is a critical enhancer factor that potentially coactivates stage-specific expression of PPAR γ and C/EBP α during adipogenesis. Induction of adipogenesis in 3T3L1 preadipocytes provokes dynamic redistribution of BRD4 to de novo super-enhancer regions including the cis-regulatory elements controlling transcription of PPAR γ and C/EBP α , thereby promoting differentiation. Displacement of BRD4 from enhancer chromatin with a BET bromodomain inhibitor blocks transcription of *Pparg* resulting in failure of differentiation. These data establish BET bromodomain proteins as essential transcriptional coactivators of the adipocyte cell state transition and implicate BET proteins in the regulation of systemic metabolic processes.

Results

BET Bromodomain Proteins Control Adipocyte Differentiation. We first tested the effect of BET bromodomain inhibition on adipocyte differentiation using the 3T3L1 murine fibroblast model system (“L1”). Induction of differentiation using dexamethasone, isobutylmethylxanthine (IBMX), and insulin (DMI) resulted in adipocyte differentiation on day 8, as reflected by uniform lipid accumulation measured by oil red O staining (Fig. 1A). Cotreatment of preadipocytes with a panel of structurally dissimilar BET bromodomain inhibitors blocked differentiation (Fig. 1A) (20–24). Effects on adipogenesis correlated with their relative IC₅₀ against BRD4 (25). PFI-1 demonstrated a more modest block in adipogenesis, while RVX-208 demonstrated no difference at the concentration tested (1 μ M) (Fig. 1A and Fig. S1A). Inhibition of L1 differentiation by JQ1 was concentration-dependent, occurred without significant cytotoxicity, and resulted in only a modest slowing of cell cycle (Fig. S1B and C).

Given these potent effects of BET bromodomain inhibition on adipogenesis, we next considered the role of BETs in gene regulation during differentiation. To address this issue, we designed a gene expression probe set (58 probes) for digital mRNA analysis (Nanostring). This curated gene set, containing probes for measuring expression of proadipogenic TFs, key transcriptional coregulators, and mature adipocyte markers, represents a molecular signature that encompasses critical stages of the differentiation process. At day 4 of L1 differentiation, we observed expected induction of master regulatory TFs including *Cebpa*, *Pparg*, members of the *Klf* family, and retinoid-x receptor family (*Rxr*) as well as downstream target genes involved in adipocyte function including lipid uptake (*Cd36*; fatty acid binding protein: *Fabp4*), lipid synthesis (monoacylglycerol lipase: *Magl*; diacylglycerol O-acyltransferase1: *Dgat1*), and systemic metabolism (*Adipsin*; *Adipoq*) (Fig. 1B and C). Cotreatment of L1s with JQ1 abrogated induction of the adipocyte gene set (Fig. 1B and C), and unsupervised hierarchical clustering grouped JQ1-treated L1s more closely with preadipocytes. Both *Pparg* and *Cebpa* mRNA levels were quantitatively similar to preadipocytes (Fig. S1D and E). BET inhibition also suppressed induction of *Adipoq* and *Fabp4*, two genes directly regulated by PPAR γ (Fig. S1F and G). Complementary studies using siRNA-mediated knockdown of *Brd4* supported a role for this BET family member in L1 differentiation (Fig. S1H and I). To explore the relevance of these findings in primary cells, we next performed adipocyte differentiation assays using the stromal vascular fraction (SVF) isolated from murine subcutaneous (s.c.) adipose depots. Cells from SVF differentiated into mature adipocytes over 10 d in the presence of a defined hormonal mixture. Concomitant BET bromodomain inhibition resulted in a concentration-dependent block in differentiation (Fig. 1D). In rodents, a short period of visceral adipose tissue expansion occurs in the first weeks of postnatal life, which may, in part, be

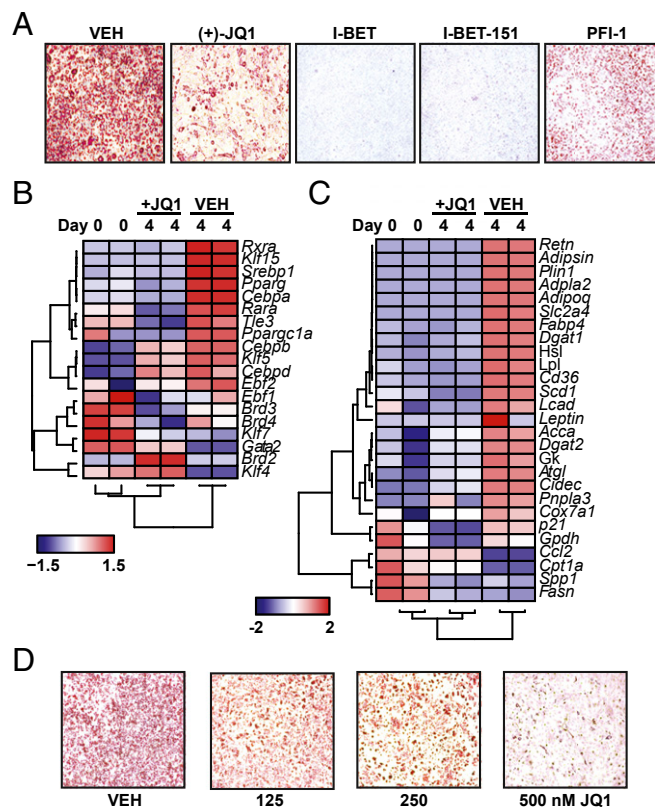


Fig. 1. BET bromodomain proteins control adipocyte differentiation. (A) Photomicrographs of oil red O stained L1 adipocytes after induction of differentiation by DMI (day 8) \pm structurally distinct BET bromodomain inhibitors (JQ1 at 500 nM; I-BET, I-BET-151, and PFI-1 at 1 μ M). (B and C) Heatmaps of expression of proadipogenic, master regulators (B), and mature adipocyte genes (C) at D0 and D4 of differentiation \pm JQ1 (500 nM). (D) Photomicrographs of oil red O staining after differentiation of cells isolated from the stromal vascular fraction of mouse s.c. adipose tissue \pm JQ1. Data represent mean \pm SEM. The statistical significance of the difference in expression between vehicle (VEH) and JQ1 was determined using a two-tailed *t* test. (Magnification: 4 \times .)

regulated by adipogenesis (26). To test the role of BET bromodomain proteins in adipose tissue, we treated 2-wk-old pups with JQ1 for 12 d and detected a significant reduction in epididymal white adipose tissue (EWAT) mass (Fig. S1J). In EWAT, *Pparg2* mRNA levels were also lower in JQ1-treated animals. Taken together, these data establish a role for BET bromodomain proteins in adipocyte differentiation in both the L1 model system and primary mouse adipogenic precursors.

BETs Control Expression of the Master TFs *Pparg* and *Cebpa*. A precise, temporally regulated cascade of TF activity drives adipogenesis. Consistent with this wave of TF function, we observed a time-dependent effect of BET bromodomain inhibition on L1 differentiation. JQ1 cotreatment on day 2 or day 4 after induction of differentiation resulted in significant reductions in lipid accumulation and *Pparg*, *Cebpa* and *Adipoq* gene expression, with these inhibitory effects diminishing when JQ1 was administered at later time points in the differentiation process (Fig. 2A and C and Fig. S2A). JQ1 treatment of fully differentiated adipocytes (day 6) had no effect on *Pparg* and *Cebpa* gene expression or the mature adipocyte phenotype measured on day 8. We next tested whether JQ1 effects were reversible during this first 96-h window by performing JQ1 washout experiments. As before, JQ1 treatment throughout the 8 d completely blocked L1 differentiation (Fig. S2B). JQ1 exposure during day 0 through day 2 also suppressed differentiation and *Pparg* expression, but partial recovery of expression did occur. This recovery was more

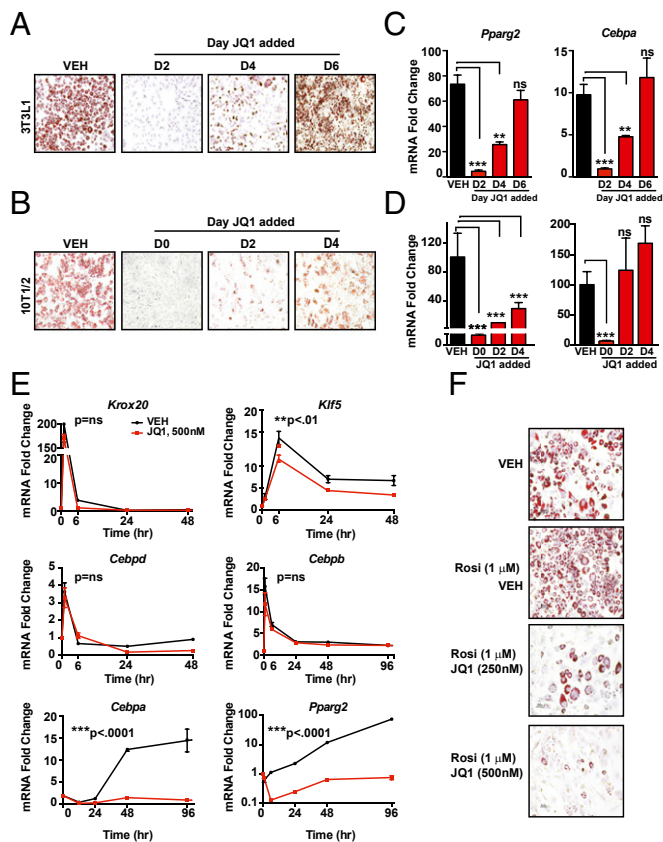


Fig. 2. BETs control expression of the master TFs *Pparg* and *Cebpa*. (A and B) Photomicrographs oil red O stained L1s (A) or C3H10T1/2 mesenchymal stem cells (B) after induction of differentiation ± JQ1 (500 nM). JQ1 was added to differentiation medium on indicated days. Staining was on D6 for L1 and D4 for 10T1/2. (Magnification: 4×.) (C and D) Bar plots of fold change in *Pparg* and *Cebpa* mRNA levels in 3T3L1 cells and 10T1/2 cells from A and B. ns, not significant. (E) Line plots of fold change in mRNA of early response TFs involved in adipogenesis measured at 1, 6, 24, 48, and 96 h following induction of differentiation ± JQ1 (500 nM). (F) Oil red O staining of L1 adipocytes 8 d after induction of differentiation in the presence or absence of rosiglitazone (1 μM) ± JQ1 (500 nM). (Magnification: 10×.) Data represent mean ± SEM. The statistical significance of the difference in expression between vehicle (VEH) and JQ1 at each time point in C and D was determined using one-way ANOVA with post hoc (Dunnett's) multiple comparison tests of the means. ** $P < 0.01$, *** $P < 0.001$. For E, two-way ANOVA was performed with *** $P < 0.001$ for interaction comparing time × treatment groups.

evident in cells exposed to BET inhibition for only 24 h between D1 and D2 (Fig. S2 B and C).

Adipogenesis proceeds in two phases: (i) early fate specification, involving commitment of stem cells to the adipocyte lineage, and (ii) terminal differentiation, in which committed cells progressively acquire features of mature adipocyte phenotype (3). As L1 cells are already committed to the adipocyte lineage (3), we asked whether BETs also played a role in the earlier events of lineage specification. Therefore, we performed an additional series of differentiation experiments with C3H10T1/2 cells (10T1/2), a cell line derived from the C3H mouse embryo that possesses mesenchymal stem cell-like properties and can be induced to differentiate into adipocytes using a hormonal mixture (27). In the 10T1/2 model, mature adipocytes are evident by day 4. Similar to our L1 experiments, BET inhibition blocked differentiation in 10T1/2 cells in a time-dependent manner (Fig. 2 B and D and Fig. S2D). Reductions in *Pparg* expression persisted in the presence of BET inhibition whether initiated on day 0 or day 2 of differentiation, while *Cebpa* mRNA levels were similar to vehicle in day 2-treated cells (Fig. 2D). In contrast to BRD4 loss-of-function experiments, enforced

expression of BRD4 in NIH3T3s—a nonadipogenic cell line—did not induce differentiation (Fig. S2E), consistent with a role for BRD4 as a coactivator that transduces signals from enhancer-bound TFs to the transcriptional machinery. Collectively, these data establish that BETs are stage-specific regulators of adipocyte differentiation and *Pparg* expression in committed preadipocytes (L1) and mesenchymal stem cells (10T1/2), but BRD4 is not sufficient to drive differentiation in cells lacking intrinsic adipogenic potential.

L1 differentiation proceeds by the coordinated action of a TF cascade that converges on *Pparg* and *Cebpa* expression. We next tested whether BET bromodomain proteins regulate the first wave of master TF expression during adipogenesis. In time-course experiments, expression of *Krox20*, *Klf5*, *Cebpb*, and *Cebpd* increased 1 h after starting L1 differentiation, peaked by 6 h, and returned to baseline by 24–48 h (Fig. 2E). Cotreatment of L1 cells with JQ1 had no effect on the magnitude or kinetics of *Krox20*, *Cebpb*, or *Cebpd* regulation, with a modest effect on *Klf5* mRNA levels. Despite no significant effects on the expression of these early TFs, BET inhibition completely abolished induction of L1 cells with a high-affinity, synthetic PPAR γ ligand (rosiglitazone, 1 μM) that further drives adipogenesis also failed to rescue the block in differentiation by BET bromodomain inhibition (Fig. 2F and Fig. S2F). Overall, these data support a model in which BET bromodomain proteins promote adipocyte differentiation by coactivating expression of *Pparg* and *Cebpa* in a cell type-specific manner.

BRD4 Is Dynamically Redistributed to Preadipogenic Super Enhancers.

To determine the role of BETs in adipocyte enhancer function, we first investigated genome-wide BRD4 binding to chromatin in L1 preadipocytes (day 0, “D0”) and differentiating adipocytes (day 2, “D2”) using chromatin immunoprecipitation coupled with high-throughput deep sequencing (ChIP-seq). Experimental data obtained for BRD4 were integrated with publicly available datasets that provide high-resolution epigenomic maps of euchromatin during adipocyte differentiation (5). First, we identified BRD4 to be enriched at preadipocyte and adipocyte promoters (13% and 4%, respectively, and as follows) and intergenic (39% and 38%) and intragenic (47% and 57%) regulatory sequences (Fig. S3A). Alignment of BRD4 ChIP-seq data with published chromatin state maps of enhancers (H3K27ac) and promoters (H3K4me3) in L1 cells demonstrated a strong correlation between H3K27ac and BRD4 at both D0 and D2 (Fig. S3B). Global alignment revealed BRD4 localized to chromatin at promoters and enhancers in both preadipocytes and adipocytes (Fig. S3C).

We next ranked BRD4 enhancers by increasing ChIP-seq signal and identified 212 and 411 super-enhancers in preadipocytes and adipocytes, respectively (Fig. 3 A and B). Differences in super-enhancer regions between preadipocytes and adipocytes (Fig. 3 A and B) prompted a systematic evaluation of the dynamic changes in super-enhancer organization. We observed significant redistribution of BRD4 to new enhancers in the D2-differentiating adipocytes. Differential enhancer analysis reclassified a significant subset of enhancers as transitioning from typical to super-enhancer (“adipocyte-gained,” $n = 365$) and from super to typical enhancer (“preadipocyte-lost,” $n = 54$) (Fig. 3C). Genes associated with adipocyte-gained super-enhancers were enriched for TFs that positively regulate adipocyte differentiation, including *Pparg*, *Cebpa*, and *Cebpb* (Fig. 3C). In contrast, other genes associated with preadipocyte-lost super-enhancers, such as *Klf3* and *Pleiotrophin* (*Ptn*), are known to interfere with differentiation and are down-regulated during adipogenesis; furthermore, prior work has revealed that enforced expression of these factors suppresses differentiation (28, 29). Super-enhancers feature high levels of coactivator protein enrichment and are also densely occupied by master regulatory TFs (8). Motif analysis revealed the GR (NR3C1) and C/EBP motifs were found at much higher density at adipocyte-gained super-enhancer regions compared with preadipocyte-lost super-enhancer regions (Fig. 3 D and E). Comparison of binding

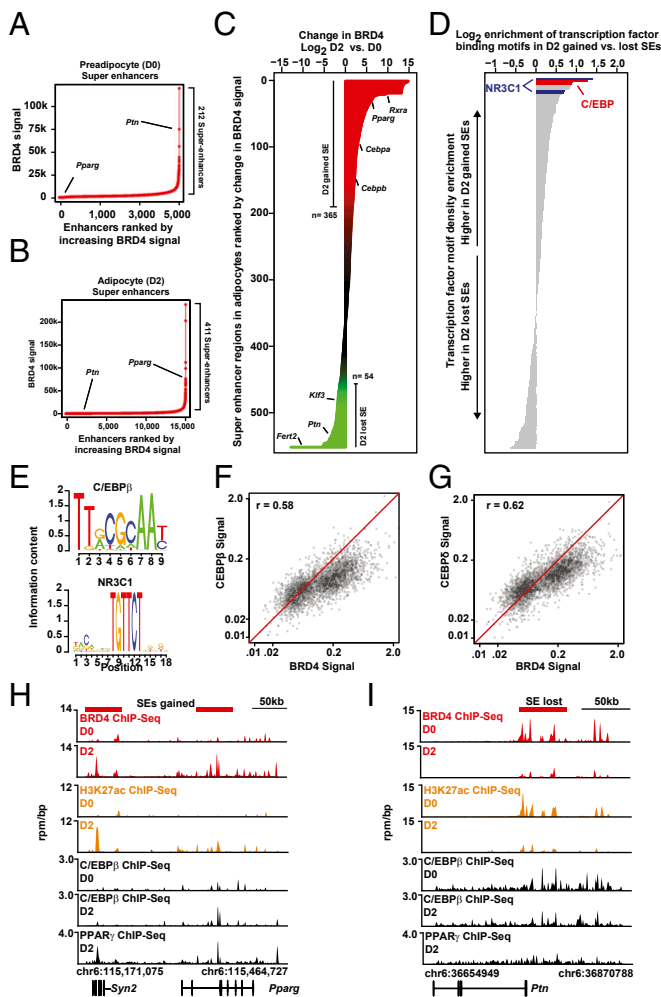


Fig. 3. BRD4 is dynamically redistributed to proadipogenic enhancers. (A and B) Plots of enhancers in preadipocytes (D0; A) and adipocytes (D2; B) ranked by increasing BRD4 signal in units of reads per million (rpm). Enhancers are defined as regions of BRD4 ChIP-Seq binding not contained in promoters. The cutoff discriminating typical enhancers from super-enhancers is shown as a dashed line. (C) Horizontal bar plot of all genomic regions containing a SE in preadipocytes (D0) or differentiating adipocytes (D2) ranked by \log_2 change in BRD4 signal. The x axis shows the \log_2 fold change in BRD4 signal. Change in BRD4 levels at SEs are colored by intensity of change (green to red). (D) Horizontal bar plot showing the ratio of TF motif density between gained (D2) and lost (D0) super-enhancers. Twenty-one TFs are displayed whose motifs occur more frequently than expected based on dinucleotide background model. The motifs are ranked by \log_2 fold change in density between D2 gained and D0 lost super-enhancers. (E) Consensus sequences of C/EBP β (Top) and NR3C1 (Bottom) DNA binding motifs. (F and G) Scatter plot of C/EBP β versus BRD4 binding signals (F) or C/EBP δ versus BRD4 signals (G) in L1 cells on D2 of differentiation. (H and I) Gene tracks of ChIP-Seq signal (rpm/bp) for BRD4, H3K27ac, C/EBP β , and PPAR γ at the *Pparg* locus (H) or *Ptn* (I) locus in 3T3L1 cells on D0 (Upper) or D2 (Lower) of differentiation.

enrichment between BRD4 and previously generated datasets for C/EBP β , CEBP δ , GR, and PPAR γ ChIP-seq also demonstrated strong statistical correlation at D2 of L1 differentiation between each of these TFs and BRD4 ($R^2 = 0.58, 0.62, 0.43, 0.57$, respectively) (Fig. 3 F and G and Fig. S3D), suggesting genomic colocalization of these factors. In addition, coimmunoprecipitation experiments revealed a modest interaction between CEBP β and BRD4 during differentiation (Fig. S3E), as previously identified in other cell types (13, 14). At the *Pparg* locus, preadipocytes feature minimal BRD4 or H3K27ac enrichment (Fig. 3H). Following induction of differentiation, L1 cells on D2 acquired

massive increases in both BRD4 and H3K27ac levels at a 5' distal regulatory element and the promoter region of *Pparg*, consistent with formation of super-enhancers (Fig. 3H). By contrast, the *Ptn* locus contained high levels of BRD4 and H3K27ac at the 5' enhancer region in preadipocytes, which were lost on D2 of differentiation (Fig. 3I). To test the signals that control recruitment of BRD4 to chromatin, we omitted individual components of the adipocyte differentiation mixture. The absence of insulin in this mixture resulted in a reduction of BRD4 recruitment to the *Pparg* locus after 24 h of differentiation (Fig. 4 and Fig. S3F). Overall, these results establish that BRD4 dynamically redistributes away from preadipocyte genes to new super-enhancers associated with stage-specific expression of master regulatory factors including proadipogenic TFs such as *Pparg*.

BRD4 Super-Enhancer Binding Sites Regulate *Pparg* Gene Expression and Adipogenesis. Recruitment of BRD4 to super-enhancers at the *Pparg* locus suggests that these cis-regulatory elements may directly control *Pparg* gene expression. To test this hypothesis, we employed clustered, regularly interspaced, palindromic repeats interference (CRISPRi) to silence individual cis-regulatory elements occupied by BRD4 (30). L1 cells were selected for stable expression of dCas9-KRAB along with individual guide RNAs (gRNAs) complementary to DNA at BRD4-enriched loci within the *Pparg* super-enhancers (Figs. 3H and 4). Targeting these sites by multiple, individual gRNAs repressed *Pparg* gene induction on D4 of L1 differentiation, compared with cells expressing dCas9-KRAB with no gRNA (Fig. 4). Notably, silencing of the distal 5' site (E1) and intragenic sites (E4, E5) resulted in the most significant reduction in gene expression, with more modest effects at the other regions tested. CRISPRi of these *Pparg* enhancers also attenuated adipogenesis, as measured by oil red O staining (Fig. 4B). These data establish an important functional role for these de novo formed, BRD4-enriched cis-regulatory elements in adipogenesis in the endogenous chromatin context of the cell.

Transcription of *Pparg* and *Cebpa* Is BET Bromodomain-Dependent. The dynamic redistribution of BRD4 to de novo super-enhancers predicts that depletion of BRD4 from chromatin would reduce transcription at super enhancer-associated genes. To analyze the transcriptional consequences of disruption of BET super-enhancers, we performed ChIP-seq analysis of RNA Pol II occupancy in L1 preadipocytes (D0) as well as differentiating L1s (D2) in the presence or absence of the selective BET bromodomain inhibitor (JQ1, 500 nM). Comparison of RNA Pol II signal from actively transcribed genes in vehicle versus JQ1-treated L1s revealed overall statistical correlation between these two groups (Fig. 5A). However, at a subset of genes, RNA Pol II signal in JQ1-treated cells was lower than vehicle (Fig. 5A). Within this cluster, reductions in RNA Pol II signal at the *Pparg* gene locus were noted in JQ1-treated L1 cells. A comparison of RNA Pol II signal at the *Pparg* and *Cebpa* loci on D0 versus D2 revealed a robust increase during differentiation, coincident with acquisition of BRD4 at super-enhancers (Figs. 3H and 5B and Fig. S4A). BET bromodomain inhibition with JQ1 depleted BRD4 from chromatin, and this resulted in reduced transcription of *Pparg* and *Cebpa*. This effect on *Pparg* and *Cebpa* expression also blocked transcription of known downstream target genes such as *Fabp4* (Fig. S4B). Finally, we considered the possibility that TF genes induced at earlier time points during L1 adipogenesis (Fig. 2E) are less sensitive to BET inhibition because they are not controlled by super-enhancers. Analysis of a previously published dataset of genome-wide binding of MED1—a constituent of super-enhancers (8)—at 4 h after the start of L1 differentiation revealed no super-enhancers assigned to *Krox20*, *Cebpd*, and *Cebpb* genes (Fig. S4C) (7). Collectively, these data reveal that BET inhibition blocks transcription of super enhancer-associated genes including *Pparg* and *Cebpa*.

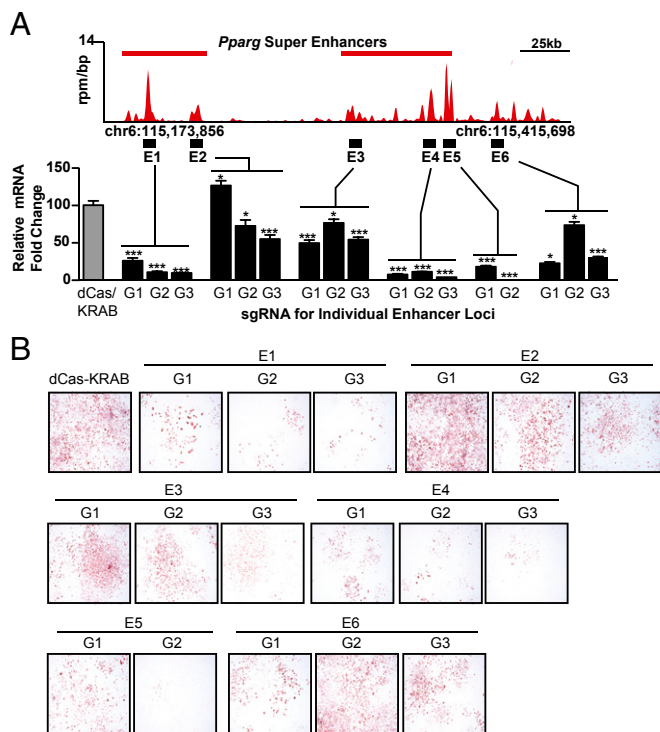


Fig. 4. BRD4 binding sites regulate *Pparg* expression and adipogenesis. (A) Gene track of ChIP-Seq signal of BRD4 on D2 of L1 differentiation at the upstream and intragenic *Pparg* super-enhancer regions (Upper) and bar plot of *Pparg* mRNA expression (D4 of L1 differentiation) in cells stably expressing dCas9-KRAB alone or with gRNAs targeting individual enhancer constituent sites (E1–E6). (Lower) Three unique gRNAs per enhancer site were used in separate cell lines. (B) Oil red O staining of cells (D7) treated as in A. The statistical significance of the difference in expression between individual gRNA and dCas9-KRAB was determined using two-tailed t test. (Magnification: 4 \times .) * $P < 0.05$; *** $P < 0.001$.

Discussion

Adipocyte differentiation requires the coordinated activity of the master TFs, C/EBP α , and PPAR γ . Disruption of their expression or function in humans profoundly alters metabolism, in part, through effects on adipocyte differentiation (31). In this study, we explored the role of chromatin-dependent signal transduction in this process. We demonstrate that the BET bromodomain-containing protein, BRD4, transduces the adipogenic program through formation of de novo super-enhancers that drive transcription of *Pparg* and *Cebpa* and promote adipocyte differentiation.

Super or stretch enhancers are clusters of enhancers where TFs and coactivator proteins are concentrated and drive transcription of genes controlling cell state (8, 9). In this study, we identified that BRD4 redistributes to de novo super-enhancers in proximity to genes encoding TFs, including *Cebpa* and *Pparg*, in response to adipocyte differentiation cues. These data build on prior reports demonstrating that TF hotspots colocalize with super-enhancers containing MED1 in differentiating L1 cells (7). Functional interactions between Mediator and BRD4 may partially explain how BRD4 redistributes to these regions (32). In addition, the colocalization of BRD4 with multiple TF-binding motifs, as observed in our study, suggests that site-specific BRD4 redistribution may occur via interactions between BRD4 and specific DNA-binding TFs. In the context of inflammation, biochemical studies have demonstrated that BRD4 can bind acetylated p65-RelA, implicating this interaction as a possible mechanism for genome-wide redistribution of BRD4 to inflammatory super-enhancers during innate immune activation (19, 33). Furthermore, a functional relationship between C/EBP β and BRD4 has been described in NIH

3T3 fibroblasts (14). Overall, these data are consistent with a model in which lineage-specific TFs activated during adipogenesis direct state-specific flux of BRD4 and other coregulators to de novo super-enhancers that control cell state.

We find that selective depletion of BRD4 from chromatin impairs adipocyte differentiation. These results occurred in three models of adipogenesis (3T3L1, 10T1/2, and SVF) in response to a panel of structurally dissimilar BET bromodomain inhibitors or siRNA-mediated knockdown of *Brd4*. Notably, two independent studies have recently emerged that used BET bromodomain inhibition or conditional *Brd4* deletion to demonstrate a role for BETs in adipogenesis (34, 35). The results presented here align with these reports while also providing additional significant insights, including the use of CRISPR interference to demonstrate directly that silencing of individual, BRD4-enriched endogenous enhancer elements at the *Pparg* locus resulted in disrupted *Pparg* expression and adipogenesis. Whether BRD4 enrichment at specific cis-regulatory elements is sufficient to drive adipogenesis and which elements are required for this will be of interest to investigate in future studies. Overall, when considered collectively, these data demonstrate a requirement for BETs and BRD4 in adipogenesis through collaboration with lineage-determining TFs and may in part explain why systemic *Brd4* haploinsufficient mice have reduced adipose tissue formation (36). The effects of BET bromodomain inhibition diminished

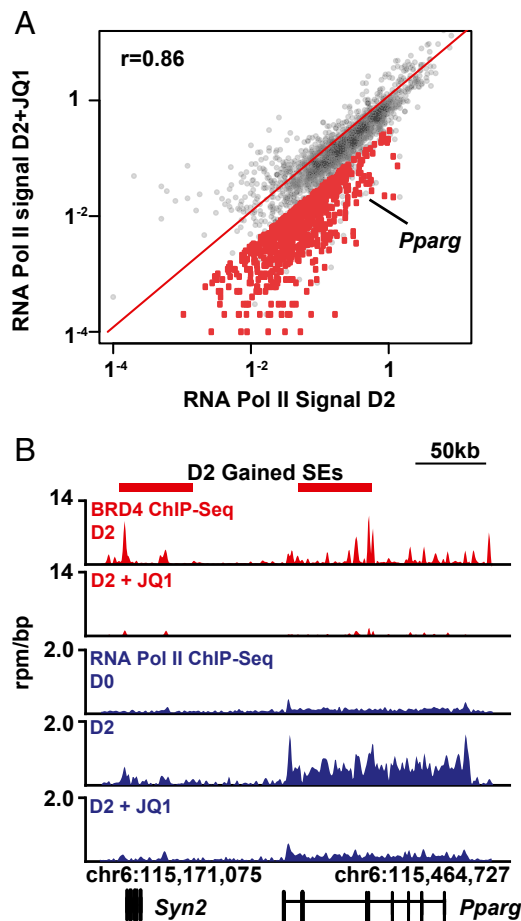


Fig. 5. Transcription of adipogenic master regulatory factors is BET bromodomain-dependent. (A) Scatter plot of RNA Pol II ChIP-Seq signal at gene bodies of all actively transcribed genes in L1 cells on D2 of differentiation versus D2 + JQ1 (500 nM). (B) Gene tracks of ChIP-Seq signal (rpm/bp) for BRD4 (D2 versus D2 + JQ1 of differentiation) and RNA Pol II on D0 (Top), D2 (Middle), and D2 + JQ1 (500 nM, Bottom) of differentiation at the *Pparg* (B) locus in L1 cells.

over time, revealing the presence of precise temporal windows during which enhancer activity is required to regulate distinct stages of adipocyte differentiation. The muted effect of BET bromodomain inhibition at later time points may reflect compensation by other coactivators such as MED1 at established super-enhancers, functional cooperativity between multiple TFs bound at these regions or bromodomain-independent recruitment of BETs to these enhancers (13, 37). Our findings in adipogenesis extend prior work describing the role of BRD4-enriched super-enhancers in cell state. In embryonic stem cells (ESCs) and in certain cancers, super-enhancers regulate expression of pluripotency factors (8, 17) or oncogenes, respectively (16, 22). In these contexts, BET bromodomain inhibition triggers differentiation, growth arrest, and/or cell death (22). The differentiation block by JQ1 in L1 and 10T1/2 cells stands in sharp contrast to the prodifferentiation effects of BET inhibition in ESCs and NUT midline carcinoma (17, 22). These differences highlight how BETs transduce signals controlling the establishment of specific, TF-driven cell states by coactivating context-specific enhancer activity to RNA polymerase II.

BET bromodomain inhibitors are a new class of drugs in cancer therapy now under investigation in human clinical trials. The data presented herein have implications for this therapeutic approach. Disruption of BET-occupied enhancers may have adverse consequences in noncancer cellular compartments including adipocytes, where stimulus-coupled transcription is an essential mechanism controlling metabolic homeostasis. Furthermore, analysis of BET-dependent super-enhancer wiring may provide insight into potential

on-target toxicities of pharmacologic BET bromodomain inhibitors that target cell-state defining genes. Considered more broadly, understanding the precise mechanisms underlying context-specific, BET bromodomain-dependent coactivation may deepen our understanding of the gene regulatory circuits governing tissue-specific responses in development and diseases outside of cancer.

Experimental Procedures

Animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School. Adipocyte differentiation was performed in media supplemented with fetal bovine serum (10%), insulin, dexamethasone, and IBMX. For 10T1/2 cells, 1 nM T3 and 0.125 mM indomethacin was also included. Summary data are presented as mean \pm SEM, and Student's *t* test was used to compare groups. For kinetic gene expression, ANOVA was conducted. Methods for ChIP-Seq, coimmunoprecipitation, CRISPRi, RNA preparation, and cell staining are provided in *SI Experimental Procedures*.

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