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Epigenetic and pharmacological control of pigmentation via Bromodomain Protein 9 (BRD9)

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Abstract

Lineage-specific differentiation programs are activated by epigenetic changes in chromatin structure. Melanin-producing melanocytes maintain a gene expression program ensuring appropriate enzymatic conversion of metabolites into the pigment, melanin, and transfer to surrounding cells. During neuroectodermal development, SMARCA4 (BRG1), the catalytic subunit of SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complexes, is essential for lineage specification. SMARCA4 is also required for development of multipotent neural crest precursors into melanoblasts, which differentiate into pigment-producing melanocytes. In addition to the catalytic domain, SMARCA4 and several SWI/SNF subunits contain bromodomains which are amenable to pharmacological inhibition. We investigated the effects of pharmacological inhibitors of SWI/SNF bromodomains on melanocyte differentiation. Strikingly, treatment of murine melanoblasts and human neonatal epidermal melanocytes with selected bromodomain inhibitors abrogated melanin synthesis and visible pigmentation. Using functional genomics, iBRD9, a small molecule selective for the bromodomain of BRD9 was found to repress pigmentation-specific gene expression. Depletion of BRD9 confirmed a requirement for expression of pigmentation genes in the differentiation program from melanoblasts into pigmented melanocytes and in melanoma cells. Chromatin immunoprecipitation assays showed that iBRD9 disrupts the occupancy of BRD9 and the catalytic subunit SMARCA4 at melanocyte-specific loci. These data indicate that BRD9 promotes melanocyte pigmentation whereas pharmacological inhibition of BRD9 is repressive.

KEYWORDS
BRD9, bromodomain, chromatin remodeling, epigenetic, melanocyte differentiation, melanoma, pigmentation, SWI/SNF

1 | INTRODUCTION

Melanocytes are cells that originate from the neural crest and migrate to the epidermis, hair follicles, epithelial surfaces of mucous membranes, meninges, heart, the inner ear, and the uvea and conjunctiva of the eye. Neural crest cells differentiate into melanoblasts, becoming committed to the melanocyte lineage upon expression of the melanocyte-inducing transcription factor (MITF). Upon differentiation, cutaneous melanocytes synthesize melanin and provide this pigment to the skin, thereby protecting against the damaging effects of ultraviolet radiation from the sun (Steingrimsson et al., 2004).

There are several transcription factors in addition to MITF which regulate pigmentation. MITF is transcriptionally regulated by Paired Box 3 (PAX3) and SRY-Box Transcription Factor (SOX10) in melanoblasts (Steingrimsson et al., 2004). SOX10 cooperates with MITF to activate downstream pigment genes (Lang et al., 2005) which include enzymes that synthesize melanin: tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1), and dopachrome tautomerase (DCT) during differentiation of melanoblasts to melanocytes. The activating-enhancer-binding protein 2 family of transcription factors (TFAP2) promote expression of a subset of pigmentation genes by facilitating MITF binding (Kenny et al., 2022), including interferon regulatory factor 4 (IRF4) which regulates TYR expression (Praetorius et al., 2013). Melanogenesis occurs in specialized organelles called melanosomes which are transported through dendrites to keratinocytes to provide pigmentation to the skin. There are hundreds of genes that influence the level of pigmentation in animal models (Baxter et al., 2019). This transcriptional circuitry impinges upon epigenetic regulators, which can also contribute to pigmentation.

SWItch/Sucrose Non-Fermentable (SWI/SNF) enzymes are epigenetic regulators that play important roles in the melanocyte lineage. SWI/SNF is a multi-subunit complex that utilizes energy from ATP hydrolysis to alter histone–DNA contacts. Various purification schemes have identified diverse complexes in mammalian cells that differ in subunit composition, termed canonical (c) BAF, Polybromo (PBAF), and noncanonical (nc) SWI/SNF (Centore et al., 2020). These complexes vary depending on the presence of one of two mutually exclusive ATPase subunits, SMARCA2 (BRAHMA, BRM) or SMARCA4 (Brahma-related gene 1, BRG1) and distinct associated factors (BAFs). Canonical BAF (cBAF) complexes contain either SMARCA4 or SMARCA2 as the central ATPase as well as either ARID1A or ARID1B as a signature subunit. Polybromo-associated BAFs (PBAF) include SMARCA4 as its central ATPase and four unique subunits, ARID2, PBRM1, DPF2, and BRD7, which distinguish it from BAF complexes. Noncanonical (nc)SWI/SNF is a smaller complex which also contains SMARCA4 as the central ATPase and unique subunits, BRD9 and BICRA or BICRAL. In melanoma cells, MITF interacts with a novel form of the PBAF complex containing not only SMARCA4 and PBRM1 but also CHD7 (chromodomain helicase DNA binding 7) (Laurette et al., 2015). SMARCA4 also interacts with SOX10 and is required for melanocyte development in vivo and differentiation in vitro (Marathe et al., 2017). However, in some contexts, the alternative ATPase, SMARCA2, can also interact with MITF and partially compensate for SMARCA4 loss in the regulation of melanocyte-specific gene expression (Keenen et al., 2010). SMARCD1, a component of cBAF, PBAF, and ncSWI/SNF complexes, directly interacts with both MITF and SOX10 to promote melanocyte-specific gene expression by facilitating recruitment of SMARCA4 to melanocyte-specific loci (Aras et al., 2019). While multiple SWI/SNF subunits have been implicated in the regulation of melanogenesis, we still lack an integrated understanding of the contribution of the different SWI/SNF subunits to this process.

Significance

The melanocyte lineage arises from neural crest cells which give rise to melanoblasts which migrate to the skin, hair, eyes, and mucous membranes and further differentiate into melanocytes. Cutaneous melanocytes play an important role in protecting skin from ultraviolet radiation by synthesizing and distributing the pigment melanin. The catalytic domain of the SMARCA4 subunit of the SWI/SNF chromatin remodeling complex is essential for melanocyte development and regulation of pigmentation. We now show that pharmacological inhibition of bromodomains contained within subunits of the SWI/SNF complex suppresses melanin synthesis and pigmentation gene expression in melanocytes and melanoma cells. We found that bromodomain protein 9 (BRD9) plays a role in regulating expression of genes involved in melanin synthesis. These results provide new insight into the function of a bromdomain-containing component of the SWI/SNF complex in melanocytes and melanoma cells.

The SWI/SNF subunits, SMARCA4, SMARCA2, PBRM1, BRD7, and BRD9 have conserved motifs called bromodomains. Bromodomains are 110 amino acid modules that bind to acetylated lysine residues on histone proteins and regulate epigenetic information. Small molecules that selectively bind bromodomains can inhibit their function by displacing them from chromatin, resulting in the suppression of gene regulation (Xu & Vakoc, 2017). These small molecules have been useful in deciphering the biological functions of the bromodomain extra-C terminal domain (BET) family of proteins (BRD2, BRD3, BRDT, and BRD4) and are in clinical trials for cancer treatment. In normal cells, BET proteins are required for muscle and brown adipose tissue development (Lee et al., 2017) and promote erythroid, osteoblast, muscle, adipocyte, and melanocyte differentiation (Brown et al., 2018; Najafova et al., 2017; Roberts et al., 2017; Stonestrom et al., 2015; Trivedi et al., 2020). More recently, the bromodomains of the SWI/SNF subunits, SMARCA2 and SMARCA4, were reported to promote muscle differentiation (Sharma et al., 2021). However, it is unknown if the bromodomains of the SWI/SNF complex are required for differentiation of other lineages.

In this study, a panel of bromodomain inhibitors was evaluated for their effects on melanogenesis. We found that drugs selective for BRD9 significantly reduced visible pigmentation and melanin

synthesis. Gene expression profiling revealed drug-induced suppression of pigmentation genes, which were also previously found to be regulated by SMARCA4. RNAi depletion of BRD9 confirmed that BRD9 was required for expression of selected pigmentation genes in melanoblasts and melanoma cells. BRD9 and SMARCA4 co-occupied melanogenic loci, both of which were disrupted with drug treatment, suggesting that inhibition of BRD9 binding, de-stabilizes occupancy of SMARCA4. In conclusion, our data indicate that the BRD9 subunit of the SWI/SNF complex plays a role in the regulation of pigmentation by promoting the expression of pigmentation genes.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Melb-a cells were obtained from the Wellcome Trust Functional Genomics Cell Bank (St. George's, University of London, UK), maintained, and differentiated (Marathe et al., 2017). Cells were maintained in RPMI, 10% fetal calf serum, 40pM fibroblast growth factor and 10 ng/ml stem cell factor and differentiated in DMEM, 10%fetal calf serum, 2 nM alpha melanocyte stimulating hormone (NDP-alpha MSH), and 200nM phorbol-myristate-acetate. Neonatal human epidermal melanocytes (NHEMs) were obtained from Thermofisher and cultured in Media 254 with Human Melanocyte Growth Supplement (HMGS) (Thermofisher) (Saladi et al., 2013). 501MEL cells were obtained from Yale Cell Culture Core Facility and cultured in DMEM with 10% fetal calf serum.

2.2 | Cell counts

Cells were trypsinized and re-suspended in media at the indicated times. Cell counts were taken using the Scepter 2.0 handheld automated cell counter (Millipore-Sigma).

2.3 | Small molecule inhibitors

N′-[1,1-bis(oxidanylidene)thian-4-yl]-5-ethyl-4-oxidanylidene-7-[3-(trifluoromethyl)phenyl]thieno[3,2-c]pyridine-2-carboximidamide was purchased from Selleck Chemicals (PubChem CID 91668541, iBRD9, I-BRD9, GSK602, Houston, Texas, USA). 4-[4-[(dimethylamino) methyl]-3,5-dimethoxy-phenyl]-2-methyl-2,7-naphthyridin-1-one (PubChem CID 118796358, BI7273) and (E)-1-(2-hydroxyphenyl)-3-((1R,4R)-5-(pyridin-2-yl)-2,5-diazabicyclo[2.2.1]heptan-2-yl)prop-2-en-1-one (PubChem CID 78243717, PFI3, PFI-3) were purchased from Cayman Chemicals.

2.4 | Melanin assay

Cells were counted, and an equal number of cells for each condition (1.5 million cells) lysed in 1 M NaOH for 30 min. Absorbance was read at 475 nM, and melanin content was calculated based on a standard curve (Marathe et al., 2017).

2.5 | RNA-seq

Total RNA was prepared from vehicle (DMSO) or i-BRD9-treated Melb-a melanoblasts by TRIzol extraction (Invitrogen) followed by the RNeasy kit (Qiagen). Next-generation transcriptome sequencing, including library preparation, quality assessment, preparation, and sequencing, was performed using the Bio Basics workflow.

2.6 | Bioinformatics

Raw paired-end next-generation sequencing files recorded on the Illumina HiSeq 4000 platform were converted into fastq files were processed using CASAVA version 1.8.2. Raw read count was filtered to remove low-quality reads and reads with adaptors. Genome alignment to the Genome Reference Consortium Mouse Build 39 (GRCm39) was performed using tophat2 version 2.1.1 and bowtie2 version 2.4.5 (Trapnell 2012, Nature Protocols). Differential gene expression analysis (Kim et al., 2013) between reference vehicle and iBRD9 drug-treated samples was determined using the DESeq2 package in bioconductor version 3.14 (Anders & Huber, 2010). The resulting *p* values were adjusted by controlling the false discovery rate (FDR) using Benjamini and Hochberg's approach with *p* values below 0.05. Pathway enrichment, gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using GOseq and the KEGG database (Young et al., 2010). (Kanehisa et al., 2008). In addition, comparative analysis with previously published gene expression data sets was performed with the CLC Genomic Workbench (Hilden, Germany). Gene orthologues were mapped using Genome Reference Consortium Human Build 37 (GRCh37) and the mouse genome database (MGD) at the Mouse Genome Informatics website accessed 03/29/2019 (The Jackson Laboratory, Bar Harbor, Maine) (Bult et al., 2019).

Publicly accessible ChIP-seq datasets in Gene Expression Omnibus (GEO) in bigWig format were visualized as custom tracks on the UCSC Genome Browser.

2.7 | RNA isolation and quantitative real-time PCR

Total RNA was reverse transcribed into cDNA using the Quantitect Reverse Transcription kit (Qiagen). Quantitative PCR (qPCR) was performed in SYBR Green master mix (Qiagen) with an Applied Biosystems 7500 PCR and analyzed with the SDS software (Keenen et al., 2010) (Thermo Fisher). The mRNA levels of human pigmentation genes *MITF*, *SOX10*, *TYR*, *TYRP1*, *DCT*, and *IRF4* were normalized to *ACTβ*. Corresponding murine mRNA levels of *Mitf*, *Sox10*, *Tyr*, *Tyrp1*, and *Dct* were normalized to *Rpl7*. Oligonucleotide primer sequences are listed in Table S1.

2.8 | Cell extracts and immunoblot analysis

Cell extracts were prepared in Triton Lysis Buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10%glycerol, 2 mM DTT, 1 mM PMSF], protease cocktail (Millipore-Sigma), phosphatase inhibitor cocktail (Millipore-Sigma). Protein transfer was performed in a buffer containing 20%methanol, and the blots were probed with primary antibodies. Bands were visualized by enhanced chemiluminescence after incubation with species-matched secondary antibodies. (Keenen et al., 2010). The BRD9 antibody (A303781) was from Bethyl Laboratories; the MITF (ab12039) and SMARCA4 (ab110641) antibodies were from Abcam. SOX10 (N20), TYR (sc-7833), and TYRP1 (sc-10,443) antibodies were from Santa Cruz Technologies. The Tubulin (21485) antibody was from Cell Signaling Technology.

2.9 | siRNA knockdown

Control siRNA (51–01-14) and siRNAs targeting mouse BRD9 (mm.RiBrd9.13.1: 5′-UAAUGAAUAUAAAUCAGUCACAGAA-3′, mm.RiBrd9.13.2: 5′-GUCCCAGUGCAAGUAGAAACUACCA-3′) and human BRD9 (hs.Ri.BRD9.13.1: GGUAAUGAAUUGUCUGUGGU AUCAT-3′) were purchased from Integrated DNA Technology. Transfection using DharmaFECT 1 was performed on cells in growth media (Thermo Fisher). After 48 h, growth media was replaced with differentiation media for Melb-a cells or fresh growth media for 501MEL cells, and cells were then harvested 48 h later.

2.10 | Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed as previously described (Keenen et al., 2010). Briefly, cells were fixed with 1% formaldehyde for 10 min at room temperature. Nuclei were isolated in a buffer containing 50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 0.25% TritonX, and protease inhibitors and then washed with a buffer containing 10 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and protease inhibitors. Nuclei were then lysed in a buffer containing 10 mM Tris–HCl, pH 8.0. 100 mM NaCl, 9 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, and 0.5% N-lauryolsarcosine. Each sample was sonicated in 30-second pulses at high power, for a total of 6 min. The average fragment size was approximately 200–1000 base pairs. Immunoprecipitations were carried out in the lysis buffer and supplemented with 1.1% Triton X-100. After overnight immunoprecipitations at 4°C, complexes were washed five times in RIPA buffer (50 mM HEPES, 500 mM LiCl, 0.1 mM EDTA, 1.0% NP-40 and 0.7% Na-Deoxycholate) and once with TE containing 50 mM NaCl. Approximately 50 µg of chromatin was used in each ChIP. The immune complexes were eluted with 50 mM Tris [pH 8.0], 10 mM EDTA, and 1.0% SDS. Crosslinks were reversed by overnight incubation at 65°C. After proteinase K digestion, DNA was purified by phenol/chloroform extraction and ethanol precipitation. Genomic regions of interest were retrieved using the UCSC Genome Browser. Primers were designed using the Primer 3.0 tool version 4.1.0 (Koressaar et al., 2018; Untergasser et al., 2012). PCR amplifications were performed on an ABI 7500 system (Thermo Fisher) using SYBR green master mix (Qiagen). Data were analyzed using ABI 7500 software to generate cycle thresholds (CT values). Delta CT values were calculated by subtracting the CT values of the ChIPs obtained with the antibodies of interest from the CT values of the "input" or IgG controls. The $2^{-\Delta\Delta Ct}$ method was then used to calculate enrichment of immunoprecipitated material. Average ChIP enrichment was calculated from two biological replicates, each amplified as three technical replicates.

The BRD9 and SMARCA4 antibodies were the same as those used for immunoblots. Histone H3 (61799) and H3K4me3 (39159) antibodies were from Active Motif, and the H3K27ac antibody (4729) was from Abcam. The control IgG antibody was from Millipore Sigma. The oligonucleotide primers for qPCR are listed in Table S1.

2.11 | Statistical analysis

Statistical significance was calculated by the Student's *t*-test using Graphpad Prism.

3 | RESULTS

3.1 | Select bromodomain inhibitors abrogate visible pigmentation and melanin synthesis in Melb-a cells

Melb-a cells are unpigmented mouse melanoblasts that become pigmented as they differentiate into functional melanocytes (Sviderskaya et al., 1995). As a strategy to identify epigenetic regulators important for melanocyte differentiation, we screened a panel of bromodomain inhibitors to evaluate their effects on pigmentation. The BET-bromodomain inhibitor, JQ1 (Filippakopoulos et al., 2010) and two drugs, i-BRD9 (Theodoulou et al., 2016) and BI7273 (Martin et al., 2016), which are selective for the BRD9 component of SWI/SNF, profoundly decreased visible pigmentation after 48 hours while treatment with PFI-3, selective for the bromodomains of SMARCA4, SMARCA2, and PBRM1, did not cause a visible color change (Figure 1a).

Differentiating Melb-a cells have been reported to continue to proliferate even as they become pigmented (Sviderskaya et al., 2001). As expected, control Melb-a cells became noticeably darker as they were induced to differentiate over four days while iBRD9-treated cells failed to become pigmented (Figure 1b). To determine whether the effects of iBRD9 treatment were due to inhibition of melanogenesis or to a reduction in the number of melanocytes, a quantitative melanin assay was performed. Treatment with iBRD9 during differentiation modestly but significantly decreased Melb-a proliferation (Figure 1c, left). However, melanin

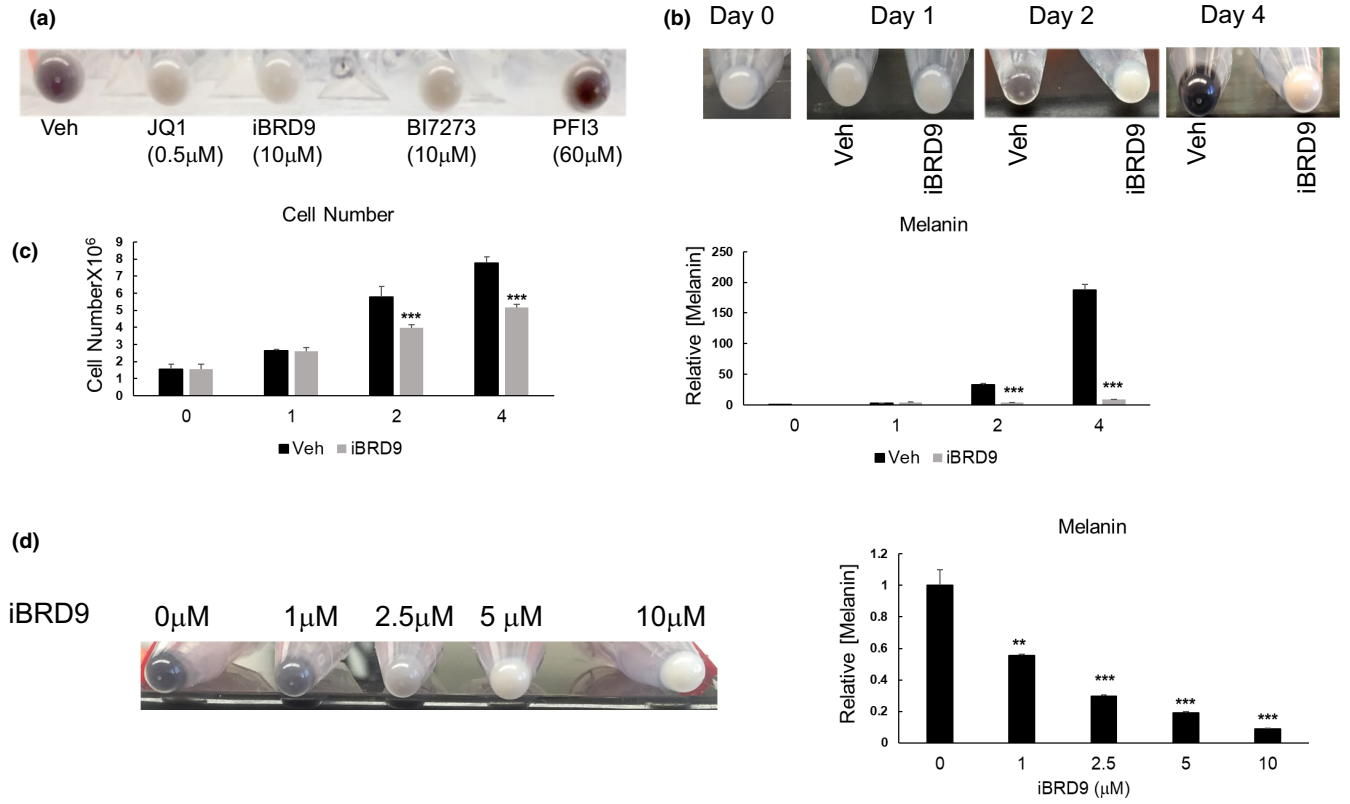

FIGURE 1 Effects of bromodomain inhibitors on melanin synthesis and proliferation. Melb-a cells were cultured in growth media then transferred to differentiation medium to which vehicle or a bromodomain inhibitor was added. (a) Melb-a cells were cultured for 48 h in differentiation medium containing vehicle (Veh), JQ1 (0.5 μM), iBRD9 (10 μM), BI7273 (10 μM), and PFI-3 (30 μM). Cells were pelleted and photographed. (b) Melb-a cells were cultured in differentiation media with vehicle (Veh) or 10 μM iBRD9 for the indicated number of days. Photographed cell pellets are shown. (c) Melb-a cells were cultured for the indicated number of days in differentiation media containing vehicle or 10 μM iBRD9, then counted. Left: Cell numbers are shown. Right: 1.5×10^6 cells were subjected to the melanin assay. Numbers were normalized to the vehicle treated control. (d) Melb-a cells were cultured in differentiation media in the presence of vehicle or the indicated concentrations of iBRD9 for 4 days. Left: Cells were pelleted and photographed. Right: 1.5×10^6 cells were subjected to the melanin assay. Numbers were normalized to the vehicle treated control. Representative pictures from three or more experiments are shown. Graphs indicate the average of three or more independent experiments. Standard error bars are shown (*$p < .05$, **$p < .01$, ***$p < .001$).

assays, which utilized a fixed number of cells, indicated that iBRD9 treatment dramatically reduced melanin content (Figure 1c, right). The inhibitory effect of iBRD9 on melanin synthesis was found to be dose dependent over a range of concentrations (Figure 1d). Treatment with BI7273 was also found to result in a dose dependent decrease in melanin synthesis, although the effect was less than with iBRD9 (Figure S1). At a higher dose, treatment with PFI-3 resulted in a smaller but significant decrease in melanin content (Figure S1). These data suggest that inhibitors of SWI/SNF bromodomain-containing proteins interfere with melanin synthesis, an important feature of melanocyte differentiation.

3.2 | iBRD9 inhibits transcription of melanogenic enzymes in Melb-a cells

Experiments were then focused on determining if iBRD9 reduces melanin synthesis by suppressing gene expression. Melanin synthesis in Melb-a cells is characterized by a time-dependent increase in the expression of TYR, TYRP1, and DCT (Dopachrome Tautomerase) (Marathe et al., 2017). Treatment of Melb-a cells with iBRD9 decreased TYR and TYRP1 at the mRNA and protein levels at early and late stages of the differentiation process, while DCT expression was not significantly altered (Figure 2a,b). In addition to the melanogenic enzymes, SOX10, a transcriptional regulator of these genes (Jiao et al., 2004; Murisier et al., 2007) was inhibited by iBRD9 treatment while the expression of other regulators, MITF (Bentley et al., 1994; Fang et al., 2002; Yasumoto et al., 1994) and SMARCA4 (Laurette et al., 2015; Marathe et al., 2017) were not affected by iBRD9 treatment.

In order to determine whether the iBRD9-induced changes in melanogenic gene expression involve histone covalent modifications, we performed chromatin immunoprecipitations (ChIPs) followed by quantitative PCR (qPCR) to evaluate changes in the levels of histone H3 tri-methylation at lysine 4 (H3K4me3), a mark of active transcription. Consistent with gene expression changes that occurred 48 h after iBRD9 treatment, there was a decrease in H3K4me3 enrichment at the *Tyr* and *Tyrp1* promoters while there was no significant change at the *Dct* or silent *IgH* enhancers (Figure 3).

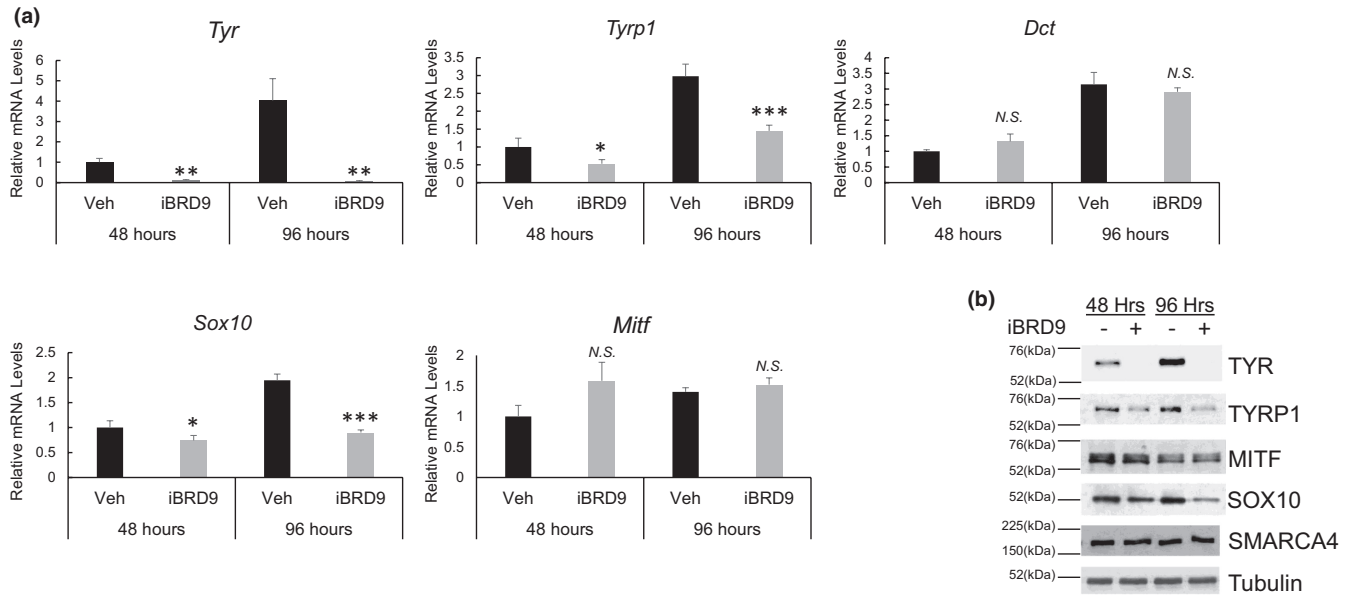

FIGURE 2 iBRD9 inhibits melanocyte-specific gene expression in Melb-a cells. Melb-a cells were cultured in differentiation media in the presence of vehicle or 10 μM iBRD9. Cells were harvested at the indicated time points and subjected to (a). qRT-PCR. *Tyr*, *Tyrp1*, or *Dct* levels were normalized to that of *Rpl7*. The data are the average of three independent experiments. Standard error bars are shown (*$p < .05$, **$p < .01$, ***$p < .001$). (b) subjected to Western blot using the indicated antibodies. Tubulin is a loading control. The figure is representative of three or more experiments.

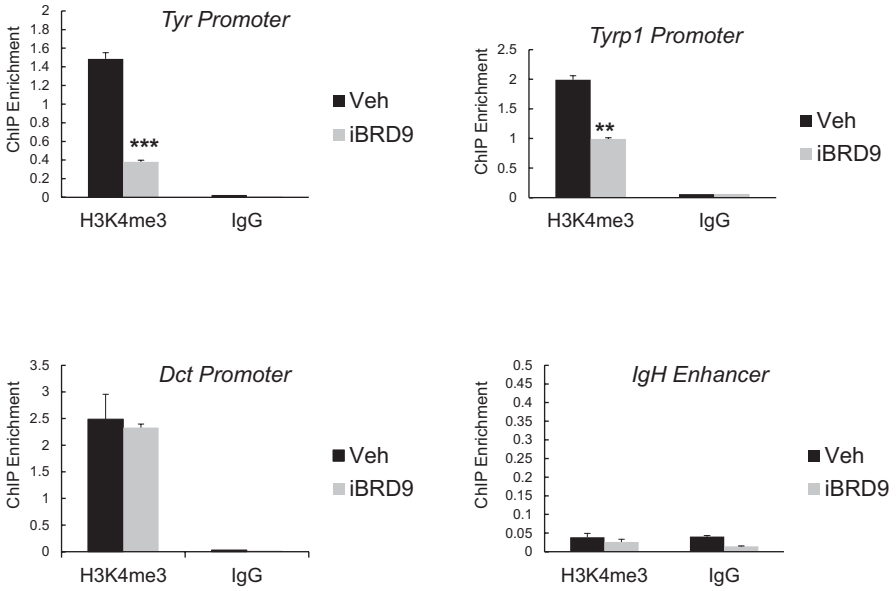

FIGURE 3 iBRD9 decreases H3K4me3 levels at melanogenic enzyme loci. Melb-a cells were cultured in differentiation media containing vehicle (Veh) or 10 μM iBRD9 for 48 h. Cells were harvested for chromatin immunoprecipitations (ChIPs) with an antibody to histone H3 trimethylated at lysine 4 (H3K3me3) or control IgG. ChIPs were quantified by qPCR using primers to the promoters of melanogenic enzyme genes and as a control, the silent IgH enhancer. Normalization was to input. The data are the average of two experiments analyzed in triplicate. Standard error bars are shown (*$p < .05$, **$p < .01$, ***$p < .001$).

3.3 | iBRD9 alters the melanocyte transcriptome

To gain additional insight into how iBRD9 suppresses melanogenesis, RNA was sequenced from Melb-a cells that had been treated with vehicle or iBRD9 for 48 h. Treatment with iBRD9 resulted in 3791 significantly up-regulated genes and 4076 significantly down-regulated genes (Figure 4a). Gene ontology (GO) indicated enrichment of the terms, pigment biosynthetic process, pigment regulation, and melanocyte differentiation, as well as cell cycle and metabolism (Figure 4b). Further analysis revealed that iBRD9 treatment positively and negatively impacted upon biological processes and pathways which could affect cellular differentiation and melanocyte function as well as pathways related to cancer progression and metabolism (Figure S2). In addition to decreased expression of melanogenic enzymes, TYR and TYRP1, iBRD9 treatment also decreased expression of other genes known to play a role in melanin synthesis, melanosome function, and transfer (Figure 4c). Interestingly, these genes were also previously found to be down-regulated by the BET-inhibitor, JQ1, in Melb-a cells (Trivedi et al., 2020) and by depletion of SMARCA4 in Melb-a cells (Marathe et al., 2017), in 501MEL melanoma cells, and/or in immortalized human melanocytes (HERMES) (Laurette et al., 2015).

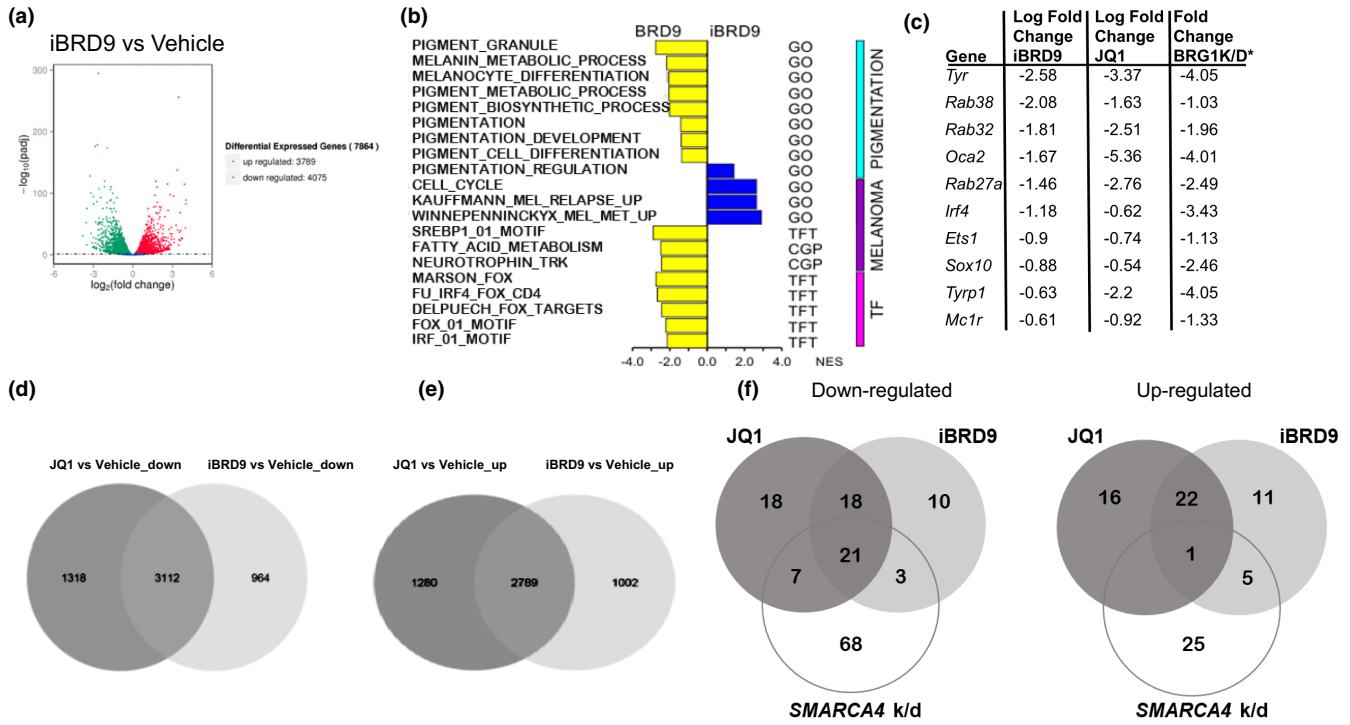

FIGURE 4 Transcriptomic changes in iBRD9-treated Melb-a cells. Melb-a cells were cultured for 48 h in differentiation media that contained either vehicle or 10 μM iBRD9. RNA from three biological replicates was subjected to RNA-seq. Differential gene expression between vehicle (DMSO) and 10 μM iBRD9-treated Melb-a cells was determined from RNA-seq data ($p < .05$). (a) Plot showing the number of up and down-regulated genes. Red dots represent genes which are significantly up-regulated by iBRD9, green dots represent genes that are significantly down-regulated by iBRD9, and blue dots represent non-significant changes in gene expression. (b) Analysis of pathways relevant to pigmentation and overlap in differentially regulated genes with the indicated data sets was conducted. Normalized enrichment score (NES) is the enrichment score for the gene set after it has been normalized across analyzed gene sets. Yellow bars represent processes that are significantly down-regulated by iBRD (negative numbers), and blue bars represent processes that are significantly up-regulated by iBRD9 (positive numbers). The *x*-axis indicates the enrichment score in either direction. (c) Chart comparing the effects of iBRD9 with JQ1 treated Melb-a cells and with SMARCA4 depleted 501Mel cells on the expression of selected pigmentation genes. (d) Venn diagram showing overlap between the total number of genes down-regulated 1.5-fold or more ($p = .05$) by iBRD9 and JQ1 in Melb-a cells. (e) Venn diagram showing overlap between the total number of genes up-regulated 1.5-fold or more ($p < .05$) by iBRD9 and JQ1 in Melb-a cells. (f) Venn diagrams showing overlap between the total number of genes down-regulated (left) or up-regulated (right) 1.5-fold or more ($p < .05$) by iBRD9 and JQ1 in Melb-a cells with eGene expression changes that occur in SMARCA4 depleted 501MEL and HERMES cells (twofold or greater). Hypergeometric analysis indicated significant overlap between iBRD9 and JQ1 down-regulated genes, iBRD9 and SMARCA4 regulated genes, and JQ1 and SMARCA4 regulated genes (left). For up-regulated genes, the overlap was significant only between iBRD9 and JQ1 (right).

Further analysis revealed that there was a high level of overlap in transcriptomic changes elicited by iBRD9 treatment and those previously found to be elicited by JQ1 treatment in Melb-a cells (Trivedi et al., 2020) (Figure 4d,e). We then compared how JQ1 and iBRD9 treatment of Melb-a cells affected pigmentation gene expression. Pigmentation-related eGenes were previously identified in an expression-quantitative locus study using primary human melanocytes (Zhang et al., 2018), and we previously found that JQ1 treatment resulted in altered expression of nearly 50% of the 379 identified pigmentation-related eGenes (Trivedi et al., 2020). iBRD9 treatment resulted in the differential expression of 167 of these eGenes, of which 94 genes were down-regulated and 73 genes were up-regulated (Table S1). There was also a high degree of overlap between eGenes modulated by iBRD9, those reported to be altered by JQ1 (Trivedi et al., 2020), and those reported to be altered by SMARCA4 depletion in human melanoma (501MEL) and Hermes immortalized melanocytes (Laurette et al., 2015) as shown in Table S1. Figure 4f shows the overlap between the most highly down-regulated and up-regulated eGenes between iBRD9, JQ1 treatment in Melb-a cells, and SMARCA4 depletion in 501Mel melanoma cells and Hermes immortalized melanocytes. Hypergeometric analysis revealed that the overlap between the most highly down-regulated eGenes (Figure 4f, left) in any of the three groups was significantly greater than would be expected by chance. However, the overlap between the most highly up-regulated eGenes (Figure 4f, right) was significant only for iBRD9 and JQ1 treated cells.

We also interrogated a recently published dataset of 501MEL cells which had been depleted of the PBAF subunit, ARID2 (Carcamo et al., 2022). Of the 94 eGenes that were significantly down-regulated by iBRD9 in Melb-a cells, 16 were down-regulated and 15 were up-regulated by ARID2 depletion in 501MEL. Of the 73 eGenes up-regulated by iBRD9 in Melb-a cells, 11 were down-regulated and 13 were up-regulated by ARID2 depletion in 501MEL cells (Table S2). Because ARID2 depletion results in down-regulation of BRD7, these

data suggest that for a subset of genes affected by iBRD9, the regulation may be attributed to BRD7. However, there were a substantial number of eGenes affected by iBRD9 that were not significantly affected by ARID2 depletion and could potentially be regulated by BRD9.

3.4 | BRD9 is required for expression of pigmentation-specific genes in Melb-a cells

To determine whether BRD9 mediates the effects of iBRD9 on pigmentation gene expression, we first evaluated BRD9 and BRD7 expression during Melb-a differentiation. Both BRD9 and BRD7 were expressed in Melb-a cells, but BRD9 expression was high until 96 h from the start of differentiation while BRD7 expression decreased 48 h after the start of differentiation (Figure 5a). We focused on BRD9 as a regulator of pigmentation genes. Depletion of BRD9 by RNAi resulted in suppression of TYR and TYRP1 at both the protein (Figure 5b) and mRNA levels (Figure 5c), indicating that BRD9 regulates key melanogenic gene expression which at least partially explains the suppressive effect of iBRD9 and BI7273 on pigmentation.

3.5 | BRD9 promotes expression of pigmentation genes in human melanocytes and melanoma cells

We next evaluated the effects of iBRD9 on primary neonatal human epidermal melanocytes (NHEM) and 501MEL melanoma cells. BRD9 and BRD7 were expressed at similar levels in NHEMs and 501MEL cells (Figure 6a). Treatment of NHEMs with iBRD9 for 14 days resulted in decreased visible pigmentation of the melanocytes (Figure 6b) and inhibited expression of TYR and TYRP1 protein (Figure 6b).

In 501MEL cells, there was also reduced expression of TYRP1 and SOX10 protein at 24 and 72 h after treatment with iBRD9 while SMARCA4 levels were not affected (Figure 6c). Although expression of MITF and TYR was not suppressed by iBRD9 in 501Mel cells (data not shown), analysis of the mRNA levels of TYRP1, SOX10, and selected eGenes revealed that iBRD9 had an inhibitory effect on their expression (Figure 6d). Interestingly, DCT was previously found to be up-regulated by ARID2 depletion while the other eGenes we evaluated were found not to be significantly affected by ARID2 depletion (Table S2). eGenes suppressed by iBRD9 but not ARID2 depletion were deemed to be good candidates for regulation by BRD9, and this was confirmed by depleting BRD9 with RNAi (Figure 6e).

3.6 | iBRD9 disrupts SMARCA4 and BRD9 occupancy on pigmentation loci in melanoma cells

To test the hypothesis that iBRD9 suppresses pigmentation gene expression by disrupting BRD9 and SMARCA4 binding at these loci, we probed publicly available datasets that had evaluated SMARCA4 and BRD9 genomic occupancy in human melanoma cells by chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq). In a SMARCA4 ChIP-seq dataset on 501Mel cells (Laurette et al., 2015), there was a strong SMARCA4 peak in an

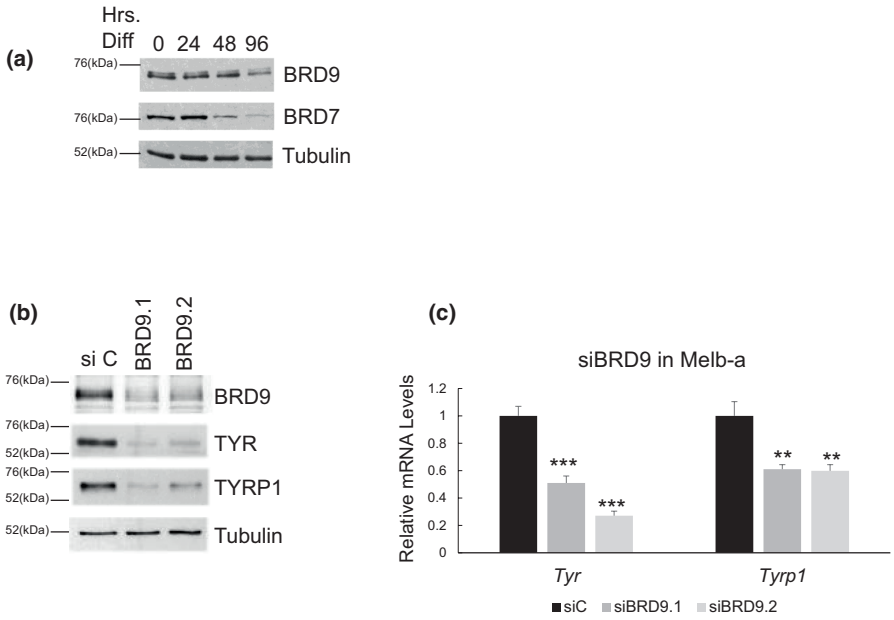

FIGURE 5 Depletion of BRD9 inhibits expression of melanogenic enzyme genes in Melb-a cells. (a) Melb-a cells were differentiated for the indicated number of hours and subjected to Western blotting with antibodies to BRD9 or BRD7. Tubulin is a loading control. (b) Melb-a cells were transfected with a control siRNA (siCtrl) or siBRD9, then cultured in differentiation media for 48 h. Westerns were performed with the indicated antibodies. Tubulin is a loading control. The figure is representative of three or more experiments. (c) Melb-a cells were transfected as in (b) then subjected to qRT-PCR. *Tyr* and *Tyrp1* levels were normalized to the level of *Rpl7*. The data are the average of three independent experiments. Standard error bars are shown (**$p < .01$, ***$p < .001$).

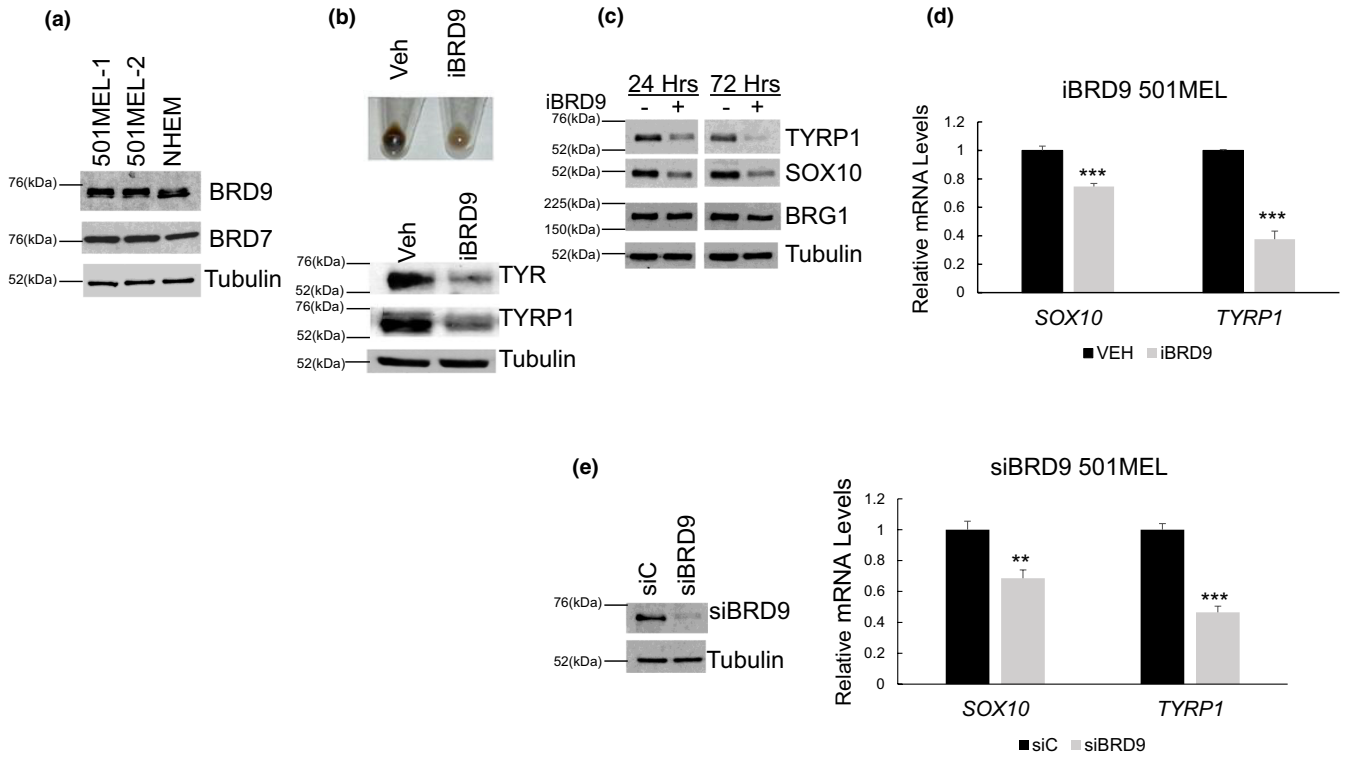

FIGURE 6 iBRD9 or siBRD9 inhibits melanocyte-specific gene expression in NHEMs and 501MEL cells. (a) Protein extracts from 501MEL cells (1%–70% confluence and 2%–100% confluence) and NHEM (70% confluence) were subjected to Western blotting with antibodies to BRD9 or BRD7. Tubulin is a loading control. The Western is representative of two or more experiments. (b) NHEMs were cultured for 14 days in the presence of vehicle (Veh) or 10 μM iBRD9. Top: Photographs of pelleted cells. Bottom: Western blots with the indicate antibodies. Tubulin is a loading control. The figures are representative of two experiments. (c) 501MEL cells were cultured in vehicle or 10 μM iBRD9 for the indicated time and subjected to Western blotting with the indicated antibodies. The figure is representative of three or more experiments. (d) 501MEL cells were cultured in vehicle (Veh) or 10 μM iBRD9 for 72 h. qRT-PCR is shown. mRNA levels were normalized to the level of *ACTβ*. The data are the average of three independent experiments. Standard error bars are shown (***$p < .001$). (e) 501MEL cells were transfected with control siRNA (siCtrl) or siBRD9 and cultured for 72 h. Left: Western blot showing BRD9 depletion. Tubulin is a loading control. The figure is representative of three experiments. Right: qRT-PCR. *SOX10* and *TYRP1* mRNA levels were normalized to ACTβ. The data are the average of three independent experiments. Standard error bars are shown (**$p < .01$, ***$p < .001$).

intronic region of *SOX10* (Figure S3, top) and a strong peak in a -8 KB upstream region of *TYRP1* as well as a smaller peak in the coding region of *TYRP1* (Figure S3, top panel). In contrast, a dataset on a uveal melanoma cell line, MEL270, which investigated both SMARCA4 and BRD9 binding, SMARCA4 and BRD9 peaks occurred in a region -12 KB upstream of the *SOX10* locus but no obvious peaks in the intronic region as were detected in 501MEL cells (Figure S3, bottom 2 panels). The MEL270 data did contain SMARCA4 and weak BRD9 peaks in the −8 KB upstream and coding regions of *TYRP1* (Figure S3. bottom two panels) which overlapped the SMARCA4 peaks in the 501MEL dataset. Although the specific region of *SOX10* occupied by SMARCA4 differed in these two datasets, they suggested that both SMARCA4 and BRD9 directly bind *SOX10* and *TYRP1* in melanoma cells.

Chromatin immunoprecipitations followed by PCR (ChIP-PCR) were conducted to assess SMARCA4 and BRD9 occupancy as a function of iBRD9 treatment. As was found in the 501MEL genomic data, SMARCA4 was enriched at the *SOX10* intron and a −8 KB upstream region of *TYRP1*. However, we did not detect enrichment of either SMARCA4 or BRD9 at the -12 KB upstream region of *SOX10* relative to the negative control region (*PRM1*) (Figure 7a,b). Thus, our data are consistent with the prior genomic 501MEL dataset regarding SMARCA4 occupancy at the *SOX10* locus. Our data are also consistent with both 501Mel and Mel270 datasets regarding SMARCA4 and BRD9 binding at the *TYRP1* locus. Importantly, BRD9 and SMARCA4 enrichment at these regions decreased in iBRD9-treated cells (Figure 7a,b).

To associate the effects of iBRD9-associated disruptions in SMARCA4 and BRD9 occupancy at the *SOX10* and *TYRP1* loci with epigenetic changes, we also performed ChIP-PCR on total histone H3, tri-methylated H3 at lysine 4(H3K4me3) which is associated with actively transcribed promoters and acetylated H3 at lysine 27 (H3K27ac), which is associated with active enhancers and promoters. The *SOX10* intronic and *TYRP1* upstream regions had lower levels of H3 enrichment than the *SOX10*-12 KB upstream region and the control *PRM1* region, and this did not change with iBRD9 treatment (Figure 7c). Enrichment of H3K4me3 was detected only at the *SOX10* intron, which is close to the start site of transcription, and this decreased upon iBRD9 treatment (Figure 7d). The *SOX10* intron and *TYRP1*-8 KB upstream region were both enriched in H3K27ac, which

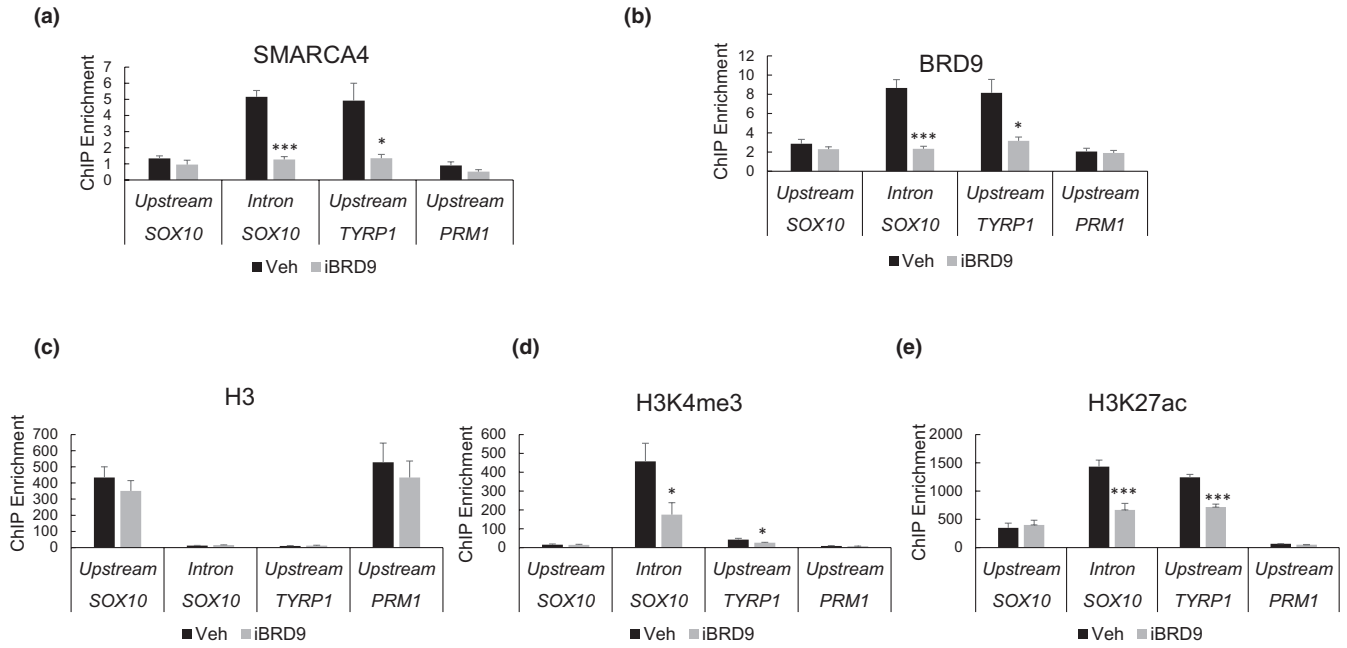

FIGURE 7 iBRD9 disrupts BRD9 and SMARCA4 binding at melanocyte-specific loci. Chromatin immunoprecipitations (ChIPs) with the indicated antibodies or IgG control were performed on 501MEL cells that had been cultured for 72 h in the presence of vehicle (Veh) or 10 μM iBRD9. ChIP-qPCR was performed with primers to the indicated genomic regions and normalized to IgG. ChIP enrichment is the fold increase relative to IgG. (a) SMARCA4 (b) BRD9 (c) Histone H3 (d) Histone H3K4me3 (e) Histone H3K27me3. The data are the average of three independent experiments. Standard error bars are shown (*$p < .05$, **$p < .01$, ***$p < .001$).

was significantly reduced by iBRD9 treatment (Figure 7e). In combination, these data suggest that BRD9 facilitates SMARCA4 binding to the selected pigmentation genes which can then promote histone modifications associated with accessible chromatin.

4 | DISCUSSION

The bromodomain (BrD) is a conserved protein module that binds to acetylated lysines on histones and other proteins, thereby mediating protein–protein interactions and participating in diverse biological processes (Filippakopoulos et al., 2012). In humans, there are 46 different BrD-containing proteins, many of which have chromatin remodeling and transcriptional functions (Zaware & Zhou, 2019). The BrDs have been classified into families based on structural similarity (Filippakopoulos, Picaud Cell, Filippakopoulos et al., 2012). The development of the first potent BrD small molecule inhibitors facilitated functional characterization of the BrD and extra terminal (BET) family members, BRD2, BRD3, BRD4, and BRDT (Filippakopoulos et al., 2010; Nicodeme et al., 2010). BET proteins have been highly studied as therapeutic targets in a diverse array of human disorders and in the regulation of normal processes such as lineage development and cellular differentiation (Brown et al., 2018; Najafova et al., 2017; Roberts et al., 2017; Stonestrom et al., 2015). We previously found a role for BRD2 and BRD4 in melanocyte differentiation (Trivedi et al., 2020), raising the possibility that other BrD-containing proteins might also be involved. Considering the established role of SWI/SNF chromatin remodeling complexes in melanocyte development (Laurette et al., 2015; Marathe et al., 2017) and the availability of small molecule inhibitors, we investigated BrD-containing subunits of the SWI/SNF complex using these inhibitors.

The BrD-containing proteins of the SWI/SNF complex include SMARCA4, SMARCA2, PBRM1, BRD7, and BRD9. The structurally similar Brds of SMARCA4, SMARCA2, and PBRM1 are in family VIII while the structurally similar BrDs of BRD7 and BRD9 are in family IV (Filippakopoulos et al., 2012). Several studies suggest that there is a context-dependent requirement for SWI/SNF BrDs. The SMARCA4/SMARCA2 BrDs are required for muscle differentiation, and the SMARCA4 BrD is required for regulation of target genes by the repressor element-1-silencing transcription factor (REST) but the BrD of the single SWI/SNF ATPase in Drosophila is not required for normal fly development (Elfring et al., 1998; Ooi et al., 2006; Sharma et al., 2021). The BrDs of PBRM1 are required for mediating interactions with p53 and tumor suppressor function but not needed for muscle differentiation (Cai et al., 2019; Sharma et al., 2021). Recent studies implicated the BrD of BRD9 in the regulation of embryonic stem cell pluripotency, androgen receptor signaling, interferon-activated gene expression, and myoblast proliferation (Ahmed et al., 2022; Alpsoy et al., 2021; Börold et al., 2021; Gatchalian et al., 2018; Padilla-Benavides et al., 2022). Interestingly, inhibition of the BRD9/BRD7 BrDs has recently been reported to suppress melanoma tumorigenicity (Mason et al., 2021).

We found that PFI-3 a small molecule inhibitor selective for the BrDs of SMARCA4, SMARCA2, and PBRM1 (Gerstenberger et al., 2016) had only modest effects on the development of visible pigmentation in an in vitro model of melanocyte differentiation

compared to JQ1, iBRD9, and BI7273. This contrasts with previous reports by us and others which demonstrated that depletion of SMARCA4 profoundly disrupts pigmentation both in vitro and in vivo (Laurette et al., 2015; Marathe et al., 2017). While this may seem contradictory, it is important to note that the findings presented here do not exclude the contribution of other domains of SMARCA4. Importantly, a prior study showed that targeting the SMARCA4/SMARCA2 ATPase domain was more effective than targeting the bromodomain for curbing the growth of some cancer cells (Vangamudi, 2015). Similarly, in combination with our prior work, showing that dominant-negative versions of SMARCA4, which harbor mutations in their ATPase domains, suppress pigment gene expression (de la Serna et al., 2006), these findings suggest that the ATPase domains may be more important for the regulation of pigmentation than the SMARCA4/SMARCA2 bromodomains (de la Serna et al., 2006). However, additional studies are required to demonstrate a differential requirement for the SMARC4/SMARCA2 bromodomains and ATPase domains in the regulation of pigmentation. Importantly, we cannot rule out a critical requirement for these bromodomains in other aspects of melanocyte development which could impinge upon pigmentation in vivo.

Our findings in Melb-a cells contrast with the recent report that PFI-3 profoundly inhibits muscle differentiation (Sharma et al., 2021). In muscle differentiation, the BrDs of SMARCA4 and SMARCA2, but not PBRM1, were implicated in the expression of a subset of SMARCA4/SMARCA2-dependent gene expression and for stable binding of the ATPases to promoters required for cell cycle exit but not directly for the expression of muscle structural genes. A requirement for the SMARCA4/SMARCA2 BrDs in the regulation of cell cycle genes may be why PFI-3 has a greater effect on myogenesis than on melanogenesis. Prior cell cycle exit is a prerequisite for expression of terminal markers of muscle differentiation during myogenesis (Novitch et al., 1996), but is not required for melanogenic genes to be expressed; pigmented Melb-a cells can still divide and proliferate (Sviderskaya et al., 2001).

Treatment of Melb-a cells with iBRD9 suppressed accumulation of melanin and visible pigmentation, suggesting an effect on melanocyte differentiation. The effect of iBRD9 on pigmentation was confirmed by RNA-seq, which indicated a broad effect on structural genes that regulate pigmentation and other aspects of melanocyte differentiation. iBRD9 also suppressed the expression of transcription factors that are important for pigmentation gene expression, including IRF4 (Interferon Regulatory Factor 4) (Praetorius et al., 2013) and ETS1 (ETS Proto-oncogene 1) (Saldana-Caboverde et al., 2015). Interestingly, there was a high degree of overlap between iBRD9 and JQ1 altered pigmentation gene expression. Although iBRD9 has been reported to have 700-fold selectivity over BET bromdomain proteins, some of the overlap could result from off target effects on BRD2 and BRD4, which have been shown to also play a role in melanocyte differentiation (Theodoulou et al., 2016; Trivedi et al., 2020). Another possibility is that BRD9, which has been shown to cooperate with the BET protein, BRD4 in the regulation of pluripotency genes in embryonic stem cells and interferon-inducible genes in macrophages (Ahmed et al., 2022; Gatchalian et al., 2018), has a similar partnership with BET family members in melanocytes and melanoma cells. Studies will be needed to identify the BRD9 interactome in melanocytes and melanoma cells.

iBRD9 has been reported to have 200-fold selectivity for the BrD of BRD9 versus the highly similar BrD of BRD7, off-target effects. Our studies showing that BRD9 depletion by siRNA suppresses expression of melanogenic enzymes and transcriptional regulators, IRF4 and SOX10 as well as demonstration of BRD9 binding at the *TYRP1* and *SOX10* loci is strong evidence that BRD9 regulates at least a subset of the genes suppressed by iBRD9. Additional evidence for the involvement of BRD9 in the regulation of pigmentation is our finding that a different drug, BI7273, also selective for BRD9 (Martin et al., 2016) inhibited melanin synthesis. However, we did note that BI7273, which was found to be more selective for BRD9 than iBRD9 (Karim et al., 2020), had a less profound effect on melanin synthesis compared to iBRD9. In combination, these data indicate that BRD9 regulates melanin synthesis but also raise the possibility that some of the pigmentation effects observed with iBRD9 may be due to off-target inhibition of the highly similar BRD7 BrD. Indeed, BRD7 is a component of PBAF, the SWI/SNF complex that interacts with MITF in melanoma cells (Laurette et al., 2015). However, interrogation of a dataset from ARID2-depleted 501MEL melanoma cells indicates that only a small subset of eGenes are regulated by the PBAF complex. However, the requirement for PBAF and by extension, BRD7, may differ during differentiation of melanoblasts to melanocytes. Additional studies will be required to determine the functional importance of BRD7 and the PBAF complex in the regulation of pigmentation during melanocyte differentiation.

Our data show a correlation between eGenes affected by iBRD9 in Melb-a cells and those affected by SMARCA4 depletion in 501MEL cells (Laurette et al., 2015). This prompted us to investigate whether iBRD9 could suppress SMARCA4 recruitment to commonly regulated genes. We saw a correlation between SMARCA4 and BRD9 binding as well as disruption of both SMARCA4 and BRD9 binding to two melanocyte loci. Chemical inhibition of class VIII BrDs of the SWI/SNF complex in muscle cells compromised SMARCA4 and SMARCA2 occupancy on target genes (Sharma et al., 2021). Our data demonstrate that chemical inhibition of class IV BrDs of the SWI/SNF complex is associated with disruption of both BRD9 and SMARCA4 binding to pigmentation loci in melanoma cells. However, we cannot rule out that BRD9 may also act independently of SMARCA4 at some pigmentation loci since it has been reported that a subset of BRD9 occupied sites do not overlap with SMARCA4 binding (Kenny et al., 2021) and that SMARCA4 binding is not sensitive to BRD9 inhibition at a subset of overlapping sites (Inoue et al., 2019). Therefore, additional genomic experiments will be needed to determine the extent to which BRD9 regulates pigmentation-specific gene expression by promoting SMARCA4 binding.

It is also not known if BRD9 has an extensive role in regulating melanoma differentiation. On a genomic scale, it was shown that the chromatin alterations which occur upon PBAF loss in melanoma cells

are contingent upon the levels of MITF and the identity of the dominant transcription factors (Carcamo et al., 2022). Therefore, the suppressive effects of iBRD9 on melanoma differentiation status may not completely parallel those that occur during melanoblast differentiation. This may explain why TYR, which was highly suppressed by iBRD9 in Melb-a cells, was not suppressed in 501MEL cells. Since bromodomain inhibitors are emerging as novel epigenetic drugs for treating cancer, it will be important to evaluate genome-wide effects of BRD9 inhibition on melanoma cells of varying transcriptional states.

AUTHOR CONTRIBUTIONS

Ivana L. de la Serna: Conceptualization, Methodology, Original Draft Preparation, Editing. **Tupa Basuroy**: Methodology, Original draft preparation, Editing. **Megan R. Dreier**: Methodology. **Fabian V. Filipp**: Data Analysis, Writing, Editing. **Thomas Blomquist**: Data Analysis, Editing. **Caitlin Baum**: Data Analysis, Editing. **Robert Trumbly**: Data Analysis, Editing.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

RNA-seq datasets were submitted to GEO. The accession number is as follows: GSE210015.

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SUPPORTING INFORMATION

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