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BAF60A mediates interactions between the microphthalmia-associated transcription factor and the BRG1-containing SWI/SNF complex during melanocyte differentiation

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Abstract

SWI/SNF chromatin remodeling enzymes are multisubunit complexes that contain one of two catalytic subunits, BRG1 or BRM and 9–11 additional subunits called BRG1 or BRM-associated factors (BAFs). BRG1 interacts with the microphthalmia-associated transcription factor (MITF) and is required for melanocyte development in vitro and in vivo. The subunits of SWI/SNF that mediate interactions between BRG1 and MITF have not been elucidated. Three mutually exclusive isoforms of a 60-kDa subunit (BAF60A, B, or C) often facilitate interactions with transcription factors during lineage specification. We tested the hypothesis that a BAF60 subunit promotes interactions between MITF and the BRG1-containing SWI/SNF complex. We found that MITF can physically interact with BAF60A, BAF60B, and BAF60C. The interaction between MITF and BAF60A required the basic helix-loop-helix domain of MITF. Recombinant BAF60A pulled down recombinant MITF, suggesting that the interaction can occur in the absence of other SWI/SNF subunits and other transcriptional regulators of the melanocyte lineage. Depletion of BAF60A in differentiating melanoblasts inhibited melanin synthesis and expression of MITF target genes. MITF promoted BAF60A recruitment to melanocyte-specific promoters, and BAF60A was required to promote BRG1 recruitment and chromatin remodeling. Thus, BAF60A promotes interactions between MITF and the SWI/SNF complex and is required for melanocyte differentiation.

KEYWORDS
BAF60A, chromatin remodeling, melanocyte differentiation, microphthalmia-associated transcription factor (MITF)

1 | INTRODUCTION

Melanocytes are cells that synthesize melanin and populate the skin, hair follicles, heart, choroid of the eye, and inner ear (Steingrimsson, Copeland, & Jenkins, 2004). During embryonic development, the neural crest gives rise to precursors called melanoblasts, which then differentiate into melanocytes. Melanocytes in the basal layer of the epidermis protect the skin against the damaging effects of ultraviolet radiation by synthesizing and transferring melanosomes containing melanin to surrounding keratinocytes (Kadekaro et al., 2003). They can transform to melanoma, an aggressive skin cancer with a poor prognosis. Key transcription factors that regulate melanocyte specification, survival, proliferation, and differentiation also have critical functions in melanoma.

The microphthalmia-associated transcription factor (MITF) is the master regulator of melanocyte differentiation and considered a

lineage addiction oncogene in melanoma (Garraway & Sellers, 2006). MITF is a helix-loop-helix leucine zipper transcription factor that binds to E boxes and regulates expression of genes important for melanocyte differentiation, survival, and proliferation (Levy, Khaled, & Fisher, 2006). MITF target genes include those that encode the tyrosinase (*Tyr*) family of enzymes required for melanin synthesis: *Tyr*, Tyr-related protein 1 (*Tyrp1*), and dopachrome tautomerase (*Dct*) as well as other melanosomal proteins. The Sry-related transcription factor, SOX10, also participates in regulating the expression of these genes (Jiao et al., 2004; Lang et al., 2005; Ludwig, Rehberg, & Wegner, 2004; Murisier, Guichard, & Beermann, 2006; Murisier, Guichard, & Beermann, 2007). SOX10 is expressed in neural crest precursors and is critical for establishing the Schwann cell and melanocyte lineages (Wegner, 2005). In melanoblasts, SOX10 activates MITF expression and interacts with MITF to regulate expression of a subset of MITF target genes required for melanin synthesis (Marathe et al., 2017). Transcriptional activation of melanocyte-specific genes also involves crosstalk between these lineage-specific transcriptional activators and SWItch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complexes, which induce chromatin modifications permissive for transcription (de la Serna et al., 2006).

SWI/SNF enzymes are multisubunit and evolutionarily conserved chromatin remodeling complexes that utilize energy derived from ATP hydrolysis to physically remodel chromatin structure. The catalytic activity of chromatin remodeling is attributed to two mutually exclusive central ATPases, namely BRG1 or BRM, which are more than 70% identical to each other (Saladi & de la Serna, 2010). These complexes also contain 8–12 BRG1 or BRM-associated factors (BAFs), which can enhance chromatin remodeling activity or interact with cellular factors to help target the complex to genomic loci. Diverse complexes composed of either BRG1 or BRM and an assortment of different BAFs have been detected in cells. Among the ATPases, BRG1 has a predominant role in melanocyte development in vivo and differentiation in vitro (Laurette et al., 2015; Marathe et al., 2017). A minimal complex consisting of BRG1 and BAF155 or BAF170 has been shown to functionally interact with zinc finger transcription factors, SP1, EKLF, and GATA1, but neither with the helix-loop-helix transcription factor, TFE3, nor with NF-κB (Kadam et al., 2000; Phelan, Sif, Narlikar, & Kingston, 1999). Additional BAFs are required to mediate interactions between helix loop helix and many other transcription factors with BRG1 and the SWI/SNF complex (Forcales et al., 2012; Hsiao, Fryer, Trotter, Wang, & Archer, 2003).

Among the BAFs, one of three variants of a 60-kDa subunit, BAF60A, BAF60B, or BAF60C, encoded by *Smarcd1*, *Smarcd2*, and *Smarcd3* respectively, mediates interactions between the SWI/SNF complex and a number of transcription factors. BAF60A interacts with a diverse set of transcriptional regulators, including nuclear hormone receptors, ppaγ-coactivator-1α (PGC-1α), JUN/c-FOS, p53, and SOX10 (Flajollet, Lefebvre, Cudejko, Staels, & Lefebvre, 2007; Fryer & Archer, 1998; Hsiao et al., 2003; Ito et al., 2001; Li et al., 2008; Weider et al., 2012). In vivo, BAF60A has been studied in the liver where conditional deletion of the *Smarcd1* gene renders mice resistant to diet-induced hypercholesteremia and atherosclerosis but does not affect viability (Meng et al., 2015). BAF60B interacts with p53, ATM, and CEBPε (Ji et al., 2017; Priam et al., 2017; Witzel et al., 2017). Deletion of *Smarcd2* compromises hematopoietic development and results in late embryonic lethality (Priam et al., 2017; Witzel et al., 2017). BAF60C was shown to interact with the helix-loop-helix transcription factors, MYOD in myocytes and USF1 in hepatocytes (Simone et al., 2004; Wang et al., 2013). Depletion of BAF60C during mouse development causes heart defects, blocks skeletal muscle differentiation, and results in embryonic lethality (Lickert et al., 2004). Muscle-specific deletion of the gene encoding BAF60C impairs glucose sensing and glycolytic metabolism in myotubes (Meng et al., 2013). Both BAF60A and BAF60B were previously found to be in a complex with MITF in melanocytes and melanoma cells (Laurette et al., 2015). BAF60A was also found to be in a complex with SOX10 in melanocytes (Marathe et al., 2017). However, a functional analysis of BAF60A or other BAF60 subunits in the regulation of gene expression in melanocytes has not previously been reported.

In this study, we report that MITF can physically interact with BAF60A, BAF60B, and BAF60C. The interaction between MITF and BAF60A required a region that includes the basic helix-loop-helix (bHLH) domain of MITF. Recombinant BAF60A pulled down recombinant MITF, suggesting that the interaction can occur in the absence of other components of the SWI/SNF complex and in the absence of SOX10. Depletion of BAF60A in differentiating melanoblasts inhibited melanin synthesis and MITF target genes that regulate melanin synthesis. MITF promoted BAF60A recruitment to a melanocyte-specific promoter, and BAF60A was required to promote BRG1 recruitment and chromatin remodeling.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Melb-a cells were obtained from the Welcome Trust (United Kingdom) and cultured in growth media (Roswell Park Memorial Institute Medium [RPMI] 1640 with 10% fetal bovine serum, 40 picoM fibroblast growth factor, and 10 ng/ml stem cell factor). Differentiation was induced when cultures were 70% confluent by replacing growth media with differentiation medium (dulbecco's modified eagle medium [DMEM] with 10% fetal bovine serum, 2 nM [Nle4, D-Phe7]-alpha melanocyte stimulating hormone, and 200 nM phorbol-myristate-acetate). Human Embryonic Kidney (HEK) 293T cells were obtained from the American Type Culture Collection (Manassas, VA). They were cultured in DMEM media containing 10% fetal calf serum. 501Mel melanoma cells were cultured as previously described (Keenen, Qi, Saladi, Yeung, & de la Serna, 2010).

2.2 | Plasmids

Vectors for expression of 3X-FLAG-tagged *BAF60* subunits in mammalian cells were previously described (Lores, Visvikis, Luna, Lemichez, & Gacon, 2010). *Mitf* deletion constructs were generated by polymerase chain reaction (PCR) and subcloned into the *Not*1/*Mlu*I sites of a pCMV-V vector. For bacterial expression, *BAF60A*

complementary DNA (cDNA) was subcloned into the *Sal*1/*Hin*dIII sites of pGEX-2T (GE Healthcare, Pittsburgh, PA) and MITF cDNA was subcloned into the *Eco*RI/*Hin*dIII sites of pRSET-B (Thermo Fisher Scientific, Waltham, MA). All constructs were verified by sequencing.

2.3 | Cell extracts and immunoblot analysis

Cell extracts were prepared and western blot was performed as described in Keenen et al. (2010). Antiserum to BRG1 was previously described (de La Serna et al., 2000). The BAF60A antibodies (cat#A301-594 for immunoprecipitations and cat#A301-595 for westerns) were purchased from Bethyl Laboratories (Montgomery, TX) and previously used in Wilson et al. (2014). For some experiments, a BAF60A antibody (cat#611728) that was previously used in Chen et al. (2012) was purchased from BD Biosciences (San Jose, CA). The BAF60B antibody (A301-596) was from Bethyl Laboratories. The BAF60C antibody (ab50556) was purchased from Abcam (Cambridge, MA) and previously described in Goljanek-Whysall et al. (2014). The MITF antibody (ab12039) was also from Abcam and previously described in Keenen et al. (2010). The Sox10 (sc17342), TYRP1 (cat#sc10443), and TYR (sc7833) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The Tubulin (2148) antibody was from Cell Signaling Technology (Boston, MA) and the FLAG antibody (M2) was from Sigma (St. Louis, MO).

2.4 | Immunoprecipitations

Melb-a cells were differentiated for 48 hr then lysed and immunoprecipitated with antibodies to BAF60A, BAF60B, and BAF60C. HEK 293T cells were transfected with pCMV or cotransfected with pCMV -MITF-M constructs and 3X-FLAG-CMV-BAF60A, B, or C (Lores et al., 2010) using Lipofectamine (Thermo Fisher Scientific), as described in Saladi et al. (2013). Cells were lysed after 48 hr and immunoprecipitated with FLAG antibody as described (Keenen et al., 2010). Elution was conducted with 3X FLAG peptide (Sigma Aldrich, St. Louis, MO) as described in Fock et al. (2018).

For glutathion S-transferase (GST) pulldowns, BL21 cells (Thermo Fisher Scientific) were transformed with empty pGEX, pGEX-2tBAF60A, or pRSET-B-MITF. Bacterial cultures were induced with isopropyl beta-D-1-thiogalactopyranoside (IPTG) and harvested after 6 hr. pGEX and pGEX-2tBAF60A were lysed and immunoprecipitated with glutathione agarose (Sigma Aldrich). The washed beads were then incubated with extracts from pRSET-B-MITF-transformed cells and immunoprecipitated.

2.4.1 | RNA isolation and quantitative real-time PCR

Total RNA was isolated using Trizol (Invitrogen, Waltham, MA), and cDNA was prepared using the Quantitect Reverse Transcription kit (Qiagen, Valencia, CA). Quantitative PCR was performed as previously described (Keenen et al., 2010).

Primer sequences to mouse genes were the following: *Baf60a*: 5′-GGA AGC TGC GAA TTT TCA TT-3′and 5′-TTT GTC CAG TTC GAT CAC CA-3′, *Mitf*: 5′-CAG ACC CAC CTG GAA AAC C-3′ and 5′-ATG GTG AGC TCA GGA CTT GG-3′, *Tyr*: 5′-TTC AAA GGG GTG GAT GAC CG-3′ and 5′-GAC ACA TAG TAA TGC ATC C-3′, *Tyrp1*: 5′-GCC CCA ACT CTG TCT TTT CTC AAT-3′ and 5′-GAT CGG CGT TAT ACC TCC TTA GC-3′, *Dct*: 5′-GGA CCG GCC CCG ACT GTA ATC-3′ and 5′-GTA GGG CAA CGC AAA GGA CTC AT-3′, *Trpm1*: 5′-CCT ACG ACA CCA AGC CAG AT-3′, and 5′-GAC GAC ACC AGT GCT CAC AC-3′ Rpl7: 5′-GGA GGA AGC TCA TCT ATG AGA AGG-3′ and 5′-AAG ATC TGT GGA AGA GGA AGG AGC-3′. Primer sequences to human genes were the following: *BAF60A*: 5′-GTA TGG GCC AGA CAA CCA TG-3′ and 5′-ACG AGT CTG GGT ATG GAT GC-3′, *MITF*: 5′-CTC GAG CTC ATG GAC TTT CC-3′ and CCA GTT CCG AGG TTG TTG TT-3′, *TYR*: 5′-GGT GGG AAC AAG AAA TCC AG-3′ and 5′-TCC TCC AAT CGG CTA CAG AC-3′, *TYRP1*: 5′-TGG GAT CCA GAA GCA ACT TT-3′ and 5′-TGT GGT TCA GGA AGA CGT TG-3′, *DCT*: 5′-GGG TTT CTG CTC AGT TGC TT-3′ and GGA TGT AGG GAC CAC TCC AG-3′, *TRPM1*: TGC TCC ATC TCA TGG TGA AA-3′ and 5′-TCC CCT ACG TGG CTG ATA AC-3′, *RPL19*: 5′-AAA CAA GCG GAT TCT CAT GG-3′ and 5′-TTG GTC TCT TCC TCC TTG GAT-3′.

2.5 | Small interfering RNA knockdown

Smart pools of small interfering RNA (siRNA) targeting mouse and human BAF60A were obtained from Dharmacon (Waltham, MA). Additional siRNAs targeting BAF60A were obtained from Integrated DNA Technologies (Coralville, IA) Undifferentiated Melb-a cells were transfected in growth medium for 48 hr. The medium was then replaced, and the cells were cultured in differentiation medium for an additional 48 hr. 501Mel cells were cultured and transfected as previously described (Keenen et al., 2010).

2.6 | Formaldehyde-assisted isolation of regulatory elements

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was performed as described in Simon, Giresi, Davis, & Lieb (2012) with some modifications. Cells were cross linked in 1% formaldehyde for 6 min at room temperature and quenched with 125 mM glycine. Cells were then lysed and sonicated as described in Keenen et al. (2010). Sonicated chromatin was subjected to two rounds of phenol/chloroform extraction, back extracted once with TE and then once with chloroform. The aqueous phase was ethanol precipitated and digested with 0.2 mg/ml Proteinase K for 1 hr at 55°C. Cross-links were then reversed by heating overnight at 65°C. DNA was then purified by an additional phenol-chloroform extraction and ethanol precipitation. Control Inputs were 10% of each sample that was heated at 65°C overnight to reverse crosslinking before purification. The primers used were as follows: mouse *Tyr* proximal: 5′-AGT CAT GTG CTT TGC AGA AGA T-3′ and 5′-CAG CCA AGA ACA TTT TCT CCT T-3′, mouse *Tyrp1* proximal region: 5′-GCA AAA TCT CTT CAG

CGT CTC-3′ and 5′-AGC CAG ATT CCT CAC ACT GG-3′, mouse, *IgH* Enhancer: 5′-GCC GAT CAG AAC CAG AAC ACC-3′ and 5′-TGG TGG GGC TGG ACA GAG TGT TTC-3′.

2.7 | Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (Keenen et al., 2010) using the same antibodies as in western blotting. Primers were the same as used in FAIRE. The primer sequences for mouse genes were as follows: *Tyrp1* promoter (−162 to +65): (forward: 5′-GCAAAATCTCTTCAGCGTCTC-3′) and (reverse: 5′-AGCCAGATTC CTCACACTGG-3′), *Tyr* promoter (−254 to −56): (forward: 5′-AGTCA TGTGCTTTGCAGAAGAT−3′) and (reverse: 5′-CAGCCAAGAACATTT TCTCCTT-3′) and *Igh* enhancer (forward: 5′-GCCGATCAGAACCAG AACACC-3′) and (reverse: 5-TGGTGGGCTGGACAGAGTGTTTC-3′).

2.8 | Propidium iodide staining and fluorescence-activated cell sorting

Cells were fixed with 100% ethanol for 1 hr, stained with propidium iodide–RNAse solution for 30 min, and loaded on a FACS-Calibur (BD Biosciences) by the University of Toledo Flow Cytometry Core Facility. Data were analyzed using Cell Quest Pro (BD Biosciences).

2.9 | Melanin quantification

Melanin was quantified as previously described (Marathe et al., 2017). Cells were counted and then lysed in 0.1 M NaOH at 37°C with vortexing for 20 min. Melanin content was calculated based on the absorbance at 475 nm as compared with the standard curve obtained using synthetic melanin (Sigma) after normalizing cell numbers.

2.10 | Statistical analysis

Statistical significance was calculated by the Student's *t* test when comparing two sets of data and a one-way analysis of variance followed by Dunnette's multiple comparison tests for comparing more than two sets of data (Graphpad Prism, version 5.03).

3 | RESULTS

3.1 | BAF60 subunits are expressed in differentiating Melb-a cells and interact with MITF

Melb-a cells are derived from the epidermis of neonatal mice and can be cultured as unpigmented melanoblasts or they can be induced to differentiate into pigmented melanocytes (Sviderskaya,

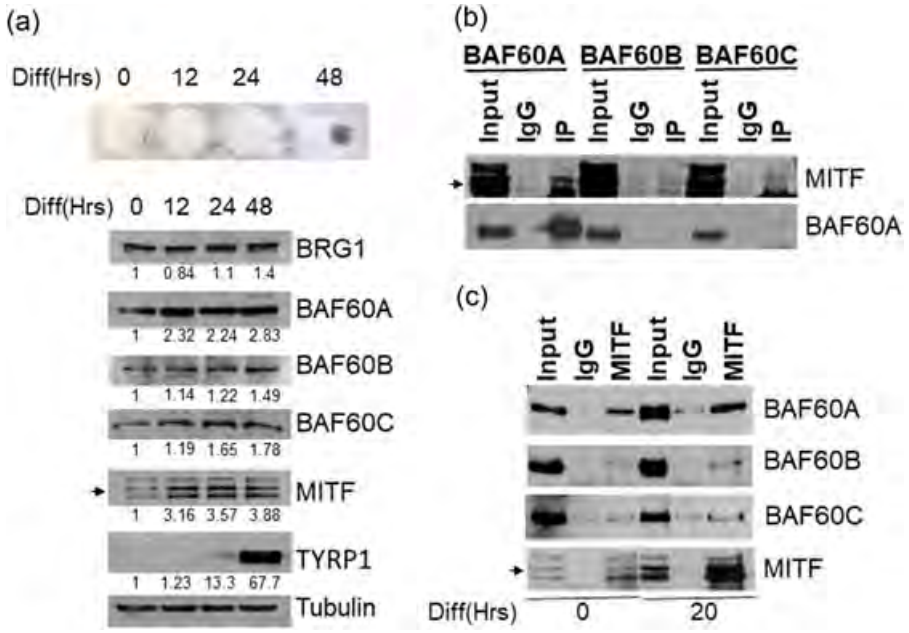

FIGURE 1 BAF60 subunit expression and interaction with MITF in Melb-a cells. (a) Top: Undifferentiated (0 hr) and differentiating Melb-a cells at 12, 24, and 48 hrs were pelleted and photographed. Bottom: Protein extracts were prepared from differentiating Melb-a cells at each time point and subjected to western blotting with the indicated antibodies. Tubulin was used as a loading control. Band intensity was determined by Image J. After subtracting background, band intensities were normalized to those of tubulin at each time point. (b) Melb-a cells that had been differentiated for 24 hr were immunoprecipitated with an irrelevant antibody (IgG) or with rabbit antibodies to BAF60A (A301-594), BAF60B (A301-596), or BAF60C (ab50566). Cell extract (Input) or the immunoprecipitated material was run on an SDS-polyacrylamide gel and blotted with mouse antibodies to MITF (ab12039) or BAF60A (611728). (c) Melb-a cells that had been differentiated for 24 hr were immunoprecipitated with an irrelevant antibody (IgG) or with an antibody to MITF. Cell extract (Input) or the immunoprecipitated material was run on an SDS-polyacrylamide gel and blotted with antibodies to BAF60A, BAF60B, or BAF60C or MITF (ab12029). The arrows indicate the unphosphorylated and phosphorylated bands corresponding to MITF-M. The figures are representative of three or more experiments. IgG: immunoglobulin G; MITF: microphthalmia-associated transcription factor; SDS: sodium dodecyl sulfate

Hill, Balachandar, Barsh, & Bennett, 2001; Sviderskaya, Wakeling, & Bennett, 1995). To begin to evaluate the function of the three BAF60 isoforms in melanocyte differentiation, we assessed expression of BAF60A, BAF60B, and BAF60C in differentiating Melb-a melanoblasts. As previously reported (Marathe et al., 2017), we found that pigmentation became visible after approximately 48 hr of differentiation (Figure 1a, top). BRG1 levels were fairly constant during differentiation, whereas MITF was expressed at a low level before differentiation and increased 12 hr after differentiation. A melanogenic marker, TYRP1, became detectable after 24 hr and increased substantially by 48 hr, coordinately with the change in visible pigmentation (Figure 1a, bottom). We found that all three BAF60 subunits were expressed in both undifferentiated and differentiated Melb-a cells. BAF60A expression increased modestly 12 hr after differentiation, whereas BAF60B and BAF60C levels changed less than twofold through the time period examined.

We performed co-immunoprecipitation experiments with antibodies to the BAF60 subunits to determine if any of them interact with MITF. We detected a strong interaction between MITF and BAF60A and a somewhat weaker interaction between MITF and BAF60C. The interaction between MITF and BAF60B was barely above the IgG control (Figure 1b). In reciprocal experiments, MITF was used to immunoprecipitate the BAF60 subunits in undifferentiated and differentiating Melb-a cells. These experiments confirm strong interactions between MITF and BAF60A in both undifferentiated and differentiating Melb-a cells (Figure 1c). Considerably weaker interactions were detected between MITF and BAF60B and BAF60C.

3.2 | MITF interacts with BAF60A through its bHLH domain

To further characterize the physical interactions between the BAF60 isoforms and MITF, HEK 293T cells were cotransfected with expression vectors for MITF and 3X-FLAG-tagged BAF60A, B, or C. Immunoprecipitations were performed with an antibody directed against the FLAG epitope of BAF60 proteins. As shown in Figure 2a, BRG1, which forms a central ATPase of the SWI/SNF complex was

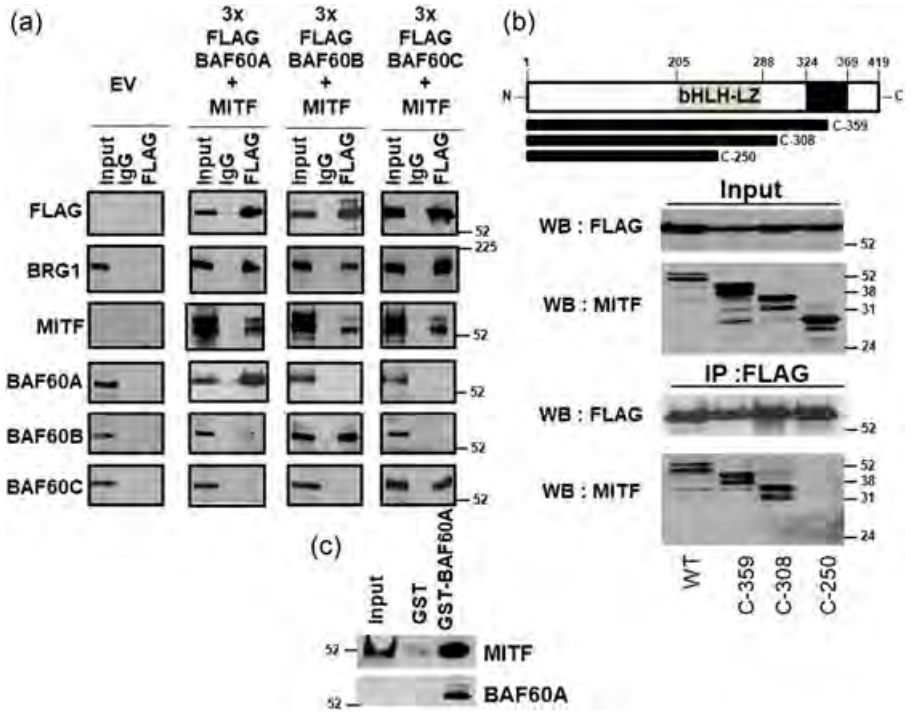

FIGURE 2 Characterization of MITF interactions with BAF60 subunits. (a) HEK 293T cells were transfected with an empty vector or a vector expressing 3X-FLAG-tagged *BAF60A*, *BAF60B*, or *BAF60C*. Cells were harvested after 48 hr. Whole cell extract was used for immunoprecipitation with IgG as a control or with FLAG antibody tagged Sigma M2 beads. The immunoprecipitated material was run on an SDS-polyacrylamide gel and immunoblotted for FLAG, BRG1, MITF, BAF60A (A301-595), BAF60B (A301-596, BAF60C (ab50566)). (b) 293T cells were cotransfected with 3X-FLAG *BAF60A* and *Mitf* deletion constructs. Immunoprecipitations were performed with an antibody to FLAG and run on an SDS-polyacrylamide gel. Western blotting was performed with FLAG and MITF antibodies. Top: Schematic showing C-terminal deletion constructs relative to the basic helix-loop-helix leucine zipper (bHLH-LZ) and the C-terminal transactivation domain from 324 to 369 shown in black. Middle: FLAG and MITF expression in cell extract (inputs). Bottom: FLAG and MITF expression detected from FLAG immunoprecipitations. (c) BL21 bacterial cells were transformed with either an MITF plasmid or a plasmid expressing GST or GST-BAF60A, cultured, and induced with IPTG for 4 hr. GST or GST-BAF60A was immunoprecipitated with GST-agarose. The beads were then incubated with the extract from MITF-transformed cells, and pulldowns were performed. Inputs are from MITF-transformed cells. GST: glutathion S-transferase; IgG: immunoglobulin G; IPTG: isopropyl beta-D-1-thiogalactopyranoside; MITF: microphthalmia-associated transcription factor; SDS: sodium dodecyl sulfate [Color figure can be viewed at wileyonlinelibrary.com]

co-immunoprecipitaed with all three BAF60 proteins, indicating that they each form a SWI/SNF complex containing BRG1. MITF also co-immunoprecipitated with all three BAF60 proteins. Thus, although MITF preferentially interacts with BAF60A in Melb-a cells (Figure 1b,c), MITF also strongly interacts with the other BAF60 subunits in 293T cells.

We proceeded to define the region of MITF that mediates the interaction with BAF60A by generating MITF C-terminal deletion constructs (Figure 2b). HEK 293T cells were cotransfected with wild type or MITF deletion constructs and 3X-FLAG-tagged BAF60A. C-terminal deletion constructs which had a portion of the C-terminal transactivation domain of MITF (Takeda et al., 2000) removed or those that completely lacked this domain retained the ability to interact with BAF60A. However, further deletion of the C terminus to encompass the bHLH region of MITF abolished the interaction with BAF60A, indicating its requirement (Figure 2b). We performed GST pulldowns from proteins that were purified from bacterial cells and found that the two proteins interact, even in the absence of other SWI/SNF components and SOX10, a neural crest transcription factor that was previously demonstrated to interact with BAF60A (Marathe et al., 2017; Weider et al., 2012; Figure 2c). Thus, we identify the BAF60A subunit of the SWI/SNF complex as an interacting partner of MITF.

3.3 | Depletion of BAF60A in Melb-a melanoblasts abrogates melanin synthesis and modestly affects cell cycle progression

Melb-a cells synthesize melanin and become pigmented when induced to differentiate (Sviderskaya et al., 2001). MITF plays a crucial role in regulating genes involved in melanin synthesis. Since BAF60A interacts with MITF, we investigated whether it is required for MITF-regulated processes. Transfection of Melb-a cells with siRNAs targeting BAF60A-depleted BAF60A but did not decrease expression of BAF60B or BAF60C, BRG1, or SOX10 (Figure 3a). However, BAF60A depletion resulted in decreased levels of MITF, and two enzymes involved in melanin synthesis, *Tyr* and *Tyrp1*. The expression of the genes encoding these enzymes is regulated by

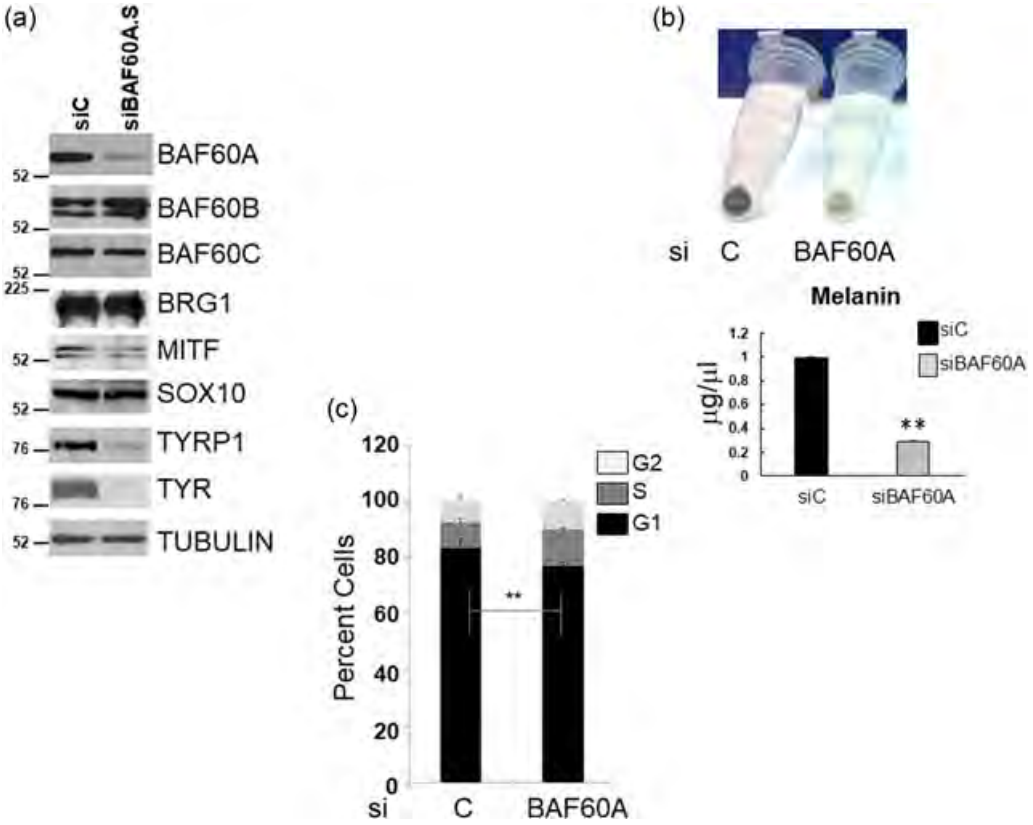

FIGURE 3 Effects of BAF60A depletion on melanoblast differentiation and cell cycle profile. (a) Melb-a cells were transfected with control siRNA or a pool of four siRNA sequences targeting BAF60A. After 72 hr, growth media was replaced with differentiation media. Protein extracts were prepared from cells that were differentiated for 48 hr and run on an SDS-polyacrylamide gel. Western blotting was performed with the indicated antibodies. Tubulin is shown as a loading control. The figure is representative of three or more experiments. (b) Top: A representative picture of cell pellets from differentiated Melb-a cultures that had been transfected control siRNA or a pool of four siRNA sequences targeting BAF60A. Bottom: Control and BAF60A-depleted Melb-a cells were subjected to the melanin assay. The data are from two experiments performed in triplicate. Statistical significance was calculated by Student's *t* test (**$p < 0.01$). (c) Control and BAF60A-depleted Melb-a cells were obtained as in (a) then stained with propidium iodide and subjected to flow cytometry. SDS: sodium dodecyl sulfate; siRNA: small interfering RNA

MITF and is an indicator of melanocyte differentiation. Consistent with decreased TYR and TYRP1 levels, depletion of BAF60A resulted in cells that were visibly lighter (Figure 3b, top) and that synthesized less melanin (Figure 3b, bottom).

Cellular differentiation is characterized by decreased proliferation and arrest in the G1 phase of the cell cycle, which in melanocytes is promoted by MITF (Carreira et al., 2005). We found that depletion of BAF60A resulted in a modest but statistically significant increase in the percent of cells in the S phase and a decrease in the percent of cells in the G1 phase of the cell cycle (Figure 3c). This modest change in the cell cycle profile may contribute to the decrease in melanocyte differentiation or may be a result of the decrease in differentiation that we observed upon depletion of BAF60A.

3.4 | Depletion of BAF60A abrogates expression of MITF and MITF target genes involved in melanin synthesis

We assessed the effects of the BAF60A knockdown on the expression of Mitf and several Mitf target genes at the messenger RNA level by transfecting Melb-a cells with control siRNA, a smart pool of siRNA that targets BAF60A (Figure 4a, left) or two additional siRNAs that target BAF60A (Figure 4a, right). Knockdown of BAF60A significantly abrogated the expression of Mitf, *Tyr*, and *Tyrp1* and two other *MITF* target genes, *Dct* and *Trpm1*, in mouse Melb-a cells (Figure 4b). A previous report found that BAF60A forms a complex with MITF in 501Mel melanoma cells (Laurette et al., 2015). However, a functional requirement for BAF60A in the regulation of MITF target genes was not established in 501Mel cells. We found that depletion of BAF60A in 501Mel cells (Figure 4c) also abrogates *MITF* and MITF target gene expression (Figure 4d). Therefore, BAF60A is required for expression of melanocyte-specific genes in differentiating mouse melanoblasts and in human melanoma cells.

3.5 | MITF is required for BAF60A recruitment to the Tyr and Tyrp1 promoters during melanocyte differentiation

We performed ChIP in undifferentiated and differentiated Melb-a cells to determine whether BAF60A is recruited to promoters regulated by MITF during melanocyte differentiation. We found that although initially low on the *Tyrp1* and *Tyr* promoters in undifferentiated cells, BAF60A occupancy increases upon differentiation (Figure 5a). To determine if BAF60A recruitment is dependent on MITF, we depleted MITF using siRNA (Figure 5b). ChIP analysis indicated that depletion of MITF significantly reduces BAF60A enrichment on the *Tyrp1* and *Tyr* promoters

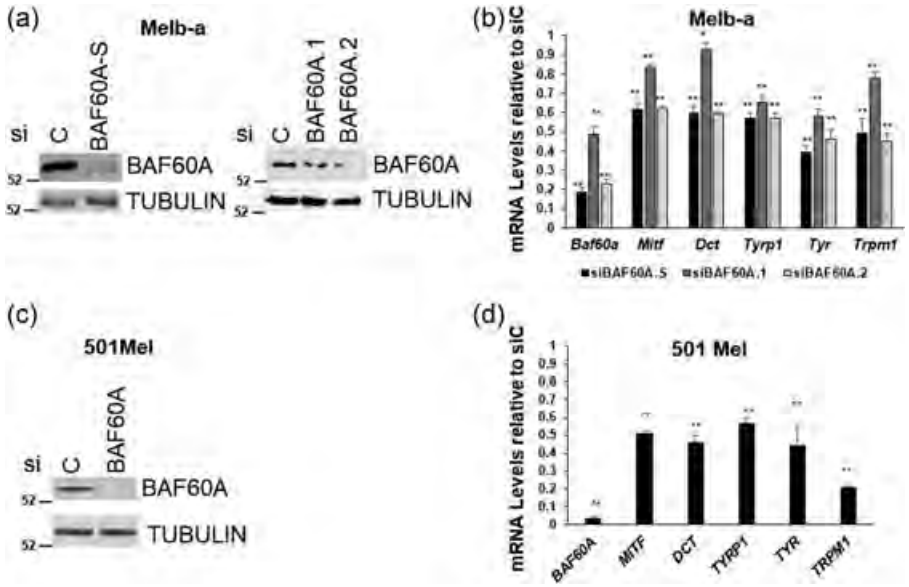

FIGURE 4 Effects of BAF60A depletion on MITF and MITF target gene expression in Melb-a cells and 501Mel melanoma cells (a) Western blots of Melb-a cells that were transfected with control siRNA, a pool of four siRNA sequences (left) or two different siRNA sequences targeting BAF60A (right) as in Figure 3a. (b) RNA was isolated from cells that were transfected with control siRNA or siRNAs targeting BAF60A as in (a), reverse transcribed, and subjected to quantitative PCR with primers to the indicated genes. Relative mRNA levels were calculated by using RPL7 as a control and normalizing to siC values. The data are from two or more experiments performed in triplicate. Statistical significance was calculated by Student's *t* test (*$p < 0.05$, **$p < 0.01$). (c) 501Mel melanoma cells were transfected with control siRNA (siC) or a pool of four siRNA sequences targeting BAF60A. Protein extracts were prepared from 501Mel cells harvested 72 hr after transfection and subjected to western blotting to detect BAF60A depletion. (d) RNA was isolated from 501Mel cells, reverse transcribed and subjected to qRT-PCR as in (c) except that values for each gene were normalized to RPL19. The data are from two experiments performed in triplicate. Statistical significance was calculated by Student's *t* test (**$p < 0.01$). MITF: microphthalmia-associated transcription factor; mRNA: messenger RNA; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; siRNA: small interfering RNA

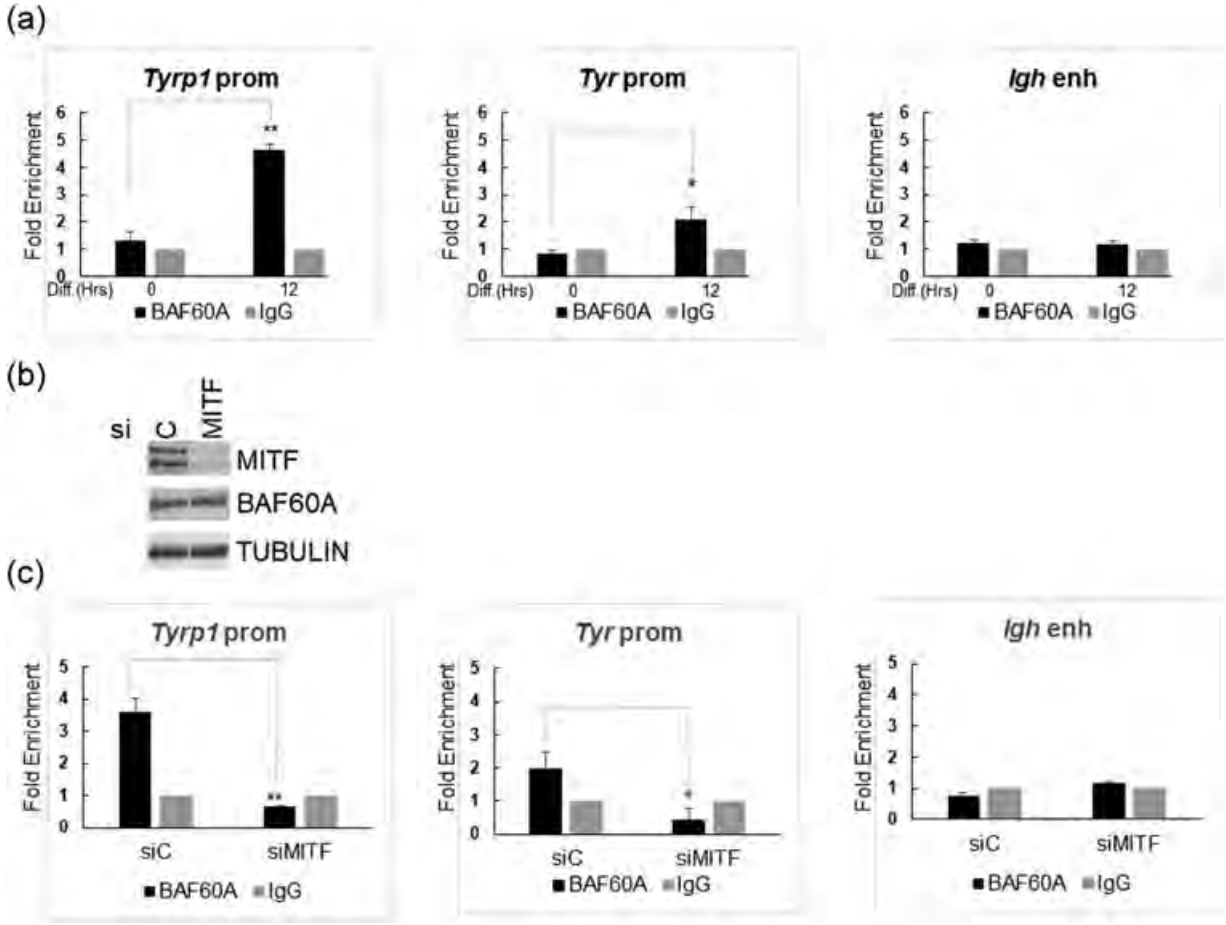

FIGURE 5 BAF60A recruitment to MITF target gene promoters. (a) Undifferentiated Melb-a cells or cells that were differentiated for 12 hr were harvested for the ChIP assay. The ChIP assay was performed using an antibody to BAF60A or as a control, IgG. BAF60A enrichment on the *Tyrp1* and *Tyr* promoters was normalized to the enrichment of control IgG. Levels of BAF60A enrichment relative to control IgG are shown on the *Tyrp1* and *Tyr* promoters, and as a control the *IgH* enhancer region. (b) Melb-a cells were transfected in growth medium with control siRNA or siRNA targeting MITF for 72 hr. Growth medium was replaced with differentiation medium, and the cells were cultured for an additional 12 hr. Protein extracts were prepared, run on an SDS-polyacrylamide gel and immunoblotted with the indicated antibodies. Tubulin is a loading control. (c) Cells were harvested for the ChIP assay, and enrichment of BAF60A was quantified as in (a). ChIP data are the averages of three or more experiments. Statistical significance was calculated by Student's *t* test (**$p < 0.01$). ChIP: chromatin immunoprecipitation; IgG: immunoglobulin G; MITF: microphthalmia-associated transcription factor; SDS: sodium dodecyl sulfate; siRNA: small interfering RNA

(Figure 5c). Thus, MITF is required for BAF60A recruitment to the promoters of melanocyte-specific genes during melanocyte differentiation.

3.6 | BAF60A is required for BRG1 recruitment to the Tyr and Tyrp1 promoters

To investigate the requirement for BAF60A in the transcriptional regulation of MITF target promoters, we performed ChIP to determine if BAF60A depletion compromises MITF and BRG1 binding. We found that BAF60A depletion had a small effect on MITF binding to the *Tyrp1* and *Tyr* promoters that was statistically significant only on the *Tyr* promoter (Figure 6a). In contrast, BAF60A depletion significantly abrogated BRG1 recruitment to the *Tyrp1* and *Tyr* promoters (Figure 6b). Thus, BRG1 recruitment is dependent on BAF60A.

3.7 | BAF60A is required for chromatin remodeling on the Tyr and Tyrp1 promoters

We previously found that chromatin accessibility at the *Tyrp1* and *Tyr* promoters increases in an MITF- and BRG1-dependent manner as Melb-a cells are differentiated (Marathe et al., 2017). To determine if BAF60A is also required for chromatin remodeling, we performed FAIRE with control and BAF60A-depleted cells. Consistent with a requirement for BAF60A in the recruitment of the BRG1 catalytic subunit of the SWI/SNF complex, we found that depletion of BAF60A significantly reduced chromatin accessibility at the *Tyrp1* and *Tyr* promoters (Figure 7).

4 | DISCUSSION

The SWI/SNF complex has previously been implicated in melanocyte development and melanoma proliferation. Several studies show that

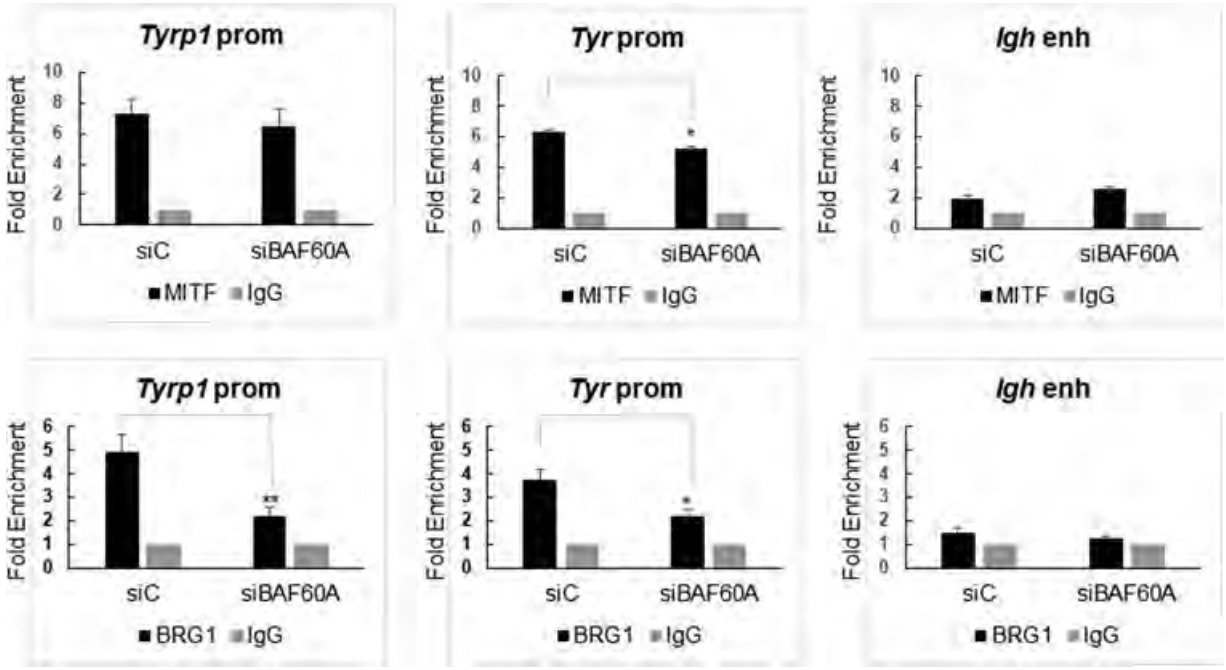

FIGURE 6 Effects of BAF60A depletion on MITF and BRG1 recruitment to MITF target gene promoters. (a) Melb-a cells were transfected in growth medium with control siRNA or a pool of siRNAs targeting BAF60A for 72 hr. Growth medium was replaced with differentiation medium, and the cells were cultured for an additional 12 hr. Cells were harvested for the ChIP assay and enrichment of MITF was quantified on the *Tyrp1* and *Tyrp1* promoters and as a control the *IgH* enhancer as in Figure 5a. (b) ChIPs were performed on Melb-a cells as in (a) except that the antibody was to BRG1. ChIP data are the averages of three or more experiments. Statistical significance was calculated by Student's *t* test (**$p < 0.01$). MITF: microphthalmia-associated transcription factor; siRNA: small interfering RNA

the catalytic subunit of the complex, BRG1, is essential for both processes (Keenen et al., 2010; Laurette et al., 2015; Marathe et al., 2017; Vachtenheim, Ondrusova, & Borovansky, 2010). BRG1 is recruited to the regulatory regions of melanocyte-specific genes by the master regulator of melanocyte development, MITF, and coactivates target genes by remodeling chromatin structure. Although previous studies have co-immunoprecipitated BAFs with MITF, there have not yet been any studies to investigate the functional role of any of these associated factors in melanocytic cells. Especially lacking is the identification of subunits that mediate direct interactions between MITF and the SWI/SNF complex and function to promote melanocyte differentiation.

The three BAF60 isoforms are mutually exclusive components of SWI/SNF complexes that mediate direct interactions with master regulators of differentiation in a lineage-specific manner (Puri & Mercola, 2012). In this study, we found that the BAF60A, BAF60B, and BAF60C are all expressed in melanocytes and physically interact with MITF. We pursued studies to further characterize the

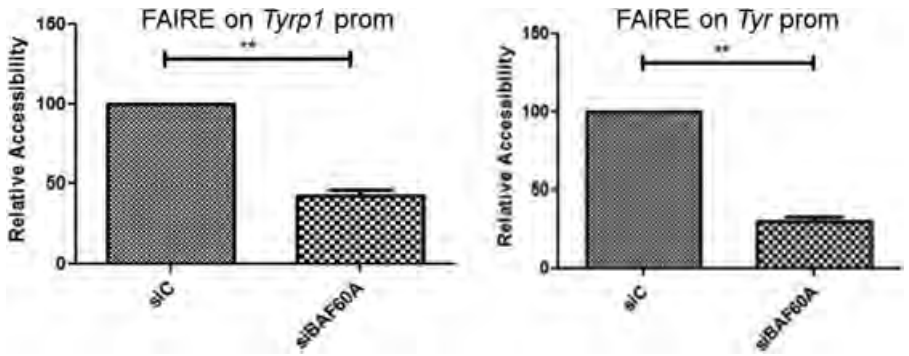

FIGURE 7 Effects of BAF60A depletion on chromatin accessibility at MITF target promoters. Melb-a cells were transfected in growth medium with control siRNA or a pool of siRNAs targeting BAF60A for 72 hr. Growth medium was replaced with differentiation medium, and the cells were cultured for an additional 12 hr. Cells were harvested for the FAIRE assay. Chromatin accessibility at the *Tyr* (left) and *Tyrp1* (right) promoters is shown relative to a control *IgH* enhancer region. FAIRE data are the averages of three or more experiments. Statistical significance was calculated by Student's *t* test (**$p < 0.01$). FAIRE: formaldehyde-assisted isolation of regulatory elements; MITF: microphthalmia-associated transcription factor; siRNA: small interfering RNA

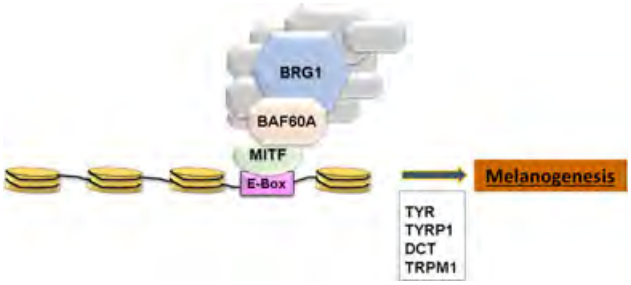

FIGURE 8 BAF60A mediates interactions between MITF and BRG1-containing SWI/SNF complexes. Schematic model depicting BAF60A as an SWI/SNF subunit that mediates interactions between MITF and BRG1-containing SWI/SNF complexes. BAF60A was found to be required for recruitment of BRG1 to promoters of MITF target genes, chromatin remodeling, gene expression, and melanin synthesis. MITF: microphthalmia-associated transcription factor

interaction between MITF and BAF60A because we found that the physical interaction between MITF and BAF60A was stronger than between MITF and the other two BAF60s in melanoblasts.

The interaction between MITF and BAF60A required the helix-loop-helix domain of MITF and occurred between proteins purified from bacterial cells, suggesting that BAF60A mediates direct interactions with MITF. To our knowledge, this is the first report that BAF60A can interact with other helix-loop-helix transcription factors. Like MITF, MYOD, the master regulator of muscle differentiation, also has a helix-loop-helix domain but BAF60A is not highly expressed in muscle (Forcales et al., 2012). Previous studies in muscle indicate that both BAF60B and C interact with MYOD but that BAF60C is the critical subunit required for the expression of a majority of MYOD target genes. Indeed, over-expression of BAF60A and to a lesser extent, BAF60B, in muscle progenitors impedes muscle differentiation by effectively diminishing BAF60C incorporation into the SWI/SNF complex (Saccone et al., 2014). Interestingly, BAF60C also promotes hepatic cell differentiation, whereas BAF60B plays a negative role by blocking lineage conversion (Ji et al., 2017). In contrast, BAF60A promotes endoderm differentiation and restricts embryonic cell pluripotency (Alajem et al., 2015). BAF60A is the only BAF60 subunit to interact with SOX10 in Schwann cells (Weider et al., 2012). Thus, although lineages often express two or more of the BAF60 subunits, one subunit seems to play a predominant role in the differentiation of a particular cell type. We found that BAF60A is required to promote melanocyte differentiation and melanin synthesis.

Our studies indicate that BAF60A promotes interactions between MITF and the SWI/SNF complex because depletion of BAF60A compromised the recruitment of BRG1 to the promoters of MITF-regulated pigmentation genes and decreased chromatin accessibility on those promoters (Figure 8). We also detected a small decrease in MITF occupancy on the TYR and TYRP1 promoters. This may reflect the decrease in MITF levels when BAF60A is depleted or it may suggest that BAF60A facilitates MITF binding. A two-step model whereby SWI/SNF components facilitate MYOD binding through interactions with a pioneer transcription factor was demonstrated to occur during muscle differentiation (de la Serna et al., 2005; Forcales et al., 2012) and could potentially explain how MITF initially gains access to its binding sites in repressive chromatin structure during melanocyte differentiation. However, since the decrease in MITF binding is small, a different SWI/SNF subunit or even a different chromatin remodeling enzyme may fulfill this role in melanocytes.

In conclusion, our studies indicate that BAF60A plays a central role in melanocyte differentiation by interacting with MITF to promote expression of pigmentation genes. Although BAF60B and BAF60C also interact with MITF when overexpressed in 293T cells, the interactions between MITF and BAF60A were markedly stronger than the interactions between MITF and BAF60B or BAF60C in Melb-a cells. Furthermore, knockdown of BAF60A had noticeable effects on pigmentation and on melanocyte-specific gene expression. Thus, our data suggest that BAF60A is the critical BAF60 subunit for melanocyte lineage specification. However, although BAF60C interacted weakly with MITF in Melb-a cells, Melba-cells transfected with siRNA targeting BAF60C were visibly lighter than controls, suggesting that BAF60C can also contribute to the differentiation process (Supporting Information Figure 1). Since MITF is not the only transcription factor (Seberg, Van Otterloo, & Cornell, 2017) to contribute to melanocyte development, we do not rule out a role for BAF60C in the regulation of pigmentation genes through interactions with other transcription factors involved in melanocyte specification. Furthermore, since all three BAFs strongly interact with MITF when overexpressed in 293T cells, the role of each BAF may be different in other contexts, such as melanoma. Indeed, an unbiased screen identified BAF60B as an MITF interacting protein in melanoma cells (Laurette et al., 2015).

In addition to pigmentation genes, MITF regulates genes involved in the cell cycle, survival, the DNA damage response, and energy metabolism (Cheli, Ohanna, Ballotti, & Bertolotto, 2010; Giuliano et al., 2010; Slade & Pulinilkunnil, 2017). These MITF functions are especially important in melanoma where MITF is considered a lineage-specific oncogene, having what often appears to be paradoxical roles at different stages of the oncogenic process. It is not well understood how MITF appropriately regulates such a diverse set of genes in response to extracellular signals. Dynamic interactions with diverse SWI/SNF subcomplexes, generated by combinatorial assembly of alternative subunits such as the BAF60 isoforms, may help MITF to regulate discrete programs of gene expression by forming a "code" that impinges upon the function of other transcriptional regulators.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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