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entitled

Bromodomain Containing Proteins in Melanocyte Differentiation and Melanoma

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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An Abstract of
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Bromodomains belong to a family of evolutionarily conserved protein modules originally found in proteins associated with chromatin and in nearly all nuclear histone acetyltransferases. Bromodomains modulate enzyme activities, protein assembly, and protein-protein interactions via lysine acetylation with broad implications in a wide variety of cellular events, such as transcriptional activation and chromatin remodeling. They are the sole protein domains which function as acetyl-lysine binding residues and are epigenetic readers which bind to the acetylated residues regulating chromatin structure and gene expression. We assessed two groups of bromodomain containing proteins with their relevance to melanocyte differentiation and melanoma.

In the first study, the function of bromodomain (BRD) and extra-C terminal domain (BET) protein family in modulating pigmentation and melanocyte differentiation was deduced. BET protein family members (BRD2, BRD3, BRD4, and BRDT) are epigenome readers that bind to acetyl-lysine residues on the tails of histones H3 and H4, and exert key roles at the interface between chromatin remodeling and transcriptional
regulation. BRD4 is a BET protein which acts as a transcriptional coactivator and was initially known as mitotic chromosome associated protein (MCAP). BRD4 associates with acetylated chromatin throughout the cell cycle and regulates transcription at targeted loci. It is implicated in cellular growth control, cell cycle progression and cancer development. However, the role of BRD4 in melanocyte differentiation has not been investigated.

In this study, we utilized an in vitro murine model of immortalized murine melanoblasts (melb-a cells) which can be differentiated by treatment with alpha-MSH. Alpha-MSH upon binding to melanocortin 1 receptor activates the microphthalmia-associated transcription factor (MITF) which is the master regulator of melanocyte differentiation and melanoma oncogene. MITF in turn activates genes required for melanin production, proliferation, and survival. JQ1 is a cell permeable small molecule inhibitor of BET proteins which binds to BRD4 with the highest affinity and specificity, inhibiting its binding to the acetylated lysine residues. JQ1 has an excellent shape complementarity with the acetyl-lysine binding cavity of bromodomains of BET family. Treating melb-a cells with JQ1 compromised MITF target gene expression, melanin synthesis, and reduced proliferation of melb-a cells as well as neonatal human epidermal melanocytes (NHEM) and melanoma cells. Furthermore, we determined that BRD4 occupied the promoters of MITF target genes that regulate pigmentation. Treatment with JQ1 inhibited the binding of both BRD4 and MITF to these promoters. Moreover, treating the melb-a cells as well as NHEMs disrupts histone H3 lysine 4 trimethylation (H3K4me3) which is an active mark of transcription. The effects on gene expression were recapitulated by siRNA mediated silencing of BRD4. We showed a physical
interaction between the two proteins. Thus, we hypothesize that BRD4 plays a crucial role in melanocyte differentiation by interacting with MITF and can be a potential novel target for treatment of hyperpigmentation disorders and in melanoma.

In the second project, the role of bromodomain containing proteins of an ATP-dependent chromatin remodeling complex SWI/SNF were assessed. SWI/SNF complexes can be categorized as BAF (BRG1/BRM-associated factor) complex bearing either one of ARID1A/ARID1B or PBAF (Polybromo-associated BAF) complex harboring ARID2, BAF180 and BRD7 subunits based on purified complexes from mammalian cells. In particular, we investigated the role of PBAF SWI/SNF complex in the context of melanoma tumorigenicity.

Ultraviolet (UV)-radiation causes DNA damage and is implicated in the etiology of cutaneous melanoma, an aggressive malignancy that is notoriously chemoresistant. SWI/SNF chromatin remodeling enzymes have critical functions in the regulation of gene expression. Importantly, PBAF specific SWI/SNF subunits are disrupted in a number of human cancers including melanoma. We were interested in assessing a potential tumor suppressive role of PBAF complexes in the context of melanocytes and melanoma cells. We hypothesized that the PBAF specific SWI/SNF complex mediates tumor suppression by activating p53 target genes in response to UV-radiation. ARID2 has been identified as a gene that is frequently mutated in melanoma. We surveyed a panel of melanoma cell lines and found that a subset of melanoma cells is deficient in the expression of the other PBAF specific subunits, BAF180 and BRD7. Our data indicate that BRG1, BAF180, BRD7, and ARID2 promote expression of multiple p53 target genes as well as cell cycle arrest in UV-irradiated melanocytes and melanoma cells. BRG1, the central ATPase in
PBAF SWI/SNF complex promoted cell cycle arrest in melanoma cells. Thus, our work suggests that a specific configuration of the SWI/SNF complex may have a tumor suppressive role in melanocytes and that aberrant expression or mutations of SWI/SNF components may contribute to melanoma tumorigenicity.
I would like to dedicate my dissertation research to my parents Mr. Rakeshkumar Trivedi (Bapu) and Mrs. Hemangini Trivedi (Ma) for their unconditional love and blessings they have showered on me. I am where I am today in life is because of their upbringing and their sacrifices. I am indebted to them for believing in me and supporting my endeavors of pursuing doctoral research in the United States of America. I would also like to take this opportunity to thank my late uncle Mr. Bhavnesh Jani (Mama) and my cousin Mr. Vishrut Trivedi (Mota Bhai) for always being a source of inspiration. One of the reasons I pursued PhD is because they wanted to call me a Doctor.
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List of Abbreviations

Alpha-MSH........................................Alpha- melanocortin stimulating hormone
ARID..........................................................AT-rich interactive domain

BAF...........................................................BRG1/BRM associated factors
BET..........................................................Bromodomain and extra terminal domain
BRG1..........................................................Brahma related gene 1
BRM............................................................Brahma

CHD..........................................................Chromodomain
CREB/CBP/CREBBP..................................Cyclic-AMP response element-binding protein
CTM..........................................................C-terminal motif

DCT...........................................................Dopachrome tautomerase
HAT..........................................................Histone acetyltransferase
HDAC........................................................Histone deacetylase
HMT..........................................................Histone methyltransferase

MCAP..........................................................Mitotic chromosome-associated protein
MC1R........................................................Melanocortin -1 receptor
MITF........................................................Microphthalmia-associated transcription factor

PBAF........................................................Polybromo-associated BAF
P-TEFb......................................................Positive transcription elongation factor

SWI/SNF.....................................................Mating type switching/sucrose non fermenting
TRP1..........................................................Tyrosinase related protein 1
TYR............................................................Tyrosinase

UVR............................................................Ultra violet radiation
List of Symbols

\( \alpha \)- Alpha
\( \beta \)- Beta
\( \kappa \)- Kappa
\( \varepsilon \)- Epsilon
\( \gamma \)- Gamma
Chapter 1

Introduction and Literature Review

1.1 Melanocytes: Proliferation and Differentiation

Melanocytes are neural-crest derived cells located in the basal layer of epidermis that produce the pigment melanin [1, 2]. Undifferentiated precursors of melanocytes are called melanoblasts, which are derived from neural crest cells in embryonic skin [2, 3]. Melanoblasts are multipotent and are believed to be differentiated into melanocytes only when it exhibits pigmentation and shows structural characteristics such as dendrite formation and small granular structures known as melanosomes which contain melanin [4, 5]. Melanin is responsible for providing photo-protection and thermoregulation by absorbing UV radiation and transforming it to harmless heat [4]. Melanocytes produce two types of melanin: brownish-black eumelanin and reddish-yellow pheomelanin. Eumelanin-containing melanosomes (eumelanosomes) are elliptical, with longitudinal pigment deposits in intraluminal fibrils whereas pheomelanin-containing melanosomes (pheomelanosomes) are spherical, with granular pigment deposits in multivesicular bodies. Thus, differences in melanosome morphology depict the differences in the type of melanin synthesized [2]. Once melanin is produced, it is stored in the melanosomes, which are transferred from melanocytes to keratinocytes, thus giving skin its
characteristic color. Melanoblast/melanocyte proliferation and differentiation are regulated by tissue microenvironment, especially by keratinocytes, which synthesize endothelins, steel factor, hepatocyte growth factor, leukemia inhibitory factor and granulocyte-macrophage colony-stimulating factor. Alpha-melanocyte stimulating hormone also stimulates melanocyte differentiation [2]. Three major enzymes regulate the melanin synthesis in melanocytes. The melanocyte-specific enzyme, tyrosinase (TYR), is required for the rate-limiting step of melanin synthesis while two other members of the tyrosinase family, tyrosinase related protein 1 (TRP1) and dopachrome tautomerase (DCT), regulate the type of melanin synthesized [6, 7]. The expression of these enzymes is highly specific to melanocytes and hence their expression is often utilized as indicators of melanocyte differentiation. Thus, melanocyte proliferation and differentiation are not only regulated by genes encoding typical growth factors and their receptors but also by genes classically known for their role in pigment formation. Disruption of normal melanocyte function can lead to skin cancer and other congenital pigmentary disorders such as Waardenburg syndrome and Tietz syndrome that affect the level of pigmentation [8-10]. Thus, understanding the mechanisms which regulate melonocyte differentiation and pigmentation could yield interesting cues regulating these processes and intervention of such players could be useful for treating pigment-related disorders.

**Microphthalmia-associated transcription factor**

The microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper transcription factor that binds to DNA recognition sequences called E-box elements in the promoter region of the target genes with the consensus sequence
CA[T/C]GTG. Related family members of this group includes transcription factor E3 (TFE3), transcription factor EB (TFEB), and transcription factor EC (TFEC) [11-13].

MITF plays a crucial role in the development, differentiation, function and survival of melanocytes and hence, is a master regulator of melanocytes [14-16]. MITF binds to the regulatory regions of many genes which regulate pigmentation, as well as genes important for melanocyte survival, and activates their expression. In regards to activating pigmentation gene expression, our lab was the first to establish a novel requirement of SWI/SNF enzymes for MITF activity [17, 18]. It was reported that MITF physically interacts with BRG1, which is the core ATPase of the SWI/SNF complex and also with other subunits of the complex. Thus, pharmacological suppression of MITF expression can decrease pigmentation. Modulation of MITF expression or activity is therefore a useful strategy for treating hyperpigmentation disorders.

In addition to melanocytes, MITF is expressed in retinal pigment epithelium cells, as well as in certain non-pigmented cell lineages including osteoclast, mast cells [19], and cardiomyocytes. MITF is transcribed from several alternative promoters, giving rise to various splice isoforms. There are nine different isoforms of MITF known till date, which exhibit tissue-specific expression patterns. In melanocytes, the MITF-M isoform is selectively expressed [20].

An autosomal dominant condition known as Waardenburg syndrome type IIA occurs in humans expressing heterozygous mutation of MITF [21]. Such individuals exhibit a congenital white forelock and sensorineural deafness. In mice, complete lack of MITF results in characteristics such as white fur, deafness, and small eyes.
(microphthalmia) [22, 23]. Loss of pigmentation in such mice is due to the absence of melanocytes, and not the defect of melanin synthesis in viable melanocytes.

MITF has been shown to be a lineage-specific survival oncogene in melanoma, which is amplified in 5-20% of total human melanoma cases. In melanoma patients, MITF amplification was associated with a decreased five-year survival [24]. MITF has also been shown to play an important role in controlling the proliferative, invasive and metastatic properties of melanoma cells [24-26].

MITF is regulated both transcriptionally and post-translationally. At the transcriptional level, MITF-M is regulated by transcription factors which include MITF itself, paired box gene 3 (PAX3), cAMP-responsive element binding protein (CREB), sex-determining region Y-box 10 (SOX10), lymphoid enhancer-binding factor 1 (LEF1/TCF), one cut domain 2 (ONECUT-2), and the mitogen-activated protein kinase (MAPK) pathway [27].

At the post-translational level, MITF is known to be phosphorylated [28, 29], sumoylated [30, 31], and ubiquitinated [32]. Phosphorylation of MITF occurs mainly by MAPK, ribosomal S6 kinase (RSK), glycogen synthase kinase-3β (GSK3β) and p38. In melanocytes, c-kit activation causes MITF phosphorylation by ERK2 and RSK. Once phosphorylated, MITF induces recruitment of the transcriptional coactivator p300, and targets itself for ubiquitination and proteolysis. Protein inhibitor of activated STAT3 (PIAS3) mediates MITF sumoylation repressing its activity.
Figure 1-1 Regulators of MITF. Reprinted from Archives of Biochemistry and Biophysics, Vol 563: p. 28-34, Copyright (2014) with permission from Elsevier.

MITF is thought to play paradoxical roles in melanoma proliferation, survival, and invasion. Whereas lower levels of MITF correspond to increased motility and invasive capacity in melanoma, higher levels are thought to impart increased proliferative capacity [33]. High levels of POU3F2 (BRN2) transcription factor, a direct repressor of MITF transcription is observed in melanoma cells that express low levels of MITF [25, 34].

MITF targets a variety of genes with wide implications in biological processes. Potentially important functional transcriptional targets of MITF include regulators of cell cycle (CDK2, p21<sup>CIP1</sup>, p16<sup>INK4A</sup>), differentiation, survival and metastasis (BCL2, BCL2A1, c-Met), cAMP levels (PDE4D3), metabolism, miRNA processing (DICER), DNA repair, invasiveness, and pigmentation [27].
DIAPH1 is a gene that encodes the diaphanous-related formin Dia1, which is involved in actin polymerization and coordinating actin cytoskeleton. MITF has been shown to regulate the expression of DIAPH1 to control invasiveness and proliferation. Knock down of MITF leads to downregulation of Dia1, a cyclin-dependent kinase inhibitor 1B (p27Kip1)-dependent G1 arrest, reorganization of the actin cytoskeleton, and increased invasiveness. Proliferation occurs when moderate MITF levels lead to actin polymerization and suppressed p27Kip1 [26]. Senescence can be triggered in melanoma cells by prolonged MITF suppression [35].

MITF can interact with chromatin remodeling enzymes and thus promote changes in chromatin structure. “Epigenetic programming” through altered chromatin structure and histone modifications are believed to dictate how cells such as melanocytes specifically respond to certain environmental cues.
1.2 Chromatin Remodeling

Eukaryotic genomes are packed into condensed chromatin fibers to fit over a meter of DNA within a microscopic volume of the nucleus. Histones are the unique proteins which govern this compaction [36]. Pierre Chambon’s laboratory was the first to name the nucleosome [37, 38], which is the basic building block of chromatin. It consists of 147 base pairs of DNA wrapped in a left-handed superhelix 1.7 times around a core histone octamer (two copies each of histones H2A, H2B, H3, and H4). Histone H1 serves as a linker histone between the adjacent nucleosomes and directs the path of DNA that makes up the chromatin fiber. This compacted structure is rendered inherently repressive to a major of cellular processes that require access to the DNA sequence. Each core histone comprises of two functional domains: “Histone-fold domain” which suffices histone-histone and histone-DNA contacts within the nucleosome and NH2-terminal and COOH-terminal “tail” domain comprising of sites essential for posttranslational modifications such as acetylation, methylation, phosphorylation, and ubiquitination [36].

The higher order chromatin structure can be divided into transcriptionally “active” euchromatin or “inactive” or “repressive” heterochromatin [39]. Thus, the chromatin structure needs to be remodeled for active gene transcription to take effect. Chromatin remodeling is the regulated alteration of the chromatin structure which is accomplished by covalent modification of the histones or by the action of ATP-dependent remodeling complexes [40].
Chromatin Remodeling Enzymes

Chromatin-modifying complexes can be broadly classified into two major groups based on their modes of action as follows.

1) ATP-dependent complexes, which uses the energy from ATP hydrolysis to locally disrupt or alter the association of histones with DNA.

2) Histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes, which regulate the transcriptional activity of genes by determining the acetylation levels of the amino-terminal domains of nucleosomal histones associated with them.
Based on a unique subunit composition and the presence of a distinct ATPase, four different classes of ATP-dependent chromatin remodeling enzymes can be recognized namely SWI/SNF, ISWI, CHD, and Ino80 [41, 42].

**Figure 1-4 ATPases of SWI2/SNF2-related chromatin remodeling complexes.**
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**The ISWI Group**

These complexes contain the ISWI (Imitation Switch) protein as the ATPase subunit and were purified from *Drosophila* extracts using biochemical methods based on their ability to disrupt and/or generate regularly spaced nucleosomal arrays [43]. The ISWI-based complexes are much smaller and have fewer subunits as compared to the SWI/SNF complexes containing between two and six peptides [40]. The presence of two unique domains namely SANT and SLIDE is specific to this group of complexes. These domains constitute a nucleosomal recognition unit which allows it to bind to DNA and unmodified H4 tails. Acetylation of H4K16 has been shown to reduce the ISWI activity.
and is targeted away from transcriptionally active chromatin. ACF (ATP-utilizing chromatin assembly and remodeling factor), NURF (Nucleosome-remodeling factor), and CHRAC (Chromatin accessibility complex) are the most extensively studied members of this group. These complexes have been implicated in regulation of several functions such as heterochromatin formation, DNA replication, and transcriptional repression [43, 44].

The CHD Group

The Chromodomain-Helicase-DNA binding (CHD) family comprises of 9 members which have been further divided into 3 sub-families namely CHD1, Mi-2 and CHD7. This family of chromatin remodeling complexes possesses two chromo domains at the amino terminal. Apart from chromatin remodeling, this complexes also possess deacetylase activites presumably by means of HDAC subunits [43]. The CHD members can function as monomers or associate with other factors to form complexes, of which the best characterized is the Nucleosome remodeling and deacetylase (NURD) complex. It has been suggested that Mi-2 could be recruited to specific genes by repressors [45, 46] implicating this complex in transcriptional repression and as a regulator of genomic stability [41, 43].

The Ino80 Group

The ATPase domain of the Ino80 (Inositol requiring 80) family of chromatin-modifying complexes is referred to as the split ATPase domain due to a split in the middle due to the presence of a spacer region [47]. This complex exhibits DNA helicase activity through Pontin (Rvb1) and Reptin (Rvb2) and absence of the activity of either of them leads to abrogation of the activity of Ino80 complexes [43]. This complex also has an affinity for H2A variants of H2AX and H2AZ. The complex mediate
eviction at double strand breaks and promotes homologous recombination repair implicating its importance in DNA repair. The complex has been shown to be critical for regulating telomere length and maintaining genomic stability [43, 47].

**SWI/SNF Chromatin Remodeling Enzymes:**

SWI/SNF enzymes are evolutionarily conserved, multisubunit complexes of 1-2 MDa that utilize the energy derived from ATP hydrolysis to alter the nucleosome structure by disrupting the histone-DNA contacts [48]. Each complex consists of a central ATPase catalytic domain which can either be Brahma (BRM) or Brahma related gene 1 (BRG1) and 9-12 accessory proteins known as BRG1/BRM associated factors (BAFs). BRG1 was characterized in yeast and was the first chromatin remodeling protein to be identified [49, 50]. BRM was first identified in *Drosophila* as a suppressor of body segment defects observed in Polycomb mutants. The SWI/SNF complex does not possess any sequence specificity and is recruited to target regions primarily via interactions between BAFs and key transcriptional modulators [17]. SWI/SNF activity has been shown to be required for a variety of physiological processes including embryonic pluripotency [51, 52], development of neural progenitors [53], cardiac development [54], liver development [55], erythropoiesis [56] and differentiation of a plethora of cell types including muscle differentiation [57, 58], hematopoietic [59], neural differentiation [60], adipogenesis [61] and very recently melanocyte differentiation[17, 18]. Two variants of the complex have been identified in mammalian cells – BAF (also known as SWI/SNF-A) and PBAF (also known as SWI/SNF-B) based on the purified complexes from mammalian cells. These two complexes have highly related but distinct subunit composition [62]. Specific components of chromatin remodelers confer specialized
activities and selective gene targeting to distinct complexes. The “core” SWI/SNF complex subunits includes BAFs 47 (SNF5/INI1), 53, 57, 60, 155, 170 and actin. Additionally “specificity” subunits like ARID2 (AT-rich interactive domain 2, BAF200) and Polybromo (BAF180) distinguish Polybromo-associated BAF (PBAF) from BAF complexes, which are specified by ARID1 (BAF250) [63]. Notably, although the complex normally consists of 9-12 subunits, only four of these – BRG1 or BRM (mutually exclusive ATPase subunits that are human homologues of the yeast Swi2/Snf2 ATPase subunit), SNF5 (INI1/BAF47), BAF155 and BAF170 – are required in vitro to remodel nucleosomes and additional BAFs are required for interactions with transcriptional activators and repressors in vivo [62]. SWI/SNF enzymes can play complex roles in the regulation of gene expression, and participate in activation as well as repression of a specific gene, owing to its heterogeneity.

Many of the BAFs have been established as bonafide tumor suppressors which get inactivated in multiple malignancies as well as in cancer cell lines [62, 64-66]. BRG1 and BRM share about 74% sequence identity and exert similar biochemical activities in vitro [67]. However, the function of these ATPases is very diverse in vivo as is indicated by their disruption/inactivation studies in mice. Whereas BRG1 inactivation is embryonic lethal in homozygotes, and heterozygotes show accelerated development of tumors, BRM inactivation exhibits mild proliferation defects and showed upregulation of BRG1 in certain tissues, suggestive of the fact that BRG1 can compensate for BRM loss in several cases [68, 69]. Whereas BRG1 was found to interact with zinc finger proteins, BRM was found to be preferentially binding with ankyrin repeat proteins [70].
Histone modifying enzymes

Covalent modifications of DNA or histones coordinate the maintenance of both local and global chromatin architecture [71, 72]. These modifications regulate DNA-templated processes, including transcription, repair, and replication [73]. Chromatin modifications determine accessibility to specific DNA loci by physically enhancing or weakening the non-covalent interactions between histones or between histones and DNA, and providing a platform for recruiting epigenetic regulators. Examples of histone modifying enzymes are Histone acetyltransferases (HATs), Histone deacetylases (HDACs), kinases, histone methyltransferases (HMT) and demethylases. These enzymes modify histone tails by means of addition or removal of methyl, acetyl or phosphate groups post-translationally [9]. Epigenetic regulators can be broadly categorized as epigenetic writers, which are responsible for addition of covalent modifications on histones and DNA, epigenetic erasers, which are responsible for removal of covalent modifications on histones and DNA, and the epigenetic readers, proteins with
specialized binding domains that recognize and bind to covalent modifications of histones and DNA [72].


**Histone Acetylation:** The Nε-acetylation of lysine residues is the histone modification which is associated with “open” chromatin conformation and transcriptional activation. Lysine acetylation is one of the most abundant post-translational modifications in the human proteome. Acetylation marks have been associated with processes like metabolism, DNA repair, protein-protein interactions and protein stability [74]. Acetylation is a highly dynamic process and is mainly regulated by competing activities of two enzymatic families, the histone lysine acetyltransferases (HATs) and the histone deacetylases (HDACs). There are two major types of HATs: (1) type-B, which are predominantly cytoplasmic and modify free histones, and (2) type-A, which are primarily nuclear and consists of CBP/p300 families. Global histone acetylation patterns are perturbed in cancers [75, 76].
**Histone Deacetylation:** HDACs are enzymes that reverse lysine acetylation and 18 such enzymes are subdivided into 4 major classes depending on the sequence homology. Class I (HDAC 1-3 and HDAC8), class II (HDAC 4-7 and HDAC 9-10) and class IV (HDAC11) share a related catalytic mechanism requiring zinc metal ion but does not involve the use of a cofactor. In contrast, class III HDACs (sirtuins 1-7) employs a distinct catalytic mechanism which is NAD⁺-dependent. HDACs target both histone and non-histone proteins and substrate specificity for these enzymes are largely mediated by components of multi-subunit complexes such as Mi2/NuRD, comprising of HDACs [75, 77].

**Histone Methylation:** Methylation in histones occurs on side chains of arginine, lysine, and histidine residues. The best-characterized sites of histone methylation are the ones on lysine residues. Some of these (H3K4, H3K36, and H3K79) are associated with active genes in euchromatin, whereas others (H3K9, H3K27, and H4K20) are associated with heterochromatin regions of the genome. Different methylation states on the same residue can also localize differently. For example, H3K4me2/3 spans the transcriptional start site (TSS) of active genes, whereas H3K4me1 is associated with active enhancers. Similarly, whereas monomethylation of H3K9 is seen at active genes, trimethylation of H3K9 is associated with gene repression [75, 78].

**Histone Demethylation:** Two classes of histone demethylases have been studied so far. LSD1 (KDM1A), demethylates lysine via an amine oxidation reaction with FAD as a cofactor, and belongs to first class. The Jumonji demethylases belong to second class of demethylases and have been studied more extensively. They have a conserved JmjC
domain, which functions via an oxidative mechanism and radical attack (involving Fe(II) and α-ketoglutarate) [75].

**Histone Phosphorylation:** Phosphorylation influences the overall structure and function of the local chromatin environment and is integral to essential cellular processes such as mitosis, apoptosis, DNA repair, replication and transcription. Histone phosphorylation sites on core histones can be divided into two broad categories: (1) those involved in transcription regulation, and (2) those involved in chromatin condensation.

**Kinases:** They are the main players of signal transduction pathways which convey extracellular cues within the cell. Coding mutations, altered expressions, and recurrent translocations involving signaling kinases are among the most frequent oncogenic phenomena described in cancer [79].

**Histone Acetylation and Histone Methylation Readers:** The primary readers of $\text{N}^\epsilon$-acetylation of lysine residues are bromodomains, which contain an evolutionarily conserved binding motif. These consists a diverse group of proteins which function as chromatin remodelers, histone acetyltransferases, histone methyltransferases, and transcriptional co-activators [80]. Bromodomains have been discussed in detail in separate sections.

Lysine methylation leads to considerable physicochemical diversity of lysine; these modification states are read and interpreted by proteins containing different specialized recognition motifs. Methylation readers are broadly classified into two major families, the Royal Family (Tudor domains, Chromo domains, and malignant brain tumor [MBT] domains) and PHD fingers [81].
Bromodomains are evolutionarily conserved 110-amino acid protein modules that recognize and bind to acetylated lysine residues (KAc) on histone proteins and regulate epigenetic information by turning genes on and off [82]. Bromodomains were first reported in the *Drosophila* protein brahma [83, 84], and are present in many chromatin-associated proteins and nearly all known nuclear histone acetyltransferases (HATs) [85]. It was long known from yeast genetic studies that bromodomains play a critical role in chromatin remodeling but specific biological functions began to emerge only after it was discovered that they function as acetyl-lysine binding domains [86]. Lysine acetylation is dynamic, as this modification directs both structural changes to chromatin and gene transcription. It is believed that the bromodomain is the sole protein domain known to recognize acetyl-lysine residues on proteins [74, 87].

Bromodomains can contribute to highly specific histone acetylation by tethering transcriptional HATs to specific chromosomonal sites, and to the assembly and activity of
multi-protein complexes of chromatin remodeling enzymes such as SAGA and NuA4
[88, 89]. Bromodomain-containing proteins have been implicated in cellular
differentiation and transcriptional regulation[90]. Detailed functions of bromodomains
have been subsequently discussed in a separate section.

Structure of the Bromodomain

The three-dimensional structure of a bromodomain was first determined by
performing nuclear magnetic resonance (NMR) spectroscopy of the bromodomain from
the human transcriptional coactivator PCAF (p300/CBP-associated factor). A number of
three-dimensional structures of bromodomains have been reported thereafter which
include bromodomains either in the free form or bound to an acetyl-lysine containing
peptide [91]. These include bromodomains from human transcriptional protein TAFII250
[91, 92], human and Saccharomyces cerevisiae GCN5, human PCAF [91, 93], human
coadvator CBP [91, 94], human BET (bromodomains and extra terminal domain) family
protein BRD2 [91, 95], human transcription factor BPTF (bromodomain and PHD
domain transcription factor) [91, 96], and human SWI/SNF chromatin-remodeling
complex protein Brg1 (Brahma-related gene 1) [91, 97]. It was evident from the
structural analysis of all these bromodomains that most of them, if not all, adopt an
atypical left handed bundle of four helices ($\alpha_Z$, $\alpha_A$, $\alpha_B$, $\alpha_C$) arranged in an antiparellel
fashion [98]. The evolutionarily conserved structural fold is termed the ‘BrD fold’ [99].
The structural arrangement places the ZA and BC loops at one end of the molecule and
the AB loop and the N- and C-terminal ends on the opposite ends of the molecule. There
is an additional secondary structural element called the ZA helix (a short single-turn
helix) within the ZA loop due to the left handed twist topology of the bundle, which is
less common as compared to the right handed twist [98]. The inter-helical ZA and BC loops are of variable length and sequence, and together form a hydrophobic pocket, which serves to stabilize the structure and interact with acetyl-lysine modifications [86, 91, 99]. It was observed from aligning the bromodomain sequences that the highest degree of conservation occurs within the ZA loop region. Significantly, several of these residues were accessible for protein-interactions. Interestingly, it also became known that ZA loop has three positions which are a common site of amino acid insertions or deletions imparting importance to this region for substrate specificity. Henceforth, the ZA loop is thought to play an important role in mediating bromodomain function in which conserved residues impart conserved substrate interactions and non-conserved residues mediate protein-specific bromodomain function [98].

**Figure 1-8 Structure of Bromodomain.** Reprinted from Gene, Vol 272(1-2):Pg:1-9 Copyright (2001) with permission from Elsevier
Acetyl-lysine recognition of bromodomain

The unique ability of NMR spectroscopy to measure changes in the local chemical environment and/or conformation of a protein induced upon binding to a ligand led to the discovery of acetyl-lysine recognition by bromodomains. NMR has the ability to detect weak but highly specific interactions between a protein and a ligand [86]. The overall sequence similarity between members of the bromodomain family is not high and there are significant variations in the sequences of the ZA and BC loops despite the conserved BrD fold [85, 99]. Tyr^{1125}, Tyr^{1167} and Asn^{1168} in CREBBP (or CBP) are amongst the most conserved residues in the large BrD family which are engaged in acetyl-lysine recognition [94, 99-101]. The alternative modes of acetyl-lysine recognition by the BrD fold came to light when it was found out that TRIM28 BrD does not bind to lysine-acetylated residues, whereas the BrD6 of Polybromo does [99, 102]. A network of water-mediated hydrogen bonds with protein backbone carbonyl groups also contributes to acetyl-lysine binding. Site-directed mutagenesis played a critical role in cementing the function of bromodomains as acetyl-lysine binding domains [86, 103]. The BET family of BrD-containing proteins (BRDT, BRD3, BRD4) have the ability to recognize two acetylation marks with a single binding pocket [99, 104-106].

Human bromodomain family

There are 61 human bromodomains contained within 46 different bromodomain containing proteins, with some proteins containing multiple bromodomains [74, 107]. Till date, depending on the structure-based classification of human bromodomains, these 61 human bromodomains have been categorized into 8 families that share similar sequence length and at least 35% sequence identity [74, 82, 87, 108]. In contrast to this, the yeast
genome encodes only 9 bromodomain-containing proteins with a total of 14 BrDs [87, 109].

The first subfamily consists of a functionally diverse group of proteins that includes the HAT p300/CBP-associated factor (PCAF). In fact, it was the BRD structure of PCAF that demonstrated the ability of BRDs to function as acetyl-lysine binding domains [82, 103]. Additional proteins which belong to this group include the general control of amino-acid synthesis 5-like 2 (GCN5L), transcription factor Fetal Alzheimer antigen (FALZ) and the chromatin remodeling factor cat eye syndrome chromosome region 2 (CECR2), all of which localize in the nucleus.

The Bromodomain and Extra Terminal (BET) proteins are grouped in the second subfamily. They share a common architecture consisting of two N-terminal BRDs that exhibit high levels of sequence conservation as well as an extra-terminal (ET) domain and a more diverse C-terminal recruitment domain. This subfamily comprises of 4 proteins namely BRD2, BRD3, BRD4 and BRDT. It has been shown that these BET proteins are recruited to transcriptional start sites during mitosis [110-112] and the BET protein BRD4 tethers the positive transcription elongation factor (P-TEFb) to these sites via its unique C-terminus [74]. Highly specific small molecule inhibitors of this family have recently emerged as promising therapeutic agents in inflammation and cancer [82, 113].

The third subfamily of BRDs contains the transcriptional regulator bromodomain containing 8B (BRD8B), the HAT enzymes CREB binding protein (CREBBP) and E1A binding protein p300 (EP300), the C-terminal domain of the chromatin remodeling factors WD repeat domain 9 (WDR9 domain 2), the bromodomain adjacent to zinc finger
domain 1B (BAZ1B), the C-terminal domain of the JAK/STAT pathway related bromodomain-containing protein disrupted in leukemia (BRWD3 domain 2) and the C-terminal domain of the insulin signaling related pleckstrin homology domain interacting protein (PHIP domain 2).

The fourth subfamily contains the transcriptional regulators bromodomain containing 7 (BRD7) [63, 74], bromodomain containing protein 1 (BRD1), bromodomain and PHD finger-containing protein 1 (BRPF1), two AAA domain containing protein (ATAD2) as well as the KIAA1240 protein (KIAA1240), the bromodomain containing 9 (BRD9) and the bromodomain and PHD finger-containing protein 3 (BRPF3) [74].

Subfamily V of the human BRDs comprises of the transcriptional repressor tripartite motif-containing 66 (TRIM66), the tripartite motif-containing 33 (TRIM33), the transcriptional regulator transcriptional intermediary factor 1 (TIF1α), the transcriptional regulators nuclear auto-antigen Sp-100 (SP100), nuclear auto-antigen Sp-110 (SP110) and SP140 nuclear body protein (SP140) as well as the SP140-like protein (LOC93349), the transcriptional repressor bromodomain adjacent to zinc finger domain 2A (BAZ2A) and the bromodomain adjacent to zinc finger domain 2B (BAZ2B).

Subfamily VI of BRDs includes the histone methyl-transferase myeloid/lymphoid or mixed-lineage leukemia (MLL) and the transcriptional co-regulator tri-partite motif-containing 28 (TRIM28).

Subfamily VII contains the transcriptional repressor zinc finger MYND domain containing 11 protein (ZMYND11), the transcriptional initiators TAF1 RNA polymerase II TATA box binding protein (TBP)-associated factor (TAF1) and TAF-1 like RNA
polymerase II TATA box binding protein (TBP)-associated factor (TAF1L) and the N-terminal BRDs of the chromatin remodeling factors WD repeat domain 9 (WDR9 domain 1), the JAK/STAT pathway related bromodomain-containing protein disrupted in leukemia (BRWD3 domain 1) and the insulin signaling related pleckstrin homology domain interacting protein (PHIP domain 1).

The last subfamily of the human bromodomains (VIII) contains the methyltransferase ash1 (absent, small, or homeotic)-like (ASH1L), the chromatin remodeling factors SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a2 (SMARCA2) and the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4 (SMARCA4) as well as the Polybromo 1 (PB1) [74].

**Function of Bromodomains**

Targeting of chromatin-modifying enzymes to specific sites is often carried out by bromodomains. In Drosophila, a methyltransferase ASH1L activates ultrabithorax expression and its mammalian homologue has been associated with actively transcribed genes. Mixed lineage leukemia (MLL), a multidomain methyltransferase, regulates self-renewal of haematopoietic stem cells by controlling HOX (homeobox) gene expression and displays haplo-insufficiency [87] meaning a single functional copy of the gene is insufficient for bringing about a wild-type condition, leading to the diseased state.

HATs CREBBP and EP300 act as transcriptional coactivators controlling a variety of biological processes, including cell growth, genomic stability, development, neuronal plasticity and memory formation, as well as energy homeostasis [114]. Homozygous knockout of CREBBP leads to severe defects in blood vessel formation in the central nervous system, developmental retardation and mice die in utero [115].
Homozygous deletion of EP300 leads to death of mice between days 9 and 11.5 of gestation with defects in neurulation, cell proliferation and heart development [116]. Gen5-knockout mice die during embryogenesis [117] whereas homozygous deletion of a closely related gene, PCAF leads to short-term memory defects and conditioned fear associated with increased plasma corticosterone levels [118].

ATAD2 has been shown to regulate expression of genes required for cell cycle progression. There is a suggestive role of ATAD2 in prostate cancer by mediating specific androgen receptor functions involved in cancer cell survival and proliferation [119].

TAF1 modulates the rate of transcription initiation by interacting with transcriptional regulators and binding to the core promoter sequence encompassing transcriptional start sites [120]. It acts as a general transcriptional activator and regulates various essential biological processes like myogenesis, DNA-damage response, cell cycle and apoptosis [87].

The WD repeat proteins of BRWD1 and BRWD3 are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis and gene regulation [121, 122]. BRWD1 associates with SWI/SNF complex component and plays a vital role in chromatin remodeling [123]. In mice, BRWD1 is required for normal spermiogenesis and a mutation in BRWD1 leads to phenotypically normal, but infertile mice [87].

TRIM24 mediates ligand-dependent activation of the androgen receptor and the retinoic acid receptor. It also interacts with nuclear receptors such as thyroid, vitamin D3 and oestrogen receptors [124]. TRIM28 plays a key role in early embryogenesis by acting
as a corepressor for Kruppel-associated box-domain-containing zinc finger proteins [125].

TRIM33 plays an important role in development which was advocated by the death of mice in utero, which are deficient of TRIM33 [126]. TRIM66 functions as a transcriptional silencer by associating with heterochromatin-associated factors and is mainly expressed in testis. [127]

SWI/SNF complexes remodel chromatin structure, leading to either transcriptional activation or repression of target genes depending on the composition of various complexes [49]. Mammalian SWI/SNF complexes have a vital role in cell differentiation and proliferation and are essential components of the embryonic stem cell core pluripotency transcriptional network [128]. PB1 is a key regulator of senescence and has roles in cell cycle regulation [129].

ISWI complexes are key regulators of transcription, heterochromatin replication and chromatin structure [130]. The DNA-replication machinery requires the activity of BAZ1A to penetrate condensed chromatin structure [131]. BAZ2A is a key subunit of NoRC (nucleolar remodeling complex), which mediates transcriptional silencing of ribosomal RNA [132].

The bromodomain extra-C terminal domain (BET) family of proteins includes BRDT, BRD2, BRD3 and BRD4 [133]. These proteins have been implicated in the regulation of transcription and proliferation [98]. BRD2 functions as a transcriptional coactivator or corepressor in a promoter-specific or tissue-specific manner. BRD2 is essential for embryonic development in mice [134, 135] and an association between BRD2 and juvenile myoclonic epilepsy in humans has been reported [136]. BRDT is the
testis-specific BET family member which is essential for spermatogenesis [137]. Deletion of BRDT in mice led to sterility and an altered histone modifications (enrichment of acetylated H3K9 and H3K27me3) in the exon region of BRDT gene of subfertile patients was observed [138]. Genome-wide association studies (GWAS) linked polymorphism in BRDT to sterility in European male [139]. BET proteins are epigenome readers that play a key role in gene expression by acting at the interface between chromatin remodeling and transcriptional regulation.

**BRD4**

BRD4, originally named MCAP (Mitotic chromosome-associated protein), binds preferentially to acetylated lysine residues found in histones (H3K14ac and H4 dual lysines K5 and K12 or K8 or K16) and non-histone proteins (RelA subunit of nuclear factor (NF)κB) [140, 141]. BRD4 is associated with acetylated chromatin throughout the cell cycle, and regulates transcription at targeted loci [140]. This association of BRD4 with acetylated chromatin is critical for the rapid expression of early G1 genes upon exiting mitosis [142]. BRD4 has been proposed to transmit epigenetic memory across cell division by bookmarking transcriptionally active genes by an unknown mechanism [143]. BRD4 is a key mediator of transcriptional elongation by recruiting the positive transcription elongation factor complex (P-TEFb) [144]. This association of BRD4 with active P-TEFb is suggestive of its role in transcription by RNA polymerase II [145]. Functional transition of BRD4 between chromatin targeting and transcriptional regulation leads to speculation of diverse biological roles of BRD4 [140, 143]. Interestingly, there seems to be multiple functionally distinct pools of BRD4. One remains associated with chromatin during mitosis responsible for accelerating post mitotic gene activation [146].
Another BRD4 pool recruits P-TEFb to the sites of active transcription [142] and a third pool which is recruited by the transcriptional Mediator complex independent of P-TEFb [145]. BRD4 stimulates G1 gene transcription and promotes cell cycle progression to S-phase [87, 147]. BRD4 activation also predicts the survival of patients with breast cancer [87, 148]. Activation of BRD4 manipulates the response of tumors to their microenvironments in vivo, resulting in reduction of tumor growth and metastasis in mice [148]. BRD4 has been implicated in the differentiation of NUT-midline carcinoma [149], as well as skeletal muscle differentiation in association with MyoD and myogenin [150].

BRD4 controls the transcription of a number of viral genes. It regulates HIV transcription by inducing the phosphorylation of CDK9 (cyclin-dependent kinase 9) in the HIV transcription initiation complex and inhibiting the kinase activity [151]. BRD4 also inhibits the proteasomal degradation of the papillomavirus E2 protein [152]. It associates with Kaposi’s sarcoma-associated herpesvirus-encoded LANA-1 (latency-associated nuclear antigen) and murine γ-herpesvirus protein which is required for establishing viral latency in vivo [153, 154].

BRD4 heterozygotes display pre- and post-natal growth defects associated with a reduced proliferation rate. These mice also exhibit a variety of anatomical abnormalities such as head malformations, absence of subcutaneous fat, cataracts, and abnormal liver cells. In primary cell cultures, heterozygous cells also display reduced proliferation rates. Embryos nullizygous for BRD4 die shortly after implantation and are compromised in their ability to maintain inner cell mass in vitro, indicating a role in fundamental cellular processes [155].
Recent studies with small molecule inhibitors of the BET proteins have revealed a critical role of BRD4 in the development of several hematopoietic and somatic cancers, such as Burkitt’s lymphoma, multiple myeloma [156], melanoma [157], colon [158] and breast cancer [159].

The chemical inhibition of BRD4 alters gene expression and results in reduced proliferation of cancerous cells. As a result, BET bromodomain proteins are being targeted for use in male contraception and in the treatment of NUT-Midline carcinoma, leukemia and other cancers, HIV, cardiac failure, inflammation, and viral infection.

**Domain organization of BRD4 family proteins**

Bromodomain-containing protein 4 (BRD4) is a member of BET family that contains two tandem bromodomains (BDI and BDII) and an extraterminal (ET) domain. In humans, four BET proteins (BRD2, BRD3, BRD4 and BRDT) exhibit similar domain organizations, gene arrangements, and some functional properties [145]. Except for BRDT, which is expressed specifically in testis and ovary, BRD2, BRD3, and BRD4 are widely distributed. Although BDI, BDII, and the ET domains are characteristics of the BET family proteins, other domains, such as motifs B and SEED (Ser/Glu/Asp-rich region), are also highly conserved. The C-terminal motif (CTM) is only present in BRD4 [160]. The ET domain mediates protein-protein interactions, which are conserved for the human BET proteins [161].
Therapeutic potential for inhibition of BRD4

The methyl ester analogue (MS417) of JQ1 selectively inhibits NF-κB transcriptional activation of proinflammatory genes in kidney cells treated with TNFα or infected by HIV. Genetic knockdown of BRD4 or inhibition with JQ1 causes a decrease in c-MYC mRNA levels in acute myeloid leukemia (AML) cells and a profound anti-leukemic effect in vitro and in vivo. I-BET151 demonstrated significant activity in mixed-lineage leukemia (MLL)-fusion leukemia through inhibition of transcription at BCL2, CDK6, and c-MYC. JQ1, I-BET762, and I-BET151 downregulates MYCN transcriptional program in neuroblastoma cell lines. JQ1 down-regulated c-MYC and induced profound changes in gene expression via both c-MYC-dependent and independent mechanisms in glioblastoma samples. Treating patient-derived samples with JQ1 led to growth arrest of the malignant cells in NUT-midline carcinoma [133].
1.4 JQ1: A selective and potent inhibitor of BET family bromodomains

Epigenetic proteins have been an intently pursued target in ligand discovery for therapeutic gain which is evident by the FDA approval of DNA methyltransferase inhibitors and HDAC-I, but with the advancement of medicinal chemistry, chromatin-associated proteins such as bromodomains are gaining importance, as modulating bromodomain/acetyl-lysine interactions with small molecules provide new avenues for tailoring therapies to specific diseases. Based on the observation by Mitsubishi Pharmaceuticals that simple thienodiazepines possessed binding activity of BRD4, a prototype ligand, known as JQ1, was developed which specifically inhibits the BET family of bromodomain proteins from interacting with chromatin [162]. The BET antagonist JQ1 is a cell-permeable small molecule that binds competitively to acetyl-lysine recognition motifs, such as bromodomains. JQ1 is highly potent and specific to a subset of human bromodomains, having excellent shape complementarity with the acetyl-lysine binding cavity. A general binding assay based on differential scanning fluorimetry (DSF) [163] was conducted to observe the specificity of JQ1 as specific substrates of most bromodomains are unknown. Binding of JQ1 significantly increases the thermal stability of all the bromodomains of the BET bromodomain and extra-terminal family, highest being that of BRD4, while no significant stability shifts are detectable outside the BET family, indicating that JQ1 is highly selective. In contrast, the stereoisomer (-)-JQ1 showed no significant interaction with any bromodomain present in the panel. To assess the competitive binding of (+)-JQ1 with acetyl-lysine, a luminescence proximity homogenous assay (alpha-screen) [164] was adapted to the BET bromodomains. (+)-JQ1 proved to be a potent, highly specific and \( K_{ac} \)-competitive inhibitor for the BET family of
bromodomains [162]. Evaluations of JQ1 have indicated that BET bromodomain proteins can be targeted for use in male contraception and in the treatment of human diseases including cancer, inflammation, and viral infection.


BET Bromodomain inhibitors in cancer

Table 1-1 BET Bromodomain inhibitors in cancer. Adapted and modified from Oncotarget. Mar 20;6(8):5501-16 Copyright (2015)
<table>
<thead>
<tr>
<th>Name</th>
<th>Cancer type</th>
<th>Target</th>
<th>Mechanism/pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>JQ1</td>
<td>Tam-R breast cancer</td>
<td>BRD3/4</td>
<td>Suppresses the classic estrogen receptor-a signaling pathway and the growth of Tam-R breast cancer cells in culture</td>
</tr>
<tr>
<td>NUT midline carcinoma (NMC)</td>
<td>BRD3/4</td>
<td></td>
<td>Suppresses different BRD4-NUT translocations</td>
</tr>
<tr>
<td>AML cells</td>
<td>BRD4</td>
<td></td>
<td>Reduce binding of BRD4 and RNA polymerase II to the DNA of c-MYC and BCL2</td>
</tr>
<tr>
<td>OCI-AML3 cell line</td>
<td>BRD4</td>
<td></td>
<td>Trigger caspase 3/7-mediated apoptosis and DNA damage response.</td>
</tr>
<tr>
<td>Erythroleukemia cell line UT7</td>
<td>BRD4</td>
<td></td>
<td>Inhibit Epo-induced UT7 proliferation and restoring terminal erythroid differentiation</td>
</tr>
<tr>
<td>B-cell non-Hodgkin lymphoma</td>
<td>BRD4</td>
<td></td>
<td>Induce cell death through MYC-CYCLON pathway</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>BRD4</td>
<td></td>
<td>Induce cell death through targeting MYCN</td>
</tr>
<tr>
<td>Primary glioblastoma xenograft lines</td>
<td>BRD4</td>
<td></td>
<td>Induced marked G1 cell-cycle arrest and apoptosis through Bcl-xL and p21(CIP1/WAF1).</td>
</tr>
<tr>
<td>Osteosarcoma cells</td>
<td>BRD4</td>
<td></td>
<td>Trigger transcriptional silencing of MYC and RUNX2, resulting from the depletion of BRD4</td>
</tr>
</tbody>
</table>

| LBET151             | Myeloma cell                  | BRD2/3/4| Induce apoptosis and exerts strong anti-proliferative effect associating with contrasting effects on oncogenic MYC and HEXIM1, and inhibit transcriptional activator PTEFsβ |
| AML                 | BRD4                          |        | Suppress cell growth in a HOX gene independent manner, but relieving upon NPM1c mutation and cyttoplasmic dislocation. |
| Erythroleukemic (HEL) cell | BRD4                      |        | Suppress myeloproliferative neoplasms by constitutively active JAK2 kinase         |
| LBET762             | Myeloma cell                  | BRD2/3/4| Inhibit myeloma cell proliferation, resulting in survival advantage in a systemic myeloma xenograft model. |
| neuroblastoma tumor models | BRD2/3/4            |        | Suppress cell growth in apoptosis signaling, and N-Myc-driven pathways, including the direct suppression of BCL2 and MYCN. |
Table 1-2 Bromodomain inhibitors in clinical development. Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS DRUG DISCOVERY] Vol 13, Pg 337-356 Copyright (2014)

<table>
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<th>ClinicalTrials.gov identifier (status)</th>
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<td>Resverlogix</td>
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<td>Atherosclerosis</td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td>Type 2 diabetes</td>
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<td>GSK 525762</td>
<td>GlaxoSmithKline</td>
<td>I</td>
<td>NUT midline carcinoma (NMC), solid tumours and haematological malignancies</td>
<td>NCT01587703 (recruiting)</td>
</tr>
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<td>OncoEthix</td>
<td>I</td>
<td>Acute leukaemia and other haematological malignancies</td>
<td>NCT01713582 (recruiting)</td>
</tr>
<tr>
<td>CPI-0610</td>
<td>Constellation</td>
<td>I</td>
<td>Progressive lymphoma</td>
<td>NCT01949883 (recruiting)</td>
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<tr>
<td>TEN-010</td>
<td>Tensha Therapeutics</td>
<td>I</td>
<td>NMC</td>
<td>NCT01987362 (recruiting)</td>
</tr>
</tbody>
</table>

1.5 SWI/SNF Enzymes in Cancer:

SWI/SNF complex lacks intrinsic DNA sequence specificity and are typically recruited to genomic sites through physical interactions with sequence-specific transcription factors. SWI/SNF chromatin remodeling enzyme functions as an epigenetic regulator assisting in gene expression by turning genes on and off. In this context, the SWI/SNF complex serves as an oncogene or a tumor suppressor, depending on the interacting protein partners [165]. Meta-analyses of cancer genome-sequencing data have revealed that mutations that inactivate SWI/SNF subunits are found in nearly 20% of human cancers [166]. These mutations are generally loss of function, implicating SWI/SNF as a major tumor suppressor in diverse cancers.

It has been shown that Microphthalmia transcription factor (MITF) which is a master regulator of melanocyte differentiation interacts with SWI/SNF enzymes [17]. Our lab has shown that BRG1 promotes melanoma differentiation and increases melanoma cell survival on exposure to a chemotherapeutic agent or to UV radiation. The
BRM subunit of SWI/SNF chromatin remodelers has been implicated in melanocyte senescence [167]. BRG1 expression is increased in human cutaneous melanoma and it is a novel binding partner of tumor suppressor p16INK4a which is an important melanoma susceptibility gene and is also associated with increased melanoma invasiveness [168-170]. Our lab has shown that down-regulation of BRM in BRG1-deficient melanoma cells significantly inhibits the expression of proproliferative and survival genes CDK2, TBX2, and BCL2, thus compromising tumorigenicity. However, the requirement for BRG1 is not compensated by BRM in several melanoma cell lines [171].

BAF180 has been identified to be mutated in 41% of renal cell carcinomas and a small minority of breast cancers [172]. It is thought to function as a tumor suppressor by interacting with the p53 protein to promote cell cycle arrest and senescence [129, 173]. ARID2, a PBAF SWI/SNF specific subunit is required for stability of BAF180 [174]. ARID2 mutations have been detected in hepatitis C virus-associated hepatocellular carcinomas, non-small-cell lung cancer, pancreatic cancer, and melanoma. Analysis of large-scale melanoma exome data has identified ARID2 as the most frequently mutated SWI/SNF component [175, 176]. Another PBAF SWI/SNF specific subunit, BRD7, is frequently deleted in a subset of breast tumors that express wild-type, but not mutant, p53. It has been implicated as a cofactor for p53 that activates oncogene-induced senescence. BRD7 along with its PBAF SWI/SNF component BAF180 are critical regulators of p53 required for replicative senescence [129]. BRD7 inhibits the G1-S progression of nasopharyngeal carcinoma (NPC) cell lines and is thought to be a tumor suppressor gene [177].
The ARID1A subunit of SWI/SNF complexes has been found to specifically mutated in primary human cancers. It is mutated in 50% of ovarian clear cell carcinoma and in 30% of endometrial carcinoma. It is occasionally mutated in medulloblastomas, primary breast cancer and lung adenocarcinoma cell line indicating its tumor suppressor activities in various lineages. ARID1B mutations have been reported to be highly frequent in breast cancer [178]. Both, ARID1A and ARID1B mutations have been reported in hepatocellular carcinoma, neuroblastoma, as well as in melanoma, at a low frequency [175].

BAF47, a core component of SWI/SNF complex acts as a classic tumor suppressor with inactivating mutations occurring in nearly all malignant rhabdoid tumors. These lethal tumors are poorly responsive to current therapies and arise in the brain, kidney and other soft tissues [178]. Down-regulation of BAF47 occurs in significant number of primary and metastatic melanomas and correlates with poor prognosis [175].

**Table 1-1 SWI/SNF mutations in cancer.** Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS CANCER] Vol 11, Pg 481-492
There are multiple studies suggestive of the important role played by SWI/SNF enzymes in melanoma. A number of SWI/SNF subunits were on a microarray that identified MAPK dependent genes in melanoma cells [179].
Chapter 2

Role of BRD4 in melanocyte differentiation

2.1 Introduction

Bromodomains belong to a family of evolutionarily conserved protein modules originally found in proteins associated with chromatin and in nearly all nuclear histone acetyltransferases. Bromodomains modulate enzyme activities, protein assembly, and protein-protein interactions via lysine acetylation with broad implications in a wide variety of cellular events, such as transcriptional activation and chromatin remodeling. They are the sole protein domains which function as acetyl-lysine binding residues and are epigenetic readers which bind to the acetylated residues regulating chromatin structure and gene expression.

BET protein family members (BRD2, BRD3, BRD4, and BRDT) are epigenome readers that bind to acetyl-lysine residues on the tails of histones H3 and H4, and exert key roles at the interface between chromatin remodeling and transcriptional regulation. BRD4 is a BET protein which acts as a transcriptional coactivator and was initially known as mitotic chromosome associated protein (MCAP). BRD4 associates with acetylated chromatin throughout the cell cycle and regulates transcription at targeted loci.
It is implicated in cellular growth control, cell cycle progression and cancer development. However, the role of BRD4 in melanocyte differentiation has not been investigated.

In this study, we utilized an in vitro murine model of immortalized murine melanoblasts (melb-a cells) which can be differentiated by treatment with alpha-MSH. Alpha-MSH upon binding to melanocortin 1 receptor activates the microphthalmia-associated transcription factor (MITF) which is the master regulator of melanocyte differentiation and melanoma oncogene. MITF in turn activates genes required for melanin production, proliferation, and survival. JQ1 is a cell permeable small molecule inhibitor of BET proteins which binds to BRD4 with the highest affinity and specificity, inhibiting its binding to the acetylated lysine residues. JQ1 has an excellent shape complementarity with the acetyl-lysine binding cavity of bromodomains of BET family. Treating melb-a cells with JQ1 compromised MITF target gene expression, melanin synthesis, and reduced proliferation of melb-a cells as well as neonatal human epidermal melanocytes (NHEM) and melanoma cells. Furthermore, we determined that BRD4 occupied the promoters of MITF target genes that regulate pigmentation. Treatment with JQ1 inhibited the binding of both BRD4 and MITF to these promoters. Moreover, treating the melb-a cells as well as NHEMs disrupts histone H3 lysine 4 trimethylation (H3K4me3) which is an active mark of transcription. The effects on gene expression were recapitulated by siRNA mediated silencing of BRD4. We showed a physical interaction between the two proteins. Thus, we hypothesize that BRD4 plays a crucial role in melanocyte differentiation by interacting with MITF and can be a potential novel target for treatment of hyperpigmentation disorders and in melanoma.
2.2 Materials and methods

Cell Culture

Human Embryonic Kidney (HEK) 293T cells were obtained from the ATCC. They were cultured in DMEM Media (Life Technologies, Grand Island, N.Y.) containing 10% fetal bovine serum. Mouse melanoblast cells (Melb-a) were obtained from Dr. Dorothy Bennett (The Welcome Trust, UK). The growth media consisted of RPMI1640 (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum (Life Technologies, Grand Island, N.Y.), 20ng/ml stem cell factor, and 20 picoM fibroblast growth factor 2. When cells grew to cover 70% of the dish, they were shifted to differentiation media. The differentiation media consisted of DMEM (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum, 200 nM phorbal 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) and 2 nM Nle4, D-Phe7-alpha-MSH (Sigma-Aldrich, St. Louis, MO). Neonatal human epidermal melanocytes (NHEMs) were isolated as described in [180] and cultured in Media 254 (Invitrogen, Carlsbad, CA, USA).

RNA isolation and quantitative real time PCR

Total RNA was isolated using Trizol (Invitrogen) and cDNA was prepared using the QiagenQuantitect Reverse Transcription kit. Quantitative PCR (qPCR) was performed in SYBR Green master mix (Qiagen) with an Applied Biosystems 7500 PCR and analyzed with the SDS software as described [18]. Mouse Bcl2, p21, Tyrosinase, TRP1, TRPM1 and BRD4 mRNA levels were normalized to mouse RPL7. Human TRP1 mRNA levels were normalized to human RPL19. All the primers were obtained from Integrated DNA Technologies (IDT).

Primer sequences for mouse melanoblasts are as follows:
Bcl2: 5'- AGT TCG GTG GGG TCA TGT GTG -3' and 5'- CCA GGT ATG CAC CCA GAG TG -3'
p21: 5'- ACA CAC AGA GAG AGG GCT AAG G -3' and 5'- AGA TCC ACA GCG ATA TCC AGA C -3'
Tyrosinase: 5'- TTC AAA GGG GTG GAT GAC CG -3' and 5'- GAC ACA TAG TAA TGC ATC C -3'
TRP1: 5'- GCC CCA ACT CTG TCT TTT CTC AAT -3' and 5'- GAT CGG CGT TAT ACC TCC TTA GC -3'
TRPM1: 5'- CCT ACA CCA AGC CAG AT -3' and 5'- GAC GAC ACC AGT GCT CAC AC -3'
BRD4: 5'- TAA AAA CTC CAA CCC CGA TG -3' and 5'- TGC TCT CCG ACT CAG AGG AT -3'
RPL7: 5'- GGAGGAAGCTCATCTATGAGAAGG -3' and 5'- AAG ATC TGT GGA AGA GGA AGG AGC -3'

Primer sequences for human melanocytes are as follows:
TRP1: 5'- TGG GAT CCA GAA GCA ACT TT -3' and 5'- TGT GGT TCA GGA AGA AGA GGA AGG AGC -3'
RPL19: 5'- AAA CAA GCG GAT TCT CAT GG -3' and 5'- TTG GTC TCT TCC TTG GAT -3'

siRNA knockdown

A SMARTPool of siRNAs targeting mouse BRD4, and a non-targeting siRNA [26] were obtained from Dharmacon. Transfection was performed according to
manufacturer’s instructions. Media was replaced with the differentiation media consisting of DMEM (Life Technologies, Grand Island, N.Y.), 10% fetal bovine serum, 200 nMphorbal 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) and 2 nM Nle4, D-Phe7-alpha-MSH (Sigma-Aldrich, St. Louis, MO) 72 hours post transfection and cells were then harvested 48 hours later and subjected to Western blotting, Quantitative real time PCR, Chromatin immunoprecipitation and FAIRE analysis.

**Cell extracts and immunoblot analysis**

Cell extracts were prepared and Western Blotting was performed as described[18]. The MITF, BRD4 and β-Actin antibodies were from Abcam (Cambridge, MA, USA). The Tyrosinase and TRP1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The Tubulin antibody was from Cell Signaling (Beverly, MA, USA).

**Co-immunoprecipitations**

HEK 293T cells obtained from ATCC were transfected with FLAG tagged MITF construct using Lipofectamine LTX (Invitrogen, Carlsbad, CA). Cells were harvested 48 hours post-transfection. Co-immunoprecipitations were performed as previously described[18]. Species matched IgG (Santa Cruz Technologies) was used as a control. Differentiated Melb-a cells were incubated with BRD4 antibody and western blotting was performed using MITF and BRD4 antibodies.

**Chromatin immunoprecipitations (ChIPs)**

ChIPs were performed with a control rabbit IgG, control mouse IgG, BRD4 antibody, MITF antibody, Histone H3 antibody, Histone 3 Lysine 4 trimethylation (H3K4me3)
antibody, Histone H4 antibody, Histone 4 Lysine 16 acetylation (H4K16ac) antibody as described[18]. Primers used were as follows:

Mouse Tyrosinase Promoter: 5’-AGT CAT GTG CTT TGC AGA AGA T-3’ and 5’-CAG CCA AGA ACA TTT TCT CCT T-3’

Mouse TRP1 Promoter: 5’-GCA AAA TCT CTT CAG GT CTC-3’ and 5’-AGC CAG ATT CCT CAC ACT GG-3’

Mouse IgH Enhancer: 5’-GCCGATCAGAACCAGAAC ACC-3’ and 5’-TGGTGGGGCTGGACAGAGTTTC-3’.

Human TRP1 Promoter: 5’-AGT GCT TCT GGC CTT TTC TTT GTT GCT GAT AA-3’

Human CD25 Promoter: 5’-AAA TAG CAA TTT CGC CGT TG-3’ and 5’-TAC GTT AGT GCT GGG GCT TT-3’

**Propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS)**

Approximately 1 x 10^6 cells were fixed with 100% ethanol for 1 h, stained with PI-RNAse solution for 30 min and loaded on a FACS-Calibur (BD Biosciences, San Jose, CA) at the University of Toledo Flow Cytometry Core Facility. Data was analyzed using Cell Quest Pro (BD Biosciences).

**Apoptosis assay**

Cells were stained with Annexin V as described in [171] and analyzed on a FACS-Calibur (BD Biosciences) at the University of Toledo Flow Cytometry Core Facility.
Melanin assay

Melb-a cells were differentiated in the presence of JQ1 or vehicle control and harvested at the time points indicated. Cells were analyzed for melanin content as described in [181]. Briefly, cells were trypsinized and counted using Scepter 2.0 handheld automated cell counter (Millipore) and equal number of cells from each plate were lysed in 0.1M NaOH and vortexed for 20 minutes. Melanin content was calculated based on the absorbance at 475nm as compared to the standard curve obtained using synthetic melanin (Sigma).

Cell Images

An equal number of mouse melanoblasts which are responsive to α-MSH were seeded onto polystyrene dishes and cultured in growth media at 37°C and 5% CO₂. The growth media consisted of RPMI1640 (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum (Life Technologies, Grand Island, N.Y.), 20ng/ml stem cell factor, and 20 picoM fibroblast growth factor 2. When cells grew to cover 70% of the dish, they were shifted to differentiation media. The differentiation media consisted of DMEM (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum, 200 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) and 2 nM Nle4, D-Phe7-alpha-MSH (Sigma-Aldrich, St. Louis, MO). 500 nM active enantiomer of JQ1 (+JQ1) or 500nM inactive enantiomer of JQ1 (-JQ1) was added to the cells that were maintained in growth media and to the cells that were shifted to differentiation media. Cells were then photographed using EVOS® microscope (AMG, Bothell, WA) at 10Xmagnification at the indicated time points.

Chromatin accessibility (FAIRE analysis)
Melb-a cells were differentiated for 48 hours in the presence of active and inactive isomer of JQ1 and harvested for FAIRE analysis. NHEMs were cultured in the presence of an inactive isomer of JQ1 (-JQ1) or the active isomer of JQ1 (+JQ1) for 96 hours and harvested for FAIRE analysis.

FAIRE analysis was performed as described in [182]. Briefly, cells were cross-linked with 1% formaldehyde for 6 minutes and neutralized with glycine for 5 mins. Chromatin was sheared by sonication for 4 minutes. 10% chromatin was removed to be used as Input or unFAIRE material and remaining chromatin material was processed for FAIRE by phenol-chloroform extraction. Samples were subjected to qPCR and normalized to inputs and IgH Enhancer.

**Cell counts**

Cells were trypsinized at the indicated times. Cell counts were taken using the Scepter 2.0 Hand-Held Automated Cell Counter (Millipore, Billerica, MA).

**Statistical analysis**

Statistical significance was calculated by the student’s t-test.
2.3 Results

**JQ1 treatment inhibits proliferation by inducing cell cycle arrest**

In order to determine if BET proteins have a role in melanocyte differentiation, immortalized mouse melanoblasts, melb-a cells were differentiated in the presence and absence of the BET protein inhibitor JQ1. An equal number of melb-a cells were seeded onto polystyrene dishes and cultured in growth media. When cells grew to cover 70% of the dish, they were shifted to fresh growth media or to differentiation media. 500nM active enantiomer of JQ1 (+JQ1) or 500nM inactive enantiomer of JQ1 (-JQ1) was added to the cells that were maintained in growth media and to the cells that were shifted to differentiation media. Cells were then photographed using EVOS FL microscope (AMG, Bothell, WA) at 10X magnification at the indicated time points. Cells that were differentiated in the presence of the inactive enantiomer of JQ1 accumulated melanin granules while cells differentiated with active JQ1 accumulated fewer melanin granules, indicating that JQ1 inhibited melanin synthesis (Fig. 1A).

The images in Fig. 1A indicated that JQ1 also caused a reduction in cell proliferation. In order to assess the effects of JQ1 on proliferation, cell counts were obtained after melanoblasts that were differentiated in the presence or absence of JQ1 for the indicated time periods. JQ1 significantly suppressed proliferation (Fig. 1B).

We then conducted an Annexin Assay in order to gain insights into the anti-proliferative effect of JQ1 on melb-a cells. Melb-a cells were cultured in presence of active and inactive JQ1 and harvested at the time points indicated and subjected to Annexin Assay. Percent Annexin V positive cells indicate the percent of cells undergoing apoptosis. As observed, the exposure of cells to JQ1 resulted in minimal cell death and
the extent of cell death which was measured by percent annexin V positive cells was similar to the cells treated with inactive JQ1 (Fig. 1C). Therefore, treatment with JQ1 did not result in a significant increase in apoptotic cells.

To determine if the effects of JQ1 on proliferation were due to cell cycle changes, cells were stained with propidium iodide and subjected to flow cytometry. JQ1 promoted cell cycle arrest in G1 (Fig. 1D).

In order to further assess the effects of active isomer of JQ1 on melanin synthesis, melb-a cells were maintained in growth media and differentiation media in presence of a vehicle control (DMSO) or active JQ1 or were left untreated. Cells were harvested at the time points indicated in eppendorf tubes and the pellets were photographed. As was visible, untreated cells and the cells treated with vehicle control showed a similar extent of pigmentation upon differentiation whereas JQ1 treatment led to reduced pigmentation (Fig. 2A).

The melanin content vehicle treated and JQ1 treated cells was measured and normalized to cell number. JQ1 treated cells had a significantly lower melanin content compared to vehicle treated cells (Fig. 2B).

Similarly, neonatal human epidermal melanocytes (NHEMs) were treated with an active or inactive enantiomer of JQ1. Treatment of NHEMs with active JQ1 reduced the number of melanin granules and altered cell morphology (Fig. 3A).

Similarly to mouse melanoblasts, JQ1 did not cause a significant increase in apoptosis of NHEMs (Fig. 3B) but did promote the cell cycle arrest at G1 (Fig. 3C).

To further characterize the effects of JQ1 treatment on pigmentation of NHEMs, cells were treated with vehicle control or JQ1 and harvested at the indicated time points.
Cell pellets were collected and photographed. Cell pellets from JQ1-treated cultures were substantially lighter than control pellets, indicating that JQ1 reduced melanin synthesis (Fig. 3D).

**JQ1 treatment abrogates the expression of a subset of MITF target genes**

The data in Fig. 1A, Fig. 2 and Fig. 3D indicated that melanin synthesis was abrogated by treatment of both melb-a cells and NHEMs with JQ1. Therefore, we assessed the transcriptional effects of JQ1 on the Microphthalmia-Associated Transcription Factor (MITF) and on the expression of MITF target genes that regulate melanin synthesis. To assay the effects of JQ1 on gene expression at the protein level, total cell extracts were prepared from melb-a cells and NHEMs after treatment with JQ1 at the indicated time points and then subjected to Western Blotting. JQ1 treatment had no significant effect on the levels of MITF whereas the protein levels of MITF target genes tyrosinase and tyrosinase related protein 1 (TRP1) were decreased upon JQ1 treatment (Fig. 4A & Fig. 4C).

To assay the effects of JQ1 on gene expression at the mRNA level, RNA was isolated from melb-a cells and NHEMs at the time points indicated, reverse-transcribed, and subjected to quantitative PCR. JQ1 inhibited expression of tyrosinase and TRP1 at the mRNA level (Fig. 4B & Fig. 4D). Transient receptor cation channel protein 1 (TRPM1) is an MITF target gene that is associated with melanocyte differentiation [183]. We found that JQ1 also abrogated TRPM1 expression (Fig. 4B).

The cyclin dependent kinase inhibitor, p21 and the pro-survival gene, BCL2 are also MITF targets. Because treatment with JQ1 inhibited proliferation and cell cycle progression, we investigated the effects of JQ1 on the expression of p21 and BCL2. We
detected a significant increase in p21 mRNA levels when melb-a cells were differentiated with JQ1 (Fig. 4E). Interestingly, JQ1 treatment did not have a significant effect on expression of BCL2 (Fig. 4F). Thus, JQ1 has differential effects on different classes of MITF target genes.

**BRD4 occupies the promoter of MITF target genes and JQ1 treatment disrupts the enrichment of BRD4 and MITF on promoters of MITF target genes**

Previous studies have shown that JQ1 binds to BRD4 with higher affinity than to other BET proteins and disrupts the acetyl-lysine binding activity of BRD4. Since JQ1 treatment inhibits the expression of MITF target genes (Fig. 4), we assessed the occupancy of BRD4 on promoters of MITF target genes. By mining publicly available data sets, we analyzed a ChIP-Seq data set which was performed in the SK-MEL-5 melanoma cell line (GSM1121099). We detected peaks of BRD4 binding on a number of loci that regulate pigmentation (Fig. 5A). We then performed chromatin immunoprecipitation with antibodies to BRD4 (Fig. 5B & Fig. 5D) and MITF (Fig. 5C & Fig. 5E) on differentiated melb-a cells and NHEMs that were cultured in the presence of an inactive isomer of JQ1 (-JQ1) or the active isomer of JQ1 (+JQ1). BRD4 and MITF enrichment was quantified by qPCR using primers to the promoters of pigmentation genes (TRP1 and Tyrosinase in melb-a cells, TRP1 in NHEMs). It was observed that the occupancy of BRD4 and MITF on melanocyte specific promoters was disrupted when the cells were cultured in JQ1 (Fig. 5B-Fig. 5E).

**JQ1 disrupts the histone mark of H3K4me3 and reduces chromatin accessibility**

H3K4me3 is a histone modification mark known to be associated with transcriptionally active genes. Chromatin immunoprecipitations were performed with an
antibody to histone H3 trimethylated on lysine 4 (H3K4me3) on melb-a cells (Fig. 6A) and NHEMs (Fig. 6B) that were cultured in the presence of an inactive isomer of JQ1 (-JQ1) or the active isomer of JQ1 (+JQ1). H3K4me3 enrichment was quantified by qPCR using primers to the indicated promoters. The data indicates that JQ1 disrupts this epigenetic mark (Fig. 6A & Fig. 6B).

As JQ1 disrupted the active mark of transcription, we evaluated the effect of JQ1 on chromatin accessibility by performing the formaldehyde assisted isolation of regulatory elements (FAIRE) assay. Melb-a cells (Fig. 6C) and NHEMs (Fig. 6D) that were cultured in the presence of an inactive isomer of JQ1 (-JQ1) or the active isomer of JQ1 (+JQ1) were subjected to FAIRE analysis. The fold accessibility of chromatin was quantified by qPCR using primers to the indicated promoters. It was observed that JQ1 led to reduced chromatin accessibility at the melanocyte specific promoters (Fig. 6C & Fig. 6D).

**Knocking down BRD4 inhibits expression of genes that regulate melanin synthesis**

To determine if the effect of JQ1 is specific to BRD4, an RNA interference approach was utilized. To assay the effects of knocking down BRD4 on gene expression at the protein level, total cell extracts were prepared from differentiated mouse melanoblasts and were subjected to Western Blotting. Knocking down BRD4 had no significant effect on the expression level of MITF whereas MITF target genes of Tyrosinase and TRP1 were inhibited (Fig. 7A).

To assay the effects of knocking down BRD4 on gene expression at the mRNA level, RNA was isolated from differentiated mouse melanoblasts, reverse-transcribed, and
subjected to quantitative PCR. BRD4 knockdown inhibited expression of tyrosinase and TRP1 at the mRNA and protein levels (Fig. 7B & Fig. 7C).

**BRD4 knock down disrupts the active mark of transcription and reduces chromatin accessibility**

Chromatin immunoprecipitations were performed with an antibody to H3K4me3 on melb-a cells that were cultured in the presence of a control siRNA or siBRD4. H3K4me3 enrichment was quantified by qPCR using primers to the indicated promoters. The data indicates that knocking down BRD4 disrupts this epigenetic mark (Fig. 7D).

We also checked the effect of knocking down BRD4 on chromatin accessibility. Melb-a cells that were cultured in the presence of siC or siBRD4 were subjected to FAIRE analysis. The fold accessibility of chromatin was quantified by qPCR using primers to the indicated promoters. It was observed that BRD4 knockdown led to reduced chromatin accessibility at the melanocyte specific promoters (Fig. 7E).

**BRD4 physically interacts with MITF**

Our data indicated that BRD4 occupies the promoters of MITF target genes and that disruption of BRD4 activity with the BET inhibitor JQ1 led to down regulation of MITF target genes. Therefore, we investigated if BRD4 physically interacts with MITF. To assay for physical interactions between BRD4 and MITF, 293T cells were transfected with epitope-tagged (FLAG) MITF using Lipofectamine LTX (Life Technologies, Grand Island, N.Y.). Total cell extracts were immunoprecipitated with an antibody to BRD4 and were subjected to Western Blotting. The Western Blot was probed with an antibody to BRD4 (Abcam, Cambridge, MA) and an antibody to the FLAG epitope (Sigma-Aldrich,
St. Louis, MO). It was observed that BRD4 co-immunoprecipitated with FLAG-MITF suggesting a physical interaction of BRD4 with MITF (Fig. 8A).

Next, we probed for physical interactions between BRD4 and MITF in melb-a cells. The BRD4 antibody pulled down MITF in differentiated melb-a cells, suggesting a physical interaction between the two proteins (Fig. 8B).

2.4 Discussion

Melanocytes are cells located in the basal layer of the epidermis that produce the pigment melanin. Once melanin is produced, it is stored in melanosomes, which are transferred from melanocytes to keratinocytes, thus giving skin its characteristic color. Disruption of normal melanocyte function can lead to skin cancer and other skin diseases that affect the level of pigmentation. Our studies identified BRD4, a BET bromodomain protein, as a possible therapeutic target for treatment of hyperpigmentation disorders. We propose a novel mechanism where BRD4 is essential for carrying out the process of melanocyte differentiation and pigmentation by regulating the transcription of genes essential for melanin synthesis. We utilized mouse melanoblasts (melb-a) cells and physiologically relevant neonatal human epidermal melanocytes (NHEMs) to test our hypothesis. JQ1, a well known BET bromodomain inhibitor was used in our studies to block the activity of BRD4. We observed that JQ1 treatment led to a reduced proliferation in our cell lines. We performed annexin assay and cell cycle assay using flow cytometry analysis to check for the cause of reduced proliferation in these cell lines and detected that JQ1 did not affect apoptosis, but caused cell cycle arrest at G1 phase. Dysfunction of melanin can result in a number of hyperpigmentation disorders. Upon differentiation, melb-a cells accumulated melanin granules and increased pigmentation
which was evident from our cell images. We observed a reduction in the number of melanin granules accumulated after treating the cells with JQ1. Inhibiting the BET bromodomain activity with JQ1 led to down regulation of melanin pigment.

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte differentiation which is activated by α-MSH binding to the MC1R receptor. MITF regulates transcription of a wide variety of genes with implications in cell survival, melanogenesis and cell cycle arrest. The melanocyte-specific enzyme, tyrosinase (TYR), is required for the rate-limiting step of melanin synthesis while two other member of the tyrosinase family, tyrosinase related protein 1 (TRP1) and dopachrome tautomerase (DCT), regulate the type of melanin synthesized. Treating the cells with JQ1 led to down regulation of pigment specific genes. However, although the effects of JQ1 on gene expression of TRP1 was profound and consistent in Melb-a as well as NHEM cell lines, tyrosinase gene expression seems to be differentially regulated in these cell lines, which was evident from the fact that tyrosinase was affected at a much later time point in case of NHEMs. We assessed the binding sites of BRD4 by analyzing a ChIP-seq data and found out that BRD4 occupied the promoters of MITF target genes. JQ1 treatment disrupts the binding of BRD4 and MITF to the promoters of pigment-specific genes.

H3K4me3 is an active mark of transcription which was disrupted by treating the cells with BET inhibitor, JQ1. Lysine acetylation is a predominant post translational modification which is characterized by an open chromatin structure. BRD4, with its bromodomain modules recognizes the acetyl-lysine residues. We detected that JQ1 treatment led to reduced chromatin accessibility. Since JQ1 inhibits BET bromodomain family members, we wanted to assess if the effects on gene expression were recapitulated
by siRNA mediated silencing of BRD4. Indeed, knocking down BRD4 led to similar effects as observed upon treating the cells with JQ1. Next, we wanted to assess the interaction between BRD4 and MITF since JQ1 treatment, as well as knocking down BRD4, led to down-regulation of MITF target genes and disrupted the occupancy of MITF on the promoters of pigment-specific genes. We carried out a co-immunoprecipitation assay in 293T’s and differentiated melb-a cells and detected a physical interaction between the two proteins. Thus, we hypothesized that BRD4 plays a crucial role in melanogenesis by interacting with MITF and can be a potential novel target for treatment of hyperpigmentation disorders.
2.5 Figure Legends

Fig.1. JQ1 treatment inhibits proliferation by inducing cell cycle arrest in mouse melanoblasts.

(A) Representative mouse melanoblasts (melb-a) cell images taken with EVOS FL microscope at 10X magnification at indicated time points in presence of inactive and active isomer of JQ1.

(B) In order to assess the effect of JQ1 on proliferation, treated Melb-a cells were harvested at the time points indicated and counted with a Scepter 2.0 Hand-Held automated cell counter. Cell counts plotted are the average of 3 independent experiments and statistical significance was calculated. * p<0.05, ** p<0.01.

(C) Melb-a cells were cultured in the presence of inactive and active isomer of JQ1 for the indicated time points. Cells were then harvested by trypsinization and stained with Annexin V and subjected to flow cytometry. Percent Annexin V positive cells indicate the percentage of cells undergoing apoptosis. The percentages plotted are the average of 3 independent experiments. Standard error bars are shown.

(D) To determine if the effects of JQ1 on proliferation were due to cell cycle changes, Melb-a cells were harvested at indicated time points and stained with propidium iodide and subjected to flow cytometry on a FACS-Calibur (BD Biosciences, San Jose, CA, USA at the University of Toledo Flow Cytometry Core Facility). Data was analyzed using Cell Quest Pro (BD Biosciences). Percentage of cells in G1, S
and G2 phases were plotted and statistical significance was calculated.

***p<0.001

Fig.2. JQ1 inhibits melanin synthesis.

(A) Melb-a cells were left untreated, treated with vehicle control (DMSO) and active isomer of JQ1 and harvested at the indicated time points. Cells were collected in eppendorf tubes and pellets were photographed.

(B) Harvested cells in Fig. 2A which were cultured with vehicle control and active isomer of JQ1 were subjected to melanin assay and melanin content per cell (µg/µl) was plotted.

Fig.3. JQ1 inhibits proliferation, pigmentation and induces cell cycle arrest in neonatal human epidermal melanocytes.

(A) Representative neonatal human epidermal melanocyte (NHEM) cell images taken with EVOS® microscope at 10X magnification at indicated time points in presence of inactive and active isomer of JQ1.

(B) NHEM cells were cultured in the presence of inactive and active isomer of JQ1 for the indicated time points. Cells were then harvested by trypsinization and stained with Annexin V and subjected to flow cytometry. Percent Annexin V positive cells indicate the percentage of cells undergoing apoptosis. The percentages plotted are the average of 3 independent experiments. Standard error bars are shown.
(C) To determine if the effects of JQ1 on proliferation were due to cell cycle changes, NHEM cells were harvested at indicated time points and stained with propidium iodide and subjected to flow cytometry on a FACS-Calibur (BD Biosciences, San Jose, CA, USA at the University of Toledo Flow Cytometry Core Facility). Data was analyzed using Cell Quest Pro (BD Biosciences). Percentage of cells in G1, S and G2 phases were plotted and statistical significance was calculated.

(D) NHEM cells were treated with vehicle control (DMSO) and active isomer of JQ1 and harvested at the indicated time points. Cells were collected in eppendorf tubes and pellets were photographed.

Fig.4. JQ1 abrogates the expression of pigmentation genes in Melb-a and NHEM cells

(A) Total cell extracts were prepared from Melb-a cells harvested at the time points indicated and were subjected to Western Blotting. Antibodies to detect Tyrosinase and TRP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to detect MITF was from Abcam (Cambridge, MA) and the loading control, Tubulin was from Cell Signaling (Beverly, MA, USA). A representative blot is shown.

(B) RNA was isolated from Melb-a cells at time points indicated, reverse-transcribed, and subjected to quantitative PCR. Primers for mouse Tyrosinase, TRP1 and TRPM1 were obtained from IDT. Relative mRNA expression levels were normalized to RPL7 as a control and are shown relative to undifferentiated sample treated with inactive isomer of JQ1. Standard error bars and statistical significance are shown. * $p<0.05$, ** $p<0.01$, ***$p<0.001$
(C) Total cell extracts were prepared from NHEM cells harvested at the time points indicated and were subjected to Western Blotting. Antibodies to detect Tyrosinase and TRP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to detect MITF and the loading control β-Actin was from Abcam (Cambridge, MA). A representative blot is shown.

(D) RNA was isolated from NHEM cells at time points indicated, reverse-transcribed, and subjected to quantitative PCR. Primers for human TRP1 were obtained from IDT. Relative mRNA expression levels were normalized to RPL19 as a control and are shown relative to sample untreated with MSH. Standard error bars and statistical significance are shown. ***p<0.001

(E) Total RNA was isolated as in Fig. 1D, reverse-transcribed, and subjected to qPCR with primers to p21 and RPL7. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown and statistical significance was calculated. * p<0.05

(F) Total RNA from Melb-a cells treated with active and inactive isomer of JQ1 were harvested at the indicated time points, reverse-transcribed, and subjected to qPCR with primers to BCl2 and RPL7. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown.
Fig.5. BRD4 occupies the promoter of MITF target genes and JQ1 treatment disrupts the enrichment of BRD4 and MITF on promoters of MITF target genes

(A) ChIP-seq (GSM1121099) shows BRD4 peaks on several loci important for melanogenesis. This ChIP-seq data was available in GEO profiles performed on SK-MEL5 melanoma cells. TSS: Transcriptional start site

(B) Chromatin immunoprecipitation (ChIPs) were performed with an antibody to BRD4 and a control antibody IgG in Melb-a cells differentiated for 48 hours in presence of active and inactive isomer of JQ1. Fold enrichment of BRD4 on the TRP1 and Tyrosinase Promoter is shown relative to a control IgH Enhancer region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. ** p<0.01

(C) Chromatin immunoprecipitation (ChIPs) were performed with an antibody to MITF and a control antibody IgG in Melb-a cells differentiated for 48 hours in presence of active and inactive isomer of JQ1. Fold enrichment of MITF on the TRP1 and Tyrosinase Promoter is shown relative to a control IgH Enhancer region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. * p<0.05, ***p<0.001

(D) Chromatin immunoprecipitation (ChIPs) were performed with an antibody to BRD4 and a control antibody IgG in NHEM cells cultured in presence of active and inactive isomer of JQ1 for 96 hours. Fold enrichment of BRD4 on the TRP1 Promoter is shown relative to a control CD25 region. The data are representative
of three independent experiments. Standard error bars and statistical significance are shown. ***p<0.001

(E) Chromatin immunoprecipitation (ChIPs) were performed with an antibody to MITF and a control antibody IgG in NHEM cells cultured in presence of active and inactive isomer of JQ1 for 96 hours. Fold enrichment of MITF on the TRP1 Promoter is shown relative to a control CD25 region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. ***p<0.001

**Fig.6. JQ1 disrupts the H3K4me3 enrichment and reduces chromatin accessibility**

(A) Chromatin immunoprecipitation (ChIPs) were performed with an antibody to H3K4me3 and a control antibody histone H3 in Melb-a cells differentiated for 48 hours in presence of active and inactive isomer of JQ1. Fold enrichment of H3K4me3 on the TRP1 and Tyrosinase Promoter is shown relative to a control IgH Enhancer region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. **p<0.01, ***p<0.001

(B) Chromatin immunoprecipitation (ChIPs) were performed with an antibody to H3K4me3 and a control antibody histone H3 in NHEM cells cultured in presence of active and inactive isomer of JQ1 for 96 hours. Fold enrichment of H3K4me3 on the TRP1 Promoter is shown relative to a control CD25 region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. ***p<0.001
(C) Chromatin accessibility assay was performed using FAIRE technique in Melb-a cells differentiated for 48 hours in presence of active and inactive isomer of JQ1. Fold accessibility on the TRP1 and Tyrosinase Promoter is shown relative to a control IgH Enhancer region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. *** p<0.001

(D) Chromatin accessibility assay was performed using FAIRE technique in NHEM cells cultured in presence of active and inactive isomer of JQ1 for 96 hours. Fold accessibility on the TRP1 Promoter is shown relative to a control CD25 region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. ** p<0.01

Fig.7. Knocking down BRD4 inhibits expression of pigmentation genes, disrupts the active mark of transcription and reduces chromatin accessibility

(A) Total cell extracts were prepared from Melb-a cells cultured in presence of a control scrambled siRNA or specific siRNA against BRD4. Melb-a cells were subjected to siRNA containing growth media for 72 hours and then the media was changed to differentiation media. Melb-a cells were harvested 48 hours post differentiation and were subjected to Western Blotting. Antibody to detect BRD4 and MITF was from Abcam (Cambridge, MA). Antibodies to detect Tyrosinase and TRP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for the loading control Tubulin was from Cell Signaling (Beverly, MA, USA). A representative blot is shown.
(B) RNA was isolated from Melb-a cells cultured in presence of a control scrambled siRNA or specific siRNA against BRD4. Melb-a cells were subjected to siRNA containing growth media for 72 hours and then the media was changed to differentiation media. Melb-a cells were harvested 48 hours post differentiation and were subjected to trizol extraction. RNA was reverse-transcribed, and subjected to quantitative PCR. Primers for mouse BRD4 was obtained from IDT. Relative mRNA expression levels were normalized to RPL7 as a control and are shown relative to sample treated with control siRNA. Standard error bars and statistical significance are shown. ***p<0.001

(C) RNA was isolated as in Fig. 7B, reverse-transcribed, and subjected to quantitative PCR. Primers for mouse Tyrosinase and TRP1 was obtained from IDT. Relative mRNA expression levels were normalized as in Fig. 7B. Standard error bars and statistical significance are shown. * p<0.05, **p<0.01

(D) Melb-a cells were cultured in presence of a control scrambled siRNA or specific siRNA against BRD4. Melb-a cells were subjected to siRNA containing growth media for 72 hours and then the media was changed to differentiation media. Melb-a cells were harvested 48 hours post differentiation and were subjected to chromatin immunoprecipitation(ChIPs). ChIP were performed with an antibody to H3K4me3 and a control antibody histone H3. Fold enrichment of H3K4me3 on the TRP1 and Tyrosinase Promoter is shown relative to a control IgH Enhancer region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. * p<0.05
(E) Melb-a cells were harvested as described in Fig 7D. FAIRE chromatin accessibility assay was performed. Fold accessibility on the TRP1 and Tyrosinase Promoter is shown relative to a control IgH Enhancer region. The data are representative of three independent experiments. Standard error bars and statistical significance are shown. ***p<0.001

Fig.8. BRD4 physically interacts with MITF

(A) 293T cells were transfected with epitope-tagged (FLAG) MITF using Lipofectamine LTX (Life Technologies, Grand Island, NY). Total cell extracts were immunoprecipitated with an antibody to BRD4 and were subjected to Western Blotting. The Western Blot was probed with an antibody to BRD4 (Abcam, Cambridge, MA) and an antibody to the FLAG epitope (Sigma Aldrich, St. Louis, MO).

(B) Melb-a cells differentiated for 48 hours were harvested and whole cell extracts were used for immunoprecipitation with an antibody to BRD4 or a control IgG. The immunoprecipitated material was subjected to Western Blotting and immunoblotted for MITF and BRD4. 10% of the input was loaded on the gel.
2.6 FIGURES

Figure 1

A. 

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B. 

Cell Count ($\times 10^6$)

- Melba - JQ1
- Melba + JQ1

Time post differentiation

C. 

Annexin Assay in Melb-a

Percent Annexin V Positive Cells

- Melba - JQ1
- Melba + JQ1

Time Intervals

D. 

- G2
- S
- G1

Time Intervals

- 24HR - JQ1
- 24HRS + JQ1
- 48HR - JQ1
- 48HRS + JQ1

Percentages: ***
**Figure 2**

A. Day 0 | Day 1 | Day 2
---|---|---
UN | VC | JQ1

Day 3
---
UN | VC | JQ1

Day 4
---
UN | VC | JQ1

Day 5
---
UN | VC | JQ1

B. Melanin Content (μg/μl)

**Figure 3**

A. UN-Utreated
VC-Vehicle Control

B. Percent Annexin V Positive Cells

---

64
**Figure 4**

### D.

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VC – Vehicle Control

### C.

![Bar chart comparing different days and treatments](chart.png)

### A.

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- MITF
- TRP1
- TYROSINASE
- TUBULIN

65
Figure 5

A.

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Figure 6

A. Fold Enrichment of H3K4me3 on TRP1 Promoter

B. Fold Enrichment of H3K4me3 on TRP1 Promoter

C. TRP1 Promoter

D. Fold Enrichment of BRD4 on TRP1 Promoter

E. Fold Enrichment of MITF on TRP1 Promoter
Figure 7

A. Differentiated

B. BRD4

C. TYROSINASE and TRP1
Figure 8
Chapter 3

The PBAF complex promotes expression of p53 target genes in response to ultraviolet radiation in melanoma

3.1 Introduction

Ultraviolet (UV)-radiation causes DNA damage and is implicated in the etiology of cutaneous melanoma, an aggressive malignancy that is notoriously chemoresistant. SWI/SNF chromatin remodeling enzymes are multi-protein complexes composed of one of two related ATPases, BRG1 or BRM, and 9-12 associated factors (BAFs), that have critical functions in the regulation of gene expression. SWI/SNF complexes have been categorized as BAF and PBAF based on purified complexes from mammalian cells. The PBAF complex contains only BRG1 as the catalytic subunit and includes specific subunits namely BAF180 (Polybromo), BRD7, and ARID2. Importantly, PBAF specific SWI/SNF subunits are disrupted in a number of human cancers including melanoma. We were interested in assessing a potential tumor suppressive role of PBAF complexes in the context of melanocytes and melanoma cells. We hypothesized that the PBAF specific SWI/SNF complex mediates tumor suppression by activating p53 target genes in
response to UV-radiation. ARID2 has been identified as a gene that is frequently mutated in melanoma. We surveyed a panel of melanoma cell lines and found that a subset of melanoma cells is deficient in the expression of the other PBAF specific subunits, BAF180 and BRD7. Our data indicate that BRG1, BAF180, BRD7, and ARID2 promote expression of multiple p53 target genes as well as cell cycle arrest in UV-irradiated melanocytes and melanoma cells. BRG1, the central ATPase in PBAF SWI/SNF complex promoted cell cycle arrest in melanoma cells. Thus, our work suggests that a specific configuration of the SWI/SNF complex may have a tumor suppressive role in melanocytes and that aberrant expression or mutations of SWI/SNF components may contribute to melanoma tumorigenicity.

3.2 Materials and methods

Cell lines

Mouse melanoblast cells (Melb-a) were obtained from Dr. Dorothy Bennett (The Welcome Trust, UK). The growth media consisted of RPMI1640 (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum (Life Technologies, Grand Island, N.Y.), 20ng/ml stem cell factor, and 20 picoM fibroblast growth factor 2. When cells grew to cover 70% of the dish, they were shifted to differentiation media. The differentiation media consisted of DMEM (Life Technologies, Grand Island, N.Y.), 10% fetal calf serum, 200 nMphorbal 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) and 2 nM Nle4, D-Phe7-alpha-MSH (Sigma-Aldrich, St. Louis, MO). Neonatal human epidermal melanocytes (NHEMs) were isolated as described in [180] and cultured in Media 254
SK-MEL5, WM-266-4, A375, SK-MEL147, MEL224 and MEL505 melanoma cells were obtained from ATCC. YUMAC melanoma cell lines were from Yale Cell Culture Core Facility (New Haven, CT, USA). SK-MEL5 cells expressing an empty vector or BRG1 were described in [18]. Melanoma cell lines were cultured as described in [18].

**UV-irradiation**

Melanoma cells were irradiated with a lamp that emits 75% in the UVB range and 25% in the UVA range (National Biologics, Twinsburg, OH, USA) at dose of 50mJ/cm². UVC was blocked with a Kodacel sheet (Eastman Kodak, Rochester, NY, USA).

**RNA isolation and quantitative real time PCR**

Total RNA was isolated using Trizol (Invitrogen) and cDNA was prepared using the Qiagen Quantitect Reverse Transcription kit. Quantitative PCR (qPCR) was performed in SYBR Green master mix (Qiagen) with an Applied Biosystems 7500 PCR and analyzed with the SDS software as described [18]. Mouse p21 mRNA levels were normalized to mouse RPL7. Human p21, GADD45A, p53, BAF180 and BRD7 mRNA levels were normalized to human GAPDH. Primers for human GADD45A and BRD7 were obtained from SABiosciences (Qiagen). All the other human primers were obtained from Integrated DNA Technologies (IDT).
Primer sequences for mouse melanoblasts are as follows:

\[ \text{p21: 5'} - \text{ACA CAC AGA GAG AGG GCT AAG G} - 3' \quad \text{and} \quad 5' - \text{AGA TCC ACA GCG ATA TCC AGA C} - 3' \]

\[ \text{RPL7: 5'} - \text{GGAGGAAGCTCATCTATGAGAAGG} - 3' \quad \text{and} \quad 5' - \text{AAG ATC TGT GGA AGA GGA AGG AGC} - 3' \]

Primer sequences for human melanocytes and melanoma cell lines are as follows:

\[ \text{p21: 5'} - \text{GGA TGT CCG TCA GAA CCC A} - 3' \quad \text{and} \quad 5' - \text{CAG GTC CAC ATG GTC TTC C} - 3' \]

\[ \text{p53: 5'} - \text{AGA ACC AAC ACG ACG TGG CTG C} - 3' \quad \text{and} \quad 5' - \text{GCC AGA CTG AGG CAA GTG AGG G} - 3' \]

\[ \text{BAF180: 5'} - \text{TGC TGC AGG CCT CTC AGG CT} - 3' \quad \text{and} \quad 5' - \text{TCA CCA GCT GAA TCC TCC CAC AGT} - 3' \]

\[ \text{GAPDH: 5'} - \text{TGG TCA CCA GGG CTG CTT TT} - 3' \quad \text{and} \quad 5' - \text{GGT GAA GAC GCC AGT GGA CT} - 3' \]

\text{siRNA knockdown}

siRNA targeting mouse ARID2 and a non-targeting siRNA were obtained from
Dharmacon. Transfection was performed according to manufacturer’s instructions. Media was replaced with the differentiation media consisting of DMEM (Life Technologies, Grand Island, N.Y.), 10% fetal bovine serum, 200 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) and 2 nM Nle4, D-Phe7-alpha-MSH (Sigma-Aldrich, St. Louis, MO) 72 hours post transfection and cells were then harvested 48 hours later and subjected to Western blotting and Quantitative real time PCR analysis. siRNAs targeting human p53, BAF180, BRD7 and ARID2 and a non-targeting siRNA were obtained from Dharmacon (Lafayette, CO, USA) and transfected according to the manufacturer’s instructions. Cells were UV-irradiated 72 hr after siRNA transfection and assayed by Western blotting and Quantitative real time PCR, 48 hr after irradiation.

**Cell extracts and immunoblot analysis**

Cell extracts were prepared and Western Blotting was performed as described [18]. The BRG1 antiserum was previously described in [17]. The BAF47 antibody was from BD Transduction Laboratories (BD Biosciences, San Jose, CA). The BAF180 antibody was from Bethyl Labs (Montgomery, TX, USA). The p21, p53 and ARID2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The BRD7 antibody was from Proteintech Group (Chicago, IL, USA). The Tubulin antibody was from Cell Signaling (Beverly, MA, USA).

**Co-immunoprecipitations**

SK-MEL5 cells expressing BRG1 were transfected with FLAG tagged BRG1 construct
using Lipofectamine LTX (Invitrogen, Carlsbad, CA). Cells were harvested 48 hours post-transfection. Co-immunoprecipitations were performed as previously described [18]. Species matched IgG (Santa Cruz Technologies) was used as a control.

**Propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS)**

Approximately 1 x 10^6 cells were fixed with 100% ethanol for 1 h, stained with PI-RNase solution for 30 min and loaded on a FACS-Calibur (BD Biosciences, San Jose, CA) at the University of Toledo Flow Cytometry Core Facility. Data was analyzed using Cell Quest Pro (BD Biosciences).

**Statistical analysis**

Statistical significance was calculated by the student’s t-test.

### 3.3 Results

**Expression levels of PBAF specific SWI/SNF components**

We assessed the expression levels of PBAF specific SWI/SNF components in a panel of established melanoma cell lines and compared them with the neonatal human epidermal melanocytes (NHEM). We found that the levels of BRD7 were down regulated to a certain extent in all the melanoma cell lines as compared to NHEMs. BRG1 expression levels were almost similar in all the cell lines with the exception of SK-MEL-5 cells expressing empty vector. ARID2 and BAF180 protein expression levels were varying in different cell lines. However, it was observed that SK-MEL-5 cells expressing BRG1 had
increased expression of all the PBAF specific SWI/SNF components suggestive of stabilizing PBAF SWI/SNF complex in this cell line (Fig 1).

**BRG1 promotes cell cycle arrest in response to UV-irradiation**

To determine whether BRG1 also protects against UV-induced DNA damage, SK-MEL-5 cells expressing either empty vector or BRG1 were subjected to sham or UV-irradiation. Cells were harvested 48 hours post irradiation and subjected to cell cycle analysis using flow cytometry. It was observed that SK-MEL-5 cells expressing BRG1 led to cell cycle arrest at G1 phase post UV-irradiation (Fig 2).

**BRG1 promotes expression of a subset of p53 target genes**

The p53 pathway activates upon induction of DNA damage. We irradiated SK-MEL-5 cells expressing either empty vector or BRG1 with a UV lamp. We performed a time course of protein expression and found that p53 accumulated equally in control and BRG1 expressing cells. We have previously determined that re-expression of BRG1 in SK-MEL-5 cells promotes expression of cyclin-dependent kinase inhibitor and cell cycle arrest gene, p21 [18]. We observed that p21 accumulated at higher levels in BRG1 expressing cells at all time points following UV-irradiation (Fig 3). At the RNA level, BRG1 promoted 3-4 fold increase in p21 levels compared to control cells that were sham-irradiated. Although p21 expression was induced by UV-irradiation in both control as well as BRG1 expressing SK-MEL-5 cells, the levels were higher in UV-irradiated BRG1 expressing cells (Fig 4). GADD45A is a p53 target gene which has been
implicated in growth arrest and DNA-damage. BRG1 significantly increased the expression of GADD45A at the mRNA level in both sham and UV-irradiated cells with the greatest effects observed in UV-irradiated BRG1 expressing SK-MEL-5 cells (Fig 4).

**Knocking down p53 abrogates BRG1 mediated activation of a subset of p53 target genes**

To determine if the observed BRG1 dependent increase in the expression of p21 and GADD45A in response to UV-irradiation was dependent on p53, we introduced a short-hairpin RNA targeting p53 in sham and UV-irradiated SK-MEL-5 cell stably expressing BRG1. We observed an increase in p53 and subsequent increase in p21 expression levels in the UV-irradiated cells treated with control siRNA. We detected a decrease in p21 expression at the protein level in both sham and UV-irradiated cells (Fig 5A). Furthermore, expression of p21 and GADD45A was significantly decreased at the mRNA level in UV-irradiated SK-MEL-5 cells expressing BRG1 (Fig 5B).

**BAF180 interacts with BRG1 and stabilizes PBAF SWI/SNF complex in melanoma cells**

BAF180 has been implicated as a tumor suppressor that is mutated in breast and renal cell carcinomas and as a SWI/SNF subunit that promotes p53 transcriptional activity [129, 172, 173]. To determine whether BAF180 is involved in BRG1 mediated activation of p53 target gene expression in melanoma cells, we compared expression of BAF180 on sham or UV-irradiated control SK-MEL-5 cells and SK-MEL-5 cells that express BRG1.
We observed that BAF180 protein expression was increased in BRG1 expressing cells but was not dependent on UV-irradiation, whereas protein expression of the BAF47 subunit of the SWI/SNF complex was unaffected (Fig 6A). Protein-protein interactions among SWI/SNF subunits have been shown to regulate the stability of the SWI/SNF components [184]. BAF180 was co-immunoprecipitated with BRG1, suggesting that BRG1 stabilized BAF180 protein levels by restoring the PBAF complex in these melanoma cells (Fig 6B).

**Requirement of BAF180 in BRG1-mediated activation of a subset of p53 target genes**

In order to investigate whether BAF180 is required for the observed BRG1-dependent increase in the expression of p21 and other p53 target gene, we introduced a short-hairpin RNA that targets BAF180 in SK-MEL-5 cells expressing BRG1. Down-regulation of BAF180 resulted in a small reduction in p53 protein levels in sham-irradiated cells but not in UV-irradiated cells, whereas p21 protein levels were affected on knocking down BAF180 in sham as well as UV-irradiated cells (Fig 7A). Moreover, knocking down BAF180 compromised the expression of p21 and GADD45A at the mRNA level in SK-MEL5- cells expressing BRG1 (Fig 7B).

**PBAF specific SWI/SNF components are required for activation of a subset of p53 target genes**

To assess if BAF180 is required for p21 expression in other cells, we utilized WM-266-4
cells which have high levels of PBAF specific SWI/SNF subunits. Upon knocking down BAF180, BRD7 (Fig 8A) and ARID2 (Fig 8C) individually in WM-266-4 cell line, we detected significant down regulation of the p53 target genes, p21, and GADD45A (Fig 8B and Fig 8D).

**Knocking down ARID2 compromised the activation of p53 target genes in human melanocytes and mouse melanoblasts**

Finally, to investigate the effects of knocking down the ARID2 component of PBAF SWI/SNF complex in NHEMs and Melb-a cells, we introduced short hairpin RNA that targets ARID2 in NHEMs (Fig 9A) and Melb-a cells (Fig 9C). Down regulation of ARID2 significantly compromised the expression of p21 (Fig 9C and Fig 9D).

### 3.4 Discussion

UV-radiation induces DNA damage and is the major environmental risk factor for all skin cancers, including melanoma [185]. The DNA damage response is a multi-step process that involves activation of cell cycle checkpoints and DNA repair. The response to DNA damage involves activation of the p53 pathway, which promotes expression of p21 and cell cycle arrest at the G1 phase. p21 is a p53 trans-activation target that mediates p53 driven G1 arrest in response to DNA damage [186]. In spite of extensive evidence from animal models in support of p53 as a tumor suppressor in melanoma initiation and progression, the majority of human melanomas retain wild-type p53. Components of the PBAF SWI/SNF complex have been implicated in the regulation of
DNA damage response by their role in transcriptional activation of p53 target genes. BRG1, the ATPase in PBAF SWI/SNF complex, has been shown to promote melanoma differentiation as well as to increase survival of melanoma cells that were exposed to a chemotherapeutic agent or to UV radiation [18, 171]. To determine if BRG1 protects against UV-induced DNA damage, we carried out a cell cycle analysis wherein SK-MEL-5 cells expressing empty vector or BRG1 were subjected to sham and UV-irradiation and harvested 48 hours post treatment. Harvested cells were processed for flow cytometry analysis and it was observed that BRG1 containing SK-MEL-5 cells exhibited a significant cell cycle arrest at G1 phase. It suggested that BRG1 promotes an anti-proliferative effect in response to UV-irradiation. p21 is one of the critical regulators of DNA damage response and a cyclin-dependent kinase inhibitor. We observed that p21 accumulated at higher level in BRG1 expressing cells at all the time points following UV-irradiation. Thus, in melanoma cells, BRG1 modulates the response to UV-radiation by promoting expression of cell cycle regulators involved in check point control and DNA damage. This function was highly dependent on the BAF180 subunit of the SWI/SNF complex. BAF180 is a bromodomain containing subunit of the PBAF complex which exclusively contains BRG1 as the catalytic subunit. Recent studies have implicated BAF180 as a tumor suppressor in several human cancers [129, 172, 173]. We detected an increase in the expression levels of BAF180 in BRG1 expressing cells independent of UV treatment. BAF180 was shown to be interacting with BRG1 suggesting the stabilization of PBAF SWI/SNF complex in these cells. We could assess the direct interactions between BAF180 and BRG1 using yeast-two hybrid assay or GST pull-down
Our data indicate that depletion of BAF180 compromises p53 target gene expression in melanoma indicating that it may play a tumor suppressive role in melanoma. BRD7 and ARID2 are also PBAF specific SWI/SNF subunits [166]. We elucidated the requirement of these components in regulating the expression of a subset of p53 target genes in melanoma cell lines. Our data indicates that BRD7 is downregulated in melanoma cell lines as compared to melanocytes suggestive of its tumor suppressive role in melanoma. Two genome-wide sequencing studies reported that mutations in ARID2 occur in melanoma at a significant frequency and may be driver mutations [176, 187]. There is a lack of knowledge into the biological functions of ARID2 in melanocytes and how ARID2 might act as a tumor suppressor. Our data with neonatal human epidermal melanocytes and mouse melanoblast indicates that knocking down ARID2 compromised p21 gene expression. Thus, our work suggests that a specific configuration of the SWI/SNF complex may have a tumor suppressive role in melanocytes and that aberrant expression or mutations of SWI/SNF components may contribute to melanoma tumorigenicity.
3.5 Figure Legends

Fig. 1. Expression levels of PBAF specific SWI/SNF components in a panel of melanoma cell lines compared to neonatal human epidermal melanocytes

Total cell extracts were prepared from NHEM cells and melanoma cell lines indicated and were subjected to Western Blotting. Antiserum to detect BRG1 was described [17]. The BAF180 antibody was from Bethyl Labs (Montgomery, TX, USA). The ARID2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The BRD7 antibody was from Proteintech Group (Chicago, IL, USA). The Tubulin antibody was from Cell Signaling (Beverly, MA, USA). A representative blot is shown.

Fig. 2. BRG1 promotes cell cycle arrest in response to UV-irradiation.

SK-MEL5 cells stably expressing either empty vector (EV) or BRG1 were sham-irradiated or UV-irradiated for 48 hours. Cells were then harvested, stained with propidium iodide and subjected to flow cytometry on a FACS-Calibur (BD Biosciences, San Jose, CA, USA at the University of Toledo Flow Cytometry Core Facility). Data was analyzed using Cell Quest Pro (BD Biosciences). Percentage of cells in G1, S and G2 phases were plotted and statistical significance was calculated.

Fig. 3. DNA damage response upon UV-irradiation.

SK-MEL5 cells stably expressing either empty vector (EV) or BRG1 were sham-irradiated or UV-irradiated. Cell extracts were prepared 0, 2, 12, 24, and 48 hrs after
irradiation and subjected to Western Blotting with antibodies to BRG1, p53 and p21. Tubulin is a loading control. A representative blot is shown.

**Fig. 4. BRG1 promotes expression of a subset of p53 target genes**

SK-MEL5 cells stably expressing either empty vector (EV) or BRG1 were sham-irradiated or UV-irradiated. Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to p21, GADD45A and GAPDH. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. **p <0.01**

**Fig. 5. Down-regulation of p53 abrogates BRG1 mediated activation of p53 target genes.**

(A) Total cell extracts were prepared from SK-MEL5 cells stably expressing BRG1 which were cultured in presence of a control siRNA or specific siRNA against p53. Cells were either sham-irradiated or UV-irradiated 72 hr after siRNA transfection and assayed by Western blotting 48 hr after irradiation for antibodies p53 and p21. Tubulin is a loading control. A representative blot is shown.

(B) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to p53, p21, GADD45A and GAPDH for samples harvested as in Fig 5A. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. **p <0.01**
**Fig. 6. Stabilization and interaction of BAF180 with BRG1.**

(A) SK-MEL5 cells stably expressing either empty vector (EV) or BRG1 were sham-irradiated or UV-irradiated. Cell extracts were prepared 0, 2, 12, 24, and 48 hrs after irradiation and subjected to Western Blotting with antibodies to BRG1, BAF180 and BAF47. Tubulin is a loading control. A representative blot is shown.

(B) SK-MEL5 cells stably expressing BRG1 were transfected with epitope-tagged (FLAG) BRG1 using Lipofectamine LTX (Life Technologies, Grand Island, NY). Total cell extracts were immunoprecipitated with an antibody to BRG1 and were subjected to Western Blotting. The Western Blot was probed with an antibody to BRG1, BAF47 and BAF180. 10% of the input was loaded on the gel.

**Fig. 7. BAF180 is indispensable in BRG1-mediated activation of subset of p53 target genes.**

(A) Total cell extracts were prepared from SK-MEL5 cells stably expressing BRG1 which were cultured in presence of a control siRNA or specific siRNA against BAF180. Cells were either sham-irradiated or UV-irradiated 72 hr after siRNA transfection and assayed by Western blotting 48 hr after irradiation for antibodies BAF180, p53 and p21. Tubulin is a loading control. A representative blot is shown.

(B) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to BAF180, p21, GADD45A and GAPDH for samples harvested as in Fig
7A. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. *p <0.05, **p <0.01

Fig. 8. PBAF specific SWI/SNF components are required in mediating activation of p53 target genes.

(A) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to BAF180, BRD7 and GAPDH for samples which were prepared from WM-266-4 cells which were cultured in presence of a control siRNA or specific siRNA against BAF180 or BRD7. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. *p <0.05, **p <0.01

(B) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to p21, GADD45A, and GAPDH for samples which were prepared from WM-266-4 cells which were cultured in presence of a control siRNA or specific siRNA against BAF180 or BRD7. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. *p <0.05, **p <0.01

(C) Total cell extracts were prepared from WM-266-4 cells cultured in presence of a control siRNA or specific siRNA against ARID2. Cells were harvested 72 hr after siRNA transfection and assayed by Western blotting for antibody against ARID2. Tubulin is a loading control. A representative blot is shown.
(D) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to p21, GADD45A, and GAPDH for samples which were prepared from WM-266-4 cells which were cultured in presence of a control siRNA or specific siRNA against ARID2. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. **p < 0.01, ***p < 0.001

Fig. 9. ARID2 is required for activation of a subset of p53 target genes in human melanocytes and mouse melanoblasts.

(A) Total cell extracts were prepared from NHEM cells cultured in presence of a control siRNA or specific siRNA against ARID2. Cells were harvested 72 hr after siRNA transfection and assayed by Western blotting for antibody against ARID2. Tubulin is a loading control. A representative blot is shown.

(B) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to p21 and GAPDH for samples which were prepared from NHEM cells which were cultured in presence of a control siRNA or specific siRNA against ARID2. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. *p < 0.05

(C) Total cell extracts were prepared from Melb-a cells cultured in presence of a control siRNA or specific siRNA against ARID2. Cells were harvested 72 hr after siRNA transfection and assayed by Western blotting for antibody against ARID2.
Tubulin is a loading control. A representative blot is shown.

(D) Total RNA was harvested, reverse-transcribed, and subjected to qPCR with primers to p21 and GAPDH for samples which were prepared from Melb-a cells which were cultured in presence of a control siRNA or specific siRNA against ARID2. The data shown are the average of 3 or more independent experiments that were subjected to qPCR. Standard error bars are shown. *p < 0.05
3.6 FIGURES

Figure 1

[Image of Western blot showing different cell lines and proteins investigated: BRG1, ARID2, BAF180, BRD7, and TUBULIN. The cell lines include NHEM, SK-Mel5, SK-Mel5 BRG1, SK-Mel147, Mel224, A375, WM-266-4, YUMAC, and Mel505.]

Figure 2

[Image of a bar chart showing the percentage of cells in different phases of the cell cycle (G1, S, G2) for EV and BRG1 treatment groups under Sham and UV conditions. The graph indicates 48 hr post UV-treatment.]
Figure 5

![SK-Mel 5 BRG1 diagram]

Figure 6

![SK-Mel 5 BRG1 diagram]
Figure 9
Chapter 4

Overall Discussion

Bromodomains are an extensive family of evolutionarily conserved protein modules that function as acetyl-lysine binding domains. This recognition serves as a pivotal mechanism for regulating protein-protein interactions in numerous cellular processes including chromatin remodeling and transcriptional activation. Two groups of bromodomain containing proteins will be discussed in this section with their relevance to melanocyte differentiation and melanoma.

In the first project, the function of BET group of bromodomains in modulating pigmentation and melanocyte differentiation was deduced. BET protein family members are the epigenetic readers that bind to acetyl-lysine residues on the tails of histones H3 and H4, and exert key roles at the interface between chromatin remodeling and transcriptional regulation. There is increasing evidence of their role in human diseases, and recently a number of small-molecule inhibitors have been reported to have remarkable pre-clinical efficacy in various malignancies. These findings have provided
for a thrust of exploring BET proteins as novel targets in cancer therapy. We dissected the role of BRD4 in melanocyte differentiation and the potential of inhibiting BRD4 by a small molecule inhibitor JQ1 in treating hyperpigmentation disorders.

Ultraviolet (UV) radiation from sunlight is an environmental risk factor involved in the etiology of skin cancer, the most dangerous being melanoma. Melanoma is a highly aggressive form of skin cancer with an extremely poor prognosis after advancing to metastatic stage. Moreover, exposure to UV radiation can result in development of hyperpigmented lesions called solar lentigines. Lentigines are pigmented macules characterized by a localized proliferation of melanocytes, which appear to replace keratinocytes in the basal layer of the epidermis. Melanocytes are the pigment producing cells which synthesize melanin and distribute the pigment to keratinocytes, thus giving skin its characteristic color. The regulation of skin pigmentation is complex and involves not only melanin synthesis by melanocytes but also proper distribution of melanin to surrounding keratinocytes. Disruption of normal melanocyte function leads to over-stimulation of melanin which results in hyperpigmentation disorders. Current treatment options for hyperpigmentation include chemical peels, intense pulse light therapy, and bleaching creams such as hydroquinone or arbutin. The topical application of hydroquinone is the most effective treatment strategy available but it has adverse side effects that include increased sensitivity to UV radiation and increased risk for developing melanoma and other skin cancers. Thus, there is a need for the development of improved therapeutic options for hyperpigmentary conditions.
We utilized a novel approach of targeting an epigenetic regulator to gain insights in the modulation of melanogenesis and melanocyte function. We found that treatment of cultured human neonatal epidermal melanocytes (NHEMs) with the BET inhibitor, JQ1 resulted in a loss of pigmentation over time. We also assessed the effects of JQ1 in an in vitro mouse model of melanocyte pigmentation. Mouse melanoblasts (melb-a) cells are unpigmented cells that can be induced to differentiate into pigmented melanocytes. We observed that treatment of melb-a cells with JQ1 suppressed pigmentation by suppressing melanin synthesis. Furthermore, we determined that JQ1 suppressed proliferation of these cells by inducing cell cycle arrest and not causing cell death. However, treatment with JQ1 may be toxic to keratinocytes. Keratinocytes are the principal cell type of the epidermis protected from UV radiation by melanin. It needs to be studied how JQ1 affects keratinocyte growth and response to UV radiation. We will perform a dose dependent response experiment and monitor cell death and melanin synthesis to determine a dose that alters melanogenesis but does not cause keratinocyte death. Alternatively, we will test other BET inhibitors, such as PFI-1, which has similar effects on pigmentation, to identify one that causes minimal cell death.

The microphthalmia-associated transcription factor (MITF) is an important regulator of pigmentation-specific genes and melanocyte proliferation. MITF is a master regulator of melanocyte differentiation that binds to the regulatory regions of many genes which regulate pigmentation, as well as genes important for melanocyte survival, and activates their expression. Thus, modulation of MITF expression or activity could be a useful strategy for treating hyperpigmentation disorders. The melanocyte specific enzyme,
tyrosinase (TYR), is required for the rate limiting steps of melanin synthesis while two other melanocyte specific enzymes, tyrosinase related protein 1 (TRP1) and dopachrome tautomerase (DCT) regulate the type of melanin synthesized. We found that treatment of melb-a cells and NHEMs with JQ1 inhibited expression of genes that encode these enzymes. However, although the effects of JQ1 on gene expression of TRP1 was profound and consistent in Melb-a as well as NHEM cell lines, tyrosinase gene expression seems to be differentially regulated in these cell lines, which was evident from the fact that tyrosinase was affected at a much later time point in case of NHEMs. Transient receptor cation channel protein 1 (TRPM1) is an MITF target gene that is associated with melanocyte differentiation. We found that JQ1 also abrogated TRPM1 expression. The cyclin dependent kinase inhibitor, p21 and the pro-survival gene, BCL2 are also MITF targets. Because treatment with JQ1 inhibited proliferation and cell cycle progression, we investigated the effects of JQ1 on the expression of p21 and BCL2. We detected a significant increase in p21 mRNA levels when melb-a cells were differentiated with JQ1. Interestingly, JQ1 treatment did not have a significant effect on expression of BCL2. Thus, JQ1 has differential effects on different classes of MITF target genes. It would also be interesting to assess the effects of how quickly is melanogenesis restored when JQ1 is removed from the culture media.

Previous studies showed that JQ1 binds to BRD4 with higher affinity than to other BET proteins and is the BET protein most likely to mediate the suppressive effects of JQ1. Although BRD4 is the BET protein most sensitive to JQ1, it also affects other BET proteins to a lesser extent. Thus, there is a small possibility that inhibition of other BET
proteins underlie the suppressive effects of JQ1 on the expression of MITF target genes, or that activities of other BET proteins can compensate for BRD4. We will down-regulate other BET proteins by siRNA approach, alone and in combination with BRD4 knockdown to deduce their effects on pigmentation. A CRISPR/Cas9 approach to mutate or flox multiple genes in vitro or in vivo can also be utilized.

Next, we wanted to identify BRD4 binding sites in melanocytes and investigate the changes in BRD4 binding that occur as a result of BET inhibition. By mining publicly available data sets, we came across a ChIP-seq data that was performed in a melanoma cell line SK-MEL-5. We detected peaks of BRD4 binding on a number of loci that regulate pigmentation. By performing chromatin immunoprecipitation in melb-a cells, we indicate that BRD4 is enriched on the promoters of pigment-specific genes of TRP1 and tyrosinase and JQ1 displaces BRD4 from these sites. BET inhibition also disrupts MITF binding onto the promoters of its target genes. Addition of a parallel gene-expression study, e.g. RNA-seq would indicate the functions of BRD4-binding sites. Performing a genome-wide study to understand the role of BRD4 in modulating chromatin architecture and gene expression simultaneously could yield a global picture of how these genes are functioning in the context of melanocytes. Furthermore, JQ1 disrupts histone H3 lysine 4 trimethylation (H3K4me3), a mark of active transcription. This suggests that BRD4 directly regulates the transcription of these genes involved in melanin synthesis. It would be useful to deduce the effect of BET inhibition on inhibitory marks of transcription like histone H3 lysine 27 trimethylation (H3K27me3) or histone H3 lysine 9 trimethylation
BRD4 has a role in regulating higher order chromatin structure by mediating communication between promoters and enhancers. A chromosome conformation capture assay can be performed in the presence of active and inactive enantiomer of JQ1 to analyze the changes in chromosome conformation and evaluate the requirement of BRD4 at the promoters of pigment specific genes. We also investigated the effect of BET inhibition on chromatin accessibility. It was observed by formaldehyde-assisted isolation of regulatory elements (FAIRE) assay that JQ1 treatment as well as knocking down BRD4 led to reduced chromatin accessibility at the melanocyte specific promoters.

Because BRD4 is an epigenetic reader and not a chromatin remodeling enzyme, the BRD4 elicited changes in chromatin structure are thought to be due to BRD4 interactions with other proteins. Our data indicated that BRD4 occupied the promoters of MITF target genes and that disruption of BRD4 activity with the BET inhibitor led to down regulation of MITF target genes, we set to investigate the interaction of BRD4 with MITF. Using co-immunoprecipitation assays, we deduced the physical interactions between these two proteins. To further analyze if the interaction between BRD4 and MITF is direct, GST-pull down assays or yeast-two hybrid assays need to be performed.

We have performed an extensive study to deduce the role of a BET protein in melanocyte differentiation and pigmentation in vitro. It needs to be assessed if JQ1 responds in a similar way on intact skin. Studying the effects of BET inhibition on human skin equivalents or animals (hk14-Kitl mice) which possess epidermal melanocytes would
strengthen the results of our study. However, the investigation of the role of BRD4 in regulating melanin synthesis in melanocytes is novel. We have combined BET inhibition by JQ1 with RNAi strategies to interrogate the function of BRD4 in melanocyte differentiation and pigmentation. The outcome of our studies has a potential to translate into novel therapeutic options for treating skin disorders and for developing products that protect against damage from solar radiation by targeting epigenetic regulators. In total, our results demonstrate that BRD4 plays an important role in melanocyte differentiation by interacting with MITF, and can be targeted by BET protein inhibitors for treatment of hyperpigmentation disorders.

In the second project, the role of bromodomain containing proteins of an ATP-dependent chromatin remodeling complex, SWI/SNF, were assessed. In particular, we investigated the role of PBAF SWI/SNF complex in the context of melanoma tumorigenicity. SWI/SNF is an evolutionarily conserved ATP-dependent multi-subunit chromatin-remodeling complex that performs fundamental roles in gene regulation, cell lineage specification, and organ development. Genomic studies have revealed a tumor-suppressive role for SWI/SNF complex, with inactivating mutations in nearly 20% of human cancers. Mammalian SWI/SNF complexes can be classified broadly as BAF (BRG1/BRM-associated factors) complex bearing either one of ARID1A/ARID1B or PBAF (Polybromo-associated BAF) complex harboring ARID2, BAF180 and BRD7 subunits. Loss-of-function mutation of genes encoding BRG1 and BAF180 are particularly common in lung and renal cell cancers, respectively. Specific mutations in BRG1 have been identified in pancreatic, breast, lung and prostate cancer cell lines.
Mutations in PBAF specific SWI/SNF components have been reported widely in various cancer types.

Melanoma is an extremely aggressive malignancy originating in pigment-producing melanocytes in the basal layer of epidermis. The alarming increase in its rate of incidence (5-7% annually) and lack of effective treatment underscores the necessity to investigate the molecular mechanisms by which melanoma develops. In that regards, studying the regulatory pathways involved in melanoma development and progression is of utmost importance. UV-radiation causes DNA damage and is the major environmental risk factor in the development of melanoma.

There is an increasing evidence of some SWI/SNF subunits being down-regulated or mutated in melanoma. Our preliminary data shows BRD7 protein expression levels to be down-regulated in a panel of melanoma cell lines as compared to neonatal human epidermal melanocytes. The studies of melanoma identified ARID2 as the SWI/SNF component that is most frequently mutated. BRG1, the ATPase in PBAF SWI/SNF complex, has been shown to promote melanoma differentiation as well as to increase survival of melanoma cells that were exposed to a chemotherapeutic agent or to UV radiation.

The tumor suppressor, p53 is activated in response to DNA damage and serves as a critical regulator of cell cycle checkpoints. The response to DNA damage involves activation of the p53 pathway, which promotes expression of p21 and cell cycle arrest at the G1 phase. p21 is a p53 trans-activation target that mediates p53 driven G1 arrest in
response to DNA damage. In spite of extensive evidence from animal models in support of p53 as a tumor suppressor in melanoma initiation and progression, the majority of human melanomas retain wild-type p53. Some studies suggest that the loss of p53 in melanoma may be unnecessary due to the existence of compensatory mechanisms that involve over-expression of p53 inhibitory proteins such as MDM2, silencing of downstream effectors, such as APAF1, and by activation of anti-apoptotic factors and inhibitors of apoptosis.

BRG1, the central ATPase in PBAF SWI/SNF complex, has been reported to be required for p53-driven transcriptional activation and cell-cycle regulation. Components of the PBAF SWI/SNF complex (BRD7 and BAF180) have been implicated in the regulation of DNA damage response by their role in transcriptional activation of p53 target genes.

We wanted to assess the tumor suppressive role of PBAF SWI/SNF complex in context of melanoma by activating p53 pathway in response to UV-radiation. To determine if BRG1 protected against UV-induced DNA damage, we carried out a cell cycle analysis wherein SK-MEL-5 cells expressing empty vector or BRG1 were subjected to sham and UV-irradiation. It was observed that BRG1 containing SK-MEL-5 cells exhibited a significant cell cycle arrest at G1 phase. It suggested that BRG1 exhibited anti-proliferative effect on melanoma cells under UV-irradiation. The requirement of PBAF specific SWI/SNF subunits could possibly be assessed by performing similar cell cycle analysis by knocking down these components individually or in combinations.

p21 is a cyclin-dependent kinase inhibitor and one of the critical regulators of DNA
damage response. We detected that BRG1 expressing cells accumulated p21 at higher levels, following UV treatment, suggestive of its role in the regulation of this gene. BAF180 is a PBAF SWI/SNF specific subunit which is implicated as a tumor suppressor in several human cancers like renal cell carcinoma and breast cancer. We detected an increase in the expression levels of BAF180 in BRG1 expressing cells independent of UV treatment. BAF180 was shown to be interacting with BRG1 suggesting the stabilization of PBAF SWI/SNF complex in these cells. We could assess the direct interactions between BAF180 and BRG1 using yeast-two hybrid assay or GST pull-down assay. Our studies suggest BAF180 to be indispensable for BRG1-mediated activation of a subset of p53 target genes. Our data indicate that depletion of BAF180 compromises p53 target gene expression in melanoma indicating that it may play a tumor suppressive role in melanoma.

SWI/SNF mutations can be mutually exclusive with other tumor suppressor mutations (e.g., PTEN and p53) in certain tumor types, suggesting that SWI/SNF performs tumor protective functions that overlap with known pathways. It would be interesting to check for the p53 independent activity of SWI/SNF complex in context of melanoma progression. Co-immunoprecipitation of p53 with PBAF specific SWI/SNF subunits along with chromatin immunoprecipitation assays on the promoters of a subset of p53 target genes could shed light on the requirement of specific sub-units for activating these p53 target genes.

We elucidated the requirement of BRD7 and ARID2 components of PBAF SWI/SNF
complex in regulating the expression of a subset of p53 target genes in melanoma cell lines. Similar to BAF180, our data indicated the requirement of BRD7 and ARID2 to mediating activation of p21 and GADD45A. There is a lack of knowledge into the biological functions of ARID2 in melanocytes and how ARID2 might act as a tumor suppressor. Our data with neonatal human epidermal melanocytes and mouse melanoblast indicates that knocking down ARID2 compromised p21 gene expression. Thus, our work suggests that a specific configuration of the SWI/SNF complex may have a tumor suppressive role in melanocytes and that aberrant expression or mutations of SWI/SNF components may contribute to melanoma tumorigenicity.

In our zeal for unraveling the tumor suppressive functions of a multi-subunit PBAF SWI/SNF complex in the context of melanoma, understanding the mechanisms by which tumor progresses, is of utmost importance. It might be due to an inactivating mutation of a specific subunit, dysfunction of the aberrant residual complex or the disintegration of the entire complex that could lead to abnormal cell growth. Understanding these aspects will give us a better outlook on the activity of PBAF SWI/SNF complex and p53 in the context of melanoma.
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