A Dissertation

entitled

SWI/SNF Chromatin Remodeling Enzymes: Epigenetic Modulators in Melanoma

Invasiveness and Survival

By

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

of Philosophy Degree in Biomedical Sciences

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Abstract

SWI/SNF enzymes are multi subunit complexes consisting of an ATPase subunit, either BRG1 or BRM. They act as epigenetic modulators and remodel the chromatin in an ATP dependent manner. The SWI/SNF complex has 9-12 BRG1 or BRM associated factors called (BAFs). Earlier studies from our lab showed that BRG1 interacts with Microphthalmia associated transcription factor (MITF) to promote melanocyte differentiation. MITF is a lineage addiction oncogene in melanoma that regulates melanoma proliferation and metastasis. Several of the SWI/SNF subunits have been demonstrated to behave as tumor suppressors in mice and been found to be down regulated in many forms of cancer. However, our lab has shown that although BRG1 or BRM is down-regulated in a small subset of melanoma cell lines, one catalytic subunit is always retained and the other SWI/SNF subunits are expressed at high levels compared to normal melanocytes. This has led to the hypothesis, that in melanoma, SWI/SNF enzymes interact with MITF and other transcription factors to modulate melanoma metastasis and survival.

In the first study we demonstrate that BRG1 is significantly upregulated in stage IV metastatic melanomas compared to stage III melanoma and normal skin. We found that BRG1 modulates the expression of genes that code for components of the extracellular matrix and regulators of adhesion. We found that BRG1 promotes adhesion of melanoma cells to laminin and collagen but inhibits adhesion to fibronectin. Furthermore, in vitro BRG1 promotes the invasive potential of the cells by upregulating matrix
metalloproteinase 2 (MMP2) expression at the transcriptional level. Thus, our studies demonstrate that BRG1 epigenetically modulates melanoma invasive ability in vitro, suggesting that BRG1 plays a role in melanoma metastasis.

SWI/SNF enzymes have been demonstrated to regulate the response to DNA damage by promoting DNA repair and cell cycle checkpoint controls. Earlier studies from the lab showed that BRG1 promotes melanoma resistance to the DNA damaging agent, cisplatin. In the present study we determined that BRG1 also promotes survival in response to DNA damage induced by UVB radiation. We found that in addition to promoting DNA repair and regulating cell cycle proteins, BRG1 directly modulates the apoptotic pathway by activating expression of the melanoma-inhibitor of apoptosis gene (ML-IAP). ML-IAP is a potent inhibitor of the apoptotic pathway, and a potential target for melanoma treatment. In melanoma cells, ML-IAP expression is regulated by a cooperative activation loop involving BRG1 and MITF that promotes permissive chromatin structure and an active epigenetic signature on the ML-IAP promoter. Our studies suggest that an effective option for treating melanoma may be the use of epigenetic drugs that target either BRG1 or chromatin remodeling enzymes that function in concert with BRG1.
Dedication

I dedicate this thesis to my parents and late grandparents, for their unconditional love, support and sacrifices.
Acknowledgement

I want to thank both my mentors Dr. Ivana de la Serna and Dr. Khew-Voon Chin for their constant guidance and support. I would like to thank firstly Dr. Chin for all the earlier studies, which I had done during the beginning of my Ph.D. I want to thank Dr. de la Serna who has been a wonderful mentor and has shown so much confidence in me and entrusted me with various projects over the years.

I also want to thank the members of my committee, Dr. Maltese for all the support, Dr. Ratnam for all the advice during the joint lab meetings, Dr. Yeung, for all his time and discussions and Dr. Trempe for his valuable time and advice.

I would like to thank all my past and present lab members and friends.

I want to thank my parents, brother and family for their love, support and being there for me always.

Last but not the least, I thank my wife (Haymanti) for always being there and for her moral support during the course of my Ph.D.
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Chapter 1.

Literature Review

Melanoma

Melanoma is one of the aggressive forms of skin cancer with very poor prognosis in advanced cases (Miller and Mihm, 2006). It has high metastatic potential and is highly resistant to current therapy (Zbytek et al., 2008). It accounts for about 3% of skin cancers. The five-year survival rate of melanoma patients is very poor (approximately 16%). Treatment of melanoma depends on the stage of the disease at the time of detection. If detected early, it can be controlled and cured by surgical excision (Tucker and Goldstein, 2003). Chemotherapy, photodynamic therapy, radiation therapy and biological therapy are other kinds of therapy that are used depending on the stage of melanoma, however they are not effective. In the year 2010 alone 68,130 new cases and 8700 deaths were reported just in the United States alone (www.cancer.gov). The incidence of melanoma is not dependent on age and is one of the main cancers that result in death of people between the ages of 20-35 (Houghton and Polsky, 2002).

Melanoma arises from the transformation of melanocytes. Melanocytes are the pigment synthesizing cells present predominantly in the epidermal layers of the skin. The pigment melanin, when synthesized, protects the skin from UV radiation and UV induced DNA
damage. There are various stages in the progression of melanoma from nevi to the highly aggressive and metastatic stage. Formation of nevi or moles present on the skin is the first stage, which is characterized by activating mutations in the BRAF gene. These nevi can remain static for decades. Additional mutations make the nevi progress into dysplastic nevi and with alterations of other genes, leads to the radial growth phase and vertical growth phase and finally into the metastatic phenotype. Each stage in the progression of melanoma is associated with various molecular events. Dysplastic nevi are characterized by the loss of p16 /CDKN2 and PTEN genes which results in aberrant growth(Curtin et al., 2005; Flores et al., 1996; Li et al., 1997; Miller and Mihm, 2006). This may take place in a benign nevus or in a normal cell. The next stage is the radial growth phase which is characterized by the ability of cells to proliferate intraepidermally. There is decreased differentiation in this stage. The next stage is the vertical growth phase where the cells acquire the ability to invade the dermis, cross the basement membrane and reach the lymph nodes(Clark et al., 1984; Pollock et al., 2003). Metastatic melanoma is the final stage where the cells migrate/spread to distant organs like the brain, liver, and bone. Many genes that play an important role in the extra cellular matrix and cellular adhesion and invasion are deregulated in this stage. E-cadherin loss, N-cadherin expression, increased MMP2 expression and \( \alpha V\beta 3 \) integrin expression are some of the genes that are deregulated(Haass et al., 2005).

**ExtraCellular Matrix and its Role in Melanoma Invasion**

There are various stages in the progression of melanoma characterized by the localization of melanoma cells. Melanoma metastasis is the last stage and it is characterized by the invasion
of melanoma cells to lymph nodes and migration to distant organs like the brain, bone or liver. In this process, the cells enter the blood vasculature and are carried to distant organs where they start invading the target organ and form tumors. After they invade target organs, there are various factors like immune surveillance, cell cycle arrest and apoptotic stimuli from the microenvironment, which decide the fate of these metastasized cells (Miller and Mihm, 2006).

There are various intrinsic factors that play an important role in melanoma invasion and metastasis like the alteration in the expression of cadherins, cell adhesion molecules (CAMs), matrix metalloproteinases (MMPs) and integrins. Cell adhesion molecules (CAMs) are expressed on the cell surface and are required for interactions between similar cells or different cell types. There are various adhesion molecules like neural-CAM (NCAM), melanoma-CAM (MCAM) and VCAM. They are required for melanoma to melanoma cell adhesion as well as melanoma-endothelial cell adhesion. Cadherins maintain homeostasis in skin by maintaining the interactions between melanocytes, keratinocytes, fibroblasts and endothelial cells. Progression from the radial growth phase to the vertical growth phase is marked by loss of expression of E-cadherin and gain of expression of N-cadherin. Loss of E-cadherin results in increased motility, proliferation and invasive potential of melanoma cells. Gain of N-cadherin enables melanoma cells to be metastatic by increasing their interaction with other N-cadherin expressing cells such as dermal fibroblasts and vascular endothelial cells (Hsu et al., 1996; Johnson, 1999). Integrins (ITGA) are transmembrane glycoproteins that mediate specific interactions between cells and the extracellular matrix (ECM) and regulate migration (ITGA4, ITGA7, ITGA3). Integrins mediate interaction with various components of the extracellular matrix, collagen, fibronectin and laminin. The
progression from radial to vertical growth phase is associated with the expression of alphaVbeta3 integrin, which induces the expression of MMP2, which in turn facilitates the degradation of collagen in basement membrane (Brooks et al., 1996; Danen et al., 1994; Kuphal et al., 2005). Matrix metalloproteinases are involved in the degradation of extracellular matrix and play an important role in embryonic development, tissue remodeling, angiogenesis, cancer progression and metastasis. Changes in synthesis and activity of MMPs can lead to diseases such as cirrhosis and arthritis. MMP-2 degrades type IV collagen and gelatin and results in dissociation of melanoma cells from the basement membrane (Hofmann et al., 2000; Sato et al., 1994a; Vincenti and Brinckerhoff, 2007).

The metastatic stage of cancer is a deadly stage in any form of cancer. During this process, cancer cells disseminate from the primary site of the tumor and start invading the neighboring tissue, undergo intravasation, where they start entering the blood stream and lymph vessels and exist as circulating tumor cells (CTC’s) and reach distant tissue, undergo extravasation, where they exit from the blood stream and start colonizing in regions like brain, liver and bone. The epithelial-mesenchymal transition (EMT) occurs at the primary tumor site where epithelial cells lose tight junctions. The architecture of the cytoskeleton is altered as well. EMT can induce stem-cell-like properties in cells. EMT is a transdifferentiation process and in recent years it has been implicated in cancer cell invasion and metastasis. The microenvironment in the region of cancer cells constitutes various kinds of cells like the granulocytes, lymphocytes, fibroblasts and other cell types, which induce the signals required for the EMT like state. These signals activate transcription factors, which
then activate EMT programs within the cancer cells (Chaffer and Weinberg, 2011; Fidler, 2003; Kraljevic Pavelic et al., 2011; Yang and Weinberg, 2008).

MITF

Microphthalmia associated transcription factor (MITF), the master regulator of melanocyte differentiation, controls the expression of different classes of genes that encode the enzymes needed for melanin synthesis, melanosome structure, and melanocyte survival such as tyrosinase, trp1 and Dct, as well as genes encoding cell cycle regulators and pro-survival genes. MITF is a basic helix-loop-helix leucine zipper transcription factor and binds to E box regions of the promoters of target genes. MITF requires functional SWI/SNF (switch for sucrose non fermentation) for the activation of its target genes, tyrosinase, TRP1 and TRP2 in melanoma.

MITF is considered a lineage addiction oncogene that is amplified in 20% of melanoma cells and is required for determining cell fate by regulating survival, proliferation and differentiation. MITF regulates survival by transcriptionally regulating the pro-survival gene Melanoma Inhibitor of Apoptosis (ML-IAP), an anti-apoptotic gene that is highly expressed in a variety of cancers including melanoma (Dynek et al., 2008). MITF also regulates BCL2 expression which is an inhibitor of apoptosis, thereby promoting the survival of melanoma cells (McGill et al., 2002).
MITF regulates Cyclin Dependent Kinase 2 (CDK2) activity at the transcriptional level, thereby regulating melanoma proliferation (Du et al., 2004). T-box factor 2 (Tbx2), which is an inhibitor of senescence, is a direct target of MITF in melanocytes. The expression of Tbx2 correlates with the expression of MITF in melanoma cell lines, melanoblasts and melanocytes (Carreira et al., 2000). MITF inhibits cell cycle proliferation by promoting cell cycle arrest in a p21 dependent manner by cooperating with Rb1 (Carreira et al., 2005) and upregulating p16 (INK4A) by binding to its promoter. MITF thus promotes terminal differentiation of melanocytes by inhibiting proliferation (Loercher et al., 2005). A rheostat model has been proposed to correlate the levels of MITF to its function, where low levels of MITF promote apoptosis, medium levels promote melanocyte proliferation and high levels promote terminal differentiation (Carreira et al., 2006).

SWI/SNF chromatin remodeling enzymes play an important role in the promotion of activation of melanocyte specific genes by cooperating with MITF. MITF promotes the recruitment of SWI/SNF to target genes involved in the synthesis of melanin pigment by remodeling the chromatin structure (de la Serna et al., 2006a; Keenen et al., 2010).

**Epigenetics**

Epigenetics is the modification of DNA or histones that affect the transcription of genes, which play an important role in various functions in cells. It involves methylation, acetylation, and phosphorylation by various methylases, demethylases, acetylases, deactylases and kinases, which play an important role in turning on or off genes. Various abnormalities
involving epigenetic modifications have been studied in various diseases. The major epigenetic marker is DNA methylation and the hypermethylation of tumor suppressor genes, global hypomethylation of DNA and inactivation of microRNA genes by methylation are well known examples. Besides the modification of DNA, histones undergo various modifications that regulate the transcription of genes, DNA repair, etc. Epigenetic modifications and abnormalities play an important role in cancer.

**Chromatin remodeling enzymes**

Chromatin exists in a highly condensed form and is composed of nucleosomes made up of an octamer of histones H2A, H2B, H3 and H4 wrapped by 146bp of DNA and a linker histone H1. It exists in a highly inactive state as heterochromatin and in an active state as the euchromatin. The highly intact chromatin organization regulates various cellular processes like growth, differentiation, replication and also maintains the integrity of the genome. The highly condensed heterochromatic state is inaccessible for various transcription factors. There are various chromatin-remodeling enzymes that remodel the chromatin by modifying the histones and opening the chromatin so that transcription factors and other transcriptional machinery can bind and activate transcription. The amino terminal tails of histones undergo various modifications. Covalent modifications on the histones alter the DNA-histone interactions, thereby promoting changes in nucleosome structure and confirmation and thus making the DNA more accessible (Bannister and Kouzarides, 2011).
Histone modifications are catalyzed by 2 classes of enzymes, enzymes that aid in the post translational modification of histones by methylation, acetylation, phosphorylation (Oki et al., 2007) and ubiquitination (Wang et al., 2004) in an ATP independent manner, and enzymes that utilize energy obtained by the hydrolysis of ATP to disrupt the DNA-histone interactions. They thereby function to alter chromatin conformation and/or reposition nucleosomes (Kingston and Narlikar, 1999). Both the classes of enzymes are thought to cooperate to modify chromatin, thereby regulating transcription of genes, by either activation or repression. Both classes of chromatin remodeling enzymes modify the chromatin and generate a histone code (Blossey, 2006).

The various chromatin remodeling enzymes that post translationally modify the chromatin structure are histone acetyl transferases (HATs), histone deactelyases (HDACs), kinases, histone methyltransferases (HMTs), and demethylases (Allfrey et al., 1964; Rea et al., 2000; Tamaru et al., 2003). These enzymes modify specific residues on amino terminal tails like the methylation on lysine 4 of H3 (H3K4) or the acetylation on lysine 12 of H4 (H4K12). These modifications serve as active or repressive marks on the chromatin. Methylation of H3K4, H3K20, and acetylation of H4 (H4K5, K8, K12, K16) serve as active marks of transcription(Chicoine et al., 1986; Li et al., 2011; Parthun, 2007; Ruthenburg et al., 2007; Sobel et al., 1995). Methylation of H3 on lysine 9 and 27 serve as major marks of repression of transcription(Hansen et al., 2008; Ng et al., 2009).
The second class of chromatin remodelers, ATP dependent chromatin remodeling enzymes are SWI/SNF, ISWI (Imitation SWI) complexes, NuRD (Nucleosome remodelling and deacteylase) and Mi-2/CHD (Chromodomain, helicase DNA binding) complexes (Boyer et al., 2004; Eberharter and Becker, 2004; Marfella and Imbalzano, 2007; Sif, 2004). ATP dependent chromatin remodeling enzymes are classified based on the presence of functional domains in the catalytic ATPase subunit. The ATPase subunit of SWI/SNF has a bromo domain which interacts with the acetyl residues present on histone tails. The ISWI family proteins contain a SANT domain (SW13, ADA2, NCoR and TFIIB). The SANT domain helps preferentially in the interaction with nucleosomes containing linker DNA to core nucleosomes. The ISWI complexes have one of the two subunits Snf2h and Snf2l and both the subunits contain a SANT domain which helps in binding to unmodified histones (He et al., 2008; Langst et al., 1999). There are 9 CHD proteins in the CHD family (CHD1-9). The CHD family of proteins have a chromodomain which facilitates the interaction with methylated histone tails. The CHD family of proteins also have a DNA binding domain, Plant Homeo Domains (PHD), BRM and Kismet domains (BRK), and SANT domains (Bannister et al., 2001; Flanagan et al., 2005; Lachner et al., 2001; Sims et al., 2005) (Fig. 1). INO80 (Inositol requiring 80) proteins are ATP dependent chromatin remodeling enzymes that play an important role in the recruitment of DNA repair proteins to DSBs (double strand breaks) by displacing the nucleosomes at the damaged site. They also play an important role in the transcription of genes. Apart from playing a role in DNA repair and transcription, INO80 proteins also aid in DNA replication (Ebbert et al., 1999; Peterson and Cote, 2004; Sarkar et al., 2010; Watanabe and Peterson, 2010).
Three different ATP dependent chromatin remodeling enzymes containing common ATPase domain. They carry distinct bromo, SANT, and chromo domains which are required for recognition of the histones.
**SWI/SNF enzymes**

SWI/SNF is a class of enzymes that modifies the chromatin structure by utilizing the energy obtained from the hydrolysis of ATP to alter nucleosome structure. SWI/SNF subunits are 1-2 MDa in size and consist of 9-12 subunits containing Brg1/Brm and their associated factors (BAFs) (Muchardt and Yaniv, 1993) (Fig 2).

SWI/SNF enzymes were first identified in yeast and are known to regulate the mating type switching and sucrose fermentation and hence the name SWI/SNF – switch for sucrose non fermenting (Breeden and Nasmyth, 1987; Neigeborn and Carlson, 1984; Stern et al., 1984). SWI/SNF are conserved multi subunit complexes with an ATPase subunit, either the bramha (Brm) or brahma related gene-1 (Brg1), which is the core or catalytic subunit (fFig. 2). This ATPase subunit utilizes the energy obtained from ATP hydrolysis to alter the nucleosome structure to facilitate the binding of transcriptional factors, either activators or repressors to nucleosomal DNA(Vignali et al., 2000). SWI/SNF enzymes have been implicated in the activation and repression of genes by their physical interactions with gene specific regulators, which are thought to recruit these enzymes to the specific loci. The various gene activation events that require SWI/SNF chromatin remodeling enzymes include regulation by nuclear receptors (Fryer and Archer, 1998), induction of stress response, activation of TCDD (2,3,7,8-tetrachloro- dibenzo-p-dioxin) inducible genes (Kalpana et al., 1994), c-myc (Cheng et al., 1999), c-fos/jun heterodimers(Ito et al., 2001),
interferon-β (Klochendler-Yeivin et al., 2000), interferon-γ (Pattenden et al., 2002), hsp70 (Sullivan et al., 2001) and CD44 genes (Klochendler-Yeivin et al., 2000).

SWI/SNF enzymes are important for developmentally regulated pathways including activation of erythroid (Armstrong et al., 1998), myeloid (Kowenz-Leutz and Leutz, 1999), muscle (de la Serna et al., 2001) and adipocyte gene expression (Pedersen et al., 2001). SWI/SNF enzymes play a role in cell cycle regulation, DNA recombination and repair (Bochar et al., 2000; Lee et al., 2010; Ray et al., 2009). Components of the complex are targets of viral regulatory proteins and are mutated in many forms of cancer (Bultman et al., 2000; Decristofaro et al., 2001; Kalpana et al., 1994; Lee et al., 1999; Miller et al., 1996). Disruption of Ini1 or Brg1 components in mice has been shown to be embryonically lethal suggesting SWI/SNF enzymes are essential for viability (Bultman et al., 2000; Guidi et al., 2001).
Figure 2

SWI/SNF enzymes containing BRG1 or BRM and associated factors, BAFs
SWI/SNF in Cancer

SWI/SNF has been shown to be involved in the development of lung, breast, colon and prostate cancers. Various SWI/SNF sub-units have been shown to be down regulated in various forms of cancer indicating their role in tumorigenesis (Roberts and Orkin, 2004; Wilson and Roberts, 2011). SNF5/Ini1 was originally identified as a protein binding to the HIV integrase (Guidi et al., 2001; Kalpana et al., 1994). It was later shown to frequently undergo rearrangement in rhabdoid tumors. One allele undergoes deletion while the other allele is mutated or silenced in rhabdoid tumors, which strongly indicates it might be playing the role of tumor suppressor (Versteeg et al., 1998). Several other subunits including the ATPase subunits are either silenced or over expressed in various types of cancers. SWI/SNF components are concomitantly silenced in primary lung tumors. BRG1 and BRM are lost in about 30-40% of lung cancers (Betz et al., 2002; Decristofaro et al., 2001). Inactivating mutations of the BRG1 gene, on chromosome arm 19p, are present in several human lung cancer cell lines (Girard et al., 2000). SWI/SNF function potently regulates core AR (androgen receptor) target gene promoter activation, with a preference for BRM-containing complexes. BAF57 an associated factor of SWI/SNF complex is an important regulator of AR function. It binds to AR and is recruited to its targets upon ligand activation and is required for AR dependent prostatic endocarcinoma cell proliferation (Link et al., 2005).

BRG1 expression is high in invasive form of prostate cancer. Gastric carcinomas with lymph node metastasis express higher BRG1 than carcinomas without metastasis (Sentani et al., 2001; Yamamichi et al., 2007). BRG1 cooperates with ZEB1 and regulates the expression of
E-cadherin and affects the epithelial to mesenchymal transition (EMT), thereby regulating tumor invasion in the initial stages (Sanchez-Tillo et al., 2010). Transgenic knockout mouse models have been highly useful in understanding the role of SWI/SNF subunits in cancer development. Knockout of a single allele of BRG1 resulted in the development of tumors in 10% of mice. Biallelic knockout of BRG1 is embryonically lethal (Bultman et al., 2000). However knocking out BRM did not result in lethality. BRM null mice are larger in size compared to wild type mice (Muchardt and Yaniv, 1999; Reyes et al., 1998). Heterozygous knockout of BAF47 in mice resulted in the development of tumors that are similar to malignant rhabdoid tumors (Roberts et al., 2002).

Recent studies have shown that blocking BAF57 recruitment inhibits AR activity. Since BAF57 recruitment occurs in response to AR agonists but not therapeutic antagonists, it led to the development of inhibitory peptide BAF57 inhibitory peptide (BIPep) for therapeutic intervention in prostate cancer (Link et al., 2008). In lung cancer cell lines, loss of BRM had been shown to be a reversible epigenetic change. Recent studies had shown that using a pharmacological inhibitor containing HDAC activity, BRM expression could be restored which then inhibited BRM dependent growth in BRM deficient cancer cells (Gramling et al., 2011).

Understanding the epigenetic abnormalities in cancer has a great potential for translational research. Designing effective pharmacological compounds targeting the epigenetic factors altered in cancers may be a good approach for treating cancer. These epigenetic therapies can be used to reverse the effects in cells by either inhibiting or activating the genes as well.
as epigenetic modifiers (Gramling et al., 2011; Link et al., 2008; Masetti et al., 2011; Mund and Lyko, 2010).

**SWI/SNF in DNA Repair**

DNA damage that occurs in cells due to environmental toxins has to be repaired to maintain genomic stability and impairment in this process leads to cancer. Ultra-violet radiation (UVR) is one of the most important environmental agents known to cause DNA damage in cells. UVR is comprised of UVA (320-400nm), UVB (280-320nm) and UVC (200-280nm). Exposure to UVA leads to the formation of free radicals which cause single strand breaks in the DNA of the cells. UVB exposure leads to the formation of pyrimidine dimers and 6-4 photo adducts. UVC radiation is obstructed by the ozone layer. UVR induced DNA damage triggers various cellular responses like cell cycle arrest, DNA repair and apoptosis. When the damage is completely repaired the cells then re-enter the cell cycle and if the damage is irreparable, the cells may undergo apoptosis (Jhappan et al., 2003).

Melanin plays a protective role in UVR induced DNA damage by absorbing the UV photons and free radicals induced by UVR. There are two types of melanin, eumelanin and pheomelanin. Eumelanin is present in the skin and hair. Pheomelanin is present in red hair and individuals with freckles (Chhajlani and Wikberg, 1992). Alpha-melanocyte stimulating hormone (a-MSH) binds to melanocortin-1 receptor (MC1-R) and induces the synthesis of eumelanin. This involves a cascade of signaling events involving MITF (Evans et al., 1988; Langley and Sober, 1997).
DNA damage caused by UVR in melanocytes has to be repaired. DNA damage may lead to mutations and if the DNA damage is not repaired and the cells do not undergo apoptosis, it might lead to the formation of mutant melanocytes which then progress to melanoma. There are various DNA repair pathways present in the cell, Base Excision repair (BER) which repairs the oxidative damage induced by UVA and the Nucleotide excision repair (NER) repairs by removing the 6-4 photo adducts and the pyrimidine dimers formed by UVB (Matsumura and Ananthaswamy, 2002).

While DNA repair is taking place in the cell, the other cellular responses like the cell cycle arrest mediated by p16 and p53 and apoptosis mediated by Bcl2 family proteins like Bax are activated. p53 is one of the major cellular stress response proteins which is stabilized and activated in response to UVR. Cells undergo cell-cycle arrest in G1 phase mediated by transcriptional activation of p21. p53 also induces Bax mediated apoptosis by activating it transcriptionally. Bax is a pro-apoptotic protein that belongs to the Bcl2 family. Cell fate is determined by the UV dosage they are exposed to, undergoing either cell cycle arrest or apoptosis (Jiang et al., 1999; Jung, 1986; Legge, 1978; Smith et al., 2000).

In recent years, the role of chromatin remodeling enzymes in DNA repair has come to light. For the repair machinery to gain access to the damaged DNA, they require chromatin remodeling enzymes like INO80 and SWI/SNF to remodel chromatin for binding of the DNA repair proteins like XPC to be able to process the DNA damage. SWI/SNF enzymes have been shown to induce gamma H2A.x phosphorylation and damage sensitivity when
there is DNA double strand breaks. Gamma H2AX undergoes phosphorylation on its serine 139 residue and is an early DNA damage response. It is induced in DNA double strand breaks thus serving as a marker for DNA damage and repair (Mah et al., 2010). UV radiation also induces the phosphorylation of gamma H2AX when UV induced lesions collide with replicative forks which result in the formation of DSBs and the activation of ATR signaling pathways (Ichihashi et al., 2003). The core and catalytic subunits of SWI/SNF have been implicated in enhancing DNA repair. SNF5, the core subunit of SWI/SNF has been shown to play a important role in NER pathways by colocalizing with XPC to the damage site, activating the phosphorylation of ATM, and thereby activating the DNA damage response (Czaja et al., 2010; Morrison et al., 2004; Park et al., 2010; Ray et al., 2009; Sarkar et al., 2010; van Attikum et al., 2004).

**Apoptosis**

Apoptosis, also termed as programmed cell death, is an important mode of cell death. There are various other forms of cell death like necrosis, entosis, and methuosis. The characteristic features of apoptosis are chromatin condensation, nuclear fragmentation and formation of plasma membrane blebs. Apoptosis is classified into two pathways based on the mode of activation, the extrinsic and intrinsic pathways. It is an important process during development of multicellular organisms (morphogenesis) by disposing of the unwanted cells. Apart from development it is important for other processes like differentiation and proliferation, by removing the defective and harmful cells. Defects in apoptosis may lead to
cancer, autoimmune diseases, etc and excessive apoptosis may lead to neurodegenerative disorders, AIDS (Thompson, 1995).

There are various molecules involved in the apoptotic pathways like the caspases classified as initiator and executioner caspases, and other regulatory proteins like the Bcl2 family and inhibitor of apoptosis proteins (IAP’s). Apoptosis is triggered by various stimuli, which then activates a series of events or cascade of proteins. Caspases are proteases, which form the major component of the apoptotic signaling network. They are synthesized as inactive zymogens or procaspases and upon activation are cleaved into a small and large subunit which then form a hetero tetramer resulting in formation of active caspase. The initiator capases are caspase 2, 8, 9, 10 and the executioner caspases are 3, 6 and 7. Once the initiator caspases become active in response to some stimuli, they activate the effector or executioner caspases by proteolytic cleavage (Green, 2005; Hotchkiss et al., 2009; Wallach et al., 2002; Wallach et al., 1999).

Bcl2 is one of the major anti-apoptotic proteins. It is also a regulatory protein in apoptosis. There are various other Bcl2 homologous proteins Bcl-XL and Bcl-W which are characterized by the presence of the BH (Bcl2 homology) domains BH1, BH2, BH3, BH4, which serve as prosurvival proteins. The pro-apoptotic proteins of the Bcl2 family are Bax, Bak, Bok and they possess BH1, BH2 and BH3 domains whereas Bid, Bim, Bad, and Noxa contain the BH3 domain alone (McKenzie et al., 2008; Yin et al., 1999; Zong et al., 2001).
MLIAP

Inhibitors of apoptosis (IAPs) proteins are anti-apoptotic regulators which block cell death in response to stimuli or stress. They inhibit apoptosis by blocking caspase activation/cleavage. The IAP family has cIAPs (cellular inhibitor of apoptosis), XIAP (X-chromosome binding IAP), ILP-2 (IAP like protein 2), NAIP (neuronal apoptosis inhibitory protein), BRUCE (Apolon), Survivin, and Livin (ML-IAP or BIRC7 or KIAP). The IAP proteins have baculovirus IAP repeat (BIR) motifs which are required for the anti-apoptotic activity. Livin was identified in malignant melanoma and hence the name melanoma IAP (ML-IAP). It inhibits caspase activity by inhibiting Smac/DIABLO activity by degradation, thereby inhibiting the activity of caspase 3, 7 and 9. Small interfering RNAs, targeting ML-IAP, inhibit tumor growth in mice (L428, a Hodgkin's disease (HD) cell line and Transformed human melanocytes (MLTR) were used in the tumorigenicity studies)(Abd-Elrahman et al., 2009; Badran et al., 2004; Chang and Schimmer, 2007; Ma et al., 2006; Salvesen and Duckett, 2002; Uren et al., 1998; Vucic et al., 2000).

Hypothesis

The role of SWI/SNF subunits in the progression of melanoma has not been studied. Earlier studies from the lab have shown that BRG1 or BRM expression is deregulated in melanoma cell lines (Keenen et al., 2010). Tissue array analysis for the expression of BRG1 has shown that BRG1 levels are significantly higher in stage 4 metastatic melanomas
compared to stage 3 and normal samples. Re-expression of BRG1 in SW13 adenocarcinoma cell lines affected the expression of a subset of genes which comprised of MMP2, E-cadherin and few other genes that may regulate the invasive phenotype. Metastatic melanoma is characterized by loss of E-cadherin and gain of expression of genes involved in ECM and cell adhesion. Based on this observation, we set out to investigate if BRG1 has a role in regulating melanoma invasion.

The SK-Mel5 melanoma cell line is deficient of BRG1. Re-introduction of BRG1 in SK-Mel5 cells conferred increased resistance to cisplatin compared to cells lacking BRG1 (Keenen et al., 2010). BRG1 was recently found to play a role in the DNA repair process involving NER (Zhang et al., 2009). We found that BRG1 regulated the expression of MITF target genes p21, a cell cycle regulator and ML-IAP, a pro-survival gene. Based on both these observations, we wanted to investigate the role of BRG1 in the UV induced DNA damage response.
Chapter 2. Modulation of extracellular matrix/adhesion molecule expression by BRG1 is associated with increased melanoma invasiveness

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Running Title: BRG1 promotes melanoma invasiveness

Published in Mol Cancer. (2010 Oct 22;9:280)
Abstract

Background: Metastatic melanoma is an aggressive malignancy that is resistant to therapy and has a poor prognosis. The progression of primary melanoma to metastatic disease is a multi-step process that requires dynamic regulation of gene expression through currently uncharacterized epigenetic mechanisms. Epigenetic regulation of gene expression often involves changes in chromatin structure that are catalyzed by chromatin remodeling enzymes. Understanding the mechanisms involved in the regulation of gene expression during metastasis is important for developing an effective strategy to treat metastatic melanoma. SWI/SNF enzymes are multisubunit complexes that contain either BRG1 or BRM as the catalytic subunit. We previously demonstrated that heterogeneous SWI/SNF complexes containing either BRG1 or BRM are epigenetic modulators that regulate important aspects of the melanoma phenotype and are required for melanoma tumorigenicity in vitro.

Results: To characterize BRG1 expression during melanoma progression, we assayed expression of BRG1 in patient derived normal skin and in melanoma specimen. BRG1 mRNA levels were significantly higher in stage IV melanomas compared to stage III tumors and to normal skin. To determine the role of BRG1 in regulating the expression of genes involved in melanoma metastasis, we expressed BRG1 in a melanoma cell line that lacks BRG1 expression and examined changes in extracellular matrix and adhesion molecule expression. We found that BRG1 modulated the expression of a subset of extracellular matrix remodeling enzymes and adhesion proteins. Furthermore, BRG1 altered melanoma adhesion to different extracellular matrix components. Expression of BRG1 in melanoma
cells that lack BRG1 increased invasive ability while down-regulation of BRG1 inhibited invasive ability in vitro. Activation of metalloproteinase (MMP) 2 expression greatly contributed to the BRG1 induced increase in melanoma invasiveness. We found that BRG1 is recruited to the MMP2 promoter and directly activates expression of this metastasis associated gene.

**Conclusions:** We provide evidence that BRG1 expression increases during melanoma progression. Our study has identified BRG1 target genes that play an important role in melanoma metastasis and we show that BRG1 promotes melanoma invasive ability in vitro. These results suggest that increased BRG1 levels promote the epigenetic changes in gene expression required for melanoma metastasis to proceed.
Background

Melanoma is an aggressive malignancy, characterized by high potential for metastasis and notoriously resistant to chemotherapeutics (Chin et al., 2006; Zbytek et al., 2008). The prognosis for patients with melanoma is dependent on the stage of the disease as measured by tumor thickness, ulceration, and the presence of metastases (Xing et al.). According to the American Joint Committee on Cancer staging system, Stage I melanomas are less than 1mm thick and localized to the skin. Stage II melanomas are greater than 1mm thick, may be ulcerated, but are still localized to the skin. In stage III, the tumor has spread to nearby lymph nodes but not yet detected at distant sites. In stage IV, the tumor has spread beyond the original area of skin and nearby lymph nodes to other organs, or to distant areas of the skin or lymph nodes. The five year survival rate for stage I, II, III, and IV is estimated to be 92%, 68%, 45%, and 11% respectively (Balch et al., 2001). The high mortality rate associated with metastatic melanoma and the lack of effective treatment underscores the necessity to understand the mechanisms that promote melanoma progression.

The progression from a primary tumor to metastatic melanoma is a multistep process that involves detachment from the primary tumor mass, invasion into the dermis, migration through the extracellular matrix (ECM), and vasculature and colonization of distant sites (Gaggioli and Sahai, 2007; Melnikova and Bar-Eli, 2009). Each of these steps involves cytoskeletal alterations as well as changes in the tumor cell’s interactions with neighboring cells and with the ECM (Johnson, 1999). The inherently high metastatic potential associated with melanoma has been attributed to the migratory nature of neural crest derived precursors that give rise to the melanocyte lineage (Gupta et al., 2005). Metastatic potential is also dependent on pro-metastatic genetic changes such as those involving NEDD9
amplification as well as epigenetic changes that modulate the expression of genes required for each step in the process (Hoek et al., 2008; Kim et al., 2006). Thus, the propensity for melanoma to metastasize may be intrinsically determined, permanently fixed by genetic alterations, and dynamically modulated at an epigenetic level by signals from the changing microenvironment.

Epigenetic regulation of gene expression often involves changes in chromatin structure that are catalyzed by chromatin remodeling enzymes (Cavalli, 2006; Keenen and de la Serna, 2009). Two classes of enzymes remodel chromatin structure by catalyzing covalent histone modifications or by hydrolyzing ATP to mobilize nucleosomes (Li et al., 2007). SWI/SNF complexes are ATP dependent chromatin remodeling enzymes that have been shown to increase DNA accessibility, allowing gene specific regulators or general transcription factors to bind and to activate or repress gene expression (Li et al., 2007). SWI/SNF enzymes play critical roles during organism development (Saladi and de la Serna). Particularly relevant to melanoma is the regulatory role that SWI/SNF enzymes play in promoting neural crest migration and differentiation as well as SWI/SNF interactions with Microphthalmia – Associated Transcription Factor (MITF), a lineage survival oncogene in melanoma (Bajpai et al.; de la Serna et al., 2006a; Matsumoto et al., 2006).

Mammalian SWI/SNF complexes are composed of the BRG1 or BRM catalytic ATPase subunit and 9-12 BRG1/BRM associated factors (BAFs) (Sif, 2004). Diverse SWI/SNF complexes are distinguished by the particular ATPase and the presence of unique BAFs (Wang et al., 1996). The BRG1 and BRM containing complexes have similar chromatin remodeling activity in vitro but do not necessarily have redundant functional roles in vivo (Bultman et al., 2000). Dependent on the cellular context, BRG1 and BRM play overlapping
or distinct roles in tumorigenesis. Both BRG1 and BRM expression is down-regulated in lung cancer (Reisman et al., 2003). However, low expression of BRM has been associated with gastric cancer while high expression of BRG1 has been associated with advanced stages of gastric and prostate cancer (Sentani et al., 2001; Sun et al., 2007; Yamamichi et al., 2007).

Reconstitution of SWI/SNF subunits into cancer cells that lack expression typically induces a change in morphology (Asp et al., 2002; Dunaief et al., 1994). Furthermore, disruption of SWI/SNF activity by the introduction of dominant negative BRG1 and BRM into normal cells dramatically alters cell size and shape and invasiveness (Hill et al., 2004). These morphological changes parallel changes in the expression of cytoskeletal regulators, cell surface proteins, adhesion molecules, and enzymes that degrade the ECM (Asp et al., 2002; Banine et al., 2005; Hill et al., 2004; Liu et al., 2001; Ma et al., 2004). Thus, SWI/SNF enzymes play an important role in regulating the expression of genes important for tumor metastasis. We previously demonstrated that BRG1 and BRM expression is variable in melanoma cell lines, such that some cell lines express elevated levels of BRG1 and BRM and a subset of cell lines are deficient in BRG1 or BRM (Keenen et al.). We found that reconstitution of BRG1 in a BRG1 deficient melanoma cell line promoted expression of MITF target genes that regulate melanogenesis and survival. Furthermore, BRG1 promoted resistance to cisplatin and down-regulation of BRG1/BRM significantly compromised tumorigenicity. An independent study determined that sequential down-regulation of BRG1 and BRM inhibits melanoma proliferation (Vachtenheim et al.). These studies suggest that SWI/SNF enzymes are important epigenetic modulators of melanoma tumorigenicity and potentially regulate metastatic potential.
To further characterize BRG1 expression in melanoma, we assayed expression of BRG1 in patient derived metastatic melanomas. We found that BRG1 mRNA levels were significantly higher in stage IV tumors compared to stage III tumors and to normal skin. Furthermore, BRG1 protein levels were elevated in highly invasive human metastatic melanoma cell lines. We expressed BRG1 in an established melanoma cell line that lacks detectable levels of BRG1 and profiled expression of extracellular matrix and adhesion molecules. We found that BRG1 modulated the expression of a subset of cell surface receptors, adhesion proteins, and extracellular matrix remodeling enzymes. Furthermore, BRG1 altered adhesion to different ECM components and promoted invasion through matrigel. Activation of matrix metalloproteinase (MMP) 2 expression in BRG1 expressing cells was determined to contribute to the BRG1 mediated increase in invasive ability. Down-regulation of BRG1 in a highly invasive melanoma cell line resulted in decreased MMP2 expression and decreased invasive ability. We investigated the mechanisms involved in BRG1 mediated activation of MMP2 expression and found that BRG1 interacts with a transcriptional regulator of MMP2, the SP1 transcription factor, and is recruited to the matrix metalloproteinase (MMP) 2 promoter. In combination, these results suggest that BRG1 plays a role in promoting melanoma progression by regulating the expression of metastasis associated genes.

Results

BRG1 is highly expressed in metastatic melanoma

To evaluate BRG1 expression during melanoma progression, we examined BRG1 mRNA levels using quantitative (qPCR) arrays (Origene) containing normalized cDNA prepared from patient derived normal skin (3 samples), from stage III (21 samples) and stage
IV (19 samples) metastatic melanoma specimen. Although BRG1 mRNA levels were lower in a subset of individual melanoma samples compared to normal skin, the average level of BRG1 was higher in stage III (1.2 fold) and stage IV melanoma (1.7 fold) compared to that in normal skin (Fig. 1A). The higher levels of BRG1 in stage IV melanoma compared to normal skin was statistically significant (p<.05). There was also a statistically significant increase in BRG1 mRNA levels in stage IV melanoma compared to stage III melanoma (p<.01). Although there was also a trend toward increased BRG1 expression in stage III melanoma compared to normal skin, the increase was not statistically significant, possibly due to an insufficient normal skin sample size. Interestingly, microarray profiling of primary melanoma tumors compared to normal skin revealed that BRG1 mRNA levels in primary melanoma is significantly higher than in normal skin (Rhodes et al., 2004; Talantov et al., 2005) (Supplementary Fig. 1). In combination, these data suggest that BRG1 mRNA levels are elevated in primary melanoma compared to normal skin and increase during disease progression (from stage III to IV).

We and others determined that SK-MEL5 cells, derived from an axillary node melanoma, are deficient in BRG1 expression (Keenen et al.; Vachtenheim et al.). To determine whether BRG1 protein levels are consistently down regulated in other metastatic melanoma cell lines, we compared BRG1 protein levels in SK-MEL5 cells with levels in two highly metastatic melanoma cell lines, A375SM and WM-266-4. The A375SM cell line was established from a lung metastasis formed by injection of parental cells into nude mice (Li et al., 1989). The WM-266-4 cell line was derived from a lymph node metastasis (Westermark et al., 1986). We found that both A375SM and WM-266-4 express high levels of BRG1 compared to SK-MEL5 cells and to normal human melanocytes (Fig. 1B). We previously reported that re-
introduction of BRG1 in SK-MEL5 cells promotes pigmentation as well as increased resistance to cisplatin (Keenen et al.). As shown in Fig. 1B, BRG1 reconstituted SK-MEL5 cells express BRG1 at similar levels as A375SM and WM-266-4, which we previously estimated to be approximately 2 fold higher than that in normal melanocytes (Keenen et al.).

**BRG1 modulates extracellular matrix and adhesion molecule expression in SK-MEL5 melanoma cells**

A previous microarray study showed that re-expression of BRG1 in a BRG1/BRM deficient human adrenal adenocarcinoma cell line (SW13 cells), activated the expression of 80 genes and repressed the expression of 2 genes (Liu et al., 2001). Many of the BRG1 regulated genes were cell surface proteins and extracellular matrix remodeling enzymes or secreted proteins such as CD44, E-cadherin, matrix metalloproteinase (MMP) 2, and osteonectin (SPARC) (Banine et al., 2005; Liu et al., 2001; Ma et al., 2004). Thus, re-expression of BRG1 in BRG1/BRM deficient adenocarcinoma cells alters the expression of a subset of genes, and in particular the expression of genes that potentially have important roles in regulating tumor metastasis.

To evaluate how re-expression of BRG1 in the BRG1 deficient melanoma cell line, SK-MEL5, alters the expression of metastasis associated gene expression, we examined BRG1 induced changes in gene expression using quantitative RT² Profiler PCR Arrays (SABiosciences) and assayed the expression of 84 genes related to cell-cell and cell matrix interactions (Supplementary Table 1). We found that the expression of 13 genes on the array was highly up-regulated by BRG1 (greater than 4-fold) (Fig. 2A). The most highly up-regulated genes (greater than 10 fold) were neural cell adhesion molecule (NCAM1), E-cadherin (CDH1), catenin delta 2/neural plakophilin related armadillo protein (CTNND2),
MMP2, and laminin b3 (LAMB3) (Fig. 2A). Other highly activated genes (greater than 4 fold) included MMP10, tissue specific inhibitor of metalloproteinase (TIMP) 3, integrins a3 and a7, two collagen genes, and genes encoding components of the basement membrane (Fig. 2A).

BRG1 activated the expression of 10 additional genes at least two fold, including CD44, MMP9 and MMP14 (MT1-MMP) (greater than 2 fold) (Fig. 2B). Interestingly, re-expression of BRG1 also significantly inhibited the expression of 8 genes (Fig. 2C), while the remaining 53 genes on the array were not significantly affected by the expression of BRG1 (Supplementary Table 1). Thus our data indicate that re-expression of BRG1 in BRG1 deficient melanoma cells affects the expression of a subset of cell surface and extracellular matrix remodeling enzymes, some of which overlap (E-cadherin, CD44, and MMP2) and some which are distinct from those reported to be modulated by reconstitution of BRG1 in BRG1/BRM deficient SW13 adenocarcinoma cells. Many of the genes we found to be modulated by BRG1 (Fig. 2A, B, and C) encode proteins that play a role in regulating melanoma invasiveness and metastatic potential (Gaggioli and Sahai, 2007; Haass et al., 2005; Johnson, 1999).

The most highly activated gene in BRG1 reconstituted SK-MEL5 cells was NCAM1 (Fig. 2A). NCAM1 is a cell adhesion molecule (CAM) in the immunoglobulin superfamily that is expressed at the cell surface and mediates cell to cell and cell matrix interactions (Brummendorf and Lemmon, 2001). High expression of NCAM1 in malignant neoplasms, including melanoma, is associated with an aggressive tumor phenotype (Gattenlohner et al., 2009). Although high levels of NCAM1 have been associated with metastatic potential, the functional role of NCAM1 in melanoma has not been demonstrated, and high levels of
NCAM1 have also been detected in benign nevi (Reed et al., 1999). Thus, the role of NCAM1 in melanoma metastasis is unclear. MCAM (MUC18), a related cell adhesion molecule is over-expressed in advanced primary and metastatic melanoma. Its expression in melanoma cell lines enhances metastatic potential in nude mice (Shih et al., 1997; Xie et al., 1997). We found that in addition to NCAM1, BRG1 significantly increased the expression of MCAM (Fig. 2D). Thus, re-expression of BRG1 in SK-MEL5 cells activated the expression of two related cell adhesion molecules that have been implicated in promoting tumor metastasis. We verified that the changes in NCAM1 and MCAM expression also occurred at the protein level (Fig. 2E). Interestingly, increased levels of the 140KD NCAM1 isoform was detected in BRG1 expressing cells. This isoform is associated with malignant neoplasms and induction of anti-apoptotic programs (Gattenlohner et al., 2009).

**E-cadherin localization to the cell junction is compromised in BRG1 reconstituted SK-MEL5 cells**

Two of the most highly activated genes in BRG1 expressing SK-MEL5 cells were E-cadherin (CDH1) and catenin delta 2/neural plakophilin related armadillo protein (CTNND2) (Fig. 2A). E-cadherin is a calcium dependent transmembrane receptor that localizes to adherens junctions and mediates cell-cell adhesion. In many cancer types, loss of E-cadherin expression coincides with acquisition of an invasive phenotype and development of metastatic disease. In normal melanocytes, E-cadherin mediates melanocyte-keratinocyte interactions and loss of E-cadherin expression or a change in its cellular distribution is associated with early phases of melanoma. Furthermore, over-expression of E-cadherin in melanoma cells reduces melanoma invasiveness (Molina-Ortiz et al., 2009). Thus, expression of BRG1 in SK-MEL5 cells could potentially reduce melanoma invasiveness through up-
regulation of E-cadherin. Interestingly, BRG1 also promoted expression of d-catenin/neural plakophilin-related armadillo protein (CTNND2) (Fig. 2A), but had no effect on the expression of b-catenin or a-catenin (data not shown), two other members of armadillo/b-catenin superfamily of cell adhesion molecules. Increased expression of CTNND2 in prostate cancer has been associated with redistribution and loss of E-cadherin at the adherens junction (Lu et al., 2005).

We verified that reconstitution of BRG1 in SK-MEL5 cells resulted in increased E-cadherin and CTNND2 expression at the protein level (Fig. 3A). To determine the status of E-cadherin at the cell surface in control SK-MEL5 cells and SK-MEL5 cells expressing BRG1, we performed flow cytometry. We found that although total E-cadherin expression increased (Fig. 3A), the localization of E-cadherin to the cell surface was reduced in cells expressing BRG1 compared to control cells (Fig. 3B). Furthermore, immunofluorescence revealed that E-cadherin was mostly cytoplasmic in BRG1 expressing SK-MEL5 cells (Fig. 3C). Reduced localization of E-cadherin to the cell surface suggested that in SK-MEL5 cells, re-expression of BRG1 may further compromise E-cadherin function.

**BRG1 alters melanoma adhesion to different ECM components**

Re-expression of BRG1 in SK-MEL5 cells resulted in an altered pattern of integrin expression (Figs. 2A and 2C). Integrins are transmembrane glycoproteins that mediate specific interactions between cells and the ECM and regulate migration (Kuphal et al., 2005). Hetero-dimers composed of a and b subunits serve as receptors with specificity for different ligands. Integrin expression is a key determinant of a cell’s ability to attach to different ECM components and to migrate on these substrates. Aberrant integrin expression has been associated with melanoma progression (Kuphal et al., 2005).
BRG1 enhanced the expression of integrins α7 and α3 and inhibited the expression of integrins α4 and β3 (Figs. 2A and 2C). Modulation of integrin expression by BRG1 suggested that reconstitution of BRG1 in BRG1 deficient melanoma cells might alter melanoma cell interactions with specific ECM components. We compared the ability of the control (empty vector) SK-MEL5 cells with that of the SK-MEL5 cells expressing BRG1 to adhere to laminin, collagen, and fibronectin. We found that BRG1 expressing cells demonstrated increased adhesion to laminin and collagen and decreased adhesion to fibronectin (Fig. 4). The observed increase in adhesion to laminin is consistent with increased expression of integrin α7, which is a component of α7β1, a complex that has high affinity for laminin (Echtermeyer et al., 1996). Increased adhesion to collagen is consistent with increased expression of α3, which is a component of the α3β1 complex that has high affinity for several ECM components, including collagen (Kuphal et al., 2005). Reduced adhesion to fibronectin is consistent with decreased expression of α4, which forms the α4β1 complex and β3 which forms the αVβ3 complex, two integrins with high affinity for fibronectin (Kuphal et al., 2005). The expression of these integrins is elevated in primary or metastatic melanomas (Hartstein et al., 1997; Kramer et al., 1991; Moretti et al., 1993; Nikkola et al., 2004), however it is not possible to designate specific integrins as “pro-neoplastic” because their effect on tumor progression is dependent on the cellular context and the specific step in tumor progression (Guo and Giancotti, 2004). Our data indicate that BRG1 may regulate metastatic potential by modulating the integrin profile and altering adhesiveness to different ECM components.

**MMP2 activity is up-regulated in BRG1 expressing SK-MEL5 cells and contributes to increased melanoma invasiveness**
In addition to changes in adhesion, metastasis also requires extensive ECM remodeling. The matrix metalloproteinases (MMPs) are the main proteases that remodel the ECM (Vincenti and Brinckerhoff, 2007). Re-expression of BRG1 in SK-MEL5 cells resulted in a dramatic increase in MMP2 and MMP10 expression and a smaller but significant increase in MMP9 and MMP14 (MT-MMP1) expression (Figs. 2 A and B) and a decrease in MMP1 and MMP16 expression (Fig. 2 C). We verified that the observed changes in the mRNA profile resulted in consistent changes in protein expression for MMP1, MMP2, MMP9, and MMP14 (Fig. 5A).

Expression of MMPs is controlled at the transcriptional and post-translational levels (Yan and Boyd, 2007). Our data indicated that BRG1 promotes expression of MMP2, MMP9, and MMP14 at the protein level (Fig.5A). MMP2 (gelatinase A, 72-kDa type IV collagenase) and MMP9 (gelatinase B, 92-kDa type IV collagenase are secreted as inactive pro-zymogens that are subsequently processed and activated. MMP14 (MT1-MMP) is a membrane bound MMP that activates MMP2 at the cell surface (Sato et al., 1994b). Furthermore, naturally occurring tissue inhibitor of metalloproteinases (TIMPs) down-regulate MMP activity (Hofmann et al., 2000). The balance between TIMP and MMP expression is critically important in determining overall MMP activity. We found that in addition to MMPs, BRG1 also activated expression of TIMP2 and TIMP3, which would be expected to down-modulate MMP activity (Figs. 2A, 2B, and 5A).

In order to determine if re-expression of BRG1 in SK-MEL5 cells resulted in increased secretion of active MMP2 and MMP9, we performed gelatin zymography on supernatants derived from control and BRG1 expressing SK-MEL5 cells. We determined that although TIMP levels were increased, there was still a substantial increase in active MMP2 and MMP9.
secreted by SK-MEL5 cells expressing BRG1 compared to BRG1 deficient SK-MEL5 cells (Fig. 5B).

The observed increase in MMP2 and MMP9 activity as well as other alterations in extracellular matrix and adhesion molecule expression suggested that BRG1 plays an important role in regulating melanoma invasiveness. To determine the overall biological consequence of BRG1 re-expression in SK-MEL5 cells, we investigated whether BRG1 promotes changes in the ability of melanoma cells to be invasive in vitro. We found that SK-MEL5 cells that express BRG1 had significantly increased ability to invade through Matrigel coated Boyden chambers (Fig. 5C).

To elucidate the mechanisms by which BRG1 promotes invasion, we treated cells with an inhibitor of MMP2/MMP9 and performed invasion assays. We found that inhibition of MMP2 and MMP9 activity partially abrogated the BRG1 mediated increase in invasive ability (Fig. 5D). Consistently, siRNA mediated down-regulation of MMP2 (Fig. 5E) also reduced the BRG1 mediated increase in invasiveness (Fig. 5F). Thus, activation of MMP2 and possibly MMP9 expression contributes to the BRG1 induced increase in SK-MEL5 invasive ability.

**Down-regulation of BRG1 in WM-266-4 cells inhibits melanoma invasiveness**

Most established melanoma cell lines express high levels of BRG1 (Keenen et al.), including two metastatic melanoma cell lines, A375SM and WM-266-4 (Fig. 1B). This raised the possibility that BRG1 is required for these cells to be invasive. To determine if loss of BRG1 compromises invasive ability in one of these highly invasive cell lines, we down-regulated BRG1 expression in WM-266-4 cells using a pool of siRNAs that target BRG1 but not the alternative ATPase, BRM (Fig. 6A and 6B). We performed a timecourse after siRNA
transfection and determined that BRG1 down-regulation was effective 120 hours after transfection (Fig. 6B). Interestingly, BRM expression was slightly lower in cells transfected with control siRNA compared to untreated but then increased in BRG1 down-regulated cells. However, expression of the BRG1/BRM associated factor, INI1, did not change as a result of siRNA transfection. Previous studies have suggested that BRM expression is highly sensitive to growth conditions (Muchardt et al., 1998). We found that in WM-266-4 cells, BRM expression but not BRG1 or INI1 expression is sensitive to changes in WM-266-2 confluency (Supplementary Fig. 2). Thus, we speculate that siRNA transfection may have an inhibitory effect on BRM expression because of non-specific effects on proliferation. Nevertheless, BRM expression was elevated in BRG1 knockdown cells compared to both untreated cells and cells that expressed control siRNA (Fig. 6B).

We found that down-regulation of BRG1 resulted in decreased MMP2 and MCAM expression (Fig. 6C) and reduced invasion through Matrigel-coated Boyden chambers (Fig. 6D). Furthermore, although BRM levels increased in BRG1 down-regulated cells, our data suggest that BRM can not compensate for these BRG1 specific functions. Thus, both a gain of function and loss of function approach show that high levels of BRG1 promote melanoma invasive ability in vitro.

**SP1 interacts with BRG1 to regulate MMP2 expression in SK-MEL5 cells**

Our data suggested that activation of MMP2 is an important mechanism by which BRG1 promotes melanoma cell invasive ability. To determine the mechanism by which BRG1 activates MMP2 expression in SK-MEL5 melanoma cells, we investigated whether BRG1 interacts with a transcriptional regulator of MMP2. BRG1 was previously shown to directly activate the MMP2 promoter through interactions with the transcription factor, SP1.
in SW13 cells (Ma et al., 2004). Similarly, we found that siRNA knockdown of SP1 (Fig. 7A) reduced the level of MMP2 that was secreted by SK-MEL5+BRG1 cells (Fig. 7B). Furthermore, we detected a physical interaction between BRG1 and SP1 (Fig. 7C) and found that BRG1 was recruited to the MMP2 promoter (Fig. 7D). As was previously demonstrated in SW13 cells, BRG1 significantly increased the binding of SP1 to the MMP2 promoter (Kadam et al., 2000) (Fig. 7E). This data suggests that BRG1 directly regulates MMP2 expression in melanoma cells through interactions with SP1 and by facilitating SP1 association with the MMP2 promoter. Interestingly, SP1 has been shown to preferentially interact with the BRG1 catalytic subunit in vitro (Kadam et al., 2000). Thus, a specific role for BRG1 in the activation of MMP2 and melanoma invasiveness may result from selective interactions with the SP1 transcriptional regulator.

**Discussion**

Melanoma progression is a dynamic process that requires tumor cells to possess decreased adhesive interactions with surrounding cells and with the extracellular matrix at some points in the metastatic cascade and increased adhesive interactions at other times (Melnikova and Bar-Eli, 2007). Metastatic potential also depends on adequate vascularization and the ability to degrade components of the ECM. These processes are regulated by reversible changes in the expression of genes involved in cell attachment, motility, and proteolytic degradation of the ECM (Fidler, 1990). Previous studies showed that SWI/SNF enzymes modulate expression of ECM related molecules in normal and cancer cells (Banine et al., 2005; Hill et al., 2004; Liu et al., 2001; Ma et al., 2004). Furthermore, alterations in the expression of SWI/SNF components have been implicated in oncogenesis and multiple subunits have been determined to play tumor suppressive roles.
We previously characterized SWI/SNF subunit expression in melanoma cell lines and found that a subset of melanoma cell lines was depleted in either the BRG1 or BRM catalytic subunit. Restoration of BRG1 in a melanoma cell line that lacks BRG1 expression enhanced the expression of MITF target genes and promoted increased resistance to cisplatin (Keenen et al.).

To further characterize BRG1 expression in melanoma, we assayed expression in melanoma tumors. In the present study, we determined that BRG1 mRNA levels are significantly up-regulated in stage IV melanoma tumors when compared to normal skin or stage III melanoma tumors. Furthermore, primary melanoma tumors and most melanoma cell lines express high levels of BRG1 (Fig. 1B, Supplementary Fig. 1) (Keenen et al.; Vachtenheim et al.). A recent study indicated that BRG1 expression is increased at the protein levels in primary melanoma tumors compared to dysplastic nevi, but that there is no significant difference in BRG1 levels between primary and metastatic melanoma samples (Lin et al.). However, this study found that there may be a tendency for negative to weak BRG1 expression to be associated with a better patient survival (Lin et al.). In contrast, a separate study suggested that BRG1 protein expression is frequently down-regulated in primary and metastatic melanoma compared to normal skin, but that a higher proportion of metastatic melanoma tumors express BRG1 compared to primary tumors (Becker et al., 2009). These studies in combination with our present study suggest that BRG1 status plays a role in melanoma progression, however further investigations that utilize larger sample sizes will be required to resolve the discrepancies between the different studies.

Re-expression of BRG1 in the BRG1/BRM deficient human adrenal adenocarcinoma cell line, SW13 preferentially alters the expression of a limited number of genes that mostly
encode cell surface and ECM interacting proteins (Liu et al., 2001). Re-introduction of
BRG1 in a BRG1 deficient breast cancer cell line, ALAB also had a high impact on the
expression of genes that encode cell surface and ECM interacting proteins (Hendricks et al.,
2004). This observation and the correlation between high BRG1 levels and melanoma
progression prompted us to study the impact of BRG1 on the expression of genes involved
in adhesion and extracellular matrix remodeling in melanoma cells.

Our study indicates that BRG1 activates the expression of both overlapping and distinct
ECM related genes in melanoma cells as those in SW13 cells (Fig. 8). Expression of BRG1
in SK-MEL5 melanoma cells resulted in the activation of MMP2, E-cadherin, and CD44 as
was also seen when BRG1 was expressed in BRG1/BRM deficient SW13 cells (Banine et al.,
2005; Liu et al., 2001; Ma et al., 2004). However, the expression of osteonectin (SPARC), a
BRG1 dependent gene in SW13 cells, was not significantly affected by re-expression of
BRG1 in SK-MEL5 cells (Liu et al., 2001) (Fig. 8, Supplementary Table 1). Furthermore,
BRG1 activated and repressed a number of cell surface and ECM interacting genes in SK-
MEL5 cells that have not been identified as being BRG1 dependent in SW13 cells.
Interestingly, BRG1 had opposite effects on MMP1 expression in SK-MEL5 cells compared
to SW13 cells (Fig. 8). Thus, the requirement for BRG1 in the activation of specific genes is
to a large extent cell context dependent. Interestingly, we found that BRG1 activated the
expression of neural cell adhesion molecule (NCAM1) and d-catenin/neural plakophilin-
related armadillo protein (CTNND2), two genes whose expression is highly enriched in
neural cells. Activation of these neural specific genes by BRG1 may reflect the neural crest
derivation of melanoma cells.
Expression of BRG1 in melanoma cells modulated the expression of a number of ECM related genes that have opposing effects on melanoma invasiveness. In particular, BRG1 activated E-cadherin expression and down-regulated the expression of MMP1 and integrins a4 and b3. Down-regulation of E-cadherin and high levels of MMP1 and integrin aVb3 are associated with transition from the radial non-invasive to the invasive vertical growth phase and the acquisition of metastatic potential in melanoma (Haass et al., 2005; Huntington et al., 2004; Li et al., 2001). However, we found that BRG1 activated expression of other MMPs and integrins as well as MCAM, all of which have been shown to be important for promoting melanoma invasive ability and tumor progression (Haass et al., 2005). Melanoma cells employ distinct strategies for invasion, each of which may differ in the degree of dependence on the different molecular regulators (Gaggioli and Sahai, 2007). Interestingly, a previous study showed that dominant negative BRG1 activates integrin aV expression but still inhibits the invasive ability of fibroblasts (Hill et al., 2004). In our studies, both a gain of function and loss of function approach indicated that BRG1 promotes melanoma invasive ability, suggesting that high levels of BRG1 promote mechanisms by which melanoma cells invade that do not rely on the induction of all known cell surface regulators.

The activation of MMP2 expression by BRG1 contributed to the increased invasive ability of BRG1 expressing SK-MEL5 cells (Figs. 5C and 5D). BRG1 was previously shown to regulate MMP2 expression in SW13 cells by a transcriptional mechanism that involves SP1 (Ma et al., 2004). Our data indicate that BRG1 activates MMP2 expression in melanoma cells by a similar mechanism involving co-activation of SP1 mediated transcription (Fig. 7). However, BRG1 inhibited the expression of integrin b3, which is also regulated by SP1 (Jin et al., 1998). The differential requirement for SWI/SNF function in the regulation of a
transcription factor’s targets has been previously observed and is not well understood (de la Serna et al., 2005; de la Serna et al., 2006a). A recent study suggests that diverse SWI/SNF complexes and sub-complexes can be recruited to different promoters and that the functional outcome of SWI/SNF activity on specific promoters may be determined by the composition of the SWI/SNF complex and the chromatin context (Ryme et al., 2009). Furthermore, the recent observation that SWI/SNF enzymes also regulate microRNA expression adds an additional layer of complexity to the overall impact made by SWI/SNF enzymes in the regulation of cellular gene expression profiles (Mallappa et al.). Further work will be required to decipher the mechanisms by which a high level of BRG1 results in a gene expression profile that promotes melanoma invasiveness and potentially dictates metastatic potential in vivo.

A number of studies have implicated SWI/SNF subunits, including BRG1, as tumor suppressors. Mutations or down-regulation of BRG1 expression occurs in multiple human tumors and haploinsufficiency of BRG1 predisposes mice to mammary tumors (Bultman et al., 2008). Furthermore, when re-expressed in SW13 cells, BRG1 interacts with the retinoblastoma protein (Rb) to induce a G1 cell cycle arrest (Dunaief et al., 1994). These studies have implicated BRG1 as a tumor suppressor that curbs proliferation. In contrast, our data suggest that BRG1 expression is elevated in melanoma and promotes melanoma invasiveness. Interestingly, higher levels of BRG1 have also been associated with prostate and gastric cancer invasiveness and tumor progression (Sentani et al., 2001; Sun et al., 2007). A recent study showing that residual BRG1 expression is required for tumorigenesis to occur in INI1 deficient mice suggests that the role of BRG1 in tumorigenesis is more complex than previously thought and that the outcome of BRG1 disruption may be lineage specific (Wang.
et al., 2009). We previously reported that BRG1 interacts with MITF, the master regulator of melanocyte differentiation and lineage addiction oncogene in melanoma (Keenen et al.). In this study, we found that BRG1 promotes expression of NCAM1 and CTNND2, two markers that are highly expressed in neural crest derived cells. Thus, the contrasting role of BRG1 in melanoma may in part result from the lineage specific derivation of this cancer type.

Conclusions: Our study suggests that over-expression of BRG1 contributes to melanoma progression. We have determined that BRG1 mRNA levels are higher in stage IV metastatic melanomas compared to stage III melanomas and to normal skin. Furthermore, we have determined that BRG1 modulates the expression of extracellular matrix and adhesion molecules that play an important role in melanoma metastasis. Our data indicate that modulation of extracellular matrix and adhesion molecule expression by BRG1 is associated with increased melanoma invasive ability in vitro. The down-regulation of SWI/SNF components in tumorigenesis has been elegantly demonstrated in numerous studies and is further supported by mouse models (Reisman et al., 2009). Our work adds to several other studies (Link et al., 2008; Sentani et al., 2001; Sun et al., 2007) that suggest the over-expression of a SWI/SNF component may also contribute to tumorigenesis.

Methods

Cell Culture

SK-MEL5 and WM-2664 melanoma cells were from the ATCC. A375SM melanoma cells were a kind gift from Dr. Menashe Bar-Eli (M.D. Anderson Cancer Center). SK-MEL5 cells expressing an empty vector or BRG1 were described in (Keenen et al.). Human
melanocytes were from Cascade Biologics (Portland, Oregon, USA) or Yale Cell Culture Core Facility (New Haven, Connecticut, USA). With the exception of melanocytes, all cells were grown in DMEM supplemented with 10% FBS. Human melanocytes were grown in Media 254 with added growth supplements (Cascade Biologics). The MMP2/MMP9 inhibitor, 4-Biphenylylsulfonyl)amino-N-hydroxy-3-phenylpropionamide (BiPS) was from Calbiochem (San Diego, CA, USA) and was used at 10mM.

**RNA Isolation and Quantitative Real-time PCR**

Total RNA was isolated with the Qiagen RNeasy mini kit and reverse transcribed as described (Keenen et al.). Quantitative real-time PCR was performed in SYBR Green Master Mix (Qiagen, Germantown, Maryland) with an Applied Biosystems Prism 7500 PCR system and analyzed with the SDS software as described (Keenen et al.). MCAM and GAPDH primers were purchased from SABiosciences (Frederick, MD, USA).

**Tumor qPCR Arrays**

The Tissue Scan Melanoma qPCR Arrays (MERT501) containing cDNAs from normal skin, stage III, and stage IV melanomas were obtained from Origene Technologies (Rockville, MD, USA). The primers used to detect BRG1 (SMARCA4) were from SABiosciences (Frederick, MD, USA). BRG1 levels were normalized by amplifying with primers to Human b-actin (Forward: CAGCCATGTACGTTGCTATCCAGG) and (Reverse: AGGTCCAGACGCAGGATGGCATG). The results were averaged from values obtained by running three PCR arrays. Statistical significance was determined by utilizing a Mann-Whitney Wilcoxon test.

**Extracellular Matrix and Adhesion focused qPCR Arrays**
Extracellular Matrix and Adhesion molecule RT² Profiler PCR Arrays were purchased from SABiosciences (Fedrick, MD). The primer sets in this array are described in http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-013A.html. CT values obtained for 84 extracellular matrix and adhesion molecule gene expression were normalized to a value obtained by averaging the CT values of four different housekeeping genes. For each primer set, the fold change in SK-MEL5+BRG1 cells was determined relative to values obtained in control SK-MEL5 cells +empty vector. Average values were obtained from four PCR arrays with cDNA from control cells (from three different samples) and an additional four PCR arrays with cDNA from SK-MEL5 cells +BRG1 (from three different samples). Statistical significance was calculated using the student’s t test.

**Antibodies**

The Tubulin antibody was from Sigma (St. Louis, Missouri, USA). FLAG M2 antibody and FLAG M2-Agarose were from Sigma. The E-cadherin, CTNND2, and MCAM antibodies were from BD Biosciences (San Jose, CA, USA). The BRG1, NCAM1 and SP1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The MMP2 and MMP9 antibodies were from Cell Signaling (Beverly, MA, USA). The MMP14 antibody was from Millipore (Temecula, CA, USA). The TIMP3 antibody was from Abcam (Cambridge, MA, USA). Control IgG antibody used for ChIPs was from Millipore (Billerica, MA, USA).

**Cell extracts and immunoblot analysis**

Cells were lysed in 20mMTris (pH 7.4),150 mM NaCl, 2mM EDTA, 1% Triton X, 10% glycerol, supplemented with a protease inhibitor cocktail (Sigma). SDS-PAGE and Western blotting were carried out as described (de La Serna et al., 2000).
**Flow Cytometry**

Cells were incubated in fetal bovine calf serum (Invitrogen, Carlsbad, CA, USA) for 10 minutes at room temperature to block nonspecific antibody binding and then with the primary antibody or an isotype matched IgG control for 20 minutes at 4°C. After one wash with FACS buffer (PBS+0.5%BSA, 5% fetal calf serum, 0.1% sodium azide, cells were incubated with secondary antibody for 20 minutes at 4°C, then washed twice with FACs buffer. Cells were re-suspended in 0.1% paraformaldehyde then loaded onto a FACS-Calibur (BD Biosciences, San Jose, CA, USA). Data was analyzed using Cell Quest Pro (BD Biosciences). Statistical significance was calculated using the student’s t test.

**Immunocytochemistry**

Immunocytochemistry was performed as described (de la Serna et al., 2006a) using an E-cadherin antibody (BD Biosciences) and goat anti-mouse-Alexa Fluor568 (Molecular Probes). Images were taken with a Nikon Eclipse TE2000-U fluorescence microscope at 60X magnification.

**Zymography**

Zymography was performed as previously described (Ma et al., 2004). Control SK-MEL5 and SK-MEL5+BRG1 cells were cultured in serum free medium for 36 hours. Conditioned medium was collected, normalized to cell number, and subjected to electrophoresis in a polyacrylamide gel containing 1mg/ml gelatin. After electrophoresis, the gel was washed in 2.5% Triton X-100 for 1 hour at room temperature to remove the SDS and then incubated for 24 hours at 37°C in a buffer consisting of 5mM CaCl₂ and 1mM ZnCl₂. The gel was stained in 0.25% Coomasie Blue for 30 minutes, de-stained in methanol/acetic acid solution.
and photographed on a light box. Proteolytic activity was detected as white bands against a blue background.

**siRNA Knockdowns**

Acell SMART Pool siRNAs targeting BRG1 (E-010431), MMP2 (E-005959), SP1 (E-026959), and red non-targeting siRNAs (D-001960) were purchased from Dharmacon Inc. (Chicago, Il., USA) and used to transfect melanoma cells according to the manufacturer’s instructions. WM-266-4 cells were transfected with control or siRNA targeting BRG1. BRG1 expressing SK-MEL5 cells were transfected with control or siRNA targeting MMP2 or SP1.

**Adhesion Assays**

Adhesion assays were performed as previously described (Wang et al., 2002). 96 well plates were coated with laminin (10ug/ml), collagen (type 1) (20ugm/ml), or fibronectin (20ugm/ml), and incubated at 4°C overnight. The plates are then washed with Wash buffer (DMEM with 0.1% BSA) and blocked in DMEM with 0.5% BSA for 45-60 minutes at 37°C. 2x10^4 cells were added to each well and incubated at 37°C for 30 minutes. Non-adherent cells were removed by washing three times with Wash buffer. The cells are then fixed with paraformaldehyde and incubated for 10-15 minutes and washed once with Wash buffer. The cells were stained with crystal violet for 10 minutes, washed with water, and dried. 2% SDS was added and the plates were incubated at room temperature for 30 minutes. Absorbance was read at 550nm. Statistical significance was calculated using the student’s t test.

**Matrigel Invasion Assay**

Invasion assays were performed using matrigel coated chambers (BD Biosciences, Bedford, MA, USA) as recommended by the manufacturer. SK-MEL5 cells expressing an empty
vector or BRG1 were seeded in serum free media at a density of $1.25 \times 10^5$ cells per well on top of control or matrigel inserts. Media containing 5% FBS was used as a chemoattractant. After incubation for 16 hours, non-invading cells were removed from the upper surface and invading cells were stained with 1% Toluidine Blue and counted. Multiple fields were counted in triplicate membranes with a microscope at 20X magnification. The data shown is from two independent experiments done in triplicate. For studies involving inhibition of MMP2/MMP9, cells were pre-treated with 10mM 4-Biphenyllysulfonyl]amino-N-hydroxy-3-phenylpropionamide (BiPS) (Calbiochem, San Diego, CA, USA) for 3 hours and then plated onto the Boyden chambers in media containing 10mM BiPS. For knockdown studies, invasion assays were performed 120 hours after transfection of control or siRNAs targeting BRG1. Statistical significance was calculated using a student’s t test.

**Co-Immunoprecipitations**

Co-immunoprecipitations were performed as previously described (Keenen et al.).

**Chromatin Immunoprecipitations**

Chromatin Immunoprecipitations were performed as previously described (Keenen et al.) using FLAG to detect FLAG-BRG1 or IgG as a control. The primers used to detect the MMP2 promoter were (Forward: GGGGAAAAGAGGTGGAGAAA) and (Reverse: CGCCTGAGGAAGTCTGGAT). CD25 primers were previously described (Doan et al., 2004). Statistical significance was calculated using the student’s t test.

**Conflict of interest:**

The authors declare no conflict of interest.

**Author Contributions**
SVS designed and performed most of the experiments. BK performed RT-PCR experiments, invasion assays, co-immunoprecipitations, and chromatin immunoprecipitations. HGM performed Westerns. HQ performed RT-PCR experiments and helped with data analysis. KVC helped design experiments and contributed resources. ILD conceived the study, designed experiments, and wrote the article. All authors approved the final version.

**Financial Support**

ILD was supported by the National Institute of Environmental Health Sciences; Grant number: 5K22ES12981, Ohio Cancer Research Associates, American Cancer Society, Ohio Division
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Figure Legends

Figure 1

**BRG1 is highly expressed in patient derived melanomas and metastatic melanoma cell lines.**

A. Tissue Scan Melanoma qPCR Arrays (Origene) containing cDNAs from patient derived normal skin, stage III melanomas, and stage IV melanomas were subjected to qRT-PCR with BRG1 specific primers. BRG1 levels were normalized to b-actin. The results were averaged from values obtained by performing three PCR arrays. B. Western blot comparing BRG1 levels in two invasive melanoma cell lines (A375SM and WM-266-4) and human epidermal melanocytes with that in BRG1 deficient SK-MEL5 cells, SK-MEL5 cells reconstituted with empty vector (EV), and BRG1 reconstituted SK-MEL5 cells. Total cell lysate was loaded. Tubulin is a loading control.

Figure 2

**BRG1 modulates the expression of extracellular matrix and adhesion molecule expression in SK-MEL5 cells.**

The expression of extracellular and adhesion molecule related genes was profiled in control SK-MEL5 (empty vector) and BRG1 reconstituted SK-MEL5 cells using a focused qPCR array. The expression level of each gene was normalized to a control value obtained by averaging the expression of four housekeeping genes. The average of three to four independent experiments is shown. All changes in gene expression are statistically significant as determined by the student’s t test (p value equal to or less than 0.05). A. Expression of genes that are activated greater than four-fold by BRG1. B. Expression of genes that are activated greater than two fold by BRG1. C. Expression of genes that are down-regulated by
BRG1. D. MCAM expression was evaluated separately from the qPCR array using gene specific primers. Normalization of mRNA levels was to GAPDH. E. Detection of NCAM1 and MCAM protein expression by Western blotting. Tubulin is a loading control.

**Figure 3**

**BRG1 promotes E-cadherin expression but compromises E-cadherin localization to the cell junction in SK-MEL5 cells**

A. Detection of E-cadherin (CDH1) and CTNND2 protein expression by Western blotting. Tubulin is a loading control. B. Cells were stained with control IgG or an antibody to E-cadherin and FITC labeled secondary antibody. E-cadherin at the cell surface was quantified by FACS analysis. Significantly less E-cadherin was localized to the cell surface in SK-MEL5 +BRG1 cells compared to control SK-MEL5 cells (p<0.05). C. E-cadherin staining of representative control SK-MEL5 cells and BRG1 expressing SK-MEL5 cells by immunofluorescence.

**Figure 4**

**BRG1 expression alters adhesion of SK-MEL5 cells to different ECM components.**

96 well plates were coated with laminin, collagen, or fibronectin and blocked with BSA. Control SK-MEL5 cells expressing empty vector (EV) and SK-MEL5 cells expressing BRG1 were seeded onto coated plates at a density of 2X10^4 cells. After incubation for 30 minutes, plates were washed and the cells stained with crystal violet. Control plates coated with BSA were also included but did not retain cells after washing (data not shown). Representative fields are shown (20X magnification). Adhesion was quantified by reading absorbance at 550 nm. The data shown is the average of two independent experiments done in triplicate. The fold change in adhesion to all three substrates was significantly altered by expression of
BRG1 (p<0.01). A. Adhesion to laminin. B. Adhesion to collagen. C. Adhesion to fibronectin.

Figure 5

MMP2 activity is up-regulated in BRG1 expressing SK-MEL5 cells and promotes invasion through matrigel.

A. Detection of MMP and TIMP protein expression by Western blotting. Tubulin is a loading control. B. MMP activity in control (EV) and SK-MEL5 expressing BRG1 was determined by zymography. Cells were cultured in serum free media for 24 hours and the supernatant collected and normalized to cell number. C. Invasion assays were performed using matrigel coated chambers with 5% FBS as a chemoattractant. Control SK-MEL5 cells (EV) and SK-MEL5 cells expressing BRG1 were seeded at a density of 1.25x10^5 cells per well on top of control or matrigel inserts. Percent invasion through matrigel was calculated relative to migration through the control insert. The data shown is the average of two independent experiments done in triplicate. Expression of BRG1 significantly increased invasion (p<0.01). D. Vehicle treated (DMSO) or BiPS (10mM) treated BRG1 expressing SK-MEL5 cells were subjected to the invasion assay as described in C. The data shown is representative of two independent experiments performed in duplicate. Treatment with the MMP2/MMP9 inhibitor, BiPS significantly inhibited invasion (p<0.01). E. SK-MEL5 +BRG1 cells were transfected with Acell SMART Pool siRNAs targeting MMP2 or red non-targeting siRNAs and analyzed by Western blotting 120 hours after transfection. F. Control and MMP2 down-regulated SK-MEL5+BRG1 cells were subjected to the invasion assay as described in C, 120 hours after transfection with siRNAs. Invasion assays were performed in triplicate. Down-regulation of MMP2 significantly compromised invasion (p<0.01).
Figure 6

Down-regulation of BRG1 inhibits melanoma invasion through matrigel.

A. WM-266-4 melanoma cells transfected with control (DY-547 labeled) siRNAs were visualized by phase-contrast microscopy.  B. WM-266-4 melanoma cells were transfected with Acell SMART Pool siRNAs targeting BRG1 or red non-targeting siRNAs and analyzed by Western blotting.  C. Control and BRG1 down-regulated WM-266-4 cells were subjected to the invasion assay as described in Fig. 5, 120 hours after transfection with siRNAs. Invasion was significantly compromised in BRG1 down-regulated cells (p<0.01).

Figure 7

SP1 interacts with BRG1 to regulate MMP2 expression

A. SK-MEL5 +BRG1 cells were transfected with Acell SMART Pool siRNAs targeting SP1 or red non-targeting siRNAs. SP1 expression was analyzed by Western blotting 144 hours after transfection.  B. MMP2 secretion in control and SP1 down-regulated cells was analyzed by zymography 144 hours after transfection.  C. Co-immunoprecipitations were performed with FLAG antibody or control IgG. Co-immunoprecipitating proteins were analyzed by Western blotting with the indicated antibodies.  D. Chromatin immunoprecipitations (ChIPs) were performed with antibodies to SP1, BRG1, or control IgG and analyzed by qPCR with primers specific for the MMP2 promoter or a control CD25 region. The results from two independent experiments were assayed twice. BRG1 was significantly enriched on the MMP2 promoter compared to the control CD25 region (p<0.01). SP1 binding to the MMP2 promoter was significantly increased in SK+MEL5+BRG1 compared to control cells (p<0.01).
Figure 8

Comparison of extracellular matrix and adhesion molecule regulation by BRG1 in SW13 and SK-MEL5 cells

Venn diagram showing genes up-regulated by BRG1 in SW13 cells (yellow), genes up-regulated by BRG1 in SK-MEL5 cells (red), and genes down-regulated by BRG1 in SK-MEL5 cells (green). Three genes were activated by BRG1 in both SW13 and SK-MEL5 cells (orange). Only genes that were assayed in both SK-MEL5 cells (present study) and SW13 cells (Banine et al., 2005; Liu et al., 2001; Yamamichi-Nishina et al., 2003) are shown.

Supplementary Figure 1.

Gene expression profiling of BRG1 (SMARCA4). The box plot is from a gene expression data set (Talantov, Clinical Cancer Research, 2005) as reported by the Oncomine microarray database. The levels of BRG1 mRNA in the indicated number (N) of malignant melanoma samples were determined to be significantly higher than those in samples from normal skin (p = 2.9 × 10⁻⁷).

Supplementary Figure 2.

Extracellular Matrix and Adhesion molecule RT² Profiler PCR Array. The expression of 84 extracellular and adhesion molecule related genes was profiled in control SK-MEL5 (empty vector) and BRG1 reconstituted SK-MEL5 cells using a focused qPCR array. For each primer set, the fold change in SK-MEL5+BRG1 cells was determined relative to values obtained in control SK-MEL5 cells +empty vector. Statistical significance was calculated using the student's t test (* indicates p < 0.05, **indicates p < 0.01). All other values were found to be not significantly different. Expression of genes that were activated by BRG1 greater than 2 fold are highlighted in red. Expression of genes that were down-regulated by
BRG1 at least 2 fold are highlighted in green. N.D. represents genes that were not detected by the qRT-PCR assay or that had CT values > 34.
Figure 1

A.

B.
Figure 2

A. Fold Increase (mRNA)

B. Fold Increase (mRNA)
Figure 3

A.

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B.

%CDH1 Positive Cells

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C.

EV

BRG1

EV

BRG1
Figure 4

A. [Graph showing absorbance at 550 nm for Laminin with bars for EV and BRG1]

B. [Graph showing absorbance at 550 nm for Collagen with bars for EV and BRG1]

C. [Graph showing absorbance at 550 nm for Fibronectin with bars for EV and BRG1]
Figure 5

A. 

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B. 

(kDa)

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C. 

Number of Invading Cells

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D. 

Number of Invading Cells

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Figure 5

E. 

si  Control  MMP2

MMP2

ERK

F. 

Number of Invading Cells

si  Control  MMP2
Figure 6
Figure 7

A. si Control SP1
   SP1
   ERK

B. si Control SP1
   (kDa)
   76
   52

C. BRG1
   Total protein
   FLAG
   IgG
   Total protein
   FLAG
   IgG
   SP1
   BRG1

MMP2
Figure 7

D.

E.
Figure 8

SW13

- SPARC
- MMP1
- ECM1
- E-cadherin
- MMP2
- CD44

SK-MEL5

- MMP9
- COL5α1
- MMP10
- COL8α1
- MMP14
- LAMA2
- TIMP2
- COL15α1
- TIMP3
- LAMA3
- ITGα3
- THBS3
- ITGα7
- CTNND2
- MCAM

- MMP1
- ITGβ3
- THBS1
- MMP16
- SPP1
Supplementary Figure 1

![Box plot showing log2-transformed transcript levels for normal and melanoma samples.](image)

- **Normal**
  - N=7

- **Melanoma**
  - N=45
Supplementary Figure 2

<table>
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Conclusions from this chapter:

• BRG1 expression is significantly higher in stage IV melanoma compared to normal skin and stage III melanoma.

• BRG1 expression modulates the expression of ECM related genes.

• BRG1 interacts with SP1 and regulates MMP-2 expression and activity.

• Activation of BRG1 increases the ability of SK-MEL5 cells adhere to laminin and collagen and also increases the invasive potential in vitro.

• Knock-down of BRG1 in metastatic melanoma cells decreases the expression of MMP2 and Mcam and decreases the invasive potential in vitro.
Chapter 3. **BRG1 epigenetically modulates the DNA damage response in melanoma cells and promotes survival through a cooperative activation loop with MITF**

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Abstract

Exposure to ultraviolet (UV) radiation causes DNA damage and is implicated in the etiology of cutaneous melanoma, an aggressive malignancy that is notoriously chemoresistant. In most cells, DNA elicits a highly conserved cellular response that involves activation of cell cycle checkpoints, DNA repair, and apoptosis. However, specialized mechanisms render melanocytes and melanomas particularly resistant to apoptosis. SWI/SNF chromatin remodeling enzymes have previously been demonstrated to promote survival in response to DNA damage by promoting cell cycle checkpoints and DNA repair. However, a direct role for SWI/SNF enzymes in the regulation of apoptosis has not yet been demonstrated. In this study, we show that in melanoma cells, BRG1 modulates the response to UV-induced DNA damage by activating expression of the melanoma inhibitor of apoptosis gene (ML-IAP). BRG1 epigenetically regulates ML-IAP expression through a cooperative activation loop with the master regulator of melanocyte differentiation and lineage addiction oncogene, Microphthalmia-Associated Transcription Factor (MITF), to promote transcriptionally permissive chromatin structure on the ML-IAP promoter. Thus, we illuminate a lineage specific mechanism by which BRG1 epigenetically modulates the cellular response to DNA damage through regulation of an anti-apoptotic gene.
**Introduction**

Cutaneous melanocytes function to synthesize and distribute melanin to surrounding cells on the skin, thus protecting against the damaging effects of ultraviolet (UV) radiation from sunlight. Excessive exposure to UV radiation causes DNA damage and is the most critical environmental risk factor for developing melanoma (23). Malignant melanoma is an aggressive malignancy that is refractory to most chemotherapeutics and thus has a high mortality rate. The aggressive nature of melanoma has been linked to expression of lineage-specific factors that are not present in other cell types (20) and to the evolution of unique pro-survival mechanisms that render melanocytes particularly resistant to death from UV radiation, thus allowing them to fulfill their role in photoprotection (23).

Sunlight emits UV radiation that can be divided into three major regions: UVC (200-280nm), UVB (280-320), and UVA (320-400nm) (23). UVC is not a risk factor for the development of melanoma because it is shielded by atmospheric ozone. Exposure of skin to UVA and UVB results in DNA damage and elicits a highly conserved cellular response that is critical for maintaining genomic integrity. The three key steps in the response include cell cycle arrest, DNA repair, and apoptosis. Each of these steps may be modulated depending on the type of damage and by cell specific mechanisms.

UVB can induce bulky DNA lesions in the form of cyclobutane dimers and 6-4 photoadducts, between the 5’ six position and the 3’ four position of two adjacent pyrimidines that can lead to genetic mutations and can initiate melanoma in mice (10).
UVA, the predominant component of sunlight, penetrates deeply in the skin and can indirectly cause DNA damage by generating reactive oxygen species. The response to DNA damage involves activation of the p53 pathway which promotes expression of p21CIP1/WAF1 and cell cycle arrest at a G1 checkpoint (23). The bulky cyclobutane dimers and photoproducts are primarily removed by nucleotide excision repair (NER) and the oxidative lesions by base excision repair (BER). If the damage can be successfully repaired, mechanisms exist for re-entry into the cell cycle, or if the damage is irreparable, cells generally undergo apoptosis, however melanocytes and melanoma cells have evolved lineage specific mechanisms that render them particularly resistant to apoptosis (23).

The Microphthalmia Associated Transcription Factor (MITF) specifies the melanocyte lineage and promotes melanocyte differentiation and survival. MITF is also considered a lineage addiction oncogene that is amplified in about 20% of melanomas and contributes to melanoma chemoresistance (16). In addition to melanocyte specific genes that regulate melanin synthesis and melanocyte function, MITF directly regulates expression of the cell cycle inhibitors p21CIP1/WAF1 and p16INK4A (5, 28). In response to DNA damage, MITF facilitates cell cycle arrest by activating p21CIP1/WAF1 and ensures genomic integrity by promoting early stages of DNA repair (27). MITF also activates the expression of the pro-survival genes, BCL2 and ML-IAP (BIRC7, livin) (14, 30). High levels of BCL2 and ML-IAP have been correlated with increased melanoma survival following UV irradiation and treatment with other DNA damaging agents (4, 22). Thus, as a regulator of pro-survival genes, MITF is a primary determinant of
melanocyte survival following UV irradiation. We previously reported that SWI/SNF enzymes interact with MITF to regulate MITF target genes in melanoma (25).

SWI/SNF enzymes are multi-subunit complexes that remodel chromatin structure in an ATP dependent manner and render chromatin permissive for transcription, replication, and DNA repair (13). Heterogeneous complexes with specific functions can be formed by the inclusion of one catalytic subunit, which is BRG1 or BRM, and 8-12 associated proteins (BAFs), including the core subunits, BAF47, BAF155, and BAF170 (38). Certain SWI/SNF subunits have tumor suppressor activity and are involved in the maintenance of genomic integrity (37). BAF47-null mouse embryonic fibroblasts exhibit abnormal nuclear morphology and are impaired in the ability to survive upon exposure to UV radiation and treatment with genotoxic agents (3). Furthermore, SWI/SNF complexes have been implicated in the regulation of the DNA damage response by their role in the transcriptional activation of genes involved in the control of proliferation and checkpoint activation (17). Several studies also reveal that the SWI/SNF complex have a direct role in the repair of double strand breaks induced by gamma irradiation as well as a critical role in repairing DNA damage induced by UV radiation (33, 36). However, SWI/SNF enzymes have not previously been demonstrated to directly affect the apoptotic pathway in DNA damaged cells.

We previously demonstrated that that re-expression of the SWI/SNF subunit, BRG1, in BRG1 deficient melanoma cells increases survival after treatment with the DNA damaging agent, cisplatin (26). Furthermore, we found that BRG1 is highly expressed in metastatic melanoma tumors, potentially contributing to the chemoresistance
that is typical of advanced melanoma (39). In this study we determined that BRG1 promotes melanoma survival in response to UV-radiation. We found that in addition to previously established mechanisms that involve cell cycle checkpoint control and DNA repair, BRG1 inhibits apoptotic pathways by activating expression of the melanoma inhibitor of apoptosis, ML-IAP gene. Our data show that activation of ML-IAP by BRG1 is highly dependent on MITF and that a cooperative activation loop involving BRG1 and MITF operates to establish permissive chromatin structure on the ML-IAP promoter and ensure high levels of ML-IAP expression. Thus, we have identified a lineage specific mechanism by which BRG1 epigenetically modulates the response to DNA damage, by promoting expression of an inhibitor of apoptosis.

**Materials and Methods**

**Cell Culture**

SK-MEL5, WM-2664, and A375 melanoma cells were from the ATCC. The 501Mel and YUMAC melanoma cell lines were from Yale Cell Culture Core Facility (New Haven, Connecticut, USA). The SKMEL5 stable cells lines expressing empty vector (EV) or BRG1 were previously described (26). All melanoma cells were grown in DMEM supplemented with 10% FBS.

**UV irradiation**

Melanoma cell cultures were washed with phosphate buffered saline (PBS) and irradiated in a small volume of PBS with an FS20 lamp that emits a spectrum which is 75% in the UVB range, and 25% in the UVA range, peaking at 313 nm (National Biologics, Twinsburg Ohio, USA). Wavelengths in the UVC range were blocked by
covering the lamp with a Kodacel sheet (Eastman Kodak, Rochester, NY, USA). The cells were subjected to doses of 21 mJ/cm\(^2\) (for gene expression studies) or 50mJ/cm\(^2\) for all other experiments. UV radiation dosage was monitored with a UVB-500C meter (National Biologics, Twinsburg Ohio, USA). Sham irradiated cells were subjected to identical conditions except that the lamp was not turned on. After irradiation, growth media was replaced and cells were incubated for designated intervals of time.

**Transfections**

A375 melanoma cells were transfected with CMV-Lac Z or CMV-MITF using Liptofectamine LTX (Invitrogen, Carlsbad CA, USA) according to the manufacturer’s recommendations. Cells were harvested 24 hours after transfection for RNA or protein.

**RNA Isolation and Quantitative Real-time PCR**

Total RNA was isolated by Trizol extraction and reverse transcribed as described in (26). Quantitative real-time PCR was performed in SYBR Green Master Mix (Qiagen, Germantown, Maryland) with an Applied Biosystems Prism 7500 PCR system and analyzed with the SDS software as described (26). Primers used for determining mRNA levels were from SABiosciences. A no RT control was included for each primer set.

**Cell extracts and immunoblot analysis**

Cells were lysed in 20mMTris (pH 7.4),150 mM NaCl, 2mM EDTA, 1% Triton X, 10% glycerol, supplemented with a protease inhibitor cocktail (Sigma). SDS-PAGE and Western blotting were carried out as described (11). The Tubulin antibody was from Sigma (St. Louis Missouri, USA). The BRG1 antiserum was previously described (12). The ML-IAP (livin) antibody was from Abcam (Cambridge, MA, USA).
p21CIP1/WAF1 and p53 antibodies were from Santa Cruz Inc. (Santa Cruz CA, USA). The ERK (p42/p44) antibody, the caspase 3 antibody, and the antibody detecting cleaved PARP were from Cell Signaling Technology (Boston MA, USA). The MITF (C5) antibody was a gift from Dr. David Fisher (Massachusetts General Hospital, Boston MA, USA).

**TUNEL Assay**

Cells were plated on chamber slides and irradiated as described above. DNA strand breaks were assayed with the the In situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis Indiana, USA) according to the manufacturer’s directions. Briefly, cells were fixed with 1% (w/v) paraformaldehyde, washed, and permeablized. DNA was labeled with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and dUTP-fluorescein. Cells were then washed and visualized at 40X magnification with a Nikon Eclipse TE2000-U fluorescence microscope.

**Annexin V Assay**

The Guava Nexin Annexin V Reagent Kit (Millipore, Billerica, MA, USA) was used to detect phosphatidylserine translocation to the external face of the cell membrane. Cells were washed and stained with Annexin V-PE and the dye, 7-ADD as recommended by the manufacturer (Millipore, Billerica, MA, USA). Stained cells were then assayed on a Guava Personal Cell Analysis System and analyzed with the Guava software (Millipore).
**Comet Assays**

Comet assays were performed under alkaline conditions as described in (41) except that DNA was stained with SYBR Orange. Comet images were captured with a Nikon Eclipse TE2000-U fluorescence microscope at 60X magnification and analyzed with TriTek Comet Score Freeware program. Average value of tail moments was determined by counting at least 100 cells per sample. Statistical significance was calculated using the student’s t test.

**Gamma H2AX foci**

Immunocytochemistry was performed as previously described (39) with an antibody to gamma H2AX (Millipore, Billerica MA, USA) and a goat anti-mouse-Alexa secondary antibody (Molecular Probes). Images were taken with a Nikon Eclipse TE2000-U fluorescence microscope at 100X magnification. Foci were counted in at least 50 nuclei. Statistical significance was calculated using the student’s t test.

**siRNA Knockdowns**

Acell SMART Pool siRNAs targeting BRG1 (E-010431) and red non-targeting siRNAs (D-001960) were as previously described. All other siRNA knockdowns were performed with Dharmafect 1 according to the manufacturer’s instructions. The siRNA targeting both isoforms of ML-IAP was 5’-(GGAAGAGACUUUGUCCACA (dTdT)-3’ as previously described in (7). The siRNA targeting ML-IAP-a was 5’-(GGGCGUGGUGGGUUCUUGA (dTdT)-3’ as previously described in (8, 45). The siRNA targeting MITF was 5’-(AGCAGUACCUUCUACCAC)(dTdT)-3’ and the scrambled siRNA used as a control for both ML-IAP and MITF knockdowns was 5’-
(UUCUCCGAACGUGUCACGU)(dTdT)-3’ as previously described in (6). Cells were UV irradiated 72 hours after siRNA transfection and assayed by Western blotting or subjected to the annexin V, and TUNEL assays, 36 hours after irradiation. Statistical significance was calculated using the student’s t test.

**Chromatin Immunoprecipitations**

Chromatin Immunoprecipitations were performed and quantified by qPCR as previously described (26) Antibodies to H3k4me3, H3ac, and H3K27me3 were from Active Motif (Carlsbad CA, USA). Control IgG and the antibody to H3K27me3 was from Millipore. The antibody to MITF (C5) used in ChIPs was from Abcam (Cambridge MA, USA). Statistical significance was calculated using the student’s t test.

**Chromatin Accessibility Experiments**

Nuclei were isolated in NP40 lysis buffer (10mM Tris-HCl (pH 7.4), 10mM NaCl, 3mM MgCl2, 0.5% NP40), washed once with MNase I digestion buffer (10mM Tris-HCl (pH 7.4), 15 mM NaCl, 0.15mM spermine, 0.5mM spermidine) and then resuspended in MNase I digestion buffer. CaCl2 was added to a final concentration of 1mM and the samples were transferred to tubes containing 3U/ml MNase I (Worthington Biochemical Corp., Lakewood NJ, USA). Digestion was stopped after 15minutes with Stop buffer (100mM EDTA, 10mM EGTA, 2.5%SDS) and the genomic DNA was extracted twice with phenol/chloroform. Control samples were treated identically except that MNase I was not added. Purified genomic DNA was subjected to qPCR and analyzed as described in (9, 35). Statistical significance was calculated using the student’s t test.
Results

BRG1 protects melanoma cells from apoptosis after UV irradiation

Our previous studies indicated that expression of the two SWI/SNF ATPase subunits, BRG1 and BRM, is variable in established melanoma cell lines, such that one ATPase is down-regulated in a subset of melanoma cell lines compared to normal human melanocytes (26). One cell line, SK-Mel 5 melanoma cells express BRM but do not express detectable levels of BRG1. We constructed SK-MEL5 cells that stably express BRG1 and found that BRG1 activated a number of MITF target genes and increased resistance to the DNA damaging agent, cisplatin (26). Ultraviolet radiation from the sun consists of wavelengths in the UVA and UVB, and UVC range, however, the UVC waveband does not reach the earth’s surface. Although UVA and UVB wavebands both cause DNA damage and have been implicated in melanoma, only the UVB waveband is capable of initiating melanoma in mice (10). Previous studies investigated the role of SWI/SNF enzymes in the cellular response to UVC (17, 36), but there have not yet been any studies on the role of SWI/SNF enzymes in the response to wavebands of ultraviolet light relevant to melanoma. To determine whether BRG1 protects against UVB-induced DNA damage, we irradiated SK-MEL 5 cells expressing empty vector and SK-MEL5 cells expressing BRG1 with a lamp that emits ultraviolet light predominantly in the UVB range. We found that p53 was activated in both control and BRG1 expressing cells and that BRG1 levels remained high after irradiation (Fig. 1A). Consistent with previous reports, MITF levels were decreased as a result of UV irradiation. UV irradiation leads to phosphorylation of MITF, which promotes transcriptional activity that is followed by
proteosome-mediated degradation (27). These data indicate that UV irradiation elicited a characteristic DNA damage response in both control SK-MEL5 cells (empty vector) and in SK-MEL5 cells expressing BRG1.

In order to determine how BRG1 modulates the response to UV radiation, we assayed proliferation of control SK-MEL5 melanoma cells and BRG1 expressing cells during steady state conditions and after UV irradiation. When an equivalent number of cells were plated and cultured under standard conditions, BRG1 expressing cells accumulated at a slower rate than control SK-MEL5 cells (Fig. 1B, top). UV irradiation resulted in a decrease in proliferation of both control and BRG1 expressing cells however, proliferation of BRG1 expressing cells was less affected than that of control cells (Fig. 1B, bottom). Furthermore, BRG1 protected the irradiated melanoma cells from apoptosis as reflected by a decreased percentage of TUNEL positive cells (Fig. 1C) and a decreased percentage of annexin positive cells compared to irradiated SK-MEL5 cells expressing only empty vector (Fig.1D). Expression of the effectors of apoptosis, cleaved caspase 3 and PARP, was detectable at higher levels in irradiated control cells but not in BRG1 expressing cells (Fig. 1E). These data suggest that after UV irradiation, the higher level of proliferation observed for BRG1 expressing cells is due to a decrease in caspase mediated apoptosis.

**BRG1 enhances DNA repair in UV irradiated melanoma cells**

Previous reports indicated that SWI/SNF enzymes promote repair of bulky lesions in UV irradiated cells by interacting with components of the NER machinery in yeast (18) and in human cells (36). To determine whether BRG1 protects melanoma cells from
apoptosis by promoting DNA repair, we performed comet assays over a time course after irradiation to detect the extent of DNA damage. We detected significant tail formation in both control and BRG1 expressing melanoma cells 30 minutes after irradiation, although the comet tails were significantly shorter in BRG1 expressing cells (Fig.2A). We previously found that BRG1 promotes expression of genes that encode pigmented enzymes and that cell pellets obtained from BRG1 expressing cells are dark compared to parental SK-MEL5 cells and SK-MEL5 cells that express only empty vector. Thus, the significant decrease in tail length exhibited by BRG1 expressing cells at the earliest time point may reflect prevention of DNA damage by increased melanin synthesis. Two hours after irradiation, comet tails were significantly shorter in BRG1 expressing cells than in control cells (Fig 2A). However, by 24 hours, there were no comet tails in either control or BRG1 expressing cells, indicating that DNA damage was ultimately repaired even in the absence of BRG1 (Fig. 2A).

SWI/SNF enzymes were previously demonstrated to promote phosphorylation of the histone variant, H2AX, in response to UV irradiation (36). H2AX phosphorylated at serine 139 (gamma H2AX) forms foci that facilitate the recruitment of repair and checkpoint proteins to sites of DNA damage arising from strand breaks and are also formed after UV irradiation (29). Consistent with a previously demonstrated role in gamma H2AX foci formation, we observed that BRG1 expressing cells had significantly more gamma H2AX foci than control SK-MEL5 melanoma cells (Fig. 2B). In combination, these data suggest that BRG1 may prevent DNA damage and also enhance
DNA repair in response to UV irradiation in SK-MEL5 melanoma cells, at least in part by promoting H2AX foci formation.

**BRG1 promotes expression of inhibitors of cell cycle progression and apoptosis**

We considered the possibility that an additional mechanism by which BRG1 protects against UV irradiation is to reinstate cell cycle checkpoint controls. Re-expression of BRG1 in the BRG1/BRM deficient adenocarcinoma cell line, SW13, revealed that in these cells BRG1 activates expression of two DNA damage responsive genes, GADD45a and p21CIP1/WAF1, and protects against UV irradiation by restoring a G1 checkpoint (17). Furthermore, p21CIP1/WAF1 is activated by p53 in response to DNA damage in a SWI/SNF dependent manner (44). Interestingly, p21CIP1/WAF1 is also activated by MITF in UV irradiated melanoma cells, elicits a temporary G1 cell cycle arrest, and this is critical for preventing apoptosis (27).

To determine the role of BRG1 in the regulation of p21CIP1/WAF1 expression in UV irradiated melanoma cells, we compared p21CIP1/WAF1 expression in sham and UV irradiated cells at 12 hours and at 24 hours after irradiation. We found that in sham irradiated cells, BRG1 promoted a 3-4 fold increase in p21CIP1/WAF1 mRNA levels compared to cells expressing empty vector (Fig. 3A). We found that although p21CIP1/WAF1 expression was induced by UV radiation both in control SK-MEL5 cells and in SK-MEL5 cells expressing BRG1, the levels of p21CIP1/WAF1 were higher in UV-irradiated SK-MEL5 cells expressing BRG1 than in UV irradiated SK-MEL5 cells lacking BRG1. However, BRG1 did not increase the fold induction of p21CIP1/WAF1 by UV radiation. Thus, in SK-MEL5 melanoma cells, BRG1 promotes constitutively
high levels of p21CIP1/WAF1, but is not required for induction of p21CIP1/WAF1 by UV irradiation.

A role for SWI/SNF enzymes in the regulation of the apoptotic pathway has not previously been demonstrated. To determine whether BRG1 can promote melanoma survival in UV irradiated cells by inhibiting apoptosis, we investigated the regulation of expression of the melanoma inhibitor of apoptosis (ML-IAP) by BRG1 in response to UV radiation. We previously found that expression of BRG1 in SK-MEL5 cells results in an increase in ML-IAP expression under steady state conditions and that on the ML-IAP promoter, BRG1 promotes a dramatic increase in histone H3 tri-methylation at lysine 4 (H3K4me3), a mark of active transcription (26). Interestingly, after multiple passages of SK-MEL5 cells that express BRG1, we found that ML-IAP mRNA levels were stably and dramatically elevated compared to cells lacking BRG1, but were not further activated by UV radiation (Fig. 3B). Other studies have also demonstrated that ML-IAP expression is not activated by treatment with DNA damaging agents, although ML-IAP promotes survival in response to these agents (7, 14).

To corroborate our analysis of the regulation of p21CIP1/WAF1 and ML-IAP expression by BRG1 in response to UV radiation, we performed chromatin immunoprecipitations (ChIPs) to detect H3K4me3 as an epigenetic mark of active transcription. We found that in cells expressing empty vector, H3K4me3 levels on the p21CIP1/WAF1 promoter were significantly increased after UV irradiation, indicating transcriptional activation (Fig.3C). In cells that express BRG1, H3K4me3 levels on the p21CIP1/WAF1 promoter were significantly higher than in cells lacking BRG1 and
increased even further after UV irradiation, although the fold increase in H3K4me3 on the p21CIP1/WAF1 promoter by UV radiation was similar in control and BRG1 expressing cells. Levels of H3K4me3 on the ML-IAP promoter were barely higher than the levels of H3K4me3 on the silent CD25 promoter in cells lacking BRG1, were dramatically induced in cells that express BRG1, but were not further elevated by UV irradiation (Fig. 3C).

Interestingly, the epigenetic changes elicited by BRG1 in sham and UV irradiated melanoma cells closely correlated with changes in the expression of p21CIP1/WAF1 and ML-IAP at the RNA level (Fig. 3A and 3B) and at the protein level (Fig.3D). The profound effect of BRG1 on the expression of ML-IAP protein was striking. Two isoforms of ML-IAP (α and β) that arise from alternative splicing have been reported to exhibit differential anti-apoptotic properties in response to DNA damage and in tumorigenesis (1, 8). We observed that BRG1 promotes expression of both ML-IAP α and β isoforms (Fig.3D) and that ML-IAP protein is virtually undetectable in SK-MEL5 cells that lack BRG1. ML-IAP expression was barely detectable at the protein level in parental SK-MEL5 cells and in SK-MEL5 cells that express only empty vector even at dark exposures (data not shown). Thus, BRG1 is required to activate ML-IAP expression to levels that result in detectable protein. This suggested that BRG1 mediated activation of this negative regulator of apoptosis is a mechanism by which BRG1 promotes increased survival in response to UV radiation.

**Protection of melanoma cells from UV-induced apoptosis is dependent on ML-IAP**
To explore whether BRG1 can modulate the response to UVR by inhibiting the apoptotic pathway through activation of ML-IAP, we down-regulated ML-IAP expression using an siRNA that targets ML-IAP α (Fig. 4A, left panel) as well as an siRNA that targets both ML-IAP isoforms (Fig.4A, right panel). We observed that specific knockdown of ML-IAPα in BRG1 expressing SK-MEL5 cells resulted in a substantial increase in PARP cleavage after UV irradiation and that knockdown of both ML-IAP isoforms also resulted in an increase in PARP cleavage. Furthermore, knockdown of ML-IAP resulted in a substantial increase in the percentage of TUNEL positive cells (Fig.4B) and knockdown of ML-IAP α as well as knockdown of both isoforms of ML-IAP resulted in significant increase in the percentage of annexin positive cells upon UV irradiation (Fig.4C). Thus, we conclude that activation of one or both isoforms of ML-IAP by BRG1 contributes to the observed resistance of BRG1 expressing melanoma cells to UV radiation induced apoptosis. Interestingly, in sham irradiated cells, down-regulation of ML-IAP also resulted in a significant increase in annexin positive cells, suggesting that activation of ML-IAP may be required for the survival of melanoma cells that express BRG1. To our knowledge, this is the first evidence that SWI/SNF enzymes can modulate the DNA damage response by directly inhibiting apoptotic pathways.

**Expression of ML-IAP is dependent on co-expression of MITF and BRG1**

ML-IAP has a restricted range of expression, being highly expressed in melanoma cells and in some lymphoma cell lines (24). In melanoma cells lines, ML-IAP expression is closely correlated with co-expression of MITF (14). We found that in a
panel of melanoma cell lines, ML-IAP protein expression was correlated with co-expression of both MITF and BRG1 (Fig. 5A). A375 melanoma cells express high levels of BRG1 but low levels of MITF and express undetectable levels of ML-IAP whereas SK-MEL5 cells express high levels of MITF but virtually undetectable levels of BRG1 and undetectable levels of ML-IAP. Several other melanoma cell lines that express both MITF and BRG1 express high levels of ML-IAP. Importantly, SK-MEL5 cells express the alternative SWI/SNF ATPase, BRM but do not express high levels of ML-IAP. We found that BRG1 activates ML-IAP expression in SK-MEL5 melanoma cells that express abundant MITF but lack BRG1 (Fig. 5A) and that depletion of BRG1 by siRNA inhibits ML-IAP expression in melanoma cells that express abundant BRG1 and MITF (Fig.5B). In contrast, we did not observe a correlation between expression of the alternative ATPase, BRM, and ML-IAP expression in this panel of melanoma cell lines (Fig.5B). Thus, although BRM may contribute to ML-IAP regulation, BRG1 plays a critical role in the activation of ML-IAP that cannot be compensated by the endogenous levels of BRM. To investigate the requirement of MITF for activating BRG1 dependent ML-IAP expression, we transiently expressed MITF in melanoma cells that express high levels of BRG1. We found that MITF activates ML-IAP expression in melanoma cells that lack MITF but express BRG1 (Fig.5C). Furthermore, depletion of MITF in SK-MEL5 cells expressing BRG1 inhibits ML-IAP expression (Fig.5D). Thus, ML-IAP expression is highly dependent on co-expression of both MITF and BRG1.

**BRG1 regulates ML-IAP expression through a cooperative activation loop with MITF**
SWI/SNF enzymes lack sequence specific binding ability and are thought to be recruited to specific promoters by interactions with gene specific transcriptional activators (13). However, gene specific transcriptional activators have limited access to their binding sites when the recognition sequences are embedded in repressive chromatin structure. As we previously determined, MITF is required to recruit BRG1 to several MITF target promoters, including ML-IAP (Fig. 6A) (25). Interestingly, we also found that MITF binding to the ML-IAP promoter was dependent on BRG1, suggesting that the recruitment of MITF and BRG1 to the ML-IAP promoter is interdependent (Fig. 6B).

To understand the mechanism by which MITF promotes the recruitment of BRG1 concomitantly with a requirement for BRG1 in promoting MITF recruitment, we performed chromatin accessibility experiments to probe the changes in chromatin structure elicited by BRG1 on the ML-IAP promoter. The ML-IAP promoter has two E boxes, both of which bind MITF and are activated by MITF (14). We assayed BRG1 induced changes in chromatin structure on the ML-IAP promoter by digesting nuclei from control and BRG1 expressing SK-Mel5 cells with micrococcal nuclease (MNase I). We then utilized a CHART-PCR assay (35) to detect changes in accessibility to MNase I at regions encompassing each of the E boxes and at an upstream region of the ML-IAP promoter (Fig. 6C). The regions surrounding both E boxes became more accessible to MNase I in BRG1 expressing cells while a 5’ upstream region was unaffected by BRG1 (Fig. 6D). Interestingly, the accessibility of the region surrounding E box 1 was increased by BRG1 to a greater extent than that of the region surrounding E box 2. These data suggest that in the absence of BRG1, MITF has limited accessibility to its recognition
sites in the ML-IAP promoter, and may unstably associate with the promoter at one or both E boxes, giving rise to low levels of ML-IAP mRNA, but virtually undetectable protein. In BRG1 expressing cells, MITF dependent recruitment of BRG1 results in chromatin remodeling that increases the accessibility of both E boxes, particularly that of E box1 and augments MITF interactions with the ML-IAP promoter. Thus, cooperative interactions between MITF and BRG1 operate to promote recruitment of both factors to the ML-IAP promoter.

**BRG1 promotes epigenetic changes on the ML-IAP promoter characteristic of active transcription**

We detected a dramatic (36 fold) increase in the levels of the active H3K4me3 mark on the ML-IAP promoter as a result of BRG1 expression in SK-MEL5 cells (Fig. 3C). To determine the mechanisms by which BRG1 activates transcription of ML-IAP, we assayed whether other chromatin modifications associated with active transcription are modulated as a result of BRG1 expression. We performed ChIPs to detect changes in histone H3 and histone H4 acetylation (H3Ac and H4Ac). Histone acetylation is important for the recruitment of bromodomain containing proteins such as BRG1, and is generally associated with transcriptional activation. MITF has been demonstrated to interact with the histone acetyltransferase, p300/CBP and can promote histone acetylation at target promoters (12, 40). We found that in control cells, lacking BRG1, the levels of H3ac were 10 times higher on the ML-IAP promoter than on the silent CD25 promoter. There was a 14 fold increase in H3 acetylation levels in BRG1 expressing cells (Fig.7A). The levels of H4ac were approximately twice as high on the ML-IAP promoter as on the
silent CD25 promoter in cells lacking BRG1 and increased 5 fold as a result of BRG1 expression (Fig. 7B). Thus, the histone acetylation state of the ML-IAP promoter was significantly increased by BRG1 but to a lesser extent than H3K4 trimethylation. This data suggest that in the absence of BRG1, the ML-IAP promoter is characterized by low levels of histone acetylation, most likely established by unstable MITF binding (Fig. 6B). We speculate that unstable MITF binding to one or both E boxes and a low level of histone acetylation on the ML-IAP promoter may facilitate the recruitment of BRG1 which would then increase the accessibility of MITF binding sites and enhances MITF association with the ML-IAP promoter as well as the association of other chromatin modifying enzymes.

Transcriptional activation by SWI/SNF enzymes can also involve suppression of inhibitory chromatin covalent modifications such as histone H3 tri-methylation at lysine 27 (H3K27me3). Interestingly, we also detected BRG1 dependent changes in the levels of H3K27me3 on the ML-IAP promoter (Fig. 7C). In control cells lacking BRG1, H3K27me3 levels on the ML-IAP promoter were high but significantly lower compared to levels on the silent CD25 promoter, suggesting a low level of promoter activity. The levels of H3K27me3 on the ML-IAP promoter were significantly reduced by BRG1, consistent with increased transcriptional activity that is dependent on BRG1. Enrichment of control IgG on both the ML-IAP and CD25 promoters was significantly lower than enrichment of all histone modifications and did not significantly change as a result of BRG1 expression, demonstrating that ChIPs were specific (Fig. 7D). In combination, these data suggest that MITF and BRG1 cooperate to recruit each other to the ML-IAP
promoter in order to generate an epigenetic signature that is consistent with transcriptional activation (Fig.8).

**Discussion**

Sunlight emits UV radiation that causes DNA damage, particularly to cells on the skin and is the major environmental risk factor for all skin cancers, including melanoma (23). The DNA damage response is a multi-step process that involves activation of cell cycle checkpoints and DNA repair. Extensive damage may result in apoptosis, thus preventing the emergence of genomically unstable cells that can give rise to cancer. However, melanocytes and melanoma cells are specialized to prevent apoptosis when challenged with DNA damage and have evolved lineage specific mechanisms to ensure cell survival (23).

SWI/SNF enzymes are multi-component complexes with subunits that have been designated to have tumor suppressor activity in mice and to be down-regulated in a subset of human cancers (37). SWI/SNF mediated tumor suppression is thought to occur by regulating the cell cycle and maintaining genomic integrity. Along these lines, SWI/SNF enzymes regulate the cell cycle by interacting with classical tumor suppressors including p53 and the retinoblastoma protein (Rb) (37). Recent studies also indicate that SWI/SNF enzymes interact with components of the NER machinery and stimulate DNA repair (36). However, we previously found that SWI/SNF enzymes interact with MITF, a lineage addiction oncogene in melanoma (26).
MITF plays a critical role in promoting melanoma survival in response to DNA damage elicited by UV radiation and by chemotherapeutics such as cisplatin (16, 27). Furthermore, our studies indicated that re-expression of BRG1 in a BRG1 deficient melanoma cell line, promotes survival in cisplatin treated cells (26). Thus, we were prompted to investigate the hypothesis that SWI/SNF enzymes modulate the DNA damage response in melanoma cells by lineage specific mechanisms that involve interacting with MITF. In particular, the response to UV radiation is most pertinent to cells that normally reside on the skin.

P53 is activated in response to DNA damage and serves as a critical regulator of cell cycle checkpoints and induction of apoptosis. p21WAF1/CIP1 is an important p53 trans-activation target that mediates p53 driven G1 arrest in response to DNA damage (21). In some cell types, p53 activity is differentially regulated by the two different SWI/SNF ATPases, BRG1 and BRM, such that BRG1 preferentially activates p21WAF1/CIP1 in response to DNA damage (44). However, BRG1 also plays a role in down-regulating p53 activity and may thereby promote proliferation (31). Thus, in response to DNA damage, BRG1 may have an important role in promoting cell cycle reentry and preventing senescence. Our data suggest that in SK-MEL5 melanoma cells, the G1 checkpoint is functional in the absence of BRG1 and that BRG1 may enhance negative regulation of cell cycle progression by promoting constitutively high expression of p21CIP1/WAF1. Interestingly, we found that expression of GADD45a, a p53 target gene that regulates a G2 checkpoint was also elevated by BRG1 in UV irradiated cells (data not shown). However, BRG1 had no significant effect on the expression of several
p53 target genes that regulate apoptosis (data not shown). In combination these data suggest that in SK-MEL5 melanoma cells, although BRG1 may enhance the expression of a subset of p53 target genes, BRG1 is not required for p53 activity. Interestingly, the regulation of p21CIP1/WAF1 by MITF has been suggested to be an important mechanism by which MITF promotes increased survival in response to UV radiation (27). Furthermore, some studies suggest the existence of lineage specific mechanisms that activate GADD45a independently of p53 (15). Thus, in melanoma cells, BRG1 may promote expression of cell cycle regulators involved in checkpoint control by lineage specific mechanisms that are independent of p53.

Biochemical studies suggest that the condensation of DNA in chromatin impedes DNA repair enzymes from accessing DNA lesions and thus requires the activity of chromatin remodeling enzymes (19). In a cellular context, SWI/SNF enzymes have been demonstrated to promote double strand break repair and NER (34). A recent study showed that in response to UV irradiation, SWI/SNF components interact with the NER machinery, are recruited to damaged sites and promote gammaH2AX formation (36). Down-regulation of BRG1 compromises repair of CPD but does not affect repair of 6-4 photoproducts (46). Our data suggest that BRG1 also contributes to DNA repair of melanoma cells. Interestingly, MITF has been implicated in DNA repair and is required for genomic integrity. Down-regulation of MITF in melanoma cells compromises gamma H2AX foci formation in response to UV radiation and ultimately results in cellular senescence or death (14, 42). The mechanisms by which MITF promotes DNA repair have not yet been clearly elucidated but are likely to be transcriptional (2). We
speculate that in addition to interacting with the NER machinery, BRG1 may also contribute to the maintenance of genomic integrity in melanoma cells through interactions with MITF.

MITF plays an important role in the regulation of apoptosis in response to DNA damage. P53 mediated cell death is an important mechanism for eliminating irreparably damaged cells and maintaining genomic integrity. However, melanomas exhibit extraordinary resistance to typical p53 mediated inducers of apoptotic death and these mechanisms often involve MITF. Our data suggest BRG1 protects melanoma cells from apoptosis following UV irradiation by cooperating with MITF to epigenetically regulate the melanoma inhibitor of apoptosis (ML-IAP).

ML-IAP (BIRC7, livin), a recently identified MITF target gene, is a member of the inhibitor of apoptosis (IAP) family of proteins (14, 43). IAP family members contain one or more repeats of a highly conserved 70-amino acid domain termed the BIR domain that can directly bind and potently inhibit caspase activity, blocking apoptosis induced by many different stimuli including UV radiation and chemotherapeutic drugs. ML-IAP also binds to SMAC and prevents SMAC from negatively regulating XIAP-mediated caspase inhibition, thus halting execution of the apoptosis pathway. Furthermore, ML-IAP expression has been clinically correlated with chemotherapeutic response, suggesting that ML-IAP may be a viable target in the treatment of melanoma.

As an activator of ML-IAP expression in melanoma cells, BRG1 diverges from its well known role in tumor suppression and positively regulates a critical lineage survival pathway by cooperating with MITF. Interestingly, high throughput chromatin analysis
combined with genome wide ChIPs indicated that MITF occupied sites are surrounded by positioned nucleosomes, suggesting that MITF binds preferentially to nucleosome-free regions (32). The mechanisms establishing and maintaining the precise chromatin conformation required for MITF to activate its target genes was not previously known. Our data indicate that MITF and SWI/SNF complexes containing BRG1 coordinately promote transcriptionally permissive chromatin structure on the ML-IAP promoter. Thus, the transcription factor context of a cancer cell establishes the transcriptional specificity of the SWI/SNF complex and its ultimate role in tumorigenesis. Concomitantly, a specific subunit of the SWI/SNF complex determines which of its target genes, a lineage specific oncogene such as MITF can activate. However, it is still not clear why BRG1 induced chromatin structural changes on the ML-IAP promoter result in a dramatic increase in H3K4me3 that is not seen to occur at other MITF target promoters (26). We are currently investigating the involvement of other factors that cooperate with MITF and BRG1 to activate ML-IAP and other potential chemotherapeutic targets in melanoma.

Figure Legends

Fig. 1. BRG1 protects melanoma cells from UV-induced apoptosis
A. SK-MEL5 cells stably expressing empty vector or BRG1 were sham irradiated or irradiated with a UVB lamp. Cell extracts were prepared 12 hours after irradiation and subjected to Western blotting with antibodies to BRG1, p53, and MITF. Tubulin is a loading control. B. 5000 cells were plated on 24 well plates and treated as in A. Cells
were counted over a six day period with a Coulter counter. C. Cells treated as in A were stained with DAPI and subjected to the TUNEL assay 36 hours after irradiation. TUNEL positive cells detected after UV-irradiation were counted and expressed as percent DAPI stained cells (left). Representative images are shown (right). The data shown is representative of two independent experiments. D. Cells treated as in A were subjected to the annexin V assay 48 hours after irradiation. Annexin V positive cells were detected with a Guava Personal Cell Analysis System. The data shown is representative of greater than two independent experiments performed in triplicate. Statistically significant differences were determined by the student’s t test. E. Protein extracts used in A were also subjected to Western blotting with antibodies to cleaved caspase 3 and cleaved PARP. Tubulin is shown as a loading control.

Fig. 2. BRG1 enhances repair of DNA damage and promotes gamma H2AX formation
A. Comet assays were performed on sham irradiated or UV-irradiated SK-MEL5 cells expressing empty vector or BRG1 at 30 minutes, 2 hours, and 24 hours after irradiation and visualized by sybr-orange staining. Representative images are shown. B. Sham and UV-irradiated cells as in A are harvested 8 hours after irradiation. Cells were stained with DAPI and with an antibody to gammaH2AX. Representative images were taken at 100X magnification.

Fig.3. BRG1 activates high levels of p21CIP1/WAF1 and ML-IAP expression in melanoma cells. A. Total RNA from sham and UV-irradiated cells were harvested at each time point, reverse transcribed, and subjected to qRT-PCR with primers to p21CIP1/WAF1 and GAPDH. Levels of P21CIP1 mRNA were normalized to those of
GAPDH. B. The cDNA from A was also subjected to qRT-PCR with primers to ML-IAP and the levels of ML-IAP mRNA at each time point were normalized to those of GAPDH. C. Cells that were sham or UV-irradiated were crosslinked at the 12 hour time point and processed for ChIPs with an antibody to H3K4me3 or with a control IgG (data not shown). The p21CIP1/WAF1 and ML-IAP promoters were detected by qPCR with primers specific for a region that contains MITF binding sites. A region of the silent CD25 promoter was amplified as a control. ChIP enrichment is expressed as % input. Enrichment of of the indicated regions with control IgG was less than 0.1% of input (data not shown).

Fig. 4. Protection of melanoma cells from UV-induced apoptosis is dependent on ML-IAP. A. Expression of ML-IAP a (left) or both isoforms of ML-IAP (right) was down-regulated with specific siRNAs or with a control scrambled siRNA. Cells were sham or UV irradiated 72 hours after transfection of siRNAs and harvested 36 hours after UV irradiation. Total cell extracts were subjected to Western blotting with antibodies to ML-IAP and cleaved PARP. Tubulin is a loading control. B. Cells treated as in A were subjected to the TUNEL assay. C. Cells treated as in A were subjected to the annexin V assay. Down-regulation of ML-IAPa and total ML-IAP resulted in a significant increase (p<.01) in annexin V positive cells as determined by the student’s t test.

Fig. 5. Co-expression of MITF and BRG1 is required for high levels of ML-IAP expression. A. Protein extracts from a panel of melanoma cells were subjected to Western blotting with antibodies to BRG1, BRM, MITF, and ML-IAP and tubulin as a loading control. B. WM-2664 melanoma cells were transfected with a pool of control
scrambled or a pool of siRNA that targets BRG1. Top: A Western blot was performed with extracts prepared from control and BRG1 knockdown cells and probed with an antibody to BRG1. Tubulin is a loading control. Bottom: qRT-PCR was performed on control and BRG1 knockdown cells. BRG1 depletion significantly inhibited ML-IAP expression (p<.01) as determined by the student’s t test. C. A CMV-LACZ or a CMV-MITF construct was transfected into A375 melanoma cells and cells were harvested 24 hours later. Top: A Western blot was performed on transfected cells with an antibody to MITF. Tubulin is a loading control. Bottom: qRT-PCR was performed on transfected cells. Transient transfection of MITF significantly activated ML-IAP expression (p<.01) as determined by the student’s t test.

Fig. 6. BRG1 regulates ML-IAP expression through a cooperative activation loop with MITF. A. Scrambled siRNA or siRNA that targets MITF was transfected in SK-MEL5 cells that express BRG1. Top: A Western blot was performed with extracts from control and MITF depleted cells and probed with an antibody to MITF. ERK is a loading control. Bottom: ChIPs were performed with an antibody to the FLAG epitope and quantified by qPCR with primers that detect the ML-IAP promoter. Enrichment of BRG1 at the ML-IAP promoter was normalized to inputs and corrected for IgG. Down-regulation of MITF resulted in a significant reduction in the enrichment of BRG1 at the ML-IAP promoter as determined by the student’s t test. B. ChIPs were performed with SK-MEL5 cells expressing empty vector and SK-MEL5 cells expressing BRG1 using an antibody specific for MITF. MITF enrichment at the ML-IAP promoter was significantly increased by BRG1 as determined by the student’s t test. CD25 is shown as a control.
region that does not bind MITF. C. Schematic of the ML-IAP promoter that illustrates the position of the two E boxes and the position of the primers used for qPCR in chromatin accessibility experiments. D. Nuclei were isolated from SK-MEL5 cells expressing empty vector or BRG1 and digested with MNase I for 15 minutes. An aliquot was treated in an identical manner except that MNase I was not added. DNA was purified and subjected to qPCR using the primers illustrated in Fig. 6C. Accessibility at E box1 and Ebox2 was significantly increased in BRG1 expressing cells as determined by the student’s t test. Accessibility at an upstream region was not significantly affected by BRG1.

Fig. 7. BRG1 promotes epigenetic changes on the ML-IAP promoter characteristic of active transcription Chromatin immunoprecipitations were performed on SK-MEL5 cells expressing empty vector and SK-MEL5 cells expressing BRG1. Enrichment on the ML-IAP promoter was quantified by qPCR and is expressed as percent of input. Enrichment of the silent CD25 promoter is shown as a control. A. ChIP analysis of H3acetylation. B. ChIP analysis of H4 tetra-acetylation C. ChIP analysis of H3 lysine 27 trimethylation, C. ChIP analysis of control IgG.

Fig. 8 A cooperative transcriptional activation loop involving BRG1 and MITF regulate ML-IAP expression. Hypothetical model for regulation of ML-IAP expression.
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A. 

![Image of experimental results for EV and BRG1 under Sham-irradiated conditions at 30 min, 2 hr, and 24 hr.]

B. 

![Images of DAPI and gH2AX staining for EV and BRG1 under Control and UV conditions.]

Figure 2
Figure 4

A. 

B. 

C. 

%Annexin V positive
Figure 5

A. Western blot analysis of BRG1, BRM, MITF, ML-IAP, and ERK in SKMel5, SKMel5+BRG1, YUMAC, A375, WM-2664, and 501MEL cell lines.

B. Quantitative analysis showing relative ML-IAP mRNA levels in siControl and siBRG1 treated samples.

C. Western blot analysis of MITF and Tubulin in LACZ and MITF transfected samples.

D. Quantitative analysis showing relative ML-IAP mRNA levels in siControl and siMITF treated samples.
Figure 7

A. H3Ac

B. H4Ac

C. H3K27me3

D. IgG
Conclusions from this chapter

• BRG1 promotes survival of melanoma cells exposed to UV radiation.

• BRG1 promotes constitutive expression of the cyclin dependent kinase inhibitor, p21CIP/WAF1

• BRG1 expression reduces the accumulation of DNA damage and promotes gammaH2AX formation.

• BRG1 dramatically activates the expression of a pro-survival gene, ML-IAP in an MITF dependent manner.

• Protection of melanoma cells from UV-induced apoptosis is partially dependent on ML-IAP in melanoma cells.

• Activation of ML-IAP is associated with changes in chromatin structure, MITF recruitment, and histone post-translational modifications.
Chapter 4.

Discussion

Cutaneous malignant melanoma, is an aggressive malignancy that arises from the transformation of epidermal melanocytes and is notoriously chemo-resistant.

Metastatic melanoma is an extremely aggressive disease that is resistant to current therapy and has a poor prognosis. The progression of melanoma involves changes in gene expression that promote metastasis to distant organs. Transcriptional activation often requires epigenetic changes that convert repressive chromatin structure to a permissive conformation. Understanding the epigenetic mechanisms involved in the deregulation of gene expression during metastasis is important for developing an effective strategy to treat melanoma. SWI/SNF enzymes are multi-subunit complexes that include BRG1 or BRM ATPase subunit and alter chromatin structure and regulate gene expression. Several SWI/SNF subunits have been shown to be aberrantly expressed in a number of human cancers. We previously demonstrated that heterogeneous SWI/SNF complexes containing either BRG1 or BRM are epigenetic modulators that regulate important aspects of the melanoma phenotype and are required for melanoma tumorigenicity in vitro. Our new studies indicate that stage IV melanoma tumors express significantly higher levels of BRG1 compared to normal melanocytes and melanoma in less advanced stages. To determine the role of BRG1 in melanoma metastasis, we over-expressed BRG1 in an established melanoma cell line that lacks BRG1 expression. We found that BRG1 dramatically altered expression of
a number of adhesion proteins and extracellular matrix remodeling enzymes. BRG1 altered melanoma adhesion to different extracellular matrix components laminin, collagen and fibronectin. Expression of BRG1 in melanoma cells that lack BRG1 increased invasive ability while down-regulation of BRG1 inhibited invasive ability in vitro. Activation of metalloproteinase (MMP) 2 expression greatly contributed to the BRG1 induced increase in melanoma invasiveness. We found that BRG1 is recruited to the MMP2 promoter and directly activates expression of this metastasis associated gene. The results suggest that high levels of BRG1 are required to promote the epigenetic changes required for melanoma metastasis.

Exposure to ultraviolet radiation (UVR) from sunlight is the most critical environmental agent involved in melanoma genesis. Melanocytes protect surrounding skin cells from UVR damage and because of this protective role, melanocytes have evolved to be particularly resistant to apoptosis when exposed to UVR. The lineage specific mechanisms that prevent cell death of melanocytes exposed to UVR are thought to contribute to melanoma chemoresistance. Re-expression of SWI/SNF subunits in cancer cell lines deficient in a particular subunit often results in cell cycle arrest and reversion of the tumorigenic phenotype. However, re-expression of the SWI/SNF subunit, BRG1, in BRG1 deficient melanoma cells increases survival to cisplatin and to UVR exposure. Furthermore, we have found that BRG1 is highly expressed in melanoma tumors and its expression increases during late stages of melanoma, potentially contributing to the chemoresistance typical of advanced melanoma. The objective of this study is to elucidate the mechanisms by which BRG1 promotes melanoma survival in response to DNA damage. Microphthalmia-
associated transcription factor (MITF), a master regulator of differentiation in melanocytes, is a lineage addiction oncogene in melanoma, which directly regulates anti-apoptotic gene expression. We previously demonstrated that MITF interacts with the SWI/SNF complex to activate MITF target gene expression. Our studies show that BRG1 promotes lineage specific activation of anti-apoptotic pathways through interactions with MITF. BRG1 and MITF expression closely correlated with expression of the melanoma inhibitor of apoptosis (ML-IAP) gene by promoting dramatic chromatin modifications on the ML-IAP regulatory region, by increasing the active histone modification marks and decreasing the repressive marks, thus strongly activating expression of this MITF target gene. ML-IAP is a potent inhibitor of apoptosis, which is highly expressed in melanomas and contributes to the survival of melanoma cells exposed to DNA damaging agents. Our current studies have shown the mechanisms by which BRG1 activates ML-IAP expression and the role of ML-IAP in mediating BRG1 induced survival of melanoma cells exposed to UVR. Down-regulation of ML-IAP significantly compromised BRG1 mediated survival of melanoma cells exposed to UVR. The results from these studies should illuminate a lineage specific mechanism by which BRG1 promotes survival of melanoma cells exposed to a DNA damaging agent and implicate BRG1 as a target for epigenetic based therapeutics to effectively treat this highly resistant malignancy.
Chapter 5.

References


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