A Dissertation
entitled

The Role of SWI/SNF Chromatin Remodeling Enzymes in Melanoma

by

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Abstract

The microphthalmia-associated transcription factor (MITF) promotes melanocyte differentiation and cell cycle arrest. Paradoxically, MITF also promotes melanoma survival and proliferation, acting like a lineage survival oncogene. Thus, it is critically important to understand the mechanisms that regulate MITF activity in melanoma cells. SWI/SNF chromatin remodeling enzymes are multiprotein complexes composed of one of two related ATPases, BRG1 or BRM, and 9-12 associated factors (BAFs). We previously determined that BRG1 interacts with MITF to promote melanocyte differentiation. However, it was unclear whether SWI/SNF enzymes regulate the expression of different classes of MITF target genes in melanoma. In this study, we characterized SWI/SNF subunit expression in melanoma cells and observed down-regulation of BRG1 or BRM, but not concomitant loss of both ATPases. Re-introduction of BRG1 in BRG1 deficient SK-MEL5 cells enhanced expression of differentiation specific MITF target genes and resistance to cisplatin. Down-regulation of the single ATPase, BRM, in SK-MEL5 cells inhibited expression of both differentiation specific and pro-proliferative MITF target genes and inhibited tumorigenicity in vitro. Our data suggest that heterogeneous SWI/SNF complexes composed of either the BRG1 or BRM subunit promote expression of distinct and overlapping MITF target genes and that at least one ATPase is required for melanoma tumorigenicity.

Dedication

I dedicate this work to my family, friends and colleagues who have encouraged me to work towards my goals and never give up.

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I want to thank my advisor, Dr. Ivana de la Serna, for the opportunity to work and learn with her. I have great gratitude for my committee members for continuous assistance, support and guidance throughout my entire graduate school experience. The Biochemistry and Cancer Biology Department and staff have been wonderful with their constant support and guidance.

My previous and current lab members have been instrumental in my progress and success. This dissertation would not have been possible without the love, support, and encouragement I received from all of my friends and family.

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List of Abbreviations

AML................Angiomyolipoma
AR...................Androgen Receptor
BAFs…............Brg1/Brm Associated Factors
BCL2…….........B-Cell Lymphoma Protein
BRAF………...Raf Kinase; Serine/Threonine-Protein Kinase
BRCA1………Breast Cancer Type 1 Susceptibility Protein
BRG1...............Brahma-Related Gene-1
BRK................BRM and Kismet domains
BRM...............Brahma
cAMP………..Cyclic Adenosine Monophosphate
CCS………….Clear Cell Sarcoma
CDK2………..Cyclin-Dependent Kinase 2
CHD…………Chromodomain, Helicase, DNA binding
ChIPs…….......Chromatin Immunoprecipitations
DCT/TRP2…..Dopachrome Tautomerase
DSB………….DNA Double-Strand Break
ECM…………Extra Cellular Matrix
EMT…………Epithelial to Mesenchymal Transition
HATs……......Histone Acetyl Transferases
HDACs……...Histone Deacetylases
Hsp-70………Heat Shock Protein 70
INO80……….Inositol requiring 80
ISWI…………Imitation SWItch
MAPK……….Mitogen Activated Protein Kinase
MBD…...........Methyl CpG-Binding Domain
MC1R.............Melanocortin Receptor 1
MITF…...........Microphthalmia Transcription Factor
MLIA………..Melanoma Inhibitor of Apoptosis
MRT……........Malignant Rhabdnoid Tumors
α-MSH………α-Melanocyte-Stimulating Hormone
N-RAS……....Neuroblastoma RAS
NuRD……......Nucleosome Remodeling and Deacetylase
PHD………….Plant Homeo Domains
POMC…..........Proopiomelanocortin
RAR………….Retinoic Acid Receptors
Rb…................Retinoblastoma
SANT……......SWI3, ADA2, N-CoR and TFIIIB B
SWI/SNF.........Switching and Sucrose Non-Fermentation
TBX2………...T-box Transcription Factor 2
TCDD……. …2,3,7,8-tetrachlorodibenzo-p-dioxin

TIMP…….. …Tissue Inhibitor of Metalloproteinase
TRP1…….. …Tyrosinase-Related Protein 1
TRP2…….. …Tyrosinase-\Related Protein 2
UV……….. …Ultraviolet Radiation
WNT………...Wingless and Int

Chapter 1

Heterogeneous SWI/SNF Chromatin Remodeling Complexes Promote Expression of Microphthalmia – Associated Transcription Factor Target Genes in Melanoma

1.1 Chromatin Organization

Cellular processes rely on strict regulation of genetic material including: cellular growth, development, differentiation, DNA repair, replication and overall genomic stability (Ehrenhofer-Murray, 2004; Peterson & Cote, 2004). The tightly compacted structure of chromatin inhibits access of essential transcriptional factors and other transcriptional machinery to recognition sequences within promoter regions of genes. The restricted nature of chromatin must become relaxed in order for gene transcription to take place (Hansen, 2002; Tsukiyama & Wu, 1997; Workman & Kingston, 1998).

The nucleosome is the fundamental component of chromatin, consisting of 147 base pairs of DNA wrapped around two molecules of the four core histone protein. The four core histone proteins are: H2A, H2B, H3 and H4 and a linker histone H1. Linker histone H1 connects adjacent nucleosomes through physically binding to linker DNA located between histone beads enhancing the level of compaction (Aoyagi & Archer, 2005; Hayes & Hansen, 2001; Wolffe, 2001). There are numerous histone modifications that have been discovered all of which effect transcriptional activity.

1.1.1 Histone Modifications

Histones are positively charged proteins that can be modified on their N-terminal/tail region. There are various histone modifications and multiple sites on which modifications can occur resulting in numerous potential combinations.   The different classes of histone modifications are: methylation (X. Zhang & Jacobsen, 2006), phosphorylation (Nowak & Corces, 2004), acetylation (Sterner 2000), ubiquitination (Shilatifard, 2006) and sumoylation (Nathan, et al., 2006).   Histone modifications regulate changes in chromatin condensation and consequently DNA accessibility. For example, lysine acetylation is associated with gene activation while methylation is linked to both gene induction and repression, depending on the specific site of modification (Fischle, Wang, & Allis, 2003; Turner, 2000).

1.1.2 Epigenetic Events in Cancer

Alterations in DNA methylation patterns are a common feature of tumors. Methylated DNA is known to engage chromatin remodeling complexes that suppress gene transcription. Hypoacetylation is generally associated with transcription repression and hyperacetylation is associated with transcriptional activation (Strahl & Allis, 2000). It is the acetylated histones that have affinity for the bromo domains found in the ATPase subunits BRG1 and BRM of the SWI/SNF complex. Histone tails can act as platforms where transcriptional regulatory factors gather (Dhalluin, et al., 1999).

1.2 Chromatin Remodeling

In order for gene transcription to take place highly compacted chromatin must be converted into a relaxed, exposed state. There are two major classes of enzymes that remodel chromatin: ATP-dependent complexes and enzymes that post-translationally and covalently modify chromatin structure. Commonly known enzymes that post-translationally and covalently modify chromatin structure are: histone acetyl transferases (HATs), histone deacetylases (HDACs), kinases, methyltransferase and demethylase (P. B. Becker & Horz, 2002; Jenuwein, 2002; Kingston & Narlikar, 1999). Each class of chromatin remodeling enzymes participates in the regulation of gene expression events and has been extensively investigated. Chromatin remodeling enzymes function by utilizing energy from ATP hydrolysis and disrupt contacts between histone proteins and DNA resulting in an altered nucleosome structure (C. N. Johnson, Adkins, & Georgel, 2005; Kingston & Narlikar, 1999).

There are four common subfamilies of ATP-dependent chromatin remodeling machines, SWI/SNF, ISWI (Imitation SWItch), NuRD (Nucleosome Remodeling and Deacetylase) and INO80 (inositol requiring 80) (Eberharter & Becker, 2004; Sif, 2004). ATP dependent chromatin remodeling enzymes are classified on the basis of the presence of functional domains in their catalytic ATPase subunit. The SWI/SNF family includes yeast Snf2 and Sth2, *Drosophilia melanogaster* brahma (BRM), and mammalian BRM and brahma-related 1 (BRG1). The ATPase subunit of the SWI/SNF family of proteins has a bromodomain which preferentially binds acetylated histones (Muchardt & Yaniv, 1993). The SWI/SNF ATPase subunit BRG1 contains a well conserved C-terminal

bromodomain that has been shown to interact with acetylated residues within histone tails (Chandrasekaran & Thompson, 2007; Shen, et al., 2007).

The ISWI family contains a SANT (SWI3, ADA2, N-CoR and TFIIIB B) domain and the absence of a bromodomain (Boyer, Latek, & Peterson, 2004). The SANT domain preferentially binds to nucleosomes containing linker DNA over core nucleosomes (Langst, Bonte, Corona, & Becker, 1999).

The CHD (chromodomain, helicase, DNA binding) family remodelers interact with methylated histone tails. Some CHD remodelers function by sliding or emitting nucleosomes resulting in enhanced transcription of genes. Members of the CHD family often contain DNA-binding domains, Plant Homeo Domains (PHD), BRM and Kismet domains (BRK), and SANT domains. Some CHD family members have repressive roles, namely Mi-2/NuRD (nucleosome remodeling and deacetylase) complex containing histone deacetylases (HDAC1/2) and methyl CpG-binding domain (MBD) proteins (Bannister, et al., 2001; Flanagan, et al., 2005; Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001; Sims, et al., 2005).

The INO80 family remodelers include more than 10 subunits and initially purified from *S. cerevisiae*. INO80 has been shown to enhance transcriptional activation and play a role in DNA repair (Downs, et al., 2004; Ebbert, Birkmann, & Schuller, 1999; Flaus, Martin, Barton, & Owen-Hughes, 2006).

1.2.1 SWI/SNF Chromatin Remodeling Complex

SWI/SNF complexes are 1-2 MDa in size and consist of 9-12 Brg1/Brm associated factors, (BAFs) (Muchardt & Yaniv, 1993). SWI/SNF was the first chromatin

remodeling complex to be described and was discovered in *Saccharomyces cerevisiae*. SWI/SNF is conserved from yeast to humans and named for its regulatory role in yeast mating type **swi**tching and **s**ucrose **n**on-**f**ermentation (Breeden & Nasmyth, 1987; Neigeborn & Carlson, 1984; Stern, Jensen, & Herskowitz, 1984). SWI/SNF enzymes are multi-subunit complexes with an ATPase subunit, either brahma (BRM) or brahma-related gene-1 (BRG1) that utilize the energy of ATP to alter nucleosome structure and facilitate binding of transcriptional activators to nucleosomal DNA (Vignali, Hassan, Neely, & Workman, 2000). Many of the SWI/SNF subunits were first identified for their role in the regulation of the *HO endonuclease* gene or the *SUC2* gene. The SUC2 gene encodes invertase, a sucrose hydrolyzing enzyme. The HO endonuclease gene is required in yeast for mate type switching (Imbalzano, 1998; Peterson, 1996).

The two ATPase subunits, BRG1 and BRM, share over 70% sequence identity and can display similar biochemical activities *in vitro*, but don't always functionally compensate for one another (Khavari, Peterson, Tamkun, Mendel, & Crabtree, 1993; Phelan, Sif, Narlikar, & Kingston, 1999; Randazzo, Khavari, Crabtree, Tamkun, & Rossant, 1994). Some of the most common BAFs are BAF250, BAF170, BAF155, BAF60a, b or c, BAF57, BAF47/INI1 and BAF53. The presence of either BRG1 or BRM along with BAF170, BAF155 and BAF45 is sufficient for optimal chromatin remodeling activity *in vitro* (Phelan, Sif, Narlikar & Kingston, 1999). The precise responsibility of each BAF has not been determined but it is clear the composition of unique SWI/SNF complexes results in interaction with various transcriptional activators and repressors (Belandia, Orford, Hurst, & Parker, 2002; Hsiao, Fryer, Trotter, Wang, & Archer, 2003; Ito, et al., 2001; Pal, et al., 2003; Phelan, et al., 1999).

SWI/SNF enzymes have been implicated in both activation and repression of genes, by their physical interactions with gene specific regulators, which are thought to recruit these enzymes to specific loci. SWI/SNF enzymes promote gene regulation by nuclear hormone receptors (Fryer & Archer, 1998), induction of stress response (de La Serna, et al., 2000), activation of TCDD inducible genes (Kalpana, Marmon, Wang, Crabtree, & Goff, 1994), C-Myc (Cheng, et al., 1999), c-fos/c-jun heterodimers (Ito, et al., 2001), interferon-β (Klochendler-Yeivin, et al., 2000), interferon-γ (Pattenden, Klose, Karaskov, & Bremner, 2002), hsp70 (Sullivan, Weirich, Guyon, Sif, & Kingston, 2001), and CD44 genes (Klochendler-Yeivin, et al., 2000). SWI/SNF has been shown to play a critical role in multiple differentiation processes including, muscle, myeloid, adipogenesis and more recently melanocyte differentiation (de la Serna, 2001; Roy, et al., 2002; Kowenz-Leutz, & Leutz, 1999; Erickson, 2001; Takahashi, et al., 2002; de la Serna, Ohkawa, et al., 2006b).

The exact mechanism by which ATP-dependent chromatin remodeling enzymes function to disrupt nucleosomes is not fully understood. The disruption of histone-DNA contacts allows for the access of transcription factors and transcriptional machinery to the promoter regions of genes. In the chromatin fiber, translocation of the nucleosome can proceed past the end of the linker, unwinding the DNA from the neighboring nucleosome (Cairns, 2007). This mechanism is termed ''sliding-mediated nucleosome disassembly'' (Lorch, LaPointe, & Kornberg, 1987; Prunell & Kornberg, 1978).

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1.3 SWI/SNF Chromatin Remodeling Enzymes and their Role in Cancer

SWI/SNF subunits act as tumor suppressors and are mutated or silenced in many forms of cancer, including prostate, lung and breast (Gregory & Shiekhattar, 2004; Roberts & Orkin, 2004). In addition, multiple cancer cell lines have been shown to be missing one or more SWI/SNF subunits (Decristofaro, et al., 2001; DeCristofaro, Betz, Wang, & Weissman, 1999; Wong, et al., 2000). Re-introduction of SWI/SNF subunits into cancer cell lines that lack expression typically induces a change in morphology, cell cycle arrest, apoptosis or senescence. Reversion of the transformed phenotype results from re-introduction of the lost SWI/SNF subunits, indicating this loss of function contributes to tumorigenicity. In mice, disruption or silencing of a number of the SWI/SNF subunits (BRG1, INI1, Baf 155, Baf 60c, and Baf 180) is embryonic-lethal, demonstrating SWI/SNF is also essential for viability. Mice having only one copy of *Brg1* or *Ini1* are predisposed to tumor development, whereas disruption of BRM expression in mice produces only mild proliferation effects (Reyes, et al., 1998).

SWI/SNF subunits have also been found to promote the inhibition of oncogenes and the activation of tumor suppressors along with interacting with tumor suppressors, such as p53, Rb, BRCA-1 and members of the Wnt signaling pathway (Barker & Clevers, 2000; Bochar, et al., 2000; Eroglu, Wang, Tu, Sun, & Mivechi, 2006). Oncogenes, such as c-Myc have also been shown to interact with SWI/SNF subunits (Rodolfo, Daniotti, & Vallacchi, 2004; H. S. Zhang, et al., 2000).

There have also been studies showing increased expression of SWI/SNF subunits leading to tumor progression. In gastric cancer increased expression of BRG1 and decreased BRM expression has been shown. Immunostaining for BRM in primary

gastric cancers has been illustrated and is associated with a loss of differentiation (Sentani, et al., 2001; Yamamichi, et al., 2007). The regulation of BRM has been tied to epigenetics. Gastric cancer cell lines treated with histone deacetylase inhibitors rescues BRM expression and reverts the phenotype of these cells back to normal (Yamamichi, et al., 2007).

Loss of both BRG1 and BRM expression correlates with poor prognosis of non-small cell lung cancer (Reisman, Sciarrotta, Wang, Funkhouser, & Weissman, 2003). BRG1 and BRM have both been shown to be lost in 30-40% of lung cancer cell lines and 10-20% of primary lung cancers and other solid tumors. Retinoic acid receptors are a type of nuclear receptor that has been shown to be important for slowing cellular proliferation and tumor development in cancer cells and in *in vivo* models. Retinoic acid receptors are functionally dependent on proper SWI/SNF function and loss of SWI/SNF eradicates RAR function (Fukuoka, et al., 2004).

BRG1, BRM and BAF57 members of the SWI/SNSF complex are involved in androgen receptor (AR) -mediated gene expression and AR is a well known participant in prostate cancer development and progression (Dehm & Tindall, 2007; Feldman & Feldman, 2001; Hong, et al., 2005; Inoue, et al., 2002; Link, et al., 2005). Interaction of SWI/SNF components with the androgen receptor have been shown to promote androgen-dependent prostate cancer proliferation (Link, et al., 2008; Link, et al., 2005). It has been previously shown that androgen receptor transactivation potential is dependent on functional SWI/SNF, specifically the BAF57 subunit. The androgen receptor is bound by BAF57 and is recruited to endogenous androgen receptor targets after ligand activation. It has also been shown that both BRM and BAF57 are necessary for the proliferation of androgen receptor-dependent prostatic adenocarcinoma cells.

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Aberrant expression of SWI/SNF Catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers. Utilizing immunohistochemistry to investigate paraffin sections of prostate cancer specimens, it was discovered that INI1 levels didn't change, BRG1 was increased and BRM was decreased when malignant tissue was compared to benign. The average level of BRM expression was lower in malignant tissue than it was in benign tissues. The pattern of increased BRG1 expression and decreased BRM expression in malignant tissue agrees with data from gastric cancers (Sentani, et al., 2001), our melanoma data (Keenen et al., 2010), but not lung cancer (Reisman, Sciarrotta, Wang, Funkhouser, & Weissman, 2003).

It has been hypothesized that increased BRG1 expression in prostate cancer might play a role in tumor development and progression by increasing the accessibility of chromatin DNA to the AR for uncontrolled gene expression or through other unknown mechanisms (Sun, et al., 2007).

1.3.1 INI1 and Tumorigenesis

The SWI/SNF complex was initially and ultimately linked to tumorigenesis when BAF47/INI-1, was established to be a tumor suppressor (Versteege, et al., 1998). INI-1 has been shown to play a role in many cellular processes associated with oncogenesis. INI-1 can regulate cell differentiation, cell cycle control, apoptosis and cell shape. Deletions or mutations in INI-1 result in malignant rhabdoid tumors, medulloblastoma and chronic myeloid leukemia (Dunaief, et al., 1994; Roberts, Galusha, McMenamin, Fletcher, & Orkin, 2000).

In mice deletion of one allele of INI-1 results in increased susceptibility to an early cancer growth (Klochendler-Yeivin, et al., 2000). Biallelic inactivation of INI-1

leads to malignant rhabdnoid tumors (MRT), fatal pediatric tumors (DeCristofaro, et al., 1999; Sevenet, et al., 1999). INI-1 has been shown to play a role in inhibition of cell migration in a Rho-A dependent manner (Caramel, Quignon, & Delattre, 2008). Studies have also shown INI-1 has an antiproliferative activity in MRTs as INI-1 overexpression leads to a G1 cell cycle arrest linked with an increase in p16INK4a, E2F, and Cyclin D (Oruetxebarria, et al., 2004; Tsikitis, Zhang, Edelman, Zagzag, & Kalpana, 2005; Versteege, Medjkane, Rouillard, & Delattre, 2002).

1.4   SWI/SNF and Differentiation

SWI/SNF and other ATP-dependent chromatin remodeling enzymes participate in differentiation through coordinating gene expression events (de la Serna, Ohkawa, & Imbalzano, 2006). BRM has been shown to play a role in intestinal differentiation in gastric cancer. Introduction of BRM expression into gastric cancer cells that are deficient in BRM expression promotes expression of villin, a known marker of intestinal differentiation (Yamamichi, et al., 2007).

1.5   SWI/SNF and Cell Cycle Regulation

SWI/SNF plays a major purpose in the regulation of cellular proliferation. Studies in the mouse have demonstrated genetic inactivation of BRG1 or BRM results in unrestrained cellular proliferation (de la Serna, Ohkawa, & Imbalzano, 2006; Klochendler-Yeivin, Muchardt, & Yaniv, 2002; Narlikar, Fan, & Kingston, 2002;

Roberts & Orkin, 2004; Sumi-Ichinose, Ichinose, Metzger, & Chambon, 1997). BRM and BRG1 are phosphorylated at the G2/M phase of the cell cycle, resulting in chromosomal exclusion of the SWI/SNF complex upon mitosis. BRG1 along with BAF155 have been shown to coimmunoprecipitates with cyclin E. The Cyclin E/ Cyclin-dependent kinase 2 (CDK2) complex phosphorylates $p27^{Kip1}$ and promotes expression of Cyclin A, allowing cell cycle progression to the S phase. The Cyclin E/CDK2 complex phosphorylates hBRG1 and BAF155 regulating the availability of SWI/SNF and its activity. CDK2 is a catalytic subunit of the cyclin-dependent kinase complex and its activity is essential for the G1/S transition of the cell cycle (Dulic, Lees, & Reed, 1992; Koff, et al., 1992; Shanahan, Seghezzi, Parry, Mahony, & Lees, 1999). These findings suggest chromosomal exclusion of SWI/SNF could be part of the mechanism critical to transcriptional arrest during mitosis (Muchardt, Reyes, Bourachot, Leguoy, & Yaniv, 1996).

As previously mentioned SWI/SNF complexes composed of distinct subunits interact with a multitude of signaling molecules. SWI/SNF complexes containing BAF250A or BAF250B subunits have contrasting roles in cell cycle-regulation. This difference in SWI/SNF subunit composition directly effects associations with activators versus repressor complexes (Nagl, Wang, Patsialou, Van Scoy, & Moran, 2007).

The two ATPase subunits BRG1 and hBRM also physically interact with Rb, resulting in cell cycle arrest (Strober, Dunaief, Guha, & Goff, 1996). The connection of SWI/SNF and cell cycle regulation is very versatile through different members of the complex having diverse relationships with cell cycle regulators.

1.6 Microphthalmia Transcription Factor (MITF)

Microphthalmia transcription factor (MITF) is the master regulator of melanocyte differentiation (Hemesath, et al., 1994). MITF is a basic helix loop helix leucine zipper transcription factor that binds to a conserved M box in the promoters of its target genes. The genes that encodes the proteins required for melanin synthesis, tyrosinase, tyrosinase-related protein 1 (Trp1) and tyrosinase-related protein 2 (Trp2 or Dct) are all regulated by MITF (Aksan & Goding, 1998; Bentley, Eisen, & Goding, 1994; Goding, 2000; Hemesath, et al., 1994). We have previously shown that MITF requires functional SWI/SNF for proper activation of tyrosinase, TRP1 and DCT (de la Serna, Ohkawa, et al., 2006b).

The MITF gene is amplified in 10-20% of human melanomas and associated with a diminished 5 year survival rate (Garraway, et al., 2005). MITF plays various roles in determining cell fate by regulating expression of anti-apoptotic genes enhancing survival and also genes that activate cell cycle arrest (Carreira, et al., 2005; Loercher, et al., 2005; Dynek, et al., 2008; McGill, et al., 2002). MITF activates expression of both T-box transcription factor 2 (TBX2) and Cyclin dependent kinase-2 (CDK2) (Du, et al., 2004). The transcription factor TBX2 inhibits senescence and is currently being studied as a target for a pro-senescence therapy for melanoma (Rodriguez, Aladowicz, Lanfrancone, & Goding, 2008). It has been established that MITF is a very powerful regulator of cell fate however, more work needs to be done to further understand the depth of its role in melanoma.

Terminal differentiation is associated with activation of p21CIP1 and p16INK4A expression and cell cycle arrest in melanocytic cells (Carreira, et al., 2005; Loercher, Tank, Delston, & Harbour, 2005). Low levels of MITF activity have been hypothesized

to promote proliferation and higher levels of MITF activity are associated with terminal differentiation (Carreira, et al., 2006).

The term "lineage survival oncogene" is used to describe the close association relating cell lineage and tumorigenesis (Garraway & Sellers, 2006). MITF has been called a "lineage survival oncogene" because is can transform primary human melanocytes. When BRAF is activated MITF can transform melanocytes by way of increasing BCL2 expression, which prevents apoptosis (Garraway & Sellers, 2006; Garraway, et al., 2005; McGill, et al., 2002). As a result of all of the different genes that MITF can regulate there are numerous neoplasms linked with *MITF* expression, including: melanoma, clear cell sarcoma (CCS), and angiomyolipoma (AML) (Dorvault, et al., 2001; Folpe, et al., 2000; Folpe, et al., 2005; Granter, Weilbaecher, Quigley, Fletcher, & Fisher, 2001; Zavala-Pompa, et al., 2001). Mutations in the *MITF* gene result in two human diseases which are both identified by pigmentary disturbances and sensineural deafness (Widlund & Fisher, 2003). Tietz syndrome is a condition characterized by deafness and albinism and Waardenburg type 2 syndrome is rare genetic disorder most often characterized by different levels of deafness and pigmentation abnormalities (Arias, 1971).

The initial studies demonstrating the role of SWI/SNF in melanocyte specific gene expression and melanocyte differentiation are elegantly illustrated in work published in 2006 (de la Serna, Ohkawa, et al., 2006b). In this study SWI/SNF was shown to be required for activation of a subset of MITF regulated genes. Using a fibroblast model where MITF is ectopically expressed with an inducible dominant negative version of BRM or BRG1, activation of tyrosinase, TRP1 and TRP2 are shown to be dependent on a functional SWI/SNF. This study also illustrates that BRG1 co-

localizes and interacts with MITF by means of immunofluorescence and immunoprecipitation experiments. It was further demonstrated that SWI/SNF is recruited to and remodels chromatin structure on the TRP1 promoter. Chromatin immunoprecipitation studies illustrate binding of both MITF and BRG1 to the TRP1 and tyrosinase promoters. Figure 1 illustrates the role of SWI/SNF in MITF regulated melanocyte specific gene expression events (de la Serna, Ohkawa, et al., 2006b).

Figure 1

Figure 1 illustrates the role of SWI/SNF in MITF regulated melanocyte specific gene expression events. In NIH 3T3 cells, ectopically expressing MITF, a functional SWI/SNF is required for expression of a subset of MITF-mediation gene expression events important for melanocyte differentiation and pigment production (tyrosinase, TRP1 and DCT).
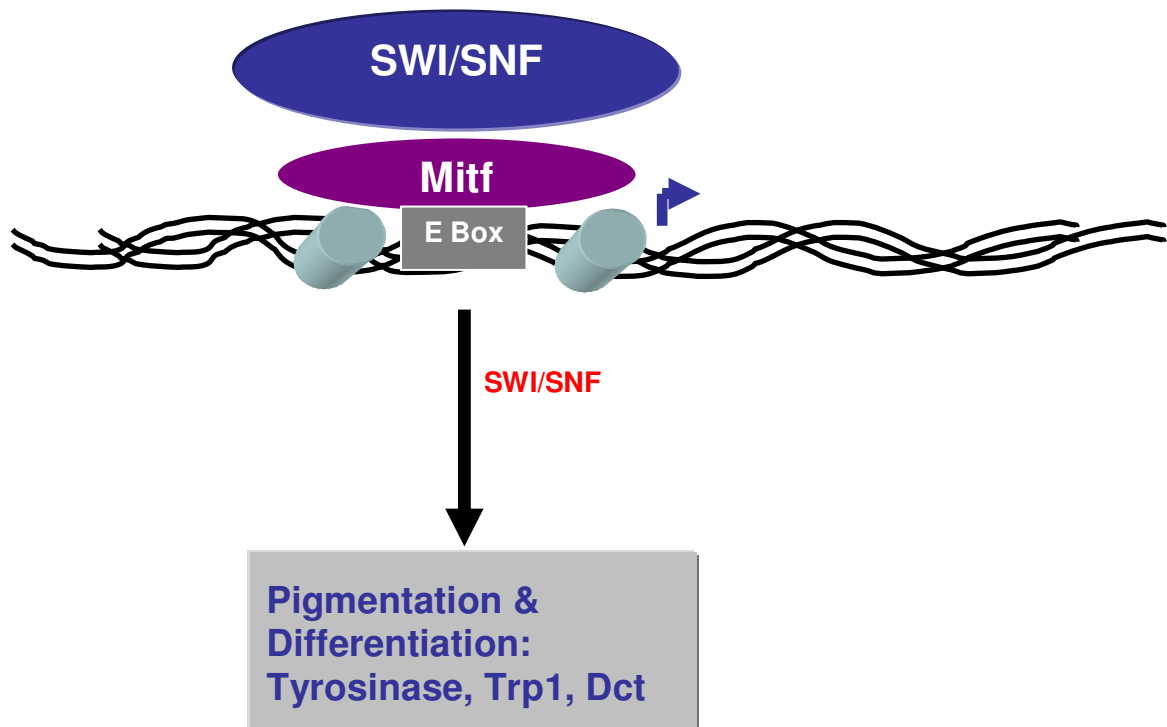
1.6.1 Melanocytes and Melanin Synthesis

Melanocytes are pigment producing cells mainly found in the epidermis that transfer melanin to surrounding cells, protecting them from DNA damage. Melanoblasts are precursor cells of melanocytes and express Microphthalmia-associated transcription factor (MITF), required for their survival during development (Steingrimsson, Copeland, & Jenkins, 2004; Vance & Goding, 2004). Melanocytes are also found in the eye and the inner ear, loss of melanocytes in the ear results in deafness (Steel & Barkway 1989).

Tyrosinase catalyzes the production of melanin from tyrosine by oxidation and is the rate limiting enzyme essential for melanin synthesis. MITF regulates the expression of tyrosinase in additional to other genes that regulate melanin synthesis and melanosome structure (Hemesath, et al., 1994). Melanin is transferred through dendritic processes of melanocytes to other melanocytes and neighboring keratinocytes in the skin. The transport of melanin takes place in the melanosomes, membrane-bound organelles (Vijayasaradhi, Xu, Bouchard, & Houghton, 1995).

The tyrosinase-related proteins, TRP1 and TRP2, are specifically expressed in melanocytes, and work with tyrosinase in the melanosome to aid in the synthesis of melanin. The development of melanocytes is illustrated below in Figure 2. During early development, melanoblasts migrate from the neural crest, along the dorsolateral pathways to colonize the dermis and epidermis (Nishimura, Yoshida, Kunisada, & Nishikawa, 1999). MITF has a role in the commitment of melanocytes during the development process. The transport of melanin between melanocytes and to keratinocytes occurs with the assistance of Rab27a, a small GTPase (Byers, Yaar, Eller, Jalbert, & Gilchrest, 2000; Hara, et al., 2000; Jordens, et al., 2006; Vancoillie, et al., 2000a, 2000b).

Tyrosinase, TRP1 and TRP2 share a similar transmembrane region which contains two metal-binding regions and a cysteine rich epidermal growth factor motif localized in the melanosomal membrane. There are distinct catalytic activities for tyrosinase, TRP1 and TRP2 all of which contribute to the biosynthesis of melanin (Widlund & Fisher, 2003).

Melanin production is the result of α-melanocyte-stimulating hormone (α-MSH) acting on the melanocortin receptor 1 (MC1R) and results in increased skin pigmentation. The tanning response, in which melanocytes produce and distribute melanin, is a defense mechanism to shield surrounding cells from the damaging effects of ultraviolet radiation (UVR). Consequentially, polymorphisms in the gene encoding MC1R and other genes that regulate melanin synthesis have been correlated with melanoma (D'Orazio, et al., 2006; Kadekaro, et al., 2005; Landi, et al., 2006; Miller & Mihm, 2006; Scott, et al., 2002).

Quantity and type of melanin production vary depending on the genetic make up of an individual. There are two types of melanin, pheomelanin and eumelanin. In epidermal melanocytes, melanocortin 1 receptor (MC1R) is a seven-transmembrane G-protein-coupled receptor that is highly variably in human populations. Variation in the MC1R receptor is responsible for the different pigmentation phenotypes in humans (Sturm, 2002; Valverde, Healy, Jackson, Rees, & Thody, 1995). MC1R has been linked to the switch between eumelanin (black) and pheomelanin (red/yellow) production (Garcia-Borron, Sanchez-Laorden, & Jimenez-Cervantes, 2005). Albinism is the result of decreased tyrosinase activity and is characterized by lack of pigmentation. The POMC gene encodes α-MSH, which binds the MC1R receptor allowing for the production of melanin which is stimulated in the presence of ultraviolet radiation (UVR).

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Adenylate cyclase production is activated in response to binding of α-MSH to its receptor, this in turn increases cAMP formation (Suzuki, Cone, Im, Nordlund, & Abdel-Malek, 1996).

Figure 2. MITF is required for Melanocyte Development

Melanocytes are derived from the neural crest and upon MITF expression are committed to the melanocyte lineage. Melanoblast are the precursor to melanocytes and transfer melanin to surrounding keratinocytes and melanocytes through the assistance of melanosomes and RAB27A. MITF target genes, DCT, tyrosinase and TRP1 are needed for pigment production and melanocyte differentiation.
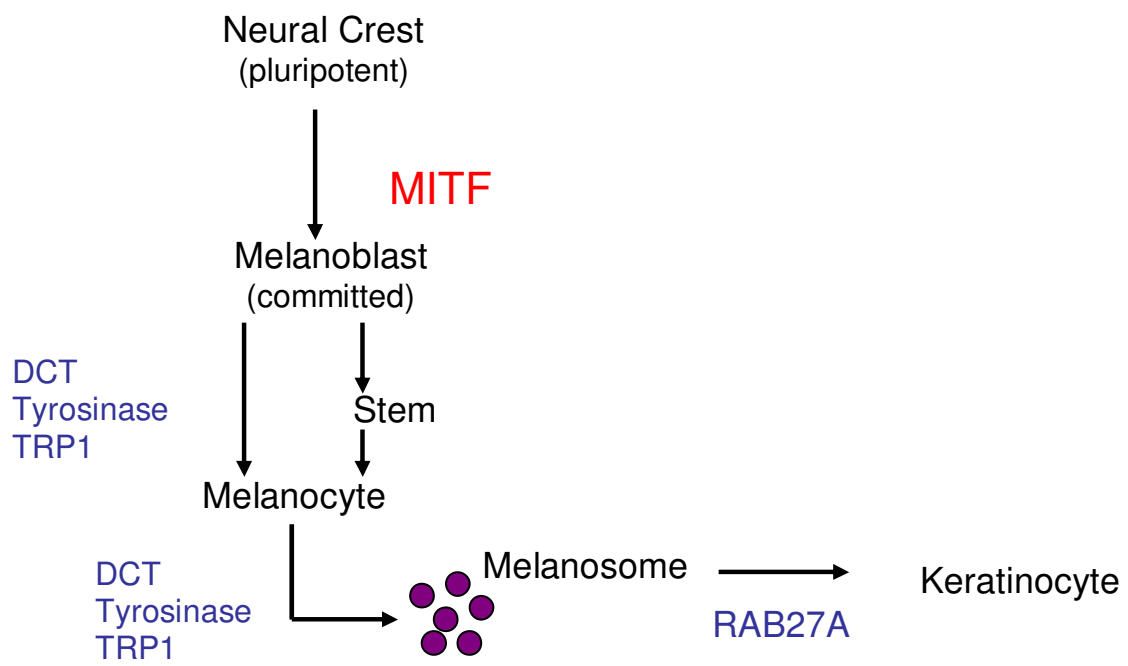
Exposure to UVR results in free radical production that can result in tumorigenesis if not properly controlled. Melanin sequesters free radicals, which have an affinity for DNA and cellular proteins.   In response to UVR exposure keratinocytes synthesize α-MSH which then binds to the MC1R receptor on the melanocyte surface (Im, et al., 1998; Pawelek, et al., 1992). The resulting increase in melanin production is due to the activation of many different signaling pathways (Hirobe, 2005). The p38 stress-signaling pathway is activated in response to UVR and induces expression of genes that are required for melanin production (Corre & Galibert, 2005). UVR is just one of the many stressors that activate the p38 kinase pathway (Galibert, Carreira, & Goding, 2001). Figure 3 illustrates a handful of the signaling pathways activated by UVR and some of the cellular effects, such as apoptosis or cell cycle arrest. There are several consequences to UVR exposure and the outcome is dependent on many epigenetic factors.

Figure 3. Cellular Effects of UVR Exposure

The p38 stress-signaling pathway is activated in response to UVR and induces expression of genes that are required for melanin production and activates Sp1 which can bind to the MMP-2 and increase expression of MMP-2. UVR can also increase binding of α-MSH to its receptor, this in turn increases cAMP formation. Increased levels of cAMP can phosphorylate CREB and increase activation and expression of MMP-2. The p53 pathway can also be activated in response to UVR exposure and result in apoptosis or cell cycle arrest and DNA repair. UVR exposure can result in DNA damage which if not properly repaired can result in survival of mutant melanocytes which can lead to melanoma.

Figure 3. Cellular Effects of UVR Exposure
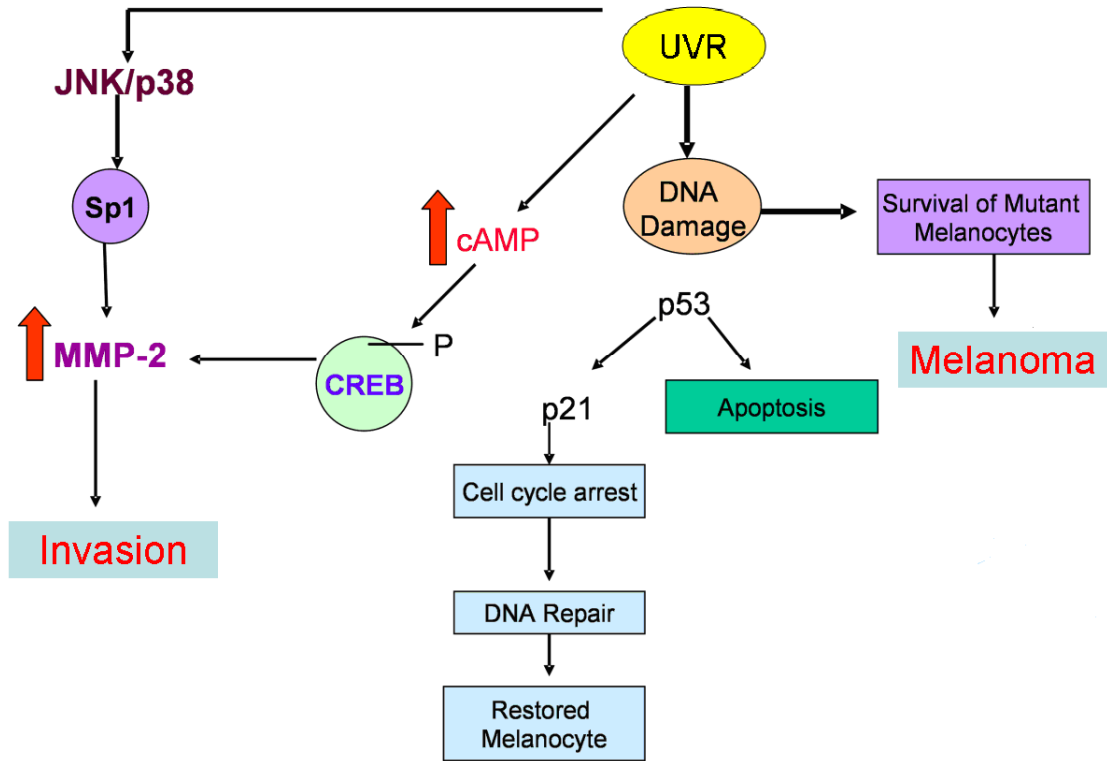
1.7 Melanoma

Over the past several decades, the mortality rate for malignant melanoma has increased by 50%, with the incidence in the US and other Western countries rising faster than any other cancer (Howe, et al., 2001). An estimated 62,480 new cases of melanoma were diagnosed in the United States during 2008, 8,420 of which resulted in death (Jemal, et al., 2008). Melanoma accounts for about 3% of skin cancer cases but causes a large majority of skin cancer deaths, with metastatic melanoma patients having a mere 2% five year survival rate. Melanoma tends to occur at a younger age than most cancers, half of which are found in people under age 57. It is the fifth most common cancer among men and the sixth most common among women (Tucker & Goldstein, 2003). The striking increase in the incidence of melanoma and resistance to treatment emphasizes the importance in defining the molecular mechanisms that regulate initiation and progression of this disease.

Melanoma is curable with early diagnosis and surgical excision (Balch, et al., 2001). Approximately 20% of patients will develop metastatic tumor due to the highly invasive and metastatic properties of melanoma (Houghton & Polsky, 2002).

Exposure to UVR is associated with an increased relative risk to the development of melanoma. There are three forms of UVR, UVA (320-400 nm), UVB (290-320 nm) and UVC (100-280 nm). UVA composes the majority of light that reaches the surface of the earth. UVB induces genotoxic and photocarcinogenic effects (Epstein, 1983; Setlow, 1974). Studies have shown that individuals obtaining irregular exposure to sun are more likely to develop melanoma in comparison to those that are exposed to sun on a more regular basis (Houghton & Viola, 1981).

There are classic stages of melanoma development that consist of the transformation of melanocytes to benign nevi through radial and vertical growth phases to metastatic disease (Clark, From, Bernardino, & Mihm, 1969). There are five definable and sequential stages of melanoma progression according to the Clark model (Miller & Mihm, 2006). According to this model, somatic mutations in the BRAF or N-RAS genes result in benign nevi or moles, these are the first noticeable changes in melanoma development (Gray-Schopfer, Wellbrock, & Marais, 2007). Activating mutations in BRAF are the most common mutations, correlated with melanoma, occurring in 50-70% of cases. The most common BRAF mutation is a glutamic acid for valine substitution at position 600 (V600E) (Davies, et al., 2002). Mutations in BRAF lead to senescence in melanocytes and are found in approximately 80% of benign nevi (Pollock, et al., 2003). Additional hits are needed for melanocytes with BRAF mutations to transform into later stages of melanoma.

During the progression of melanoma, melanocytes transform from noninvasive radial growth phase to vertical growth phase, having high metastatic potential and eventually possessing the ability to cross the basement membrane and invade the dermis. Numerous molecular lesions transpire throughout the course of melanoma progression, such as loss or mutation of key tumor suppressors, resulting in decreased differentiation, increased proliferation and invasiveness (Gray-Schopfer, et al., 2007; Miller & Mihm, 2006). BRAF is considered an oncogene and can transform immortalized melanocytes, in part by activation of the MAPK pathway (Garnett & Marais, 2004; Wan, et al., 2004; Wellbrock & Marais, 2005). MITF, the master regulator of melanocyte differentiation is a target of the mitogen activated signaling pathway (MAPK).

Many types of cancers, including melanoma, are the result of a combination of genetics and environmental factors (Goldstein & Tucker, 2001). A family history of melanoma greatly increases a person's risk for later development (Houghton & Polsky, 2002). Sensitivity to ultraviolet light is a genetic trait that affects susceptibility to melanoma. Within the basal layer of the epidermis melanocytes can undergo transformation to cutaneous malignant melanoma through excessive exposure to UVR (Jhappan, Noonan, & Merlino, 2003).

The high levels of morbidity and mortality associated with melanoma are largely due to the ability of melanoma cells to infiltrate the basement membrane, along with proliferating and metastasizing to distant organs (Haass, Smalley, Li, & Herlyn, 2005; Miller & Mihm, 2006). This metastatic phenotype is characterized by alterations in the expression of cellular adhesion molecules (Miller & Mihm, 2006). Cellular adhesion controls cell migration, tissue organization and organogenesis, disturbances in which contribute to tumor invasion and tumor cell signaling (J. P. Johnson, 1999).

Melanoma is highly resistant to current therapies. Options for treatment are mediocre including chemotherapy and immunotherapy and provide minimal response rates and only brief efficacy (Houghton & Polsky, 2002; Jhappan, et al., 2003). There have been studies illustrating the connection between the level of melanoma differentiation and resistance to melanoma therapeutics. Melanomas are recognized as very resistant to cytotoxic drugs that are commonly used for treatment. Several studies suggest melanoma cells possess lineage-specific mechanisms for drug resistance. Dopachrome tautomerase (DCT/TRP2) is a melanocytic enzyme that allows resistance to DNA damaging agents used in the treatment of melanoma. As described previously,

DCT/TRP2 expression is regulated by MITF and important for melanin and therefore pigment biosynthesis.

Melanosomes are the membrane-bound organelles located in melanocytes responsible for synthesizing melanin (Vijayasaradhi, et al., 1995) and have also been shown to increase resistance to cytotoxic drugs by increasing transport of the cytotoxic drugs from the cells (K. G. Chen, et al., 2006; Chu, et al., 2000).

1.7.1 Melanoma and SWI/SNF Chromatin Remodeling Complex

Investigating the levels of BRG1 and BRM expression in human melanoma samples we found that levels of BRG1 increase in the later stages of melanoma metastasis and levels of BRM decrease. BRG1 levels were observed to be significantly up-regulated in stage IV melanoma samples compared to normal skin and stage III melanoma (p<.05).

BRM levels were found to be significantly down-regulated in melanoma cells compared to normal skin (p=.008). This data is illustrated below in Figure 4.

Data from a group in Australia also suggests BRG1 expression is increased in metastatic melanomas compared to primary although this increase was not significant using Mann-Whitney Wilcoxon test (T. M. Becker, et al., 2009).

Figure 4 A. Levels of BRM expression in human melanoma samples compared to normal skin.
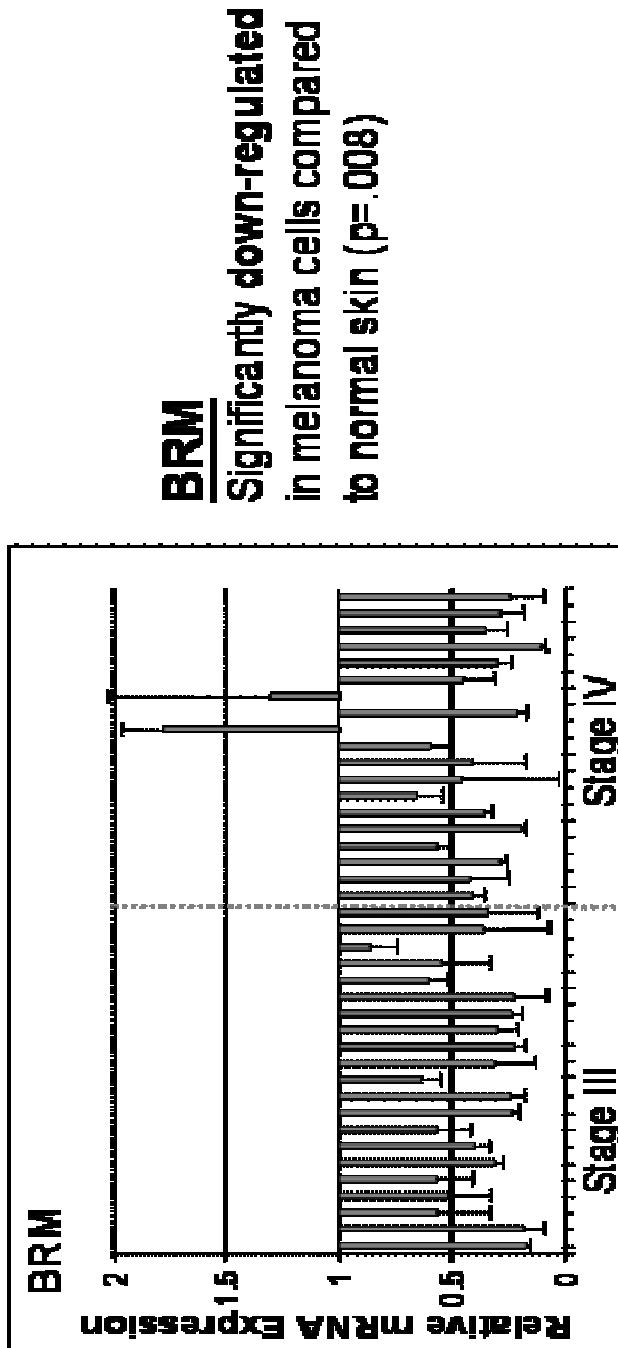
Figure 4 B. Raw Real Time PCR data from three separate experiments investigating BRM expression. Fold change values are illustrated below in the table and normalized to normal tissue samples.

	BRMa	BRMb	BRMc
Normal	1	1	1
III	0.169	0.2	0.15
III	0.157	0.3	0.113
III	0.734	0.4	1.092
III	0.58	0.3	0.667
III	0.445	0.5	0.756
III	0.338	0.3	0.301
III	0.487	0.4	0.316
III	0.588	0.7	0.414
III	0.22	0.2	0.281
III	0.282	0.3	0.167
IIIA	1.217	0.7	0.569
IIIB	0.304	0.5	0.12
IIIB	0.195	0.3	0.185
IIIB	0.27	0.4	0.21
IIIB	0.214	0.3	0.19
IIIB	0.147	0.4	0.131
IIIB	0.609	0.7	0.499
IIIC	0.286	0.7	0.649
IIIC	0.744	1	0.851
IIIC	0.199	0.7	0.176
IIIC	0.201	0.6	0.219
IV	0.352	0.4	0.482
IV	0.247	0.6	0.405
IV	0.249	0.3	0.307
IV	0.556	0.5	0.645
IV	0.171	0.2	0.208
IV	0.315	0.4	0.373
IV	0.556	0.8	0.606
IV	0.424	0.9	0.071
IV	0.256	0.7	0.277
IV	0.694	0.5	0.589
IV	1.91	0.9	1.643
IV	0.276	0.2	0.157
IV	2.757	0.8	1.811
IV	0.63	0.4	0.318
IV	0.362	0.3	0.248
IV	0.134	0.1	0.062
IV	0.413	0.4	0.241
IV	0.292	0.4	0.167
IV	0.433	0.2	0.114

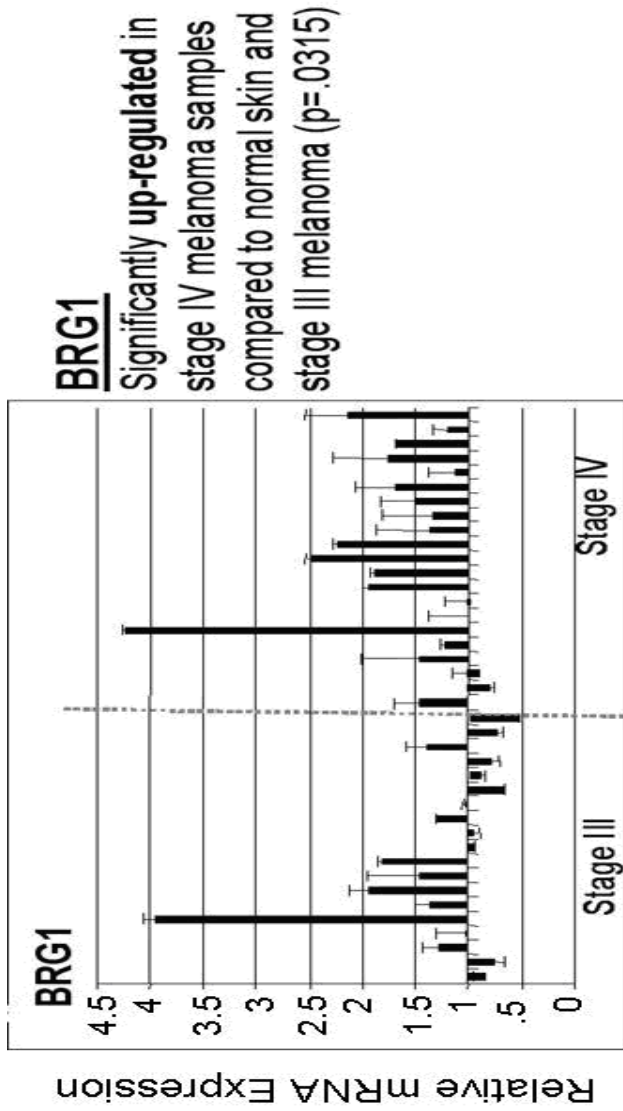**Figure 4 C.** Levels of BRG1 expression in human melanoma samples compared to normal skin.

Figure 4 D. Raw Real Time PCR data from three separate experiments investigating BRG1 expression. Fold change values are illustrated below in the table and normalized to normal tissue samples.

	BRG1a	BRG1b	BRG1c
Normal	1	1	1
III	0.72	0.914	0.918
III	0.843	0.697	0.725
III	1.104	1.329	1.382
III	1.341	0.847	0.881
III	6.79	3.864	4.019
III	1.53	1.231	1.28
III	2.163	1.803	1.875
III	2.047	1.157	1.195
III	1.845	1.79	1.836
III	0.955	0.979	0.964
IIIA	1.009	0.908	0.905
IIIB	1.295	1.283	1.298
IIIB	0.961	1.035	1.047
IIIB	0.709	0.655	0.667
IIIB	0.929	0.847	0.875
IIIB	0.885	0.753	0.777
IIIB	1.628	1.257	1.298
IIIC	0.797	0.697	0.725
IIIC	0.548	0.529	0.542
IIIC	1.733	1.32	1.353
IIIC	0.734	0.835	0.857
IV	1.176	0.779	0.783
IV	0.825	1.753	1.811
IV	1.26	1.223	1.203
IV	6.79	4.199	4.248
IV	1.437	0.768	0.788
IV	1.26	0.829	0.839
IV	4.418	1.919	1.982
IV	3.325	1.853	1.914
IV	5.04	2.46	2.526
IV	3.638	2.189	2.261
IV	1.95	1.035	1.062
IV	1.896	1.05	1.069
IV	1.896	1.301	1.289
IV	2.134	1.464	1.451
IV	1.408	0.959	0.944
IV	2.367	1.464	1.471
IV	2.645	1.682	1.678
IV	1.36	1.102	1.084
IV	2.591	1.932	1.927

1.7.2 INI-1 and Melanoma

INI-1 expression has been shown to be down regulated in melanoma in contrast to dysplastic nevi and also in advanced primary melanoma compared to less metastatic melanoma. There is also a strong association between lack of INI-1 expression and a decreased 5-year survival in melanoma patients. INI-1 has been proposed to be a marker for human melanoma progression and prognosis as well as a potential therapeutic target (Lin, Wong, Martinka, & Li, 2009).

1.8 DNA Damage, Repair & SWI/SNF

There have been reports in the literature illustrating a role for SWI/SNF in DNA repair in response to UV induced DNA damage. SWI/SNF has been shown to be involved in DNA repair by altering the accessibility DNA repair proteins to DNA lesions (Dinant, Houtsmuller, & Vermeulen, 2008). Park et al 2006 demonstrated inactivation of the SWI/SNF complex results in inefficient DNA double-strand break (DSB) repair and increased sensitivity to DNA damage (Gong, Fahy, Liu, Wang, & Smerdon, 2008; Gong, Fahy, & Smerdon, 2006; Park, et al., 2006).

Manuscript One:

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Heterogeneous SWI/SNF Chromatin Remodeling Complexes Promote Expression of Microphthalmia –Associated Transcription Factor Target Genes in Melanoma

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Running Title: SWI/SNF Chromatin Remodeling Enzymes in Melanoma

Abstract

The microphthalmia-associated transcription factor (MITF) promotes melanocyte differentiation and cell cycle arrest. Paradoxically, MITF also promotes melanoma survival and proliferation, acting like a lineage survival oncogene. Thus, it is critically important to understand the mechanisms that regulate MITF activity in melanoma cells. SWI/SNF chromatin remodeling enzymes are multiprotein complexes composed of one of two related ATPases, BRG1 or BRM, and 9-12 associated factors (BAFs). We previously determined that BRG1 interacts with MITF to promote melanocyte differentiation. However, it was unclear whether SWI/SNF enzymes regulate the expression of different classes of MITF target genes in melanoma. In this study, we characterized SWI/SNF subunit expression in melanoma cells and observed down-regulation of BRG1 or BRM, but not concomitant loss of both ATPases. Re-introduction of BRG1 in BRG1 deficient SK-MEL5 cells enhanced expression of differentiation specific MITF target genes and resistance to cisplatin. Down-regulation of the single ATPase, BRM, in SK-MEL5 cells inhibited expression of both differentiation specific and pro-proliferative MITF target genes and inhibited tumorigenicity in vitro. Our data suggest that heterogeneous SWI/SNF complexes composed of either the BRG1 or BRM subunit promote expression of distinct and overlapping MITF target genes and that at least one ATPase is required for melanoma tumorigenicity.

Introduction

Microphthalmia-associated transcription factor (MITF), the master regulator of melanocyte differentiation, promotes melanocyte lineage survival and plays a key role in melanoma progression. MITF is a basic helix loop helix leucine zipper transcripton factor that binds to a conserved M box in the promoters of tyrosinase and other melanocyte specific genes that regulate melanin synthesis and melanosome structure (Hemesath, et al., 1994). MITF also promotes expression of genes that regulate melanoma proliferation and survival and has been termed a "lineage survival oncogene," being amplified in 10-20% of human melanomas (Garraway, et al., 2005).

The role that MITF plays in melanoma has been controversial. MITF can inhibit proliferation by directly activating expression of the cyclin dependent kinase inhibitors, p21CIP1 and p16INK4A (Carreira, et al., 2005; Loercher, et al., 2005). MITF can also promote survival and proliferation by activating anti-apoptotic factors, BCL2 and MLIAP (Dynek, et al., 2008; McGill, et al., 2002), TBX2 (Carreira, Liu, & Goding, 2000), and the cell cycle dependent kinase, CDK2 (Du, et al., 2004). It has been proposed that the levels of MITF determine whether MITF promotes differentiation and cell cycle arrest or proliferation (Carreira, et al., 2006). In addition, signaling pathways and interactions with other regulatory proteins may regulate MITF activity. We have previously determined that MITF interacts with SWI/SNF chromatin remodeling enzymes to activate differentiation specific genes (de la Serna, Ohkawa, et al., 2006a).

SWI/SNF enzymes are ATP dependent multisubunit complexes that disrupt histone-DNA contacts and promote chromatin structural changes (Sif, 2004). Mammalian SWI/SNF complexes are composed of the BRG1 or BRM catalytic ATPase subunit and

9-12 BRG1/BRM associated factors (BAFs). BRG1 and BRM have overlapping functions but are not always interchangeable and can interact with distinct transcription factors in vitro (Kadam, et al., 2000). Heterogeneous SWI/SNF complexes, generated by the alternative presence of either the BRG1 or BRM ATPase as well as by a specific BAF composition, may have distinct functions. For example, BRG1 and BRM can differentially regulate gene expression (Flowers, Nagl, Beck, & Moran, 2009; Xu, Zhang, & Chen, 2007) and SWI/SNF complexes containing either the BAF250A or BAF250B subunit have opposing roles in cell cycle regulation (Nagl, et al., 2007). Thus, SWI/SNF subunit composition is an important determinant of SWI/SNF specificity.

BRG1 and BRM and other components of the SWI/SNF complex have been implicated in cancer. BRG1 or BRM expression is down-regulated in a wide array of human cancers. Loss of both BRM and BRG1 expression correlates with poor prognosis of non-small cell lung cancer (Fukuoka, et al., 2004; Reisman, et al., 2003). Other SWI/SNF components are mutated, deleted, or not expressed in many human cancer cell lines (Decristofaro, et al., 2001). Inactivating mutations in the INI1 subunit occur in the majority of malignant rhabdoid tumors (Versteege, et al., 1998). Re-introduction of SWI/SNF subunits into cancer cells that lack expression induces a flat cell morphology, cell cycle arrest, apoptosis or senescence, with reversion of the transformed phenotype, indicating that loss of SWI/SNF function contributes to tumorigenicity (Dunaief, et al., 1994; Wang, et al., 2005). Conversely, components of the SWI/SNF complex interact with the androgen receptor to promote androgen dependent prostate cancer proliferation (Link, et al., 2008; Link, et al., 2005). Thus, the role that SWI/SNF plays in cancer is complex and dependent on cellular context and potentially on subunit composition.

SWI/SNF enzymes are important regulators of cellular differentiation (de la Serna, Ohkawa, & Imbalzano, 2006). In normal cells, key regulatory factors interact with SWI/SNF enzymes to promote myogenic, myeloid, erythropoietic, adipogenic, and neuronal differentiation. SWI/SNF enzymes can also promote differentiation of cancer cells. Introduction of the BRM subunit into gastric cancer cells that are deficient in BRM promotes expression of markers of intestinal differentiation and introduction of the INI1 subunit into malignant rhabdoid tumor cells differentiates these cells along the adipogenic pathway (Caramel, Medjkane, Quignon, & Delattre, 2008; Yamamichi, et al., 2007).

The status and activity of SWI/SNF components have been characterized in many cancer types, but poorly characterized in human melanoma. A recent report indicates that BRG1 is down-regulated in primary and metastatic melanomas (T. M. Becker, et al., 2009). Moreover, it has recently been reported that down-regulation of the INI1 subunit of the SWI/SNF complex is important for bypassing oncogenic BRAF induced senescence in melanocytes and that the BRM subunit is associated with heterochromatic foci in senescing melanocytes (Bandyopadhyay, et al., 2007; Wajapeyee, Serra, Zhu, Mahalingam, & Green, 2008). Because our previous work indicated that SWI/SNF enzymes are required to promote MITF mediated activation of melanocyte specific gene expression (de la Serna, Ohkawa, et al., 2006a), we hypothesized that SWI/SNF enzymes are also critical regulators of melanoma differentiation and tumorigenicity.

We previously determined that in a tissue culture model of melanocyte differentiation, MITF promotes recruitment of BRG1 to the promoter of a melanocyte specific gene, chromatin remodeling, and activation of melanocyte specific gene expression (de la Serna, Ohkawa, et al., 2006a). To determine the ramifications of the previously observed SWI/SNF/MITF interaction in melanoma, we characterized

SWI/SNF subunit expression in normal human melanocytes and in established human melanoma cell lines. We found that normal melanocytes express high levels of all SWI/SNF subunits, including both the BRG1 and BRM catalytic subunits whereas several melanoma cell lines were markedly down-regulated in either BRG1 or BRM.

To characterize the role of SWI/SNF enzymes in the regulation of MITF target gene expression in melanoma and to distinguish between the roles played by BRG1 and BRM, we expressed BRG1 in BRG1 deficient SK-MEL5 cells. We found that expression of BRG1 enhanced melanoma differentiation and increased expression of MITF target genes that regulate pigmentation. Down-regulation of BRM in BRG1 deficient SK-MEL5 cells inhibited expression of many MITF target genes and reduced growth on soft agar. Our data indicate that both BRG1 and BRM containing SWI/SNF complexes interact with MITF to promote optimal expression of a subset of overlapping and distinct MITF target genes and that a functional SWI/SNF ATPase is required for aspects of melanoma tumorigenicity in vitro. Thus, we propose that SWI/SNF enzymes through their partnership with MITF are important epigenetic modulators in melanoma cells.

Results

BRG1 and BRM are not concomitantly down-regulated in melanoma cells

The loss of both BRG1 and BRM has been observed in some cancer cell lines (Decristofaro, et al., 2001) while only BRG1 or BRM is down-regulated in the pancreatic cancer cell line, MiaPaCa2, and in gastric cancer (Rosson, et al., 2005; Yamamichi, et al., 2007). It was recently shown that BRG1 is downregulated in a subset of primary melanomas (T. M. Becker, et al., 2009), however, this study did not address whether melanomas are concomitantly down-regulated for both the BRG1 or BRM ATPases nor did it evaluate the status of other SWI/SNF subunits.

We characterized the expression of BRG1 and BRM and the expression of other SWI/SNF proteins in normal human melanocytes and established human melanoma cell lines. We found that melanocytes expressed high levels of both BRG1 and BRM when compared to HeLa cells (known to express high levels of many SWI/SNF components) and SW13 cells (known to be deficient in both BRG1 and BRM) but that BRG1 and BRM expression was variable in a number of melanoma cell lines (Fig. 1A). SK-MEL5 cells, isolated from an axillary node metastasis (Pollack, Heagney, Livingston, & Fogh, 1981), did not express BRG1 but had levels of BRM comparable to melanocytes. PMWK, derived from a primary melanoma (Vink, et al., 1993), and YUMAC, derived from metastatic melanoma (Hoek, et al., 2004), had virtually undetectable levels of BRM but expressed high levels of BRG1 (Fig. 1A). All the melanoma cells expressed high levels of several other SWI/SNF components, including the core subunits, INI1 and BAF155. Therefore, if the expressed SWI/SNF components are functional, then overall SWI/SNF activity in a subset of melanoma cells may be compromised by down-

regulation of one SWI/SNF ATPase but not completely eliminated because the alternative ATPase is retained and the other core SWI/SNF components are also highly expressed.

In SK-MEL5 cells, BRG1 is down-regulated at the transcriptional level

Expression of BRG1 at the protein level was consistently undetectable in SK-MEL5 cells even when cultured under many different serum conditions and at different confluencies (data not shown). To determine if BRG1 is downregulated at the transcriptional level in these cells, we compared expression of BRG1 mRNA in SK-MEL5 cells with expression in normal human melanocytes and in other melanoma cells (Fig.1B). SK-MEL5 cells expressed significantly lower levels of BRG1 mRNA than melanocytes and that there was not a compensatory increase in BRM expression. The level of BRG1 mRNA in SK-MEL5 cells was similar to that in BRG1 deficient SW13 cells (Fig. 1B).

Ectopically expressed BRG1 localizes to the nucleus and assembles into a SWI/SNF complex that includes MITF

Re-introduction of SWI/SNF components into cancer cells that lack expression has been demonstrated to result in cell cycle arrest, apoptosis, or differentiation, depending on the cancer cell type and the identity of the missing SWI/SNF subunit (Caramel, Medjkane, et al., 2008; Dunaief, et al., 1994; Wang, et al., 2005; Yamamichi, et al., 2007). To determine how BRG1 regulates the melanoma phenotype, we expressed epitope tagged BRG1 into BRG1 deficient SK-MEL5 cells by retroviral infection and selected a pool of cells. We obtained expression levels of BRG1 that were approximately twice that present in normal melanocytes (Fig. 2A and B). SK-Mel28 melanoma cells

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express BRG1 at approximately twice the levels present in melanocytes (Fig. 1A and B), thus the level of BRG1 expression that we obtained in SK-MEL5 cells is within a physiologically relevant range for melanocytic cells. We found that the selected cells grew slower (50% over a 6 day period) (data not shown) but could be propagated without loss of BRG1 expression (data not shown).

We did not see a compensatory decrease in BRM levels as a result of BRG1 over-expression. Interestingly, we observed a small increase in BRM mRNA and protein, suggesting that the BRG1 expressing SK-MEL5 cells had increased levels of functional SWI/SNF complexes containing both of the catalytic subunits (Fig. 2A and B). Crosstalk between different subunits of the SWI/SNF complex has previously been reported but the mechanisms are not completely understood (J. Chen & Archer, 2005; Kang, Cui, & Zhao, 2004). Moreover, BRM levels have been shown to increase during differentiation (Machida, Murai, Miyake, & Iijima, 2001). The increased levels of BRM that we observed correlated with a more differentiated phenotype (see below).

We detected epitope tagged BRG1 in approximately 100% of the selected cells and found that BRG1 localized to the nucleus (Fig. 2C). Furthermore, ectopically expressed BRG1 interacted with other SWI/SNF subunits to form potentially active SWI/SNF complexes that also contained MITF (Fig.2D). This indicated that the established SWI/SNF complexes containing the BRG1 ATPase might act as co-regulators of MITF activity in these melanoma cells.

Expression of BRG1 in SK-MEL5 cells increases pigmentation and resistance to cisplatin

Melanoma cells are often less differentiated than their normal counterparts (Eberle, Garbe, Wang, & Orfanos, 1995).   SK-MEL5 cells retain MITF expression but are amelanotic, suggesting that differentiation specific gene expression is suppressed downstream of MITF (note MITF expression, Fig. 2D).   We found that ectopic expression of BRG1 in these cells led to a noticeable increase in pigmentation (Fig. 3A). Consistently, we detected increased expression of tyrosinase and tyrosinase related protein 1 (TRP1), two enzymes important for melanin synthesis (Fig. 3B). These results suggest that BRG1 can promote melanoma differentiation by activating MITF target gene expression. Over-expression of BRM to approximately four times the endogenous levels (supple. Fig. 1A, B, and C) did not increase visible pigmentation nor did it activate expression of most MITF target genes (supple. Fig. 1D and 1E),   suggesting that when expressed at these levels, BRM cannot compensate for BRG1 in promoting differentiation of SK-MEL5 melanoma cells.

MITF is a lineage survival oncogene that is amplified in 10%-20% of human melanoma cells and can promote melanoma chemoresistance (Garraway, et al., 2005). The extent of melanoma differentiation can have an impact on chemoresistance because melanin acts as a sink that sequesters cytotoxic compounds (K. G. Chen, et al., 2006; Chu, et al., 2000; Pak, Li, Kerbel, & Ben-David, 2000; Svensson, Lindgren, Powell, & Green, 2003). The MITF gene is not amplified in SK-MEL5 cells and these cells exhibit intermediate sensitivity to the alkylating agent, cisplatin (Garraway, et al., 2005; L. Shen, et al., 2007). Because ectopically expressed BRG1 interacted with MITF and led to an

increase in pigmentation (Fig. 3A), we tested whether BRG1 could promote melanoma chemoresistance. We treated control and BRG1 expressing SK-MEL5 cells with cisplatin and found that expression of BRG1 significantly increased resistance as measured by a cell survival assay (Fig. 3C). Thus, consistent with previous studies (K. G. Chen, et al., 2006; Chu, et al., 2000; Svensson, et al., 2003), we have correlated the status of melanoma differentiation with resistance to chemotherapeutic agents. Furthermore, our data is consistent with reports in other cancer types that have shown an increase in resistance to DNA damage upon re-introduction of BRG1 (Gong, et al., 2008; Park, Park, Hur, Kim, & Kwon, 2009). These data suggest that the status of SWI/SNF enzymes in melanoma cells can have a significant impact on chemoresistance, an important issue in developing therapeutics against melanoma.

BRG1 is recruited to the promoters of differentiation specific MITF target genes and activates their expression

We performed chromatin immunoprecipitations (ChIPs) to determine whether ectopically expressed BRG1 in SK-MEL5 cells is recruited to different classes of MITF target genes that regulate differentiation, proliferation, and survival. We detected BRG1 on the promoters of genes that encode enzymes needed for melanin synthesis, tyrosinase, tyrosinase related protein 1 (TRP1), and dopachrome tautomerase (DCT) (Fig.4A and B). We also detected BRG1 on the RAB27A promoter. RAB27A is a recently identified MITF target gene that regulates peripheral transport of mature melanosomes (Chiaverini, et al., 2008). We also detected BRG1 on the p21CIP1 promoter, an anti-proliferative MITF target gene, associated with increased melanocyte differentiation (Carreira, et al.,

2005). Thus, BRG1 was recruited to multiple promoters that regulate genes important for pigmentation and overall differentiation status.

We investigated whether BRG1 is recruited to the promoters of MITF target genes that regulate survival and proliferation. BRG1 was significantly enriched on the promoter of the melanoma inhibitor of apoptosis (MLIAP) (Fig. 4A and B), an MITF target gene and a potent anti-apoptotic factor that is highly expressed in melanoma (Dynek, et al., 2008). However, we did not detect significant enrichment of BRG1 on the CDK2 promoter (Fig.4A and B), an MITF target gene that promotes proliferation (Du, et al., 2004), nor on TBX2 or BCL2, other MITF target genes that promote melanoma proliferation and survival.

MITF plays an important role in the activation of each of the genes to which BRG1 was recruited, but is not the only transcription factor involved. To determine if MITF is required to recruit the BRG1 containing SWI/SNF complexes to these promoters, we down-regulated MITF expression with siRNA (supple. Fig. 2A and B), and performed ChIP analysis to detect BRG1. We found that down-regulation of MITF partially inhibited BRG1 recruitment to all MITF target promoters (Fig. 4C). This suggested that an important mechanism by which MITF activates gene expression is by the recruitment of BRG1 based SWI/SNF complexes to target promoters.

To determine the consequences of BRG1 recruitment to MITF target promoters, we performed ChIP analysis to detect changes in histone modifications and RNA polymerase II recruitment. BRG1 recruitment to target promoters correlated with increased levels of histone 3 tri-methylation at lysine 4 (tri-MeH3K4), an epigenetic mark associated with actively transcribed promoters and/or increased recruitment of RNA polymerase II

phosphorylated at S2 (Fig. 4D). Thus, BRG1 recruitment to MITF target promoters led to changes in promoter structure and function indicative of increased transcriptional activity.

Ectopic expression of BRG1 resulted in increased tyrosinase, TRP1, DCT, RAB27A, p21CIP1, and MLIAP mRNA expression but did not have significant effects on the expression of other MITF target genes that promote proliferation (Fig.4E). Thus, for the selected MITF target genes, recruitment of BRG1 to MITF target promoters primarily led to an increase in the expression of differentiation related genes as well as to increased expression of MLIAP, but not to other MITF target genes that promote proliferation.

BRM interacts with MITF and is required for MITF target gene expression

Although ectopic expression of BRG1 promoted expression of MITF target genes associated with differentiation, these genes are not completely silenced in BRG1 deficient SK-MEL5 cells. To determine whether SWI/SNF complexes containing BRM also cooperate with MITF and contribute to the regulation of MITF target gene expression, we investigated whether BRM interacts with MITF. Fig. 5A shows that BRM and other SWI/SNF subunits can be co-immunoprecipitated with MITF in SK-MEL5 cells.

BRM and the associated subunit, BAF57, were enriched at several MITF target promoters. Interestingly, while ectopically expressed BRG1 was not recruited to the CDK2 promoter, we detected significant levels of BRM, and while BRM was not significantly enriched on the p21 promoter, we detected significant levels of BRG1 (compare Fig.4A and 4B with Fig. 5B and 5C). Down-regulation of MITF with siRNA (Supple. Fig. 2A and B) also partially inhibited the recruitment of BRM to its set of MITF target promoters (Fig. 5D), indicating that MITF contributes to the recruitment of BRM to the MITF target promoters examined, including CDK2. Therefore, heterogeneous

SWI/SNF complexes containing either BRG1 or BRM may cooperate with MITF to regulate overlapping and distinct subsets of MITF target genes.

To determine whether SWI/SNF complexes containing BRM are required for expression of MITF target genes, we down-regulated BRM expression by retroviral transfer of small interfering RNAs (shRNAs) that target a sequence common in both BRG1 and BRM (Ramirez-Carrozzi, et al., 2006). Introduction of these shRNAs significantly down-regulated BRM expression in SK-MEL5 cells while BRG1 levels remained undetectable (Fig. 6A). We found that in the absence of BRG1, down-regulation of BRM significantly inhibited the expression of multiple MITF target genes (Fig. 6B). Interestingly, while over-expression of BRM in the absence of BRG1 did not activate pigmentation related gene expression, down-regulation of BRM inhibited expression of these genes. Thus, although BRM cannot compensate for BRG1, BRM is also important for expression of pigmentation related genes. The expression of MITF target genes that regulate survival and proliferation was also inhibited in BRM knockdown cells, including genes that were not affected by over-expression of BRG1. Although we did not detect significant enrichment of BRM on the TBX2 and BCL2 promoters, we found that down-regulation of BRM inhibited expression of these genes. Thus, the association of BRM with the TBX2 and BCL2 promoters may be transient or BRM may be indirectly required for expression of these genes. Together, these data suggest that either or both the SWI/SNF ATPases are required directly or indirectly for expression of multiple classes of MITF target genes.

We previously reported that BRG1 and other SWI/SNF subunits interact with MITF and can be detected at MITF target promoters in B16 mouse melanoma cells (de la Serna, Ohkawa, et al., 2006a). To confirm that both BRG1 and BRM are required for

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expression of MITF target genes in these melanoma cells, we down-regulated both BRG1 and BRM in B16 mouse melanoma cells and found that the expression of multiple MITF target genes was also inhibited (supple. Fig.3). Thus, SWI/SNF activity is required for expression of MITF target genes in multiple melanoma cell lines.

While expression of BRG1 activated p21CIP1 expression, down-regulation of BRM resulted in a slight increase in p21CIP1 mRNA. A significant stimulatory effect on p21CIP1 expression was noted when BRG1 and BRM were down-regulated in B16 mouse melanoma cells (supple. Fig.2). Regulation of p21CIP1 expression is complex and dependent on multiple transcription factors, including p53 (Gartel & Tyner, 1999). Further work will be required to determine the precise role that SWI/SNF enzymes play in regulating p21CIP1 expression in melanoma cells.

We found that all the melanoma cells we assayed retained expression of either BRG1 or BRM and expressed high levels of several other SWI/SNF subunits. Furthermore, down-regulation of SWI/SNF activity by depletion of the sole ATPase, BRM, in SK-MEL5 cells and down-regulation of both ATPases in mouse melanoma cells, significantly inhibited expression of MITF target genes that promote proliferation and survival but not p21CIP1, an inhibitor of proliferation. Based on these data and the known role of MITF in promoting melanoma proliferation and the ability to grow on soft agar (Garraway, et al., 2005), we hypothesized that retention of functional SWI/SNF enzymes with at least one ATPase would also be required for melanoma tumorigenicity. Consistent with this hypothesis, we found that down-regulation of the sole ATPase, BRM, in BRG1 deficient cells significantly inhibited their ability to grow in soft agar (Fig.6C and D). Therefore, similar to MITF, a critical level of SWI/SNF activity may be required to sustain aspects of melanoma tumorigenicity.

Discussion

MITF is the master regulator of melanocyte differentiation and is also a lineage addiction oncogene in melanoma (Levy, Khaled, & Fisher, 2006). Because we previously found that SWI/SNF enzymes can interact with MITF to promote melanocyte differentiation, we investigated whether SWI/SNF enzymes regulate multiple classes of MITF target genes that have been shown to be important for melanoma oncogenicity. In this study, we have characterized SWI/SNF subunit expression in established melanoma cell lines and have determined that the BRG1 or BRM ATPAse is downregulated in a subset of these cells. We found that expression of BRG1 in BRG1 deficient melanoma cells promoted melanoma differentiation and visible pigmentation. BRG1 interacted with MITF, was recruited to MITF target promoters that regulate differentiation, as well as to the promoter of the melanoma inhibitor of apoptosis (MLIAP). Furthermore, BRG1 enhanced expression of these genes. These data reveal that SWI/SNF enzymes promote expression of MITF target genes important for pigmentation and survival in human melanoma cells.

The role that SWI/SNF enzymes play in regulating the status of melanoma differentiation may have a direct bearing on melanoma therapeutics. Melanomas are notoriously resistant to cytotoxic drugs and several studies suggest that melanoma cells possess lineage specific mechanisms for drug resistance. For example, the melanocytic enzyme, DCT, confers resistance to DNA damaging agents and melanosomes contribute to resistance by sequestering cytotoxic drugs and by increasing drug export (K. G. Chen, et al., 2006; Chu, et al., 2000; Pak, et al., 2000). Furthermore, MITF promotes expression of survival genes that also contribute to chemoresistance (Garraway, et al., 2005).

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Consistent with our hypothesis that SWI/SNF enzymes promote pigmentation and MITF target gene expression, we found that BRG1 also increased the resistance of melanoma cells to cisplatin. In other cell types, SWI/SNF enzymes have also been shown to facilitate DNA repair and to protect cells against UV induced DNA damage (Gong, et al., 2008; Gong, et al., 2006; Park, et al., 2006). Thus, SWI/SNF enzymes may regulate melanoma chemoresistance by multiple mechanisms.

In addition to promoting differentiation, MITF activates expression of pro-proliferative genes such as TBX2 and CDK2, and is critically important for melanoma proliferation (Carreira, et al., 2000; Du, et al., 2004). In melanocytic cells, terminal differentiation is associated with activation of p21CIP1 and p16INK4A expression and cell cycle arrest (Carreira, et al., 2005; Loercher, et al., 2005). Thus, MITF's role in promoting both differentiation and proliferation is contradictory. Although the mechanisms have not been clearly elucidated, it has been proposed that low levels of MITF activity promote proliferation while higher levels of MITF activity are required for terminal differentiation (Carreira, et al., 2006). Consistent with this model and with our hypothesis that SWI/SNF enzymes are important regulators of MITF activity, we found that higher levels of SWI/SNF activity (achieved by the expression of BRG1 in deficient melanoma cells) promoted melanoma differentiation as well as high levels of p21CIP1 expression. A lower level of SWI/SNF activity that included one functional ATPase was necessary and sufficient to promote optimal expression of proliferation related genes. Because BRM did not compensate for BRG1 in promoting melanoma differentiation and because BRG1 did not promote pro-proliferative gene expression, an intriguing possibility is that heterogeneous SWI/SNF complexes composed of a specific ATPase regulate the different classes of MITF target genes. However, additional studies

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investigating the role of BRG1 in BRM deficient melanoma cells will be required to determine if the two ATPases play differential roles in regulating the different classes of MITF target genes or whether the observed results are due to alterations in total SWI/SNF activity.

Like the androgen receptor (AR) in prostate cancer, MITF has been classified as a lineage addiction oncogene that can activate survival and pro-proliferative gene expression (Garraway & Sellers, 2006). AR has been shown to recruit the SWI/SNF complex to transactivate target genes that promote proliferation and tumorigenicity (Link, et al., 2005). Disruption of this interaction inhibits androgen dependent prostate cancer cell proliferation (Link, et al., 2008). Similar to prostate cancer, we find that in melanoma, although SWI/SNF activity may be down-regulated to some extent by down-regulation of particular subunits, at least one functional SWI/SNF ATPase must be retained to promote aspects of melanoma tumorigenicity.

Materials and Methods

Cell lines

Melanoma cells, SW13, and HeLa cells were from ATCC or from the Yale Cell Culture Core Facility (New Haven, Connecticut). Human melanocytes were from Cascade Biologics (Portland, Oregon) or Yale Cell Culture Core Facility. 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).

siRNA and shRNA

Control siRNA and siRNAs targeting MITF were purchased from Dharmacon Inc. (Chicago, Illinois). Control and shRNA constructs targeting BRG1/BRM were provided by Stephen Smale (Howard Hughes Medical Institute, UCLA). The knockdowns are described in supplemental methods.

Antibodies

Antibodies used for Westerns and chromatin immunoprecipitations are described in supplemental methods

Immunocytochemistry

Immunocytochemistry was performed as described (de la Serna, Ohkawa, et al., 2006a) using M2 FLAG antibody (Sigma) and goat anti-mouse-FITC (sc3699) (Santa Cruz).

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Images were taken with a Nikon Eclipse T2000-U fluorescence microscope at 40X magnification.

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).

Co-immunoprecipitations

Co-immunoprecipitations were performed as described (de la Serna, Ohkawa, et al., 2006a) with FLAG M2-Agarose, MITF (C5) hybridoma supernatant, or BRM (rabbit) antibody. Species matched IgG (Upstate, Billerica, MA) was used as a control.

Chromatin immunoprecipitations

Chromatin immunoprecipitations were performed as described (de la Serna, et al., 2005) with the modifications described in Supplemental Methods. The purified DNA was amplified and analyzed by real-time PCR or run on an agarose gel, stained with ethidium bromide, and scanned with the Alpha Innotech FluorChem HD2 imaging system. Primers used for amplification are listed in Supplemental Methods.

RNA Isolation and Quantitative Real-time PCR

Total RNA was isolated with the Qiagen RNeasy mini kit and reverse transcribed as described (de la Serna, Roy, Carlson, & Imbalzano, 2001). 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. Human 18S rRNA gene was used as a control. Primer sequences are available upon request.

Cisplatin sensitivity and cell viability assay

SK-MEL-5 cells, SK-MEL-5 cells with empty vector (pBABE), and SK-MEL-5 cells with BRG1 (pBABE-BRG1) were seeded at a density of 6000 cells per well in a 96-well plate. After 24 h, cells were treated with 20 μM cisplatin for 72 h. Subsequent to drug treatment, cell viability was analyzed using the CellTiter 96® Aq$_{ueous}$ One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin).

Soft Agar Colony Formation Assay

Each well in a 6-well plate was coated with 1 mL of 10% FBS supplemented media containing 0.6% agarose. 5000 cells/well SK-MEL-5 cells expressing shBRG1/BRM or shLuciferase were plated in triplicate in DMEM containing 0.3% agarose, overlayed onto 0.6% agarose. Cells were incubated for 21 days, stained with 10mg/ml thiazolyl blue, photographed and scored. For each cell type, ten fields were counted and averaged.

Supplemental Methods

Antibodies

Antisera to BRG1 (de La Serna, et al., 2000), INI1 (de La Serna, et al., 2000), and BAF57 (Wang, et al., 2005) were previously described. BRM, BAF60c, and RNA Polymerase II (phosphoserine 2) antibodies were from Abcam (Cambridge, MA). BAF 155, BAF250A, Tyrosinase, TRP1, and ERK antibodies were from Santa Cruz (Santa Cruz, California). The triMeH3K4 antibyody was from Active Motif (Carlsbad, California). The Tubulin antibody was from Sigma (St. Louis, Missouri). The BAF250B antibody was from Bethyl Laboratories (Montgomery Texas). FLAG M2 antibody and FLAG M2-Agarose were from Sigma. MITF (C5) mouse hybridoma supernatant was provided by David Fisher (Dana Farber).

Retroviral Production and Infection

293GPG packaging cells were transfected with Empty Vector (pBABE), FLAG tagged pBABE-BRG1 or FLAG tagged pBABE-BRM constructs (Sif, Saurin, Imbalzano, & Kingston, 2001) using LipofectAmine 2000 Plus (Invitrogen, Carlsbad, California). Two rounds of infection were performed. Twenty-four h after the second infection, cells were selected with puromycin.

Olignonucleotides encoding siRNA hairpin sequences targeting BRG1 and BRM (Ramirez-Carrozzi, et al., 2006) or luciferase (Lu, et al., 2007) were subcloned downstream of the H1 promoter in the pQsupR vector (Brummelkamp, Bernards, &

Agami, 2002). Melanoma cells were infected with shRNA constructs and selected (Ramirez-Carrozzi, et al., 2006).

Chromatin Immunoprecipitations (ChIPs)

After formaldehyde crosslinking, cells were washed with PBS and nuclei isolated in a buffer containing 50mM HEPES-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP40, 0.25% TritonX, and protease inhibitors. Nuclei were washed with a buffer containing 10mM Tris-HCl, pH 8.0, 200mM NaCl, 1mM EDTA, 0.5mM EGTA and protease inhibitors. Lysis and immunoprecipitations were carried out in a buffer containing 10mM Tris-HCl, pH 8.0. 100mM NaCl, 9mM EDTA, 0.5mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauryolsarcosine and 1.1% Triton X-100. Complexes were washed five times in RIPA buffer (50 mM HEPES, 500 mM LiCl, 0.1 mM EDTA, 1.0% NP-40 and 0.7% Na-Deoxycholate) and once with TE containing 50mM NaCl. The immune complexes were eluted with 50 mM Tris [pH 8.0], 10 mM EDTA and 1.0% SDS.

siRNA mediated downregulation of MITF

Acell SMART Pool siRNAs targeting MITF (E-008674-00-0050) and red non-targeting siRNAs D-001960-0150) were purchased from Dharmacon Inc. (Chicago, Il.). SK-MEL5 and SK-MEL5+ BRG1 cells were transfected according to the manufacturer's intstructions and harvested for chromatin immunoprecipitations 96 hours later.

Primers used in ChIP Assays

hTyrosinase
F 5'-CAGTTCCTGCAGACCTTGTG-3'
R 5'-CAGGAACCTCTGCCTGAAAG-3'

hTRP1
F 5'-TTCTCTTGCCTGGTTGCTCT-3'
R 5'-TGTGGATTGCTGCCTGATAA-3'

hDCT
F 5'-CCTGACCATTCTCCAGAAGC-3'
R 5'-CAGCACATCTACGCAGCAAT-3'

hRAB27A
F 5'-TTAATAAAGCCGCCCTCTGA-3'
R 5'-GAAAAGCAGTTCGCTCCATC-3'

hp21CIP1
F 5'-TTTCCCTGGAGATCAGGTTG-3'
R 5'-AGAAGCACCTGGAGCACCTA-3'

hMLIAP
F 5'-CCTCTCTGCCTGTTCTGGAC-3'
R 5'-CTCCAGGGAAAACCCACTTT-3'

hCDK2
F 5'-TGAGCATCCCACCACTAG-3'
R 5'-GGCTGGAGAGATAGGCAGAG-3'

hCD25
F 5'-TTCTTGGTAAGAAGCCGGGAAC-3'
R 5'-TCCTCTTCAACGGCGAAATTGC-3'

BCL2 (spanning a region 152 bp upstream of the transcriptional start site) and TBX2

primers (spanning 162 bp ustream of the transcriptional start site) were purchased from

*SA*Biosciences (Federick, MD).

Conflict of interest:

The authors declare no conflict of interest.

Figure Legends

Figure. 1.

A. Detection by Western blotting of SWI/SNF subunit expression in melanocytes from two different donors, eight human melanoma cell lines, SW13, and HeLa cells. Tubulin is a loading control. B. Detection of BRG1 and BRM mRNA levels by real time RT-PCR. mRNA levels were standardized to 18S rRNA and expressed as values relative to those in human melanocytes.

Figure 2.

A. Detection by Western blotting of ectopically expressed FLAG-BRG1 protein in BRG1 deficient SK-MEL5, SK-MEL5+empty vector (EV), or SK-MEL5+BRG1. FLAG-tagged BRG1 was detected with an antibody to the FLAG epitope and an antibody to BRG1. BRM levels in BRG1 deficient and BRG1 expressing cells were also probed. Tubulin is a loading control. B. Detection of ectopically expressed FLAG-BRG1 and BRM mRNA levels by real-time RT-PCR. BRG1 and BRM mRNA levels were standardized to 18S rRNA and are shown relative to those in melanocytes. C. Detection of FLAG-BRG1 by immunocytochemistry using a FLAG antibody. D. Co-immunoprecipitation of SWI/SNF components and MITF in BRG1 deficient and BRG1 expressing SK-MEL5 cells. Top panel: Cell lysates were immunoprecipitated with M2-FLAG agarose or control mouse IgG and blotted with the indicated antibodies. Bottom panel: Cell lysates were immunoprecipitated with MITF hybridoma supernatant or control mouse IgG and blotted with the indicated rabbit antibodies.

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Figure 3.

A. SK-MEL5, SK-MEL5+empty vector (EV), SK-MEL5+BRG1 cell pellets. Cells were grown to 90% confluency, pelleted, and photographed. B. Detection by Western blotting of tyrosinase and TRP1 in human melanocytes, SK-MEL5, SK-MEL5+empty vector (EV), and SK-MEL5+BRG1. Tubulin is a loading control. C. Sensitivity of BRG1 deficient and BRG1 expressing melanoma cells to cisplatin. SK-MEL5, SK-MEL5+empty vector (EV), SK-MEL5+BRG1 cells were treated with 20 μM cisplatin for 72 h and assayed. These results were analyzed by Student's *t* test and are representative of two independent experiments performed in triplicate.

Figure 4.

A. Chromatin immunoprecipitations (ChIPs) were performed in BRG1 deficient SK-MEL5+empty vector (EV) or SK-MEL5+BRG1 cells using a FLAG antibody or control IgG, and run on an agarose gel stained with ethidium bromide. 1% input is shown. MITF target promoters were amplified with primers that span the MITF binding site. CD25 is a negative control. B. ChIPs from A were quantified by real-time PCR. The results presented are an average of two or more independent experiments done in triplicate. C. Change in BRG1 recruitment to MITF target promoters in SK-MEL5 +BRG1 cells expressing siRNAs that target MITF compared to SK-MEL5+BRG1 cells expressing a control siRNA. BRG1 recruitment was analyzed by ChIP analysis as in Fig. 4B. The data is the average of two independent experiments performed in triplicate. All values are statistically significant at p<.01. D. Fold increase in trimethylation at histone H3 (K4) and RNA polymerase II (phsophoS2) recruitment at MITF target promoters in

SK-MEL5 expressing BRG1 compared to that in control SK-MEL5 cells. The data is the average of two independent experiments performed in triplicate. E. MITF target gene expression in SK-MEL5+empty vector (EV) and SK-MEL5+BRG1 cells. mRNA levels were quantified by real-time RT-PCR. These results are an average of greater than three independent experiments performed in triplicate.

Figure 5.

A. Co-immunoprecipitation of SWI/SNF components and MITF in BRG1 deficient SK-MEL5 cells. Top panel: Cell lysates were immunoprecipitated with an antibody specific for BRM or with control rabbit IgG and blotted with the indicated antibodies. Bottom panel: Cell lysates were immunoprecipitated with MITF hybridoma supernatant or control mouse IgG and blotted with the indicated rabbit antibodies. B. Chromatin immunoprecipitations (ChIPs) were performed in BRG1 deficient SK-MEL5 cells using antibodies to BRM, BAF57, or control IgG, and run on an agarose gel stained with ethidium bromide. 1% input is shown. MITF target promoters were amplified with primers that span the MITF binding site. CD25 is a negative control. C. ChIPs from B were quantified by real-time PCR. These results are an average of two independent experiments performed in triplicate. D. Change in BRM recruitment to MITF target genes in SK-MEL5 cells expressing siRNAs that target MITF compared to SK-MEL5 cells expressing a control siRNA. These results were analyzed by Student's *t* test and are representative of two independent experiments performed in triplicate. MLIAP was statistically significant at p<.05, all other values are significant at p<.01.

Figure. 6.

A. Detection by Western blotting of BRM and BRG1 in BRG1 deficient SK-MEL5 cells that express shluciferase or shBRG1/BRM. BRG1 expressing cells were included as a positive control for BRG1. Tubulin is a loading control. B. MITF target gene expression in control SK-MEL5 (shLuciferase) cells and in cells expressing shBRG1/BRM. RNA levels were quantified by real-time RT-PCR. The results presented are an average of greater than three independent experiments done in triplicate and analyzed statistically by Student's t test. shBRG1/BRM had a significant effect on all MITF target genes tested except for p21CIP1. C. The effect of BRG1/BRM down-regulation on the ability to grow in soft agar. These results were analyzed by Student's *t* test and are representative of two independent experiments performed in triplicate.

D. Quantification of control and BRG1/BRM down-regulated cells on melanoma growth in soft agar. These results were analyzed by Student's *t* test and are representative of two independent experiments performed in triplicate.

Supplemental Figure 1.

A. Detection of FLAG-BRM in SK-MEL 5 cells with an antibody to the FLAG epitope and with an antibody to BRM. BRG1 was also assayed but undetected in deficient SK-MEL5 cells. Tubulin is a loading control. B. Detection of BRM over-expression by real-time RT-PCR. C. Detection of FLAG-BRM by immunocytochemistry using a FLAG antibody. D. A. SK-MEL5+ empty vector (EV), SK-MEL5+BRM, SK-MEL5+BRG1 cell pellets. Cells were grown to 90% confluency, pelleted, and photographed. E. MITF target gene expression in SK-MEL5+empty vector (EV) and SK-MEL5+BRM cells. mRNA levels were quantified by real-time RT-PCR. The results presented are an average of greater than three independent experiments performed in triplicate.

Supplemental Figure 2.

SK-MEL 5 +BRG1 and SK-MEL5 cells were transfected with control RNA (conjugated with a red fluorescent label, DY547) and a pool of siRNAs that target MITF (Dharmacon Inc.) Cells were harvested for ChIPs 96 hours later. Cells transfected with the control labeled siRNAs were visualized by A. phase-contrast microscopy or B. fluorescence microscopy and C. Western blots showing MITF levels in control and MITF depleted SK-MEL5+BRG1 and SK-MEL5 cells. Total ERK is shown as a loading control.

Supplemental Figure 3.

A. Western blot showing down-regulation of BRG1 and BRM (shBRG1/BRM) in mouse B16 cells. shLuciferase was used as a control. B. MITF target gene expression in control (shLuciferase) and BRG1/BRM down-regulated (shBRG1/BRM) B16 melanoma cells. mRNA levels were quantified by real-time RT-PCR. The results presented are an average of greater than three independent experiments performed in triplicate.

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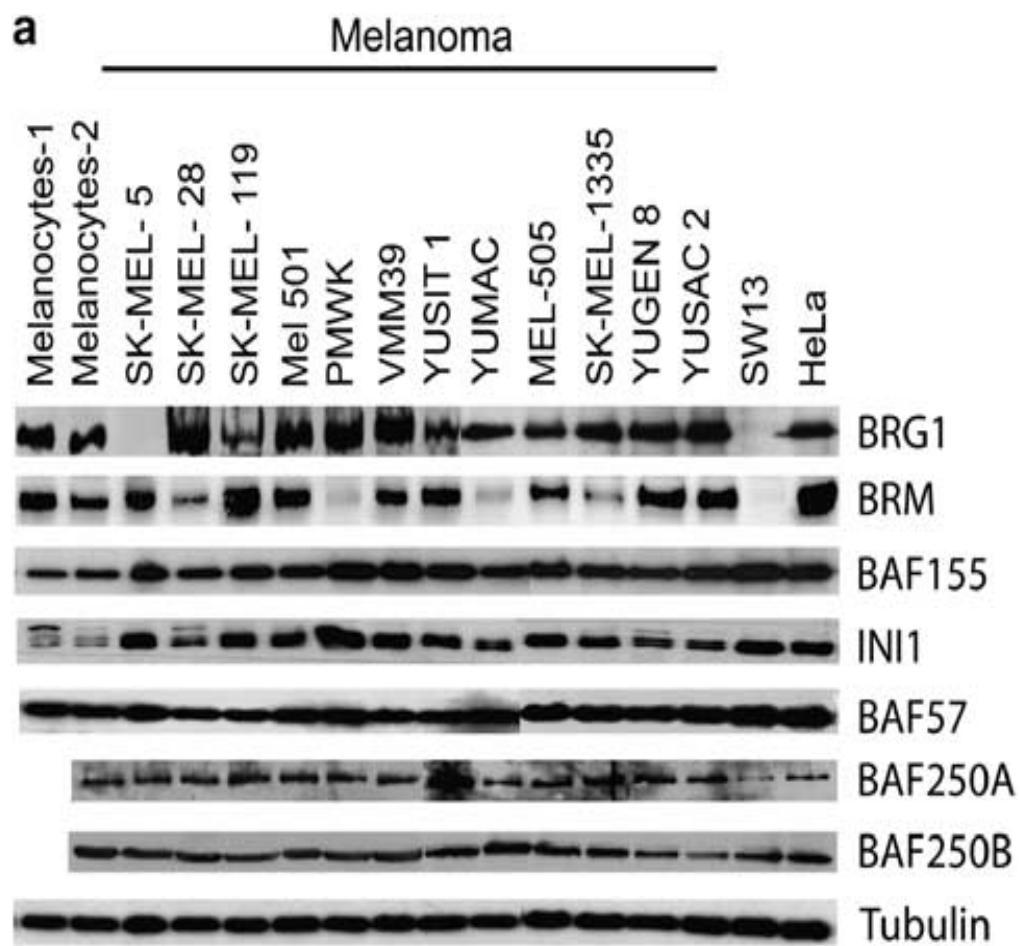
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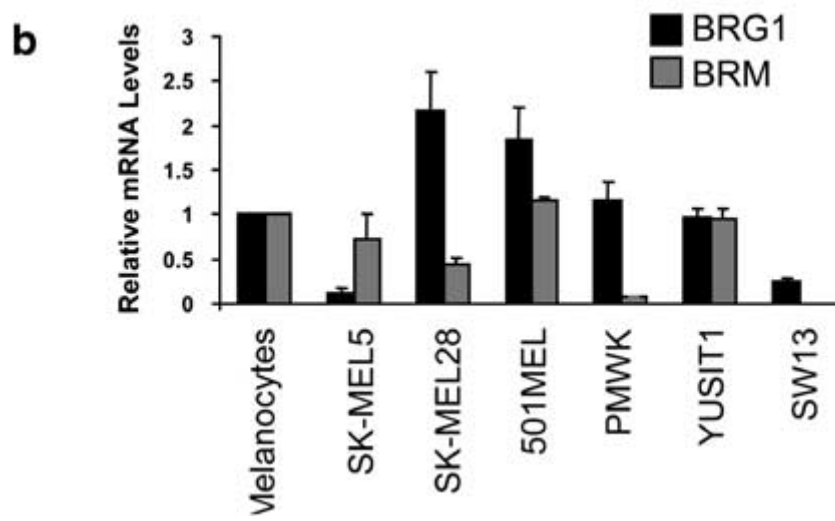
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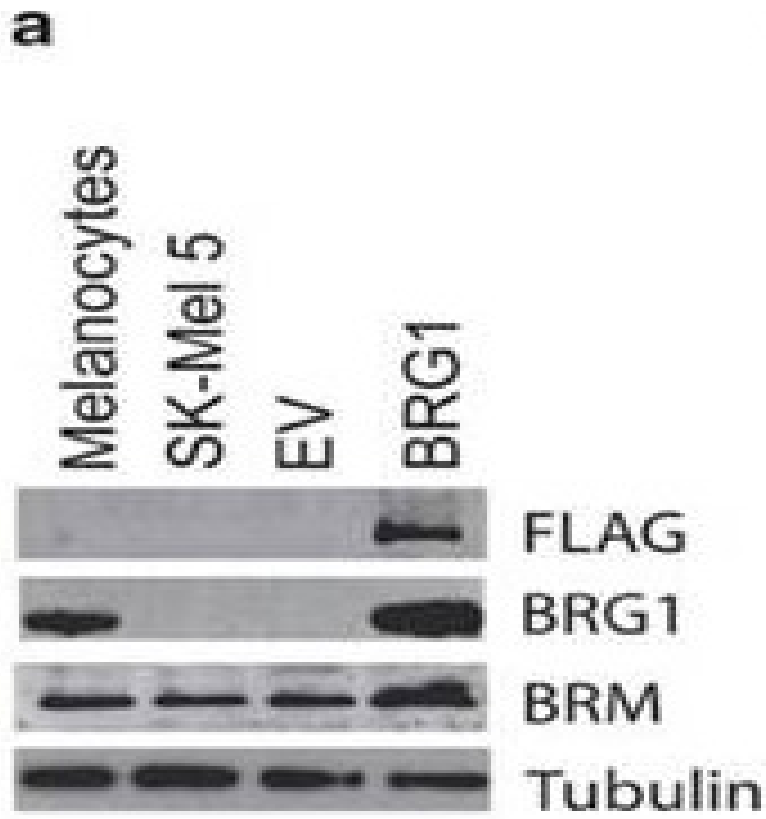
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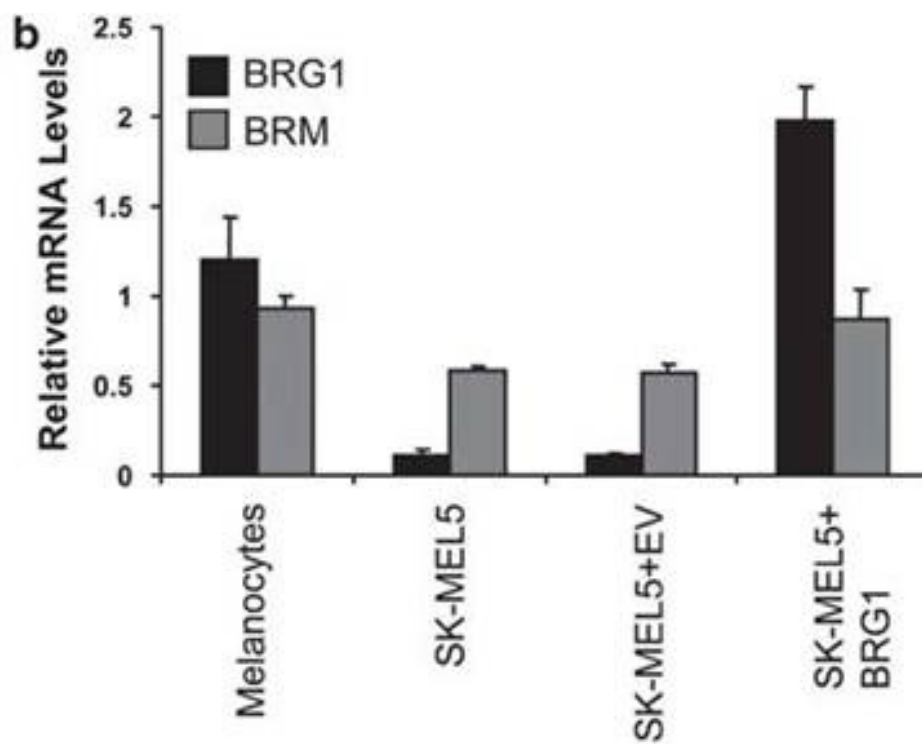
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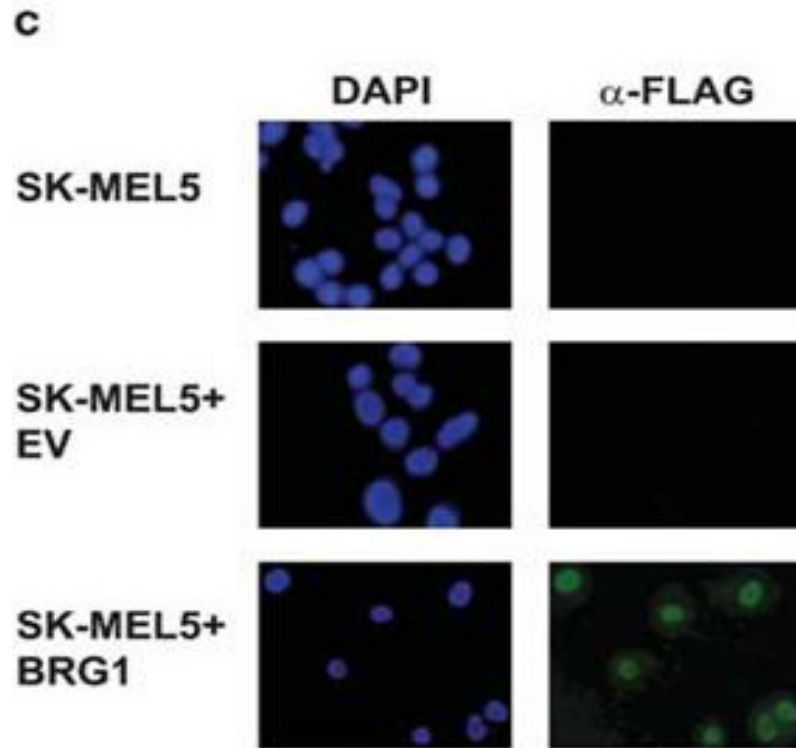
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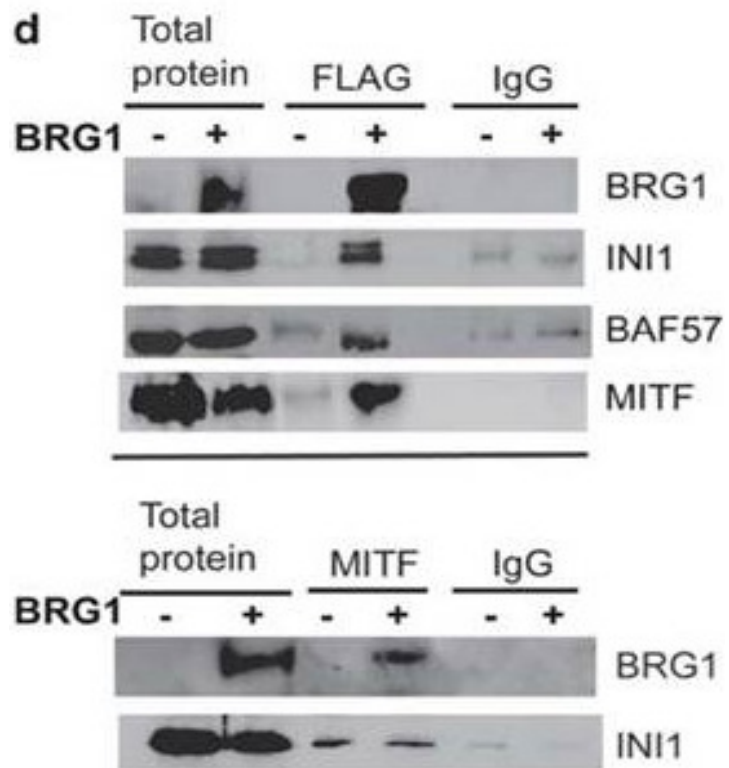
Figure 3

a

SK-MEL5   SK-MEL5+   SK-MEL5+
EV   BRG1
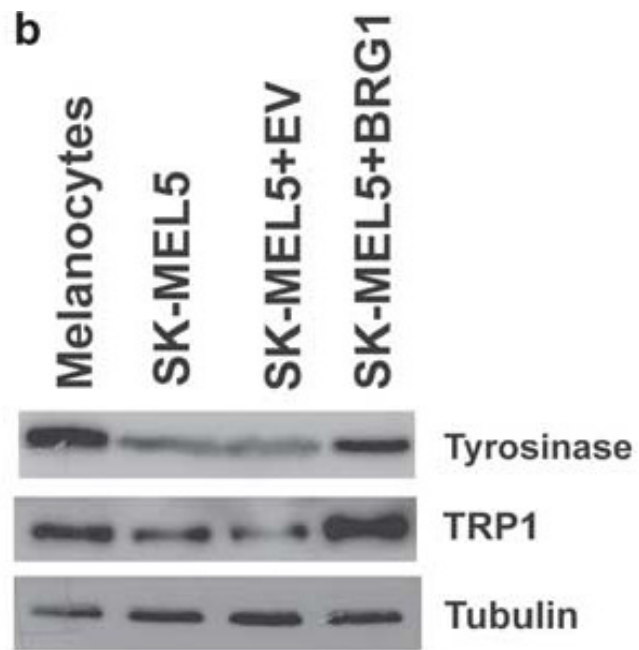
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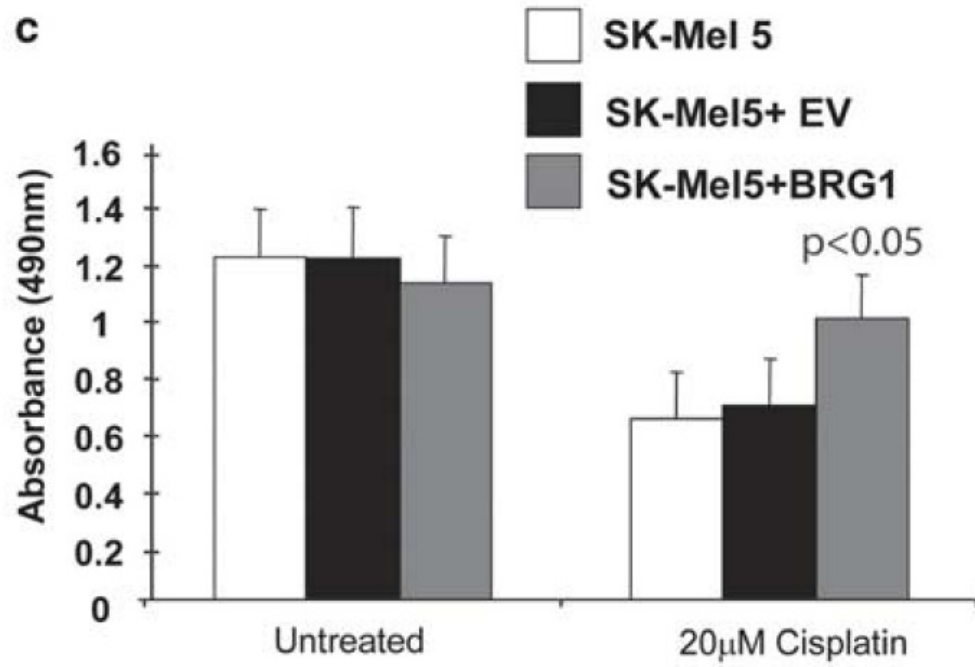
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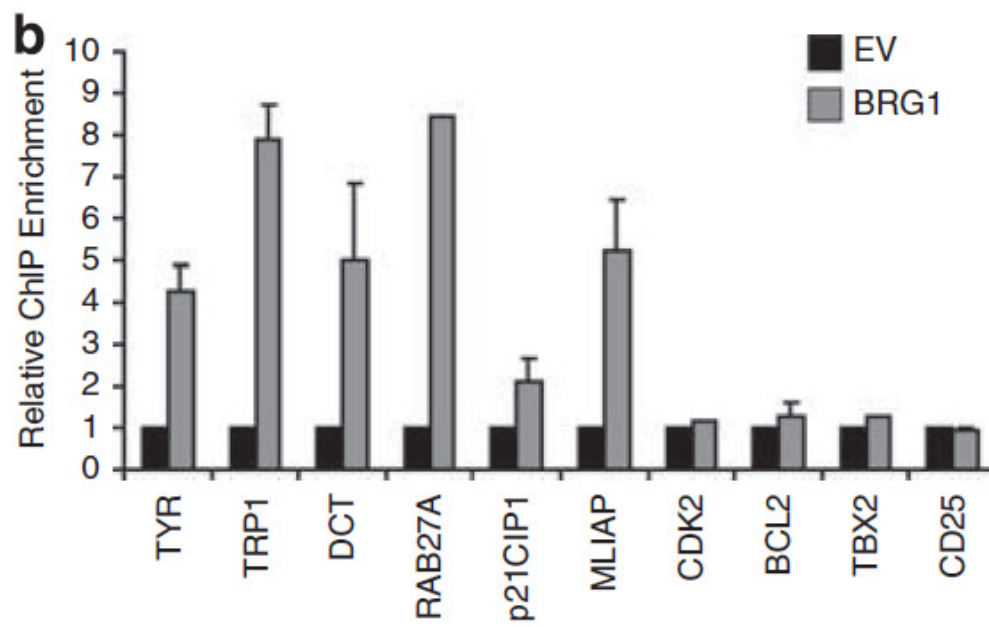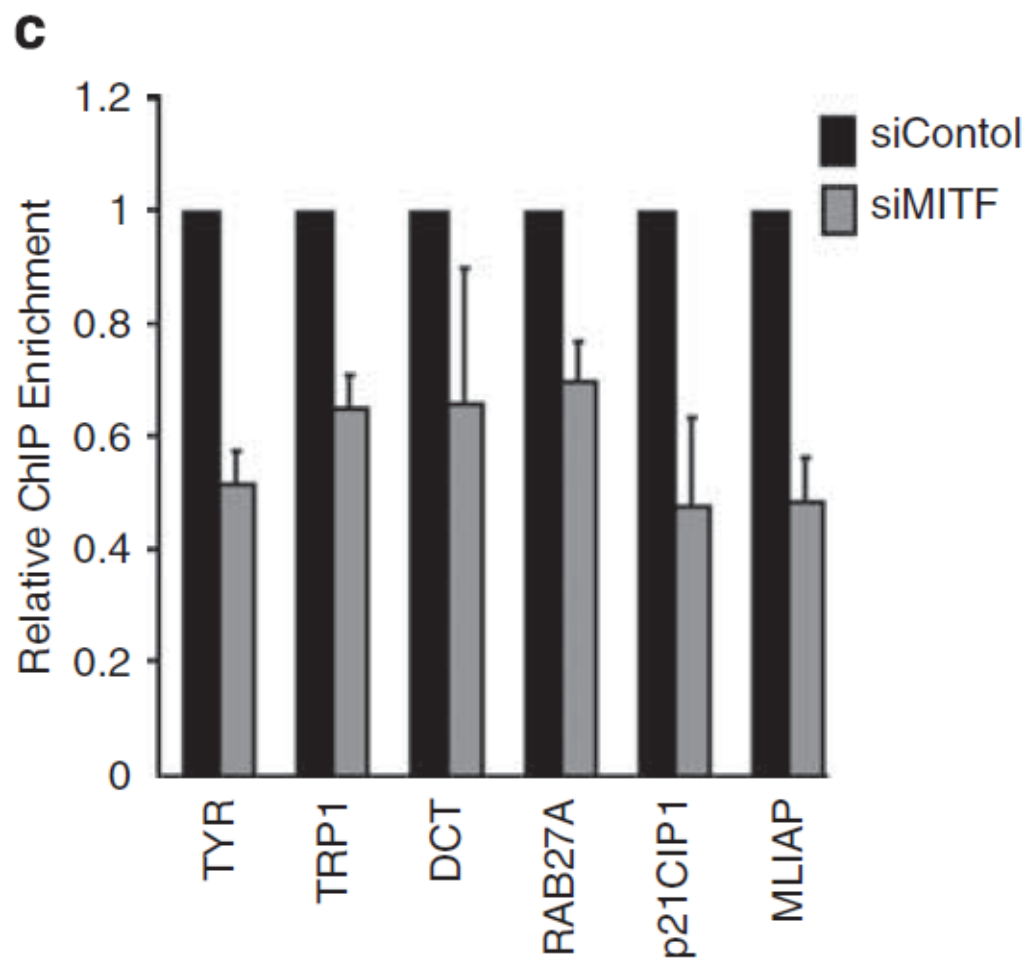
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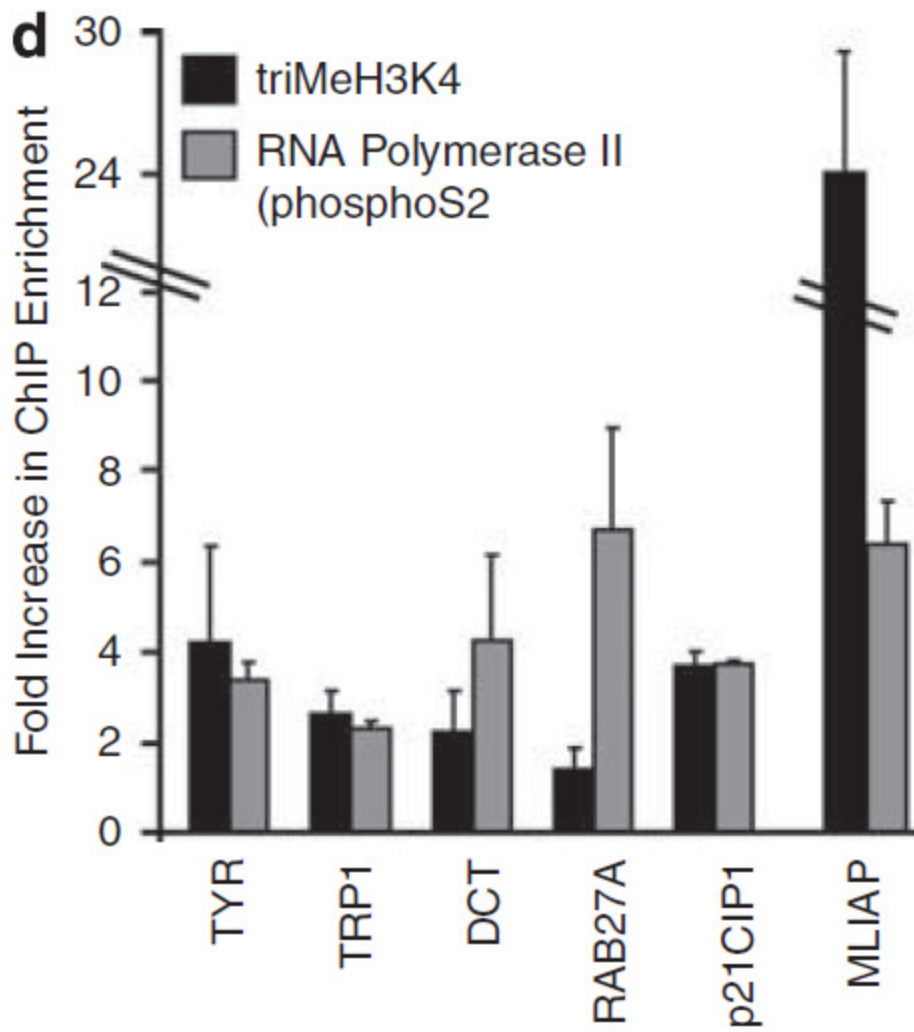
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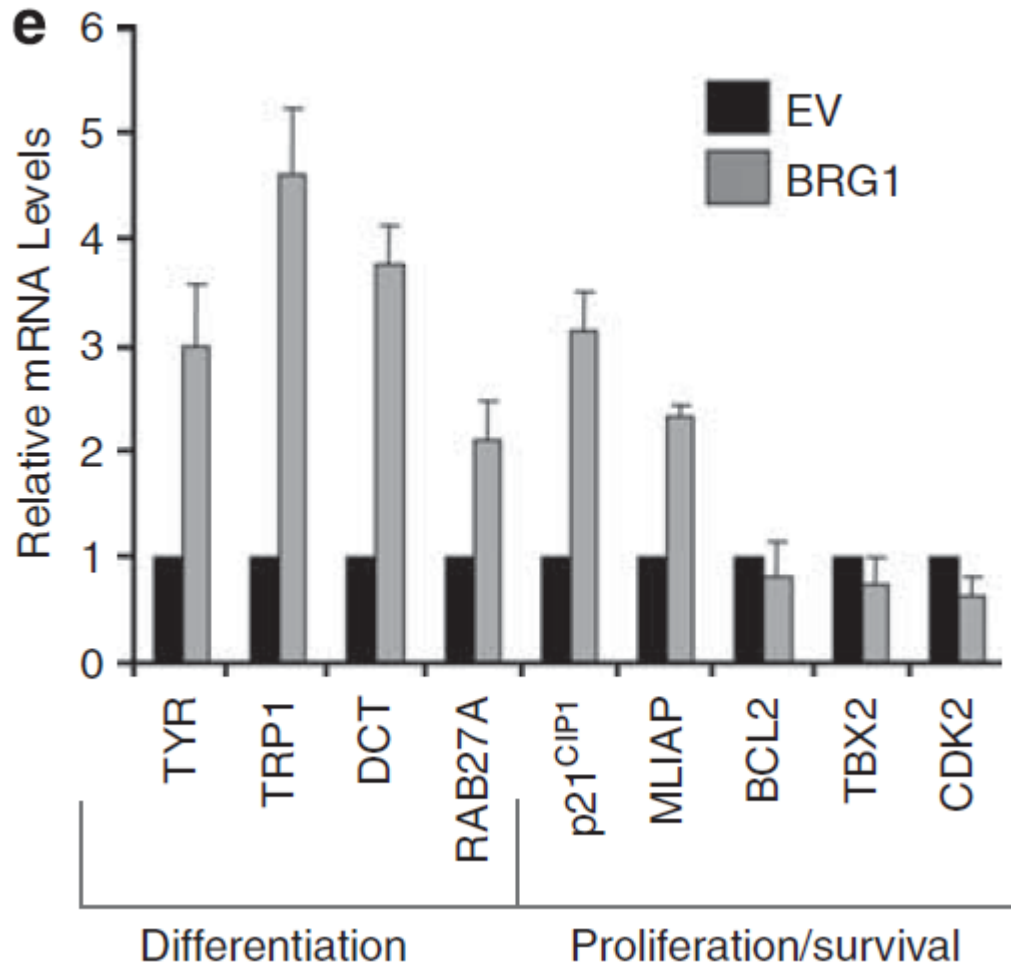
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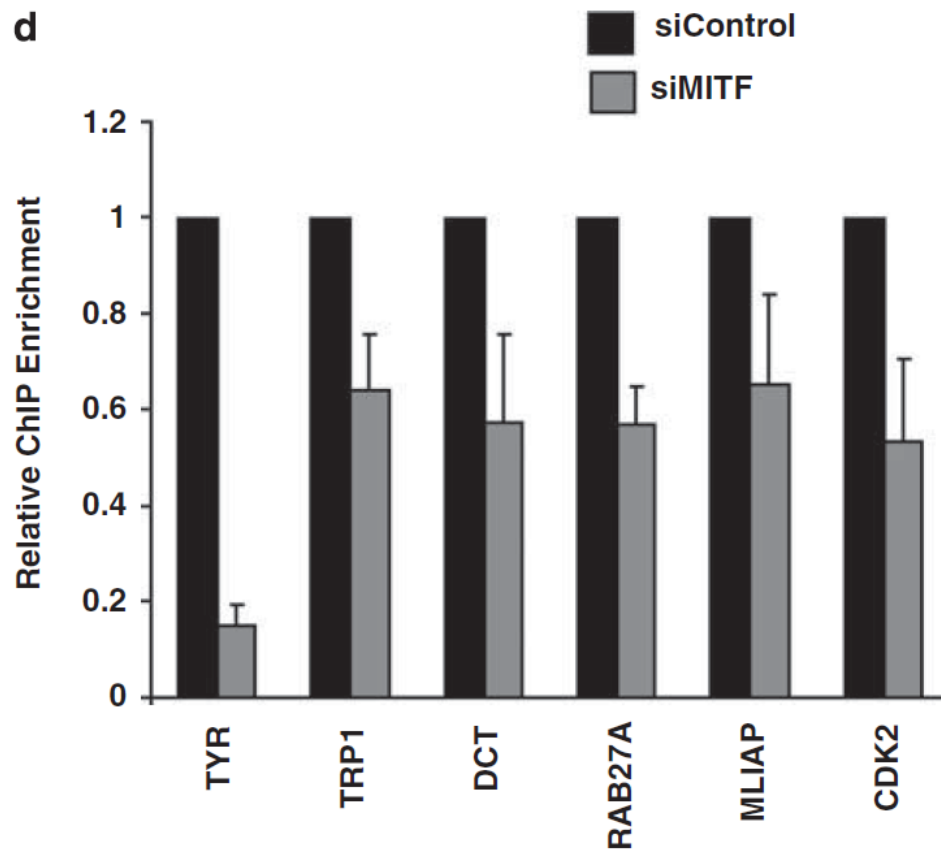
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Figure 5
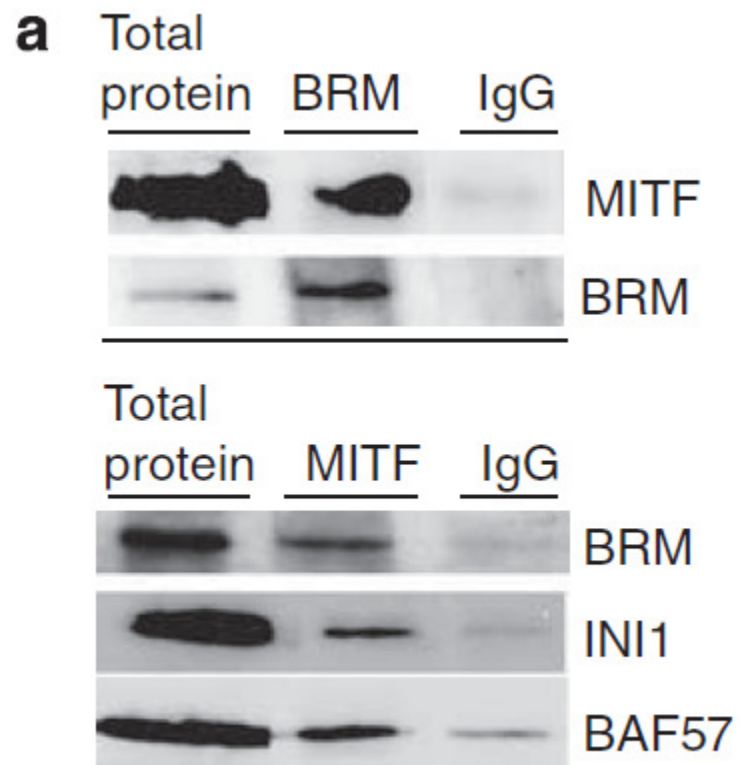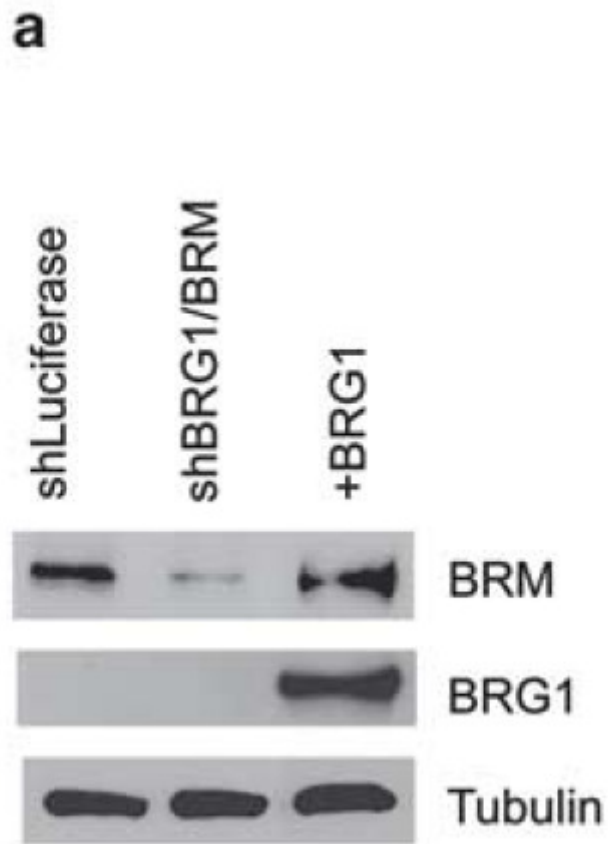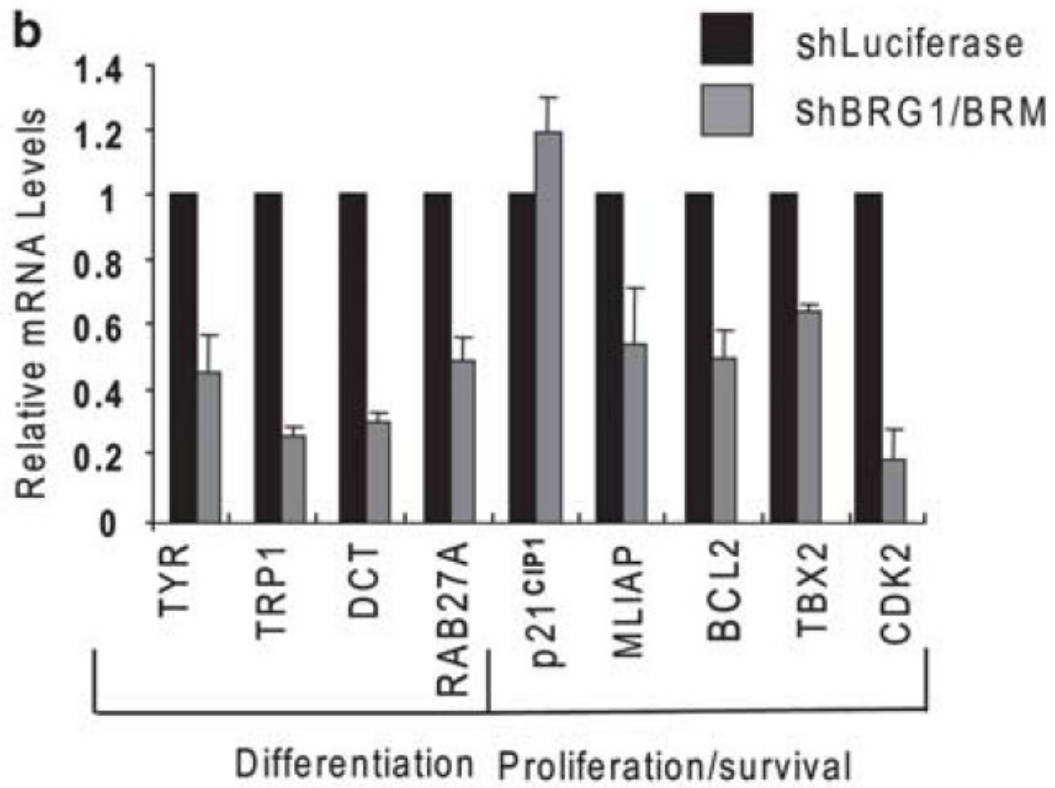
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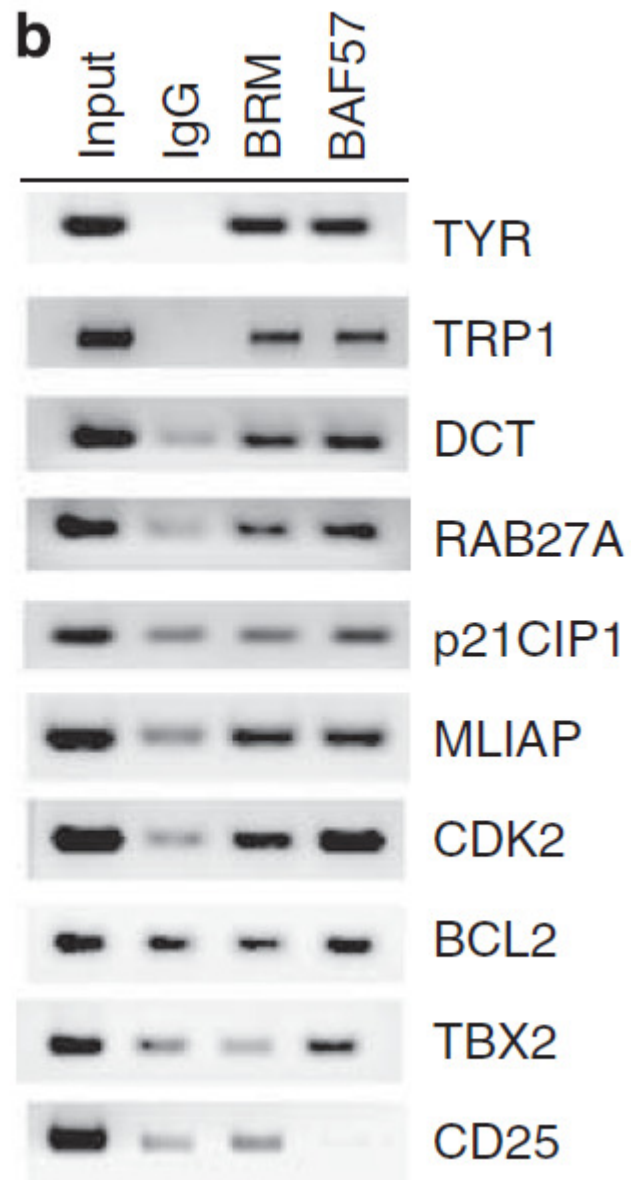
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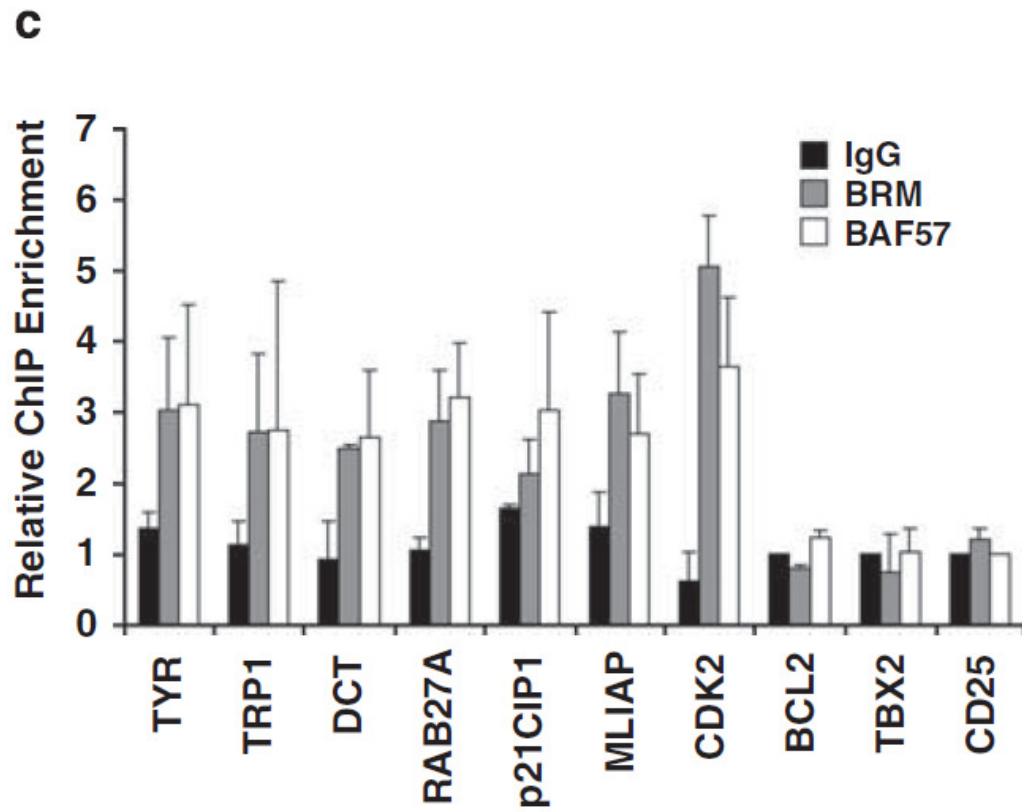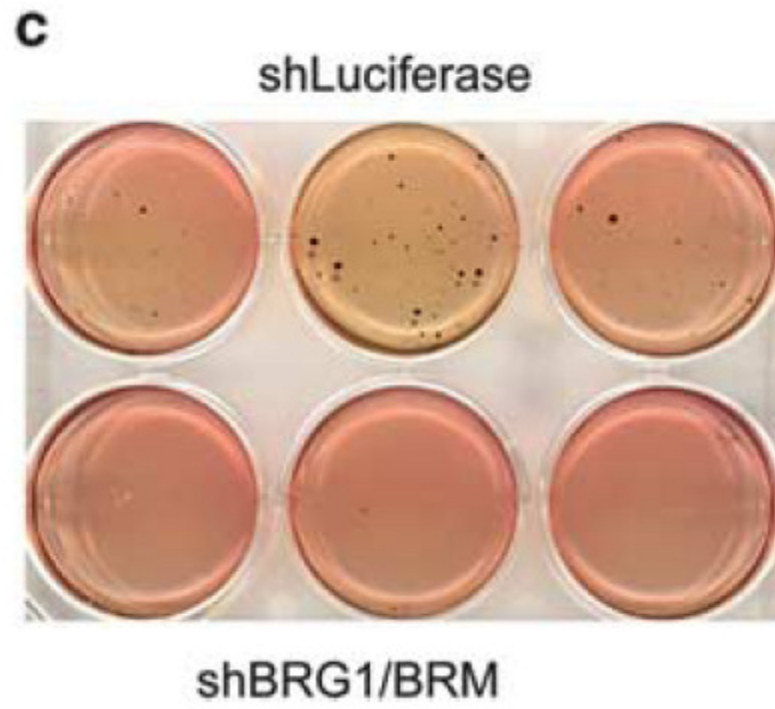
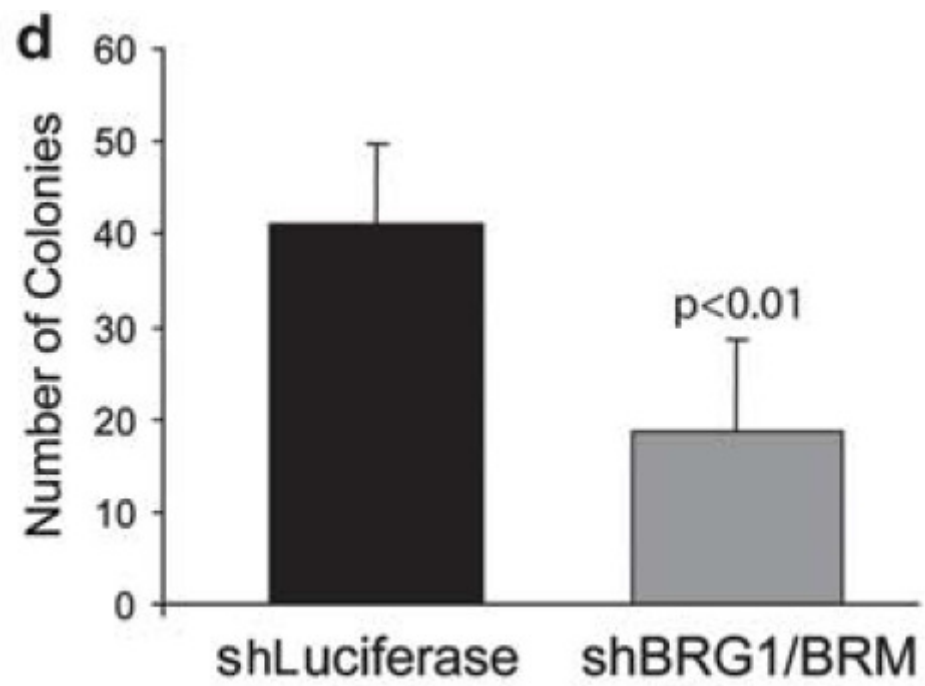**Figure 5**

Figure 6

This experiment was done by Huiling Qi.

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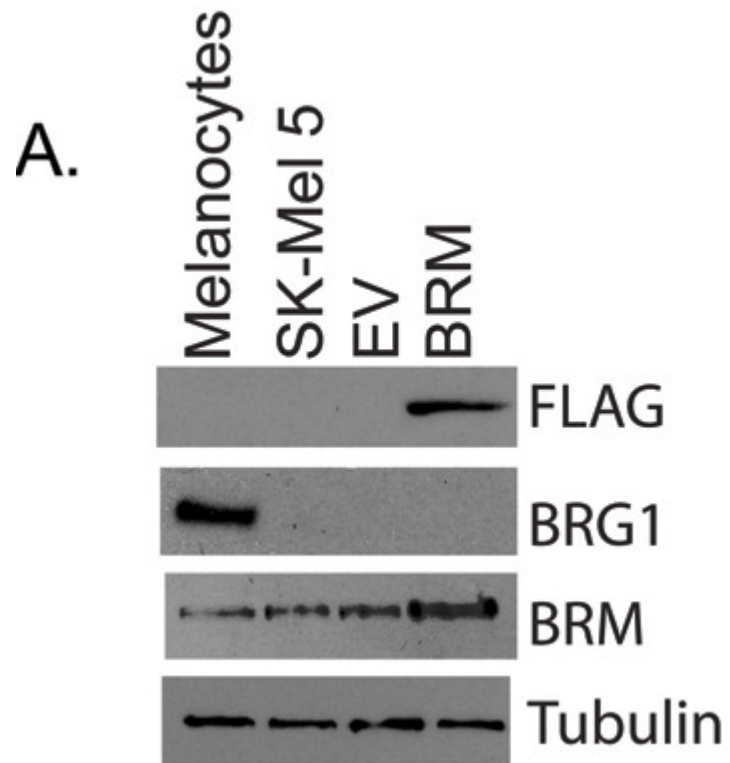**Figure 6**

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Supplemental Figure 1

Supplemental Figure 1
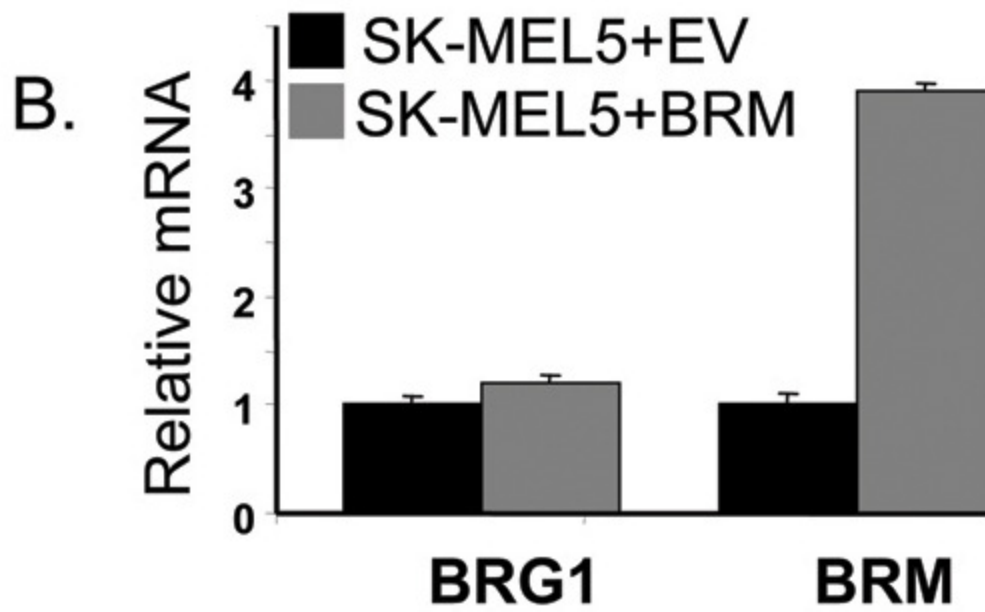
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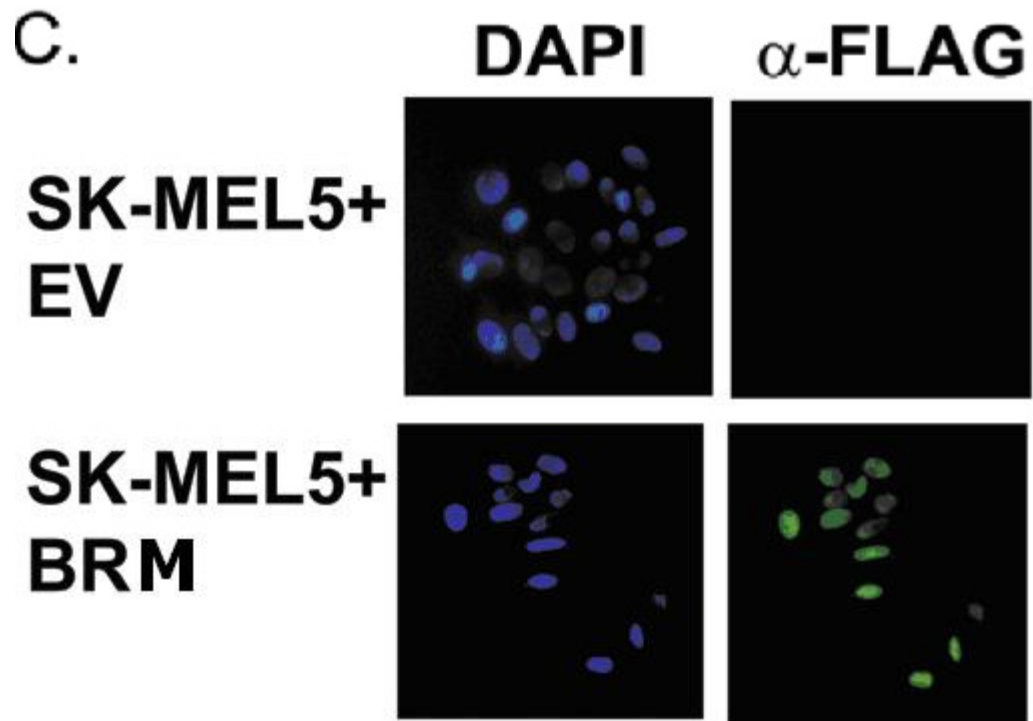
Supplemental Figure 1

D.

+EV   +BRM +BRG1

Supplemental Figure 1

Supplemental Figure 2

A.

SK-MEL5 +BRG1

SK-MEL5

Supplemental Figure 2

B.

SK-MEL5 +BRG1   SK-MEL5

Supplemental Figure 3

A.

This experiment was done by Huiling Qi.

Supplemental Figure 3

B.
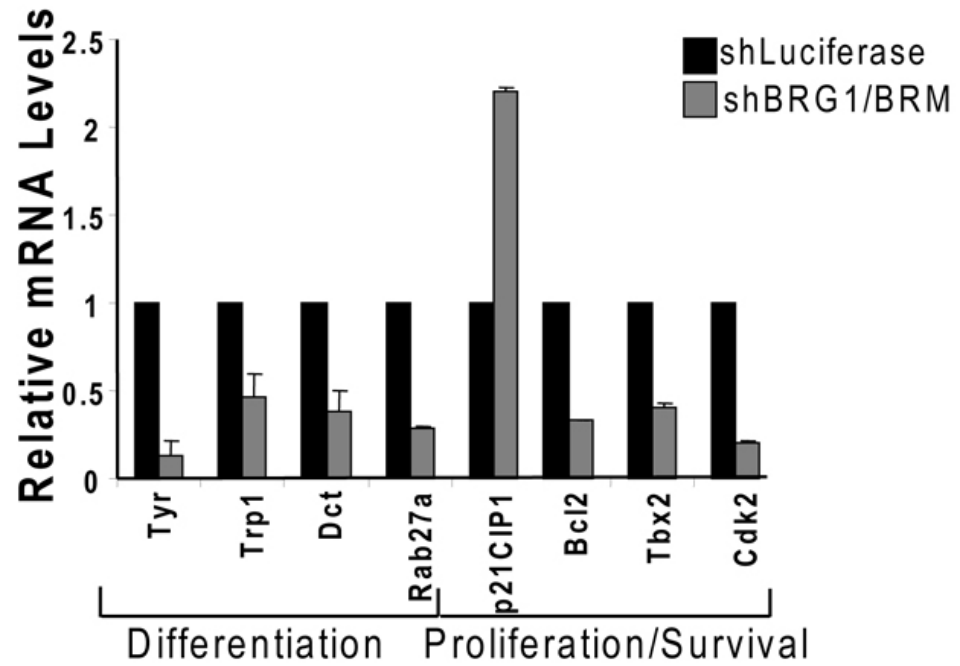
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Abstract

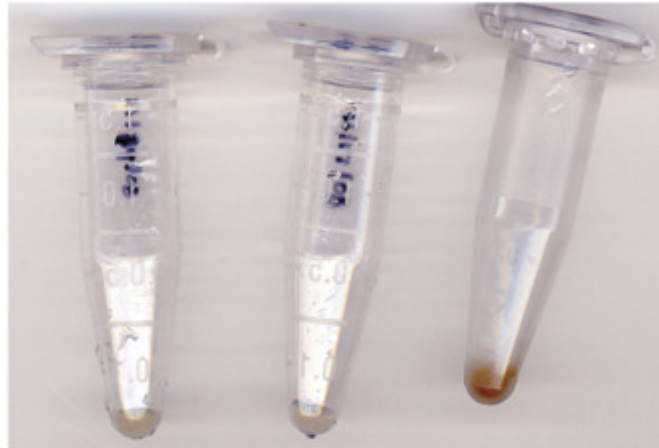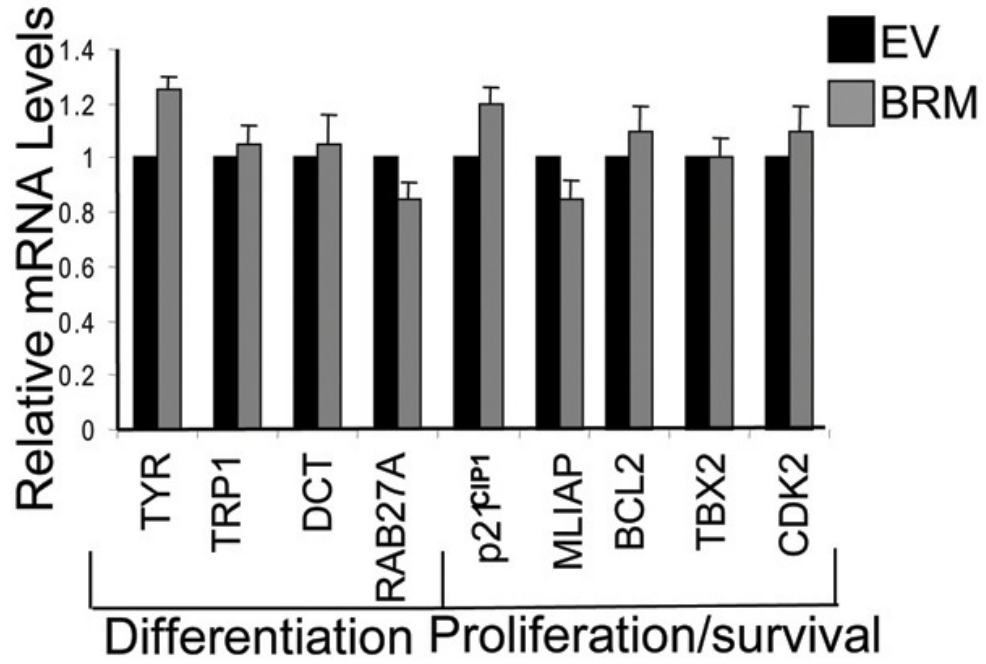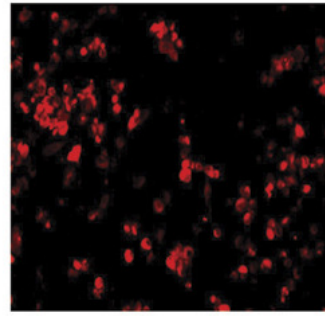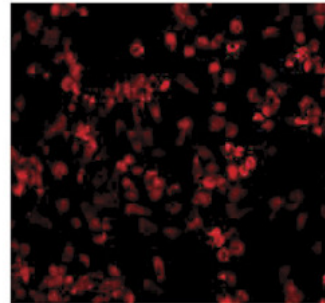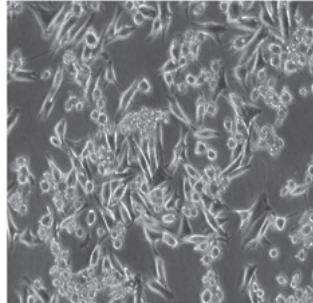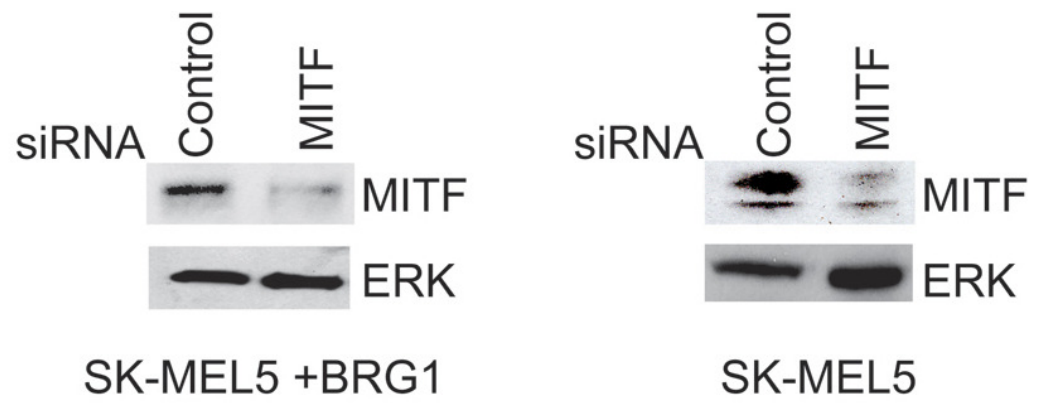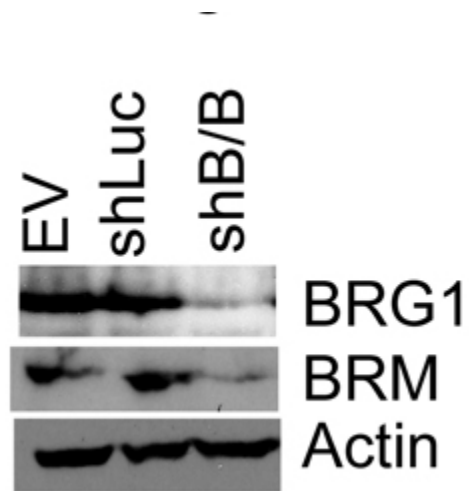As a model system to investigate the role of SWI/SNF chromatin remodeling enzymes in melanoma we used the human melanoma cell line, SK-MEL5. The SK-MEL5 cell line expresses very low levels of the BRG1 ATPase subunit of the SWI/SNF complex. We ectopically expressed BRG1 in this cell line to investigate the effect of BRG1 expression and activity in melanoma. We investigated the effect of ectopic expression of BRG1 in the SK-MEL5 cell line on the expression of many adhesion molecules and extracellular matrix proteins. Ectopic expression of BRG1 in this cell line resulted in altered expression of multiple genes known to be important for metastasis of cancer, specifically in melanoma. The expression of a number of matrix metalloproteinases (MMPs) was increased when BRG1 was ectopically expression in SK-MEL5 cells. MMPs participate in many physiological events that require ECM breakdown and modification, such as, cell migration, proliferation, morphogenesis, and apoptosis. MMPs also participate in pathological processes, such as cancer growth, metastasis, and invasion. The ectopic expression of BRM in the SK-MEL5 cell line did not have the same effect on the MMPs that we choose to further investigate, MMP-2 and MMP-9. Similar to many biological systems, it appears that the exact effect of the presence or absence of SWI/SNF and the exact composition of the SWI/SNF complex present in the cells has a variable effect on downstream gene expression events. We have also illustrated an increase in MMP-2 at the protein level and an increase in the activity of MMP-2 when BRG1 is ectopically expressed in the SK-MEL5 cell line. Our findings of BRG1 binding to the MMP-2 promoter in human melanoma cells and increasing the

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invasive phenotype of these cells gives insight into the possible role of SWI/SNF in melanoma.

Chapter 2

The Role of SWI/SNF in Matrix Metalloproteinase-2 Expression, Activity and Cellular Invasion in Melanoma

2.1 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases that degrade the extracellular matrix (ECM). The extracellular matrix is composed of a complex mixture of insoluble molecules some of which include: collagens, laminins and fibronectins (Kalluri, 2003). There are more than 25 MMPs that have been identified and they are categorized based on substrate specificity, domain organization and sequence similarities. There are six main groups of MMPs that have been defined: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs (Visse & Nagase, 2003).

Matrix metalloproteinases are activated during embryogenesis and required for wound healing/tissue repair, bone and cartilage remodeling, corneal repair and inflammation (McCawley & Matrisian, 2001; Woessner, 1991). MMPs participate in many physiological events that require ECM breakdown and modification, such as, cell migration, proliferation, morphogenesis, and apoptosis (Mannello, 2006; Page-McCaw, Ewald, & Werb, 2007; Vu & Werb, 2000). MMPs also participate in pathological processes, such as cancer growth, metastasis, and invasion (Corbitt, Lin, & Lindsey, 2007; Egeblad & Werb, 2002; Mannello, Tonti, & Papa, 2005).

Matrix metalloproteinases are secreted as inactive enzymes and activated upon cleavage of their activation domain. Post-cleavage the active site is available to bind to different target substrates. In general, MMPs cleave a peptide bond positioned just before a hydrophobic side chain (i.e. leucine or tyrosine) in the target molecule. The hydrophobic residue must fit within the active site of the MMP. The active site is flexible and most MMPs have a wide range of substrates they interact with *in vivo*.

MMP's can be activated in a number of ways. The production and activity of pro-MMPs can be rapidly induced when active tissue remodeling is essential. In some cell types the expression of MMPs is induced in response to exogenous signals, such as growth factors (IL-1, IL-4, and IL-6), transforming growth factors (EGF, HGH, and TGFβ), or tumor necrosis factor alpha (TNFα) (Cawston & Wilson, 2006; Spinale, 2007). Matrix metalloproteinases can be produced through activated endothelial cells, which can be stimulated by the presence of angiogenic growth factors secreted from tissues. Endothelial cells can proliferate into the bordering matrix and produce solid sprouts joining adjacent vessels. As sprouts continue in the direction of the source of the angiogenic stimulus, endothelial cells migrate using integrins, in addition to MMPs degrading the ECM (Inuzuka, et al., 2009). Integrins are composed of an alpha and beta subunit, the cytoplasmic domain of the β subunit is in control of signal transduction (Morgan, Thomas, Russell, Hart, & Marshall, 2004). Changes in expression of both integrins and MMPs assist in cell migration and invasion in cancer metastasis.

To control connective tissue breakdown, the activity of MMPs is regulated by multiple means and their activity is under tight control. Matrix metalloproteinases are initially synthesized as inactive zymogens with a pro-peptide domain that must be

removed before the enzyme is active. MMPs are generally expressed at low levels in latent forms, the latency of the pro-MMP is preserved by the interaction of the conserved cysteine residue in the prodomain with the catalytic zinc in the active center (Cawston & Wilson, 2006). Matrix metalloproteinases can be regulated by native inhibitor proteins found in the ECM and the serum. These physiological inhibitors are called **t**issue **i**nhibitors of **m**etallo**p**roteinases (TIMPs) (Danilewicz, Sikorska, & Wagrowska-Danilewicz, 2003). There is a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMPs bind strongly to active MMPs in a 1:1 ratio and physically bind the active site leading to an inhibition of interaction with substrates (Brew, Dinakarpandian, & Nagase, 2000). The overall shape of the TIMP molecule is similar to a wedge, which fits into the active-site cleft of an MMP in a manner similar to that of the substrate (Fernandez-Catalan, et al., 1998). Increases in the abundance of TIMPs in relation to MMPs can decrease MMP activity and have been illustrated to decrease metastatic capabilities (Li, et al., 2001).

2.2   Gelatinases and Collagenases

Gelatinases are essential in the final degradation of collagens after cleavage by collagenases. The major substrates of gelatinases are type IV collagen and gelatin (hydrolyzed form of collagen), both of which are mainly found in the basal lamina (Allan, et al., 1995). There is a gelatin-binding region positioned immediately before the zinc binding motif in gelatinases. MMP-2 and MMP-9 are both gelatinases and have three repeats of fibronectin-type II domains inserted in their catalytic domain, these regions interact with collagens and gelatins (Allan, et al., 1995; Steffensen, Wallon, & Overall, 1995).

Matrix metalloproteinase-2 has a substrate preference of denatured collagens, types IV and V collagen, elastin, fibronectin, gelatin, laminin, and proteoglycan. As previously mentioned, MMPs can be activated in multiple manners and MMP-2 has been shown to be activated by MMP-14, -16, -17 and -25. Activation of MMP-2 can also occur through PAF-induced CREB phosphorylation which results in overexpression and activation of MMP-2 (Melnikova, Mourad-Zeidan, Lev, & Bar-Eli, 2006).

MMP-2 is produced in a large number of cell types including fibroblasts, keratinocytes, chondrocytes, endothelial cells and in transformed cells (Guo, Zucker, Gordon, Toole, & Biswas, 1997; Romanic & Madri, 1994). MMP-9 is produced by keratinocytes, monocytes, macrophages and neutrophils, and carcinoma cells (Gilles, Polette, Seiki, Birembaut, & Thompson, 1997; Malik, Greenfield, Wahl, & Kiener, 1996; Seltzer, et al., 1994; B. Xie, Laouar, & Huberman, 1998). MMP-2 and MMP-9 have both been found to be important for the metastasis of many different types of cancer including metastatic melanoma.

Collagenases have the ability to disrupt the triple helix structure of collagen and have a high affinity for a specific site ¾ from the N-terminus of interstitial collagen types I, II, and III (Callejas, Casado, Diaz-Guerra, Bosca, & Martin-Sanz, 2001; Qin, Sun, & Benveniste, 1999; Uhm, Dooley, Villemure, & Yong, 1996). Collagenases have a multidomain MMP composition consisting of a signal peptide, propeptide, catalytic domain, hinge region, and hemoplexin domain. The propeptide domain contains a conserved cysteine residue, which forms a covalent bond with the catalytic zinc ion in the catalytic site, preserving the dormant state (Springman, Angleton, Birkedal-Hansen, & Van Wart, 1990). The catalytic domain contains a well conserved zinc binding sequence, critical for the proteolytic activity of MMPs.

Materials and Methods

Invasion Assay

Melanoma cells were grown for 3 days in regular growth media and re-plated with a matrigel insert, along with positive controls that contain no matrigel insert. Invasion chambers were incubated in a humidified tissue culture incubator for 22-48 hours; the cells that did not invade were removed and invading cells fixed and stained. The cells were then scored using an Olympus CKX41 microscope at 40X magnification. Fields were chosen in the center of the membrane as well as the periphery of the membrane for true representation of the cell number across the membrane. Data on cell invasion was obtained from duplicate plates in two independent experiments with approximately 500 cells counted in total.

PCR Array

Real-time PCR reactions were analyzed in total RNA using the Human Extracellular Matrix and Adhesion Molecules RT Profiler PCR Array (SuperArray Biosciences Corporation, USA) according to the manufacturer's protocol. Briefly, cDNA was prepared from 1µg total RNA (as previously described). QPCR reactions were conducted in a 25µL mixture, which included 12.5µL of 2 X QPCR master mix, 11.5µL of nuclease-free H_2O, and 1 µL of template cDNA. QPCR amplification was conducted with an initial 10-min step at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 1

min. Real time quantitations were conducted using the ABI 7500 detection system (Applied Biosystems).

The Human Extracellular Matrix and Adhesion Molecules RT Profiler PCR array profiles 84 key genes important for cell-cell ad cell-matrix interactions. This array contains extracellular matrix (ECM) proteins including collagens, matrix metalloproteinases and genes playing a role in ECM structure. Data were analyzed using the ddCT method of analysis, normalizing data to endogenous controls and the Pbabe empty vector control used in the experiment.

Gel Zymography

Gelatin zymography was performed by electrophoresing samples on a 10% (wt/vol) polyacrylamide gel containing .1% (wt/vol) gelatin. After electrophoresis, the gel containing .1% gelatin was washed twice for 30 min in 2.5% (vol/vol) Triton X-100 at room temperature and then incubated in substrate reaction buffer (50 mM Tris-HCl, 5 mM CaCl$_2$, 0.02% [wt/vol] NaN$_3$, pH 8.0 at 25°C) for 17 h at 37°C with gentle shaking. The gel was then stained with Coomassie Blue R250 in 10% (vol/vol) acetic acid, 30% (vol/vol) methanol for 1 h and destained briefly in the same solution without dye. Proteolytic activity was detected by a clear band indicating the lysis of the substrate.

Results:

One of our first observations when we ectopically expressed FLAG-tagged BRG1 in the SK-MEL5 human melanoma cell line was a change in the morphology of the cells. The altered morphology of the SK-MEL5 human melanoma cell line after retroviral BRG1 expression is illustrated in Figure 7.

To further investigate the effect of BRG1 expression in the SK-MEL5 human melanoma cell line, which innately has very low levels of BRG1 expression, we performed a PCR array for human ECM and adhesion molecules from SuperArray. The results of the RT Profiler PCR Array revealed that ectopic expression of BRG1 in the SK-MEL 5 cell line increased the expression of many molecules known to be important for metastasis of cancer, specifically in melanoma. The results of the array are illustrated in Figure 8. A short list of genes upregulated at the mRNA level in response to ectopic expression of BRG1 in the SK-MEL5 cell line: e-cadherin, catenein-δ2, vascular cellular adhesion molecule 1 (VCAM1), integrin-α-7. Genes found to decrease in expression at the mRNA level in response to ectopic expression of BRG1 in the SK-MEL5 cells were: integrin- α-4, integrin- α-8, integrin-β-4 and MMP-1.

One of the genes greatly up-regulated in response to ectopic expression of BRG1 in SK-MEL5 cells is MMP-2. There are a number of reports in the literature of increased levels of MMP-2 in metastatic cancer when compared to benign and normal tissue.

Figure 7   Morphology of SK-Mel5 human melanoma cells, empty-vector control and ectopically expressed BRG1 cells.

SK-Mel5
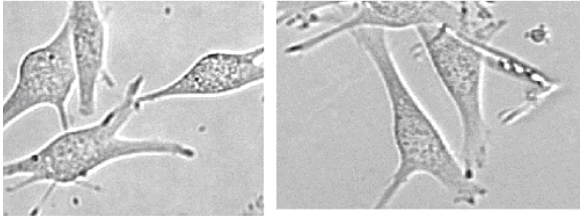
SK-Mel5-empty vector

SK-Mel5-BRG1

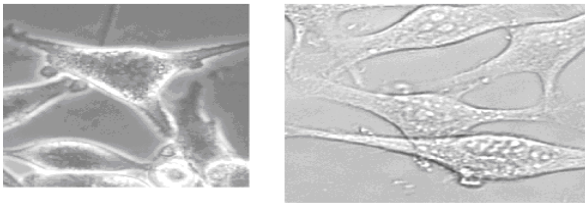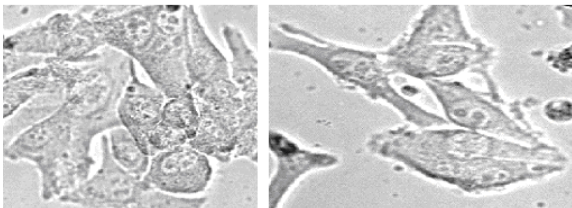To confirm MMP-2 was also elevated at the protein level we performed a western blot, results illustrated in Figure 9. We also investigated the activity of MMP-2 in the SK-MEL5 melanoma cells when BRG1 was ectopically expressed. Matrix metalloproteinase activity is demonstrated by a zymography experiment, results also demonstrated in Figure 9. It is essential to investigate the activity and not only the expression of MMPs when assessing their role in tumorigenesis.

To investigate the effect of increased MMP-2 expression and activity on the cellular phenotype of the SK-MEL5 cells we next investigated the ability of these cells to invade a matrigel matrix. Figure 10 demonstrates the increase in the invasive phenotype in the SK-MEL 5 cells when BRG1 is ectopically expressed in SK-MEL5 cells. The increased ability of the SK-MEL5 melanoma cells to be invasive, along with increased expression and activity of MMP-2 indicate the likelihood that elevated levels of BRG1 expression effect cellular changes that can result in increased metastasis of melanoma cells.

To examine if BRG1 is directly binding the MMP-2 promoter in SK-MEL5 human melanoma cells, we performed ChIP analysis. As demonstrated in figure 11, when BRG1 is ectopically expressed in SK-MEL5 cells BRG1 binds to the MMP-2 promoter, this is not seen in the empty vector control or when BRM is ectopically expressed.

Figure 8 A

Results from Human Extracellular Matrix and Adhesion Molecules PCR
Array: Genes that have Increased Expression when BRG1 is Ectopically
Expressed in SK-MEL5 cells and Normalized to HPRT, RL13A, GAPDH
and b-Actin

CD44	2.86
CDH1 (E-Cadherin)	19.67
CNTN1 (Contactin 1)	4.55
CTNND1 (Catenin-δ2)	11.29
ICAM1 (Intercellular adhesion molecule 1)	1.69
NCAM1 (Neural cell adhesion molecule 1)	1.81
THBS3 (Thrombospondin 3)	1.76
VCAM1	14.4
SELE (Selectin E)	3.33
LAMB1 (Laminin β 1)	1.72
FN1 (Fibronectin 1)	3.4
ITGA3 (Integrin-α-3)	3.13
ITGA7 (Integrin α 7)	6.48
MMP12	3.91
MMP13	16.42
MMP14	3.19
MMP2	12.71
MMP8	2.32
MMP9	3.33
TIMP1	2.15
TIMP2	2.29
TIMP3	2.7
TGFB1	1.61
ADAMTS13	3.35
TNA (Tetranectin)	2.24

Figure 8 B

Results from Human Extracellular Matrix and Adhesion Molecules PCR
Array: Genes that have Decreased Expression when BRG1 is Ectopically
Expressed in SK-MEL5 cells and Normalized to HPRT, RL13A, GAPDH
and b-Actin

ITGA4	0.1
ITGA8	0.19
ITGAL	0.45
ITGB2	0.44
ITGB3	0.36
ITGB4	0.33
ITGB5	0.37
COL12A1	0.28
COL14A1	0.02
COL5A1	0.05
COL6A1	0.22
MMP1	0.1
ADAMTS1	0.2
CTGF (Connective tissue growth factor)	0.47

Figure 8 C

Results from Human Extracellular Matrix and Adhesion Molecules PCR
Array: Genes that do not have a Change in Expression when BRG1 is
Ectopically Expressed in SK-MEL5 cells and Normalized to HPRT, RL13A,
GAPDH and b-Actin

MMP16	1.0
ADAMTS8	1.0
COL15A1	1.0
COL15A1	1.0
COL16A1	1.0
COL1A1	1.0
COL4A2	1.0
COL6A2	1.0
COL7A1	1.0
COL8A1	1.0
CSPG2	1.0
CTNNA1	1.0
CTNNB1	1.0
CTNND1	1.0
ECM1	1.0
HAS1	1.0
ITGA1	1.0
ITGA2	1.0
ITGA5	1.0
ITGA6	1.0
ITGAM	1.0
ITGAV	1.0
ITGB1	1.0
ITGB5	1.0
KAL1	1.0
LAMA1	1.0
LAMA2	1.0
LAMA3	1.0
LAMB3	1.0
LAMC1	1.0
MMP10	1.0
MMP11	1.0
MMP15	1.0

SELP	1.0
SGCE	1.0
SPARC	1.0
SPG7	1.0
SPP1	1.0
THBS1	1.0
THBS2	1.0
TNC	1.0
MMP16	1.0
MMP3	1.0
MMP7	1.0
PECAM1	1.0
SELL	1.0

Figure 8 D

Results from Human Extracellular Matrix and Adhesion Molecules PCR Array: Genes that do not have an a Change in Expression when BRG1 is Ectopically Expressed in SK-MEL5 cells and Normalized to HPRT, RL13A, GAPDH and b-Actin
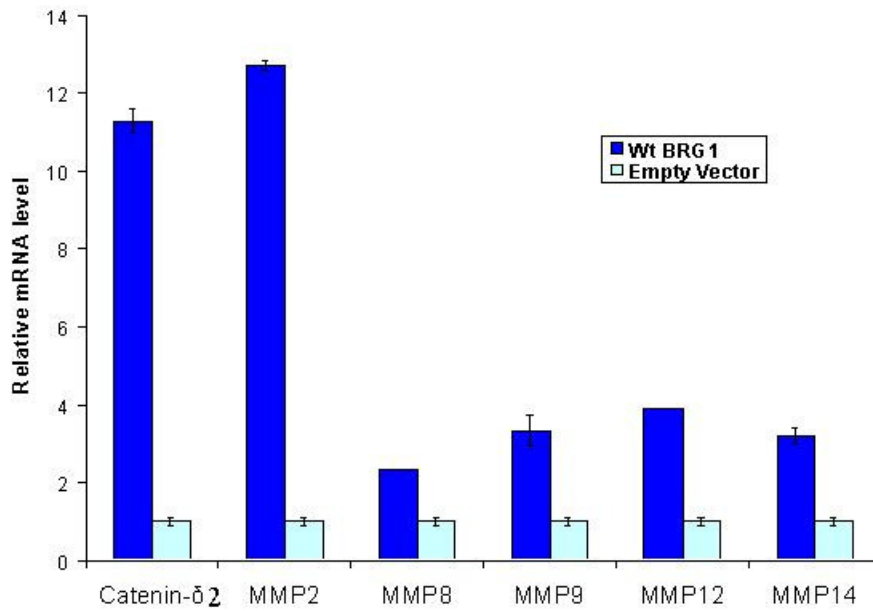
Figure 9   Western blot analysis and zymography results of SK-MEL5 human melanoma cells, empty vector control and ectopically expressed BRG1 cells.
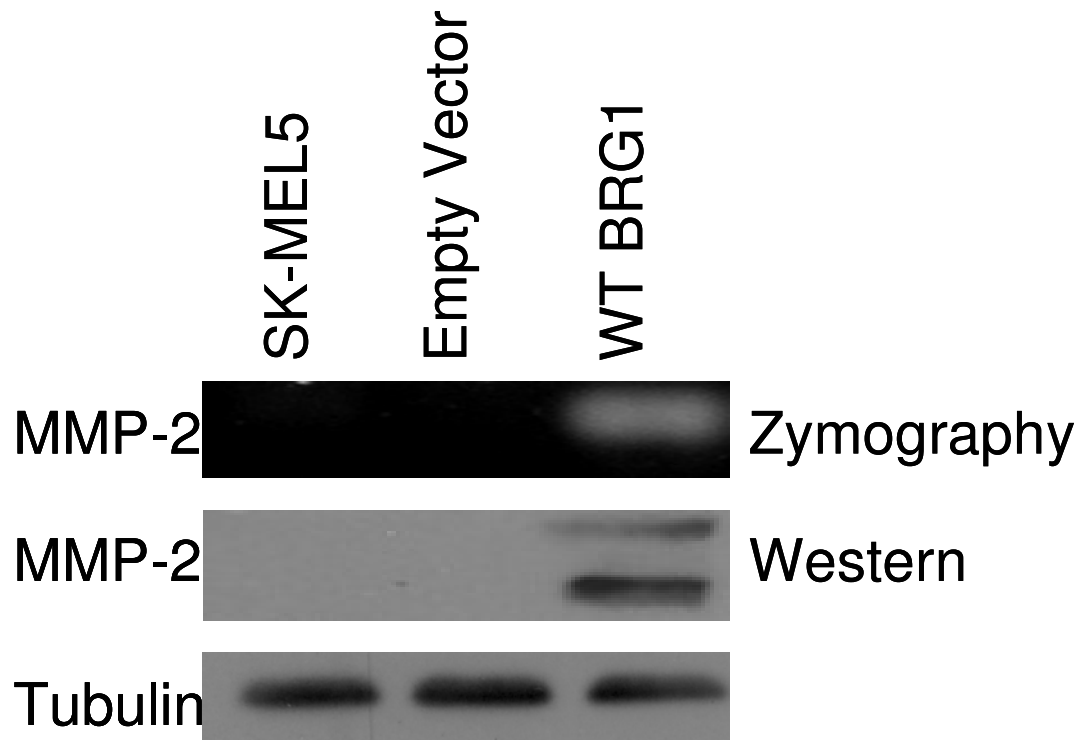
Figure 10   Expression of BRG1 increases the ability of SK-MEL5 cells to invade in a gelatin matrix when compared to empty vector control.

Invasion Assay-SK-Mel 5 melanoma cells
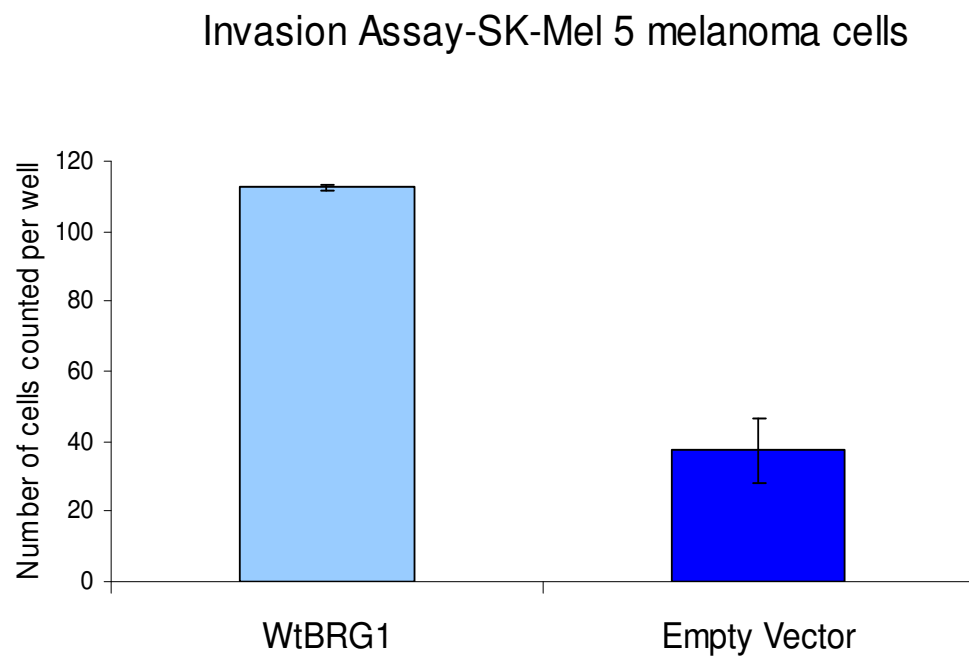
Figure 11   ChIP analysis of ectopically expressed BRG1 and BRM in SK-MEL5 human melanoma cells and binding of BRG1 and BRM to the MMP-2 promoter

Association of BRG1 on the MMP-2 promoter (by ChIP)
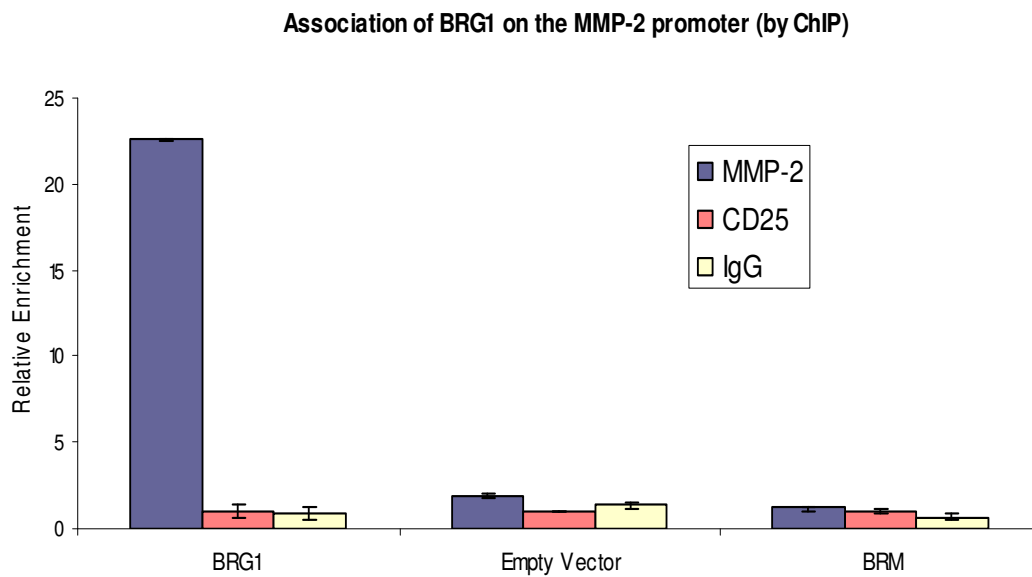
Discussion

Misregulation of chromatin remodeling enzymes results in erroneous gene activation and/or repression events altering the innate homeostatasis of a cell, leading to carcinogenesis. Expression of BRG1, BRM and other components of the SWI/SNF complex have been reported to be misregulated in a broad assortment of human cancers. The exact role of SWI/SNF in cancer appears to be very complex and dependent on multiple factors. Stage/level of progression of cancer, epigenetic factors, tissue of origin, along with possible environmental factors all have an effect on the role SWI/SNF has on the cancer susceptibility/survival outcomes.

Similar to many biological systems, it appears that the exact effect of the presence or absence of SWI/SNF and the exact composition of the SWI/SNF complex present in the cells has a variable effect on downstream gene expression events. Our findings of BRG1 to binding the MMP-2 promoter in human melanoma cells and increasing the invasive phenotype of these cells gives insight into the possible role of SWI/SNF in melanoma.

As shown by our recent publication, BRG1 and BRM do not always compensate for each others' activities. In our model system, investigating the role of SWI/SNF in promoting melanoma differentiation BRM was not able to compensate for BRG1 in its effect on pro-proliferation. It is possible that heterogenous SWI/SNF complexes consisting of a certain ATPase subunit could have a specialized effect on the different classes of MITF targets and classes of transcription factors. The effect of heterogenous SWI/SNF complexes on biological systems could be dependent on many factors such as the cellular environment or dependent on the tissue context.

SWI/SNF is required for expression of a subset of MITF regulated genes (de la Serna, Ohkawa, et al., 2006b). Evidence suggests the activity and expression level of MITF is powerfully influential on the balance between proliferation and differentiation in melanoma. With the substantial number of genes that MITF is known to regulate, differences in expression levels and activity of MITF can lead to effects on numerous signalling pathways and cellular fate. Our data suggests that heterogeneous SWI/SNF complexes composed of either BRG1 or BRM subunit promote expression of distinct and overlapping MITF target genes and that at least one ATPase is required for melanoma tumorigenicity. MITF is further involved in melanoma through its ability to promote expression of survival genes that also contribute to chemoresistance (Garraway et al 2005).

MITF is the master regulator of melanocyte differentiation (Hemesath, et al., 1994). Melanocytes originate in the neural crest and gain the ability to migrate to locations such as the eye, inner ear and some mucosal membranes. Certain transcription factors are required for this enhanced ability of melanocytes to travel during embryogenesis. After embryogenesis the ability of the melanocytes to travel long distances is lost. During embryogenesis melanocytes start off in the neural crest as melanoblasts and travel to their final destination in the epidermis and hair follicles. Throughout carcinogenesis melanocytes again gain the ability to travel long distances and this is thought to be one of the reason why melanoma is so metastatic.

The high levels of morbidity and mortality associated with melanoma are largely due to the ability of melanoma cells to infiltrate the basement membrane, along with proliferating and metastasizing to distant organs (Haass, et al., 2005; Miller & Mihm, 2006). This metastatic phenotype is characterized by alterations in the expression of

cellular adhesion molecules (Miller & Mihm, 2006). Cellular adhesion controls cell migration, tissue organization and organogenesis, disturbances in which contribute to tumor invasion and tumor cell signaling (J. P. Johnson, 1999).

The metastatic capabilities of tumor cells are reliant upon their ability to separate themselves from environmental cues that regulate their ability to migrate and become metastatic. Successful metastasis requires changes in cytoskeletal architecture, expression of surface adhesion molecules and degradation and penetration of the ECM and basement membrane.

Tumors will only metastasize if a number of events occur to allow the metastatic cells the ability to first detach from the primary tumor. Disassociation is the process by which individual tumor cells break away from the primary tumor and infiltrate surrounding stroma. Tumor cells that have successfully entered the vasculature or lymphatic system are carried to distant sites where they can invade target organs.

MMP-2 plays a role in assisting melanoma cells to degrade type IV collagen in the basement membrane, an essential step for tumor cell dissociation and invasion (S. Xie, et al., 1997). There is an epithelial-to-mesenchymal transition (EMT), in which cells gain the ability to become motile, that occurs during cancer metastasis (Hay, 1995; Radisky, 2005).

Inhibition of cell migration is important for inhibiting the progression of cancer in most cases. In melanoma specifically cells will gain the ability to migrate by altering expression of multiple genes that are initially important during embryogenesis. One of the classes of genes that are highly expressed at times of migrations are matrix metalloproteinases. Current evidence strongly implicates SWI/SNF as a key player in melanoma, given the existing interaction between SWI/SNF and MITF and the increase

in MMP-2 activity and expression in response to BRG-1 expression in a melanoma cell line. Taken together, these data demonstrate a critical role for SWI/SNF in melanoma that merits further investigation.

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Conclusions

- BRG1 promotes melanoma differentiation by interacting with MITF.

- BRM can not compensate for BRG1 loss in promoteing melanoma differentiation.

- At least one SWI/SNF ATPase is required for expression of pro-proliferative MITF target genes and for melanoma tumorigenicity.

- MITF is partially required for SWI/SNF recruitment to target promoters, suggesting that other factors may be involved.

- At least one SWI/SNF ATPase is required for expression of pro-proliferative MITF target genes and for melanoma tumorigenicity.

- BRG1 promotes melanoma differentiation by interacting with MITF.
    - BRM can not compensate for BRG1 loss in promoting melanoma differentiation.

- BRG1 promotes activation of MITF target genes including those important for pigmentation, survival and proliferation.

- BRG1 is important for MMP-2 activation & BRM can not compensate for this function.
    - Ectopic Expression of BRG1 results in an increased capacity of the SK-MEL5 human melanoma cells to be invasive *in vitro*.

- Down-regulation of BRG1/BRM in melanoma cells inhibits oncogenicity *in vitro*.

- Over-expression & knock down of SWI/SNF subunits in human melanoma cell lines effects expression of genes involved in melanocyte differentiation, malignant tumor invasion genes & chemosensitivity.

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