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Modulation of BRAHMA expression by the mitogen-activated protein kinase / extracellular signal regulated kinase pathway is associated with changes in melanoma proliferation

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Abstract

Brahma (BRM) and Brahma-related gene 1 (BRG1) are catalytic subunits of SWItch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complexes. BRM is epigenetically silenced in a wide-range of tumors. Mutations in the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) gene occur frequently in melanoma and lead to constitutive activation of the mitogen-activated protein kinase (MAPK) / extracellular signal regulated kinase (ERK1/2) pathway. We tested the hypothesis that BRM expression is modulated by oncogenic BRAF and phosphorylation of ERK1/2 in melanocytes and melanoma cells. Expression of oncogenic BRAF in melanocytes and melanoma cells that are wild-type for BRAF decreased BRM expression and increased BRG1 expression. Inhibition of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) or selective inhibition of BRAF in melanoma cells that harbor oncogenic BRAF increased BRM expression and decreased BRG1 expression. Increased BRM expression was associated with increased histone acetylation on the BRM promoter. Over-expression of BRM in melanoma cells that harbor oncogenic BRAF promoted changes in cell cycle progression and apoptosis consistent with a tumor suppressive role. Upon inhibition of BRAF(V600E) with PLX4032, BRM promoted survival. PLX4032 induced changes in BRM function were correlated with increased acetylation of the BRM protein. This study provides insights into the epigenetic consequences of inhibiting oncogenic BRAF in melanoma through modulation of SWI/SNF subunit expression and function.

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Keywords

SWI/SNF Chromatin remodeling enzymes; BRG1/BRM; melanoma; BRAF(V600E); mitogen-activated protein kinase / extracellular signal regulated kinase (ERK1/2) pathway; vemurafenib

Introduction

The mitogen-activated protein (MAP) kinase / extracellular signal regulated kinase (ERK1/2) pathway regulates cell cycle progression, cellular growth, survival, differentiation, and senescence by responding to extracellular signals. Signal transduction occurs by a cascade of kinase activity that involves the activation of RAS proteins which in turn activate the RAF family of kinases leading to the phosphorylation of the downstream mitogen-activated protein kinase kinase (MEK), and ultimately to the phosphorylation of extracellular signal regulated kinases (ERK1/2) which then phosphorylate many targets that elicit cellular changes, with effects on gene expression [1].

A high percentage of tumors exhibit constitutively high ERK1/2 signaling, most frequently resulting from mutations in rat sarcoma (RAS) genes or the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) gene [2]. Activating mutations in the BRAF gene occur in approximately 50–70% of melanomas, 90% of which have a valine to glutamic acid substitution at position 600 (BRAFV600E), leading to constitutively high ERK1/2 activity [3, 4]. Constitutive activation of the ERK1/2 pathway alters gene expression to promote proliferation and metastasis [5]. Selective inhibition of oncogenic BRAF(V600E) with vemurafenib (PLX4032) suppresses ERK signaling, causes melanoma tumor regression, and increases patient survival [6]. However, patients become resistant within a year of treatment [7]. Thus, a better understanding of the molecular mechanisms by which oncogenic BRAF(V600E) transforms melanocytes and the cellular response to BRAF(V600E) inhibition in melanoma are needed.

Although BRAFV600E supports melanoma proliferation, benign melanocytic nevi also harbor BRAF mutations. While introduction of BRAFV600E into immortalized melanocytes is sufficient for transformation [8], BRAFV600E in primary melanocytes elicits a biphasic response that includes an initial proliferative response followed by cell cycle withdrawal and ultimately senescence [9]. Oncogene induced senescence is a process that is thought to suppress tumorigenesis. Disruption of p16 and other regulators associated with oncogene induced senescence allow melanoma cells to proliferate and contributes to tumorigenesis [10]. Interestingly, components of the SWItch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex has been found to be required for oncogenic BRAF induced senescence in melanocytes and have also been associated with heterochromatic foci in melanocytes that are undergoing replicative senescence [11, 12].

SWI/SNF enzymes are multi-subunit complexes that utilize the energy derived from ATP hydrolysis to remodel chromatin structure and regulate cellular processes such as transcription, DNA repair, cell proliferation, and differentiation, [13]. Distinct SWI/SNF complexes are composed of either the Brahma (BRM) or Brahma related protein 1 (BRG1) catalytic ATPase subunit and 9–12 BRM/BRG1 associated factors (BAFs). BRM and BRG1

have similar chromatin remodeling activities in vitro but can distinctly regulate gene expression and proliferation in cells [14–19]. In normal tissues, BRG1 is primarily expressed in cell types that proliferate and self-renew while BRM is expressed in cell types that are quiescent [20]. Moreover, BRM is associated with heterochromatic foci in melanocytes [12]. Biallelic disruption of murine BRG1 is embryonic lethal while disruption of BRM results in a mild proliferative defect. Thus, there are considerable differences between the cellular functions of BRM and BRG1 in mammalian cells.

SWI/SNF components have been implicated in cancer development [21]. Mutations in BRG1, BAF200 (ARID2), and BAF180 (polybromo) have been detected in patient-derived melanoma [22, 23]. A hotspot mutation in BRM has been reported to occur in non-melanoma skin cancers [24]. Furthermore, disruption of murine BRM increases the incidence of skin and ocular tumors that result from exposure to ultraviolet radiation [24, 25]. However, mutations in BRM have not been reported to occur in melanoma nor do they occur frequently in a number of other cancers. Instead, BRM expression is epigenetically silenced in 10–20% lung, bladder, gastric, esophageal, and head/neck tumors (Glaros, 2007 #2030; Yamamichi, 2007 #2134; Shen, 2008 #2241; Reisman, 2003 #2227; [26].

Transformation of immortalized fibroblasts with oncogenic RAS suppresses BRM expression and the restoration of BRM partially reverts the transformed phenotype [27]. In this study, we tested the hypothesis that BRM expression is modulated by BRAF(V600E) through activation of the ERK1/2 pathway in melanocytes and melanoma cells. We found that introduction of BRAF(V600E) into primary neonatal human epidermal melanocytes and into melanoma cells that express wild-type BRAF resulted in a decrease in BRM expression. Treatment of human melanoma cells that harbor the BRAF(V600E) mutation with MEK inhibitors or with the BRAF(V600E) selective inhibitor, PLX4032, stimulated BRM expression and concomitantly decreased expression of the alternative SWI/SNF ATPase, BRG1. The enhancement in BRM expression was found to occur through an epigenetic mechanism that involves increased histone acetylation on the BRM promoter. Over-expression of BRM in BRAF(V600E) expressing melanoma cells that were cultured in the absence of PLX4032 suppressed proliferation as evidenced by changes in the cell cycle profile and increased apoptosis. However, in cells cultured in the presence of PLX4032, BRM expression was associated with enhanced melanoma survival. An increase in BRM acetylation was detected in PLX4032 treated melanoma cells. Thus, BRM expression is induced by PLX4032 and its activity may be altered by a post-translational modification.

Materials and Methods

Cell Culture

Neonatal human epidermal melanocytes (NHEMs) were isolated as described [28] and cultured as described [14]. B16, SK-MEL-28, SK-MEL-24, and SK-MEL5 melanoma cells were obtained from the American Type Culture Collection. YUGEN8 was obtained from the Yale Cell Culture Core Facility and described in [29]. SK-MEL5+BRG1 cells were previously described [14]. Melanoma cells were cultured as described [14]. U0126 was from Promega and used at a concentration of 20 μ M. PD0325901 was from Cayman and used at a concentration of 10 μ M. PLX4032 was from Selleck and used at a concentration of 1 μ M.

Transfections

NHEMs were transfected with an empty vector (pBABE) or pBABE-BRAF(V600E) using Lipofectamine LTX (Invitrogen) as described [16]. B16 melanoma cells were infected with control retrovirus (pBABE) or pBABE-V600E as previously described [14]. Cells were harvested 72 hours after transfection. SK-MEL-28 melanoma cells were transfected with pBABE or pBABE-BRM as described [16]. Media was replaced 48 hours after transfection with fresh media containing vehicle or PLX4032. Cells were harvested 48 hours later.

RNA isolation and Quantitative Real Time PCR

Total RNA was isolated using Trizol (Invitrogen) and cDNA was prepared using the Qiagen Quantitect Reverse Transcription kit. Quantitative PCR (qPCR) was performed in SYBR Green master mix (Qiagen) with an Applied Biosystems 7500 PCR and analyzed with the SDS software as described [14]. Primers for human BRM, BRG1, and GAPDH were obtained from SABiosciences (Qiagen). Primers that detect the human BRM 3'-UTR were (5'-GAATTCCTTCCTCCCCTGTC-3') and (5'-TGAATCTTTGAGGCCCATTT-3'). Human BRM and BRG1 mRNA levels were normalized to GAPDH. Primers for mouse BRM were (5'-CGGACCTCCCAGCGTCTCAC-3') and (5'-CCCTGGCCAACATTTTGTA-3'). Primers for mouse BRG1 were (5'-TCTGAGGTGGACGCCGACACATTA-3') and (5'-TAAGGACCTGCGTCAACTGTCAGTG-3'). BRM and BRG1 mRNA levels were normalized to mouse RPL7: 5'-GGAGGAAGCTCATCTATGAGAAGG-3' and 5'-AAGATCTGTGGAAGAGGAAGGAGC-3'.

siRNA Knockdown

siRNA targeting human BRM (5'-GTCATTGCTGAGGCTTT-3') as used in [17] and a non-targeting siRNA (5'-TTCTCCGAACGTGTCACGT-3') were obtained from Dharmacon. Transfection was performed according to the manufacturer's instructions. Media was replaced with media containing vehicle or PLX4032 48 hours post transfection. Cells were then harvested 48 hours later.

Cell extracts and immunoblot analysis

Cell extracts were prepared and Western blotting was performed as described [14]. For histone Westerns, proteins were isolated according to the Abcam protocol. Antisera to BRG1 were previously described [30]. The BRM antibody was from Abcam. The histone H3, and tetra-acetylated histone H4 antibodies were obtained from Active Motif. Antibodies to phosphorylated ERK1/2 (P-ERK1/2), total ERK1/2, BRAF, phosphorylated RB at amino acid 780, and tubulin were from Cell Signaling Technology. Antisera to acetylated BRM as described in [31] was a gift from Dr. Christian Muchardt (Institut Pasteur). Western blots were scanned and bands were quantified by densitometry using Image J software.

Chromatin immunoprecipitations (ChIPs)

ChIPs were performed with a control rabbit IgG (Santa Cruz), an antibody to histone H4 (Active Motif), and an antibody to tetra-acetylated histone H4 (Active Motif) as described [14]. Primers detected the BRM promoter (-741) (5'-TTTGGAAGCTTGCAGTCCTT-3')

and (5'-TTTGTGGCACAGTGTGGACT-3'), and a BRM upstream region (-2700) (5'-cctttaccctccaaccaca-3') and (5'-AAGGCCTCAACACAGGAGAA-3'). Primers to the CD25 promoter were previously described [14].

Immunoprecipitations

Co-immunoprecipitations were performed with an antibody to acetylated lysine (Abcam) or with species matched control IgG (Santa Cruz) as described [14].

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

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

Apoptosis Assay

Cells were stained with Annexin V as described [16] and analyzed on a FACS caliber (BD Biosciences) at the University of Toledo Flow Cytometry Core Facility.

Statistical Analysis

Statistical significance was calculated by the Student's t test.

Results

BRAF(V600E) decreases BRM expression and increases BRG1 expression in primary neonatal human epidermal melanocytes and melanoma cells

To determine if BRAF(V600E) modulates the relative expression of the SWI/SNF catalytic subunits, BRM and BRG1, we infected primary neonatal human epidermal melanocytes with a BRAF(V600E) retrovirus. BRAF(V600E) stimulated ERK phosphorylation, suppressed BRM protein expression and promoted an increase in BRG1 protein (Fig. 1A). There was also a decrease in BRM and increase in BRG1 mRNA levels (Fig. 1B). The observed changes in the relative expression of BRM and BRG1 also occurred when BRAF(V600E) was expressed in a melanoma cell line that is wild type for BRAF (B16) (Fig. 1C and D) and in immortalized murine melanocytes (melan-A cells) (data not shown). Thus, BRAF(V600E) increased ERK1/2 phosphorylation and concomitantly shifted the relative expression of BRM and BRG1 in both normal melanocytes and melanoma cells that have wild-type BRAF.

Suppression of ERK phosphorylation in BRAF(V600E) expressing melanoma cells by treatment with MEK1/2 or selective BRAF(V600E) inhibitors is associated with increased BRM expression and decreased BRG1 expression

We then investigated the effect of inhibiting ERK phosphorylation in BRAF(V600E) expressing melanoma cells on the relative expression of BRM and BRG1. Treatment of SK-MEL-28 cells with the MEK inhibitor, U0126 markedly repressed ERK phosphorylation and the relative expression of BRM and BRG1. An increase in BRM protein levels was observed

within 24–48 hours of treatment while a modest decrease in BRG1 protein levels was observed after 48 hours of treatment (Fig. 2A). BRM mRNA levels were also induced by U0126 at 24 and 48 hours whereas a transient and modest decrease in BRG1 mRNA levels was observed only at 24 hours (Fig. 2B). Similarly, suppression of ERK phosphorylation with the MEK inhibitor, PD0325901 and the BRAF(V600E) selective inhibitor, PLX4032, was associated with enhanced BRM expression at 24 and 48 hours (Fig. 2C). BRG1 protein levels also decreased modestly with these inhibitors. BRM was highly induced by both inhibitors at the mRNA level whereas there was a transient and modest decrease in BRG1 mRNA levels at 24 hours and a smaller effect at 48 hours (Fig. 2D). These data suggest that inhibition of ERK signaling in melanoma cells by either MEK inhibition or BRAF(V600E) inhibition is associated with changes in the relative expression of the two SWI/SNF catalytic subunits.

Inhibition of BRAF(V600E) promotes BRM expression and suppresses BRG1 expression in a panel of melanoma cells

BRAF(V600E) cooperates with the phosphatase and tensin homolog (PTEN) silencing to transform normal melanocytes to melanoma cells [32]. We evaluated the effects of BRAF(V600E) inhibition on the relative expression of BRM and BRG1 in multiple cell lines that harbor BRAF(V600E) and have alterations at the PTEN locus: SK-MEL-28 (Fig. 3A), SK-MEL-24 (Fig. 3B), and YUGEN8 (Fig. 3C) as well as in SK-MEL-5 (Fig. 3D), a cell line that is wild type for PTEN. Although the kinetics and extent of BRM induction varied over a time course of 24 hours following treatment with PLX4032, an increase in BRM protein levels was detected at the end of this time period in all cells. Thus, induction of BRM by PLX4032 does not depend on PTEN status.

The expression levels of SWI/SNF subunits have been shown to be stoichiometric and a change in the expression level of one SWI/SNF subunit is accompanied by changes in the levels of other SWI/SNF subunits [33, 34]. We compared the effects of PLX4032 on BRM expression in SK-MEL-5 cells, which were previously determined to be BRG1 deficient (Fig. 3D) [14, 35] with SK-MEL-5 cells that stably express BRG1 (Fig. 3E). Although the kinetics varied between the cells, BRM was induced to similar levels by PLX4032 in BRG1 deficient SK-MEL-5 cells as in BRG1 expressing SK-MEL-5 cells. Thus, BRM induction by inhibition of BRAF(V600E) is not dependent on BRG1 expression in SK-MEL-5 cells. Interestingly, BRG1 levels were reduced by PLX4032 to varying extents in all cells including SK-MEL5+BRG1 (Figs. 3A, 3B, 3C, 3D, and 3E).

The increase in BRM levels and the decrease in BRG1 levels that occur upon inhibition of BRAF(V600E) varies across melanoma cell lines and is correlated with decreased phosphorylation of the retinoblastoma protein (RB)

We compared the initial levels of BRM and BRG1 in the different melanoma cell lines and the extent of induction after treatment with PLX4032 for 48 hours (Fig. 4). At the end of this time period, phosphorylated ERK was inhibited to a similar extent in all cell lines. Densitometry (bottom panel) revealed that BRM was induced to the greatest extent in SK-MEL-24 cells (266% increase) which initially expressed the lowest levels of BRM and to the least extent in YUGEN8 cells (14% increase), which initially expressed BRM at the

highest levels. However, comparison of SK-MEL-28 with SK-MEL-5 and SK-MEL5+BRG1 indicated that the greatest induction of BRM does not necessarily occur in the cells that have the least initial levels of BRM. Interestingly, the greatest reduction of BRG1 occurred in SK-MEL-5 cells that were engineered to express BRG1 (94% decrease). BRG1 expression plummeted to levels that were virtually as low as in parental SK-MEL-5 cells. Because BRM has been associated with RB mediated cell cycle regulation [36], we investigated the phosphorylation status of the retinoblastoma protein (RB). We found a decrease in RB phosphorylation in all PLX4032 treated cells (Fig. 4). In combination, these data indicate that although the increase in BRM levels by PLX4032 is correlated with decreased phosphorylation of RB, the change in BRG1 and BRM expression can vary in different melanoma cells.

Induction of BRM expression by inhibition of BRAF (V600E) signaling is associated with changes in histone acetylation at the BRM promoter

Previous studies indicated that BRM expression can be induced by histone deacetylase (HDAC) inhibitors [31, 36]. Hence, we investigated whether PLX4032 could alter histone acetylation in melanoma cells and thereby induce BRM expression. PLX4032 as well as the MEK inhibitor, PD0325901 promoted an increase in acetylated histone H4 in SK-MEL-28 cells (Fig. 5A) and in YUGEN8 cells (Fig. 5B). We chose SK-MEL-28 cells to study further and found that H4 acetylation was also increased by PD0325901 (Fig. 5C). Treatment of these cells with sodium butyrate over a five day period resulted in a progressive increase in acetylated histone H4 and an increase in BRM expression (Fig. 5D). This result correlates suppression of ERK1/2 signaling by inhibition of BRAF(V600E) or by inhibition of MEK and BRM induction with changes in histone acetylation.

The induction of BRM expression by HDAC inhibitors is driven by transcriptional and post-transcriptional mechanisms [37, 38]. HDAC3 and HDAC9 have been shown to regulate BRM expression [39]. Furthermore, two promoter polymorphisms at -741 and at -1321 have been associated with epigenetic silencing of BRM through a mechanism that involves transcriptional regulation by histone deacetylases [40]. Hence, we investigated whether suppression of ERK signaling by inhibition of BRAF(V600E) induces BRM expression by promoting changes in histone acetylation at the BRM promoter. We observed a marked increase in histone H4 acetylation at -741 relative to the start site of the BRM promoter after 24 hours treatment with PLX4032 and a further increase after 48 hours treatment with PLX4032 (Fig. 5E). As a control, we assayed an upstream site at the BRM locus. There was also a small increase in histone H4 acetylation at an upstream site (-2700), however, the overall level of acetylation was much less at this site than at -741. Furthermore, acetylation on the control CD25 locus did not change with PLX4032 treatment. We saw a similar effect on histone acetylation on the BRM promoter when ERK1/2 signaling was suppressed with the MEK inhibitor, PD0325901 (data not shown). Thus, suppression of ERK1/2 signaling by inhibition of BRAF(V600E) or MEK promotes changes in histone acetylation at the BRM promoter that are associated with increased transcriptional activity.

The role of BRM in cell cycle regulation and survival is contingent on the status of ERK1/2 signaling

Inhibition of BRAF(V600E) suppresses melanoma cell proliferation, leading to cell cycle arrest and apoptosis [41]. To determine how the induction of BRM expression by BRAF(V600E) inhibition affects melanoma proliferation, we transfected SK-MEL-28 cells with an empty vector (EV) or with a BRM construct and cultured the cells in the presence or absence of PLX4032. An increase in BRM protein levels was observed in BRM transfected cells and a further increase in BRM protein levels was detected upon treatment with PLX4032 (Fig. 6A).

We also quantified BRM expression at the mRNA level with a primer set that detects the coding region of both the endogenous and transfected BRM genes as well as a primer set that detects the 3' untranslated region (3' UTR) that is present only in the endogenous gene. The two primer sets generated equal quantities of PCR products using RNA obtained from cells transfected with empty vector (EV), indicating that BRM mRNA was transcribed from the endogenous BRM gene (Fig. 6B). Furthermore, there was robust induction of BRM mRNA in these PLX4032 treated cells. In BRM transfected samples, the higher levels of BRM mRNA detected by the coding region primers compared to the 3'UTR primers indicated that the transfected BRM gene contributed to the increase in BRM mRNA in PLX4032 treated cells. Interestingly, the PCR signal generated by the 3'UTR primers was higher in the EV transfected cells that were treated with PLX4032 compared to the BRM transfected cells that were treated with PLX4032, suggesting that ectopically expressed BRM led to a decrease in the expression of the endogenous gene. Furthermore, overall BRM mRNA levels increased only slightly in PLX4032 treated cells that ectopically expressed BRM, indicating that expression from the endogenous gene was decreased. Thus, the decrease in expression of the endogenous BRM gene reduced the extent to which BRM could be over-expressed in these cells. However, we proceeded with functional assays based on the observed overall increase in BRM protein levels.

As expected, PLX4032 promoted the accumulation of cells in the G1 phase of the cell cycle and caused a decrease in the number of cells in S phase (Fig. 6C). Over-expression of BRM in vehicle treated cells resulted in a small but statistically significant increase in the number of cells in G1 and a decrease in the number of cells in S phase. This effect was paralleled by a reduction in cell numbers (data not shown), suggesting that BRM over-expression suppressed proliferation. In contrast, in cells treated with PLX4032, BRM over-expression resulted in a decrease in the accumulation of cells in G1 and an increase in the accumulation of cells in S phase. The effect of BRM over-expression in PLX4032 treated cells was paralleled by an increase in cell numbers (data not shown), suggesting that BRM promotes proliferation in BRAF(V600E) inhibited melanoma cells.

Although statistically significant, the effects of BRM over-expression on cell cycle progression were small. Hence, we investigated whether BRM over-expression affects apoptosis. An increase in Annexin V staining was detected when cells expressing only empty vector were treated with PLX4032 (Fig. 6D). Interestingly, over-expression of BRM had opposite effects on melanoma survival in cells grown in the absence of PLX4032 as in cells grown in the presence of PLX4032. BRM promoted an increase in apoptosis when cells

were cultured without PLX4032 and a decrease in apoptosis when cells were cultured with PLX4032 (Fig. 6D). To further evaluate the possibility that BRM promotes survival of cells treated with PLX4032, we transfected control siRNA and siBRM into SK-MEL28 cells (Fig. 6E). Depletion of BRM did not significantly affect apoptosis when cells were cultured in the absence of PLX4032 (Fig. 6F). However, depletion of BRM resulted in a marked increase in apoptosis when cells were cultured in the presence of PLX4032. Thus, induction of BRM expression helps prevent death of melanoma cells when BRAF(V600E) is inhibited and ERK1/2 signaling is compromised.

Acetylation of the BRM protein has been shown to suppress the growth inhibitory effects of BRM [31]. To better understand the contrasting effects of BRM on cell cycle control and apoptosis when melanoma cells were cultured in the presence and absence of PLX4032, we compared the acetylation status of BRM in vehicle and PLX4032 treated cells. In Figure 7A, we detected increased acetylation of BRM protein in extracts from SK-MEL-28 cells cultured in PLX4032 that were immunoprecipitated with an antibody to acetylated lysine. We confirmed the observed effects of PLX4032 on BRM acetylation in SK-MEL-28 cells over a time course during which BRM is induced (Fig. 3A) with an antibody that detects acetylated BRM (Fig. 7B). We also found that BRM acetylation increases with PLX4032 treatment in other melanoma cell lines (Fig. 7C). Thus, although BRM expression increases with PLX4032 treatment, there is also an increase in the acetylation of BRM which may decrease its transcriptional activity and ability to suppress growth, potentially causing it to act in a dominant negative manner.

Discussion

Prior studies suggested that targeting SWI/SNF enzymes is an important mechanism by which oncogenes elicit changes in gene expression. Oncogenic RAS inhibits expression the SWI/SNF catalytic subunit, BRM, during cellular transformation and restoring BRM expression partially reverses the transformed phenotype [27]. It was recently demonstrated that BRM expression is also compromised in RAS transformed mammary epithelial cells and that restoration of BRM suppresses malignancy [42]. Furthermore, BRM can be induced by MEK inhibitors in epigenetically silenced lung cancer cells [39]. Our findings indicate that BRM expression can be suppressed by oncogenic BRAF(V600E) in melanocytes and melanoma cells and that suppression of ERK1/2 phosphorylation achieved either by pharmacological inhibition of MEK or by selective inhibition of BRAF enhances BRM expression. Therefore, BRM is suppressed by multiple components of the mitogen-activated protein kinase / extracellular signal regulated kinase (ERK1/2) pathway in a range of cancer cell types.

Interestingly, whereas RAS does not alter the expression of the alternative ATPase, BRG1, [27] our findings indicate that in melanocytes, BRAF(V600E) enhances BRG1 expression and that inhibiting MEK or BRAF reduces BRG1 expression in melanoma. The effect of MEK and BRAF inhibition was modest and transient at the mRNA level. BRG1 protein expression was also highly affected in SK-MEL-5 cells that were engineered express BRG1 from a heterologous promoter. These observations suggest that post-transcriptional mechanisms are involved. Furthermore, in some of our experiments, we detected a mobility

shift in BRG1 depending on the status of ERK signaling (Fig. 2C). A prior study showed that BRG1 hyper-phosphorylation by ERK is associated with inactivation of the SWI/SNF complex [43]. Thus, in addition to expression, BRG1 activity may be altered in melanoma cells that harbor BRAF(V600E) and by PLX4032 treatment. We are currently investigating whether BRG1 phosphorylation is regulated by ERK signaling.

Epigenetic silencing of BRM can be reversed by HDAC inhibition and several HDACs have been implicated as repressors of BRM transcription [37]. Interestingly, we found that inhibiting the ERK1/2 pathway with MEK or BRAF(V600E) inhibitors promoted an increase in global histone acetylation as well as increased acetylation on the BRM promoter. A high level of enrichment was observed at a region of the BRM promoter (-742) that is polymorphic in the human population and is associated with loss of BRM expression as well as risk for lung and aerodigestive tract cancers [26, 40]. It will be interesting to determine if BRM promoter polymorphisms also affect melanoma risk and/or the response to BRAF inhibitors. BRM and BRG1 are thought to have tumor suppressive roles by their ability to interact with the retinoblastoma protein (RB) and restrict cell cycle progression [44]. Our data show that induction of BRM by PLX4032 is correlated with RB hypophosphorylation and that over-expression of BRM can suppress proliferation by promoting G1 cell cycle arrest and apoptosis in melanoma cells that harbor BRAF(V600E) and exhibit constitutively activated ERK1/2. However, PLX4032 reverses this tumor suppressive effect and converts BRM to a pro-survival factor. Post-translational acetylation of BRM dampens its growth-inhibitory effects [31]. Thus, the increased levels of histone acetylation that occur in PLX4032 treated melanoma cells may alter BRM activity by increasing BRM acetylation.

The observed shift in the effect of BRM on proliferation might also arise due to the suppression of BRG1 expression by PLX4032. We previously demonstrated that depletion of BRM in BRG1 deficient melanoma cells compromises tumorigenicity [14]. Recent studies indicate that a synthetic lethality approach which targets BRM in BRG1 deficient cancers may be an effective therapeutic strategy [45, 46]. Our observations suggest that disruption of BRM may increase the sensitivity of melanoma cells to BRAF inhibitors, potentially through a synthetic lethality effect.

Both BRM and BRG1 interact with the Microphthalmia-Associated Transcription Factor and co-activate MITF-target gene expression in melanoma [14]. MITF is considered a lineage addiction oncogene that is required for melanoma survival [47]. Thus, the tumor suppressive role of BRM and BRG1 may be offset by this interaction. Some of our studies indicate that BRM and BRG1 differentially regulate subsets of MITF target genes [14, 16]. Interestingly, a prior report demonstrated that ERK1/2 signaling differentially regulates expression of MITF 6a/b splicing isoforms [48]. The two MITF isoforms (+) and (-) differ by the presence of six amino acids and have differential effects on cell cycle regulation [49]. Suppression of ERK1/2 activity with MEK inhibitors shifts the relative ratio of MITF(+) and MITF(-) in favor of MITF(-). This shift correlates with our observed findings that suppression of ERK1/2 with MEK and BRAF inhibitors shifts the relative ratio of BRM and BRG1 in favor of BRM. Future studies will investigate whether different MITF isoforms interact differentially with BRM and BRG1 based complexes.

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Abbreviations

MAPK	mitogen-activated protein kinase
ERK1/2	extracellular signal regulated kinase 1 and 2
P-ERK	phosphorylated ERK1/2
MEK	mitogen-activated protein kinase kinase
RAS	rat sarcoma
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRAF(V600E)	BRAF with a valine to glutamic acid substitution at position 600
SWI/SNF	SWItch/sucrose non-fermentable
BRM	Brahma
BRG1	Brahma-related protein 1
BAF	BRM/BRG1 associated factors
PTEN	phosphatase and tensin homolog
HDAC	histone deacetylase
qPCR	quantitative polymerase chain reaction
ChIP	chromatin immunoprecipitation
RB	retinoblastoma protein
MITF	Microphthalmia-Associated Transcription Factor

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Research Highlights

- Expression of BRM and BRG1 is modulated by the ERK1/2 pathway in melanoma.
- Suppression of ERK1/2 activity by MEK or BRAF inhibitors enhances BRM expression.
- Activation of BRM by ERK1/2 inhibition is associated with chromatin modifications.
- BRM has an anti-proliferative effect in melanoma cells that harbor oncogenic BRAF.
- BRM becomes a pro-survival factor when ERK1/2 activity is suppressed.

Fig 1. BRAF(V600E) decreases BRM expression and increases BRG1 expression in primary neonatal human epidermal melanocytes (NHEM) and B16 melanoma cells

(A) NHEMs were transfected with empty vector (EV) or a BRAF(V600E) construct. Cell extracts were subjected to Western blotting with antibodies to BRAF, BRM, BRG1 and P-ERK1/2. Total ERK1/2 and tubulin were used as loading controls. The percent change in BRM and BRG1 protein levels in BRAF(V600E) expressing cells relative to levels in the EV control is shown in the bottom graph. (B) Total RNA from control (EV) and BRAF(V600E) transfected NHEMs was reverse-transcribed, and subjected to quantitative PCR (qPCR). BRM and BRG1 mRNA levels were normalized to GAPDH. The percent change in BRM and BRG1 mRNA levels in BRAF(V600E) expressing cells relative to levels in the EV control is shown in the graph. (C) B16 melanoma cells were infected with an empty retrovirus (EV) or a BRAF(V600E) retrovirus. Cell extracts were subjected to Western blotting with antibodies to BRAF, BRM, BRG1, and P-ERK1/2. The percent change in BRM and BRG1 protein levels in BRAF(V600E) expressing cells relative to levels in the EV control is shown in the bottom graph. (D) Total RNA from EV and BRAF(V600E) infected B16 cells was reverse transcribed and subjected to qPCR. BRM and

BRG1 mRNA levels were normalized to GAPDH. The percent change in BRM and BRG1 mRNA levels in BRAF(V600E) expressing cells relative to levels in the EV control is shown in the graph. The data are the average of two or more independent experiments that were subjected to qPCR three times. Standard error bars are shown. (**p<0.01).

Fig 2. Inhibition of ERK1/2 phosphorylation by treatment of BRAF(V600E) expressing melanoma cells with MEK inhibitors or a selective BRAF(V600E) inhibitor increases BRM expression and decreases BRG1 expression

(A) Cell extracts from SK-Mel-28 melanoma cells treated with vehicle or with U0126 (20 μM) for 24 and 48 hours were subjected to Western blotting with antibodies to BRM, BRG1, and P-ERK1/2. Total ERK1/2 was used as a loading control. The percent change in BRM and BRG1 protein levels in U0126 treated cells relative to levels in the vehicle control is shown in the bottom graph. (B) Total RNA from vehicle and U0126 treated cells was harvested, reverse-transcribed, and subjected to qPCR. BRM and BRG1 mRNA levels were normalized to GAPDH. The percent change in BRM and BRG1 mRNA levels in U0126 treated cells relative to levels in the vehicle control is shown in the graph. (C) Cell extracts from SK-Mel-28 melanoma cells that had been treated with vehicle, PD0325901 (10 μM), or PLX4032 (1 μM) for the indicated times were subjected to Western blotting with antibodies to BRM, BRG1, and P-ERK1/2. Total ERK1/2 was used as loading control. The percent change in BRM and BRG1 protein levels in PD0325901 and PLX4032 treated cells compared to levels in the vehicle control is shown in the bottom graph. (D) Total RNA from vehicle treated, PD0325901, and PLX4032 treated cells was reverse-transcribed, and

subjected to qPCR. BRM and BRG1 mRNA levels were normalized to GAPDH. The percent change in BRM and BRG1 mRNA levels in PD0325901 and PLX4032 treated cells relative to levels in the vehicle control is shown in the graph. The data are the average of two or more independent experiments that were subjected to qPCR three times. Standard error bars are shown. (** $p < 0.01$ and * $p < 0.05$).

Fig 3. Inhibition of BRAF(V600E) signaling with PLX4032 results in an increase in BRM expression and a decrease in BRG1 expression in a panel of melanoma cell lines (A) SK-MEL28 (B) SK-MEL-24, (C) YUGEN8, (D) SK-MEL5, and (E) SK-MEL5+BRG1 were cultured in PLX4032 and harvested after 0, 4, 8, 12, and 24 hours. Cell extracts were subjected to Western blotting with antibodies to BRM, BRG1, and P-ERK1/2. Total ERK1/2 was a loading control. The percent change in BRM and BRG1 protein levels in PLX4032 treated cells at each time point relative to levels at 0 hours is shown in the bottom graph

Fig 4. The fold increase in BRM expression and decrease in BRG1 expression in PLX4032 treated melanoma cells varies among melanoma cell lines

(A) Cell extracts from SK-MEL-28, SK-MEL-24, YUGEN8, SK-MEL-5, and SK-MEL-5+BRG1 cells that had been treated with vehicle or with PLX4032 (1 μ M) for 48 hours were subjected to western blotting with antibodies to BRM, BRG1, P(780)-RB and P-ERK1/2. Total ERK1/2 and Tubulin were used as loading controls. Quantification of BRM and BRG1 protein levels in each cell line is shown relative to Tubulin (bottom graphs). The percent change in BRM and BRG1 protein levels in PLX4032 treated cells relative to levels in the vehicle control is indicated for each cell line.

Fig 5. The increase in BRM expression that occurs with suppression of ERK1/2 phosphorylation is correlated with changes in histone acetylation at the BRM promoter

(A) SK-MEL-28 cells and (B) YUGEN8 cells were treated with vehicle or with the selective BRAFV600E inhibitor, PLX4032 (1 μ M) and harvested 48 hours later. Cell extracts were subjected to Western blotting with an antibody to acetylated histone H4 (AcH4). Total H3 was used as loading control. Quantification of AcH4 levels is shown (bottom graphs) (C) SK-Mel-28 cells were treated with vehicle or with the MEK1/2 inhibitor PDO325901 (10 μ M) and harvested 48 hours later. Cell extracts were subjected to Western blotting with an antibody to acetylated histone H4 (AcH4). Total H3 was used as loading control.

Quantification of AcH4 levels is shown (bottom graph). (D). SK-Mel-28 cells were treated with sodium butyrate (1mM) and harvested for protein after the indicated times. The cell extracts were subjected to Western blotting with antibodies to BRM and AcH4. Tubulin was used as loading control. BRM levels are relative to tubulin and expressed as percent change compared to BRM levels at day 0 (bottom graph). (E) Chromatin immunoprecipitations (ChIPs) were performed on SK-MEL-28 cells that were treated with vehicle or with PLX4032 using a non-specific IgG antibody and antibodies specific for AcH4 and total H4. Enrichment was quantified by qPCR. Enrichment with IgG was virtually undetectable (data not shown). The AcH4 signal was normalized to that of total H4 at the -741, -2700 upstream and as a control, a region of the CD25 promoter. The data is representative of three independent experiments that were subjected to qPCR three times. Standard error bars are shown (**p<0.01, *p<0.05).

Fig 6. BRM regulates cell cycle distribution and survival

(A) SK-MEL-28 cells were transiently transfected with empty vector (EV) or a BRM construct and were cultured in media containing vehicle or PLX4032 (1 μ M) for 48 hours. Cell extracts were subjected to Western blotting with antibodies to BRM, BRG1, and P-ERK1/2. Total ERK1/2 is a loading control. The percent change in BRM and BRG1 levels relative to vehicle treated cells that express empty vector (EV) is shown in the bottom graph. (B) SK-MEL-28 cells were transiently transfected with empty vector (EV) or a BRM construct and cultured in media containing vehicle or PLX4032 (1 μ M) for 48 hours. Total RNA was harvested, reverse-transcribed, and subjected to qPCR. BRM mRNA levels were detected with primers that anneal to the coding region or to the 3'UTR and normalized to GAPDH. The percent change in BRM mRNA levels is relative to levels in cells transfected with empty vector (EV) and cultured in vehicle. The data are the average of three independent experiments. Standard error bars are shown. (**p<0.01). (C) SK-MEL-28 cells as in A were stained with propidium iodide and sorted by flow cytometry. The data are the average of three independent experiments. Standard error bars are shown. (**p<0.01, *p<0.05). (D) Cells treated as in A were harvested 48 hours after treatment and subjected to the annexin V assay. The data are the average of three independent experiments performed in triplicate. Standard error bars are shown (**p<0.01, *p<0.05). (E) SK-MEL-28 cells transfected with si control (siC) or siBRM and cultured in media containing vehicle or PLX4032 (1 μ M) for 48 hours. Cell extracts were subjected to Western blotting with antibodies to BRM, BRG1, and P-ERK. Total ERK1/2 is a loading control. The percent change in BRM and BRG1 levels relative to vehicle treated cells that express siC is shown in the bottom graph. (F) Cells treated as in D

were subjected to the annexin V assay. The data are the average of three experiments performed in triplicate. Standard error bars are shown (** $p < 0.01$).

Fig 7. PLX4032 increases the levels of acetylated BRM

(A) SK-MEL-28 cells were cultured in media containing vehicle or PLX4032 (1 μ M) for 48 hours. Cell extracts were immunoprecipitated with an antibody to acetylated lysine or with rabbit IgG and subjected to Western blotting with an antibody to BRM. B. The extracts from SK-MEL-28 cells shown in Fig. 3A were subjected to Western blotting with antisera to acetylated BRM. ERK1/2 is shown as a loading control. C. The extracts from melanoma cells shown in Fig. 4A were subjected to Western blotting with antisera to acetylated BRM. ERK1/2 is shown as a loading control.