ACTIVATION OF GRM1 EXPRESSION BY INDUCIBLE MUTATED B-RAF *IN VITRO* AND *IN VIVO*

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ABSTRACT OF THE THESIS

Activation of Grm1 Expression by inducible mutated B-RAF *in vitro* and *in vivo*

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Melanoma is a form of skin cancer that arises from the neoplastic transformation of melanocytes. Constitutive activation of the MAPK pathway due to mutations in the serine-threonine protein kinase B-RAF or N-RAS has been reported to play important roles in melanomagenesis. Previously, our laboratory identified that ectopic expression of metabotropic glutamate receptor 1 (Grm1) in melanocytes was sufficient to induce spontaneous melanoma development in transgenic mouse models (TG-3 and EPv). We also demonstrated that Grm1 expression activates the MAPK pathway. Recently, we detected Grm1 expression by immunohistochemical staining of tumors derived from transgenic mice harboring a B-RAF (V600E) mutation on PTEN null background. Similarly, Grm1 expression was also detected in tumor samples derived from transgenic mice with an N-RAS (Q61R) mutation on INK4a null background. In addition, several stable immortalized mouse melanocytic clones with exogenous mutant B-RAF (V600E) cDNA showed Grm1 expression *in vitro*. Since normal mouse melanocytes acquire loss of p16INK4a in order to become immortalized, we investigate whether loss of PTEN or INK4a/ARF was required for Grm1 expression by mutated B-RAF (V600E). We made use of a transgenic mouse model (BJB), which has an inducible B-RAF (V600E) genotype only in melanocytes. Here we show that induction of B-RAF (V600E) stimulates skin hyperpigmentation, presumably from increased proliferation of melanocytes. Results

showed detectable Grm1 expression in BJB skin samples, suggesting that the induction of mutated B-RAF (V600E) may be sufficient to promote activation of Grm1 expression, and that ablation of PTEN expression/function but not of INK4a may be required.

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DEDICATION

I dedicate this work to the loving memory of my father Domingo Sierra and to my mother Sixta Sierra for her unconditional love and encouragement.

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INTRODUCTION

PART I: MELANOMA

A. Melanoma

Melanoma is an aggressive form of skin cancer that arises from the aberrant transformation of melanocytes, the pigment-producing cells of the skin (Chudnovsky et al. 2005). Risk factors for melanoma includes nevi, exposure to UV radiation, familial predisposition, fair skin type, and weakened immune systems (Greene et al. 1985, Hawryluk and Tsao 2014). Melanoma incidence is on the rise in the United States, where according to 2014 American Cancer Society estimates, ~76,100 individuals were expected to be diagnosed with melanoma, with ~9,710 of these cases resulting in death (American Cancer Society. 2014). When detected early, melanoma patients have a good chance to survive the disease, if tumors are removed by surgical resection. However, metastatic melanoma in its late stages is largely refractory to existing therapies and has a poor prognosis, with a median survival rate of 9 months and 5 years survival rate of less than 5% (Balch et al. 2009, Gray-Schopfer et al. 2007).

Three essential molecular pathways have been found to be dysregulated in melanoma. These include the mitogen-activated protein kinase (MAPK) pathway, due to activating mutations in RAS, RAF and KIT; the PI3K/AKT pathway, as result of mutations in RAS, mutations or loss of PTEN and aberrant expression of AKT; p16INK4a as consequence of defects in CDKN2A, ARF, and p53 (Wangari-Talbot and Chen 2012). Analysis of these pathways have led to discovery of novel molecular targets in melanoma, giving rise to the development of small molecule therapies and

immunotherapy. Agents such Ipilimumab, a monoclonal antibody that blocks the cytotoxic T-lymphocyte (CTLA-4) responsible for slowing the body's immune system, and Vemurafenib a specific inhibitor of mutated B-RAF (V600E) have emerge as promising therapies (Solus and Kraft 2013,Conde-Perez and Larue 2014). The problem with these treatments is that tumor cells become resistant to their effects and patients relapse. Thus, additional research to elucidate the molecular mechanisms of melanomagenesis and evolution of the disease is necessary for generating more effective therapeutic strategies.

PART II: METABOTROPIC GLUTAMATE RECEPTOR 1 (GRM1)

A.TG-3 Mouse Model

In our laboratory, a transgenic mouse model (TG-3) with the predisposition to develop melanoma was generated with a genomic fragment (clone B) (Chen et al. 1996). This 2-kb genomic DNA fragment has been shown to commit fibroblasts to adipogenesis *in vitro* (Chen et al. 1989, Colon-Teicher et al. 1993). Instead of developing the expected obese phenotype, one out of the five founder mice, TG-3, at 7-8 months after birth displayed pigmented lesions that were determined to be melanoma by histological analysis (Chen et al. 1996). Offspring of TG-3 developed similar observable exterior melanocytic lesions at organs where the epidermal melanocytes reside, the perianal region, legs, ears, eyelid, tail, and snout with100% penetrance (Chen et al. 1996). Molecular analysis of TG-3 genomic DNA revealed that a single concatemer of head-to-head, head-to-tail, and tail-to-tail of clone B was integrated into the third intron of Grm1, the gene that encodes Grm1 and a concurrent deletion of about 70-kb of host genomic sequences at the same site (Pollock P. M. et al. 2003). Comparison of gene expression between tumor and normal ear samples revealed Grm1 expression only in the tumor but not normal ear samples. We hypothesized that the ectopic expression of Grm1 initiated the transformation of melanocytes and led to tumor formation. To test our hypothesis that Grm1 was a driver of melanomagenesis, another mouse line Tg (Grm1)EPv (E), was developed with the expression of Grm1 cDNA under the control of a melanocyte-specific promoter, dopachrome tautomerase (Dct) (Pollock P. M. et al. 2003). Hyper-proliferating melanocytes that developed into primary melanomas with 100% penetrance were observed in this second transgenic line, and confirmed that ectopic expression of Grm1

was sufficient to induce the transformation of melanocytes *in vivo* (Pollock P. M. et al. 2003).

B. Metabotropic glutamate receptor 1 (Grm1)

Grm1 (gene: Grm1 for mouse, and GRM1 for human) belongs to the class C G protein-coupled receptor (GPCR) superfamily (Conn and Pin 1997, Teh and Chen 2012). Structurally, Grms consists of a seven-transmembrane domain structure, LIVBP-like domain, and a cysteine rich domain (Conn and Pin 1997). There are eight Grms subtypes (Grm1 through Grm8), and are classified into three separate groups based upon their sequence homology, signal transduction mechanisms and pharmacologic properties: Group I includes Grm1 and Grm5, Group II includes Grm2 and Grm3, and Group III includes Grm4, Grm6, Grm7 and Grm8 (Conn and Pin 1997).

Grm1 is normally expressed in the central nervous system and is activated by its natural ligand, L-glutamate (Teh and Chen 2012). Grm1 is involved in mediating neuronal excitability, synaptic plasticity, and feedback inhibition of neurotransmitter release (Speyer et al. 2012). All of which promotes learning and memory formation in the central nervous system (Hermans and Challiss 2001). Grm1 encodes five alternative splice variants (1a, 1b, 1c, 1d, and 1e) (Zhu et al. 1999). All five variants contain the same N-terminal, but differ in the amino acid composition of their C-terminal domains due to the alternative splicing (DiRaddo et al. 2013).

Structurally, Grm1 has various domains that are necessary for its functions. The N-terminus forms two large extracellular lobes separated by a cavity where the ligand glutamate binds to and is referred to as the amino terminal domain (ATD) or “Venus Fly Trap” (Beqollari and Kammermeier 2010, O'Hara et al. 1993). ATD is separated from

the trans-membrane region of Grm1 by a 70 amino acid cysteine rich domain (CRD), which is essential for dimerization, and activation of the receptor (Huang et al. 2011). This cysteine rich region is followed by the seven alpha-helical transmembrane domains (TMD). Following the TMD is the carboxyl terminus of Grm1, also known as the intracellular cytoplasmic tail domain (CTD). CTD is involved in modulating G-protein coupling and selectivity (Pin et al. 2003, Seebahn et al. 2013). The CTD is also the region subjected to alternative splicing, regulated by phosphorylation, and modulatory protein-protein interactions (Niswender and Conn 2010).

The CRD is consisted of three beta-pleated sheets and nine cysteine residues. CRD plays a key role in facilitating the allosteric coupling between the ATD and the TMD regions during ligand binding and receptor activation (Niswender and Conn 2010). Upon activation of Grm1 by glutamate, the signal induced is transmitted from the ATD through the CRDs, by way of a disulfide bridge formed between the 9th cysteine of the CRD and a cysteine residue in lobe 2 of the ATD (Muto et al. 2007, Rondard et al. 2006). As a result, a conformational change takes place that brings the C-terminal regions of the CRDs closer to one another and elicits cysteine-cysteine interaction in the e2 loop of the TMD (Muto et al. 2007). This conformational change produces a shift in the TMD to induce G-protein activation (El Moustaine et al. 2012).

Upon activation, Grm1 couples to $G_{\alpha/q11}$ proteins to induce phosphatidylinositol (4,5)-biphosphate (PIP2) hydrolysis leading to the formation of two-second messengers, inositol 1,4,5-triphosphate (IP3) and diacyglycerol (DAG) (Conn and Pin 1997, Hermans and Challiss 2001). These second messengers stimulate intracellular calcium release from the endoplasmic reticulum (ER) stores and activate Protein Kinase C (PKC),

resulting in the stimulation of G-protein-independent signal transduction pathways (Goudet et al. 2009, Hermans and Challiss 2001). Such pathways include the Mitogen Activated Protein Kinase pathway (MAPK) and the Phosphatidylinositol-3-Kinase (PI3K)/AKT pathway (Marin et al. 2006, Shin S., S. et al. 2010).

C. Grm1 Signaling in Melanoma

To examine the mechanism by which Grm1 induces melanocyte transformation, we previously created several stable Grm1-expressing melanocytic clones (MASS clones) by transfecting normal melanocytes (Melan-A) derived from C57BL/6 mice with mouse Grm1 cDNA (Shin S. S. et al. 2008a). As a result, these clones did not required 12-O-tetradecanoylphorbol-13-acetate (TPA) supplement for proliferation, one of the known features of melanocytic cell transformation (Shin S. S. et al. 2008a). When allografted into both immunodeficient nude and immunocompetent syngeneic C57BL/6 mice, robust tumors were formed within 5 days and with invasion in muscles and intestines (Shin S. S. et al. 2008a).

Our laboratory has also detected ectopic expression of GRM1 in human melanoma cell lines and biopsy samples, but none in normal melanocytes (Namkoong et al. 2007, Pollock P. M. et al. 2003). To date, we have analyzed ~175 cell lines and biopsy samples, and found GRM1 mRNA and protein to be expressed in ~60% of the samples (Marin et al. 2006, Yip 2009).

Expression and activation of Grm1 in melanoma cells has been shown to activate the MAPK and P13K/AKT pathways, two of the most frequently stimulated signaling cascades in melanoma (Namkoong et al. 2007, Shin S. S. et al. 2008a). The MAPK pathway consists of the RAS-RAF-MEK-ERK signaling cascade, and its constitutive

activation has been shown to have a considerable influence in the development of melanoma (Lee et al. 2008). Previous induction studies using GRM1 positive human melanoma cell lines confirmed that in human melanoma, activation of the MAPK pathway is dependent upon GRM1 expression (Namkoong et al. 2007). When GRM1 was stimulated with its agonist, L-quisqualate, there were increased levels of phosphorylated ERK, indicating the pathway is activated (Namkoong et al. 2007). Furthermore, when pretreating the cells with GRM1 antagonist, LY367385, followed by stimulation with the agonist, the MAPK pathway can no longer be activated as indicated steady levels of pERK (Namkoong et al. 2007). How and what activates the expression of GRM1 only in melanoma but not normal samples remain an unanswered question.

PART III: MELANOMA CELL PROLIFERATION PATHWAY

A. MAPK/ERK

The mitogen-activated protein kinase pathway (MAPK) consists of the RAS-RAF-MEK-ERK(1/2) signaling cascade that regulates extracellular signals to control cell growth, differentiation, and survival (Wellbrock et al., 2004). Earlier reports of human melanoma cell lines and biopsies have revealed that there is aberrant activation of the MAPK pathway and increased levels of phosphorylated extracellular signal-kinase (ERK), due to activating mutations in the oncogenes B-RAF and N-RAS. Notably, B-RAF and N-RAS mutations are mutually exclusive, and both oncogenic activities deregulate the MAPK pathway (Ackermann et al., 2005).

B. RAS

The human RAS protein family consists of small GTPase molecular switches of which there are three isoforms H-RAS, K-RAS, and N-RAS (Henis et al. 2009). They are crucial for mediating signals from activated cell surface receptors to regulate cellular processes such as proliferation, cell survival and differentiation (Massi et al. 2014, Trahey et al. 1987). RAS proteins are located in the inner leaflet of the plasma membrane, in response to stimulation of specific cell surface receptors by extracellular ligands, Guanine nucleotide Exchange Factors (GEFs) induce the dissociation of RAS bound GDP for the exchange of GDP for GTP, eliciting the phosphorylation of a cascade of mitogen activated protein kinases (MAPK) and trigger the activation of downstream targets (Herrmann et al. 1996). Such proteins activated by RAS include RAF and Phosphatidylinositol-3-OH kinase (PI3K) through which RAS is able to regulate cell growth and survival (Marais et al. 1995, Wangari-Talbot and Chen 2012). The

inactivation process is facilitated by GTPase activating proteins (GAPs), which enable the formation of RAS-GDP complexes, as a result of hydrolysis of GTP to GDP (Cales et al. 1988, Massi et al. 2014).

Mutations at position 12, 13, or 61 of H-RAS, K-RAS, and N-RAS, hinder the GTPase activity of these proteins by reducing the sensitivity to GAPs, which prevents the dissociation of GTP from RAS (Mor and Philips 2006, Wittinghofer et al. 1997). Consequently, mutations at these sites cause the constitutive activation of the RAS proteins, leading to tumorigenic cell transformation. In melanoma samples that were analyzed, mutations in N-RAS, K-RAS and H-RAS were detected at 20%, 2%, and 1% respectively (Massi et al. 2014). Activating mutations in N-RAS are the second most common alterations in melanoma pathogenesis, occurring frequently in nevi and early-stage melanomas (Trahey et al. 1987, Wangari-Talbot and Chen 2012). The activating N-RAS (Q61R) mutation is the most common, which is represented by the substitution of glutamine (Q) to an arginine (R) at codon 61, leading to constitutive activation of MAPK signaling (Prior et al. 2012).

C. RAF

RAF proteins are serine/threonine kinases, key components of the MAPK pathway and downstream targets of RAS. Three isoforms have been identified, A-RAF, B-RAF, and C-RAF, which are related to the murine retrovirus 3611-MSV, responsible for inducing sarcoma in newborn MSF/N mice (Rapp et al. 1983, Roskoski 2010, Wellbrock et al. 2004). Activated RAS binds to RAF proteins causing their translocation to the cell membrane where RAF activation takes place (Moodie and Wolfman 1994). Following the activation by RAS, RAF proteins phosphorylate MEK1/2, which consequently

phosphorylates ERK 1/2 (Mercer and Pritchard 2003).

Aberrant expression of B-RAF brings about atypical cell proliferation and cancer development through activation of the MAPK pathway. Mutations in B-RAF occur at a very high frequency in human cancers, most notably in melanoma (30-60%), thyroid cancer (30-50%), colorectal cancer (5-20%) and ovarian cancer (~30%) (Davies H. et al. 2002, Wellbrock et al. 2004). The activating B-RAF (V600E) is the most common mutation observed in melanoma, and it accounts for ~92% of the ~65 known B-RAF mutations in malignant melanoma and 82% in nevi (Davies H. et al. 2002, Pollock P. et al. 2003b).

B-RAF (V600E) is a result of a substitution of glutamic acid for valine at codon 600 of exon 15, produced by a single base missense substitution resulting in a T to A transition at nucleotide 1799 (Davies H. et al. 2002). This results in a conformational change in the protein structure of B-RAF that is created by a phosphomimetic glutamic acid present between the threonine 698 and serine 601 sites (Inamdar et al. 2010). Therefore, this obstructs the interaction between the P-loop and the activation segment of B-RAF, leading to its constitutive activation thus increasing the phosphorylation of MEK and ERK (Davies H. et al. 2002, Kolch 2000, Oikonomou et al. 2014). B-RAF (V600E) is frequently found in melanocytic nevi and metastatic melanoma, and has been found to arise early in melanoma pathogenesis, which is then preserved through tumor progression (Ackermann et al. 2005, Omholt et al. 2003).

D. PI3K/AKT

The PI3K (phosphatidylinositol 3-kinase pathway)/AKT signaling pathway is an important regulator of normal physiological processes in the cell. Dysregulation of the

PI3K/AKT pathway is one of the most common events in human cancers, leading to increased tumor cell growth and tumor cell survival. The pathway can be activated through several mechanisms including binding of ligands to receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), as well as by GTP bound RAS proteins (Davies M. A. 2012)**.** Upon stimulation by these signals, activated PI3K phosphorylates the 3' position of the inositol ring of lipids to produce the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP3). As result, PIP3 interacts with the pleckstrin homology (PH) domains of PDK1 (phosphoinositide-dependent kinase 1) and AKT (also known as protein kinase B) to recruit them to the inner leaflet of the plasma membrane (Davies M. A. 2012).

AKT is a serine-threonine protein kinase, of which there are 3 isoforms: AKT1, AKT2 and AKT3. Upon phosphorylation by PIP3, PDK1 phosphorylates AKT at residue Thr308 (catalytic domain) or PDK2 phosphorylates AKT at Ser473 (regulatory domain) (Alessi et al. 1997, Toker and Newton 2000). Activated AKT phosphorylates many essential downstream proteins that have the R-X-R-X-X-S/T recognition motif in order to regulate key cellular processes (Lawlor and Alessi 2001). Activated AKT, promotes cell cycle progression through the phosphorylating inactivation of FOXO transcription factors; the metabolic enzyme GSK3 (Davies M. A. 2012, Manning and Cantley 2007), and through its phosphorylation of MDM2 to promote the degradation of p53 (Zhou et al. 2001). AKT prevents apoptosis, by phosphorylating the pro-apoptotic protein BAD, to inhibit the binding of BAD to the anti-apoptotic proteins Bcl-2 and Bcl-xL (Datta et al. 1997). Moreover, AKT activates mTOR by its inactivating phosphorylation of the tuberous sclerosis complex 2 (TSC2), leading to increased cellular growth and protein

translation (Ma et al. 2005, Sarbassov et al. 2005).

Aberrant activation of the PI3K/AKT pathway in cancer may be mediated by mutations in PI3KCA the gene that encodes the p110 subunit of PI3K (Samuels et al. 2004), alterations in AKT (Bellacosa et al. 1995, Cheung et al. 2008), loss or activating mutation(s) of PTEN (Hopkins and Parsons 2014), and activating mutations in RAS. In melanoma, mutations in PI3K are less common (Davies M. A. 2012), whereas loss or activating mutation(s) of PTEN is more common and has been detected in 10% to 30% of cutaneous melanoma, resulting in an overactive PI3K/AKT pathway (Wu et al. 2003).

E. PTEN

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor that functions as the negative regulator of the PI3K/AKT pathway and is commonly deleted or mutated in melanoma. PTEN utilizes its phosphatase activity to remove the 3 phosphates on phosphatidylinositol (3,4,5)-triphosphate (PIP3) to produce phosphatidylinositol (4,5)-biphosphate (PIP2) thereby inhibiting PIP3 mediated activation of PDK1 and AKT/PKB (Myers et al. 1998, Stambolic et al. 1998). Multiple PTEN alterations have been detected in melanoma, including allelic loss in 20% of melanomas tumors, hemizygous deletions and inactivation in ~60% of melanoma cell lines (Aguissa-Toure and Li 2012, Pollock P. M. et al. 2002, Yin and Shen 2008). These defects in PTEN function render the dysregulation of the PI3K/AKT, resulting in oncogenic cellular transformation.

F. CDKN2A (INK4a/ARF)

The cyclin-dependent kinase 2A (CDKN2A) located on chromosome 9p21 encodes two essential alternative spliced tumor suppressor proteins: the cyclin-dependent kinase

inhibitor p16INK4a and the p53 activator p14ARF (Quelle et al. 1995, Wangari-Talbot and Chen 2012). Both proteins play important roles in regulating the cell cycle and alterations in the INK4a/ARF locus due to its dual coding capacity thus markedly increases the risk for developing cancer (Rizos et al. 2001). Inherited germline mutations in the INK4a/ARF locus have been associated with melanoma susceptibility in 20-40% of families having multiple cases of melanoma (Kefford et al. 1999). Moreover, somatic mutations in INK4a/ARF have also been observed in sporadic melanoma cases (Gast et al. 2010, Walker et al. 1998). p16INK4a, which is encoded by exon 1α (exons 1α, 2, 3) of CDKN2A, binds to cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) to inhibit the phosphorylation of the retinoblastoma susceptibility gene product (Rb), in order to block the G1 to S phase transition (Koh et al. 1995, Serrano et al. 1993). Rb phosphorylation by CDK4 and CDK6 results in the release of the E2F1 transcription factor resulting in the initiation of DNA replication in the cell cycle (Ohtani et al. 2004, Stevaux and Dyson 2002). Therefore, mutations or loss of p16 impairs its ability to interact with CDK4 and CDK6 in the regulatory arrest of the cell cycle, allowing unchecked progression of the cell cycle and promote cell transformation and tumor formation (Goldstein et al. 2000, Mehnert and Kluger 2012).

The 1β (exons 1β, 2, 3) transcript of CDKN2A encodes p14ARF, which binds to the human double minute-2 (HDM2) protein, promotes its degradation and blocks HDM2 mediated ubiquitination of p53, thus enhancing the levels of p53 (Tao and Levine 1999, Midgley et al. 2000). When p14ARF is mutated, there is an increase in HDM2-meadiated ubiquitination and degradation of p53 leading to uncontrolled cell growth (Kurki et al. 2004).

MATERIALS AND METHODS

A. Cell Culture

Normal mouse melanocytes (MelanA) were provided by Dr. Dorothy Bennett (St. George's University of London, UK) and grown in RPMI-1640 medium (Sigma, St. Louis MO) with 10% fetal bovine serum (FBS) (Sigma, St. Louis MO), 100 U/ml of penicillin/streptomycin (Gibco, Grand Island, NY), and 200nM 12-O-tetradecanoyphorbol-13-acetate [TPA] (Sigma, St. Louis MO).

B. DNA Transfection

DNA transfections were executed using DOTAP transfection reagent (Roche, Basel, Switzerland) following manufacturer's instructions. pEFmycB-RAF (V600E) plasmid was provided by Dr. Richard Morales (Sheridan et al.,2008). The plasmid was co-transfected with geneticin plasmid PCIneo used for drug selection of stable clones (Yu and Chen, 2012). The mutant B-RAF plasmid (4μg) and PCIneo plasmid (2ug) or PCIneo plasmid alone (4 μg) for vector control was transfected into MelanA cells ($4x10^5$ per 60-mm plate) (Yu and Chen, 2012). Stable clones were selected by resistance to G418 (200 μg/ml) (Yu and Chen, 2012).

C. BJB Mouse Strain

We made used of a transgenic mouse model that harbors an inducible B-RAF (V600E) genotype using LoxP-stop-LoxP (LSL)/Cre recombinase technology. The transgenic mouse model (BJB) (loxP-Braf(V600E)-loxP;Cre^{ERT2}), was generated by crossing a B-RAF^{CA} mouse (loxP-B-RAF (V600E)-loxP) which encodes a germline conditional B-RAF (V600E) allele, with a B6CST mouse (Cre^{ERT2}) which harbors conditionally active Cre recombinase only in melanocytes (Bosenberg et al. 2006, Dankort 2009). In order to

induce the rearrangement of the loxP-B-RAF (V600E)-loxP locus in melanocytes of BJB mice, 15 mg/ml of 4-Hydroxitamoxifen (Sigma, St. Louis, MO) was directly applied to their ears for 4 days. BJB mice were sacrificed at 4, 8, and 12 weeks after 4-hydroxytamoxifen treatments. Protein was extracted from the ears and used to perform Western immunoblots.

D. Genomic DNA Extraction

Genomic DNA was extracted from BJB mice tails for genotyping. 50 µl of (10mg/mL) Proteinase K (Promega, Madison, WI) was used to digest the tails overnight in a 55°C water bath. The next day, 50 µl of 3M NaOAc, 275 µl of chloroform/isoamyalcohol (24:1), and 275 µl of phenol was added to each sample, and supernatant was collected by centrifugation at 25,000 x g for 10 minutes. Supernatant was transferred to a fresh tube containing 300 µl of isopropyl alcohol. Samples were centrifuged at 25,000 x g for 10 minutes. The resulting supernatant was discarded, and 1000 µl of cold 70% ethanol was added, and samples were centrifuged for 10 minutes. The 70% ethanol was then discarded, and 1000 µl cold 100% ethanol was added followed by a 10 minute centrifugation at 25,000 x g. The resulting pellet was air dried and re-suspended in 150 µl of Tris EDTA buffer [100mM Tris, 10mM EDTA (pH=8.0)] at 55 °C over night. One microliter of the resulting genomic DNA was electrophoresed in a 0.8% agarose gel with ethidium bromide, and compared to a 200 ng Hind III digested lambda DNA ladder to determine the concentration based on the intensity of the DNA bands.

E. Genotyping by PCR

The extracted genomic BJB mice DNA (10ng) were used to perform PCR using primers flanking the B-RAF gene (Forward primer 5'-TGAGTATTTTTGTGGC AACTGC-3';

Reverse primer 5'- CTCTGCTGGGAAAGCGGC-3'). Using a PTC-100 programmable thermo cycler, the PCR reactions were performed under the following conditions: 1 minute at 96 °C, 30 seconds at 95 °C, 45 seconds at the annealing temperature 52 °C, and 45 seconds at 72° C for 40 cycles and 11 minutes at 72° C. The genotype of the B-RAF gene was determined by agarose gel electrophoresis (1.5% agarose gel with ethidium bromide) of PCR products compared to a 200 ng ϕX174 RF/HaeIII DNA ladder.

F. RNA extraction

Frozen BJB skin samples were made to powder by mortar and pestle in liquid nitrogen. TRI Reagent LS RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Cincinnati, OH) was added to the sample and homogenized using a polytron (Brinkmann Instruments, Westbury, NY). RNA isolation of the homogenized samples was done as specified by the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). The concentration and integrity of the resulting RNA was determined by running two microliters of RNA in a 1% RNAse free agarose gel and compared to a 2µg RNA ladder.

G. cDNA generation and PCR

One microgram of RNA was incubated with 1µl of random primers (3µg/µl) [Invitrogen, Grand Island, NY], 1µl of dNTP mix (10mM each) [Roche, Basel, Switzerland] and RNAse-free ddH_20. The samples (10µl) were heated at 65 °C for 5 minutes and then placed on ice for one minute. 2µl of M-MLVRT 5X buffer [Promega, Madison, WI], 4µl 25mM $MgCl_2$ [Boehringer Mannhein, Germany], 2µl 0.1M DTT [Invitrogen, Grand Island, NY], 1µl rRNasin (40u/µl) [Promega, Madison, WI], and 1µl Superscript II reverse transcriptase (200 u/µl) [Invitrogen, Grand Island, NY] was added. Samples were

incubated at 50 °C for 50 minutes, then at 85 °C for 5 minutes. The cDNA was then used to do PCR using primers flanking the gene encoding GAPDH (forward primer 5'-GTGATGGGTGTGAACCACGAG-3'; reverse primer 5'-CTGCTTCACCACCTTCTTGAT-3') as control, or Grm1 (foward primer 5'-CTGCAGTACACAGAGGCTAATC-3'; Reverse primer 5'-CCACTCGAGGTAACGGATAGTA-3'). Using a PTC-100 programmable thermo cycler, the PCR reactions were performed under the following conditions: 1 minutes at 95 °C, 45 seconds at 94 °C, 45 seconds at the annealing temperature 63 °C, 4 minutes at 72° C, 45 seconds at 94 °C for 14 cycles, 45 seconds at 50 °C, 4 minutes at 72° C, 45 seconds at 94 °C for 29 cycles, and 10 minutes at 72° C.

H. Protein Extraction, Western immunoblots

Cells were grown to 80-90% confluence. Protein lysates were prepared by wash with cold 1X PBS, extraction buffer [1X laemmli sample buffer (Bio Rad, Hercules, CA), 2-mercaptoethanol (Sigma, St. Louis, MO)] was added to collect cells. The volume of extraction buffer added was dependent on the size of the plate, for a 60 mm plate 200µl of extraction buffer was used. Cells were incubated at 99 °C in a PTC-100 programmable thermo cycler for 10 minutes for further cell lysis. Next, supernatants were collected by centrifugation at 25,000 x g for 10 minutes. 10µl of protein lysate were loaded per lane in the Western immunoblots.

Frozen BJB mice skin samples were turned to powder by mortar and pestle in liquid nitrogen. Extraction buffer [1X laemmli sample buffer (Bio Rad, Hercules, CA), 2-mercaptoethanol (Sigma, St. Louis, MO)] was added to the samples and homogenized using a polytron (Brinkmann Instruments, Westbury, NY). Samples were then incubated

for 2 hours on a shaker at 4 °C, then incubated at 99 °C in a PTC-100 programmable thermo cycler for 10 minutes. Next, supernatants were collected by centrifugation at 25,000 x g for 10 minutes. 25µl of protein lysate were loaded per lane in 7.5% or 10% polyacrylamide gels (Bio Rad, Hercules, CA). Gel electrophoresis was done at 150 V. The proteins were then transferred to a nitrocellulose membrane at 150 mA at 4 °C. After transfer Ponceau S Red (Sigma, St. Louis, MO) was used to stain the membrane to confirm that equal amounts of proteins were loaded in each lane. Membranes were blocked with 0.25% non-fat dry milk in TBST (Tris buffered saline + Tween 20) using a Snap ID (Millipore corporation, Billerica, MA). Membranes were then incubated over night with primary antibodies as indicated in figure legends. Bands were detected using Lumina Crescendo Western HRP substrate reagents (Millipore corporation, Billerica, MA).

I. Antibodies

Anti-Grm1 antibody was acquired from BD Biosciences (Sparks, MD). Anti-myc, HA, total-AKT, phopho-AKT (Thr308), total-ERK1/2, phospho-ERK1/2, total-PDK1, phospho-PDK1 and PTEN, were purchased from Cell Signaling Technology (Danvers, MA); anti- total IGF-IR, phospho-IGF-IR were obtain from Invitrogen (Frederick, MD); anti- p16INK4a antibody was obtain from Neo Markers (Fremont, CA); anti- B-RAF (V600E) antibody was purchased from New East Biosciences (Malvern, PA); monoclonal α-Tubulin antibody was acquired from Sigma (St. Louis, MO)

RESULTS

PART I: Mutated B-RAF (V600E) and Mutated N-RAS (Q61R) Transgenic mice

In our experimental model system the activation of the MAPK pathway in human melanoma seems to be dependent on Grm1 expression, we want to see if there is a relationship between B-RAF/N-RAS and GRM1 activation in melanoma. We previously investigated whether mutations in either B-RAF or N-RAS may initiate events leading to Grm1 expression. We have looked at tumors derived from transgenic mice harboring a mutated N-RAS (Q61R) genotype in an INK4a-null background that developed metastatic melanoma with high penetrance (Ackermann et al., 2005; Yu and Chen, 2012). These transgenic mice with mutated N-RAS in an INK4a-null background showed strong expression of Grm1 by immunohistochemical staining of the excised tumor tissues (Figure. 1D) (Yu and Chen, 2012). By similar methods, Grm1 expression was also observed in excised tumors from transgenic mice harboring mutated B-RAF (V600E) in a PTEN null background (Figure. 1C), but Grm1 expression was reduced in tumors with mutant B-RAF (V600E) in heterozygous PTEN background (Figure. 1B) (Yu and Chen, 2012).

PARTII: Transfection of Mutated B-RAF (V600E) into Normal Melanocytes

A. MelanA clones expressing B-RAF (V600E) express Grm1

To further examine whether mutant B-RAF (V600E) expression in melanocytes is capable to activate Grm1, we transfected MelanA cells, a normal melanocytic cell line, with a myc-tagged B-RAF (V600E) plasmid. We assessed the incorporation of B-RAF (V600E) in the isolated clones by looking at the myc-tag expression at the protein level by Western blot, and compared to a MelanA vector control without the myc-tagged B-RAF (V600E) plasmid (Figure. 2) (Yu and Chen, 2012). Only the transfected clones exhibited myc-tagged B-RAF (V600E) (Figure. 2). Western blot analysis of the MelanA B-RAF (V600E) clones, showed a number of these clones were positive for Grm1 expression as compared to the parent MelanA clone, which was used as a negative control (Figure. 3) (Yu and Chen, 2012).

Next we wanted to see if ectopically expressed Grm1 would promote mutation of wild type B-RAF. To do this, we transfected Grm1 c-DNA into MelanA cells to produce MASS20 cells, which have been shown to be tumorigenic in *vivo* (Shin S. S. et al. 2008a). DNA sequencing of the B-RAF gene in MASS20 cells, found wild type sequence at the site of the B-RAF (V600E) mutation (data not shown) (Yu and Chen, 2012). This observation indicates that induction of Grm1 expression in MelanA cells does not induce mutation in B-RAF.

B. B-RAF (V600E) modulates Grm1 expression in MelanA B-RAF (V600E) clones

MelanA B-RAF (V600E) clone 25, was treated with PLX 4032 a small molecule inhibitor specific to B-RAF (V600E) (0.1 μM/ 0.3 μM) for 24 and 48 hours, to assess the

influence of B-RAF (V600E) activity on the expression of Grm1 in this cell line. Protein extracts were prepared after each time point and used to performed Western immunoblots using antibodies against Grm1. Grm1 levels were significantly reduced in a concentration dependent manner at 24 and 48 hours after treatment (Figure. 4). This result demonstrates that inhibition of B-RAF function down regulates Grm1 expression, thus supporting our previous observation that B-RAF (V600E) promotes Grm1 expression in MelanA cells.

C. PTEN and INK4a expression in MelanA B-RAF (V600E) clones

It is possible that induction of Grm1 expression by B-RAF (V600E) may require another genetic alteration such as loss of PTEN or INK4a, since more Grm1 expression was observed in the IHC staining of the mutated B-RAF model in a PTEN null background than in the heterozygous PTEN background. Therefore, we evaluated the expression of PTEN and p16INK4a in the MelanA B-RAF (V600E) clones. Western blot analysis were negative for p16INK4a expression (Figure. 5), and this is not a surprise because it has been shown before that MelanA cells tend to loss INK4a in order to become immortalized (Sviderskaya et al. 2002). On the other hand, we detected PTEN expression (Figure. 5) indicating that loss of PTEN may not be necessary for Grm1 expression to become activated by mutated B-RAF. We cannot fully come to this conclusion because PTEN has various mutations that might affect its functionality. Consequently, we need to assess the functionality of PTEN in these clones before we can confirm that loss of PTEN expression/function is not necessary for Grm1 expression to become activated by mutated B-RAF.

D. P-PDK1 expression in MelanA B-RAF (V600E) clones

PTEN negatively regulates the PI3K/AKT pathway, by facilitating the removal of a phosphate at the 3 position of the inositol ring of phosphatidylinositol (3,4,5)-triphosphate (PIP3), to prevent the phosphorylation of PDK1, which phosphorylates and activates AKT (Hopkins and Parsons 2014). To assess the functionality of PTEN in MelanA B-RAF (V600E) clones, we examined the phosphorylation of PDK1 by Western blot. Phosphorylation of PDK1 was detected in all MelanA-mutated B-RAF cell lines and at much reduced levels in the MelanA parent (Figure. 6). This confirms that even though PTEN is expressed in all mutated B-RAF-MelanA clones, it may not be functional as suggested by the appearance of phosphorylated PDK1 and elevated basal pAKT (see below).

E. Elevated levels of basal expression of P-AKT and P-ERK in MelanA B-RAF (V600E) clones.

Our group has reported that the activation of Grm1 mediates oncogenic transformation of melanocytes by activating the MAPK and P13K/AKT pathways (Figure. 7) (Namkoong et al. 2007, Shin S. S. et al. 2008a). Since Grm1 expression was detected in protein extracts from MelanA B-RAF (V600E) clones, we investigated the functionality of Grm1 in these cells lines. We looked at the basal level of phosphorylation of AKT and ERK in these clones and a MelanA vector clone by Western blot. Strong phosphorylation of AKT and ERK was detected in these cultured MelanA-mutated –B-RAF clones whereas none was observed in the MelanA vector (Figure. 8). These data suggest that activation of Grm1 expression in these clones was sufficient to

upregulate basal levels of activated AKT and ERK in the absence of stimulation.

F. P-IGF-1R expression in MelanA B-RAF (V600E) clones

The insulin-like growth factor (IGF-1) is an upstream regulator of AKT; its activity has been shown to trigger activation of AKT in MelanA expressing Grm1 clones (MASS20) (Shin S., S. et al. 2010). Furthermore, we demonstrated that Grm1 trans activates the IGF-1R receptor (Figure. 9), which in turn activates AKT in cultured MASS20 clones (Teh et al. 2014). Based on these earlier observations we were interested to know whether Grm1 modulates the activation of AKT in the MelanA B-RAF (V600E) clones by trans activating the IGF-1R receptor. Western blot analysis of protein lysates from MelanA vector, -mutated B-RAF and positive control, MASS20, treated with the Grm1 agonist, L-quisqualate (Q) (10 μM), were performed using antibody against phosphorylated IGF-1R receptor. Phosphorylation of IGF-1R was detected in MelanA B-RAF (V600E) clones and in MASS20 treated with quisqualate (10 μM); Phosphorylation of IGF-1R was not observed in the MelanA vector (Figure. 10).

PARTIII: B-RAF (V600E) expressing mice (BJB)

We detected Grm1 expression by immuno staining in excised tumors from the mutated B-RAF mouse model in a PTEN null background, but reduced in the heterozygous PTEN background (Yu and Chen, 2012). It is possible that induction of Grm1 expression by B-RAF (V600E) in melanocytes requires another genetic alteration such as loss of PTEN or p16INK4a/p19ARF. This notion cannot be tested in *vitro* because melanocytes tend to acquire the loss of p16INK4a/p19ARF in culture conditions in order to become immortalized (Sviderskaya et al. 2002). In culture conditions we were only able to demonstrate the expression of PTEN but functionally inactive by the appearance of phospho-PDK1 only in MelanA-mutated –B-RAF clones not in MelanA vector control, indicating that PTEN is not suppressing the phosphorylation of PIP3.

We then turned to the *in vivo* system to examine if induction of Grm1 expression by B-RAF (V600E) in melanocytes requires the loss PTEN or p16INK4a/p19ARF. We took advantage of a genetically modified mice with an inducible B-RAF (V600E) genotype off the endogenous B-RAF gene using LoxP-stop-LoxP (LSL)/Cre recombinase technology (Dhomen et al. 2009). The BJB mouse (loxP-B-RAF (V600E)-loxP;CreERT2) was generated by crossing a B-RAF^{CA} mouse (loxP-Braf(V600E)-loxP) which encodes a germline conditional B-RAF (V600E) allele, with a B6CST mouse (Cre^{ERT2}) which harbors conditionally active Cre recombinase only in melanocytes (Bosenberg et al. 2006, Dankort 2009) (Figure. 11). BJB mice carrying the mutated allele, express normal B-RAF prior to Cre-mediated recombination. PCR of genomic DNA from BJB mice using primers flanking the B-RAF gene show a heterozygous B-RAF genotype as indicated by bands at 385 and 185 base pairs (Figure. 12).

In order to induce B-RAF (V600E) expression in melanocytes, 15 mg/ml of 4-hydroxitamoxifen was directly applied to the ears of BJB mice for 4 days at one month after birth, to induce the activation of Cre recombinase (Cre^{ERT2}). In the absence of 4-hydroxytamoxifen BJB mice display normal phenotypes. At eight weeks post treatment, the treated mice exhibit skin pigmentation in their ears and tails, presumably from increased proliferation of melanocytes due to B-RAF (V600E) induction (Figure. 13). Protein was extracted from ears at 4, 8 and 12 weeks after treatment to assess B-RAF (V600E), Grm1, PTEN and p16INK4a expression by Western immunoblot analysis.

B. Grm1 expression in B-RAF (V600E) expressing mice

The observation that BJB mice show a hyper-pigmented phenotype on their skin after 4-hydroxytamoxifen administrations, suggests B-RAF (V600E) expression in melanocytes. To further confirm this, Western immunoblots were performed on protein lysates extracted from the ear of 4-hydroxytamoxifen treated and non-treated mice using an antibody specifically against B-RAF (V600E). Non-treated samples were negative for B-RAF (V600E); however strong B-RAF (V600E) expression was detected in all the 4-hydroxytamoxifen treated samples (Figure. 14).

Similarly, we examined Grm1 expression in the B-RAF (V600E) induced samples. Only 4-hydroxytamoxifen treated samples were positive for Grm1 expression as compare to protein lysates from a Grm1 positive transgenic mouse (LLA) (Figure. 15). Mice not-treated with 4-hydroxytamoxifen were negative for Grm1 expression (Figure. 15). Western immunoblots were also used to confirm PTEN and p16INK4a expression in BJB mice. As expected, PTEN and p16INK4a expression was detected in all the analyzed samples (Figure. 16). However, PTEN was functionally inactive as demonstrated by the

appearance of phospho-PDK1 (Figure. 17) in both 4-hydroxytamoxifen treated and not-treated mice, suggesting other unknown factors but not the exchange of wild type B-RAF with the mutated one by 4-hydroxytamoxifen may be involved in the deregulation of PTEN

To further confirm Grm1 expression in BJB mice expressing mutated B-RAF, immunohistochemical staining was performed on skin samples of 4-hydroxytamoxifen induced B-RAF (V600E) BJB mice and not induced mice. LLA skin samples were used as positive control for. Skin samples of B-RAF (V600E) expressing mice showed strong Grm1 expression whereas the non-expressing B-RAF (V600E) mice showed markedly reduced Grm1 expression (Figure. 18). Taken together, these results suggests that induction of mutated B-RAF (V600E) may be sufficient to promote activation of Grm1 expression in *vitro* and *in vivo*, and that ablation of PTEN expression/function but not of INK4a may be required.

DISCUSSION

In melanocytes, the MAPK pathway regulates cell proliferation and differentiation in response to signals coming from stimulated RTKs (receptor tyrosine kinases) and GPCRs (G-protein coupled receptors) (Busca and Ballotti 2000, Wellbrock et al. 2002). Constitutive activation of the MAPK pathway as a result of activating mutations in either B-RAF or N-RAS trigger the aberrant transformation of melanocytes, and have been identified as the most frequent genetic alterations in melanoma. Approximately 50% of melanoma patients carry mutations in B-RAF and ~15-20% in N-RAS (Wellbrock and Hurlstone 2010). Although the oncogenic expressions of B-RAF or N-RAS elicit anomalous melanocyte proliferation, they are insufficient to induce melanoma, due to activation of senescence, which results in the formation benign nevi only (Chin 1999, Michaloglou et al. 2005, Sviderskaya et al. 2002). However, various groups have previously reported that mutated B-RAF or N-RAS accompanied by the silencing of either PTEN or p16INK4a bypass senescence and promote progression to malignant melanoma in mice (Ackermann et al. 2005, Bardeesy et al. 2001, Dankort 2009, Ferguson et al. 2010, Nogueira et al. 2010).

Our group has identified a novel potential oncogene in melanoma, a G-protein coupled receptor, metabotropic glutamate receptor 1 (GRM1). We showed earlier that aberrant expression of the mouse Grm1 in melanocytes alone was sufficient to induce spontaneous melanoma development *in vivo*. In addition, the human relevance of this discovery was demonstrated by revealing that >60% of human melanoma, particularly those in the late stages exhibit ectopic expression of human GRM1 (Namkoong et al. 2007, Pollock P. M. et al. 2003). Moreover, GRM1 in human melanoma cell lines were

shown to be functional by the responsiveness to GRM1 agonist and antagonists using components of the MAPK pathway as read-outs (Namkoong et al. 2007). Interestingly, strong Grm1 expression was detected in immunohistochemical staining of excised tumor tissues from transgenic mice harboring a mutated N-RAS (Q61R) genotype in an INK4a null background. Similarly, Grm1 expression was also observed in tumor tissues from transgenic mice harboring mutated B-RAF (V600E) in a PTEN null background, but Grm1 expression was reduced in tumor tissues with B-RAF (V600E) in a heterozygous PTEN background. These exciting preliminary findings suggest that mutated B-RAF or N-RAS along with loss of PTEN/INKa may initiate events leading to activation of Grm1 expression.

The notion that B-RAF (V600E) alone is not sufficient to drive melanomagenesis has been well established. Patton et al. demonstrated that over expression of B-RAF (V600E) in zebrafish melanocytes, was only able to induce nevi formation that did not progress to melanoma (Dhomen et al. 2009, Patton et al. 2005). In addition, the fact that B-RAF (V600E) is found in 80% of human nevi that remain unchanged for many years, indicates that mutated B-RAF induces senescence in melanocytes (Davies H. et al. 2002b, Dhomen et al. 2009, Pollock P. et al. 2003b). Therefore, it is apparent that B-RAF (V600E) requires additional genetic modifications in order to induce malignant melanocytic transformation.

We postulate that Grm1 may be an intermediate signaling molecule in B-RAF (V600E) expressing melanomas that contribute to melanomagenesis. The observation that our founder transgenic mice (TG-3) does not harbor a B-RAF (V600E) mutation (unpublished data) is consistent with our finding that transfection of Grm1 cDNA into

MelanA cells (MASS20), did not induce mutation of B-RAF. Interestingly, B-RAF (V600E) transfected into MelanA cells induced the expression of Grm1. Wellbrock et al. have shown that MelanA cells expressing B-RAF (V600E) induce tumor development in nude mice (Wellbrock et al. 2004b). This phenomenon can be explained by the fact that MelanA cells are p16INKa deficient (Sviderskaya et al. 2002). In addition, analysis of MelanA B-RAF (V600E) demonstrates that the functionality of PTEN is inactive as confirm by the appearance of phosphorylated PDK1. Therefore, it is possible that together with the loss of INK4a and inactive PTEN, Grm1 may function as mediator in the tumor formation of these cells. Furthermore, we show evidence that activation of Grm1 by B-RAF (V600E) in MelanA B-RAF (V600E) modulates the activation of AKT, by trans-activating the IGF-1R receptor. This supports earlier reports of the transactivation of IGF-1R by Grm1 in MASS20 cells to activate AKT (Teh et al. 2014).

Activation of Grm1 expression by mutated B-RAF was further confirmed in our *in vivo* system. Contrary to our *in vitro* system, BJB mice harbor INK4a, but PTEN is not fully functional as confirm by phosphorylation of PDK1. The inducible expression of B-RAF (V600E) in melanocytes of BJB led to expression of Grm1. Taken together, our current findings suggest that induction of B-RAF (V600E) promotes activation of Grm1 expression in mouse melanocytes and that loss or functionally inactivation of PTEN but not of INK4a may be necessary for Grm1 activation. Future studies will focus on the molecular mechanisms underlying the pathway by which Grm1 becomes activated as suggested by the results of this study.

FUTURE DIRECTIONS

In our system, activation of Grm1 expression seems to be dependent on B-RAF (V600E), but the mechanism by which this occurs remains unknown. Here we showed that inhibiting the functionality of B-RAF (V600E) with PLX4032 in MelanA B-RAF (V600E) cells reduced Grm1 expression. Therefore, we are currently doing transfections with si-B-RAF (V600E) to knockdown mutated B-RAF in MelanA B-RAF (V600E) cells, to see if inhibition of B-RAF (V600E) expression modulates Grm1 expression. Finally, we will begin to assess what transcription factor(s) are activated by B-RAF (V600E) that led to the expression of Grm1. This can be done by comparing MelanA B-RAF (V600E) cells with that of MelanA vector control in cDNA arrays made with cDNA derived from several time points after introduction of mutated B-RAF into MelanA. Finally to validate the participation of particular transcription factor(s) we will introduce the transcription factor(s) into MelanA vec cells and assess if that was sufficient to activate Grm1 expression. As a complementary approach we can suppress the transcription factor(s) expression by silencing RNA and assess if Grm1 expression is reduced or abolished.

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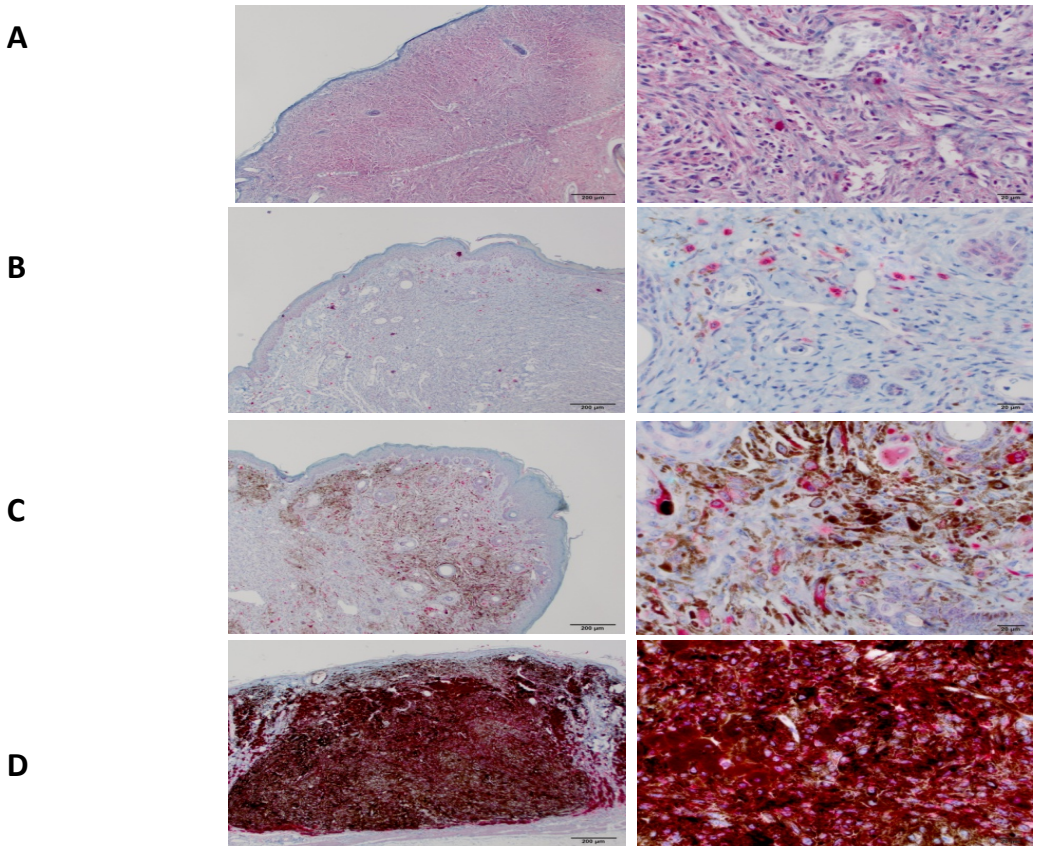
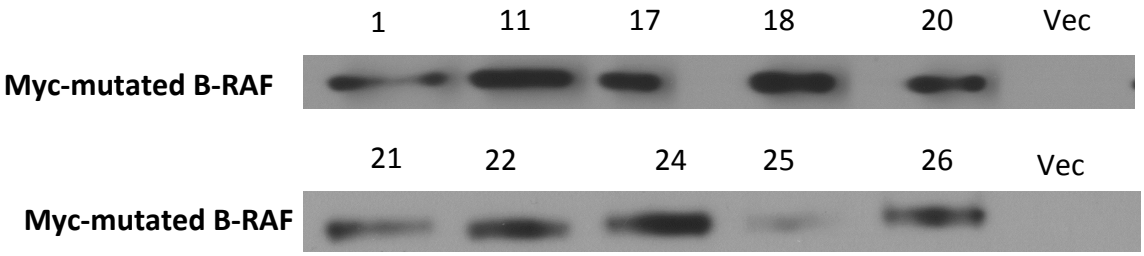
FIGURES



(Yu and Chen, 2012)

Figure 1. Immunohistochemistry staining of tumors from transgenic mice for Grm1

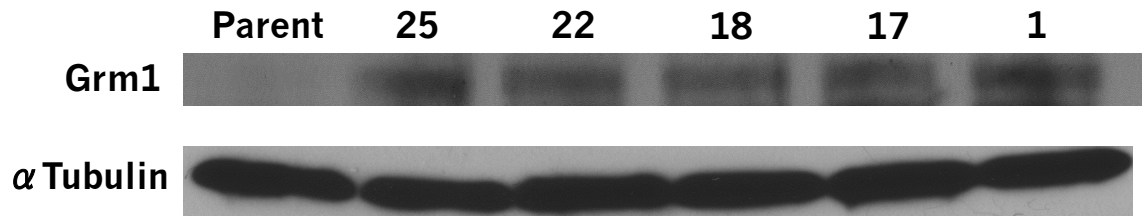
Grm1 expression in melanoma tumors from transgenic mice. **A.** Positive control TG3 mice. **B.** Mutated B-RAF (V600E) in heterozygous PTEN background. **C.** Mutated B-RAF (V600E) in PTEN null background. **D**. Mutated N-RAF (Q61R) in an INK4a null background. Tumors from the mutant N-RAS mouse displays strong Grm1 expression. Tumors from the mutant B-RAF in the PTEN null background also show significant Grm1 expression. The mutant B-RAF tumors in the heterozygous PTEN background had reduced Grm1 expression.



(Yu and Chen, 2012)

Figure 2. MelanA mutated B-RAF clones

Immortalized mouse melanocytes, MelanA, were transfected with pEF-myc-B-RAF (V600E) plasmid. Stable clones were isolated by resistance to G418 (200 μg/ml). Protein extracts from the transfected clones were isolated and used for Western immunoblots of myc-tagged mutated B-RAF protein to confirm integration of mutated B-RAF (V600E) protein. Protein lysates from MelanA vector clones were used as control.



(Yu and Chen, 2012)

Figure 3. Grm1 expression in MelanA mutated BRAF clones

Protein extracts of MelanA expressing B-RAF (V600E) clones were positive for Grm1 expression. The parent MelanA cell line is negative for Grm1 expression prior to transfection with pEF-myc-B-RAF (V600E). Levels of α-Tubulin were used as loading controls. This shows that Grm1 is expressed following the acquisition of mutated B-RAF (V600E).

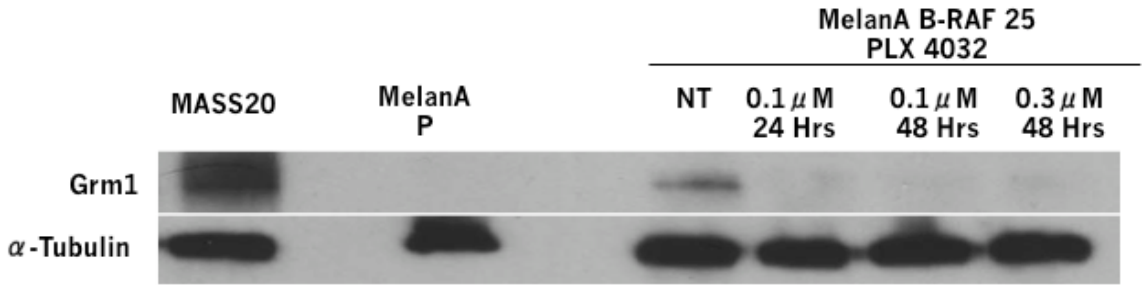


Figure 4. B-RAF (V600E) modulates Grm1 expression *in vitro*

Cultured MelanA B-RAF (V600E) clone 25, was treated with PLX 4032 a small molecule inhibitor specific to B-RAF (V600E) (0.1 μM/ 0.3 μM) for 24 and 48 hours. Protein extracts were prepared after each time point and used to performed Western immunoblots using antibodies against Grm1.

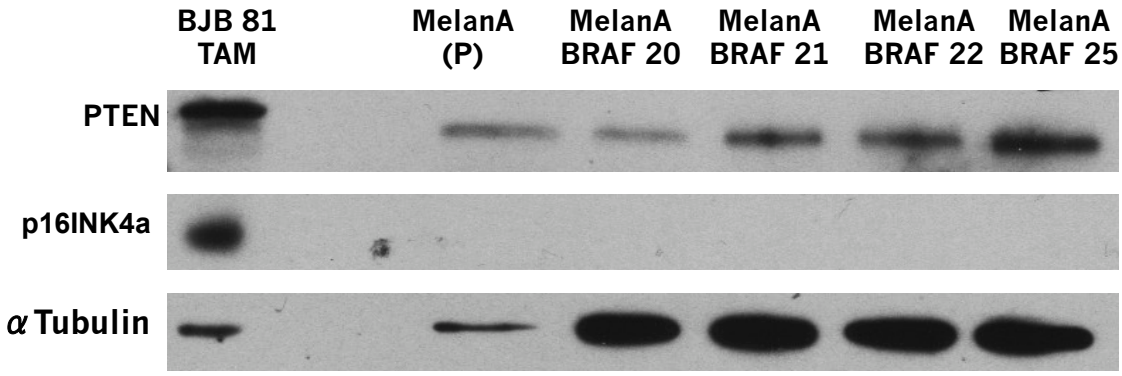


Figure 5. PTEN and INK4a expression in MelanA B-RAF V600E clones

Western immunoblots of endogenous levels of PTEN and p16INK4a in MelanA parent and in the MelanA B-RAF (V600E) clones. Protein lysate from transgenic mouse BJB 81 was used as positive control for PTEN and p16INK4a expression. Levels of α-Tubulin were used as loading controls.

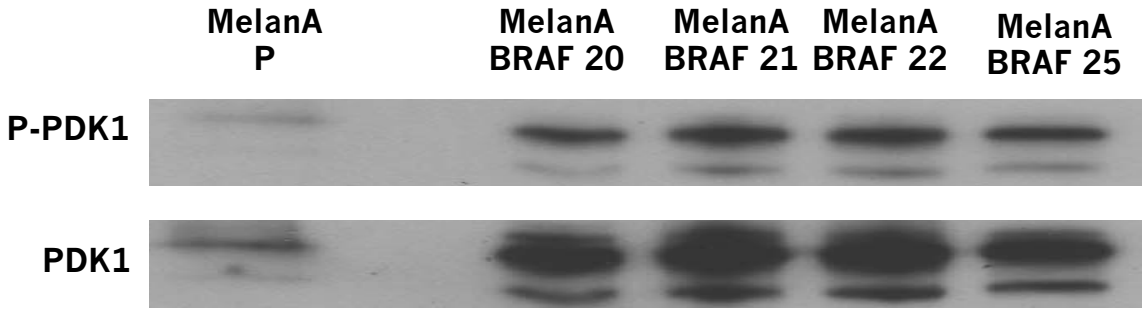
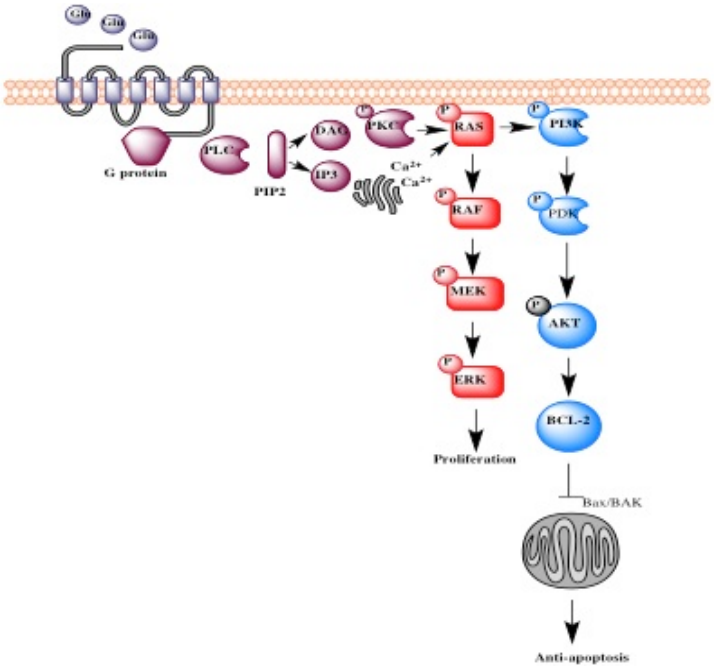


Figure 6. PDK1 activation in MelanA parent and in MelanA expressing B-RAF (V600E) clones.

Cell lysates from MelanA parent and MelanA B-RAF (V600E) clones were prepared and Western immunoblots of phospho-PDK1 were performed. The membrane was then stripped and probed with total PDK1.



Adapted from Teh and Chen, 2012

Figure 7. Signal transduction pathways elicited by stimulated Grm1.

Grm1 activation by its ligand, glutamate, in melanocytes elicits downstream signals that activate the MAPK and PI3K/AKT pathway two of the most stimulated signaling cascades in melanoma. Activation of the MAPK pathway stimulates cell proliferation, and activation of the PI3K/AKT promotes cell survival.

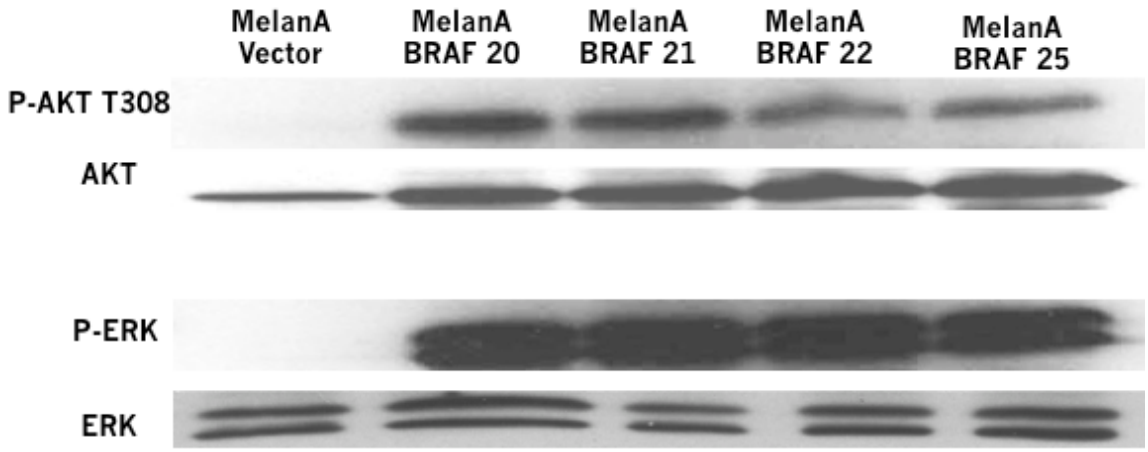
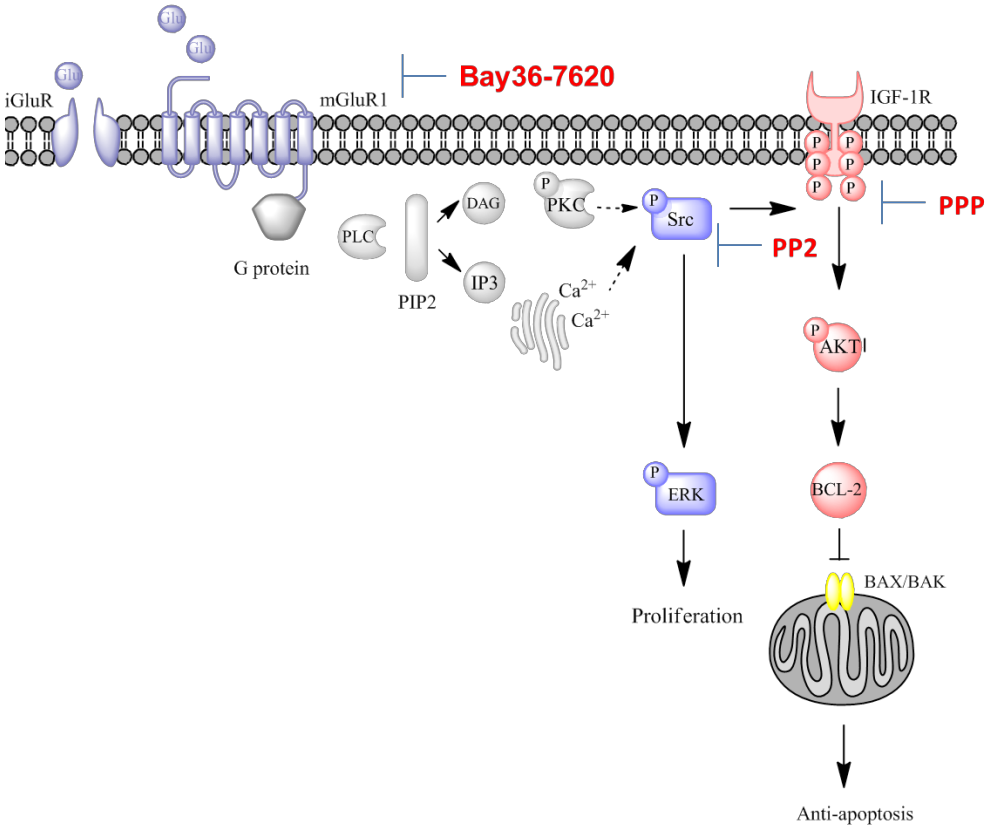


Figure 8. Elevated basal expression of P-AKT and P-ERK in MelanA BRAF (V600E) clones.

Cell lysates from MelanA vector and MelanA B-RAF (V600E) clones were prepared and Western immunoblots of basal phospho-AKT (T308) and basal Phospho-ERK were performed. The membranes were then stripped and probed with total AKT and total ERK.



Adapted from Teh and Chen, 2014

Figure 9. Proposed signaling pathway activated by Grm1 and mediated by IGF-1R transactivation.

The stimulation of the insulin-like growth factor (IGF-1) has been shown to trigger activation of AKT. Our group has demonstrated that Grm1 activity modulates the activation of AKT by trans-activating the IGF-1R receptor.

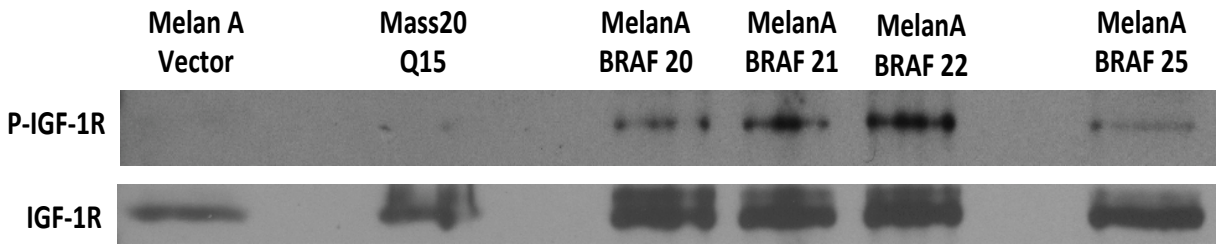


Figure 10. P-IGF-1R expression in MelanA B-RAF (V600E) clones.

Protein extracts of MelanA vector, Mass20 stimulated with L-Quisqualate (10 μM; 15 mins) and MelanA B-RAF (V600E) clones, were prepared and Western immunoblots of phospho-IGF-1R were performed. The membrane was then stripped and probed with total IGF-1R.

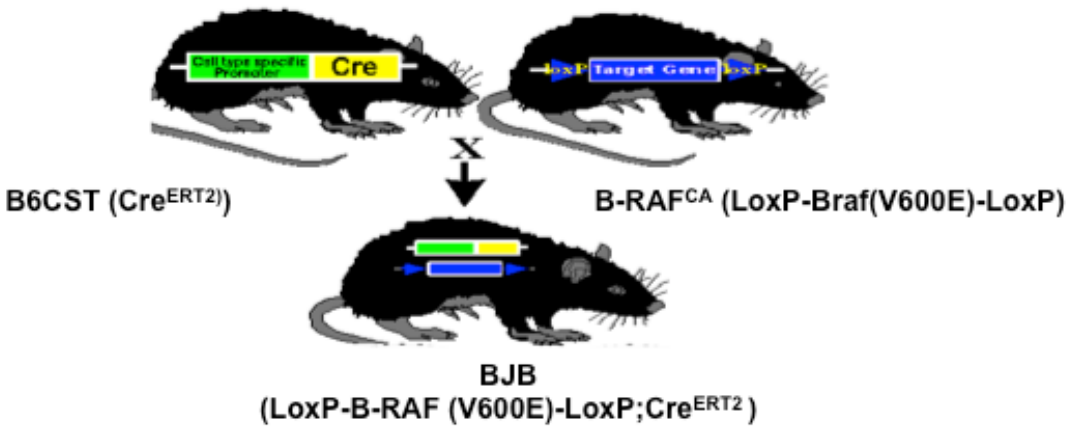


Figure 11. B-RAF (V600E) induction in BJB mice.

The BJB mouse (loxP-B-RAF (V600E)-loxP;Cre^{ERT2}) was generated by crossing a B-RAF^{CA} mouse (loxP-B-RAF (V600E)-loxP) which encodes a germline conditional B-RAF (V600E) allele, with a B6CST mouse (Cre^{ERT2}) which harbors conditionally active Cre recombinase only in melanocytes (Dhomen et al., 2009). 15 mg/ml of 4-Hydroxitamoxifen was applied to the ears of BJB mice for four days to induce the rearrangement of the LoxP-B-RAF (V600E)-LoxP locus in melanocytes of BJB mice.

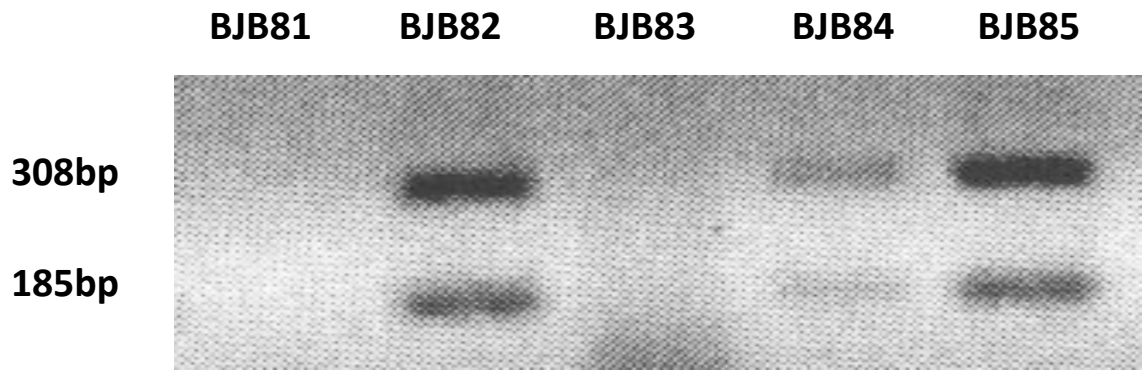


Figure 12. BJB B-RAF genotype.

RT-PCR was performed on genomic DNA extracted from the tails of BJB mice, using primers that flank the B-RAF gene. Bands at 308bp and 185bp are indicative of a heterozygous genotype for the B-RAF gene.

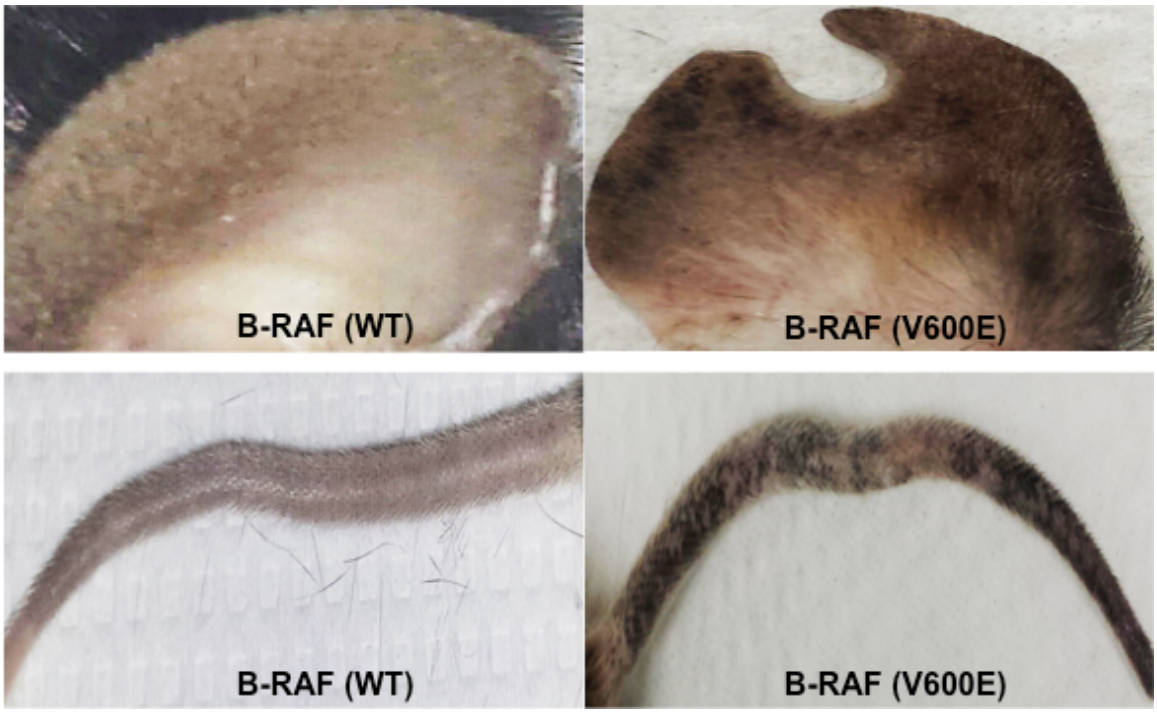


Figure 13. Phenotype of B-RAF (V600E) expressing mice.

8 weeks post 4-hydroxitamoxifen treatment, BJB (B-RAF (V600E): Cre^{ERT2}) mice developed skin hyperpigmentation around their ears and tail, presumably from increased proliferation of melanocytes caused by the induction of B-RAF (V600E).

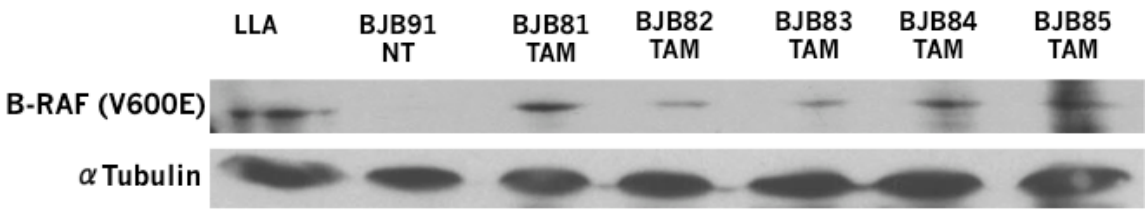


Figure 14. B-RAF (V600E) induction in BJB mice.

Protein was extracted from skin samples of BJB mice at 4, 8, and 12 weeks after mice were treated with 4-hydroxitamoxifen (TAM) (15 mg/ml) to induce B-RAF (V600E) in melanocytes. Western immunoblots using antibody against B-RAF (V600E), detected B-RAF (V600E) expression in skin samples obtained from BJB mice treated with 4-hydroxitamoxifen (TAM). BJB 91 not treated with 4-hydroxitamoxifen is negative for B-RAF (V600E) expression. Levels of α-Tubulin were used as loading controls.

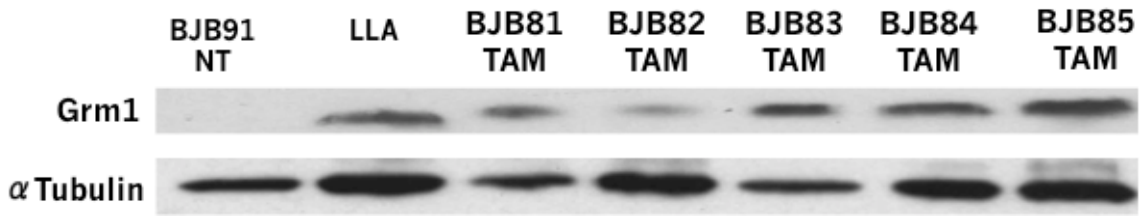


Figure 15. Grm1 expression in B-RAF (V600E) expressing mice.

Protein was extracted from skin samples of BJB mice at 4, 8, and 12 weeks after mice were treated with 4-hydroxitamoxifen (TAM) (15 mg/ml) to induce B-RAF (V600E) in melanocytes. Skin samples from BJB mice after 4-hydroxitamoxifen treatments show positive Grm1 expression only in B-RAF (V600E) expressing mice. BJB 91 NT was used as a negative control. Protein lysates from skin of LLA mice were used as positive control for Grm1 expression. Levels of α-Tubulin were used as loading controls.

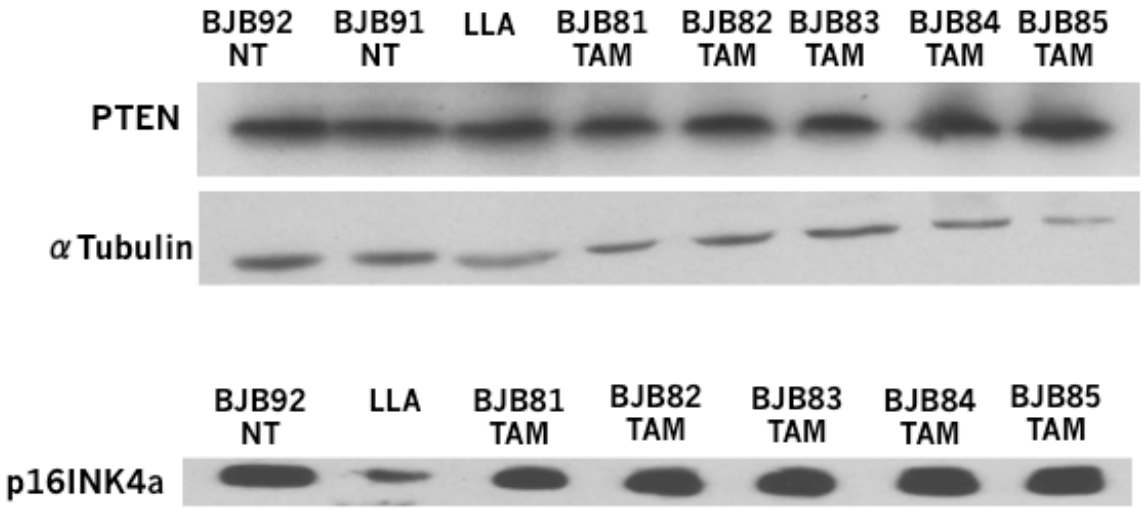


Figure 16. PTEN and INK4a expression in BJB mice

PTEN and p16INK4a expression was evaluated in BJB mice since this study aims to see whether loss of PTEN is a necessary condition of Grm1 expression by mutated B-RAF (V600E). Western immunoblots were performed with protein lysates from skin samples of BJB mice and show positive PTEN and p16INK4a expression.

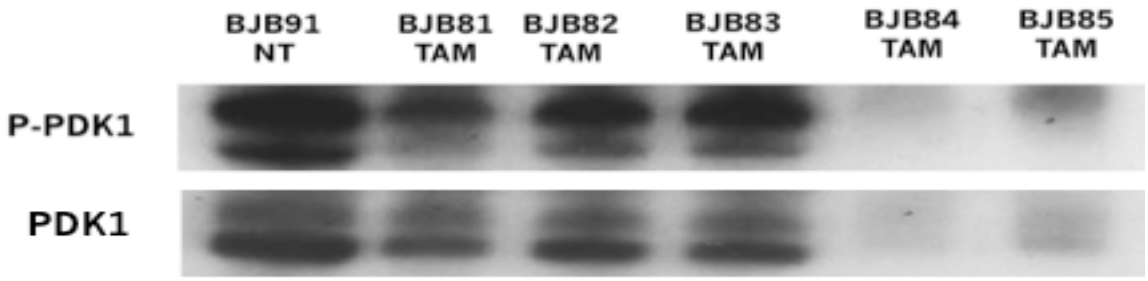


Figure 17. PDK1 activation in BJB mice

Protein lysates from skin samples of BJB mice were prepared and Western immunoblots of phospho-PDK1 were performed. The membrane was then stripped and probed with total PDK1.

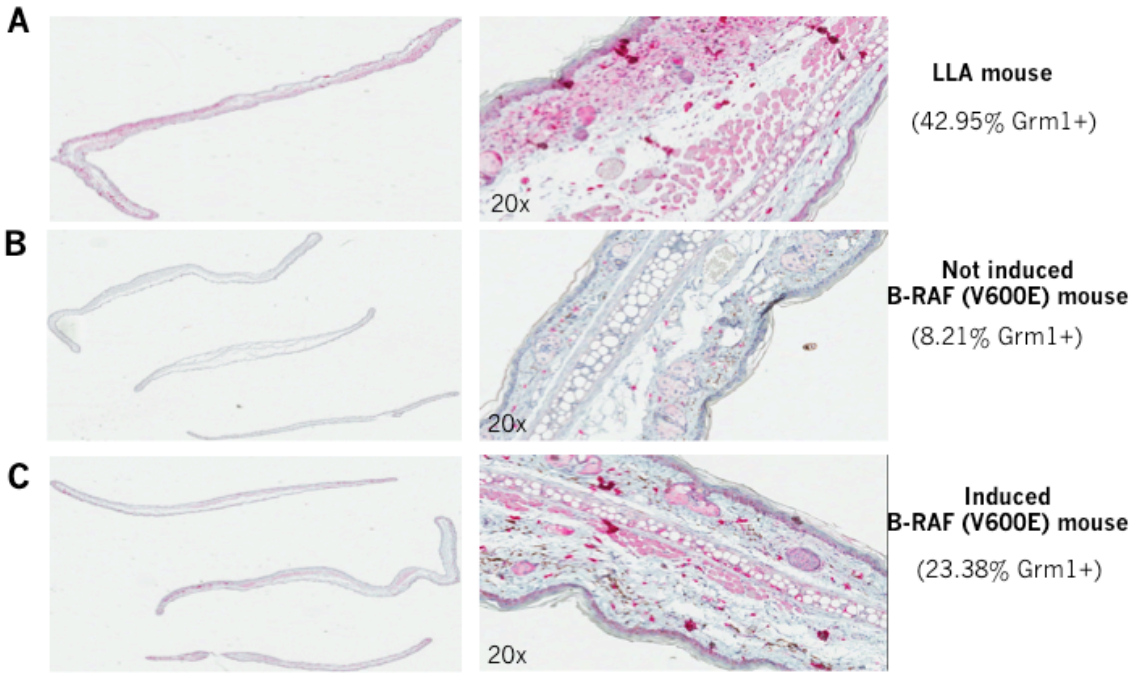


Figure 18. Immunohistochemistry staining of skin samples from transgenic mice for Grm1.

Grm1 expression in skin samples from transgenic mice expressing B-RAF (V600E). **A.** Positive control LLA mouse. **B.** Not treated mouse, does not express B-RAF (V600E). **C**. Induced mutated B-RAF (V600E) mouse after treatment with 4-hydroxytamoxifen shows strong Grm1 expression as compared to the non-treated control.

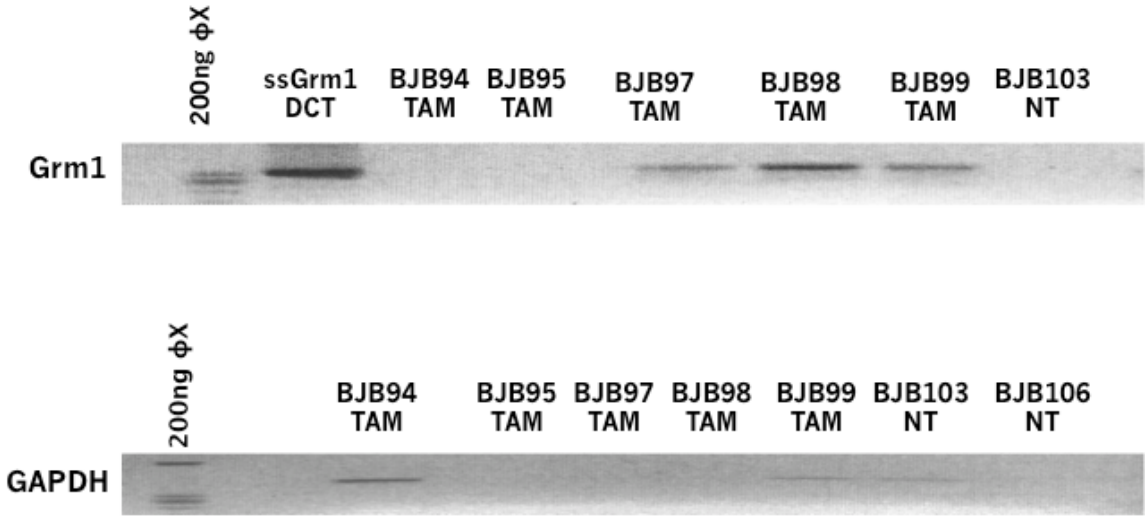


Figure 19. RT-PCR of Grm1 in B-RAF (V600E) expressing mice

RT-PCR(s) of Grm1 and GAPDH, were performed using cDNA made from extracted RNA from skin samples of BJB mice before and after 4-hydroxytamoxifen treatment (TAM). Primers that flank the Grm1 and GAPDH genes were used.