Expression of mutated BRAF (V600E) in melanocytes activates metabotropic glutamate receptor 1 expression

by

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Melanoma is the most aggressive form of skin cancer, which is caused by the neoplastic transformation of melanocytes. The precise genetic aberrations that initiate the transformation process remain largely unknown. Several genetic mutations have shown to be critical in maintaining the transformed phenotypes and the temporal expression of these mutated proteins is also vital on the onset of dysregulated cell growth.

The current study is to explore a relationship between two genetic aberrations, both have been shown to play a role in melanoma development. Our group has identified the ectopic expression of a normal neuronal receptor, metabotropic glutamate receptor 1 (Grm1) in melanocytes was sufficient to induce spontaneous metastatic melanoma development *in vivo* by the establishment of two transgenic mouse models (TG-3 and Epv). Mutated BRAF at residues 600 was shown in about 60% of nevi and melanoma specimens. Mouse models with mutated BRAF alone led to hyper-proliferated melanocytes in the absence of tumor development.

Unexpectedly, we detected Grm1 expression in tumor samples harboring mutated B-RAF(V600E) mutation on PTEN null background by immunohistochemical staining.

We hypothesize that one of the consequences of mutated BRAFis activation of Grm1 expression. We took advantage of an inducible BRAFmouse model, BJB, which has an inducible BRAF mutation (V600E) genotype only in melanocytes. We observed hyperpigmentation on their ears, tails, and skin samples, Grm1 expression was also detected. There was no tumor formation even after 13 months, while Grm1 expression persisted. We hypothesize that the order of the expression of mutated BRAF and Grm1 must play a role in the onset of melanocytic transformation.

In *in vitro* cumltured cell experiments, we also detected Grm1 expression after introduction of mutated BRAF into immortalized non-tumorigenic mouse melanocytes, MelanA cells. Moreover, siRNA decrease in mutated BRAF expression by silencing RNA resulted in parallel reduction in Grm1 expression. Taken together, these results point to an association between mutated BRAF and Grm1 expression *in vivo* and *in vitro*.

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INTRODUCTION

A. Melanoma:

Melanoma is the most life-threatening type of skin cancer, which is caused by mutations induced by various environmental factors and unrepaired DNA damage or genetic predispositions in the normal melanocytes. Cutaneous melanoma resulted from the epidermal melanocytes is the most common site of initiating growth, whereas noncutaneous melanomas are relatively rare which consist of ocular and distinct mucosal sites. According to the statistics of 2015 American Cancer Society, approximate 74000 new cases of melanoma was diagnosed, with about 9940 of death due to deadly disease. [1] Studies have identified several risk factors, including UV radiation exposure, family history and immunosuppression. [2] [3] It is not clear if the majority of melanoma develop from moles, which are the pigmented and proliferative melanocytes that have undergone several rounds of proliferation, perhaps transformed by an "oncogene" that was not sufficient to be fully transformed before become senescence, if a second mutation occurs then the mole may progress to melanoma. The characteristics of melanoma are irregular shape, various colors and raised as pigmented lesions on the skin.

Many of the therapeutic strategies to treat patients with melanoma are based on targeting mutated protein in various dysregulated pathways in melanoma. Several

proteins when become mutated activate these pathway includes G-regulatory proteins (RAS and Rac subfamily of Rho GTPases), cell surface receptor (ERBB and Src family kinase) or tumor suppressor proteins. [3] Three major pathways have been identified as critical players in melanoma, including mitogen-activated protein kinase (MAPK), due to mutation in RAS, RAF and KIT, related to the cell proliferation; PI3K/AKT pathway, due to loss of function of PTEN or dysregulation of AKT, engaged in cell apoptosis; $p16^{INK4a}$ and p14, due to mutation in cyclin-dependent kinase inhibitor 2A (CDKN2A), involved in cell cycle. [3] [4] [5] Small molecule inhibitors are to target one of the mutated proteins in these signaling pathways. Several drugs have been approved by FDA for the treatment of melanoma, such as Vemurafenib, Dabrafenib and Trametinib [6]. Recently, immunotherapies have been shown to be effective for a subpopulation of melanoma patients. For the immunotherapy, anti-CTLA-4 antibody, Ipilimumab, in combination with Dacarbazine can prolong stage IV melanoma patient's life for 3 years-survival. [6] This antibody is capable of increasing immune activity which against cytotoxic T lymphocyte antigen 4 (CTLA-4), a receptor, functioning as an immune checkpoint, downregulate the immune system. [7] [8]

However, tumor cells evolve resistance to the drug treatment and patients

relapse after months. The first explanation might be heterogeneity property of melanoma. [4] [9] Another explanation might be the cross-talk between multiple signaling pathways. [10] The recent reports indicated that AKT phosphorylates BRAF at two sites to down-regulate its catalytic activity, which lower the MAPK pathway activity. Moreover, PI3K can induce RAF and MEK expression. [10] Therefore, advanced strategies, combination therapy of Dabrafenib with Trametinib which is MEK inhibitor, is applied to treat melanoma and the result showed that combination therapy had longer progression-free survival. [11] [12]

B. Metabotropic Glutamate Receptors (mGluRs):

The role of glutamate can be as a neurotransmitter for cations influx and as a ligand to stimulate downstream proteins for survival. Therefore, two classes of glutamate receptor have been discovered, ionotropic glutamate receptors including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite (KA) subfamily; the second class of receptor is metabotropic glutamate receptors, member of G protein-coupled receptor, and is divided into eight subtypes and three groups based on sequence homology and second messengers. [13]

The metabotropic glutamate receptors belong to G-protein couple receptors (GPCRs) superfamily featured by seven transmembrane domain structure. GPCRs

are activated by extracellular ligands such as peptide, growth factor and neurotransmitter. Then, Activated GPCRs transmit extracellular signal to intracellular G protein via conformational change. G protein is composed of heterotrimeric complex of G_α, G_β, and G_γ subunits. In the inactivated state, G proteins are bound to guanosine 5' – diphosphate (GDP). When GPCRs are activated, acting as guanine nucleotide exchange factor (GEF) to promote exchange of guanosine 5' – triphosphate (GTP) for GDP within α subunit. After G_α bound to GTP, it dissociates with $G_{\beta\gamma}$ which can activate second messenger molecules.

The general structure of mGluRs contains three crucial domains, venus flytrap domain (VFD), cysteine-rich domain (CRD) and heptahelical domain (HD). VFD domain is glutamate binding site, composed of two lobes. Previous result showed that the mGluRs are activated by VFD dimerization. CRD contains nine crucial cysteine residues, each of cysteine are linked by disulfide bonds. The function of CRD is to transduce conformational change caused by extracellular signaling to intracellular domain, C terminus. HDs are made up of seven transmembrane helices interconnected by three extra- and three intra-cellular loops. [14]

Metabotropic glutamate receptor family includes eight subtypes and divided into three groups according to their sequence homology. Group I consists of

mGluR1 and mGluR5 which couple to $G_{q.}$ Group II comprises of mGluR2 and mGluR3 and Group III includes mGluR4, mGluR6, mGluR7, mGluR8. In contrary to group I, group II and group III are coupled to $G_{i/o}$ which inhibits adenylyl cyclase. [15] [16]

C. Metabotropic glutamate receptor 1 (Grm1)

Transgenic mouse models:

A transgenic mouse model (TG-3) is predisposed to develop spontaneous melanoma by insertional mutagenesis of 2-kb genomic fragment, clone B. [17] This genomic fragment has been shown commit fibroblasts to adipocyte differentiation in vitro. [18] The genetic mapping indicated that multiple tandem copies of clone B insert into the intron 3 of Grm1 and this integration led to deletion of 70 kb of host intronic sequence which most likely regulate the expression of Grm1. TG-3 displays aggressive metastasis and short latency melanoma development. The offspring of TG-3 are inherent in apparently visible melanocytic lesions in many regions, including pinnae of ear, eyes, legs, tail and skin within 2-4 weeks after birth. [3] In order to confirm the ectopic expression of Grm1 sufficient to cause melanoma, another transgenic mouse model was established, Tg(Grm1)EPv (E). [19] The Tg(Grm1)EPv (E) has expression of Grm1 in melanocytes only as the transgene, mouse Grm1 cDNA was placed under the regulation of DCT, a

melanocyte-specific promoter. [3] We observed the similar phenotype with TG-3, which developed melanocytic hyperproliferation at regions where epidermal melanocytes reside, progressed to melanoma after 4-6 months with 100% of penetrance. These data showed that ectopic expression of Grm1 is sufficient to transform melanocyte into melanoma *in vivo.* In both transgenic mouse models, the expression of Grm1 is only detectable in the tumors derived from melanocyte, but not in normal tissue.

Grm1 is an oncogene in *in vitro* and in *in vitro* settings:

Our laboratory has shown that introduction of exogenous mouse Grm1 cDNA alone into immortalized normal mouse melanocytes made from C57BL/6 mice was sufficient to alter these melanocytes into one with transformed characteristics. The clones are named MASS clones. [20] The functionalities of Grm1 in these cells were shown by its responsiveness to agonists or antagonists of Grm1 as demonstrated by the accumulation of second messengers. Furthermore, the tumorigenic potential of MASS clones was assessed by inoculation of three different MASS clones into immune-deficient nude mice or C57BL/6 syngeneic mice. Tumor formation was visible after 3-5 days of inoculation and sacrificed after 3 weeks. [20]

D. Grm1 signaling pathway in melanoma:

The natural ligand of Grm1 is L-glutamate, which is highly expressed in the central nervous system and neuropathologies. L-glutamate is a major neurotransmitter in synapse, functioning in binding and activating two types of receptor: metabotropic glutamate receptors and ionotropic receptors. The group I mGluRs are localized post-synaptically on dendrites spines and perisynaptic sites. Activation of Grm1 can increase calcium flowing into cells, and inhibit potassium currents, thus augment excitability of neurons. [21] Our lab identified Grm1 expression was detected in 80% of melanoma cell lines and 65% of primary to secondary metastatic organs samples but Grm1 expression is not detected in normal melanocytes or benign nevi. [22]

Activated Grm1 by glutamate, belong to group I mGluRs, couples to phospholipase C (PLC) via coupled G protein, $G_{\alpha/q11}$. PLC is able to cleave phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to two second messengers into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG is still bound to the membrane due to its hydrophobicity and IP_3 is released from PIP2 as soluble molecule diffusing in the cytosol. [15] DAG can activate protein kinase C (PKC). [16] IP3 can stimulate the release of calcium from the smooth endoplasmic reticulum.

Stable expressing-Grm1 mouse melanocytic clones, MASS clones, were

established to elucidate the mechanism underlying Grm1-mediated signaling pathways in melanoma. Activation of Grm1 is able to activate MAPK pathway but not PI3K pathway in cultured cells. [22] [23] [24] However, PI3K pathway is activated *in vivo* in the tumors formed by MASS clones via insulin-like growth factor -1 receptor (IGF-1R). Furthermore, IGF-1R is regulated by Grm1 as demonstrated, by Grm1 agonist and antagonist. [25]

Activation of members of PI3K/AKT pathway has detected in initiation and progression of melanoma. Positive-Grm1 human melanoma cell lines, UACC903 and C8161, also showed elevated basal levels of activated phosph-AKT compared to human cell line, C81-61, and normal melanocytes without Grm1 expression. Recently our group detected in melanoma cells with higher Grm1 expression with enhanced expression of HIF-1α, a transcription factor that regulate the Vascular Endothelial Growth Factor (VEGF) and Interleukin-8 (IL-8) expression, by PI3K/AKT/ mTOR pathway. Evidence indicated that reagent that modulate glutamatergic signaling from Grm1 also alter the expression of HIF-1α, IL-8 and VEGF. [20] However, how Grm1expression is turned on in normal melanocytes and promote cell transformation and tumor formation still remain puzzled.

E. MAPK pathway:

The mitogen-activated protein kinase pathway (MAPK) is involved in cellular

proliferation, migration, survival, tumor angiogenesis and metastasis, containing RAS-RAF-MEK-ERK (1/2) signaling cascade. MAPK pathway can be activated by several kinds of receptor, including receptor tyrosine kinase (RTKs), cytokine receptor and G protein-coupled receptor (GPCR). The extracellular signaling via cell surface receptors is transmitted into nucleus in a way of series of phosphorylation events. Dysregulation of MAPK pathway has been revealed in 90% human melanoma, due to constitutive activation by the mutation of either NRAS or BRAF kinase protein without ligand bound to receptors. In melanoma, mutation rates for BRAF is 50-70% and NRAS is 15-30%. [5]

In order to activate Ras by receptors, two cytosolic proteins, GRB2 and Sos, must be recruited first to provide a link between the receptor and RAS. GRB2 is an adaptor protein with SH2 domain and SH3 domain. It can exploit SH2 domain to bind to specific phosphorylation site on receptors and employ SH3 domain to bind to Sos protein. Sos protein is an guanine nucleotide exchange protein facilitate the conversion of inactive GDP-RAS to active GTP-RAS.

Active GTP-RAS is able to bind to the N-terminal regulatory domain of RAF protein and then activate it. In the unstimulated cells, RAF is phosphorylated and inhibited kinase activity by the phosphoserine-binding protein 14-3-3. However, interaction of the active RAS-GTP with inactive BRAF dephosphorylates the

serine bound to 14-3-3 and restores RAF kinase activity. RAF subsequently phosphorylates and activates MEK. Active MEK also phosphorylates and activate ERK1/2. Finally, active ERK1/2 enters nucleus and phosphorylates several transcription factor that regulate cellular response.

F. Mutated BRAF (V600E) in melanoma:

BRAF is one of RAF family of serine/threonine kinase, including three isoforms, A-RAF, B-RAF and C-RAF. BRAF mutated in kinase domain is dominant in the majority of cancers. RAF family has three conserved regions, CR1, CR2 and CR3, which are variable sequence and length. CR1 contains RAS-binding domain and a cysteine-rich domain. CR2 contains phosphorylated serine and threonine residues for regulating BRAF activity. CR3 contains the kinase domain and phosphorylation sites for modulating kinase activity. [26]

Mutated BRAF (V600E) is the commonest mutant protein in melanoma, leading to hyperactivation of MAPK pathway, above 60% in cancer and > 90% in benign nevi. Mutated BRAF is caused by the single missense mutation T substituted by A, changing valine to glutamic acid at codon 600 in exon 15. [27] [5] Recent studies have shown that BRAF (V600E) is not sufficient to transform melanocytes into melanoma, rather it triggers cells to undergo senescence. [28] [29] Further genetic mutation in PTEN or p16INK4a is necessary to induce these

mutated BRAF induced senescent cell to enter the cell cycle and form aggressive melanoma. [30]

Mutated BRAF (V600E) led to constitutive activation, hyperproliferation of cells via phosphorylated ERK in MAPK pathway. Activated ERK upregulate several growth factor receptors, such as epidermal growth factor receptor, c-KIT, platelet-derived growth factor receptor and vascular endothelial growth factor receptor. [31] Moreover, earlier report showed that mutated BRAF (V600E) upregulated hypoxia inducible factor-1α (HIF-1α) in melanoma, involved in angiogenesis and cell survival. Mutated BRAF (V600E) may regulate HIF-1α by posttranslational modification through suppressing VHL tumor suppressor protein. [32] Overexpression of MAPK pathway also increased cell proliferation via suppressing tumor suppressor, p16 or p53, and downregulation of cyclin-dependent kinases. These evidences indicated that mutated BRAF (V600E) is necessary for initiation of melanomagenesis.

Recently, BRAF fusion protein is discovered in melanoma patients tumors through genomic sequencing as part of personalized medicine, most of them are resulting from chromosome translocation. *BRAF* gene was rearranged with another gene, the C-terminal portion of the BRAF exons were fused with start codon of another protein, leading to the truncated BRAF protein. Characterization

of the BRAF fusion protein is that 5' N-terminal partners are binding domain and C-terminal of BRAF protein keep its kinase domain. The BRAF fusion proteins have shown constitutively activate MAPK pathway which is involved in many types of cancer, such as melanoma, prostate cancer and thyroid cancer. [21] [33]

G. BRAF dimerization:

Structure of monomer BRAF contains an N-lobe of five antiparallel β-strands and a regulatory αC helix and a C-lobe with a key loop called activation segment. Active BRAF kinase should maintain in the closed conformation by forming a dimer. The characteristics of the dimer are that the alignment of two parallel columns of conserved hydrophobic amino acid sequence, called catalytic (C-) and regulatory (R-) spines. Several modifications might change the stability of closed state, such as phosphorylation of active segment and protein-protein interactions. Inward movement of the α−C helix and the in position movement of a Phe residue on the DFG motif which is located on the activation segments. DFG motif has been discovered in the activation loop of many kinase and is crucial for protein catalysis. In active kinase, the DFG motif adopts an "in" conformation and the Asp residue is oriented toward bound ATP whereas the conformation of DFG motif is flipped outward in inactive kinase. [34] [35] Evidence shows that Arg509 located on C-terminal tip of the αC helix is the interface for side-to-side

dimerization. [36] It is believed that BRAF dimerization is because of allosterically coupled with conformational changes of the activation segment. [37]

It has been shown that mutated BRAF (V600E) has the ability to remain activated because it has increased dimerization potential and activated MAPK pathway without ligand binding to stimulate any receptors. Mutated BRAF (V600E) is kinase-impaired BRAF mutant, which has intact dimerization interface and transactivation activity. PLX 4032 was a promising specific BRAF (V600E) small molecule inhibitor. At first six to eight months of treatment, patients showed recovery and in control of tumorigenesis. However, continuing on the inhibitor caused reactivated the MAPK pathway because the wild type BRAF bound with PLX4032 could dimerize with other RAF family protein such as KSR and CRAF. [38] [39] [40] BRAF was recruited to the membrane by GTP-RAS and then dimerized with CRAF followed by the conformational change. CRAF was activated by transactivation through BRAF and then CRAF phosphorylated MEK. Therefore, even if the mutant BRAF (V600E) was inhibited by PLX4032, melanoma cells still could activate the MAPK pathway through the wild type BRAF.

H. PI3K/AKT/mTOR pathway:

The phosphoinositide-3-kinase-protein kinase B/AKT/mTOR (PI3K/AKT) pathway is involved in normal cell growth, proliferation, angiogenesis and in

malignant tumor for anti-apoptosis. Activation of PI3K/AKT pathway inhibits cell apoptosis through positive regulation of G1/S phase in melanoma by blocking cell cycle inhibitors. Several growth factors are able to activate PI3K/AKT pathway via receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs) and active GTP-RAS proteins bound on the inner cell membrane. Class IA PI3K is directly activated by stimulated receptors via binding their regulatory subunit or adaptor protein, insulin receptor substrate (IRS). PI3K then converts phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) by phosphorylation. Class IA PI3K consists of catalytic subunit, p100 heterotrimer, and regulatory subunit, p85 heteropentamer [41]. AKT is one of major downstream effectors of PI3K.

AKT activation begins by recruitment of phosphoinositide-dependent kinase 1 (PDK1) and AKT to the membrane area. Three AKT isomer and PDK1 have PH domain which is a binding site to PIP3 on the membrane. [41] Once AKT binds to PIP3 lead to the conformational change in AKT, exposing two important amino acids for phosphorylation. First site, threonine 308 (Thr 308), is phosphorylated by (PDK1) and second site, Serine 473 (Ser 473), is by PDK2. [42] AKT phosphorylates downstream substrates consisting of R-X-R-X-X-S/T-B motif in cytoplasm and nucleus. [5] X represents any amino acid and B represents bulky

hydrophobic residues. AKT promotes cell survival by phosphorylating and inhibiting Bcl-2 antagonist of cell death (BAD) which is a pro-apoptotic protein. AKT also can phosphorylates transcription factor, FOXO which translocates out of nucleus after phosphorylation. FOXO mediates transcription of proteins involved in apoptosis, cell cycle arrest and cellular metabolism. Moreover, AKT is able to inhibit p53 induced apoptosis by phosphorylation of MDM2, an E3 ubiquitin ligase that promotes p53 degradation. One of roles of AKT signaling pathway is promoting cell growth. AKT phosphorylates and inactivate GTPase-activity of tuberous sclerosis complex 2 (TSC2) which inhibits the activity of mTOR complex. mTORC1 regulates the protein synthesis through phosphorylation of S6 ribosomal protein kinase (S6K) and phosphorylation and inactivation of inhibitor of mRNA translation initiation factor 4E binding protein 1 (4EBP1). AKT signaling promote angiogenesis via increased production of HIF-1α resulting in secretion of VEGF. [43]

Dysregulation of PI3K/AKT pathway is predominant in melanoma. Increased AKT phosphorylation is detected in melanoma compared to normal melanocytes. Activated AKT3 is present in 50% of dysplastic nevi and 70% of melanoma. Aberrant activation of phosphorylated S6K might change the protein expression such Grm1 expression in melanoma. [44] Mutation in gene encoding the p100

catalytic subunit of PI3K, loss of tumor suppressor PTEN, overexpression of AKT or mutation in NRAS are some of the routes that could lead to constitutive activation of PI3K/AKT pathway.

I. Phosphatase and tensin homologue (PTEN)

PTEN is a 403 amino acid protein containing five functional domains: a PIP2-binding domain (PBD), a phosphatase domain, a C2 domain, a carboxy-terminal tail and a PDZ-binding domain. [45] PTEN has a common catalytic featured motif of tyrosine phosphatases, HCXXGXXR (X is any amino acid). [46] the active site of PTEN is wider than other protein tyrosine phosphatase (PTPs) for accommodating for binding larger molecule PIP3. [45] The structure of phosphatase domain has five beta-stands bundling with two alpha-helices on one side and four on the other side. PDZ domain is protein-protein interaction area, consisting of five or six β-strands. The function of PDZ is to scaffold many proteins together and organize signaling complexes at inner membrane. [47]

PTEN is a negative regulator in PI3K/AKT pathway, serving as a tumor suppressor protein located at chromosome 10q23-24. It acts as lipid phosphatase dephosphorylating the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the 3'-position of the inositol ring, which prevent PDK1 and AKT from binding to PIP3 via the PH domain. PTEN also acts as a phosphatase dephosphorylating

phosphoserine and phosphotyrosine in many proteins, regulating cell-to cell adhesion and migration. [48] Many researches have been focused on the loss of PTEN activity because it is involved many perspectives of cellular events. Loss of PTEN promotes protein glycosylation and folding, resulting in the metabolic reprogramming in cancer cells. Moreover, loss of PTEN activity might change the microenvironment of cells and cause the transcriptional reprogramming. Given the property of anti-PI3K/AKT pathway, PTEN deletion is necessary to transform melanocyte harboring mutated BRAF (V600E) or mutated NRAS (Q61R) into melanoma. Several proteins negatively regulate activity of PTEN, including mitogen activated protein kinase kinase 4 (MKK-4), NFkB, TGF-beta and c-JUN in many type of cancers. Moreover, phosphorylated PTEN is more stable but less active than unphosphorylated PTEN. [49]

PTEN is detected inactivated or mutation in about 29-43% of melanoma cell lines and biopsies which led to aberrant activation of PI3K/AKT pathway. PTEN missense mutation affecting the phosphatase activity or frameshift mutation changing translation of PTEN were observed in melanoma with high levels of activation of PI3K/AKT pathway. [50] PTEN null background with BRAF (V600E) mouse model showed metastatic melanoma and loss PTEN led to elevated AKT2 expression. Therefore, loss activity of PTEN is an important driver to cross the

oncogene-induced senescence barrier and cause tumorgenesis. [29] [51] [52]

Materials

A. Cell Culture:

Immortalized mouse melanocytes (MelanA) were provided by Dr. Dorothy Bennett (St. George's University of London, UK) and grown in RPMI-1640 medium (Sigma) with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml of penicillin/streptomycin (Gibco, Grand Island, NY) and 200 nM 12-O-tetradecanoyphorbol-13-acetate (TPA) (Sigma). MelanA BRAF clones are also grown in the same condition. Selection medium contains 10% FBS, 100U/ml of penicillin/streptomycin, G418 and hygromycin.

B. Plasmid Purification:

Plasmid purification was needed for TetR, siMUBRAF and siWTBRAF. The QIApeep Spin Miniprep Kit was used for preparation of purified plasmid. The procedure was operated following in the QIAGEN protocol.

The concentration of each plasmid DNA was determined by performing DNA gel electrophoresis on a 0.8% of agarose gel compared to the known concentration of DNA marker, Hind III. The DNA was precipitated with 250 mM ammonium acetate and 100% cold ethanol.

	Duplex sequence
siMUBRAF	5'-rCrArCrUrCrCrArUrCrGrArGrArUrUrUrCrUrCTT 5'-rGrArGrArArArUrCrUrCrGrArUrGrGrArGrUrGTT
siWTBRAF	5'-rCrArCrUrCrCrArUrCrGrArGrArUrUrUrCrArCTT 5'-rGrUrGrArArArUrCrUrCrGrArUrGrGrArGrUrGTT

Table 1. Inducible siMUBRAF and siWTBRAF insertion sequence

C. DNA Transfection:

DNA transfection was conducted using DOTAP transfection reagent (Roche, Basel, Switzerland) following the manufacturer's instructions. Tet-R plasmid (4ug) is co-transfected with pTinRNA mutated BRAF (V600E) plasmid (4ug) or pTinRNA wild type BRAF plasmid (4ug) into the MelanA BRAF clones. Stable clones with silencing mutated BRAF (V600E) were selected by resistance to G418 (20 ug/ml) and Hygromycin (0.25ug/ml). Stable clones with silencing wild type BRAF were selected by resistance to G418 2 (ug/ml) and Hygromycin (0.01ug/ml).

D. BJB mouse strain:

We used Cre-LoxP recombination technology to make a transgenic mouse model harboring melanocytic-specific conditional induced-mutated BRAF

(V600E) in melanocytes. The transgenic mouse model (BJB) (loxP-BRAF(V600E)-loxP: Cre ^{ERT2}) was generated by crossing a BRAF^{CA} mouse (loxP-BRAF (V600E)-loxP) with a B6CST mouse (Cre ^{ERT2}) which encodes active Cre recombinase only in melanocytes. Cre recombinase, a site-specific DNA recombinase, recombines a pair of short target Lox sequence and the double strand DNA is cut at both loxP sites. The strands are rejoined by DNA ligase in a quick and efficient process. In order to activate Cre recombinase and rearrange the loxP-BRAF (V600E)-loxP, we treated with synthetic estrogen receptor ligand, 4-hydroxytamoxifen (OHT) 15mg/ml, to the ears tissue for 4 days. Protein and genomic DNA were extracted from the ears for Western immunoblots and genetic analysis.

E. Protein Extraction, Western immunoblots:

Protein extraction buffer was made with 4x Laemmli sample buffer (Bio Rad, CA), nano-purified water and 2-Mercaptoethanol (Sigma) with proportion of 5:15:1, respectively. The volume of protein extraction buffer was varied with the size of plate, for example 200 ul of protein extraction buffer for one 60-mm plate. Cells were grown to about 90% confluence and they are then ready for protein extraction. First, plates were washed with cold 1X PBS twice, protein extraction buffer added and a scraper is used to collect the mixture. Secondly, mixture was

heated at 99 ℃ for 10 minutes. Next, tube with heated mixture was centrifuged at 14,000 RPM for 20 minutes and collect the supernatants.

For frozen tissue protein extraction, tissue sample should be kept in liquid nitrogen and be grounded to powder by mortar and pestle. Protein extraction buffer was then added to the samples and homogenized by a polytron (Brinkmann Instruments). Sample were incubated for 2 hours on a shaker at 4 ℃ and then heated at 99 ℃ for 10 minutes. Finally, protein lysates were collected from supernatants after centrifuging at 14,000 RPM for 20 minutes.

Protein lysates were loaded per lane in 7.5% or 10% polyacrylamide gels (Bio Rad, CA). Gel electrophoresis was performed by applying 150 Voltage to separate the proteins for around one hour. Next, Gels were transferred to a nitrocellulose membrane at 150 mA at 4 ℃. After transferring process, Ponceau S Read (Sigma) was used to stain the proteins to confirm that equal amounts of proteins were loaded in each lane. Membrane was then blocked with 0.25% non-fat dry milk in Tris buffered saline + Tween 20 (TBS-T). We also blocked membrane with 5% of non-fat dry milk for probing Grm1 antibody. Membrane was incubated with primary antibody overnight on the shaker at 4℃. Second day, membrane was washed with TBS-T and probed with secondary antibody, Rabbit or Mouse. Finally, protein bands were detected by using Lumina Crescendo

Western HRP substrate reagents (Millipore corporation, MA)

F. Functionality Assay of Grm1:

Cells were grown in 100-mm plates incubated with K10 medium which is glutamine/glutamate free medium in dialyzed serum for 3 days. Then cells were split to 60-mm plates with around 50% confluence, approximately $4x10^5$ cells per plate. After cells were attached to plates, we replaced K10 medium with K medium which is without serum for 1 day. Then one set of cells was treated with Grm1 agonist, Quisqualate (10uM), for various time points. Another set of cells were pre-treated with antagonist, Bay 36-7620 (10uM), for 30 minutes and then treated with Quisqualate (10uM) for different time points. Finally, we collected the protein lysate and performed Western immunoblot for protein analysis.

G. Treatment with inhibitors:

Cells were grown to 90% confluence in 60-mm plate. Then the cells were split into several plates to achieve 50% confluence. Once cells attached, we changed RPMI-1640 medium (Sigma) with 10% FBS to serum free RPMI-1640 medium for 1 day. Next day, cells were treated with inhibitors, including mutated BRAF inhibitor (PLX 4032), PTEN inhibitor (bpV(phen), IGF-1R inhibitor (OSI-906) and Src inhibitor (PP2). Finally, we collected the protein lysate and did Western

immunoblot for protein analysis.

H. Antibody:

Total-AKT, phospho-AKT (Thr308), total-ERK1/2, phospho ERK1/2, total PDK1, phospho-PDK1, Phospho-IGF-I Receptor β (Tyr1135/1136)/Insulin Receptor β (Tyr1150/1151) (19H7), total IGFR, PTEN and BRAF (55C6) were purchased from Cell Signaling Technology (Danvers, MA); Anti-p16INK4a antibody was purchased from Neo Markers (Fermont, CA); anti-mutated BRAF (V600E) antibody was purchased from New East Biosciences (Malvern, PA); anti-Grm1 antibody was purchased from R&D Systems (Minneapolis, MN); monoclonal α- Tubulin antibody was obtained from Sigma (St. Louis, MO); anti-rabbit secondary was purchased from Merck ; anti-mouse secondary was purchased from Sigma (St. Louis, MO). Anti-sheep secondary was purchased from R&D Systems (Minneapolis, MN).

RESULTs

A. Histological Staining of Grm1 in BRAF (V600E) and NRAS (Q61R) Transgenic Mice:

Mutations in BRAF (V600E) and NRAS (Q61R) are two of the most common mutations in melanoma. Aberrant Grm1 expression was detected in ~65% of human melanoma biopsy samples but not in normal melanocytes, sequence analysis did not show hot spot mutations, we suspected the alterations likely to occur in noncoding regions. We were curious to know if mutation in either BRAF (V600E) or NRAS (Q61R) could activate Grm1 expression. Grm1 histological staining was performed on tumors specimens from transgenic mice harboring BRAF (V600E) in PTEN heterozygous or null background. TG3 tumor was served as a positive control for Grm1 (Figure 1A). Transgenic mice with BRAF (V600E) in PTEN heterozygous showed moderate Grm1 expression (Figure 1B) compared to the transgenic mice with BRAF (V600E) in PTEN null background (Figure 1C). The tumors with NRAS (Q61R) in p16INK4a null background showed strong Grm1 expression (Figure 1D). Based on these preliminary results, we established *in vitro* experiment to further test our hypothesis that BRAF (V600E) expression in melanocytes could upregulate Grm1 expression. (Yu and Chen, 2012)

B. MelanA BRAF (V600E) clones expressed Grm1 expression:

We isolated stable MelanA clones that have been transfected with a myc-tagged BRAF (V600E) plasmid. We confirmed that MelanA BRAF (V600E) clones produced BRAF (V600E) protein by Western immunoblots compared to the negative control, the parental MelanA cells (Figure 2).

Next step, we would like to test whether the cells with exogenous BRAF (V600E) upregulated the ectopic expression of Grm1. We used the same protein lysate extracted from these clones in immunoblots and we detected Grm1 expression only in MelanA clones that have the mutated BRAF not the parental MelanA, which was Grm1-negative (Figure 3) (Yu and Chen, 2012).

C. Basal level of p-AKT and p-ERK1/2 are elevated in MelanA BRAF (V600E) clones:

PI3K/AKT and MAPK pathways are two of the signaling cascades critical in melanoma pathogenesis. We examined the basal level of p-AKT and p-ERK1/2 in MelanA BRAF (V600E) clones. As shown in figure 4, these clones showed higher level of phosphorylated AKT and ERK1/2 compared to the parental MelanA. We also include MASS 20, MelanA with exogenous Grm1, exhibits modest transformed phenotypes *in vitro* but formed robust tumors *in vivo* [13]. Basal p-

ERK was higher than the parental MelanA but p-AKT was not detected *in vitro* confirming earlier results. P-AKT was only elevated in tumors *in vivo.* In addition, MASS20 retained the wild type BRAF as MelanA [13].

D. PTEN and p16^{INK4a} were expressed in MelanA BRAF (V600E) clones:

p16^{INK4a} and PTEN are tumor suppressors. Loss PTEN activity is one of ways that PI3K/AKT pathway is dysregulated in cancer including melanoma. [50] [52] Moreover, loss of heterozygosity or mutation at p16 locus encoded two proteins, p16^{INK4a} and p19ARF [5] [53], is susceptible to melanoma. Therefore, we tested PTEN and p16INK4a expression in MelanA BRAF (V600E) clones. Western immunoblot analysis showed all of MelanA BRAF (V600E) clones were p16^{INK4a} negative (Figure 5) as expected because p16^{INK4a} was deleted in MelanA in order to become immortalized cells [53]. We detected PTEN expression in these clones but this measured the total PTEN regardless if there are mutations. We detected elevated basal level of p-AKT indicating that PTEN may not be functional. PTEN is a phosphatase function to remove the phosphate group on PIP3 render its activity to phosphorylated PDK1 result in inhibition of AKT signaling. We examined if PDK1 is phosphorylated in MelanA BRAF (V600E) clones. In Figure 6, we detected phosph-PDK1 expression in the MelanA BRAF

(V600E) clones by Western blots compared to MelanA parent. Taken together our results so far suggesting PTEN is likely to be nonfunctional in the MelanA BRAF (V600E) clones because we observed high levels of p-AKT and p-PDK1. The phosphatase activity of PTEN might become inactive and then it couldn't dephosphorylate PIP3.

E. PTEN functionality test:

In the previous section by Western immunoblots, we determined that the phosphatase activity of PTEN is not functional because we detected phosph-AKT and phosph-PDK1 expression in the MelanA BRAF (V600E) clones. Therefore, we carried a functional assay for PTEN by treating these clones with PTEN inhibitor, potassium bisperoxo (1,10-phenanthroline) oxovanadate (bpV(phen)) in various concentration (1uM, 2uM and 5uM) for 30 and 60 minutes [54]. bpV (phen) binds to the catalytic domain of PTEN inhibiting its phosphatase activity thus prevent the dephosphorylation of PIP3. In Figure 7, there is a dose dependent increase of p-AKT levels suggesting that more PTEN was inhibited, more activation of AKT occurs in the cells. Therefore, based on these results, the phosphatase activity of PTEN was at least partially functional because suppression of PTEN by its inhibitor led to enhanced level of p-AKT. It might be the reason

why the cells in BJB mice couldn't form tumors.

F. BRAF (V600E) modulates Grm1 expression in MelanA BRAF (V600E):

We examined the relationship between BRAF (V600E) and Grm1 by modulating mutated BRAF levels by BRAF (V600E) inhibitor, PLX 4032, and by genetically silencing either BRAF (V600E) and wild type BRAF (WTBRAF) in these clones. We applied various concentration of PLX 4032 (0.1uM and 0.3uM) to clones for 24 and 48 hours and evaluated if Grm1 expression is affected by BRAF (V600E) activity. Protein samples were extracted after each time point. The result from Western blot showed that Grm1 expression was significantly decreased after 24 or 48 hours in the presence of PLX 4032 (Figure 8), demonstrating that mutated BRAF activity is linked with the regulation of Grm1 expression.

In the short interference RNA (siRNA) experiments, we used inducible the TetR system, transfecting two plasmids, TetR and pRNA Tin-H1.2/Hygro siRNA. pRNA Tin-H1.2/Hygro vector contains H1.2 promoter with tetracycline operator (TetO1) and specific siRNA production. TetR plasmid was used to produce tetracycline repressor protein (TetR) which binds to TetO1, in the absence of the inducer, Doxycycline, an analog of tetracycline, the transcription of siRNA is

repressed. In the presence of Doxycycline, the inducer binds to TetR and release it from the TetO1 site, and permit the transcription of siRNA. The successfully transfected clones are resistant to G418 and hygromycin antibiotics carried by the two plamids. Several stable clones were isolated and silencing of target protein was evaluated by including the inducer, Doxycyclin, in the growth media for various time points, 4 days or 6 days. Reduced expression of BRAF (V600E) is confirmed by Western Blots in several clones (Figure 9). Next, same protein lysates were probed with anti-Grm1 antibody. We saw a parallel reduction of Grm1 expression in MelanA siBRAF(V600E) clones (Figure 10). From these results, we concluded that Grm1 expression was depending on mutated BRAF expression.

G. WTBRAF is involved in the activation of Grm1 expression:

Similar short interference RNA experiment was conducted by using TetR inducible system. To silence endogenous wild type BRAF by using pRNA Tin-H1.2/Hygro siRNA in the MelanA BRAF (V600E) cells. In Figure 11, we confirmed a reduction in WTBRAF expression compared to non-treatment with the inducer, Doxycycline. Next using the same protein lysates, we probed for Grm1 expression in these cells. We were surprised to see a parallel reduction in both Grm1 expression and wild type BRAF in Figure 12. In light of these results

that a decrease in either wild type or mutated BRAF protein expression led to reduction of Grm1 expression. We proposed that dimerization of BRAF is required for its kinase activity and activation of Grm1 expression. A decrease in the wild type BRAF led to a parallel decrease in the heterodimer formation, which may influence activation of Grm1 expression in these clones. Homo- and hetero-dimerization of BRAF and the isotypes are more complicated than originally thought and have been under intense investigation by several laboratories, however, the precise mechanism and the consequences of modulation remain largely unresolved.

H. Grm1 functionality Test:

Activation of AKT and ERK are two major downstream signaling cascades upon stimulation of Grm1 by its ligand / agonist. We were interested to know if the Grm1 protein in MelanA BRAF (V600E) clones is functional. First, we treated MelanA BRAF clones with Grm1 agonist, Quisqualate (Q) to stimulate the activity of Grm1. Secondly, pre-incubation of these cells with Grm1 antagonist, Bay 36-7620 (Bay) for 30 minutes followed by stimulation with Q. In order to minimize the effect of glutamate, the natural ligand of Grm1, we used glutamate/glutamine free RPMI-1640 and 10% dialyzed FBS to culture the cells

for three days. At 24 hours before the induction experiment, cells were grown in glutamate/glutamine free media without serum. Western blots were performed to analyze the level of changes in p-ERK and p-AKT.

We used levels of p-AKT and p-ERK1/2 as readouts as we have done in our previous studies. [55] [56] p-AKT and p-ERK1/2 in MelanA BRAF (V600E) clones was upregulated by Q treatment with the maximum level at 5 min and gradually back to basal level by 30 min, confirming our earlier results [56]. However, pre-treatment of these clones with Bay followed by stimulation with Q led to the similar modulation of Grm1 regulation of its downstream pathway as Q alone, in contrast to our earlier results [55] [56] (Figure 13). In our earlier studies, we showed that in human melanoma cells harbored mutated BRAF showed constitutively activated p-ERK and treatment of these cells with agonist/antagonist of Grm1 did not lead to modulation of p-ERK levels [55]. In the current studies, treatment of these cells with a specific Grm1 antagonist, Bay 36-7620, failed to render the receptor inactive as the agonist Q was still able to stimulate the receptor following the same patterns as in the absence of the antagonist. These results suggested that perhaps the activated Grm1 receptor in MelanA mutated BRAF clones functionally is somewhat different.

I. Activation of insulin-like growth factor 1 receptor (IGF-1R) expression in MelanA BRAF (V600E) clones:

The insulin-like growth factor-1 (IGF-1) is an activator AKT pathway. Previous study in our lab, we discovered that Grm1 transactivated IGF-1R in MASS clones. We were interested to know if activated p-AKT in MelanA BRAF (V600E) clones were also mediated through IGF-1R. Activation of IGF-1R expression was detected in MelanA BRAF (V600E) clones by Western blots (Figure 14). We then treated these clones with IGFR inhibitor, OSI-906. P-AKT was completely knockdown after treatment of 24 and 48 hours (Figure 15). Earlier study we showed that Src family tyrosine kinases served as intermediates between Grm1 and IGF-1R as suggested by others [25]. Here we also tested if treatment with Src inhibitor would also modulate p-AKT. We applied a concentration of Src inhibitor, PP2, in MelanA BRAF (V600E) clones for 15 minutes, displaying suppression of p-AKT expression (Figure 16). Next we used the same protein lysate from the induction experiments (Figure 13) and probed for p-IGF-1R to see whether Grm1 modulators alter IGF-1R expression. In Figure 17, p-IGFR showed similar trend of variations as p-AKT and p-ERK in the presence of Grm1 agonist and antagonist as shown in Figure 13. These results suggest that molecules that alter Grm1 function are reflected in the readouts of downstream effectors.

We made stable mutated BRAF clones in immortalized MelanA cells and shown that known downstream PTEN targets, PDK1 and AKT, were activated, suggesting that PTEN was nonfunctional in these cells, however, in the presence of PTEN inhibitor, PTEN appeared to be modulated by the inhibitor, suggesting PTEN is at least partially functional (Figure 7). We speculate that activation of Grm1 expression by mutated BRAF may mediate IGFR activity to stimulate PDK1 and AKT via an alternate route not directly involve PTEN.

J. Transgenic BJB mice harboring inducible BRAF (V600E):

Studies described above using cultured melanocyte (MelanA) showed that introduction of exogenous mutated BRAF led to Grm1 expression. However, it is known that all immortalized melanocytes have deleted $p16^{INK4a}$, therefore, in order to know if $p16^{INK4a}$ plays a role in Grm1 expression, we turned to the in vivo system by making BJB mice.

BJB mouse is an engineered transgenic mouse line using LoxP-stop-LoxP/ Cre recombinase technology. The BJB mouse (LoxP-Braf(V600E)-LoxP/CreERT2) is developed by cross-breeding two type of mice: $Braf^{CA}$ mice harboring a germline conditional Braf(V600E) allele (LoxP-Braf(V600E)-LoxP); B6CST mice harboring Tyr::CreER transgene, which can produce conditionally active Cre

recombinase specifically in melanocytes (Figure 18A). We examined the genotype of mice by doing PCR of genomic DNA extracted from the tails. The result showed the heterozygous BRAF genotype as indicated at two bands: 385 and 185 base pairs.

In the absence of 4-hydroxytamoxifen (4-HT), all tissues express wild type BRAF protein due to the absence of Cre recombination events. After treatment of 4-HT, only melanocytes will turn on the Cre recombinase and should express BRAF (V600E) mRNA and protein. We applied 15 mg/ml of 4-HT to the ears of one month old BJB mice for 4 days to induce the activation of Cre recombinase. Skin pigmented- lesion in the ears and tails were observed at eight weeks after treatment, suggesting that hyperproliferation of melanocytes as shown earlier [18] (Figure 18B). We monitored 15 4-HT treated mice over 15 months, and did not detect any tumor formation in any of them confirmed earlier studies that BRAF (V600E) is the initial phase of cell hyper-proliferation characterized by pigmented nevi and cell cycle arrest of senescence. [29]

K. Grm1 expression in BJB mice:

BJB mouse expressed hyper-pigmented lesion on the skin after treatment of 4-HT because of activation of BRAF (V600E) in melanocytes. Proteins were extracted from 4-HT treated ears and confirmed that BRAF (V600E) expressed in

the melanocytes by Western Blots. Non-treated samples were BRAF (V600E) negative. Treated samples showed strong expression of BRAF (V600E) (Figure 19).

Next, we tested Grm1 expression in BJB mouse protein lysates. We detected Grm1 expression in 4-HT treated sample but not in the not-treated samples (Figure 19). Furthermore, PTEN and $p16^{INK4a}$ expression were confirmed by Western blots in the non-treated and treated 4-OH samples. (Figure 20)

DISCUSSION

We showed that ectopic expression of Grm1 alone in melanocytes was sufficient to induce spontaneous melanoma development with 100% penetrance in transgenic mouse model [19]. We also showed that introduction of exogenous Grm1 in immortalized mouse melanocytes, mammary epithelial cells or kidney epithelial cells led to modest *in vitro* transformed phenotype but robust *in vivo* tumorigenesis [13] [56]. The human relevance of these results was revealed in similar human cancers, melanoma, breast and renal cell carcinoma, suggesting that Grm1 is a player in numerous malignancies. How and what turns on the expression of Grm1 remains an interesting question. Detection of Grm1 expression in a series of slides made from different mouse model of melanoma led us to ask if mutated BRAF or NRAS expression may also trigger Grm1 expression. Our first set of experiment was to use the immortalized mouse melanocytes and manipulated expression of mutated or wild type BRAF using downstream signaling cascades as readouts.

In downstream protein detection, p-ERK and p-AKT were exhibited higher level in MelanA BRAF (V600E) clones than MASS 20 (MelanA with exogenous Grm1 expression) and immortal mouse melanocytes (MelanA). Both MASS 20 and MelanA harbor wild type BRAF, while MelanA BRAF (V600E) has one copy

of mutated BRAF (V600E) which constitutively activate MAPK pathway without extracellular signal stimulation. Therefore, it is not surprising that mutated BARF (V600E) in MelanA BRAF (V600E) clones showed elevated levels of activated p-ERK1/2. However, it is not clear why enhanced levels of activated p-AKT was also detected.

BRAF is a member of the RAF family of kinase, a component of the MAPK signal transduction pathway, so is NRAS, which in involved in regulating cell survival and proliferation. Activation of BRAF by hetero- or homo-dimerization of kinase domains is the current hypothesis how BRAF phosphorylate downstream protein, MEK, and activate the MAPK pathway. In melanoma, mutated BRAF is detected in nevi and melanoma and is the most common mutation with the replacement of valine to glutamic acid at codon 600. This site of point mutation is in BRAF kinase domain, which enhance the BRAF kinase activity. Arg 509 is the heart of interface, connecting two BRAF monomers by hydrogen bonds. Based on our results and as suggested by others that mutated BRAF (V600E) forms heterodimer with wild type BRAF or homodimer with itself and activates the MAPK pathway. We hypothesize that disturbance in the levels of wild type or mutated BRAF as we did by silencing RNA will result in alteration of dimer formation, which in turn could influence downstream effectors,

such as Grm1 expression. Similar outcomes were observed in the presence of a specific small molecular inhibitor of BRAF (V600E), PLX 4032. A reduction in mutated BRAF activity led to a decrease of Grm1 expression. These results corroborated that Grm1 expression was modulated at least in part by the activity of mutated BRAF.

In our hypothesis, we proposed that Grm1 is upregulated by mutated BRAF (V600E). *In vivo*, BJB mouse showed Grm1 expression only after activating mutated BRAF (V600E) by 4-HT in the melanocytes only. In the untreated BJB mice there is no detectable Grm1 expression. *In vitro*, introduction into MelanA the exogenous mutated BRAF also led to Grm1 expression. Next, we demonstrated that Grm1 was functional in these cells by the responsiveness exhibited by these cells in the presence of Grm1 agonist, Quisqualate, and antagonist, Bay 36-7620 using downstream effectors of p-AKT and p-ERK as read-outs. However, Grm1 antagonist, Bay 36-7620, was not completely effective in rendering the receptor nonfunctional as shown by modulation of p-ERK/p-AKT by Quisqualate even after pretreatment with Bay 36-7620 unlike in cells with wild type BRAF. [55] [56]

Expression of mutated BRAF in melanocytes was not sufficient to induce spontaneous melanoma development unless PTEN is altered or deleted [52]. We

now know that in mice with mutated BRAF and Grm1 expression at 13-15 months after turning on the expression of both was not sufficient to induce spontaneous melanoma development in contrast to our earlier report that ectopic expression of Grm1 in melanocytes *in vivo* led to spontaneous aggressive melanoma development. It has been postulated that mutation in proteins within the MAPK pathway resulted in cell senescence. Oncogene overexpression might upregulate p16 and p53, leading to oncogene-induced senescence (OIS). It is known that p53 is activated through DNA damage signaling, which could be induced by BRAF mutation. Therefore, Grm1 expression induced by mutated BRAF is not sufficient to overcome other gene(s) / pathways already activated by mutated BRAF in driving tumor formation. However, if the Grm1 expression is turned on even in the presence of wild type BRAF background, robust tumors form.

The functionality of PTEN is an important issue. We observed elevated p-AKT and p-PDK1 expression in MelanA BRAF (V600E) clones. We also detected PTEN expression but were unable to distinguish between wild type or mutated PTEN by Western immunoblots. The primary function of PTEN is lipid phosphatase that dephosphorylates PIP3, which cannot be bound by PDK1 thus counteracting stimulation of PI3K/AKT pathway. These results, led us to assume that PTEN might be downregulated by mutated BRAF or nonfunctional in the

cells. We treated the clones with PTEN inhibitor, bpV (phen) for various time points and concentrations and we detected dose- and time-dependent activation of AKT. Therefore, we concluded that PTEN is at least partially functional in these cells. However, how the p-AKT is stimulated in cells with functional PTEN background remains unsolved. It is possible that mutated BRAF could phosphorylate PTEN which lead to more stable but inactive form of PTEN.

Expression of mutated BRAF led to Grm1 expression also constitutively activated MAPK cascade, it is not known if Grm1 expression is regulated via MAPK cascade or mutated BRAF directly altered transcription/translation in Grm1. If Grm1 mRNA is present in untreated BJB mouse ear or immortalized mouse melanocytes, MelanA, and mutated BRAF expression alone leads to Grm1 expression, then a hypothesis could conclude that the regulation was at translational level. Possible involvement of miRNA, regulators of translation such as initiation factor 4E (eIF4E) are putative candidate. [57]

The next question is why Grm1 expression induced by mutated BRAF is not sufficient to induce melanoma. We have an ongoing experiment by cross-breeding BJB mice with TG3 mice. TG3 mice expresses ectopic Grm1 expression and BJB mice harbored conditional activation of mutated BRAF in melanocytes. We speculate that the progeny of such cross probably will develop spontaneous

melanoma because of Grm1 expression. We will turn on the mutated BRAF expression at various time points and see if there is a critical time with mutated BRAF expression that would result in the absence of tumor formation, suggesting temporal regulation of drive(s) of melanomagenesis. Another way is to create a mice harboring conditional activation of Grm1 expression with mutated BRAF background. In this case, we know the activation of Grm1 has to be at least less than one month because in BJB mice, at four weeks after Cre recombinase mediated the reciprocal exchange between wild type and mutated BRFA, the Grm1 expression was detectable and was not sufficient to induce tumor formation, perhaps earlier activation of Grm1 expression is needed.

Finally, how AKT is activated in cells with mutated BRAF expression. Results from the current studies suggest that despite partially functional PTEN, PI3K/AKT signaling is stimulated. It is possible that mutated BRAF could bypass PTEN to stimulate p-AKT or lower phosphatase activity of PTEN. This could be done by using wild type and mutated PTEN in mutated BRFA cells and assess how levels of wild type or mutated PTEN modulated activation of AKT under such conditions.

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FIGURES

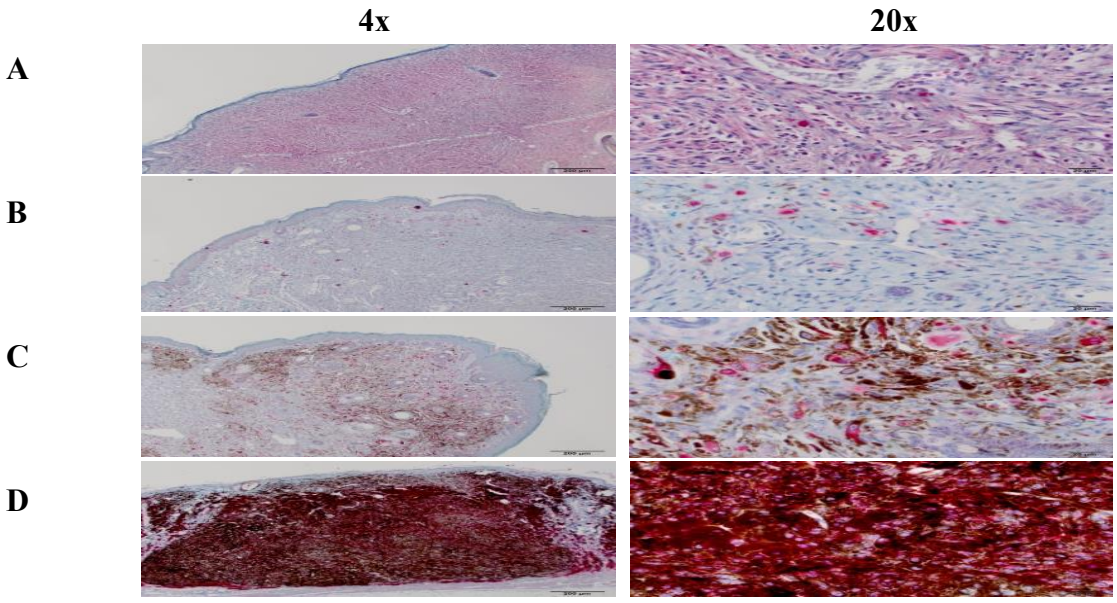

Figure 1. Immunohisotological staining of Grm1 in tumors from transgenic mice with different background

A. Grm1-Positive control TG3 mice.

B. Mutated BRAF (V600E) in heterozygous PTEN background.

C. Mutated BRAF (V600E) in PTEN null background

D. Mutated NRAS (Q61R) in an p16INK4a null background

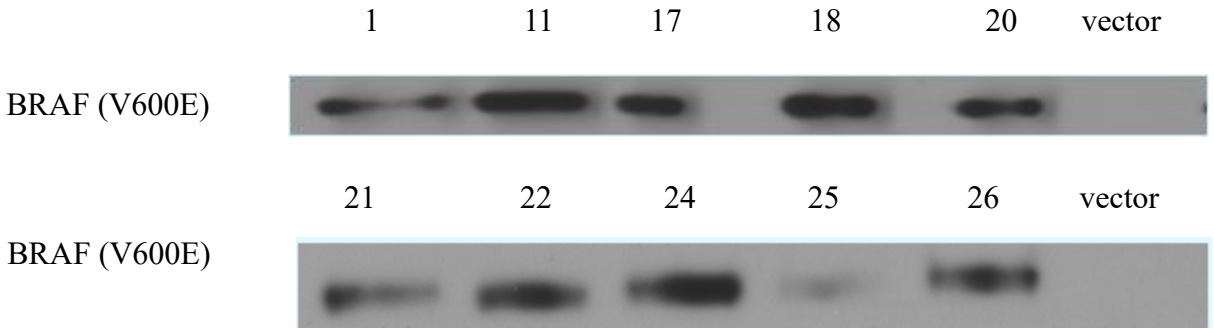

Figure 2. MelanA mutated BRAF clones

Immortalized mouse melanocytes, MelanA, were transfected with pEF-myc-BRAF (V600E) plasmid. Stable clones selected were resistant to geneticin (G418) (200ug/mg) in RPMI-1640 medium with FBS and TPA. Proteins was extracted from these clones and then confirmed the BRAF (V600E) produced in the transfected cells by Western blot.

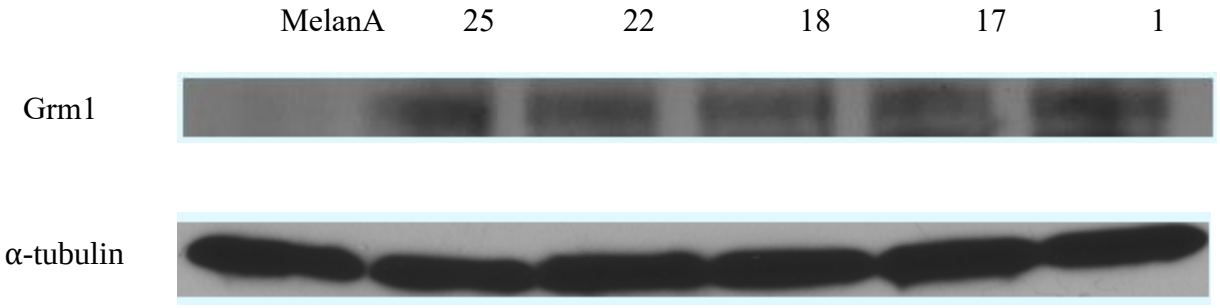

Figure 3. Grm 1 expression in MelanA mutated BRAF clones

Five MelanA BRAF (V600E) clones expressing BRAF (V600E) showed Grm1 expression compared to the negative control, MelanA. α-tubulin was used as normalization. The result indicated that Grm1 expression is induced by BRAF (V600E) in the cells.

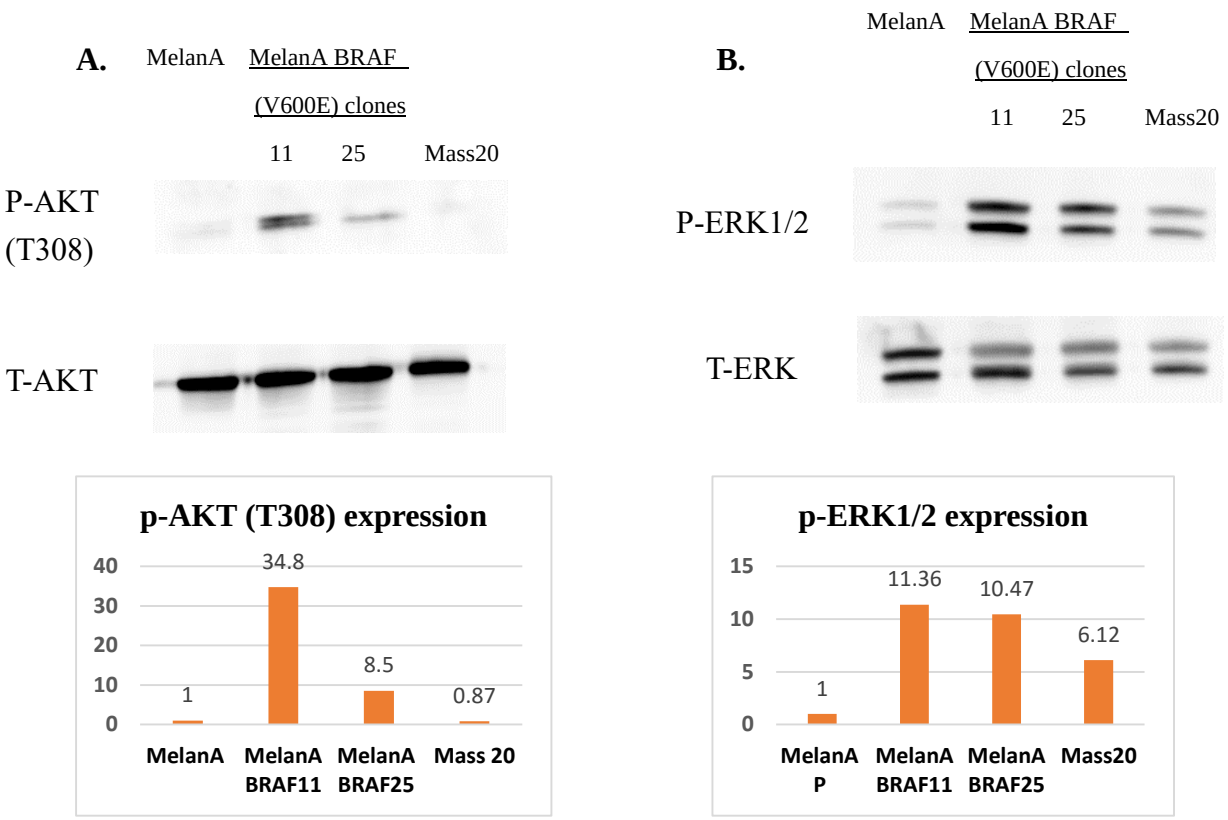

Figure 4. phosph-AKT and phosph-ERK expression in MelanA mutated BRAF clones

Cell protein lysates were extracted from MelanA BRAF (V600E) clone 11 and clone 25. Probing basal level of p-AKT (T308) and basal level of p-ERK1/2 as reas-outs to examine the activation of PI3K/AKT and MAPK pathways. Total AKT and total ERK1/2 were used as normalization. The results showed that higher basal level of p-AKT and p-ERK1/2 expressed in the cells producing BRAF (V600E) compared to the parental MelanA.

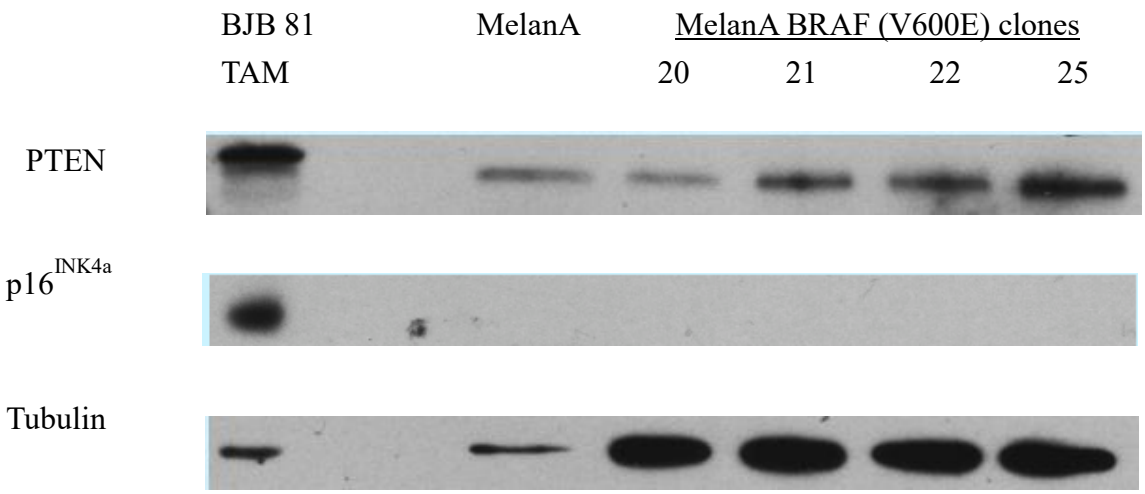

Figure 5. PTEN and p16^{INK4a} expression in MelanA BRAF clones

Probing anti-PTEN and anti-p16^{INK4a} in protein lysates extracted from the ears of BJB mouse treated with 4-HT, MelanA and MelanA BRAF (V600E) clones. In *in vitro* cell culture, p16^{INK4a} was deleted in the cells for immortalization while BJB mice was positive for p16^{INK4a}. PTEN was expressed in MelanA BRAF (V600E) clones and BJB 81 TAM. α-tubulin was used as normalization.

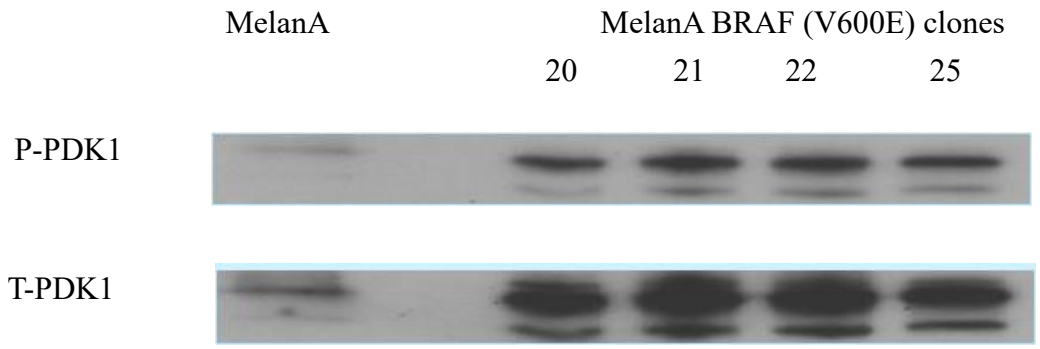

Figure 6. Phosph-PDK1 expression in MelanA mutated BRAF clones

p-PDK1 were probed in protein lysates of MelanA and MelanA BRAF (V600E) clones. p-PDK1 was able to phosphorylate AKT proteins (T308). Therefore, higher expression of phosph-PDK1 meant higher activation of PI3K/AKT pathway. The membrane was stripped and re-probed with total PDK1 as normalization.

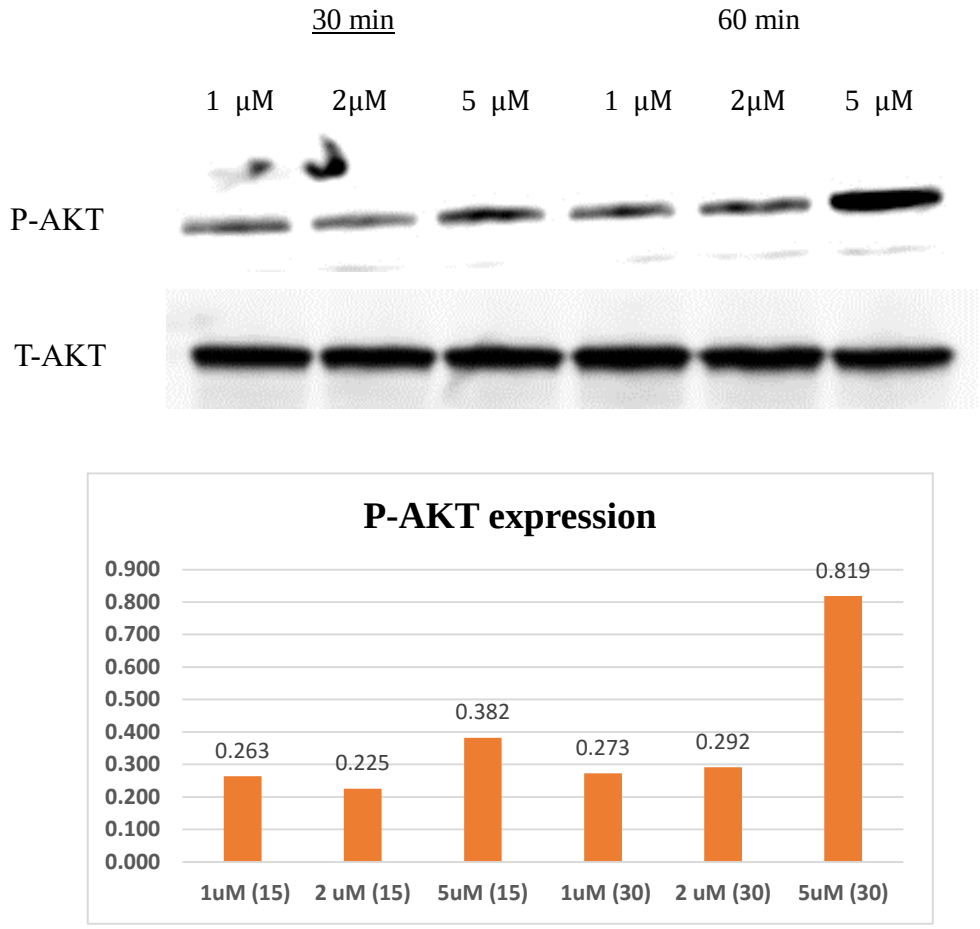

Figure 7. PTEN functionality test by PTEN inhibitor, bpV (phen)

MelanA BRAF (V600E) clones were treated with PTEN inhibitor, bpV(phen), in three different concentrations (1uM, 2uM and 5uM) for 30 and 60 minutes respectively. PTEN is an antagonist of PI3K/AKT pathway so p-AKT was probed as an outcome of function of PTEN. As the higher concentration of bpV(phen) applied, the higher activation of AKT was detected.

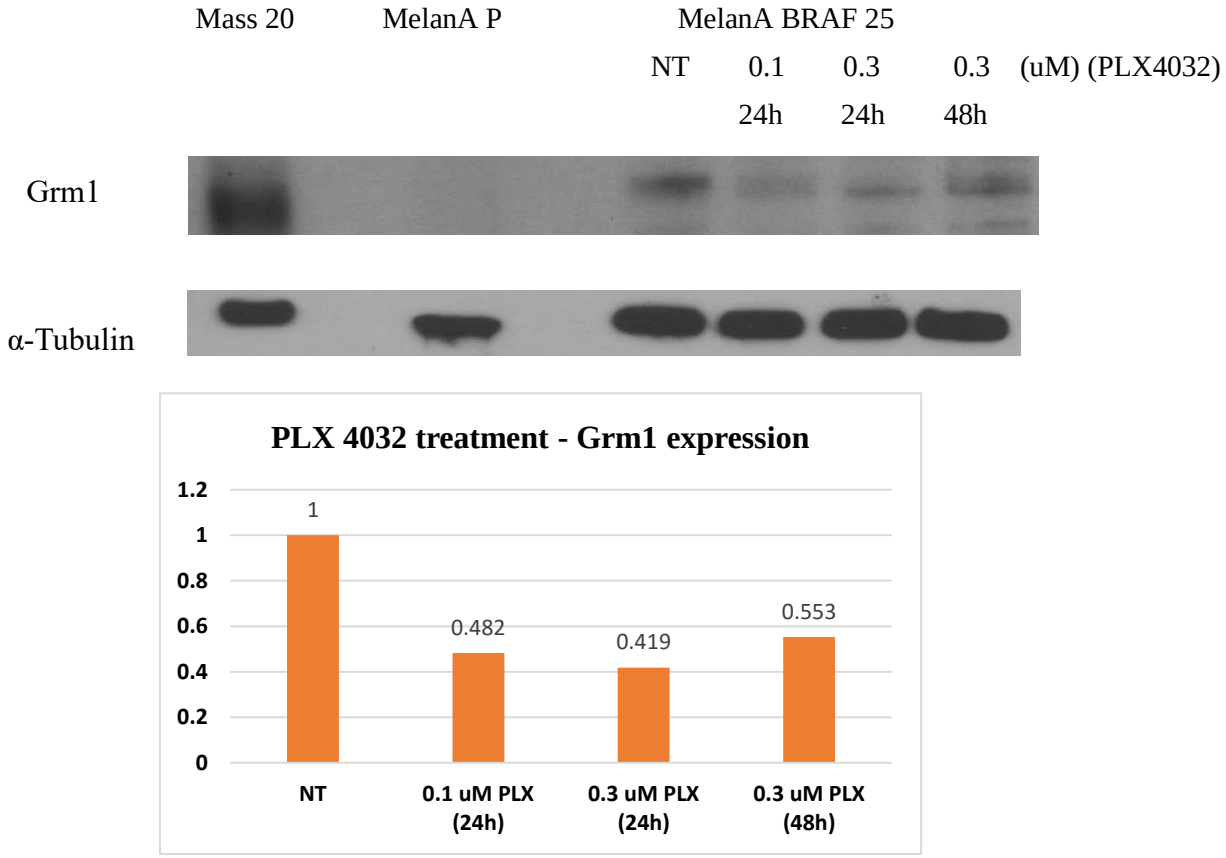

Figure 8. Grm1 expression was modulated by BRAF (V600E) activity

MelanA BRAF (V600E) cells were treated with PLX 4032 which is a small molecule inhibitor specifically for mutated BRAF (V600E) inhibitor with various concentration (0.1 μM and 0.3 μM) for 24 and 48 hours. Protein lysates were extracted after each time point and Grm1 was probed. The result demonstrated that Grm1 expression was proportional to BRAF (V600E) activity.

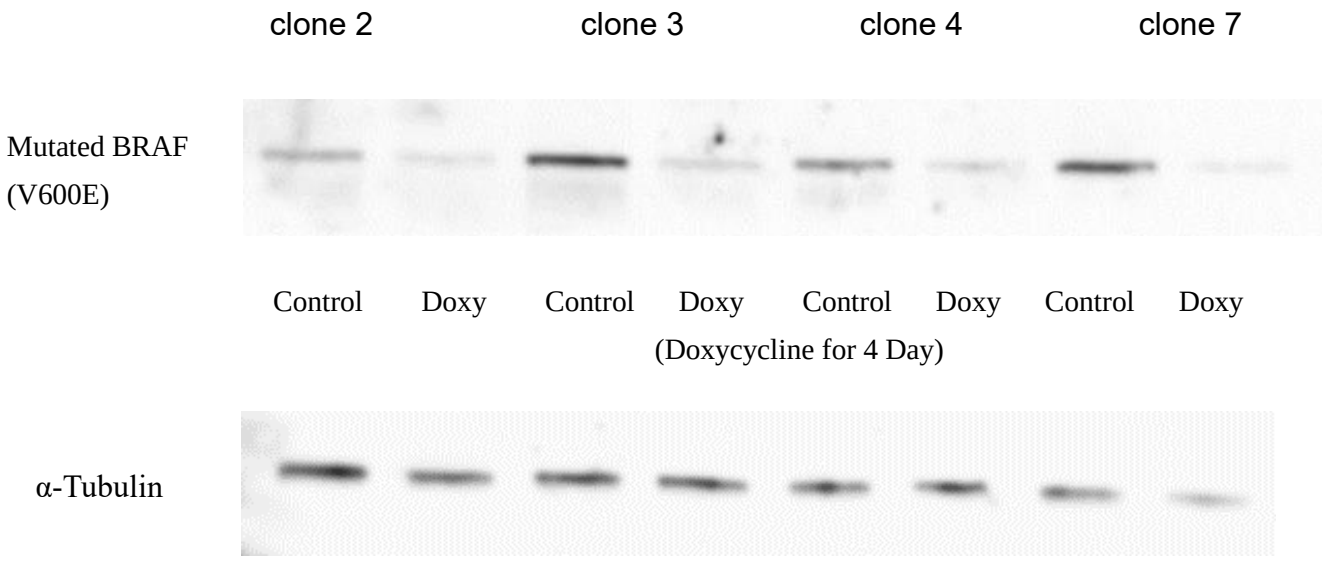

Figure 9. Generation of MelanA siBRAF (V600E) clones

MelanA BRAF (V600E) clones were transfected with Tet^R and siBRAF (V600E)-Tet^O plasmids and generated inducible MelanA siBRAF (V600E) clones, which were isolated in the RPMI-1640 media with G418 and hygromycin antibiotics. Western blots assessed suppression of BRAF (V600E) induced by Doxycycline. Control sample were the absence of doxycycline. After adding of doxycycline for 4 days, BRAF (V600E) expression was decreased.

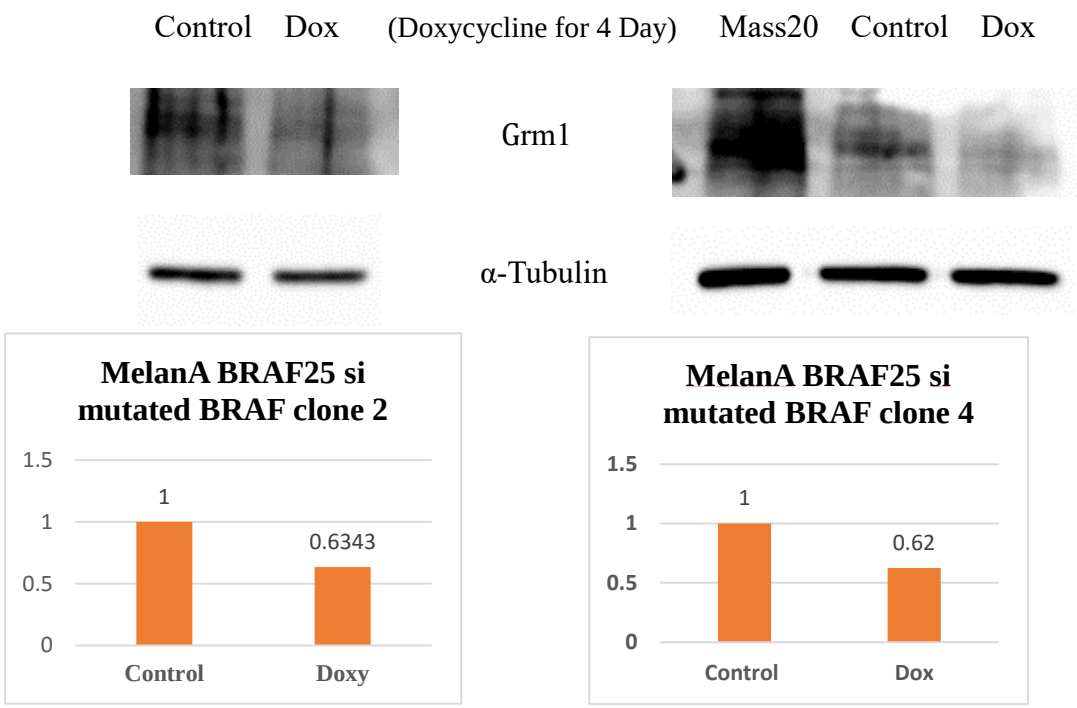

Figure 10. Grm1 expression in MelanA siBRAF (V600E) clones

Grm1 expression was evaluated in the MelanA siBRAF (V600E) clones. The result showed that Grm1 expression decreased when BRAF (V600E) expression also decreased. α-tubulin were used as normalization.

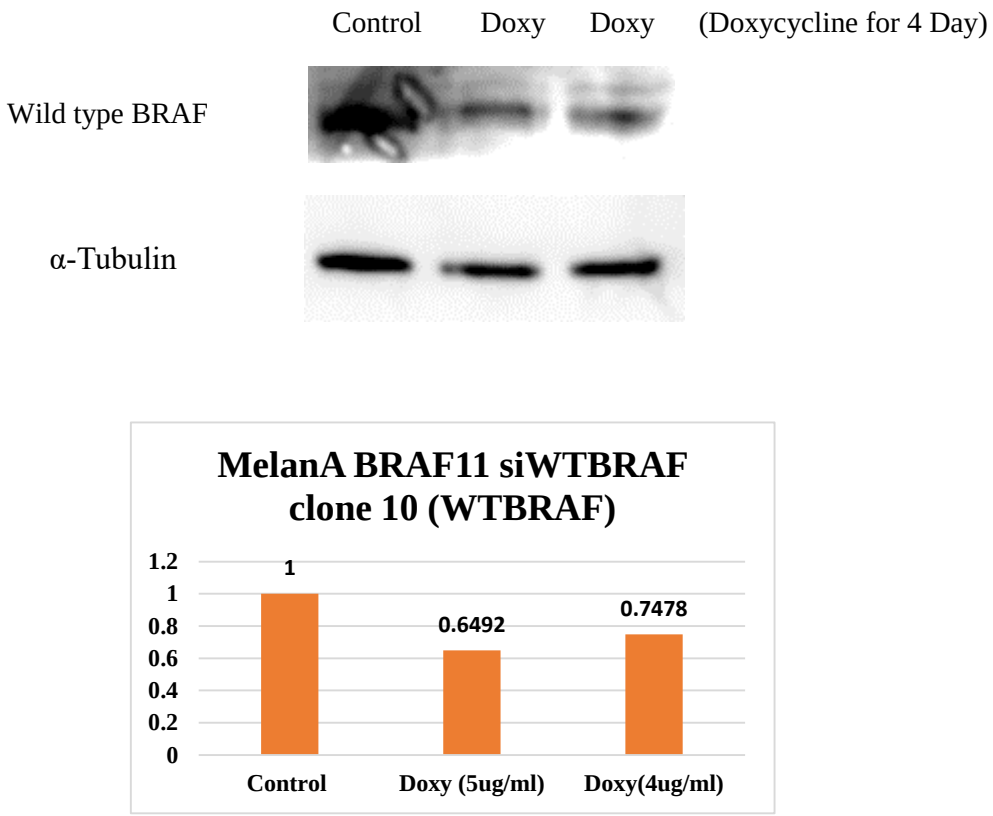

Figure 11. Generation of MelanA siWTBRAF clones

MelanA BRAF (V600E) clones were transfected with Tet^R and siWTBRAF -Tet^O plasmids and generated inducible MelanA siWTBRAF clones which were isolated in the RPMI-1640 media with G418 and hygromycin antibiotics. Western blots assessed suppression of WTBRAF induced by Doxycycline. Control sample were absence of doxycycline.

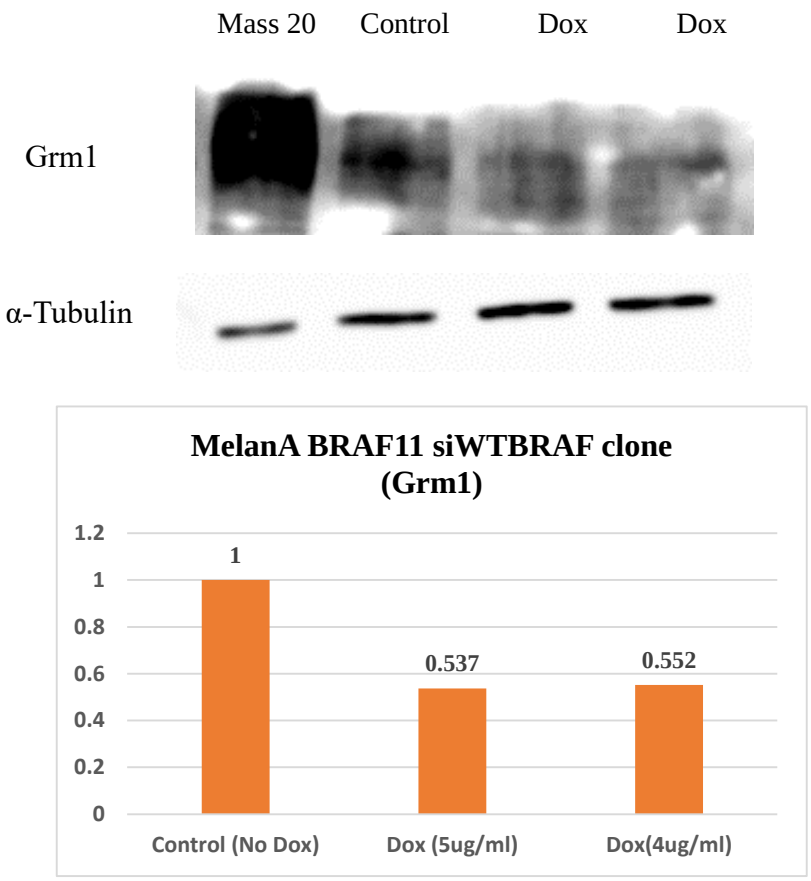

Figure 12. Grm1 expression in MelanA siWTBRAF clones

Grm1 expression was evaluated in the MelanA siWTBRAF clones. The result showed that Grm1 expression decreased correlated with a decrease in WTBRAF expression. α-tubulin was used as normalization.

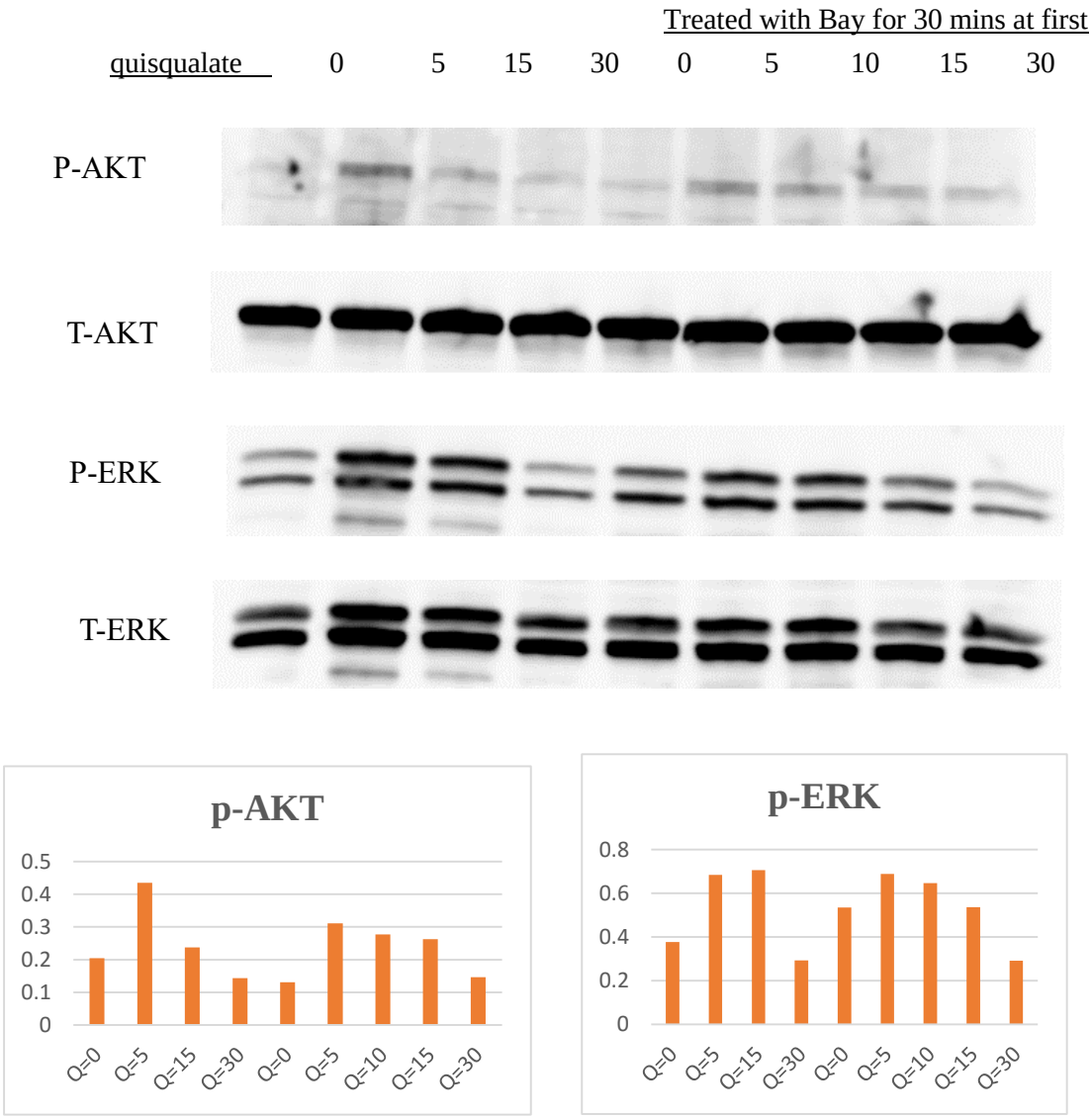

Figure 13. Modulation of AKT and ERK in MelanA BRAF (V600E) clones by Grm1 agonist and antagonist

Stimulation of mGlu1 by its agonist, L-Quisqualate (10 μM) led to the activation of AKT and ERK in MelanA BRAF (V600E) clones. Second setting was pretreatment of Grm1 antagonist, Bay 36-7620 (10 μM) for 30 minutes followed by various time point of treatment of Q. After inhibition of Grm1 activity, the function of Grm1 quickly responded to the stimulation by agonist, Q.

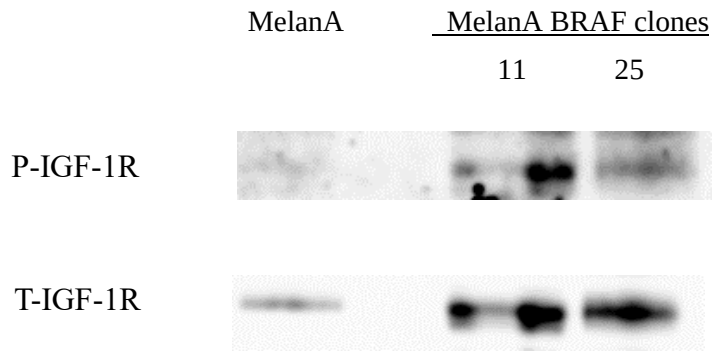

Figure 14. Phosph-IGF-1R is expressed in MelanA BRAF (V600E) clones

Phosph-IGF-1R was probed in MelanA and MelanA BRAF (V600E) clones. MelanA is negative for activated IGF-1R. MelanA BRAF (V600E) clone 11 and 25 expressed phosph-IGF-1R which might be involved in the activation of PI3K/AKT signaling pathway in these clones.

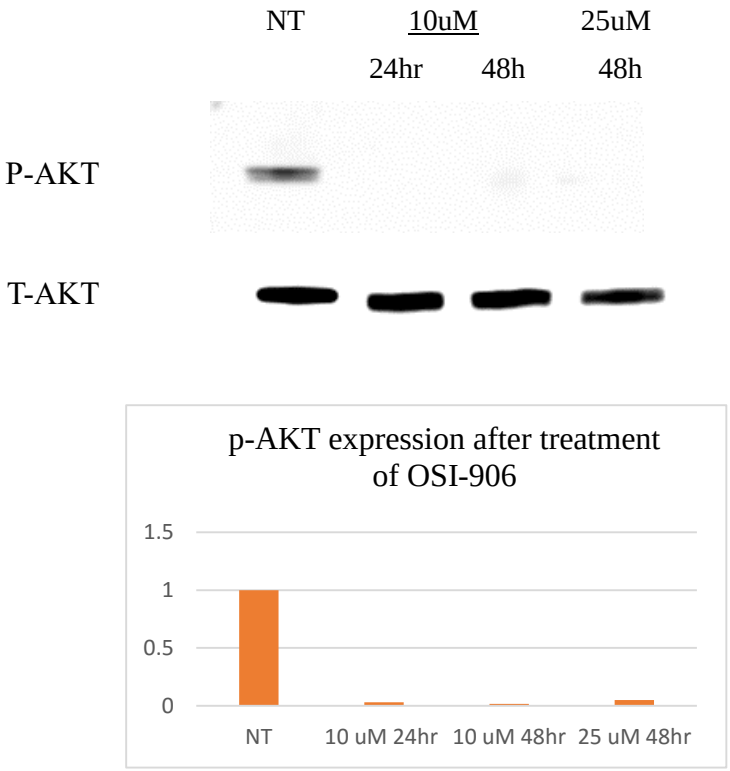

Figure 15. Activation of PI3K/AKT pathway was mediated by IGF-1R

MelanA BRAF (V600E) clones were treated with different concentrations of IGF-1R inhibitor, OSI-906. We examined whether activation of PI3K/AKT pathway was activated via IGF-1R. After treatment of IGF-1R inhibitor for 24 and 48 hours, p-AKT expression was completely knockdown. The membrane was stripped and re-probed with total AKT as normalization.

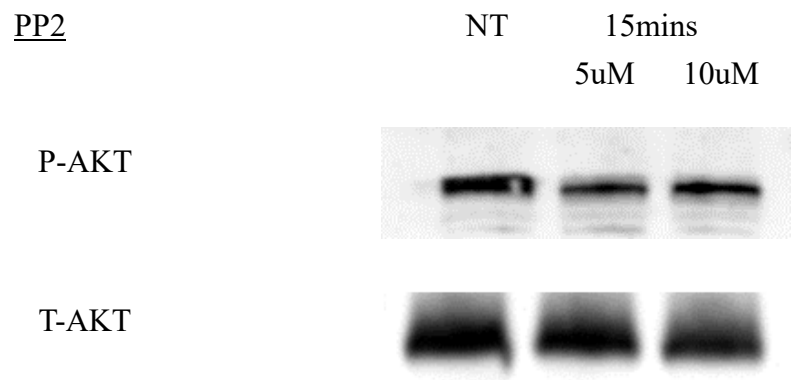

Figure 16. MelanA BRAF (V600E) treated with Src inhibitor, PP2

Treatment of MelanA BRAF (V600E) clones with 5uM and 10 μM of Src inhibitor, PP2 for 15 mins, protein lysates prepared and probed for pAKT. The proposed signaling pathway activated PI3K/AKT pathway was through the Src, non-receptor tyrosine kinase. And Src kinase protein phosphorylated IGF-1R for activating PI3K/AKT pathway. The results showed that down expression of p-AKT after treatment of Src inhibitor, PP2, for 15 mins. The membrane was stripped and re-probed with total AKT as normalization.

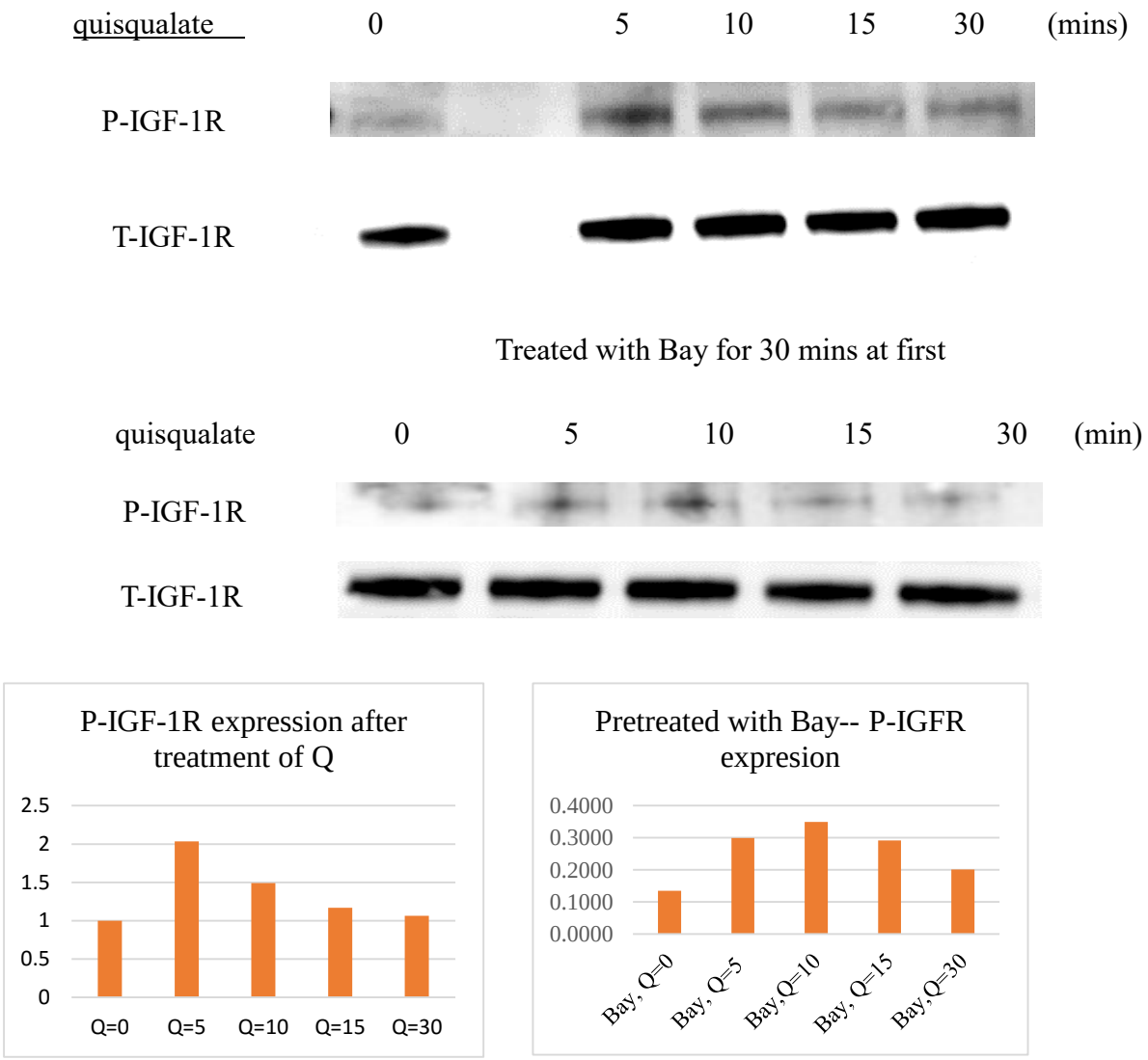

Figure 17. p-IGF-1R expression is modulated by Grm1 activity by treated with quisqualate or pre-treated with Bay followed by quisqualate

The same protein lysate as testing Grm1 functionality. MelanA BRAF (V600E) was treated with agonist, quisqualate, and antagonist, Bay followed by quisqualate. P-IGF-1R was regulated by Grm1 activity similar as the Grm1 downstream protein, p-AKT and p-ERK. The membrane was stripped and re-probed with total IGF-1R as normalization.

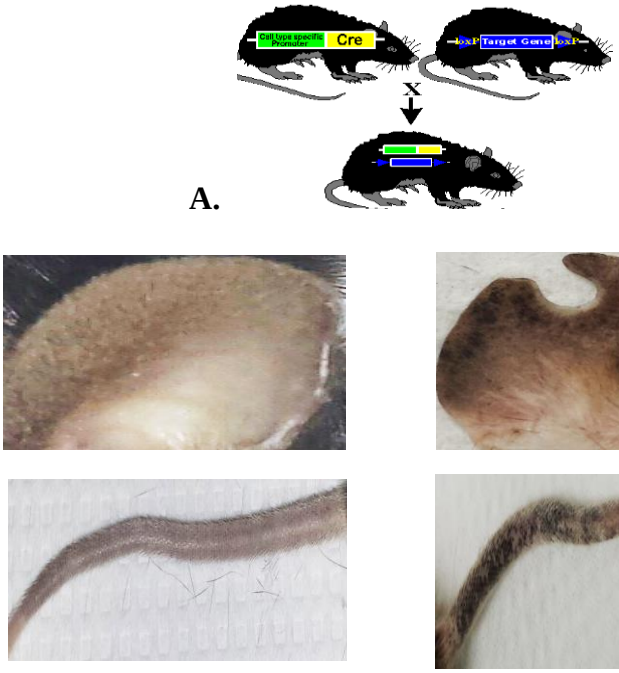

Figure 18. BJB mouse model with inducible mutated BRAF

(A) The BJB mouse was a transgenic mouse model carrying conditional gain-of-function mutations in specific tissue, melanocyte harboring loxP-BRAF (V600E)-loxP ; Cre^{ERT2} sequence. Absence of 4-hydroxytamoxifen (4-HT), Cre recombinase could not bind to loxP site and execute recombination. In the presence of 4-HT, Cre recombinase is active and BRAF (V600E) is produced in only melanocytes.

(B) 4-hydroxytamoxifen (15 mg/ml) was treated to ears of BJB mice for 4 days to induce the recombination of the loxP-BRAF (V600E)-loxP in melanocytes. After the treatment, skin hyperpigmentation around the ears and tail.

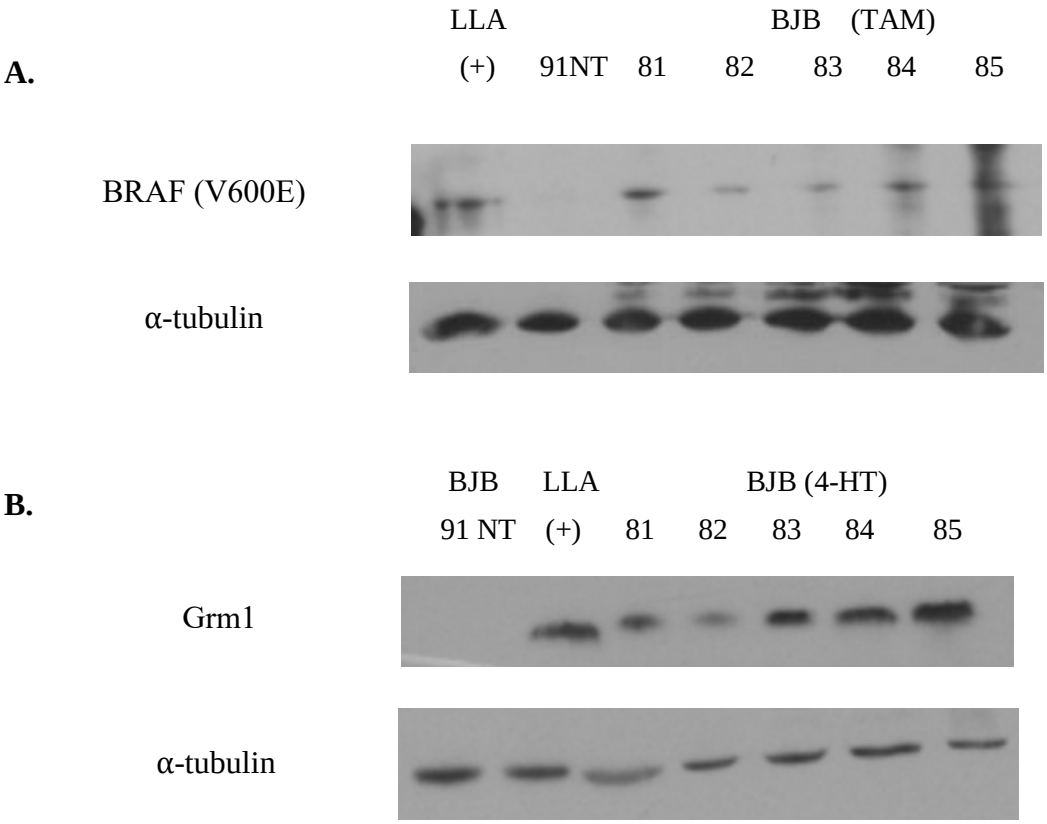

Figure 19. BRAF (V600E) and Grm1 expression in melanocytes after treatment of 4-hydroxytamoxifen

(A) Protein was extracted from the ears of BJB mice after mice were treated with 4-hydroxytamoxifen (15 mg/ml) to induce BRAF (V600E) in melanocytes. BRAF (V600E) expression was confirmed by Western blot after treatment of 4-hydroxytamoxifen. BJB 91 non-treatment of 4-hydroxytamoxifen (NT) is negative control for BRAF (V600E).

(B) Protein lysates were extracted from the ears of BJB mice after treatment of 4-hydroxytamoxifen to induce BRAF (V600E) in melanocytes. Grm1 expression was

positive in the melanocytes producing BRAF (V600E) after treatment of 4-HT. BJB 91 non-treatment of 4-hydroxytamoxifen (NT) is negative control for Grm1.

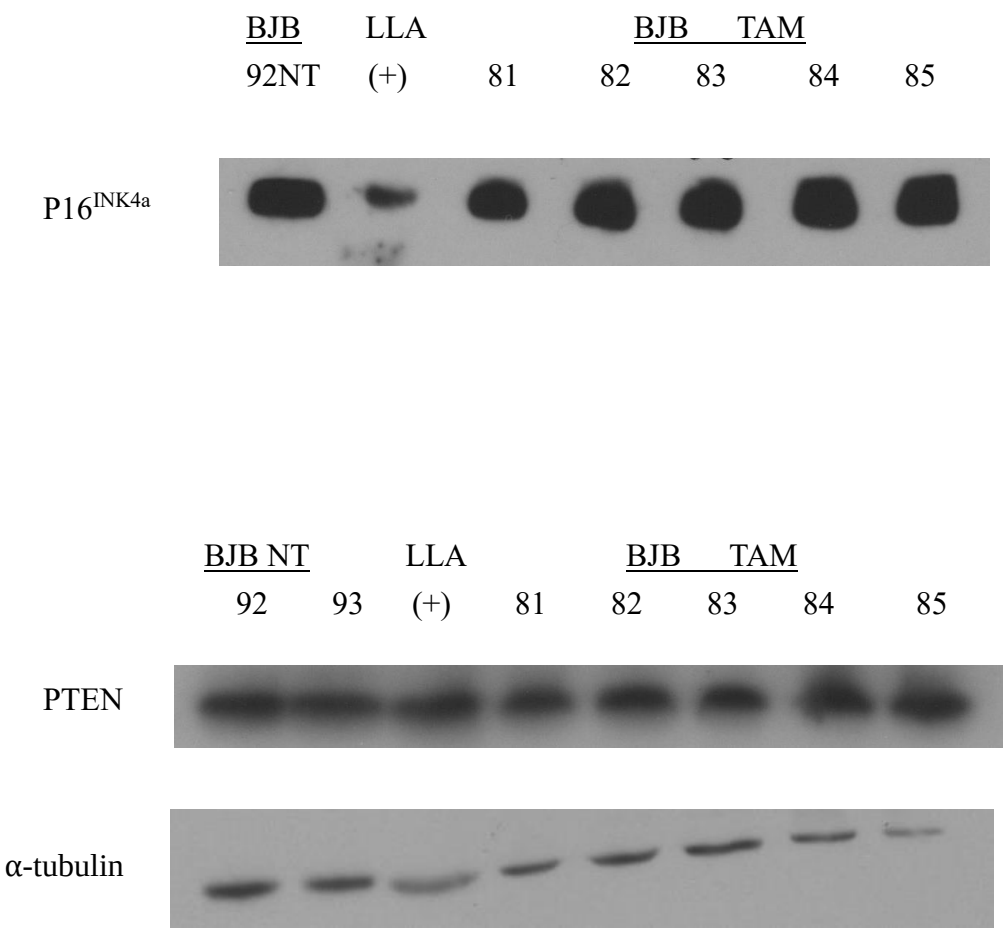

Figure 20. p16^{INK4a} and PTEN expression in melanocytes after treatment of 4-hydroxytamoxifen

Protein was extracted from the ears of BJB mice after mice were treated with 4-hydroxytamoxifen (15 mg/ml) to induce BRAF (V600E) in melanocytes. PTEN and p16^{INK4a} expression were assessed by Western immunoblots. Both of non-treatment and treatment of 4-hydroxytamoxifen showed PTEN and p16^{INK4a} expression.

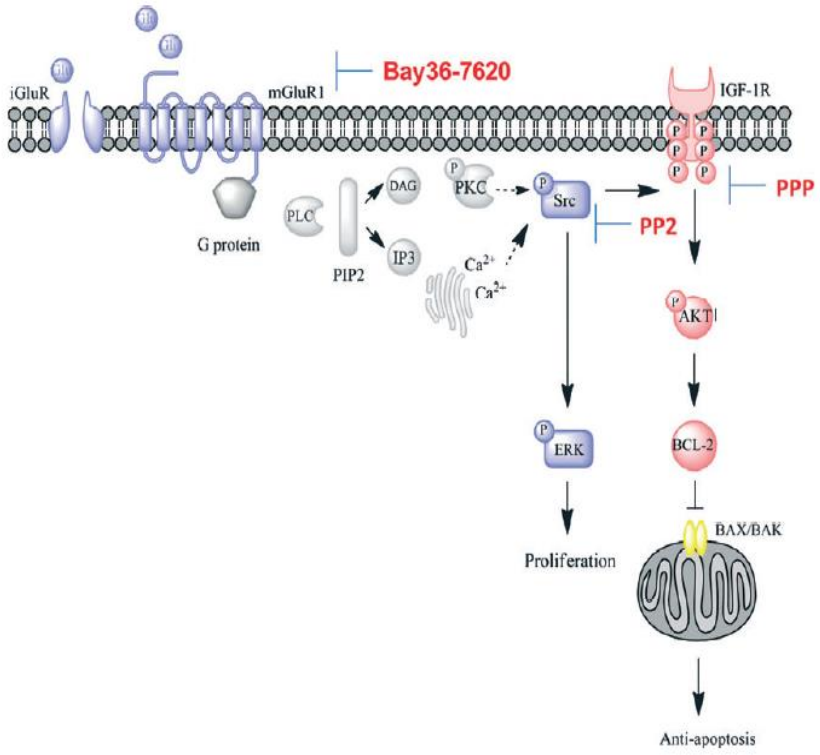

Figure 21. Signaling transduction of MAPK and PI3K/AKT pathways activated by Grm1

Grm1 activated PI3K/AKT pathway through activation of insulin-like growth factor-1 receptor. Treatment of IGF-1R inhibitor, PPP or OSI-906, inhibited the activation of AKT in MASS clones and MelanA BRAF (V600E) clones. In the induction experiment, our group showed that Grm1 activity modulated the activation of IGF-1R.