Dysregulation of the PKA Pathway in Cutaneous Melanoma

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Table of Contents

Abstract

Cutaneous melanoma originates from melanocytes, which are the pigment producing cells of a neural crest origin found in the basal layer of the epidermis. Understanding the genetics of melanoma has led to significant therapeutic breakthroughs for this malignancy. Specifically, the discovery of the hotspot *BRAF* V600E found in approximately 50% of cutaneous melanoma patients led to the development of BRAF targeted inhibitors that have produced significant initial responses and survival benefits for a subset of patients. However, clinical resistance remains a challenge. Dysregulation of the cAMP-PKA signaling pathway plays an important role in melanomagenesis. However, genetic alterations that targeted this pathway have remained relatively unclear. We have identified that the gene *PRKAR1A*, which encodes the regulatory subunit 1A of PKA haloenzyme, is significantly mutated in approximately 2% of cutaneous melanomas. Loss of the *PRKAR1A* regulatory subunit is predicted to lead to activation of PKA catalytic kinase subunits to promote phosphorylation of downstream targets in cancer. This is supported by the fact that germline mutations in *PRKAR1A* are found in tumor syndrome called Carney complex, which is characterized by the development of several tumors of neural crest origin. Furthermore, sequencing studies and genome-wide ORF screens have implicated a role for PKA signaling in BRAF inhibitor resistance. In this thesis, we provide functional evidence that *PRKAR1A* silencing alone does not lead to BRAF inhibitor resistance. However, combination silencing of *PRKAR1A* and a second regulatory subunit *PRKAR2B* leads to resistance. This work sheds light on a novel mechanism of BRAF inhibitor resistance in melanoma and suggests that PKA inhibitors should be investigated as a therapeutic strategy in melanoma.

Résumé

Le mélanome cutané provient des mélanocytes, qui sont les cellules productrices de pigments d'origine crête neurale présentes dans la couche basale de l'épiderme. La compréhension de la génétique du mélanome a conduit à des percées thérapeutiques importantes pour cette tumeur maligne. Plus précisément, la découverte du hotspot BRAF V600E trouvé chez environ 50% des patients atteints de mélanome cutané a conduit au développement d'inhibiteurs ciblés par BRAF qui ont produit des réponses initiales et des avantages de survie significatifs pour un sous-ensemble de patients. Cependant, la résistance clinique reste un défi. La dérégulation de la voie de signalisation cAMP-PKA joue un rôle important dans la mélanomagénèse. Cependant, les altérations génétiques qui ciblaient cette voie sont restées relativement peu claires. Nous avons identifié que le gène PRKAR1A, qui code pour la sous-unité régulatrice 1A de l'haloenzyme PKA, est significativement muté dans environ 2% des mélanomes cutanés. La perte de la sous-unité de régulation PRKAR1A devrait entraîner l'activation des sous-unités de kinase catalytique PKA pour favoriser la phosphorylation des cibles en aval dans le cancer. Ceci est soutenu par le fait que des mutations germinales dans PRKAR1A se trouvent dans le syndrome tumoral appelé complexe Carney, qui se caractérise par le développement de plusieurs tumeurs d'origine crête neurale. De plus, des études de séquençage et des tests ORF à l'échelle du génome ont impliqué un rôle pour la signalisation de PKA dans la résistance aux inhibiteurs de BRAF. Dans cette thèse, nous fournissons des preuves fonctionnelles que le silençage PRKAR1A seul ne conduit pas à une résistance aux inhibiteurs de BRAF. Cependant, la désactivation combinée de PRKAR1A et d'une deuxième sous-unité de régulation PRKAR2B conduit à une résistance. Ce travail met en lumière un nouveau mécanisme de résistance aux inhibiteurs de BRAF dans le mélanome et suggère que les inhibiteurs de la PKA devraient être étudiés comme stratégie thérapeutique dans le mélanome.

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Contribution of Authors

I would like to declare the contribution of Dr. Mathieu Lajoie and Rached Alkallas who performed all the bioinformatic analysis detailed in the first aim of this thesis. I would also like to declare the contribution of Karen Vo Hoang for preparing the cell lysates that were used to screen for protein expression in 13 human melanoma cell lines in the second aim of this thesis. I declare my contribution to the rest of the experimental techniques used in the second aim of this research project.

Abbreviations

AC – adenyl cyclase

ACTH – adrenocorticotropic hormone

AKAP – PKA anchoring protein

AMP – adenosine monophosphate

ARAF – A-raf

ATP – adenosine triphosphate

BRAF – B-raf

BSA – bovine serum albumin

CADD – Combined Annotation-Dependent Depletion

cAMP – cyclic adenosine 3',5'-monophosphate

CDK – cyclin dependent kinase

CRAF – C-raf

CREB – cAMP-response element binding protein

DMSO – dimethyl sulfoxide

DTT – dithiothreitol

ECL – enhanced chemiluminescence reagent

EDTA – ethylenediaminetetraacetic acid

ERK – extracellular signal-regulated kinase

FBS – fetal bovine serum

FDR – false discovery rate

GDP – guanosine diphosphate

GPCR – G-protein coupled receptor

GSK-3 – glycogen kinase synthase 3

GTP – guanosine-5`- triphosphate

HER2 – human epidermal growth factor receptor 2

HRP – horseradish peroxidase

KIT – KIT proto-oncogene, receptor tyrosine kinase

LoF – loss-of-function

MAPK – mitogen activated protein kinase

MDM2 – mouse double minute 2 homolog

MEK – mitogen activated protein kinase kinase

MITF – microphthalmia-associated transcription factor

NF1 – neurofibromin 1

NMF – negative matrix factorization

NRAS – neuroblastoma RAS viral oncogene

OFML – OncodriveFML

ORF – open reading frame

OXPHOS – oxidative phosphorylation

PBS – phosphate buffered saline

PDE – phosphodiesterase

PGC1α – peroxisome proliferator-activated receptor γ coactivator 1α

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

R1A – PKA regulatory subunit 1α

R1B – PKA regulatory subunit 1β

R2A – PKA regulatory subunit 2α

R2B – PKA regulatory subunit 2β

RIPA – radioimmunoprecipitation assay

RPMI – Roswell Park Memorial Institute

SDS-PAGE – sodium dodecyl sulphate- polyacrylamide gel electrophoresis

SMG – significantly mutated gene

STR – short tandem repeat

TCGA – The Cancer Genome Atlas

TSG – tumour suppressor gene

TWT – triple wild-type

UV – ultraviolet

Introduction

Melanoma Review and Genetics

Melanocytes are neural crest-derived cells that exist throughout the human body, most commonly in the skin, hair, and iris, but also in the nervous system, heart, and inner ear [1]. Melanocytes produce melanin, a compound which gives pigment to the skin, hair, and iris [1]. Skin melanocytes are found in the basal layer of the epidermis and communicate with keratinocytes through dendritic processes to form the epidermal melanin unit [1]. Melanin is synthesized in melanosomes, which are organelles present in the cytoplasm of melanocytes [1]. The melanosomes are transported through the dendrites of melanocytes to reach keratinocytes [1]. The melanin produced not only determines the skin colour, but also protects skin cells from ultraviolet (UV) radiation [1].

Melanoma is a cancer that develops from melanocytes [2]. In contrast to most cancers where incidence rates are declining, melanoma is one of the fastest rising cancers, especially in Western countries [2]. A major risk for cutaneous melanoma is UV exposure [3]. UV introduces signature mutations, which are characterized by cytidine to thymidine (C > T) transitions [3]. These signature mutations are found in melanoma oncogenes and tumor suppressors [4]. Key driver mutations in v-raf murine sarcoma viral oncogene homolog B (*BRAF*) and neuroblastoma RAS viral oncogene (*NRAS*) are fundamental to activate the mitogen activated protein kinase (MAPK) pathway in promoting proliferation of melanocytes, and melanomagenesis occurs when melanocytes accumulate additional secondary driver mutations [3,5].

BRAF mutations occur in 40-50% of patients with cutaneous melanoma and result in constitutive activation of the MAPK pathway, leading to promotion of unrestricted cell growth [3]. *BRAF* is part of a family of serine/threonine kinases with three isoforms, A-raf, B-raf, and C-raf

[6]. Normally, wild-type B-raf requires activation by the small GTPase RAS [3]. However, mutant B-raf is constitutively active and will phosphorylate mitogen-activated protein kinase kinase (MEK), which then phosphorylates extracellular signal-related kinase (ERK), to propagate the MAPK signalling cascade [3]. The most common *BRAF* mutation is *BRAF* V600E, which substitutes the amino acid valine 600 with a glutamic acid residue [3]. 70-88% of *BRAF* mutations are p.V600E [3]. *BRAF* p.V600K and p.V600R are less common variants [3]. The discovery of BRAF mutations has led to the development of MAPK targeted therapies in the form of BRAF and MEK inhibitors that are now included as part of standard of care treatment for BRAF mutant patients [3]. Although there are effective MAPK inhibitors available, therapy resistance remains a challenge [3].

The *NRAS, HRAS,* and *KRAS* are part of the RAS family of oncogenes [3]. In cutaneous melanoma, *NRAS* is the most frequently mutated family member, found in approximately 20% of patients [3]. Mutations in *NRAS* trigger constitutive activation of the MAPK and phosphoinositide 3-kinase (PI3K) pathways, among others [3]. However, currently there are no effective targeted therapies for patients with *NRAS* mutant melanomas [3]. Large-scale sequencing studies in 2015 identified *NF1* as a significantly mutated gene found in approximately half of *BRAF/NRAS* wild-type melanomas [7,8]. NF1 functions as a RAS GTPase Activating Protein (GAP) that downregulates RAS activity [3]. Loss-of-function mutations in *NF1* leads to nonsense-mediated mRNA decay and subsequent activation of the MAPK pathway [3,7]. Similar to RAS mutant melanomas, there are no effective targeted therapies for melanoma patients with *NF1* mutations [3]. However, immune checkpoint inhibitors, which include anti-PD-1 and CTLA-4, have produced improved survival benefits for 30-50% of melanoma patients of all MAPK genotypes and remains the standard of care treatment for melanoma patients[9] .

Cutaneous melanoma patients that lack *BRAF*, *NRAS* and *NF1* mutations are categorized as Triple Wild-Type (TWT) melanomas [7]. These melanomas are characterized by increased copy-number alterations compared to the other mutant melanoma subtypes, which include cyclin D1 and mouse double minute 2 homolog (*MDM2*) [3,7]. TWT cutaneous melanoma also contains mutations/amplifications in the oncogene *KIT*, a receptor tyrosine kinase that activates pathways affecting cell growth and survival [3,7]. It is still unclear how gene amplifications commonly observed amplifications in TWT can be used to devise new therapeutic regiments to treat melanoma patients, and numerous studies are underway to address this [7].

Melanoma metabolism

In the landmark review article, Hanahan and Weinberg have categorized the hallmarks of cancer that include sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death [10]. More recent hallmarks that have been recognized by the cancer community as vital from decades of research include the reprogramming of energy metabolism [11]. In the 1920s, Otto Warburg first discovered that cancer cells primarily use glycolysis for energy metabolism, even in the presence of oxygen, and this is known as the Warburg effect [11,12,13,14,15]. A hypothesis that may explain why cancer cells upregulate glycolysis is that the intermediate products of glycolysis could be used to synthesize amino acids and nucleotides, which would facilitate cell division [11].

Melanoma cells with *BRAF* V600E mutations are associated with decreased oxidative phosphorylation (OXPHOS) and increased glycolysis [16,17,18,19]. One study observed that knockdown of *BRAF* V600E caused glycolysis to decrease in melanoma cell lines, which

strongly suggests that the upregulation of glycolysis in melanoma is a result of the dysregulation of the MAPK pathway [20].

Additionally, *BRAF* V600E downregulates the expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), which is a driver of mitochondrial biogenesis and is involved in oxidative phosphorylation [17]. Mechanistically, *BRAF* V600E suppresses the expression of microphthalmia-associated transcription factor (*MITF*), which binds to the promoter of *PGC1α*, to directly downregulate its expression [17]. Through this connection to PGC1α, MITF is a regulator of mitochondrial respiration [17]. Tissue extracted from tumours of melanoma patients who received treatment with vemurafenib (also known as PLX4032), a potent *BRAF* V600E inhibitor, displayed increased expression of PGC1α, further suggesting that *BRAF* V600E is responsible for the downregulation of OXPHOS in melanoma [17].

Second Messenger Signalling Review

G-protein coupled receptors (GPCRs) are cell surface receptor proteins that respond to a wide variety of stimuli [21]. The three subunits of GPCRs are Gα, Gβ, and Gγ [21]. Once bound to a ligand, the GPCR undergoes a conformational change that promotes the exchange of GDP for GTP at the Gα subunit, which then releases the Gα subunit from the complex [21]. The dissociated GTP-bound Gα subunit triggers signalling cascades downstream [21]. Inactivation of the Gα subunit occurs once its own GTPase activity hydrolyzes the bound GTP to GDP [21].

A classic example of GPCR signalling is the activation of adenyl cyclase (AC) [22]. AC is stimulated by first messenger molecules such as adrenocorticotropic hormone (ACTH), which activates G-protein coupled receptors [22,23]. Released Gα subunits bind to AC to promote production of the second messenger cyclic adenosine 3',5'-monophosphate (cAMP) [22]. While AC produces cyclic AMP, 3′,5′-cyclic nucleotide phosphodiesterase (PDE) degrades it [22,24].

Discovered in 1958, cAMP is responsible for the modulation of pathways related to processes such as metabolism, gene regulation, and immune function [22,25].

Protein kinase A (PKA) is one of the main effectors of cAMP [26]. The PKA pathway is involved in the regulation of cellular metabolism [26]. PKA anchoring proteins (AKAPs), which are bound to cytoskeletal proteins and organelles, sequester PKA so that the kinase may be readily available when required [27]. PKA exists in its inactive form as a tetramer with two regulatory and two catalytic subunits [26]. In the activation of PKA, two molecules of cAMP are bound to each regulatory subunit, which allows for the release of the catalytic subunits [27]. The catalytic subunits are then free to phosphorylate downstream targets [27]. In total, there are four isoforms of the regulatory subunit (R1A, R1B, R2A, R2B) and three isoforms of the catalytic subunit (Cα,Cβ, Cγ) of PKA [27]. The expression of the regulatory and catalytic subunits vary depending on the cell type [27].

One of the major phosphorylation targets of PKA is cAMP-response element binding protein (CREB), a transcription factor involved in promoting the expression of genes which control cell proliferation and survival [26,28]. PKA activates CREB by phosphorylation on serine 133 [28]. Another downstream effector of PKA is glycogen kinase synthase 3 (GSK-3), a serine/threonine kinase that regulates glycogen synthesis and is involved in pathways responsible for cell survival and proliferation [29]. Phosphorylation of GSK-3α on serine 21 and serine 9 on GSK-3β inactivates the kinases [29].

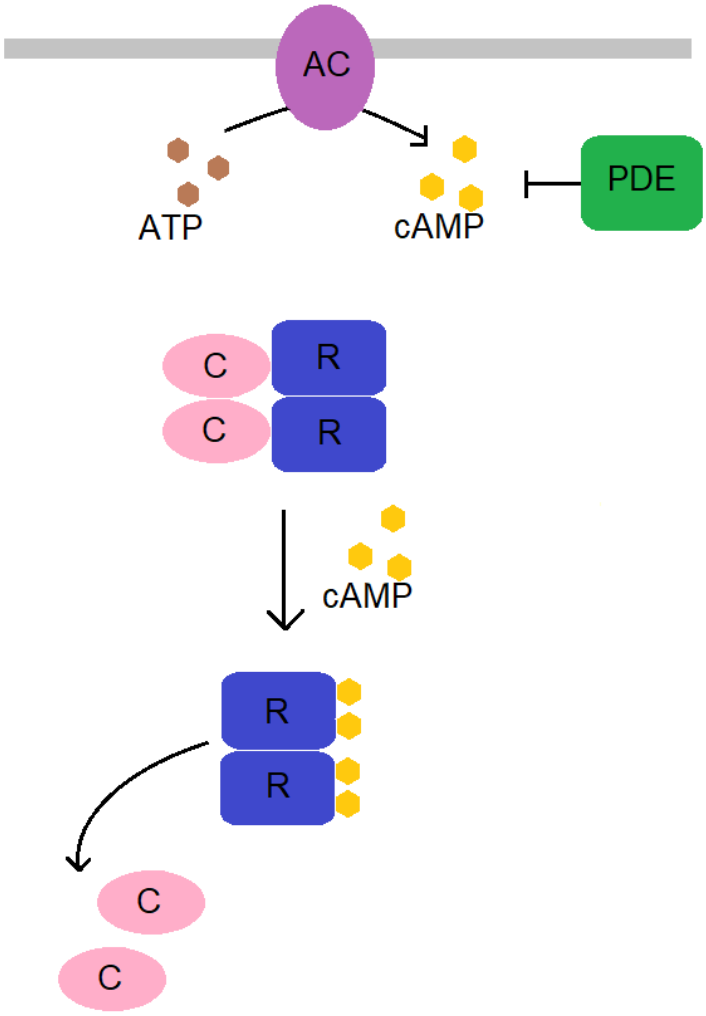

Figure 1. Diagram of PKA regulation. Adenyl cyclase (AC) is stimulated to produce cAMP from ATP, which then binds to the regulatory subunits of the PKA holoenzyme. Phosphodiesterase (PDE) enzymes degrade cAMP. As a result of cAMP binding, the regulatory subunits release the active catalytic subunits to propagate PKA signalling on downstream substrates. When there is loss of the regulatory subunits, the catalytic subunits are rendered constitutively active.

PKA and Cancer

Dysregulation of the PKA pathway is implicated in tumorigenesis and cancer [27]. The most notable example is that an inherited autosomal dominant mutation that leads to the loss of

regulatory subunit R1A of PKA is the cause of Carney Complex [30]. Patients with Carney Complex are predisposed to the development of endocrine tumours, spotty skin pigmentation, and melanotic schwannomas [30]. Without the regulatory subunit R1A, the PKA catalytic subunit becomes constitutively active, leading to unregulated PKA activity that eventually results in tumorigenesis [30]. Through this link to Carney Complex, it was found that *PRKAR1A*, the gene coding for R1A, is a tumour suppressor [30].

Interestingly, a recent report found that overexpression of the PKA catalytic subunit alpha (*PRKACA*) in a whole genome overexpression screen led to resistance to BRAF inhibitors in melanoma cells [31]. Another report observed that *PRKACA* expression is upregulated in breast cancer [32]. Breast cancer cells that were resistant to anti-HER2 therapy had increased expression of *PRKACA* and provides resistance to breast cancer cells through activation of the anti-apoptotic protein BAD [32].

One study using lymphocytes of Carney Complex patients that contained an inactivating mutation in *PRKAR1A* showed increased PKA activity due to the decreased expression of *PRKAR1A* [33]. Cells carrying the inactivating mutation in *PRKAR1A* had increased proliferation compared to cells with wild-type *PRKAR1A*, indicating that *PRKAR1A* regulates cell growth [33].

Mouse model studies demonstrated that mouse embryonic fibroblasts containing a null *PRKAR1A* genotype had increased expression of the PKA catalytic subunit and the oncogene cyclin D1 [34]. Treatment of wild-type and *PRKAR1A*-null cells with PKA inhibitor H89 decreased total cyclin D1 levels, suggesting that PKA activity is one of the many factors that regulate cyclin D1 expression [34]. This report found that inactivation of *PRKAR1A* leads to increased cell proliferation through overexpression of the PKA catalytic subunit and that cyclin D1 may play a role in resisting senescence as a result of PKA pathway dysregulation [34].

A lung adenocarcinoma study found that adenocarcinoma cells which *PRKAR1A* knockdown had decreased tendency to metastasize compared to control group [35]. Mechanistically, *PRKAR1A* was found to be a tumour suppressor in lung adenocarcinoma by regulating E-cadherin, Snail1, and ERK1/2 expression [35]. shRNA Knockdown of *PRKAR1A* led to increased Snail1 mRNA expression and increased phosphorylated ERK1/2 protein levels [35]. The study suggested that since Snail regulates E-cadherin expression, *PRKAR1A* also regulates E-cadherin by regulation of Snail1 [35].

These investigations all provide evidence that *PRKAR1A* is a tumour suppressor and that knockdown of R1A results in increased cell proliferation due to unregulated PKA activity. However, some studies propose the contrary. The investigations outlined below suggest that dysregulation of PKA regulatory subunits can result in increased proliferation of cancer cells, which is contradictory to most of the literature and in cases of disease such as Carney Complex.

Studies have shown that the PKA regulatory subunits affect breast cancer cell proliferation [36]. One investigation observed that both type R1 and type R2 of PKA regulatory subunits are overexpressed in breast cancer, but there is higher expression of type R1 compared to type R2 [36]. The ratio between protein levels of type R1 and type R2 affects breast cancer cell proliferation [36]. Overexpression of type R1 in breast cancer was associated with aggressiveness of tumour growth [36]. The effects of R1A overexpression in the breast cancer cell line MCF-7 were shown to be reversible [36]. When knockdown of R1A was performed by antisense oligonucleotides, cell numbers decreased compared to the no treatment control [36].

In human cholangiocarcinoma, *PRKAR1A* has been reported to be overexpressed [37]. Stable lentiviral knockdown of R1A in cholangiocarcinoma cell lines resulted in decreased PKA activity and decreased cell proliferation [37]. Conversely, in immunohistochemistry staining of

healthy cells from human donors, *PRKAR2B* was detected much more than *PRKAR1A* [37]. Furthermore, R2B expression was downregulated in cholangiocarcinoma cells, whereas R1A was overexpressed, which indicated that the ratio of PKA regulatory subunit expression plays a physiological role in the context of disease [37].

An earlier study from 1996 showed that overexpression of R2B in human colon carcinoma cells led to decreased proliferation, DNA synthesis, and invasion [38]. In contrast, overexpression of R1A led to increased DNA synthesis, shown by thymidine incorporation into DNA [38]. Furthermore, lentiviral overexpression of R2B in LS-147T colon carcinoma cells resulted in decreased cell numbers in serum-starved conditions, which did not occur with parental cells or cells that overexpressed R1A or R2A [38]. This indicated that R2B overexpression had caused the cells to revert to an untransformed phenotype [38].

From a 1993 report, R1A was shown to be overexpressed in melanoma [39]. In a 2008 investigation regarding the overexpression of *PRKAR1A* in melanoma, knockdown of R1A by siRNA interference led to an increase in R2B protein expression [40]. Following the increase in R2B expression, decreased cell proliferation and induced apoptosis through caspase-3 activation was observed [40]. The study reported that the high-R1A to low-R2B ratio tips the balance towards increased cell proliferation in melanoma, indicating that manipulation of the ratio between R1A and R2B expression may serve as a therapeutic option [40].

Taken together into consideration, these investigations indicate that dysregulation of the PKA pathway leads to pathological phenotypes.

Objectives

The dysregulation of the PKA pathway is known to be the cause of Carney Complex, a disease involving the growth of tumours of neural crest origin [30]. CREB, which is activated upon phosphorylation at serine133 by PKA, binds directly to the promoter of *MITF* to regulate its expression in melanocytes [41]. The development of melanoma, although driven by key mutations in the MAPK pathway, is also reliant on the accumulation of other driver mutations that contribute to the progression of the disease [3]. Although the PKA pathway is linked to the MAPK pathway through crosstalk and is implicated in other cancers, the role of the PKA pathway in the development of melanoma is unclear [41].

Our lab has performed a mutation significance analysis of over 1,000 melanomas and identified *PRKAR1A* as a significantly mutated gene (SMG), with loss-of-function mutations in approximately 2% of cutaneous melanomas. The cAMP-PKA signaling pathway has been known to play an important role in the progression of melanoma [41]. Studies have shown that overexpression of *PRKACA* is also linked to MAPK inhibitor resistance in melanoma [31]. The activation of the PKA pathway leads to increased CREB expression, and the cAMP-PKA-CREB pathway is suspected to be related to acquired resistance to MAPK inhibition due to overlapping activation of the same transcription factors, such as *MITF*, that are regulated by both the PKA-CREB and MAPK pathways [31]. This evidence strongly suggests that dysregulation of the PKA pathway leads to MAPK inhibitor resistance in cutaneous melanoma.

The objectives of this project are to 1) determine which regulators of the PKA pathway are dysregulated in melanoma and 2) determine the link between BRAF inhibitor resistance and *PRKAR1A* signalling.

Methods

Cell Lines

Human melanoma cell lines used for siRNA transfection and drug studies were: Malme-3M parental and A375 parental cell lines. A previous graduate student prepared frozen cell lysate from human melanoma cell lines which were used for western blotting in Figures 2 and 3 of the Results section: Malme-3M, A375, UACC-62, 1205-LU*, Colo829, WM793A, Meljuso, GAK, IGR1, MeWo, WM3918F, CHL1, and WM3211*. Refer to Table 1 below for the mutational signatures of the various cell lines.

Table 1. Mutational signatures of melanoma cell lines

Mutational Signature	**Human Melanoma Cell Lines**
BRAF V600E	Malme-3M, A375, UACC-62, 1205-LU*, Colo829, WM793A
BRAF V600K	IGR1
NRAS Q61L	Meljuso, GAK
NF1	MeWo, WM3918F
Triple Wild-Type	CHL1, WM3211*

*Following STR profiling, our lab is no longer using the cell lines WM3211 and 1205-LU in experiments.

Cell Culture

Cells were grown in 10 cm tissue culture treated plates in 5% CO_2 at 37°C. Cells were cultured in Roswell Park Memorial Institute (Wisent) medium supplemented with 5% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). The cell media was changed once every 3-5 days and cells were passaged when confluency reached 70-100%. 0.25%

Trypsin-EDTA (Gibco) was used to lift cells (1 mL trypsin-EDTA for each 10 cm plate). Dulbecco's phosphate-buffered-saline (D-PBS, Wisent) was used to wash the cells before adding 0.25% trypsin-EDTA. Extra cells were frozen for storage by resuspending in BAMBANKER™ (Lymphotec) serum-free medium after the cells were counted and centrifuged. The resuspended cell mixture was aliquoted by the millilitre into cryovials (Nalgene) for storage in liquid nitrogen. Cells were frozen at a concentration range of approximately 1 million cells/mL – 3 million cells/mL.

Transfection of siRNA

Freshly thawed cells were used to keep the passage number as low as possible so as to not affect transfection efficiency. Cells were pulled from liquid nitrogen and cultured for approximately one week before seeding in 6-well tissue culture treated plates at a density of 300 000-400 000 cells/well in a total volume of 2 mL media per well. Transfection took place the next day to allow cells to settle overnight. The transfection method used is the siRNA DharmaFECT procedure found online from Dharmacon. The volume of DharmaFECT transfection reagent used in experiments varied between 5 uL, 7.5 uL, and 10 uL, depending on the siRNA used in the transfection. Following transfection, the cells were placed in the incubator for approximately 48 hours before lysis or drug treatment. If the cells appeared to have morphologic changes indicating stress 24 hours after transfection, the media was replaced to reduce cytotoxicity. The siRNA used were from Dharmacon and are listed as follows: siGenome control siRNA 1, ON-TARGETplus PRKAR2B siRNA, ON-TARGETplus PRKAR2B siRNA, ON-TARGETplus PRKAR1A siRNA, and ON-TARGETplus PRKAR1A siRNA, and these siRNAs all targeted the open reading frame (ORF) of the target genes. siGenome GAPD control

siRNA from Dharmacon was used as the positive control siRNA when optimizing siRNA transfections.

Time-Dependent Drug Treatment of siRNA-Transfected Cells

Following 48 hours of siRNA transfection, the cells were treated with vemurafenib (also known as PLX4032, Selleckchem). For the time-dependent drug treatment, cells in 6-welled plates were treated with 0.1uM final concentration of vemurafenib in 2 mL RPMI 1640 media for 1 hour, 2 hours, 4 hours, and 8 hours. Vemurafenib dilutions in 100% DMSO were prepared beforehand at a concentration of 333.3uM, and this was then diluted again in RPMI 1640 media before adding to the cells. This double-dilution method was performed to ensure that the concentration of DMSO in the cell media would not be toxic to the cells. The vemurafenib-treated cells had a final concentration of 0.03% DMSO in their media. The negative control was treating the cells with a final concentration of 0.03% DMSO for 1 hour. After aspirating the drug-mixed media, each well was washed once or twice with 1 mL D-PBS before the plate was double-wrapped in Parafilm and frozen at -20°C for lysis the following day.

Lysis of Cells

The lysis buffer used was Pierce RIPA Lysis and Extraction buffer, mixed with Halt™ protease and phosphatase inhibitor cocktail (100X) (reagents from ThermoFisher Scientific). For a 6-well plate, 50 uL of lysis buffer was used to lyse each well. Cells were scraped with a cell scraper and collected in microfuge tubes for rotation at 4°C for 40 minutes. After rotation, the cell debris was spun down for 10 minutes at 15000 rpm in a pre-chilled 4°C centrifuge. The cell

pellet was discarded, and the cell lysate was collected in a fresh microfuge tube and placed in ice for protein quantification.

Protein Quantification

The DC assay from Bio-Rad was used to quantify the amount of protein present in cell lysate samples before western blotting. Protein standards were made ahead of time from bovine serum albumin (BSA) diluted in RIPA buffer to decreasing concentrations from 1.45 ug/uL to 0 ug/uL and thawed before use. Dilutions were made for each cell lysate by mixing a small amount of cell lysate with RIPA buffer for a 1:15 dilution. In a clean 96-well plate, 5 uL of the protein standards were pipetted in triplicate. As well, 5 uL of the cell lysate dilutions were pipetted into each well in triplicate. 20 uL of Reagent S (Bio-Rad) was added to each millilitre of Reagent A (Bio-Rad) to make the light-sensitive working solution Reagent A'. 25 uL of Reagent A' was then added to each well that contained protein standards and cell lysate dilutions. Following the addition of Reagent A', 200 uL of Reagent B (Bio-Rad) was pipetted into each well. The 96-well plate was then incubated in a dark drawer for 15 minutes. After incubation, the absorbance was read at 750 nm. Any large bubbles present in the wells were popped with gel-loading pipette tips before the absorbance was read. The absorbance values were used to plot a protein standard curve to determine the amount of protein present within each cell lysate sample. Samples were diluted with RIPA lysis buffer to normalize protein concentrations to the sample with the lowest protein concentration. 950 uL of NuPAGE LDS 4X Sample Buffer (ThermoFisher) was mixed with 50 uL of 1M DTT to make every millilitre of 4X sample buffer. The 4X sample buffer was added to the normalized cell lysate samples to get an end concentration of 1X sample buffer

within the total sample volume. Samples were then boiled at 95°C for 10 minutes on a heat plate and stored at -20°C.

Western Blotting

Samples were loaded onto 10 or 15-welled 1mm 8% SDS-PAGE denaturing gels, and 40 ug of protein was loaded into each well. The gels were run for 30-40 minutes at 95V until the dye front ran past the stacking gel, then the voltage was changed to 110V and the gels ran for an additional 30-40 minutes until the dye front reached the bottom of the gels. The protein molecular weight marker used was Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (Bio-Rad). The protein in the gels were transferred onto nitrocellulose membrane (Bio-Rad) using the Bio-Rad Trans-Blot Turbo Transfer System. Following transfer, the membrane was blocked for 1 hour with 5% milk in phosphate-buffered-saline-tween (PBST, 8g NaCl, 0.2g KCl, 0.24g KH_2PO_4, 1.44g Na_2HPO_4, 0.1% Tween 20, pH 7.4) at room temperature on a shaker. The membrane was then probed with primary antibodies diluted in primary antibody solution (PBST, 5% BSA, 0.05% NaN_3) overnight at 4°C with shaking. After washing three times with PBST for 5 minutes each, the membrane was incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in 2.5% milk in phosphate-buffered-saline-tween for 40 minutes at room temperature with shaking. After secondary incubation, the membrane was washed three times with PBST for 10 minutes each time. The membrane was placed on a flat plastic tray, treated with enhanced chemiluminescence reagent (ECL, GE Healthcare) and weighed down with a flat glass plate for 1-2 minutes. Then, the membrane was quickly submerged in phosphate-buffered-saline (PBS, 8g NaCl, 0.2g KCl, 0.24g KH_2PO_4, 1.44g Na_2HPO_4) and gently wiped dry on clean paper towel before being placed on an imaging

cassette. Imaging took place in the dark room with film (Diamed). If necessary, a membrane would be stripped once to re-probe for proteins that had the same molecular weights. The nitrocellulose membrane was stripped by submerging in Restore™ Western Blot Stripping Buffer (ThermoFisher) for 10 minutes while shaking at room temperature and re-probed following the procedure detailed above. Refer to Table 2 for a list of all antibodies used.

Table 2. Primary and Secondary Antibodies used in Western Blotting

Primary Antibodies					
Antibody	Source	Clonal	Clone/Catalogue	Company	Dilution
HSP70	Rabbit	Polyclonal	4872	Cell Signalling	1:1000
PRKAR1A	Rabbit	Polyclonal	A303-683A	Bethyl	1:2000
PRKAR2A	Mouse	Monoclonal	Clone 40	BD Biosciences	1:2000
PRKAR1B	Rabbit	Polyclonal	HPA026719	Millipore Sigma	1:2500
PRKAR2B	Mouse	Monoclonal	Clone 45	BD Biosciences	1:2000
PKA Catalytic Subunit	Mouse	Monoclonal	Clone 5B	BD Biosciences	1:2000
Phospho-PKA C-sub T197	Rabbit	Polyclonal	4781	Cell Signalling	1:1000
GAPDH	Rabbit	Monoclonal	14C10	Cell Signalling	1:3000
Phospho-ERK1/2 T202/Y204	Rabbit	Polyclonal	9101	Cell Signalling	1:1000
ERK1/2	Rabbit	Polyclonal	9102	Cell Signalling	1:1000
Phospho-CREB S133	Rabbit	Monoclonal	87G3	Cell Signalling	1:1000
CREB	Rabbit	Polyclonal	06-863	Millipore Sigma	1:1000
Secondary Antibodies					
Mouse HRP	Horse	N/A	7076	Cell Signalling	1:5000
Rabbit HRP	Goat	N/A	7074	Cell Signalling	1:5000

Results

Aim 1: Identification of the Dysregulation of the PKA Pathway in Melanoma Progression

The results for this aim were produced by Rached Alkallas and Dr. Mathieu Lajoie from the Watson Lab. Their contribution to this thesis is greatly appreciated.

To identify significantly mutated genes (SMGs), mutation calls for 1,014 melanoma whole exomes/genomes from 5 published studies were uniformly annotated [42]. Significantly mutated genes were identified using OncodriveFML (OFML) [43]. OFML is a statistical tool that uses a Combined Annotation-Dependent Depletion (CADD) score, which combines multiple annotations (such as conservation measures such as phyloP and protein-level scores such as SIFT) into a single metric to reflect the relative functional impact [44,45,46]. OFML uses a permutation approach that detects positive selection by comparing the average impact score of the mutations in a gene with its expected distribution under the hypothesis of neutral evolution [43]. This approach is very similar to another tool used to identify significantly mutated genes in melanoma, called InVEx [4]. To better detect tumor suppressor genes (TSGs), an additional score that considers high confidence loss-of-function (LoF) mutations (frameshifts, loss of translation start sites, premature stop codons, and splice site mutations) was created [42]. The combined OFML (CADD and LoF) analyses identified 38 SMGs (false discovery rate (FDR) < 1%) [42]. *PRKAR1A* was identified as a SMG in approximately 2% of cases [42].

Furthermore, using gene expression data from 468 samples provided by The Cancer Genome Atlas, negative matrix factorization (NMF) data analysis was performed to sort out patterns in the mRNA expression of melanoma tumour cells [42]. Five subgroups of gene expression signatures were identified among the samples [42]. Two subgroups had gene expression consistent with normal skin cells and immune cells [42]. The three other subgroups had gene

expression consistent with melanoma intrinsic expression changes [42]. One subgroup exhibited decreased expression of *MITF* mRNA [42]. The second subgroup had increased expression of genes regulating oxidative phosphorylation (the OXPHOS group) [42]. The third subgroup included the largest number of melanoma samples and had increased *MITF* expression [42]. We observed that loss-of-function mutations in *PRKAR1A* were exclusive to the OXPHOS group [42]. Furthermore, mRNA expression of *PRKACA* was also highest in this OXPHOS mRNA expression group [42]. This analysis provided evidence to implicate the dysregulation of the PKA pathway in contributing to the progression of melanoma.

Aim 2: Determine Link Between PRKAR1A Signalling and MAPK Inhibitor Resistance

The results of this aim were produced by my own work.

We wanted to establish melanoma models to interrogate the effects of *PRKAR1A* and other PKA signaling members on *in vitro* melanoma phenotypes, including response to BRAF inhibitors. To do this we performed western blots on a panel of human melanoma cell lines to compare protein expression levels of regulators of the PKA pathway (Figure 2).

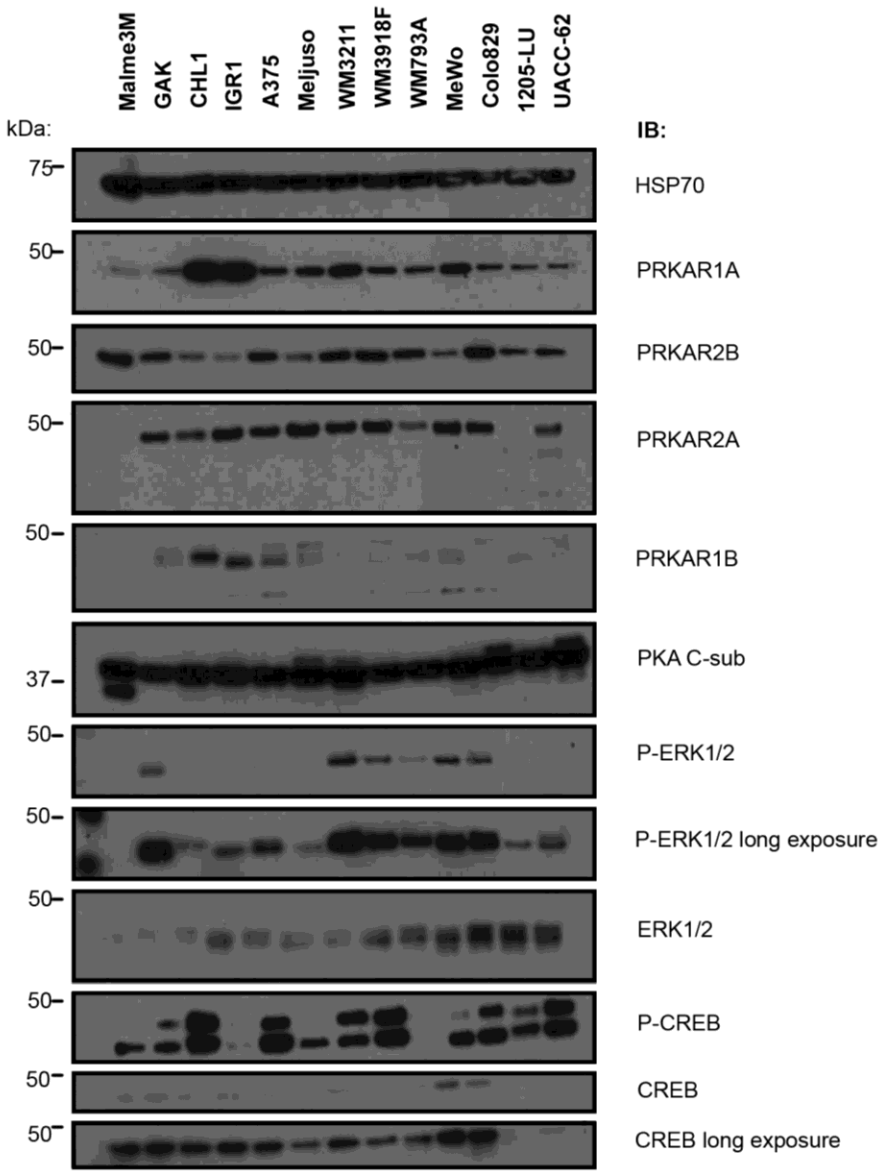

Figure 2. Western blot of PKA pathway-related protein expression in 13 human melanoma cell lines. Frozen cell lysates of human melanoma cell lines were used for this western blot. Varying expression of proteins can be compared between different cell lines. Flash exposure, unless indicated otherwise. Longer exposure times were 5 seconds. The samples were loaded onto 15-welled 8% SDS-PAGE gels and these images were taken from different gels. This is a composite image combining western blot images from different gels that were loaded with the same sample set. Note: Following STR profiling, our lab is no longer using the cell lines WM3211 and 1205-LU in experiments.

Specifically, we examined protein expression of *PRKAR1A* across our panel of *BRAF*, *NRAS* and *NF1* mutant cell lines. After comparing the protein expression levels in different human melanoma cell lines (Figure 2), we decided A375 would be the first cell line used for siRNA silencing of the PKA regulatory subunits, since it contains the *BRAF* V600E mutation and R1A and R2B were expressed at a relatively high level and at equal levels. Additionally, the A375 cell line had relatively high expression of most the signaling components of the PKA pathway, which would provide good reference points when detecting changes in protein expression. These western blot images provided insight into the baseline level of protein expression in different human melanoma cell lines.

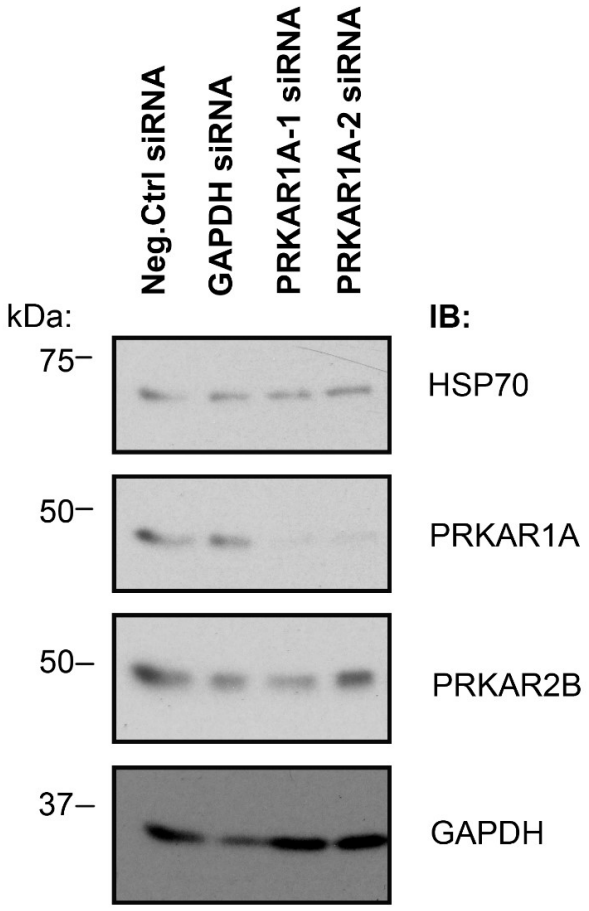

Figure 3. Western blot of siRNA silencing optimization in A375 cells. Flash exposure. 300000 cells/well were seeded in a 6-well plate. 5 uL DharmaFECT reagent was used to transfect 10 uL of 5 uM siRNA per well. *PRKAR1A* and *PRKAR2B* were probed overnight to verify knockdown effectiveness. HSP70 served as the loading control protein. The samples were loaded into two 10-welled 8% SDS-PAGE gels to run simultaneously so that *PRKAR1A* and *PRKAR2B* could be probed for at the same time. In the bottom gel, the *GAPDH* positive control siRNA seems to have efficient knockdown of *GAPDH*.

Next, we performed siRNA optimization experiments to knockdown the regulatory subunits, *PRKAR1A* and *PRKAR2B* (Figure 3 and 4). We observed significant *PRKAR1A* silencing using a ratio of 1:2 DharmaFECT to siRNA (5uL DharmaFECT to 10uL of 5uM siRNA) (Figure 3). siRNA Knockdown targeting *PRKAR2B* required further optimization. siRNA silencing of *PRKAR2B* required a greater ratio of DharmaFECT transfection reagent to siRNA observing good knockdown with 7.5 uL and 10 uL DharmaFECT (to 10uL of 5uM siRNA). *PRKAR2B* siRNA 1 silences effectively with 7.5uL and 10uL DharmaFECT, while *PRKAR2B* siRNA 2 silences only under conditions of adding 10uL DharmaFECT. Since *PRKAR2B* siRNA 2 was not as efficient as *PRKAR2B* siRNA 1 at knockdown of *PRKAR2B*, subsequent experiments used only *PRKAR2B* siRNA 1. Further transfections of *PRKAR2B* siRNA 1 used 7.5 uL DharmaFECT reagent, as adding more DharmaFECT increased the risk of cytotoxicity in cells. Additionally, the *GAPDH* siRNA control effectively silenced *GAPDH* as a positive control to compare protein knockout results. The *GAPDH* positive control was only required for optimization of siRNA transfection, therefore, it was not used for subsequent experiments.

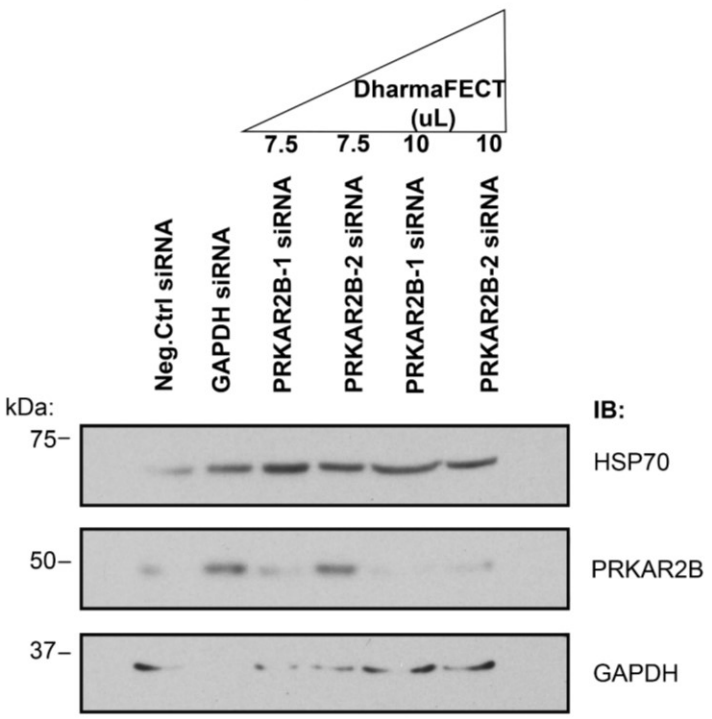

Figure 4. Western blot showing results of siRNA silencing of *PRKAR2B* in A375 cells. A375 cell lysate samples were loaded into 10-welled 8% SDS-PAGE gels. Different combinations of DharmaFECT reagent and siRNA were tested to compare the knockdown effectiveness. 7.5 uL and 10 uL of DharmaFECT were used to transfect each of the two siRNAs targeting *PRKAR2B*. Non-targeting siRNA was used as the negative control (5 uL DharmaFECT) and *GAPDH* siRNA was used as the positive control (5 uL DharmaFECT). HSP70 was probed for the loading control.

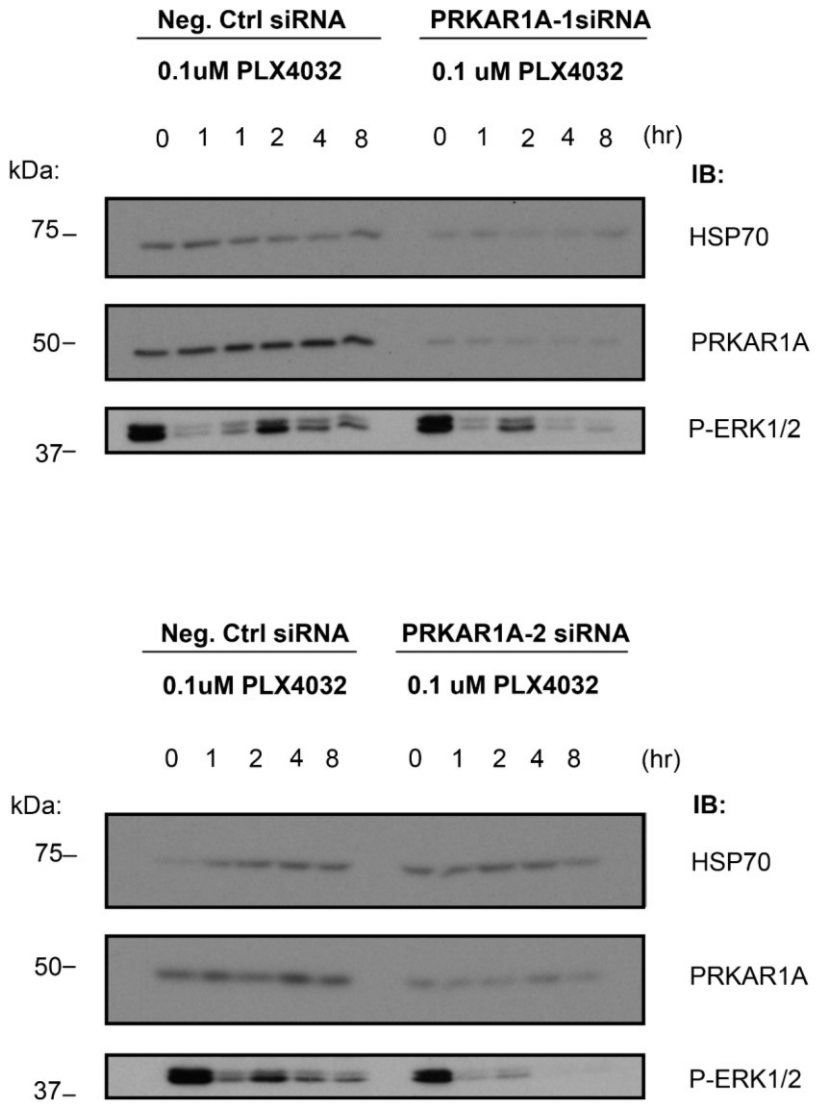

Figure 5. Western blot showing results of the time-dependent drug treatment study of *PRKAR1A*-silenced A375 cells. 400000 cells/well were seeded in 6-well plates and transfected with *PRKAR1A* siRNA. Two days following transfection, the cells were treated with 0.1 uM final concentration of PLX4032 (vemurafenib) for 1 hour, 2 hours, 4 hours, and 8 hours. DMSO served as the no treatment control and was added to the cell media for 1 hour. Total concentration of DMSO in the cell media for all samples was 0.03%.Note: In the top western blot, the negative control sample for 1-hour treatment with vemurafenib was loaded twice by accident.

Subsequently, we examined the effect of PRKAR1A silencing on BRAF inhibitor signaling using the inhibitor vemurafenib (PLX4032, Figure 5). *BRAF* mutant A375 cells were treated with 0.1 uM vemurafenib following PRKAR1A knockdown and examined effects on downstream ERK1/2 phosphorylation. We observed no consistent effect of on phospho-ERK1/2 following PRKAR1A knockdown (Figure 5). The PRKAR1A knockdown samples and control samples both had activated ERK1/2 expression after 2 hours of treatment with 0.1 uM vemurafenib, but the ERK1/2 expression was much less in *PRKAR1A* knockdown samples. This indicated that there might have been loss of negative feedback signalling from the MAPK pathway following treatment with vemurafenib which led to reactivation of the MAPK pathway, thereby increasing ERK1/2 activation after 2 hours of exposure to vemurafenib [6]. However, the knockdown of *PRKAR1A* did not appear to result in any increase in MAPK signalling as time of treatment lengthened beyond 2 hours compared to the cells transfected with non-targeting siRNA. For the negative control samples, it appeared that ERK1/2 activation was increasing at 4 and 8 hours of treatment, whereas for the *PRKAR1A* knockdown samples, ERK1/2 activation continued to decrease after 2 hours of treatment with vemurafenib.

Next, we performed knockdown of *PRKAR1A* and *PRKAR2B* simultaneously in A375 cells and examined changes in cell signalling after treatment with the BRAF inhibitor vemurafenib (PLX4032, Figure 6). Approximately two days following siRNA transfection of A375 cells in 6-well plates, 333.3 uM vemurafenib in DMSO was diluted in RPMI 1640 media and then added to the wells for a final concentration of 0.1 uM. The cells were incubated with the vemurafenib for 1 hour, 2 hours, 4 hours, and 8 hours before aspirating the media and freezing the plates. Interestingly, we observed that following *PRKAR1A/PRKAR2B* knockdown there was less BRAF inhibition of phospho-ERK following vemurafenib treatment. This result suggested

that simultaneous *PRKAR1A* and *PRKAR1B* loss leads to BRAF inhibitor resistance. Furthermore, probing for phospho-CREB S133 revealed that there was increased expression of phosphorylated CREB as well, suggesting that the PKA pathway remained activated as the treatment with vemurafenib lengthened in contrast to control samples where CREB phosphorylation decreased over treatment time (Figure 6). However, we should note that phospho-PKA Cα Thr197 was not upregulated compared to the control samples, so additional experiments are required to determine if PKA activation was increased following *PRKAR1A/PRKAR2B* knockdown.

In summary, these results implicate loss of *PRKAR1A* in BRAF inhibitor resistance. Interestingly, loss of R1A alone in A375 cells did not result in BRAF inhibitor resistance. These results suggest that there is a ratio of R1A/R2B expression required to modulate the PKA signaling in human melanoma cells. As knockdown of both R1A and R2B lead to BRAF inhibitor resistance, future studies should examine PKA inhibitors to overcome therapy resistance.

To confirm these results, we attempted to examine the effects of *PRKAR1A* and *PRKAR2B* inhibition on BRAF inhibitor resistance in a second *BRAF* mutant cell line, Malme-3M. However, the siRNA transfection was unsuccessful, and optimization of the ratio of DharmaFECT to siRNA must be achieved before subsequent experiments in Malme-3M cells could continue.

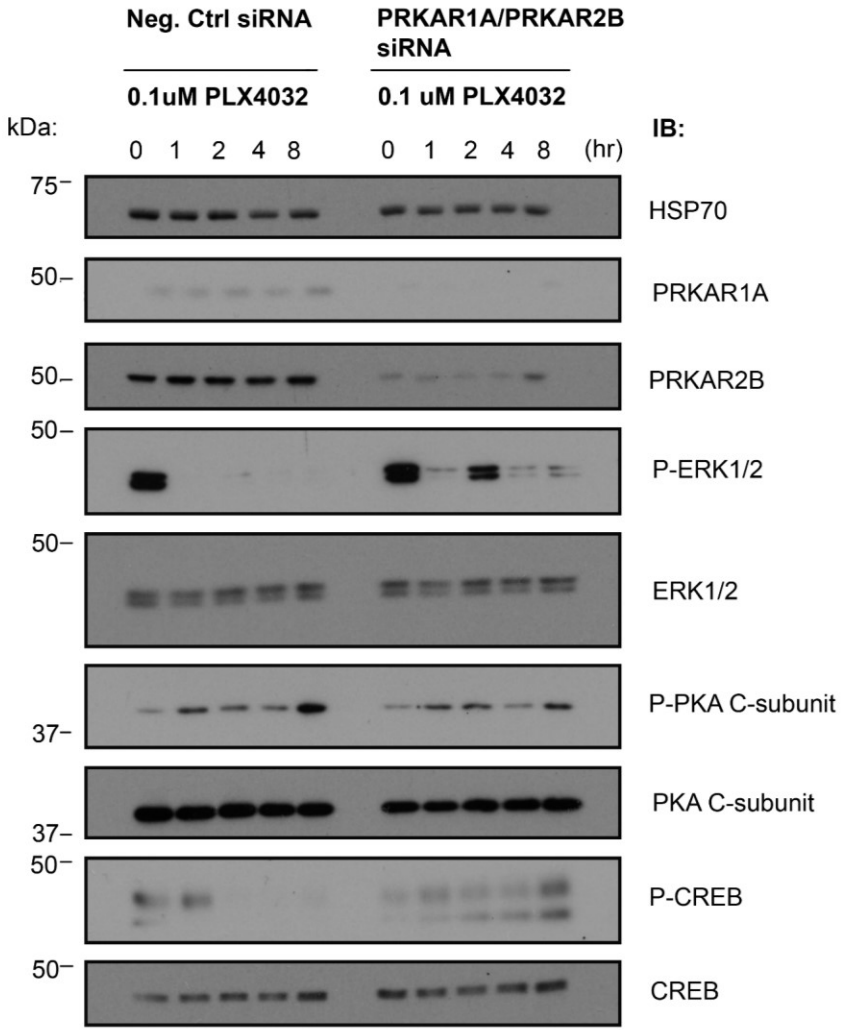

Figure 6. Western blot showing the results of probing for signalling changes in *PRKAR1A/PRKAR2B*-silenced A375 cells that were treated with 0.1 uM PLX4032 (vemurafenib) for 1, 2, 4, and 8 hours. Treatment of cells with DMSO for 1 hour served as the negative control. Due to the less effective knockdowns by R1A-2 siRNA and R2B-2 siRNA, only samples containing R1A-1 and R2B-1 siRNAs were used here. HSP70 served as the loading control. Total concentration of DMSO in the cell media for all samples was 0.03%.

Discussion

Our mutation significance analysis identified *PRKAR1A* as a significantly mutated gene possessing an enrichment of loss-of-function mutations. Our genetic data suggest *PRKAR1A* is a tumor suppressor in melanoma. This is consistent with the observation that germline mutations in *PRKAR1A* are found in patients with Carney Complex that are predisposed to the development of a number of neural crest-derived tumors. Loss of *PRKAR1A* is predicted to lead to the constitutive activation of the PKA pathway. When examining published sequencing data from a study of BRAF inhibitor pre- and post-resistance melanoma samples, *PRKAR1A* mutations were found in 2 of 45 post-treatment resistant cases [47]. Interestingly, here we observed that siRNA knockdown of *PRKAR1A* in alone did not confer resistance to BRAF inhibitor resistance. However, silencing of both *PRKAR1A* and *PRKAR2B* resulted in resistance to vemurafenib, a BRAF inhibitor, demonstrated by the abrogation of inhibition of phosphor-ERK. This suggested that both *PRKAR1A* and *PRKAR2B* work together to regulate PKA activity. Knockdown of these regulatory subunits may have led to constitutive activation of the PKA pathway as we observed increased phosphorylation of the PKA target CREB increased following treatment with vemurafenib.

According to a 2008 investigation by Mantovani *et al*, *PRKAR1A* and *PRKAR2B* are expressed in a balanced ratio in melanoma cells [40]. The study found that siRNA knockdown of *PRKAR1A* caused an increase in *PRKAR2B* expression, which was shown to reduce cell proliferation [40]. Knockdown of *PRKAR1A* did not seem to promote resistance to vemurafenib or increase cell viability (Figure 5). The reason for this could be due to *PRKAR2B* compensating for the loss of *PRKAR1A*. If the ratio of *PRKAR1A* to *PRKAR2B* were to normally maintain a balance, a loss of *PRKAR1A* may lead to increased expression of *PRKAR2B* [40]. The 2008

investigation into the overexpression of *PRKAR1A* in melanoma concluded that a high-R1A to low-R2B ratio in melanoma causes increased cellular growth rate [40]. Theoretically, when the ratio is subverted to have low-R1A and high-R2B, a decrease in cell growth occurs [40]. Our results presented here indicate that *PRKAR1A* and *PRKAR2B* work together to suppress PKA activity, and loss of these two regulatory subunits may lead to increased PKA activity, as suggested by increased CREB phosphorylation.

It has been reported that PKA mediates cAMP stimulated ERK1/2 signalling in thyroid cells, which is a point of crosstalk with the MAPK pathway [48]. Additionally, a 2013 investigation reported that MAPK inhibitor resistance may be dependent on the PKA pathway when it was found that overexpression of the PKA Cα subunit led to acquired resistance to MAPK inhibitors [31]. Therefore, it is likely that constitutive activation of the PKA pathway would lead to activation of ERK1/2 signalling in melanoma and result in acquired resistance to *BRAF* V600E inhibition.

Overexpression of *PRKAR2B* has been implicated in decreasing cellular growth in cancer cells, as detailed by a 1996 study [38]. In human colon carcinoma cell lines, overexpression of *PRKAR2B* resulted in decreased cell growth and slowed DNA synthesis [38]. This indicates that reverting the balance between *PRKAR1A* and *PRKAR2B* leads to decreased cell growth [38]. Even though the 1996 investigation focused on colon carcinoma, the findings are in the same vein as the 2008 study regarding melanoma [40,38]. This raises the possibility that silencing of *PRKAR1A* in melanoma cell lines could result in restricted cell growth. It is possible that silencing *PRKAR1A* would lead to *PRKAR2B* compensating for its loss and restrict PKA pathway signalling. Restriction of PKA signalling could cause a decrease in cell growth due to the reduction in phosphorylated CREB.

CREB is a major downstream target of PKA, and activation of CREB is known to promote tumorigenesis in melanoma [49,50]. A 2010 study reported that CREB binds to the promoter of known tumour suppressor AP-2α and downregulates its expression in melanoma [50]. AP-2α is the regulator of several genes related to cell proliferation, and it is downregulated during melanoma metastasis [51]. Furthermore, the 2010 investigation reported that phosphorylated CREB correlates positively with E2F-1 transcription factor expression [50]. E2F-1 is part of the E2F transcription factor family, which is involved in cell cycle control and apoptosis, and is a possible oncogene [50]. Most importantly, CREB promotes the expression of *MITF*, and both genes are implicated in resistance to MAPK inhibition and melanoma progression [31,52]. *MITF* encodes a transcription factor that regulates genes responsible for cell proliferation and differentiation in normal melanocytes, and this gene acts as an oncogene in melanoma [52]. CREB is a downstream effector of the cAMP-PKA pathway, which is implicated in the development of MAPK inhibitor resistance in melanoma [31]. Given that CREB seems to play a critical role in melanoma metastasis, it is possible that it may contribute to the development of resistance to BRAF inhibitors, although the mechanism should be further explored.

In conclusion, the results presented here indicate that dysregulation of the PKA pathway confers resistance to BRAF inhibition in the context of melanoma. However, activation of the PKA pathway in melanoma cells seems to be dependent on knockdown of both *PRKAR1A* and *PRKAR2B*, as these two regulatory subunits may be expressed in a balanced ratio within melanoma cells. There may be potential therapeutic benefits of manipulating the expression of *PRKAR1A* and *PRKAR2B*, as high levels of *PRKAR2B* over low levels of *PRKAR1A* could possibly lead to decreased cell viability in the presence of BRAF inhibitors.

Our results support the investigation of PKA inhibitors to overcome BRAF inhibitor resistance. In addition, future experiments should focus on bringing more insight into the mechanism between PKA signalling and BRAF inhibition. For instance, the increase in activated CREB seen after knockdown of *PRKAR1A* and *PRKAR2B* may imply a link between CREB expression and BRAF inhibitor resistance. Changes in *MITF* expression could also be probed for in response to the increased expression of phosphorylated CREB. Additionally, any metabolic changes in melanoma cells should be examined, such as the downregulation of proteins involved in OXPHOS. If the signalling crosslinks between the PKA and MAPK pathways could be clarified, that would be a valuable resource for the design of medications to treat melanoma and possibly other cancers.

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