



## Targeting effector pathways in RAC1<sup>P29S</sup>-driven malignant melanoma

Cristina Uribe-Alvarez, Sandra Lucía Guerrero-Rodríguez, Jennifer Rhodes, Alexa Cannon, Jonathan Chernoff & Daniela Araiza-Olivera

To cite this article: Cristina Uribe-Alvarez, Sandra Lucía Guerrero-Rodríguez, Jennifer Rhodes, Alexa Cannon, Jonathan Chernoff & Daniela Araiza-Olivera (2020): Targeting effector pathways in RAC1<sup>P29S</sup>-driven malignant melanoma, *Small GTPases*, DOI: [10.1080/21541248.2020.1728469](https://doi.org/10.1080/21541248.2020.1728469)

To link to this article: <https://doi.org/10.1080/21541248.2020.1728469>

 [View supplementary material](#) 

 Accepted author version posted online: 11 Feb 2020.

 [Submit your article to this journal](#) 

 Article views: 13

 [View related articles](#) 

 [View Crossmark data](#) 

**Publisher:** Taylor & Francis & Informa UK Limited, trading as Taylor & Francis Group

**Journal:** *Small GTPases*

**DOI:** 10.1080/21541248.2020.1728469

## **Targeting effector pathways in RAC1<sup>P29S</sup>-driven malignant melanoma**

Cristina Uribe-Alvarez<sup>1</sup>, Sandra Lucía Guerrero-Rodríguez<sup>2</sup>, Jennifer Rhodes<sup>1</sup>, Alexa Cannon<sup>1,3</sup>, Jonathan Chernoff<sup>1\*</sup>, & Daniela Araiza-Olivera<sup>2\*</sup>

<sup>1</sup>Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA, USA

<sup>2</sup>Instituto de Química, Universidad Nacional Autónoma de México. Circuito Exterior, Ciudad Universitaria 04510. México City, México.

<sup>3</sup>Drexel University School of Medicine, Philadelphia, PA, USA

\*Corresponding authors

E-mail address:

[daniela.araiza@iquimica.unam.mx](mailto:daniela.araiza@iquimica.unam.mx)

[Jonathan.Chernoff@fccc.edu](mailto:Jonathan.Chernoff@fccc.edu)

## Abstract

Malignant melanoma is characterized by mutations in a number of driver genes, most notably *BRAF* and *NRAS*. Recent genomic analyses revealed that 4-9% of sun-exposed melanomas bear activating mutations in *RAC1*, which encodes a small GTPase that is known to play key roles in cell proliferation, survival, and migration. The RAC1 protein activates several effector pathways, including Group A p21-activated kinases (PAKs), phosphoinositol-3-kinases (PI3Ks), in particular the beta isoform, and the serum-response factor/myocardin-related transcription factor (SRF/MRTF). Having previously shown that inhibition of Group A PAKs impedes oncogenic signaling from RAC1<sup>P29S</sup>, we here extend this analysis to examine the roles of PI3Ks and SRF/MRTF in melanocytes and/or in a zebrafish model. We demonstrate that a selective Group A PAK inhibitor (Frax-1036), a pan-PI3K (BKM120), and two PI3K $\beta$  inhibitors (TGX221, GSK2636771) impede the growth of melanoma cells driven by mutant RAC1 but not by mutant BRAF, while other PI3K selective inhibitors, including PI3K $\alpha$ ,  $\delta$  and  $\gamma$ , are less effective. Using these compounds as well as an SRF/MRTF inhibitor (CCG-203971), we observed similar results *in vivo*, using embryonic zebrafish development as a readout. These results suggest that targeting Group A PAKs, PI3K $\beta$ , and/or SRF/MRTF represent a promising approach to suppress RAC1 signaling in malignant melanoma.

KEYWORDS: melanoma, PAK, PI3K, RAC1, small GTPase, SRF/MRTF

## Introduction

Malignant melanoma is a highly aggressive cancer associated with poor overall survival. Recent genomic analyses have uncovered a variety of new driver mutations in malignant melanoma including an activating mutation in *RAC1*.<sup>1,2</sup> *RAC1* encodes a small ubiquitously expressed GTPase known to play key roles in embryonic development, immune response, cell proliferation, survival, and rearrangement of cytoskeleton by actin filament remodeling.<sup>3-5</sup> In sun-exposed cutaneous melanomas, *RAC1*<sup>P29S</sup> is the third most common oncogenic driver mutation, following *BRAF*<sup>V600E</sup> and *NRAS*<sup>Q61L/K/R</sup>.<sup>1,2</sup> *RAC1*<sup>P29S</sup> structure analysis and biochemical studies have shown that the proline to serine substitution in the hydrophobic pocket of the switch I domain of the GTPase results in an increased cycling rate from the GDP-bound inactive state to the GTP-bound active state, triggering downstream effectors and promoting melanocyte proliferation and migration.<sup>1</sup>

While RAC1 itself represents a challenging therapeutic target, its effectors might be more tractable. Many of RAC1's downstream biological effects are propagated by p21-activated kinases (PAKs). PAKs are serine/threonine specific intracellular kinases that phosphorylate downstream effector substrates regulating apoptosis, cell motility, cell morphology and cytoskeleton rearrangement.<sup>6</sup> Overexpressed PAKs result in oncogenic effects including increased cell proliferation and cell cycle progression, evasion of apoptosis, angiogenesis, and promotion of invasion and metastasis.<sup>7</sup> RAC1<sup>P29S</sup> induces PAK1 activation, which in turn phosphorylates and activates MEK1 at the Serine 298 site, facilitating ERK activation and transcription of various target genes. The PAK/MEK/ERK pathway is essential for RAS-driven transformation in a mouse model of skin cancer<sup>8</sup> and represents a potential therapeutic target for sun-driven melanomas. Zebrafish embryos injected with *RAC1*<sup>P29S</sup> mRNA displayed abnormal development and PAK and ERK elevated activity.<sup>9</sup> Defective growth was reversed by PAK and MEK inhibitors, suggesting that these may be useful to prevent the developmental effects of RAC1<sup>P29S</sup> mutations.<sup>9</sup> In addition, we and others have reported that tumors and human melanoma cell lines bearing *RAC1*<sup>P29S</sup> mutations are resistant to BRAF inhibitors but are sensitive to PAK and MEK inhibitors.<sup>9, 10</sup>

In addition to PAKs, PI3Ks represent a second recognized effector for RAC1.<sup>11</sup> PI3Ks are lipid signaling kinases that play key regulatory roles in cell survival, proliferation and differentiation.<sup>12</sup> Class I PI3K isoforms can be divided into two families based on their regulation mode: Class IA are heterodimeric proteins composed of a p110 catalytic subunit (PI3K $\alpha$ /p110 $\alpha$ , PI3K $\beta$ /p110 $\beta$  or PI3K $\delta$ /p110 $\delta$ ) whose enzymatic activity depends on their binding to a regulatory p85 subunit (p85 $\alpha$ , p55 $\alpha$ , p50 $\alpha$ , p85 $\beta$  or p55 $\gamma$ ); and Class IB which do not need to interact with a regulatory subunit to be active (PI3K $\gamma$ /p110 $\gamma$ ).<sup>12, 13</sup> PI3Ks transduce external signals from growth factors and cytokines into phospholipids that activate various downstream effector pathways such as the serine/threonine kinase AKT, and guanine-nucleotide exchange factors (GEFs).<sup>14</sup>

The PI3K/AKT pathway is an important regulator of normal cell physiology and is activated in 70% of sporadic melanomas including those containing the *RAC1*<sup>P29S</sup> mutation.<sup>15-18</sup> Activated AKT phosphorylates its protein targets inhibiting apoptosis and promoting cell survival. The AKT protein kinase family consists of three isoforms, while the targeted inhibition of AKT1 and AKT2 show little effect on combating melanoma, AKT3 inhibition resulted in increased apoptosis, reduced cell survival and decreased tumor development providing a new therapeutic target for patients with advanced stages of melanoma.<sup>15, 17</sup>

Recent transcriptome analysis from *RAC1*<sup>P29S</sup> melanocytes identified an enrichment of the SRF transcription factor targets and the epithelial to mesenchymal transition genes (EMT).<sup>11</sup> The nuclear transcription factor serum response factor (SRF) and its myocardin-related transcription factor (MRTF) co-factor are regulated by actin polymerization. Upon GTPase activation of the WAVE regulatory complex (WRC), the ARP2/3 complex catalyzes the polymerization of G-actin into F-actin liberating the SRF/MRTF transcription factor which may

now bind to DNA and induce the transcription of epithelial to mesenchymal transition (EMT) genes. SRF/MRTF inhibitors could therefore represent an attractive approach to tackling resistance in melanoma bearing the *RAC1*<sup>P29S</sup> mutation.<sup>11</sup>

Vemurafenib and dabrafenib, a BRAF and a MEK inhibitor, respectively, inhibit growth and promote tumor regression in *BRAF*<sup>V600E</sup> mutant melanomas.<sup>19-21</sup> The effect of these drugs is limited due to an eventual reactivation of the MAPK pathway and the PI3K/AKT signaling pathway that leads to drug resistance.<sup>22-28</sup> BRAFi and AKTi combinatorial therapies in human melanoma cells display promising effects on abatement of tumor growth. Additionally, combination of MEK, BRAF and AKT inhibitors delayed signs of drug resistance.<sup>29</sup> In melanoma cells, the combination of PI3K $\beta$  and PI3K $\alpha$  inhibitors is necessary to inhibit the PI3K signaling in long-term treatments.<sup>30</sup> BKM120, a pan-PI3K inhibitor, prevented AKT activation, cell cycle arrest in the G2-M phase, and induced apoptosis in human melanoma cells that metastasize to the brain in *in vitro* and *in vivo* assays. The combination of BKM120 with the MEK inhibitor binimetinib, further inhibited melanoma cell line proliferation.<sup>31</sup> PAK and MEK inhibitors may also cause tumor regression, and loss of ERK and AKT activity in a RAS-mediated skin cancer mice model.<sup>8</sup>

Here, we compare the results of inhibiting three distinct classes of RAC1 effectors – PAKs, PI3Ks, and SRF/MRTF – on the growth and survival of RAC1-mutant melanoma cell lines and on RAC1-driven changes in zebrafish embryonic development.

## Materials and Methods

### *Reagents.*

PI3K inhibitors: TGX221, GSK2636771, AS252424, GSK2269557, BKM120 and BYL719; AKT 1/2/3 inhibitor: MK 2206; and MRTF/SRF inhibitor: CCG-203971 were purchased from Selleckchem. PAK1 inhibitor Frax-1036 was generously provided by Genentech.

### *Cell culture.*

501mel, YUROB, YUFIC, YURIF and YUHEF melanoma cell lines were generously provided by Ruth Halaban (Yale University) and maintained in OptiMEM media (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS and Penicillin-Streptomycin. 451Lu, WM1791 and WM1960 were generously provided by Meenhard Herlyn (Wistar Institute) and maintained in 80% MCDB153, 20% Leibovitz's L-15, supplemented with 2% FBS, 5  $\mu$ g/ml insulin, and 1.68 mM CaCl<sub>2</sub>. All cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37° C. Cells were tested for mycoplasma and authenticated by sequencing the *BRAF*, *PREX2*, and *RAC1* genes.

### *Cell viability (mitochondrial activity).*

Melanoma cell lines were plated in 96-well plates at 5000 cells/well in the corresponding medium. After 24 hours, the cells were treated with increasing concentrations of PI3K inhibitors. Cell viability was evaluated after 72-hour incubation with drugs. Culture media was replaced by 100  $\mu$ L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a final concentration of 0.33 mg/mL dissolved in culture media and incubated at 37 °C with 5% CO<sub>2</sub> for 1 h in the dark. Supernatant was removed and replaced with DMSO to dissolve formazan crystals. Absorbance value was measured at 570 nm. Experiments were done in triplicate and 0.1% DMSO was used as negative control. IC<sub>50</sub> was calculated from three independent experiments.

### *Immunoblotting.*

Cells were grown in 6-well plates until 80% confluence was reached. Inhibitors were added at a final concentration of 100 nM and cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37° C for 24 h. Cells were washed with Dulbecco's PBS media and scrapped with 200  $\mu$ l of TNS buffer. Protein concentration was determined by Bradford and the samples were normalized to protein content. Western blot assays were performed using standard techniques. Primary antibodies used in this study were: anti-phospho-PAK1 (pSer144) (#2606), -total PAK1 (#2602), -phospho-ERK1/2 (pThr202/pTyr204) (#9101), -total ERK1/2 (#9102), -phospho-AKT (Ser473) and -total AKT (#9272) from Cell Signaling Technology.

### *Proliferation Assays.*

Proliferation was evaluated with the xCELLigence technology (Acea Bioscience, San Diego, CA, USA, distributed by Roche) in E-16-well plates. Melanoma cells were monitored during 72 h. The impedance value of each well was automatically monitored by microelectrodes placed on the bottom of plates. The impedance changes detected were proportional to the number of adhering cells and expressed as the cell index value. The experiments were conducted in triplicate. The results are shown as mean  $\pm$  standard deviation (SD) of at least three independent experiments (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001 *versus* control).

### *Wound healing assay.*

Melanoma cells were seeded on six-well plates and manually scrapped with a 200  $\mu$ L pipette tip. Cells were washed once with growth media, and then grown in fresh growth media with the IC<sub>50</sub> of the inhibitors for 24 h. Images were acquired at 100x magnification using an EVOS fluorescence microscope and the number of cells that cross into the wound area from their reference point at time zero was analyzed. To determine the migration rate of the cells, the wound areas were quantified using ImageJ software. Percentage of migration was

quantified by measuring the size of the cell free area. Data represent the mean (SD) of three independent experiments. (\*  $p < 0.05$ , \*\*  $p < 0.01$  versus Control).

### *Zebrafish Microinjection Experiments.*

Wild-type AB zebrafish embryos were collected and maintained in the Zebrafish Core Facility of Fox Chase Cancer Center under standard conditions<sup>39</sup>. Capped mRNA was obtained as follows: RAC1 (P29S) cDNA was subcloned into the pSGH2 vector. Transcripts were made *in vitro* for antisense (*Hind*III and T3 RNA polymerase) or sense (*Sac*II and SP6 RNA polymerase) full-length mRNA using the mMessage Machine kit (Ambion). Eight pairs of fish were bred and the resulting one-cell-stage embryos were injected with Phenol red or Phenol red + *Rac1*<sup>P29S</sup> mRNA directly in the cytoplasm using a nitrogen-powered Picospritzer III injector (Intracel) conjugated to a Nikon SMZ 1000 stereomicroscope at a final concentration of 35 ng/ $\mu$ l, as described previously<sup>40</sup>. Eggs were randomly separated in 50 egg-batches and kept in E3 medium at 28°C. 4 hpf mRNA injected embryos were incubated in E3 medium containing 1  $\mu$ M of PI3K, PAK1, AKT or Rho/MRTF/SRF inhibitors for 1 h and then washed thoroughly to avoid unwanted abnormalities caused by the inhibitor. Control embryos were incubated in E3 with DMSO at the same final concentration as the small molecule inhibitors. To analyze zebrafish morphology, 24 h dechorionated embryos were placed on a glass depression slide in 1% methylcellulose to stabilize the embryo. Morphology was assessed visually using a light transmission Nikon SMZ 1500, and representative images were recorded using a Nikon digital sight DS Fi1 camera. Embryonic phenotypes were scored as normal if they presented an elongated body axis, eye and heart development. Experiment was repeated three times using different breeders (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. control).

All animal procedures were performed in accordance with IACUC guides and regulations.

### *Statistics.*

Statistical analyses were carried out using the paired Student's *t*-test. All values reflect the mean (SD), with a significance cutoff of \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . All statistical analyses were completed in GraphPad Prism 6.0 or 7.0 (La Jolla, CA, USA).

## **Results**

Hotspot mutations of *BRAF*, *NRAS* and *RAC1* genes have been identified as the most frequent drivers in melanoma. In this study we analyze the effect of diverse inhibitors on melanoma cell lines that harbor different mutations. Table 1 shows the target of each small molecule inhibitor.

Viability, proliferation, migration and signaling assays were performed in 8 different cell lines: YUROB (WT), 501mel (*BRAF*<sup>V600E</sup>), YURIF (*BRAF*<sup>V600K</sup>/*RAC1*<sup>P29S</sup>), 451Lu (*BRAF*<sup>V600E</sup>), YUFIC (*NRAS*<sup>Q61R</sup>), YUHEF (*RAC1*<sup>P29S</sup>), WM1791 (*K-Ras*<sup>G12D</sup>/*RAC1*<sup>P29S</sup>) and WM1960 (*N-RAS*<sup>Q61K</sup>/*RAC1*<sup>P29S</sup>). Table 2 displays the genotype of each melanoma cell line used in this study.

### ***Cytotoxic effects of PI3K inhibitors on melanoma cell lines.***

We evaluated the viability of melanoma cell lines when exposed to increasing concentrations of the isoform-selective and the pan-PI3K inhibitors using the reduction of MTT to formazan at 72 hours as readout. Viability was significantly reduced in all the melanoma cell lines exposed to 0.1  $\mu$ M of the pan-PI3K inhibitor BKM120, and when exposed to higher concentrations of this drug (0.5  $\mu$ M), cell survival dropped below 50% (Fig. 1A). When cell lines were exposed to 0.01  $\mu$ M of the specific PI3K $\alpha$  isoform inhibitor (BYL719), viability of cells that harbor mutations in *BRAF* and *NRAS* decreased to 60%. Cell viability was reduced as the concentration of BYL719 was increased. In contrast, PI3K $\alpha$  inhibitors had no significant effect on cells that harbor *RAC1* mutations (Fig. 1B). When adding the isoform PI3K $\beta$  selective inhibitors (TGX221, GSK2636771) cell lines containing *RAC1* mutations were preferentially affected (Fig. 1C and 1D). When exposed to 0.05  $\mu$ M of TGX221, *RAC1* mutant cells survival was reduced to less than 50%. When increasing the concentration to 0.1  $\mu$ M the survival rate decreased to 20%. The viability of cells that harbor mutations in *BRAF* and *NRAS* was reduced to 70% survival with the highest concentration tested (Fig. 1C); following the same behavior pattern, when cells were exposed to 0.1  $\mu$ M of the GSK2636771 inhibitor, *RAC1* mutant cell survival was reduced to less than 40%, but when cells that harbor mutations in *BRAF* and *NRAS* were treated with this drug, no significant viability effects were observed (Fig. 1D). The PI3K $\gamma$  inhibitor AS252424 slightly reduced the survival of all cell lines to at least 80% at a concentration of 0.05  $\mu$ M, control cell line viability at a concentration of 0.05  $\mu$ M was around 40% (Fig. 1E). Finally when cells were treated with the PI3K $\delta$  inhibitor GSK2269557 no effects in any of the melanoma cell lines were observed (Fig. 1F). The calculated IC<sub>50</sub> values for each cell line treated with the tested drugs are shown in Supplementary Table 1.

### ***Cell proliferation and migration in response to PI3K inhibitors.***

We next evaluated the consequences of PI3K inhibition on cell proliferation and migration. Proliferation was studied in three melanoma cell lines with different genotypes that have only one mutation: YUROB (WT), YUHEF (*RAC1*<sup>P29S</sup>), and 501mel (*BRAF*<sup>V600E</sup>). Consistent with our previous results, WT cell proliferation was not significantly affected by the exposure to any of the inhibitors used in this study (Fig. 2A). Treatment of *RAC1*<sup>P29S</sup> mutant cell lines with the Group A PAK inhibitor Frax-1036, nearly abolished cell proliferation (Fig. 2B) which is consistent with data previously reported by our group.<sup>9</sup> PI3K $\beta$  inhibitors (TGX221, GSK2636771) also reduced *RAC1*<sup>P29S</sup> mutant cell proliferation, while all the other drugs tested caused a milder decrease in cell

proliferation. 501mel cells bearing the *BRAF*<sup>V600E</sup> mutation, displayed a significant reduced proliferation rate by the addition of the pan-PI3K and the PI3K $\alpha$  inhibitor, and a milder reduction when exposed to PI3K $\beta$  inhibitors (Fig. 2C).

RAC1 is well-known as a regulator of cell motility through its effects on the actin cytoskeleton. A wound-healing assay was used to test whether PI3K inhibitors would differentially affect the migration of melanoma cells depending on their driver mutation(s). None of the inhibitors impeded migration in WT cell lines (YUROB). The migration of *RAC1*<sup>P29S</sup> mutant cells (YUHEF) was severely diminished by the pan-PI3K and the PI3K $\beta$  inhibitors, and partially reduced by the PI3K $\alpha$  inhibitor. The *BRAF*<sup>V600E</sup> mutant cell (501mel) migration was reduced by exposure to the pan-PI3K and the PI3K $\alpha$  inhibitor, and partially reduced by the PI3K $\beta$  inhibitors. When cell lines had both *RAC1* and *BRAF* mutations (YURIF), cell migration was strongly diminished by the pan-PI3K, the PI3K $\alpha$ , and the PI3K $\beta$  inhibitors. PI3K $\gamma$  and  $\delta$  inhibitors had only a mild inhibitory effect in YURIF cell line (Fig. 3A, B).

#### **RAC1-mutant signaling response to PI3K inhibitors.**

To assess the functional role of PI3K inhibition, we examined RAC1 downstream effector proteins signaling in response to PI3K inhibitors in melanoma cell lines. Western blot analysis showed that treatment with specific PI3K drugs ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and pan-PI3K inhibitor) did not affect signaling in the WT cell line, but modified signaling in the mutant cell lines (Fig. 4). In non-treated cells, PAK and AKT activity was increased in *RAC1* and *NRAS* mutant cells while ERK activity remained constant in all the cell lines. The phosphorylation of PAK and AKT was significantly decreased in *RAC1* mutants when exposed to the pan-PI3K inhibitor (BKM120) and to the PI3K $\beta$  inhibitors (TGX221 and GSK2636771). The PI3K $\alpha$  inhibitor (BYL719) significantly reduced the activation of AKT in all cell lines. No effects were observed when cells were treated with the PI3K $\gamma$  and  $\delta$  inhibitors. ERK activity was nearly abolished in all mutant cell lines by the pan-PI3K inhibitor, and was suppressed by the PI3K $\alpha$  inhibitor (BYL719) in cell lines that have only one *BRAF* or *NRAS* mutation. PI3K $\beta$ -inhibitors (TGX221 and GSK2636771) reduced ERK phosphorylation in *RAC1*-mutant cell lines. These results suggest that PI3K regulates signaling of AKT, PAK and ERK through different isoforms (Fig. 4).

#### ***Effect of RAC1 signaling pathway inhibitors on zebrafish embryonic development.***

To examine *in vivo* signaling roles for RAC1 effectors, we employed a zebrafish embryonic development assay. This system has been deployed by us and others in previous studies to elucidate signaling pathways from human oncogenes such as *NRAS*, *BRAF*, and *RAC1*. Overexpression of *RAC1*<sup>P29S</sup> hinders zebrafish embryonic development and activates ERK signaling.<sup>22</sup> We previously showed that these defects were blocked by inhibiting components of the RAS/MAPK pathway with small molecule PAK or MEK inhibitors.<sup>22</sup> Therefore,

we asked whether these developmental defects could be suppressed by small molecule inhibitors of additional RAC1 effectors such as PI3K and SRF/MRTF. To determine if these inhibitors affect development, we introduced mRNA encoding *RAC1*<sup>P29S</sup> into one-cell stage zebrafish embryos. Abnormal RAC1 phenotype is characterized by pericardial edema, small/absent eyes, and reduced head size in ~97% of the embryos (Fig. 5). Specific small molecule inhibitors of PI3K, SRF/MRTF, AKT and PAK were diluted in the embryonic water during an hour at 4 hpf, and development was followed for 24 hours. We demonstrated that a Group 1 PAK inhibitor (Frax-1036: 40/38 normal, 40/2 abnormal), the PI3K $\alpha$  (BYL71: 40/35 normal, 40/5 abnormal), the MRTF/SRF inhibitor (CCG-203971: 40/33 normal, 40/7 abnormal), the pan-PI3K inhibitor (BKM120: 40/33 normal, 40/7 abnormal), and the PI3K $\beta$  (TGX221: 40/31 normal, 40/9 abnormal) almost completely prevented the Rasopathy like phenotype induced by activated RAC1. In contrast, the PI3K $\beta$  and the AKT inhibitors (GSK2636771:40/25 normal, 40/15 abnormal and MK2206: 40/24 normal, 40/16 abnormal) did not successfully prevent the Rasopathy-like phenotype (Fig. 5A, B). These results suggest that, of the three main groups of RAC1 effectors, Group A PAKs and/or SRF/MRTF represent the most effective targets to antagonize the developmental defects induced by RAC1<sup>P29S</sup>.

## Discussion

Melanoma is the most aggressive form of skin cancer and it is strongly associated with poor prognosis, low overall survival and drug resistance. *RAC1*<sup>P29S</sup> is the third most common mutation found in sun-exposed melanoma. We and others have shown that the RAC1<sup>P29S</sup> protein activates downstream effector proteins such as PAK, PI3K $\beta$  and SRF/MRTF, among others. Furthermore, PI3Ks activate AKT, which have a positive feedback effect on RAC1 that may in turn also stimulate certain carcinogenic properties in cells.<sup>14</sup>

As *RAC1* mutations confer poor prognoses, the inhibition of RAC1<sup>P29S</sup> and its downstream effectors might provide new therapeutic targets for appropriate selected drug resistant tumors and advanced stages melanoma. To date, few effective targets for *RAC1*-mutated melanoma other than PAK have been reported, prompting us to examine and compare the effect of PAK, PI3K, and SRF/MRTF inhibition in melanocyte signaling and zebrafish development.

Cell viability and migration assays revealed that, among all PI3K inhibitors tested, selective inhibitors for PI3K $\beta$  were the most effective against *RAC1* mutants, whereas selective PI3K $\alpha$  inhibitors were most effective in the setting of *BRAF*<sup>V600K</sup> mutations. As expected, the PI3K $\delta$  and  $\gamma$  inhibitors had fewer effects on melanocyte viability since these isoforms are mainly expressed in leukocytes.<sup>12</sup> The pan-PI3K inhibitor showed a non-selective decline in cell viability, growth and migration in all cell lines tested. These results are consistent with the idea that the main PI3K isoform engaged by RAC1 is PI3K $\beta$ , and that targeting other PI3K isoforms does

not add benefit in this setting. As a selective PI3K inhibitor would be expected to have less toxicity to cells, this finding could have therapeutic implications.

In the embryonic zebrafish development assays, the rasopathy-like phenotype induced by mutant *RAC1* was most successfully reversed when using PAK or SRF/MRTF inhibitors, while PI3K $\beta$  and AKT inhibitors were in general less effective, with some isoform specificity. As the zebrafish developmental assay is driven by high level, transient expression of transgenes, it may represent a more stringent assay for drug studies than *in vitro* studies using melanoma cell lines in culture. However, in most aspects both the developmental and *in vitro* data are in general agreement, arguing that both systems can be used to evaluate RAC1 signaling. Using these systems, our results, like those recently reported in a *Rac1*<sup>P29S</sup> mouse model,<sup>11</sup> suggest that targeting Group A PAKs and/or SRF/MRTF, and possibly also PI3K/AKT signaling, could become a promising approach to suppress RAC1 signaling in malignant melanoma.

#### **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

#### **Funding**

This work was supported by grants from the NIH (JC), the Melanoma Research Foundation (JC), and Presupuesto Interno del Instituto de Química/UNAM (DAO) as well as Cátedras CONACYT and NCI Core Grant P30 CA06927 (to Fox Chase Cancer Center).

#### **Acknowledgments**

We thank Drs. Ruth Halaban and Meenhard Herlyn for providing melanoma cell lines, Genentech for providing Frax-1036, and the Fox Chase Cancer Center Animal Facility for assistance with zebrafish experiments.

#### **Declaration of Interests**

The authors claim no conflicts of interest.

#### **References**

1. Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, et al. A landscape of driver mutations in melanoma. *Cell* 2012; 150:251-63.
2. Krauthammer M, Kong Y, Ha BH, Evans P, Bacchiocchi A, McCusker JP, et al. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nature genetics* 2012; 44:1006-14.
3. Didsbury J, Weber RF, Bokoch GM, Evans T, Snyderman R. rac, a novel ras-related family of proteins that are botulinum toxin substrates. *The Journal of biological chemistry* 1989; 264:16378-82.

4. Haataja L, Groffen J, Heisterkamp N. Characterization of RAC3, a novel member of the Rho family. *The Journal of biological chemistry* 1997; 272:20384-8.
5. Marei H, Malliri A. GEFs: Dual regulation of Rac1 signaling. *Small GTPases* 2017; 8:90-9.
6. Ye DZ, Field J. PAK signaling in cancer. *Cellular logistics* 2012; 2:105-16.
7. Radu M, Semenova G, Kosoff R, Chernoff J. PAK signalling during the development and progression of cancer. *Nature reviews Cancer* 2014; 14:13-25.
8. Chow HY, Jubb AM, Koch JN, Jaffer ZM, Stepanova D, Campbell DA, et al. p21-Activated kinase 1 is required for efficient tumor formation and progression in a Ras-mediated skin cancer model. *Cancer research* 2012; 72:5966-75.
9. Araiza-Olivera D, Feng Y, Semenova G, Prudnikova TY, Rhodes J, Chernoff J. Suppression of RAC1-driven malignant melanoma by group A PAK inhibitors. *Oncogene* 2018; 37:944-52.
10. Watson IR, Li L, Cabeceiras PK, Mahdavi M, Gutschner T, Genovese G, et al. The RAC1 P29S hotspot mutation in melanoma confers resistance to pharmacological inhibition of RAF. *Cancer research* 2014; 74:4845-52.
11. Lionarons DA, Hancock DC, Rana S, East P, Moore C, Murillo MM, et al. RAC1(P29S) Induces a Mesenchymal Phenotypic Switch via Serum Response Factor to Promote Melanoma Development and Therapy Resistance. *Cancer cell* 2019; 36:68-83 e9.
12. Liu S, Knapp S, Ahmed AA. The structural basis of PI3K cancer mutations: from mechanism to therapy. *Cancer research* 2014; 74:641-6.
13. Amzel LM, Huang CH, Mandelker D, Lengauer C, Gabelli SB, Vogelstein B. Structural comparisons of class I phosphoinositide 3-kinases. *Nature reviews Cancer* 2008; 8:665-9.
14. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nature reviews Drug discovery* 2009; 8:627-44.
15. Stahl JM, Sharma A, Cheung M, Zimmerman M, Cheng JQ, Bosenberg MW, et al. Deregulated Akt3 activity promotes development of malignant melanoma. *Cancer research* 2004; 64:7002-10.
16. Cheung M, Sharma A, Madhunapantula SV, Robertson GP. Akt3 and mutant V600E B-Raf cooperate to promote early melanoma development. *Cancer research* 2008; 68:3429-39.
17. Davies MA, Stemke-Hale K, Tellez C, Calderone TL, Deng W, Prieto VG, et al. A novel AKT3 mutation in melanoma tumours and cell lines. *British journal of cancer* 2008; 99:1265-8.
18. Shao Y, Aplin AE. Akt3-mediated resistance to apoptosis in B-RAF-targeted melanoma cells. *Cancer research* 2010; 70:6670-81.
19. Sondergaard JN, Nazarian R, Wang Q, Guo D, Hsueh T, Mok S, et al. Differential sensitivity of melanoma cell lines with BRAFV600E mutation to the specific Raf inhibitor PLX4032. *Journal of translational medicine* 2010; 8:39.
20. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 2010; 467:596-9.
21. Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* 2012; 380:358-65.
22. Fedorenko IV, Paraiso KH, Smalley KS. Acquired and intrinsic BRAF inhibitor resistance in BRAF V600E mutant melanoma. *Biochemical pharmacology* 2011; 82:201-9.
23. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 2010; 468:973-7.
24. Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 2010; 468:968-72.
25. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011; 29:3085-96.
26. Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, et al. MEK1 mutations confer resistance to MEK and B-RAF inhibition. *Proceedings of the National Academy of Sciences of the United States of America* 2009; 106:20411-6.

27. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *The Journal of clinical investigation* 2008; 118:3065-74.
28. Su F, Bradley WD, Wang Q, Yang H, Xu L, Higgins B, et al. Resistance to selective BRAF inhibition can be mediated by modest upstream pathway activation. *Cancer research* 2012; 72:969-78.
29. Lassen A, Atefi M, Robert L, Wong DJ, Cerniglia M, Comin-Anduix B, et al. Effects of AKT inhibitor therapy in response and resistance to BRAF inhibition in melanoma. *Molecular cancer* 2014; 13:83.
30. Herkert B, Kauffmann A, Molle S, Schnell C, Ferrat T, Voshol H, et al. Maximizing the Efficacy of MAPK-Targeted Treatment in PTENLOF/BRAF<sup>MUT</sup> Melanoma through PI3K and IGF1R Inhibition. *Cancer research* 2016; 76:390-402.
31. Niessner H, Schmitz J, Tabatabai G, Schmid AM, Calaminus C, Sinnberg T, et al. PI3K Pathway Inhibition Achieves Potent Antitumor Activity in Melanoma Brain Metastases In Vitro and In Vivo. *Clin Cancer Res* 2016; 22:5818-28.

**Table 1. PI3K, AKT and SRF/MRTF selective inhibitors and biological activity.**

Inhibitor	Target
BKM120 (Buparlisib)	PI3K $\alpha/\beta/\delta/\gamma$
BYL719 (Alpelisib)	PI3K $\alpha$ , minimal effect on PI3K $\beta/\gamma/\delta$
TGC221	PI3K $\beta$ , minimal effect on PI3K $\alpha$
GSK2636771	PI3K $\beta$
AS-252424	PI3K $\gamma$ , minimal effect on PI3K $\beta/\delta$
GSK2269557 (Nemiralisib)	PI3K $\delta$
MK-2206 2HCL	AKT1/2/3
CCG-203971	Rho/MRTF/SRF

**Table 2. Genetic profiles of melanoma cell lines.**

Cell line	Mutation
YUROB	WT
501mel	<i>BRAF</i> <sup>V600E</sup>
YURIF	<i>BRAF</i> <sup>V600E</sup> / <i>Rac1</i> <sup>P29S</sup>
451Lu	<i>BRAF</i> <sup>V600E</sup>
YUFIC	<i>Nras</i> <sup>Q61K</sup>
YUHEF	<i>Rac1</i> <sup>P29S</sup>
WM1791C	<i>Kras</i> <sup>G12D</sup> / <i>Rac1</i> <sup>P29S</sup>
WM1960	<i>Nras</i> <sup>Q61K</sup> / <i>Rac1</i> <sup>P29S</sup>

### Figure Legends

**Figure 1. Effect of selective PI3K inhibitors on melanoma cell viability.** Melanoma cells were treated for 72 h with concentrations ranging from 0.01  $\mu$ M to 0.5  $\mu$ M of A) pan-PI3K (BKM120), B) PI3K $\alpha$  (BYL719), C) PI3K $\beta$  (TGX221), D) PI3K $\beta$  (GSK2636771), E) PI3K $\gamma$  (AS252424), F) PI3K $\delta$  (GSK2269557). Cell viability was determined by an MTT assay. Data represent the mean (SD) of three independent experiments.

**Figure 2. Proliferation of WT, *BRAF* and *RAC1*-mutant melanoma cell lines in presence of targeted inhibitors.** A) WT cells (YUROB), B) *RAC1* mutant (YUHEF) and C) *BRAF* mutant (501mel) were treated with 100 nM of pan-PI3K (BKM120), PI3K $\alpha$  (BYL719), PI3K $\beta$  (TGX221, GSK2636771), PI3K $\gamma$  (AS252424), PI3K $\delta$  (GSK2269557) and PAK1 (Frax-1036) small molecule inhibitors. Cell number was measured during 72h using an XCELLigence device. Representative data of 3 independent experiments. The results are shown as mean (SD) of at least three independent experiments (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus control).

**Figure 3. Consequences of PI3K inhibitors on cell migration.** A cell culture wound-healing assay was developed in six-well confluent plates. After creating a gap by scratching a confluent plate of cells, WT (YUROB), *RAC1* (YUHEF), *BRAF* (501mel) and *BRAF/RAC1* (YURIF) cell lines were treated with 100 nM pan-PI3K (BKM120), PI3K $\alpha$  (BYL719), PI3K $\beta$  (TGX221, GSK2636771), PI3K $\gamma$  (AS252424) and PI3K $\delta$  (GSK2269557) inhibitors. A) Images were taken in an inverted microscope at 0 and 24 h. Scale bar 400  $\mu\text{m}$  for all images. B) Migration was quantified by measuring the length of the cell-free area. The 100% of migration was determined by the control. Percentage of migration of each treated cell line was measured against untreated cells (control). The results are shown as mean (SD) of at least three independent experiments (\*  $p < 0.05$ , \*\*  $p < 0.01$  versus control).

**Figure 4. PI3K inhibitors cell signaling modification in melanoma cell lines bearing *RAC1* and *BRAF* mutations.** Melanoma cell lines with different genotypes were grown under standard conditions. Cells were treated with vehicle (control) or 100 nM of the indicated PI3K inhibitors for 24 h. Lysates were analyzed by Western blot for PAK, AKT, and ERK phosphorylation. Representative data of 3 independent experiments.

**Figure 5. *RAC1* overexpression in zebrafish embryonic development and its treatment with PI3K, AKT and Rho/MRTF/SRF inhibitors.** Eight pairs of fish were bred and the resulting embryos were injected with Phenol red or Phenol red + *Rac1*<sup>P29S</sup> mRNA during the one-cell stage. 1  $\mu\text{M}$  inhibitors were added at 4 hpf, then removed after one hour and washed thoroughly. Embryonic morphology was scored at 24 hpf by a blinded observer. A) Representative images of developmental abnormalities were performed with a Nikon digital sight DS Fi1 camera. Scale bar 100  $\mu\text{m}$  for all images. B) Embryo phenotypes were scored as normal if they presented an elongated body axis, eye and heart development. The results are shown as mean (SD) of at least three independent experiments (\*  $p < 0.05$ , \*\*  $p < 0.01$  versus control).

IC <sub>50</sub> (μM)							
Inhibitor	YUROB	501mel	451Lu	YUHEF	YURIF	YUFIC	WM1791
BKM120	0.4	0.1	0.1	0.4	0.3	0.2	0.4
BYL719	≥1	0.01	0.01	≥1	0.05	0.02	≥1
TGX221	≥1	≥1	≥1	0.01	0.01	≥1	0.01
GSK2636771	≥1	≥1	≥1	0.05	0.08	≥1	0.05
AS252424	0.5	0.5	0.5	≥1	0.5	0.5	≥1
GSK2269557	≥1	≥1	≥1	≥1	≥1	≥1	≥1

**Supplementary Table 1. IC<sub>50</sub> of each PI3K inhibitor on different melanoma cell lines.** 24 hour melanoma cell cultures were incubated with different PI3K inhibitors for 72 h using concentrations ranging from 0.01 μM to 0.5 μM. Viability was determined by an MTT assay.

Inhibitor	IC <sub>50</sub> (μM)						
	YUROB	501mel	451Lu	YUHEF	YURIF	YUFIC	WM1791
BKM120	0.4	0.1	0.1	0.4	0.3	0.2	0.4
BYL719	≥1	0.01	0.01	≥1	0.05	0.02	≥1
TGX221	≥1	≥1	≥1	0.01	0.01	≥1	0.01
GSK2636771	≥1	≥1	≥1	0.05	0.08	≥1	0.05
AS252424	0.5	0.5	0.5	≥1	0.5	0.5	≥1
GSK2269557	≥1	≥1	≥1	≥1	≥1	≥1	≥1

**Supplementary Table 1. IC<sub>50</sub> of each PI3K inhibitor on different melanoma cell lines. 24 hour melanoma cell cultures** were incubated with different PI3K inhibitors for 72 h using concentrations ranging from 0.01 μM to 0.5 μM. Viability was determined by an MTT assay.

Accepted Manuscript

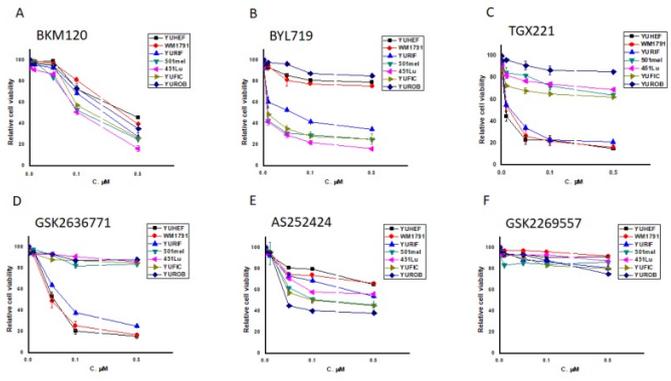


Figure 1

Accepted Manuscript

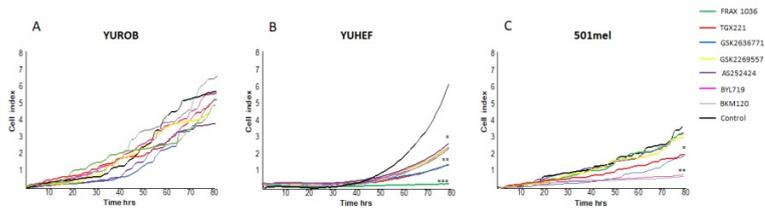


Figure 2

Accepted Manuscript

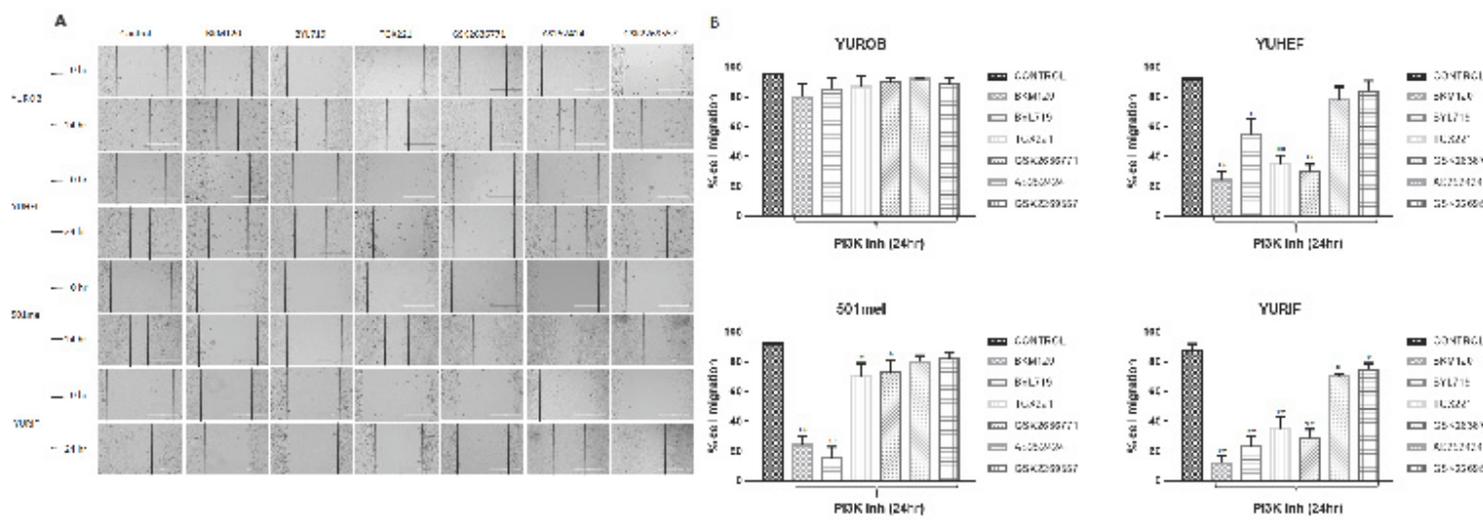


Figure 3

Accel



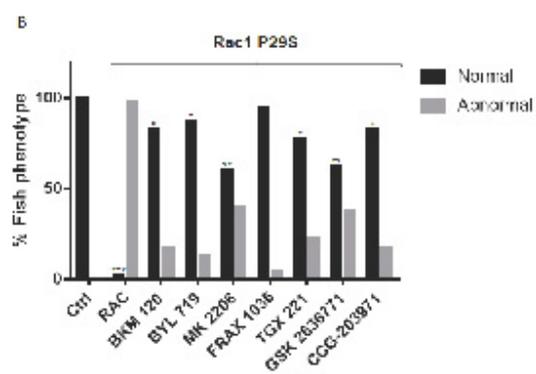
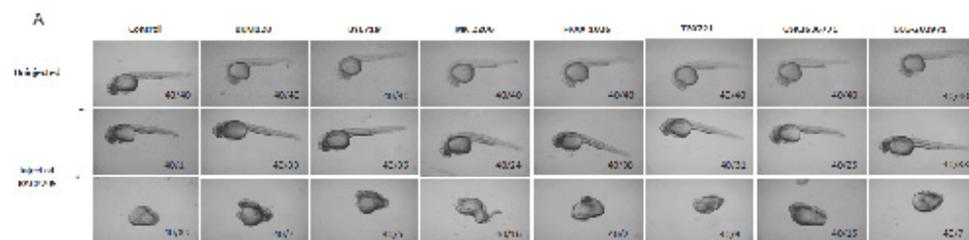


Figure 5

Accel