Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Wistar Institute

2. **Reporting Period (start and end date of grant award period):** 01/01/2012 – 06/30/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Russel E. Kaufman, M.D.

4. **Grant Contact Person's Telephone Number:** 215-898-3926

5. **Grant SAP Number:** 4100057690

6. **Project Number and Title of Research Project:** 3: Developing Potential Strategies to Overcome Drug Resistance in Melanoma

7. **Start and End Date of Research Project:** January 1, 2012 – June 30, 2013

8. **Name of Principal Investigator for the Research Project:** Jessie Villanueva, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ $465,399.48

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Villanueva, Jessie	Principal Investigator	58%	$115,267.82
Samanta, Minu	Research Assistant	100%	$ 94,928.16
Reyes-Uribe, Patricia	Research Assistant	100% 1-2/12	$ 7,931.56
Chen, Hsin-Yi	Postdoctoral Fellow	100% 6-12/12	$ 32,176.76

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Chen, Thomas	Student	100

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Baker Co. SterilGARD and UV light	Allowed us to perform cell culture, including generation of drug resistant cells, maintenance, and expansion of cell cultures necessary to accomplish all the aims of the project as well as experiments needing sterile conditions (e.g. cell viability assays).	$ 8,585

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes ____X_____ No_____

If yes, please indicate the source and amount of other funds:

The V Foundation for Cancer Research - $48,080

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No ___x_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes _X_____ No_____

If yes, please describe your plans:

I plan to submit a new proposal to a private foundation requesting funding to further explore the role of S6K in melanoma biology and therapy.

12. Future of Research Project. What are the future plans for this research project?

Data from this project has revealed a potential role of mTOR and S6K as targets for melanoma therapy. We will now investigate the role of S6K in melanoma, determine how it is regulated, and what downstream effectors of this kinase could be involved in proliferation, survival and drug resistance.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes ____X_____ No_____

If yes, how many students? Please specify in the tables below: 5

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1	1		
Female		1	1	1
Unknown				
Total	**1**	**2**	**1**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic		1		
Non-Hispanic	1	1	1	1
Unknown				
Total	**1**	**2**	**1**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White			1	
Black				
Asian	1	1		1
Other		1		
Unknown				
Total	**1**	**2**	**1**	**1**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No _____X____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No ____X_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

We have continued an on-going collaboration with the University of Pennsylvania and established a new collaboration with Sarah Cannon Cancer Center in Memphis, TN.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No ____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No _____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance

Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

The overall goal of this project was to characterize mechanisms of drug resistance to inhibitors of the MAPK pathway in melanoma and to delineate potential therapeutic strategies to overcome this resistance.

Resistance to therapy is by far the limiting factor for successful cancer treatment. Effective strategies to overcome anti-cancer drug resistance are sorely needed and can only be developed by understanding the molecular mechanisms of resistance in models that mimic the chronic administration of anti-cancer drugs used in the clinic. BRAF inhibitors have remarkable clinical activity in BRAFV600E mutant melanomas; however, responses are transient and tumors invariably develop drug resistance. During this funding cycle, we investigated the mechanisms mediating drug resistance in preclinical models of melanoma developed by chronic treatment with BRAF and MEK inhibitors both *in vitro* and in tumors derived from patients undergoing therapy. We expect that the knowledge generated from our studies will be useful to: 1) better understand the mechanisms underlying acquired resistance to BRAF and MEK inhibitors; 2) identify markers of sensitivity and resistance to inhibitors of the MAPK pathway which may help identify potential responders and non-responders; and 3) design rational combination therapies that could either be used as second line treatment to overcome drug resistance or as upfront therapies leading to more durable responses. We expect that our studies will provide useful preclinical information that will inform the design of future clinical trials for patients with melanoma, the most deadly form of skin cancer.

Background and Specific Aims:

Despite the recent approval of BRAF inhibitors such as vemurafenib (PLX4032) and dabrafenib based on striking anti-tumor activity in BRAF mutant melanomas, responses are short-lived and overall patient survival remains dismal. Thus, novel therapeutic approaches that could increase patient survival are urgently needed. Effective strategies to overcome anticancer drug resistance can only be developed by understanding the molecular mechanisms of resistance in models that closely mimic the heterogeneity of melanoma found in patients. The goal of this project was to determine mechanisms underlying resistance to BRAF and MEK inhibitors and develop strategies to overcome it. Our working hypothesis is that optimal combination therapies targeting the deregulated resistance pathways will result in durable responses and increased survival rates. We have investigated the mechanisms mediating acquired resistance to BRAF/MEK inhibitors in experimental models and validated our findings in tumor samples derived from patients undergoing therapy. We have also investigated alternative therapies to overcome resistance, which we expect will provide pre-clinical basis for future clinical trials. The original specific

aims were:

Aim 1: Molecular profiling of BRAF-mutant melanomas resistant to BRAF/MEK inhibitors. We are testing the hypothesis that melanomas acquire drug resistance through different mechanisms involving activation of alternative signaling pathways or genetic changes. We propose to model and study acquired resistance to BRAF and MEK inhibitors and to define their molecular profiles. Specifically, we will:

a. Generate and characterize melanoma cell lines resistant to BRAF inhibitors.
b. Generate and characterize melanoma cell lines resistant to MEK inhibitors.

Aim2: Design and evaluate the efficacy of combination strategies based on the tumor's molecular profile.

a. Design and test combination therapies in melanoma cell lines with acquired resistance to BRAF inhibitors.
b. Design and evaluate combination therapies in melanoma cell lines with acquired resistance to MEK inhibitors.
c. Design and evaluate selected therapies *in vivo.*

Major Achievements.

During the term of the grant, we accomplished the following milestones:

- Generated cell lines resistant to BRAF and MEK inhibitors
- Characterized mechanisms of resistance to MEK and BRAF inhibitors
- Identified a novel MEK2 mutation
- Discovered that MEK2 mutations and BRAF amplification confer resistance to BRAF, MEK, and the combination of both inhibitors
- Demonstrated that MEK2 mutations and BRAF amplification confer drug resistance in *in-vivo* mouse models
- Validated our *in vitro* findings in samples derived from patients undergoing therapy
- Found that sustained MAPK leads to persistent phosphorylation of S6K
- Demonstrated that triple combination strategies with BRAF, MEK, and PI3K/mTOR inhibitors can inhibit the growth of drug resistant tumors *in-vivo*

Summary of Results:

Aim 1: Molecular profiling of BRAF-mutant melanomas resistant to BRAF/MEK inhibitors

Deregulation of MAPK signaling is a hallmark of melanoma. Particularly, mutant BRAF- V600 melanoma cells dependent on MEK/ERK signaling and inhibitors of this pathway are being used to treat melanoma patients. BRAF inhibitors have recently received FDA approval for the treatment of unresectable metastatic BRAFV600E melanoma. Similarly, the MEK inhibitor trametinib (GSK1120212) has been approved for the treatment of metastatic melanoma. Trametinib has demonstrated clinical activity in clinical trials, and is currently being evaluated in combination with the selective BRAF inhibitor dabrafenib (GSK2118436) in BRAF-mutant

melanoma patients. Nonetheless, the long-term efficacy of these compounds is commonly limited by the emergence of drug resistance. Several resistant mechanisms to BRAF and MEK inhibitors have been recently identified. Resistance to MEK inhibitors has been particularly linked to mutations in MEK1 (Emery et al, 2010; Wagle et al, 2011); however, some MEK1 mutations in exon 3 are pre-existent and do not confer drug resistance (Shi, et al, 2012). More recently, a MEK2-E207K was identified in Lau63 melanoma cells with decreased sensitivity to MEK inhibitors (Nikolaev et al, 2012). Given the heterogeneity of melanoma, additional genetic mechanisms are expected to arise in the clinical setting. Hence, it is important to understand the determinants of resistance in this setting.

We modeled the emergence of drug resistance in BRAF-V600E melanoma cells by chronically exposing five BRAF-V600E melanoma sublines to increasing doses of trametenib (0.1 – 1000 nM) for four months in a stepwise manner as previously described (Villanueva, et al, 2010). Resistant cell populations were independently generated. Although trametinib readily decreased viability of parental cells, those cells that were chronically exposed to the MEK inhibitor were substantially less sensitive to the drug (IC50>1000 nM) (Figure 1). Melanoma cells selected for resistance to trametinib were also cross-resistant to selumetinib (AZD6244), PLX4720, and dabrafenib. Sensitivity to chemotherapeutic drugs was similar in parental and trametinib resistant (MR) sublines (Figure 2), indicating that resistance was selective for BRAF and MEK inhibitors. We then examined the effect of MEK and BRAF inhibitors on MAPK signaling. MEK and BRAF inhibitors efficiently blocked ERK phosphorylation in parental but not in the resistant cells (Figure 3), indicating that chronic treatment with MEK inhibitors selects cells that can adapt to the drug pressure by persistently activating the MAPK pathway.

To identify potential mechanisms of drug resistance we sequenced the MEK genes, as secondary mutations in the drug target are frequently associated with drug resistance. Sanger sequencing of the *MAPK2K1* and *MAPK2K2* genes identified a *de novo* MAP2K2 Q60P (MEK2Q60P) mutation in both resistant cell populations (Figure 4). All parental and resistant cell lines were further evaluated by a custom iPlex panel for a targeted panel of 65 mutations in 11 genes, including BRAF, NRAS, KIT, AKT1/3, and MAP2K1/2 among others. The iPlex analysis confirmed the presence of BRAF-V600E mutation in parental and resistant cells and the MEK2-Q60P mutation in the resistant cells; no additional mutations were identified.

To determine the biological significance of the MEK2-Q60P mutation, we ectopically expressed wild-type-MEK2 (WT) or MEK2-Q60P in BRAF-V600E melanoma cells (Figure 5). The MEK2 cDNA clone was obtained from OpenBiosystems (Lafayette, CO), and the MEK2 point mutation was generated using Stratagene's QuickChangeII XL site-directed mutagenesis kit (Invitrogen) according to the manufacturer's instructions. Primers used to generate mutant MEK2-Q60P were:

MEK2mut forward
5'-CGGCTGGAAGCCTTTCTCACCCCGAAAGCCAAGGTCGGCGAACTC-3'

MEK2mut reverse
5'-GAGTTCGCCGACCTTGGCTTTCGGGGTGAGAAAGGCTTCCAGCCG-3'.

The mutant construct was subcloned into the lentiviral vector pLU-EF1a-PGK-GFP. Both constructs were fully sequenced to confirm that the engineered mutation was present and that no additional mutations had been randomly introduced.

Treatment of control cells or cells expressing WT-MEK2 with either MEK or BRAF inhibitors led to a dose dependent inhibition of MAPK activity (Figure 5). In contrast, cells expressing exogenous MEK2-Q60P required higher concentrations of trametinib (~1000 nM) for pMEK and pERK inhibition (Figure 5A). PLX4720 had virtually no effect on pERK inhibition.

To determine if mutant MEK2 could confer drug resistance, Mel1617 mock-infected cells and cells expressing MEK2-Q60P were treated with either MEK or BRAF inhibitors (Figure 5B). The parental or mock-infected cells were highly sensitive to MEK and BRAF inhibitors; however, cells expressing MEK2-Q60P were substantially less sensitive to these compounds, suggesting that this mutation could be associated with a diminished response to these inhibitors.

Treatment of serum-starved Mel1617-MEK2-Q60P cells with BRAF inhibitors had only a small effect on phospho-MEK levels and almost no effect on phospho-ERK levels in MEK-inhibitor-resistant cells or cells ectopically expressing MEK2-Q60P even in low serum conditions (Figure 6). These data suggest that MEK2-Q60P does not require substantial mitogenic stimulation or activation by BRAF.

To further examine the role of MEK2-Q60P modulating sensitivity to MEK and BRAF inhibitors, we silenced MEK2 using shRNA.

Lentiviral MEK1 and MEK2 shRNA in pLKO1 backbone were obtained from OpenBiosystems. Lentiviruses were produced by transfection of 293T cells with the packaging plasmids encoding CMV and VSV-G along with the lentiviral shRNA vector using Lipofectamine 2000 (Invitrogen) reagent following the manufacturer's instructions. Melanoma cells were exposed to virus in the presence of 8 µg/mL polybrene for 16-18h. Infected cell populations were selected with antibiotics or sorted for GFP using flow cytometry. Expression of mutant genes or shRNA knockdown efficiency was determined by western blot analysis for the respective proteins using specific antibodies.

Treatment of Mel1617-MR cells expressing a non-targeting shRNA with MEK or BRAF inhibitors did not affect pERK levels or cell viability (Figure 7). However, treatment of Mel1617-MR cells expressing MEK2 shRNA with MEK or BRAF inhibitors decreased phospho-ERK levels and partially restored sensitivity to these drugs. In contrast, almost complete silencing of MEK1 (shMEK1-3/1) had no substantial effect on phospho-ERK levels and viability of the resistant cells treated with MEK or BRAF inhibitors (data not shown). These data suggest that mutant MEK2 is specifically associated with decreased sensitivity to MEK and BRAF inhibitors.

Although MEK2 silencing partially re-sensitized drug resistant cells to MEK and BRAF inhibitors, the decrease in cell viability was not the same as in the parental cells, suggesting that additional factors could be modulating sensitivity to these compounds. To explore this possibility, we performed array based comparative genomic hybridization (aCGH). We found

that the resistant cells had a localized amplification on chromosome 7, targeting the BRAF locus. The 20-fold amplification spanned from 139,421,284-140,910,285, and included 21 genes. Furthermore, we determined that the mutant BRAF-V600E allele was amplified compared to the wild-type allele using the peak height ratios on the iPlex assay. BRAF mRNA and protein levels were higher in the resistant cells (Figure 8). BRAF amplification has been previously observed as a mechanism of acquired resistance to MEK inhibition in colorectal cell lines and causing a small decrease in sensitivity to BRAF inhibitors in melanoma (Shi et al, 2012). Taken together, these data suggest that concurrent MEK2 mutations and BRAF-V600E amplification hyperactivate the MAPK pathway and confer resistance to both BRAF and MEK inhibitors.

Aim2: Design and evaluate the efficacy of combination strategies based on the tumor's molecular profile.

One potential approach to overcome resistance associated with reactivation of the MAPK pathway is to simultaneously target BRAF and MEK; preliminary clinical data suggests that this combination is more effective than BRAF inhibitors alone. To test if combined BRAF and MEK inhibition could be effective in the context of MEK2 mutations and BRAF amplification, melanoma cells were treated with MEK or BRAF inhibitors as single agents or in combination (Figure 9). While neither BRAF nor MEK inhibitors were able to substantially block MAPK activity in drug resistant cells or cells ectopically expressing MEK2-Q60P, MAPK signaling was almost completely inhibited when cells grown in 2D monolayers were treated with both inhibitors simultaneously. Furthermore, simultaneous treatment with both inhibitors resulted in partial decrease in viability of drug resistant cells (Figure 9B) and cells overexpressing MEK2-Q60P (Figure 9C) when grown as 2D monolayers. However, we noted that about 20-30% of cells remained viable despite co-inhibition of BRAF + MEK, raising the possibility that cells escaping combination therapy could eventually lead to tumor relapse.

To evaluate the potential significance of drug resistance *in vivo*, we injected parental cells, resistant cells, into NOD-SCID IL2-γ-null mice.

Mice were injected with $1x10^6$ melanoma cells per site (left and right flank) in a suspension of matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) / complete media at a ratio of 1:1. Tumor growth was assessed twice weekly by caliper measurement. Once tumors reached an average tumor volume of 200 - 300 mm3, mice were randomized into two treatment groups. Tumor volume was estimated using the formula (length x width x width)/2. Trametinib, Dabrafenib and 458 were suspended in 0.5% hydroxypropylmethylcellulose (Sigma) and 0.2% Tween-80 in dH2O (pH 8.0). Mice were dosed once daily by oral gavage (po qd). Animals were sacrificed four hours after the last dosing. Relative tumor weight was calculated as the individual measured tumor weight divided by the average tumor weight in the vehicle group of the same cell line and used for data analysis. Shapiro-Wilk test was used to examine data normality, and variance ratio test was used to examine the equality of variances between two groups. ANOVA (or Kruskal-Wallis test for non-normal distribution data) and post-hoc test with Bonferroni procedure were performed to examine the cell line effect on relative tumor weight. The trends of mean tumor volume over time were compared between treatments groups within same cell line using a mixed-effect model with the random effect at mouse level. A mixed-effect

model with linear or quadratic function, with or without random slopes of follow-up time (days), was compared and determined to provide the best fit. A likelihood ratio testing nested models (with versus without the interaction term of cell lines and days) was used to examine if trends were significantly different among treatment groups. Similar mixed-effect models were used to examine the tumor growth trends among cell lines.

Whereas trametinib inhibited MAPK signaling and growth in tumors derived from parental cells, it had virtually no effect on tumors derived from drug resistant cells (Figure 10).

Interestingly, we found that combined treatment with BRAF and MEK inhibitors, although partially effective *in vitro*, did not decrease the growth of trametinib-resistant tumors *in vivo* (Figure 11).

To determine if other compensatory pathways were activated in the resistant cells, we examined PI3K/mTOR activation in the trametenib resistant cells. Whereas pAKT levels were not differentially affected in the parental and resistant cells, MEK or BRAF inhibition led to inhibition of pS6K in the parental cells but not in the trametinib-resistant cells (Figure 12). Persistent phosphorylation of S6K raised the possibility that targeting this kinase could inhibit MAPK downstream signaling. To determine the effect of targeting S6K, we used the PI3K/mTOR inhibitor GSK2126458 (458) to block this pathway (Figure 13). Treatment of xenograft tumors with 458 as a single agent substantially inhibited the growth of trametinib-resistant tumors (Figure 14). However, the effects of inhibiting PI3K/mTOR were transient, as these tumors resumed growth after two weeks of treatment. In contrast, treatment with a triple combination of dabrafenib, trametinib, and 458 led to sustained tumor growth inhibition with no apparent toxicity. These studies provide proof-of-principle that effective triple combinatorial strategies targeting two or more pathways may have a favorable risk benefit profile and should be further explored as a valuable strategy to treat melanoma.

We have identified the same MEK2-Q60P mutation in a patient-derived sample, a xenograft tumor derived from a biopsy taken from a patient who relapsed while on the combination of dabrafenib and trametinib (CRPDX; combination relapsed patient-derived xenografts).

To generate CRPDX (Combination therapy resistant patient-derived xenografts), a fresh tumor biopsy was minced using cross blade technique, digested in collagenase IV for 30 minutes with repeated trituration. Approximately 100mm3 of digested tissue was implanted subcutaneously in the flank of NSG mice in matrigel. Serial transplantation was performed in a similar fashion. Tumor grafts reached ~1000 mm3 approximately eight weeks post implantation for every mouse passage. A short-term culture was generated from this tumor.

Analysis of a short-term culture derived from this CRPDX showed high BRAF expression levels, persistent MAPK signaling, and sustained phosphorylation of S6K in the presence of BRAFi, MEKi, and the combination of BRAF and MEK inhibitor (Figure 15). These cells were resistant to trametinib, dabrafenib, and the combination of both drugs. This observation further supports our findings and underscores the clinical relevance of our work, as it is likely that combination therapy with BRAF and MEK inhibitors will be used to treat melanoma patients in the community.

Our findings suggest that analysis of patient samples will need to include both genetic and genomic characterization of tumors so that all potential types of associated aberrations, such as MEK mutations and BRAF amplifications which can lead to increased MAPK signaling, can be identified. This molecular information may be useful to guide selection of therapy for patients with metastatic melanoma and improve patient outcome.

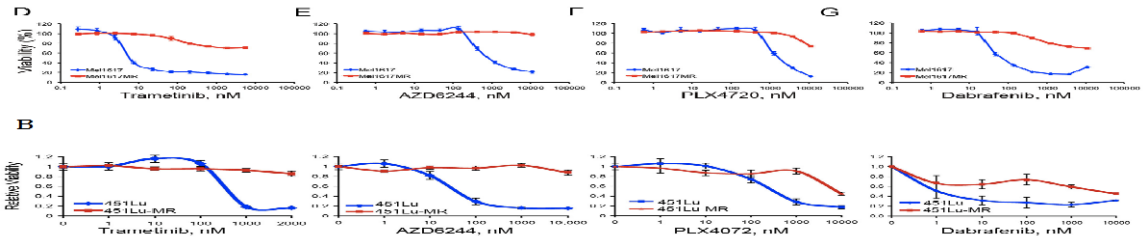


Figure 1. Parental and trametinib resistant melanoma sublines were treated with the indicated concentrations of MEK (Trametinib or AZD) or BRAF (PLX4720 or Dabrafenib) small molecule inhibitors for 72h. Cell viability was assessed by MTT assays and calculated relative to the DMSO (vehicle control)-treated cells. Data are represented as mean ± SEM with n=6

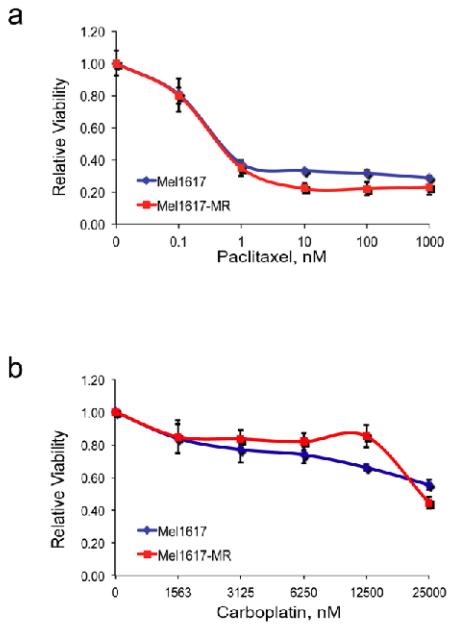


Figure 2. Mel1617 parental and Mel1617-MR trametinib resistant melanoma cells were treated with the indicated concentrations of paclitaxel (a) or carboplatin (b) for 72h. Cell viability was determined by MTT assays. Data are represented as mean ± SEM with n=6.

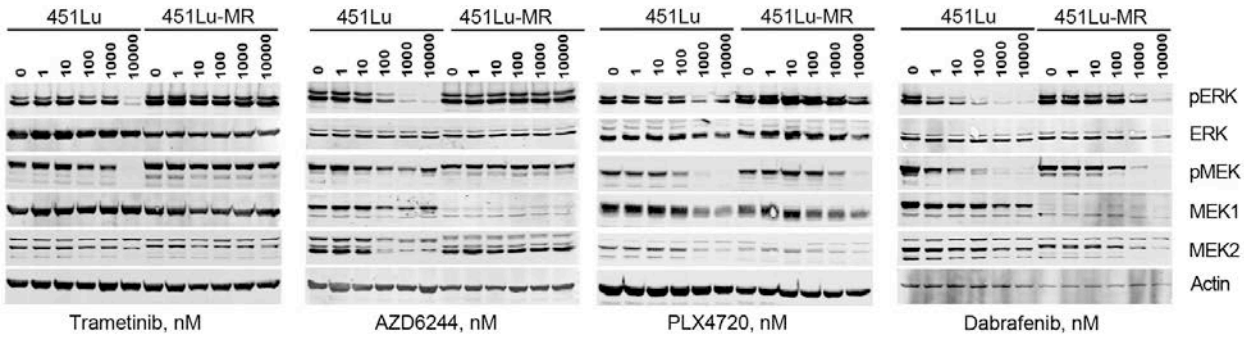


Figure 3. 451Lu parental and isogenic trametinib-resistant (MR) melanoma cells were treated with increasing concentrations of the indicated MEK (Trametinib, AZD6244) or BRAF (PLX4720 or Dabrafenin) inhibitors for 20h. Equal amounts of protein lysates were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

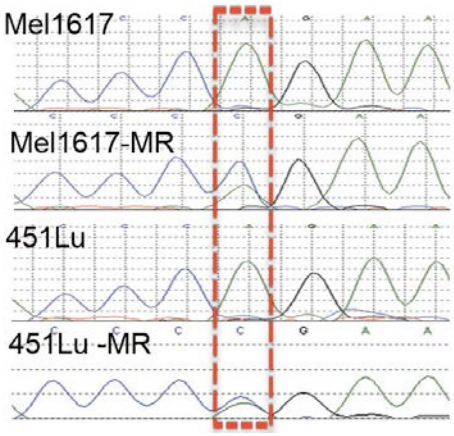
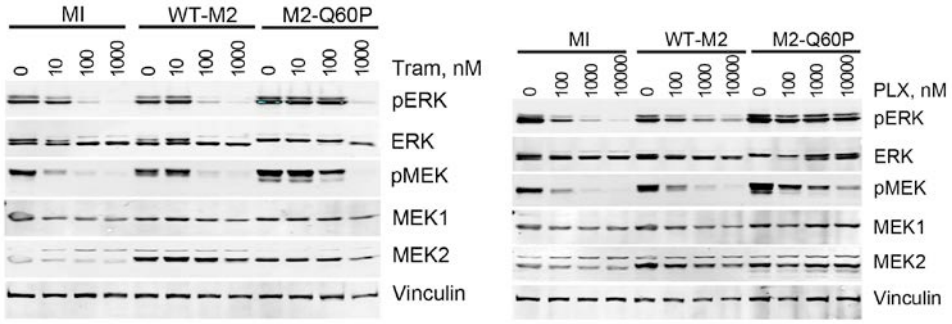


Figure 4. Chromatogram of sanger sequencing depicting the de novo Q60P mutation (c.179A>C, p.Q60P) identified in exon 2 in BRAF-V600E melanoma cells resistant to trametinib.

A



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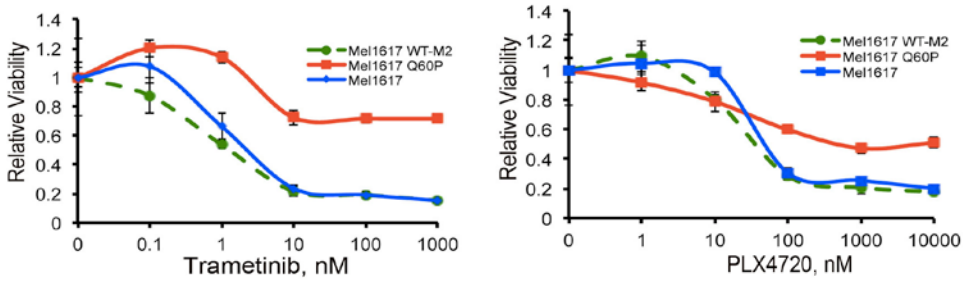

Figure 5. Mel1617 parental cells were mock-infected (MI), infected with a lentivirus carrying wild type MEK2 (WT-M2) or mutant MEK2-Q60P (M2-Q60P). (A) Immunoblotting analysis of cells treated with trametinib (Tram) or PLX4720 (PLX) for 20h. (B) Cell viability was determined by MTT assays and calculated relative to the untreated cells. Data are represented as mean ± SEM, n=6.

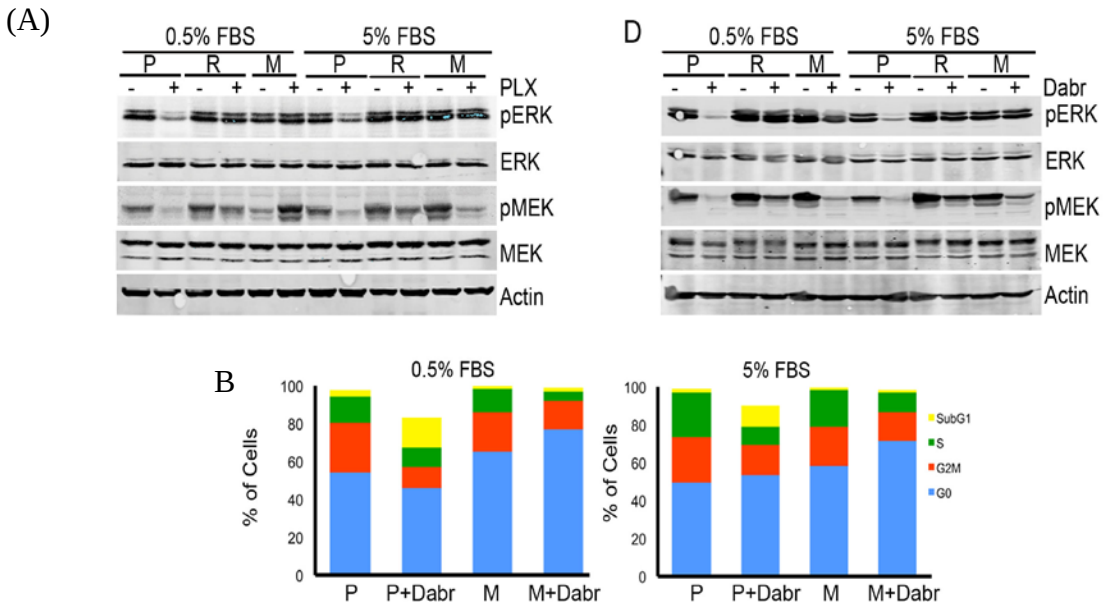

Figure 6: Mel1617 parental (P), trametinib-resistant (R) or cells expressing mutant MEK2 (M) were serum starved for 48h. Serum-starved cells were pre-treated with BRAF inhibitors for 30 minutes before addition of 0.5% or 5% fetal bovine serum (FBS). (A) Cells were further incubated for 24h, collected and protein lysates analyzed by immunoblotting with the indicated antibodies. (B) Cells were incubated for 48 h after addition of serum, stained with propidium iodide and analyzed by flow cytometry. Percentages of cells in each phase of the cell cycle from one representative experiment are shown.

A B

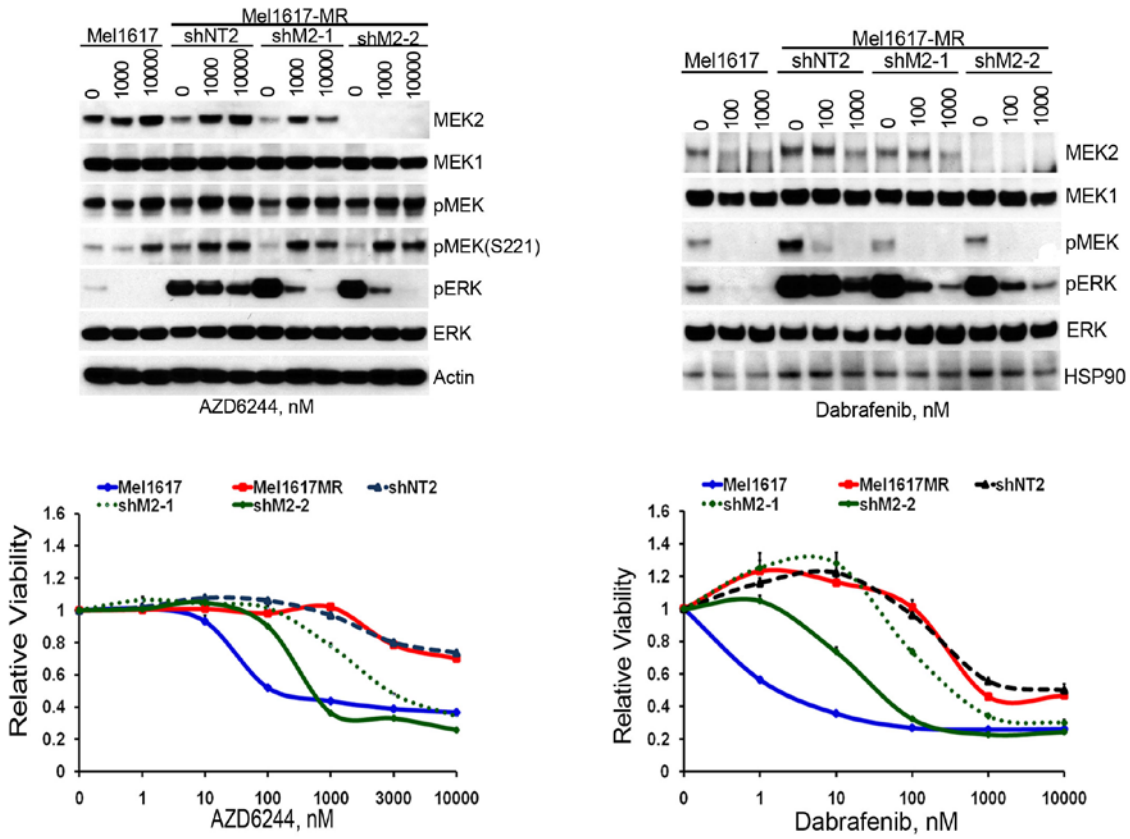

Figure 7. Mel1617-MR cells were infected with lentiviral vectors expressing a non-targeting shRNA (shNT2) or MEK2 shRNA (shM2-1, shM2-2). Transduced cells were selected with puromycin. Cells expressing MEK2 shRNA were treated with the MEK inhibitor AZD6244 (A) or the BRAF inhibitor dabrafenib (B) at the indicated doses for either 21h to determine the effect of MEK2 depletion on MAPK signaling by western blotting or for 72h for analysis of cell viability. Cell lysates were analyzed by immunoblotting (top panels). Relative cell viability was determined by MTT assays (bottom panels). Data are represented as mean ± SEM, n=5.

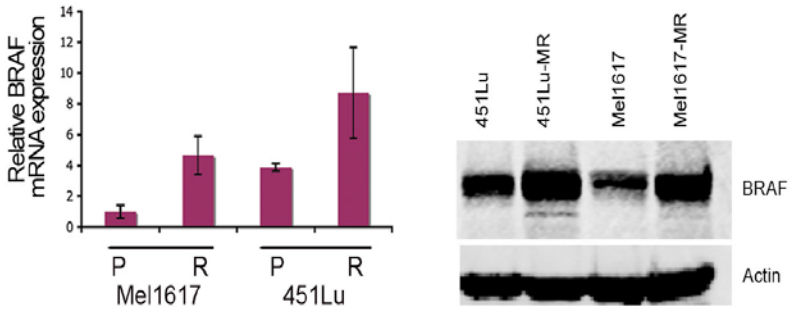

Figure 8: BRAF mRNA levels in Mel1617 and 451Lu sublines assessed by quantitative real time PCR (left panel). Data are represented as mean ± SEM (n=3); $p<0.05$ (C) BRAF protein levels were analyzed by immunoblotting in Mel1617 parental and resistant sublines (right panel).

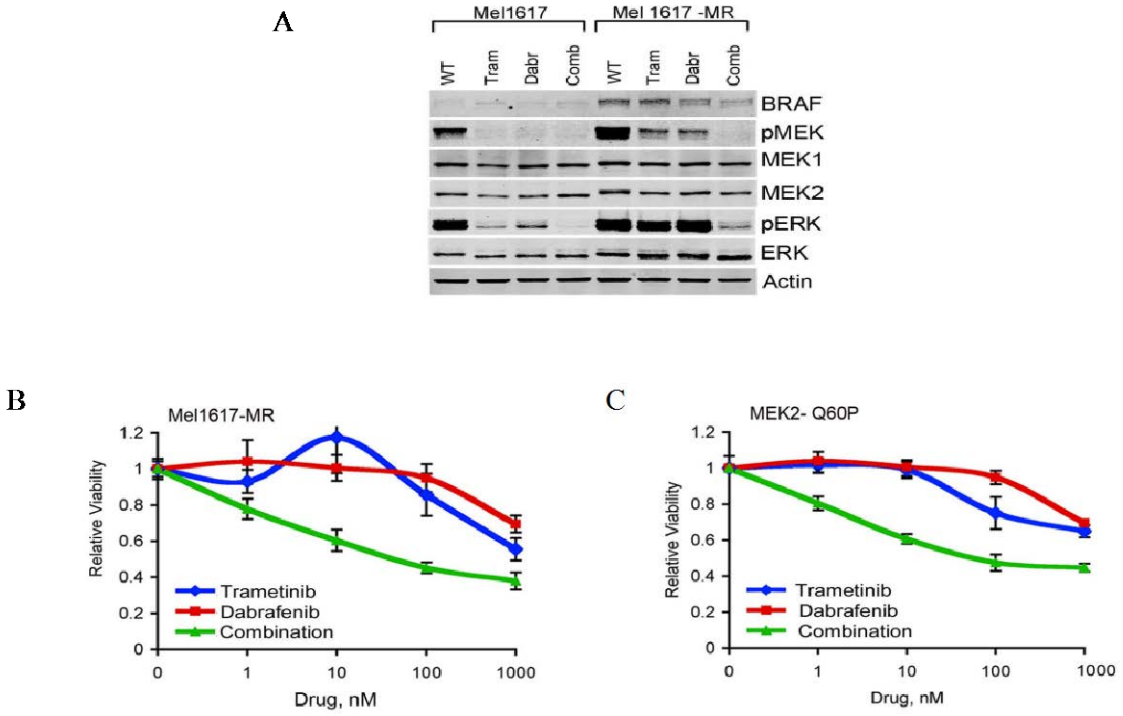

Figure 9. (A) Unlike treatment with dabrafenib or trametinib alone, treatment of Mel1617-MR cells with the combination of dabrafenib (Dabr) and trametinib (Tram) reduced ERK phosphorylation to similar levels as those observed in the parental cells expressing wild-type MEK2 treated with dabrafenib alone. (B, C) Cell viability was assessed by MTT assays in Mel1617-MR (B) and Mel1617 expressing MEK2-Q60P (C) cells treated with single agent trametenib, dabrafenib, or the combination of both drugs at the indicated doses.

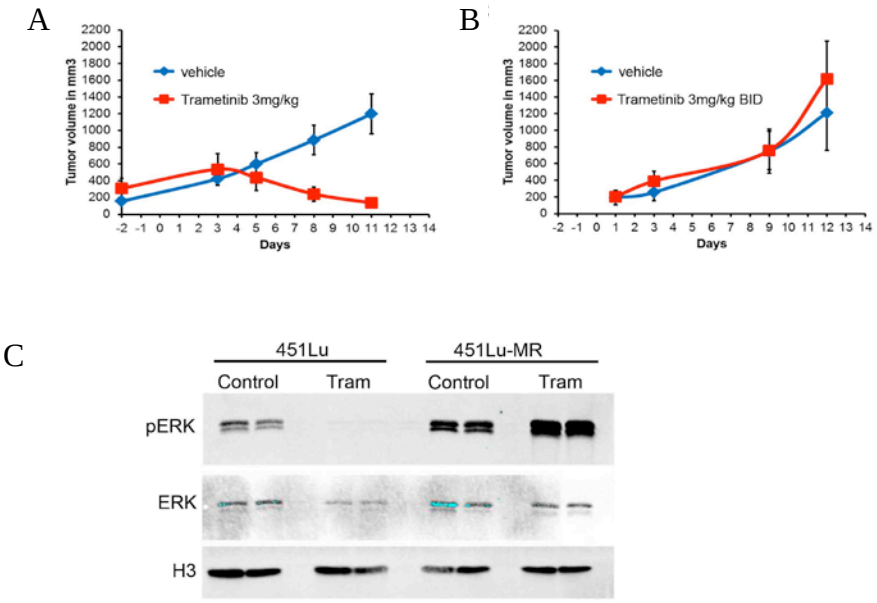

Figure 10. (A) Tumor volume of 451Lu parental (A) and 451Lu-MR drug resistant (B) xenografts treated with vehicle or Trametinib (3 mg/kg po qd) (mean ± SEM, n=5). Tumors were extracted 4h after the last dose and total protein lysates analyzed by immunoblotting with the indicated antibodies (C). A likelihood ratio testing nested model showed that the trends of relative tumor growth are significantly different between the two sublines (p=0.002). A mixed-effect model analysis indicated that tumor growth is significantly slower after treatment with Trametinib in the parental cells (p=0.033) compared with the resistant cells. Wilcoxon rank sum test shows that the tumor weight at the end of experiment is significant lower in the parental cells treated with Trametinib compared with vehicle control (p=0.008), whereas there is no significant difference in tumor weight at the end of experiment in resistant cells treated with Trametinib compared with vehicle control (p=0.117).

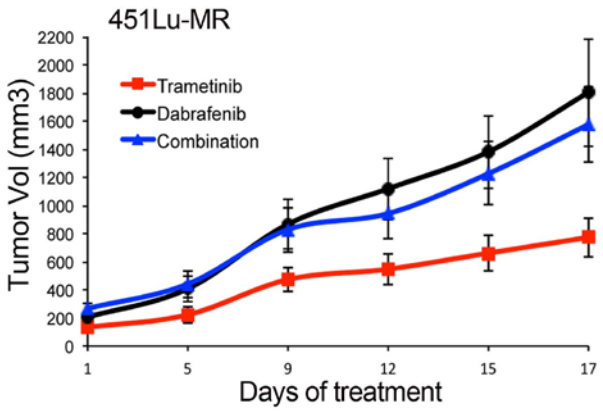

Figure 11. Mice bearing 451Lu-MR (trametinib-resistant) tumors were treated with dabrafenib (30 mg/kg), trametinib (3 mg/kg), or the combination of both drugs at the same doses for the indicated times. Compared with the group treated with trametinib as single agent, the trend of tumor growth was significantly faster in the group treated with dabrafenib 436 (p=0.015) or Trametinib + Dabrafenib (p=0.047). There was no significant difference in tumor growth between dabrafenib and the combination of trametenib plus dabrafenib (p=0.654).

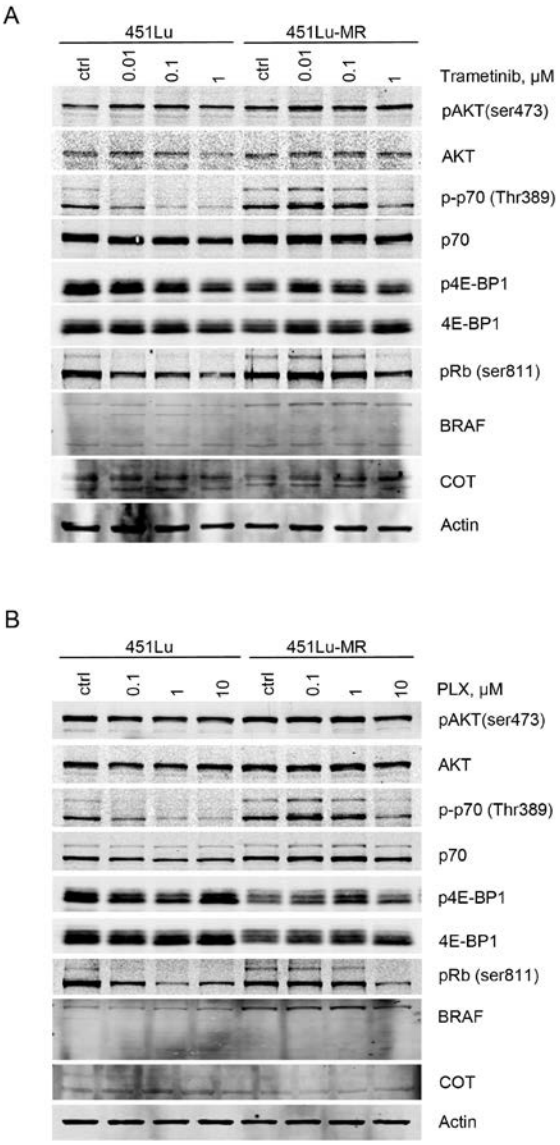

Figure 12. Parental and MR sublines were treated with trametinib (A) or PLX4720 (B) or for 22 h. Cell lysates were analyzed by immunoblotting to assess activation of the PI3K/mTOR/S6K pathway.

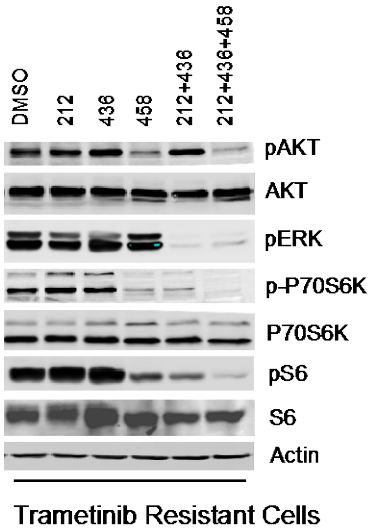

Figure 13. Phosphorylation of p70S6K can be inhibited by the dual PI3K/mTOR inhibitor 458 in vitro. Mel1617-MR cells were treated with the indicated drugs (0.1 μM) for 24h. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

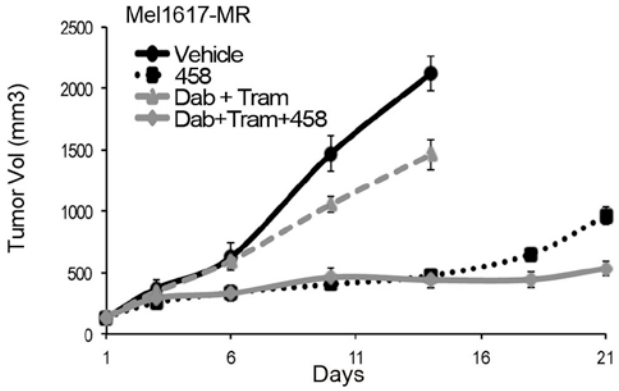

Figure 14. Mice bearing trametinib-resistant tumors were treated with vehicle, 458 (3 mg/kg), dabrafenib (Dab; 30 mg/kg) plus trametinib (Tram; 0.3 mg/kg) or a combination of three drugs at the same doses. Tumors in mice treated with vehicle or dabrafenib plus trametinib grew more rapidly and animals were sacrificed at day 15. Mice were treated with 458 or the triple combination for 21 days. Tumor growth was significantly faster in the Dab + Tram group ($p<0.0001$) and the 458 single agent group (*$p=0.005$) compared with the triple combination (Dab+Tram+458). Tumor growth rate of the Dab + Tram group was significantly faster than the group treated with 458 ($p<0.001$).

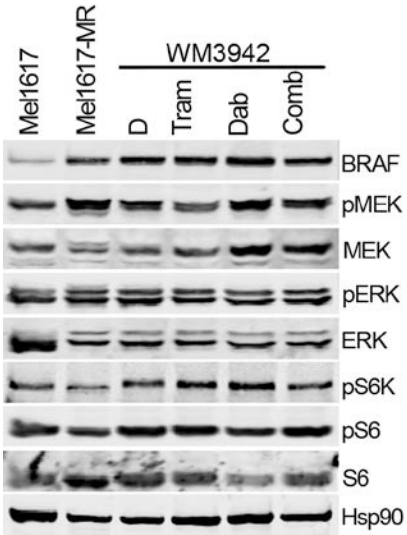

Figure 15. A short-term culture derived from a CRPDX, WM3942, was treated with the indicated compounds for 24h and analyzed by immunoblotting.

References:

Emery, C. M., Stransky, N., Cogdill, A. P., Barretina, J., et al. (2010). COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. Nature 468, 968-972.

Nikolaev, S. I., Rimoldi, D., Iseli, C., Valsesia, A., Robyr, D., Gehrig, C., Harshman, K., Guipponi, M., Bukach, O., Zoete, V., et al. (2012). Exome sequencing identifies recurrent somatic MAP2K1 and MAP2K2 mutations in melanoma. Nature genetics 44, 133-139.

Shi, H., Moriceau, G., Kong, X., Koya, R. C., Nazarian, R., Pupo, G. M., Bacchiocchi, A., Dahlman, K. B., Chmielowski, B., Sosman, J. A., et al. (2012). Preexisting MEK1 exon 3 mutations in V600E/KBRAF melanomas do not confer resistance to BRAF inhibitors. Cancer Discov 2, 414-424.

Shi H, Moriceau G, Kong X, Lee MK, Lee H, Koya RC, Ng C, Chodon T, Scolyer RA, Dahlman KB, Sosman JA, Kefford RF, Long GV, Nelson SF, Ribas A, Lo RS. (2012) Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. Nat Commun. 3:724.

Villanueva, J., Vultur, A., Lee, J. T., Somasundaram, R., Fukunaga-Kalabis, M., Cipolla, A. K., Wubbenhorst, B., Xu, X., Gimotty, P. A., Kee, D., et al. (2010). Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. Cancer cell 18, 683-695.

Wagle, N., Emery, C., Berger, M. F., Davis, M. J., Sawyer, A., Pochanard, P., Kehoe, S. M., Johannessen, C. M., Macconaill, L. E., Hahn, W. C., et al. (2011). 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.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___x__ No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___x__ No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____ Yes
___X___ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one

publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Concurrent MEK2 mutation and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma	Jessie Villanueva, Jeffrey R. Infante, Clemens Krepler, et al.	Cell Reports	July 2013	☐Submitted ☒Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No ____X_____

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Our studies provide proof-of-principle that combination strategies targeting two or more resistance pathways can be developed. Through further studies, combination therapy with BRAF, MEK, and PI3K/mTOR inhibitors will serve to improve patient treatment and mortality in patients presenting with melanoma.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention,

diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Results of our studies underscore the clinical relevance of our work, as it is likely that combination therapy with BRAF and MEK inhibitors will be used to treat melanoma patients in the community. Our studies indicate that combination therapies targeting two or more pathways warrant further evaluation as a strategy to overcome drug resistance.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No ____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

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BIOGRAPHICAL SKETCH

NAME **VILLANUEVA, Jessie**	POSITION TITLE **Assistant Professor**
eRA COMMONS USER NAME **JVILLANUEVA**	

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Universidad Peruana Cayetano Heredia, Lima, Peru	B.S.	1994	Biology
University of Miami School of Medicine, Miami, FL	Ph.D.	2003	Molecular Cell and Developmental Biology
University of Pennsylvania, Dept of Pharmacology, Philadelphia, PA	Postdoc	2003-2005	Pharmacology
University of Pennsylvania, Abramson Family Research Cancer Institute, Philadelphia, PA	Postdoc	2005-2006	Cancer Biology
The Wistar Institute, Philadelphia, PA	Postdoc	2006-2008	Molecular and Cellular Oncogenesis

Positions and Honors:

Research and Professional Experience:

2011 – present: Assistant Professor – Molecular and Cellular Oncogenesis Program, Member Melanoma Research Center - The Wistar Institute, Philadelphia, PA

2010 – 2011: Staff Scientist – The Wistar Institute, Philadelphia, PA

2009 – 2010: Associate Staff Scientist - The Wistar Institute, Philadelphia, PA

Postdoctoral:

2006 – 2008: The Wistar Institute: Molecular and Cellular Oncogenesis Program, Advisor: Meenhard Herlyn, D.V.M., D.Sc.

2005 – 2006: Abramson Family Cancer Research Institute, Department of Cancer Biology, University of Pennsylvania School of Medicine, Advisor: David A. Tuveson, MD Ph.D., "Role of B-RAF in melanoma initiation and progression"

2003 – 2005: University of Pennsylvania School of Medicine, Department of Pharmacology, Advisor: Richard K. Assoian, Ph.D., "Role of Rac and ERK for cyclin D1 expression and cell cycle progression in quiescent and cycling cells"

Graduate:

1996 – 2003: Graduate Student, University of Miami School of Medicine, Department of Cell Biology and Anatomy, Graduate Program in Molecular Cell and Developmental Biology, Advisor: Richard K. Assoian, Ph.D., "Regulation of cyclin D1 expression and G1 phase cell cycle progression by ERK and PI3K signaling pathways"

1994 – 1996: Enrolled in the Ph.D. program in Molecular and Cellular Pathology, University of Pittsburgh School of Medicine, Research Specialist III: Molecular Pathology, Department of Pathology, Advisor: Joseph Locker, M.D. Ph.D., "Regulation, gene expression and transcriptional control of the alpha-fetoprotein (AFP) gene"

Undergraduate:

1992 – 1993: Research Assistant: Molecular Biology, Universidad Peruana Cayetano Heredia, Lima, Peru, Institute of Tropical Medicine

1990 – 1992: Research Assistant: Biochemistry, Universidad Peruana Cayetano Heredia, Lima, Peru, Department of Biochemistry, Molecular Biology and Pharmacology

Fellowships and Awards:

- 2012 V Scholar
- AACR Minority Scholar in Cancer Research Award, April 2012
- National Cancer Institute: Research Training Fellowship, Nov 2004-Sep 2005
- Honorable Mention, 1999 Eastern Student Research Forum, Miami FL

- Graduate Fellowship, 1996-1997 University of Miami School of Medicine

Selected Peer-reviewed Publications:

1) **Villanueva, J**., Infante, J., Krepler, C., Reyes-Uribe, P., Samanta, M., Chen, H-Y, Li, B., Swoboda, R., Wilson, M., Vultur, A., Fukunaga-Kalabis, M., Wubbenhorst, B., Chen T., Liu, Q., Sproesser, K., DeMarini, D., Gilmer, T., Martin, A-M., Marmorstein, R., Schultz, D., Speicher, D., Karakousis, G., Xu, W., Amaravadi, R., Xu, X., Schuchter, L., Herlyn, M., Nathanson, K.: Concurrent MEK2 mutation and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma. *Cell Reports*, in press, 2013
2) Roesch A, Vultur A, Bogeski I, Wang H, Zimmermann KM, Speicher D, Körbel C, Laschke MW, Gimotty PA, Philipp SE, Krause E, Pätzold S, **Villanueva J**, Krepler C, Fukunaga-Kalabis M, Hoth M, Bastian BC, Vogt T, Herlyn M. Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B (high) cells. *Cancer Cell*. 2013;23(6):811-25. (PMID: 23764003)
3) Vultur A, **Villanueva J**, Krepler C, Rajan G, Chen Q, Xiao M, Li L, Gimotty PA, Wilson M, Nathanson KL, Herlyn M. MEK inhibition affects STAT3 signaling and invasion in human melanoma cell lines. *Oncogene*. 2013; Apr 29. doi: 10.1038/onc.2013.131. [Epub ahead of print] PMID: 23624919
4) Krepler C, Chunduru SK, Halloran MB, He X, Xiao M, Vultur AM, **Villanueva J**, Mitsuuchi Y, Neiman EM, Benetatos C, Nathanson KL, Amaravadi R, Pehamberger H, McKinlay M, Herlyn M. The novel SMAC mimetic Birinapant exhibits potent activity against human melanoma cells. *Clin Cancer Res*. 2013 Feb 12. [Epub ahead of print] (PMID: 23403634)
5) Desai BM *, **Villanueva J ***, Nguyen T-T K, Lioni M, Xiao M, Kong J, Krepler C, Vultur A, Flaherty KT, Nathanson KL, Smalley KSM, and Herlyn M. The anti-melanoma activity of dinaciclib, a cyclin-dependent kinase inhibitor, is dependent on p53 signaling. *Plos One*. 2013;8(3):e59588. (PMID: 23527225). (* Co-first authors).
6) Balaburski GM, Leu JI, Beeharry N, Hayik S, Andrake MD, Zhang G, Herlyn M, **Villanueva J**, Dunbrack RL Jr, Yen T, George DL, Murphy ME. A modified HSP70 inhibitor shows broad activity as an anticancer agent. *Mol Cancer Res*. 2013 Jan 9. (PMID: 23303345) Mol Cancer Res. 2013 Mar;11(3):219-29. doi: 10.1158/1541-7786.MCR-12-0547-T. Epub 2013 Jan 9. (PMID: 23303345).
7) Schayowitz A, Bertenshaw G, Jeffries E, Schatz T, Cotton J, **Villanueva J**, Herlyn M, Krepler C, Vultur A, Xu W, Yu GH, Schuchter L, Clark DP. 2012) Functional Profiling of Live Melanoma Samples Using a Novel Automated Platform. *PLoS ONE* 7(12): e52760. (PMID: 23285177)
8) Chae YC, Caino MC, Lisanti S, Ghosh JC, Dohi T, Danial NC, **Villanueva J**, Ferrero S, Vaira V, Santambrogio L, Bosari S, Languino L, Herlyn M and Altieri DC. Control of Tumor Bioenergetics and Survival Stress Signaling by Mitochondrial HSP90s. *Cancer Cell*. 2012; 22(3):331-344. (PMID: 22975376)
9) Somasundaram R, **Villanueva J**, Herlyn M. Intratumoral heterogeneity as a therapy resistance mechanism: role of melanoma subpopulations. *Adv Pharmacol*. 2012; 65:335-59. (PMID: 22959031)
10) Schott S, Niessner H, Sinnberg T, Venturelli S, Berger A, Ikenberg K, **Villanueva J**, Meier F, Garbe C, Busch C. Cytotoxicity of new duplex drugs linking 3'-C-ethynylcytidine and 5-fluor-2'-deoxyuridine against human melanoma cells. *Int J Cancer*. 2012;131(9):2165-74. (PMID: 22323315)
11) Herlyn M, **Villanueva J**. Sorting through the many opportunities for melanoma therapy. Pigment Cell Melanoma Res. 2011 Sep 8. [Epub ahead of print] (PMID: 21899726)
12) **Villanueva J**, Vultur A, Herlyn M. Resistance to BRAF inhibitors: unraveling mechanisms and future treatment options. *Cancer Res*. 71:7137-40, 2011. (PMID: 22131348).
13) Vultur, A, **Villanueva J**, Herlyn, M. Targeting BRAF in advanced melanoma: a first step towards manageable disease. Clin Cancer Res. 2011;17(7):1658-63. (PMID: 22131348)
14) **Villanueva J**, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, Wubbenhorst B, Xu X, Gimotty PA, Kee D, Santiago-Walker AE, Letrero R, D'Andrea K, Pushparajan A, Hayden JE, Brown KD, Laquerre S, McArthur GA, Sosman JA, Nathanson KL, Herlyn M. Acquired Resistance to BRAF Inhibitors Mediated by a RAF Kinase Switch in Melanoma Can Be Overcome by Cotargeting MEK and IGF-1R/PI3K. *Cancer Cell*. 2010; 18(6):683-95. (PMID: 21156289)
15) Vultur A, **Villanueva J**, Herlyn M. BRAF inhibitor unveils its potential against advanced melanoma. *Cancer Cell*. 2010; 18(4):301-2. (PMID: 20951940)
16) Smalley KS, Xiao M, **Villanueva, J**, Nguyen TK, Flaherty KT, Letrero R, Van Belle P, Elder DE, Wang Y, Nathanson KL, Herlyn M. CRAF inhibition induces Bcl-2 dependent apoptosis in melanoma cells with non-V600E BRAF mutations. *Oncogene*. 2009; 28(1):85-94. (PMID: 18794803)