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):** 01/01/2013 – 06/30/2014

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:** 4100062226

6. **Project Number and Title of Research Project:** 5: Developing Rational Strategies for Therapeutic Targeting of NRAS-Mutant Melanomas

7. **Start and End Date of Research Project:** 01/01/2013 – 06/30/2014

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:

   $ 175,333.00

   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
Chen	Postdoctoral Fellow	69%	$51,838
Reyes-Uribe	Research Assistant	93%	$38,842
Samanta	Research Assistant	38%	$24,137
Villanueva	Principal Investigator	16%	$20,682

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
None		

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
None		

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:

National Institutes of Health (NIH): $139,628, annual direct costs

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:

The studies performed during this funding period will enable us to apply for federal funding. I plan to submit an application for an R21 or an R01 early 2015 and further investigate the role and therapeutic value of targeting some of the candidate genes or pathways identified through this project.

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

Our initial studies have allowed us to identify some genes or pathways that appear to be critical for survival of NRAS mutant melanomas. We plan to further investigate the role of these candidate genes for melanomagenesis. Furthermore, we plan to evaluate the value of targeting candidate genes for melanoma therapy.

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:

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

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

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

If yes, please describe the collaborations:

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.

Background and Significance:

Melanoma is the most aggressive form of skin cancer and its incidence continues to rise worldwide. Although primary melanoma is generally cured through surgery, treatment of advanced disease remains challenging. Almost a decade after the discovery of mutations in the BRAF gene in 50% of all melanomas, the FDA has approved Zelboraf (vemurafenib/PLX4032) for the treatment of advanced metastatic BRAF-^{V600E} melanoma. However, there are still no good options to treat the other 50% of non-BRAF mutant patients.

Neuroblastoma RAS viral oncogene homolog (NRAS) mutations have been identified in more than 20% of melanomas. Moreover, although BRAF and Rous sarcoma (RAS) mutations have long been considered to be mutually exclusive, concurrent BRAF and RAS mutations have been identified in some melanomas, particularly in recurrent tumors of patients who progress on vemurafenib. Thus, therapies tailored to curb NRAS in this subtype of tumors are urgently needed.

The proposed studies will concentrate on melanomas containing mutations in NRAS. NRAS is a poorly understood RAS family member that is frequently mutated in melanoma. Most of our knowledge of oncogenic RAS signaling is based on studies on H- and Kirsten rat sarcoma viral oncogene homolog (KRAS), which are frequently mutated in other malignancies, but seldom in melanoma. Notably, it has recently become clear that different Rous sarcoma (RAS) family members have distinctive and non-overlapping functions, and the activity of these proteins can be determined by the cellular context. Moreover, notwithstanding the remarkable progress made in this field, the molecular and cellular pathways governed by mutant NRAS in melanoma remain inadequately understood. Furthermore, there are no effective therapies presently available for patients with melanomas containing mutant NRAS. Gaining a clearer picture of the molecular pathways controlled by mutant NRAS in melanoma will be critical to understanding ways to manage and treat this class of tumors.

Some efforts have been made to identify potential therapeutic avenues for the treatment of NRAS mutant tumors. Targeting RAS directly has thus far not proven successful; an alternative approach is to target downstream effectors of the RAS pathway. Oncogenic NRAS hyperactivates the mitogen- activated protein kinase (MAPK) pathway, creating dependence on RAS for cell survival. Although, BRAF inhibitors have demonstrated exceptional results in BRAF-mutant melanomas, NRAS-mutant tumors are resistant to BRAF inhibitors because the latter trigger paradoxical MAPK activation and increase tumor growth. MEK inhibitors have potential value for therapeutic treatment of RAS mutant tumors. However, in our preliminary studies we see a heterogeneous response; MEK inhibition decreases viability in some NRAS mutant cells but not all. NRAS can activate an array of downstream effectors, including the PI3K pathway, and this could diminish dependence on MEK. We posit that effective targeting of NRAS mutant melanomas will require the identification of key downstream mediators of NRAS

signaling that are critical for tumor cell survival. We also postulate that targeting multiple points within the RAS signaling network may be required to combat this class of tumors. In this project, we will study the molecular outcomes of NRAS silencing in NRAS mutant melanomas aiming to identify the key effectors that are required for tumor survival. We also propose to identify genes that cooperate with MEK to induce survival in NRAS mutant melanoma. This will help us to delineate effective therapeutic strategies to target NRAS in a subset of melanomas that harbor this mutant oncogene.

This proposal will fill a significant knowledge gap in the field with important clinical and public health implications. Our studies will have a significant impact because the identification of critical NRAS effectors will provide targets to induce potent apoptosis in NRAS mutant tumors; we anticipate that these data will be essential to develop effective therapies to control these neoplasms.

Specific Aims:

Aim 1. Define the molecular consequences of silencing oncogenic NRAS in melanoma. While NRAS mutations are present in a large number of melanomas, there are no systematic studies investigating the dependency on oncogenic NRAS signaling. The goals of this aim are:
a) To identify human melanoma cells that are dependent on NRAS signaling for proliferation and survival
b) To determine the biochemical effects of silencing NRAS in melanomas harboring mutations in this oncogene.

Aim2. Identify genes or pathways that when targeted can potently kill NRAS mutant melanomas. Although NRAS mutant melanomas activate the MAPK and PI3K pathways, not all RAS mutant melanoma cells are sensitive to inhibitors of these pathways. Furthermore, it is very unlikely that inhibiting only one pathway will have therapeutic benefits for NRAS mutant tumors. We will test the hypothesis that additional genes cooperate with the MAPK pathway to promote survival in NRAS mutant melanomas. We will determine the effect of silencing or inactivating selected NRAS downstream effectors on proliferation and viability of NRAS mutant melanomas either alone or in combination with MEK or PI3K inhibitors

Major Achievements

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

- Established conditions for efficient NRAS silencing using lentiviral mediated shRNA
- Generated melanoma cell lines expressing NRAS shRNA
- Identified a panel of melanomas dependent on NRAS
- Demonstrated that NRAS silencing restrains cell proliferation
- Showed that depletion of NRAS in melanoma inhibits the MAPK pathway
- Found that depletion of NRAS induces increased phosphorylation of AKT
- Determined that inhibition of MEK produces a heterogeneous response in NRAS mutant melanomas

- Demonstrated that MEK inhibition reduces viability in a subset of NRAS mutant melanomas
- Determined that some NRAS mutant melanomas are not substantially affected by inhibitors of the MAPK pathway

Summary of results

The goal of this project was to determine the molecular consequences of silencing NRAS, aiming to identify signaling molecules or pathways that are essential for survival of NRAS mutant melanomas. Towards this goal, we have identified a panel of NRAS mutant melanoma cell lines. All cell lines were subject to quality control, including microsatellite fingerprinting to verify the identity of each cell line, mycoplasma testing, and genetic validation of NRAS mutation.

During the term of this grant, substantial progress was achieved as summarized below:

Generation of cell lines expressing NRAS shRNA.

1. NRAS shRNA:

Hairpins shRNA targeting human NRAS in lentiviral vectors were obtained from Openbiosystems (ThermoFisher; Table I). These hairpins were originally generated by The RNAi Consortium (TRC) and are now commercially available and already cloned into the lentiviral vector pLKO.1. The pLKO.1 constructs can be packaged into virus particles for use in transduction experiments. Specifically, the replication-incompetent viral particles can be efficiently produced using lentiviral packaging plasmids co-transfected in 293T packaging cells. Transduction of melanoma cells with lentiviruses was conducted according to the manufacturer's guidelines. Briefly, lentiviral NRAS shRNA plasmids were isolated using Invitrogen Maxiprep Kits following the manufacturer's instructions. To package the lentiviral vectors, 2ug lentiviral plasmids, along with 2 ug packaging plasmids consisting of 1:8 ratio of VSV-G vs dr 8.2 packaging plasmids and 21 ug Arrest-in agent (Openbiosystems), were respectively diluted into 400 ul serum-free DMEM medium. Diluted DNA was then added to the diluted Arrest-in, mixed quickly and incubated at room temperature for 20 minutes. After that, 800 ul DNA/Arrest-in complex were applied to $1X10^6$ 293T cells in 1.2 ml serum-free DMEM in a 60 mm dish, and the 293T cells were incubated at 37°C for five hours. After aspirating the transfection medium, 3 ml DMEM medium containing 10% fetal bovine serum was added; and the transfected 293T was incubated at 37^{0}C for 48 hours. The lentivirus- containing supernatant were harvested and filtered (using 0.45 um low binding protein filters) and stored at -80^{0}C or immediately used.

To transduce cells, lentiviral supernatant was diluted at MOI of 1-5 with medium and then incubated with melanoma cells with 8 ug/ml polybrene for five hours. After 48 hours of incubation with regular growth medium, the transduced cells were selected with puromycin for 48 hours. NRAS depletion was assessed by Western blotting. None of the tested hairpins efficiently knocked down NRAS (Figure 1). In order to improve the degree of NRAS depletion, we next combined two different hairpins. For these experiments, we infected melanoma cells

using two different hairpins simultaneously or sequentially (Figure 2). Combining two hairpins resulted in >90% depletion of NRAS; therefore, all subsequent experiments are performed using two hairpins against NRAS.

2. Silencing NRAS in melanoma.

To evaluate the biological consequences of silencing NRAS in melanoma, we selected a panel of NRAS mutant cells. All cell lines have been sequenced to determine the NRAS mutation status. Depletion of NRAS in these cell lines was confirmed by immunoblotting (Figure 1); NRAS levels were decreased by >75% after lentiviral infection in all cell lines.

Silencing NRAS inhibits proliferation of melanoma cells

To determine the effect of NRAS silencing on cellular growth, we compared relative cell growth in NRAS mutant melanoma cells expressing an shRNA control or NRAS-shRNA. Same number of cells expressing shRNA control or shNRAS (2500-3000 cells) were seeded onto 96-well plates and grown for six days. Relative cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays every 24 hours by normalizing absorbance of NRAS-depleted cells (shNRAS) to the isogenic cell line expressing non-targeting shRNA. We observed that NRAS depletion halted the growth of five of seven melanoma cell lines (Figure 4).

The effect of NRAS depletion was further assessed on cell proliferation by comparing bromodeoxyuridine BrdU incorporation in NRAS mutant isogenic cell lines expressing non-targeting shRNA (control) or NRAS shRNA using a BrdU Cell Proliferation Assay Kit (Cell Signaling Technologies, MA). Briefly, cells were plated at a density of 5,000 to 10,000 cells per well (depending on cell line growth properties) onto 96-well plates and allowed to adhere overnight incubating at 37°C. The following day, the cells were labeled with BrdU for different times, and the plates were then fixed and processed according to the manufacturer's standard protocol. BrdU incorporation was detected using a mouse anti-BrdU antibody. Consistent with the results obtained by MTT assays on cell growth, we found that NRAS depletion led to a decrease in BrdU incorporation (Figure 5).

To determine the effect of NRAS silencing on proliferation, we assessed the expression of the proliferative marker Ki67 by immunofluorescence in NRAS mutant melanoma cells expressing an shRNA non-targeting control or NRAS-shRNA. Approximately $2x10^4$ cells expressing shRNA control or shNRAS were seeded onto cover slips, allowed to attach and spread, and grown for 24h. The next day the media was removed and the cells fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.2% w/v Triton X-100 for five minutes and then blocked with 1% BSA in PBS for 30 minutes at room temperature followed by incubation with anti-Ki-67 antibody for 1h at 37°C. After washing, secondary antibody was added to the coverslips and incubated for 1h at 37°C, followed by three washes with PBS, and finally transferred to a slide by placing them over a drop of ProLong Gold with DAPI and sealed. The slides were examined by fluorescence microscopy using a Nikon TE2000 -U microscope with a 20x Nikon Plan Fluor, NA .30 objective, and photographed using a Digital camera (Q imaging EX I). We further recorded, processed, and quantified the number of Ki67 positive cells

using image analysis software Image Pro Plus 7.0 (Media Cybernetics); image dimensions were 1360 x 1036 px. To this end, we used two channels:

- Channel 1, Dapi with Exposure: 533ms, Binning: 1 x 1, Gain: 4.000000.
- Channel 2, Ki67 with exposure: 500 ms, Binning: 1 x 1, Gain: 4.000000.

The total number of cells was determined and quantitated using the channel 1 (Dapi), whereas Ki67 positive cells were determined and quantitated using channel 2. Both images were merged using the same software (Figure 6). We observed that NRAS mutant melanomas transduced with a control shRNA were positive for Ki67, whereas depletion of NRAS substantially decreased the number of Ki67 positive cells (Figure 6), indicating that loss of NRAS inhibits proliferation of NRAS mutant melanomas. Similar results were obtained in all cell lines tested (data not shown). To further validate the effect of silencing NRAS on melanoma proliferation, we evaluated growth rate of NRAS mutant melanomas transduced with a non-targeting shRNA or NRAS shRNA. For these experiments, $5X10^4$ cells were seeded onto 12-well plates. Cells were fixed with methanol every 24 hours for five days. At the end of the time course, the cells were stained with crystal violet followed by elution of the dye with 0.1 M trisodium citrate in 50% ethanol for 10 min. Optical density at 595 nM was monitored on a microtiter plate reader in duplicates. The crystal violet assay further confirmed that depletion of NRAS decreases cell proliferation (Figure 7).

NRAS depletion inhibits the MAPK pathway

To determine the biochemical effects of silencing NRAS in melanomas harboring mutations in this oncogene, total protein lysates from NRAS mutant melanoma cells expressing a non-targeting shRNA or NRAS shRNA were analyzed by immunoblotting to determine the effect of NRAS depletion on levels or activity of potential NRAS effectors (Figure 8). We found that NRAS depletion inhibited MAPK signaling evidenced by decreased phosphorylation of MEK and ERK in all cell lines tested. However, NRAS depletion did not inhibit phosphorylation of AKT at Ser473 or Thr308. Moreover, NRAS depletion appears to increase phospho-AKT levels.

Aim2. Identify genes or pathways that when targeted can potently kill NRAS mutant melanomas.

During this grant period, we evaluated strategies aimed at blocking RAS (Rous sarcoma virus) effector molecules and evaluating the biological consequences of pharmacological inhibitors.

Since our initial studies indicated that NRAS depletion decreased MAPK signaling as described above, we wished to investigate the effect of inhibiting the MAPK pathway using small molecule inhibitors. To this end we treated NRAS mutant melanoma cells with the allosteric MEK inhibitor Trametinib at different concentrations for 24, 48, or 72 hours (Figure 9). Trametinib effectively inhibited the MAPK pathway as evidenced by decreased phosphorylation of MEK, ERK, and RSK (ribosomal S6 kinase). MAPK inhibition was coupled to inhibition of Cyclin D1 expression and phosphorylation of the retinoblastoma (Rb) protein, indicative of inhibition of cell cycle progression. MAPK inhibition at 48 and 72h also led to accumulation of cleaved lamin A, a marker of apoptosis. To further evaluate the effect of MAPK inhibition on proliferation, we seeded $5X10^4$ cells onto 12-well plates. Cells were fixed with methanol every 24 hours for ten days. At the end of the time course, the cells were stained with crystal violet as described in Aim 1A. The relative number of cells in each plate was assessed by reading

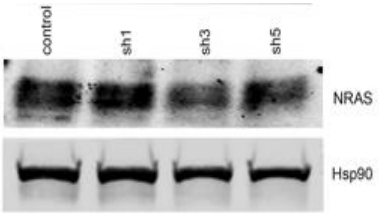
absorbance at 595 nM. Treatment of NRAS mutant melanoma cells with trametinib decreased growth compared with cells treated with vehicle control (Figure 10).

Table 1: *Hairpins targeting human NRAS (Open Biosystems).*

Clone No.	Sh #	Mature Antisense sequence:
TRCN0000033255	2	ATGACTTGCTATTATTGATGG
TRCN0000033258	5	AATGGAATCCCGTAACTCTTG
TRCN0000033254	1	ATTAGCTGGATTGTCAGTGCG
TRCN0000033256	3	TATGTCCAACAAACAGGTTTC
TRCN0000033257	4	TATTGGTCTCTCATGGCACTG

Figure 1. A melanoma cell line was infected with lentivirus carrying three different shRNA (sh1, sh3, and sh5) against NRAS as described in the text. Transduced cells were selected with puromycin 48h following infection. Puromycin resistant-transduced cells were collected, lysed and analyzed by immunoblotting to determine the efficiency of NRAS knockdown. HSP90 was used as loading control (A). Levels of NRAS and HSP90 were quantified using an Odyssey system. Relative quantification is shown in the table on the right (B).

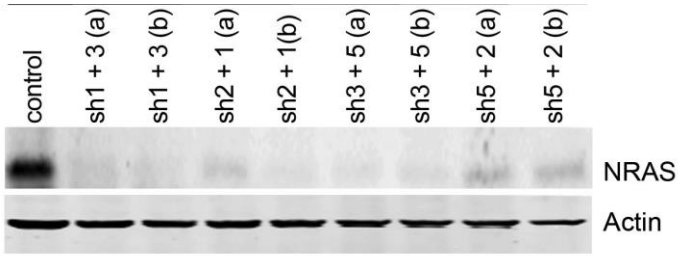
A



B

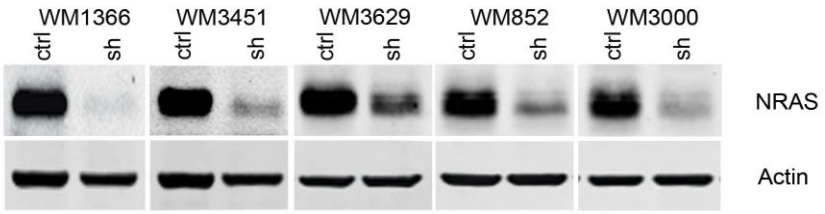
	NRAS shRNA			
	ctrl	sh1	sh3	sh5
NRAS	4.87	4.06	3.72	4.48
Hsp90	115.59	116.29	94.26	93.80
NRAS/Hsp90	4.21	3.49	3.95	4.78
Relative	1.00	0.83	0.94	1.13
% KD		17.13	6.33	-13.36
% NRAS	100	82.8657309	93.671356	113.361733

Figure 2. A melanoma cell line was infected with viruses carrying two different shRNAs either simultaneously (a) or sequentially (b). Transduced cells were selected with puromycin 48h following infection. Cells were collected, lysed and analyzed by immunoblotting to determine the efficiency of NRAS knockdown. Actin was used as loading control. Levels of NRAS and actin were quantified using an Odyssey system. Relative quantification is shown in the table below.



	control	sh1+3 (a)	sh1+3 (b)	sh2+1 (a)	sh2+1 (b)	sh3+5 (a)	sh3+5 (b)	sh5+2 (a)	sh5+2 (b)
NRAS	1.33	0.08	0.11	0.24	0.08	0.08	0.01	0.26	0.34
Actin	121.57	79.21	68.33	79.18	75.39	65.91	66.68	72.39	60.16
NRAS/Actin	1.09	0.10	0.16	0.30	0.11	0.12	0.01	0.36	0.57
Relative [ ]	1.00	0.09	0.15	0.28	0.10	0.11	0.01	0.33	0.52
% KD		90.77	85.29	72.29	90.30	88.91	98.63	67.17	48.34

Figure 3. NRAS mutant melanoma cell lines were infected with lentiviruses carrying two different shRNAs. Transduced cells were selected with puromycin 48h following infection. Cells were collected, lysed and analyzed by immunoblotting to determine the efficiency of NRAS knockdown. Actin was used as loading control. Levels of NRAS and actin were quantified using an Odyssey system. Relative quantification is shown in the table below.



	WM1366		WM3629		WM3451		WM852		WM3060	
	control	sh1+3	control	sh1+3	control	sh1+3	control	sh1+3	control	sh1+3
NRAS	5.49	0.21	36.45	8.04	10.78	0.56	17.05	4.19	15	2.9
Actin	75.1	54.06	81.94	90.16	76.99	57.81	105.8	107.74	70.97	80.87
NRAS/Actin	7.31	0.39	44.48	8.92	14.00	0.01	16.12	3.89	21.14	3.59
Relative	1.00	0.05	1.00	0.20	1.00	0.00	1.00	0.24	1.00	0.17
% of KD		94.69		79.96		99.91		75.87		83.03

Figure 4. The effect of NRAS silencing on NRAS-mutant melanoma cells was assessed on cell growth and viability by MTT assays over six days. Relative viability was calculated relative to the isogenic cell line expressing a control shRNA. Data shown as average +/- SEM (n=7)

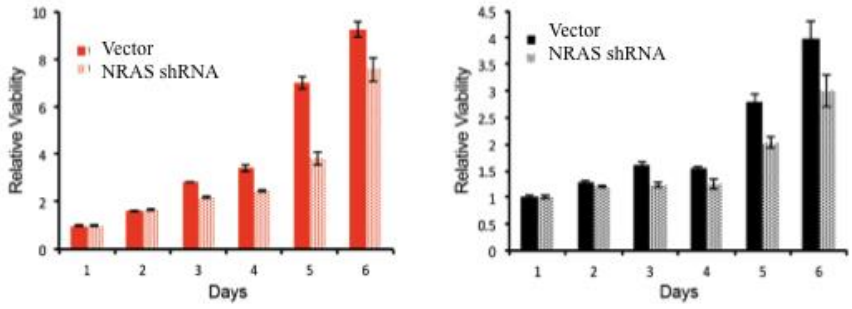


Figure 5. The effect of NRAS silencing on NRAS-mutant melanoma cells was assessed on cell proliferation by BrdU incorporation.

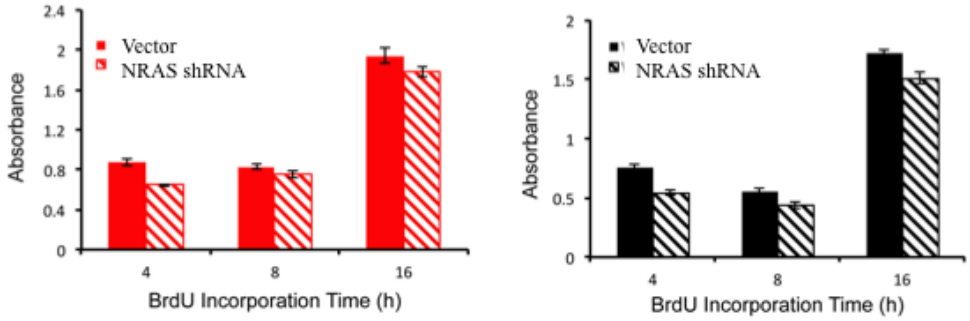


Figure 6. Depletion of NRAS decreases cell proliferation. NRAS mutant melanoma cells were transduced with a non-targeting shRNA vector control or NRAS shRNA. Transduced cells were stained with the proliferative marker Ki67 (green) and DAPI (blue) to visualize the nucleus. More than 80% of the cells transduced with a vector control were Ki67 positive. Depletion of NRAS caused a decrease in the total number of cells; furthermore, NRAS depletion caused a substantial decrease in proliferation evidenced by the loss of Ki67 positive cells.

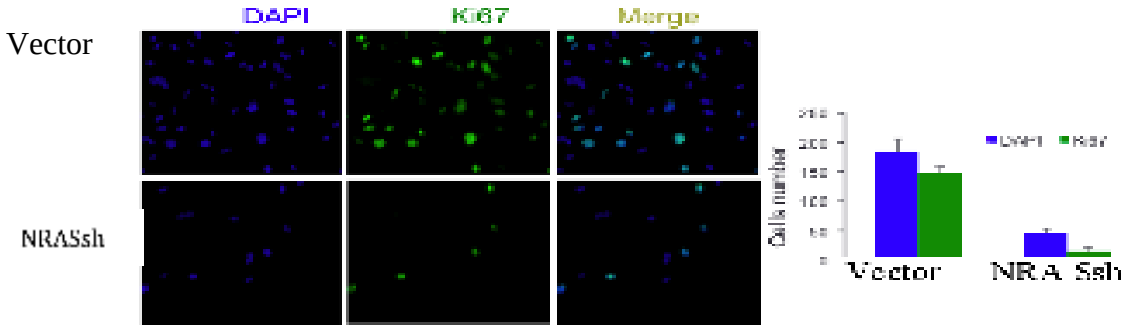


Figure 7. NRAS depletion inhibits proliferation of melanoma cells. $5X10^4$ melanoma cells transduced with either a non-targeting shRNA or a lentiviral vector carrying a NRAS shRNA construct were seeded onto 12-well culture plates in duplicate, collected every 24 hours for five days. Cells were fixed with methanol and stained with crystal violet. Representative images are shown for D0 and D5 (left panels). Relative number of cells was assessed by measuring absorbance at 595 nm. Absorbance was plotted against time, and results of a representative cell line are shown in the graph on the right panel.

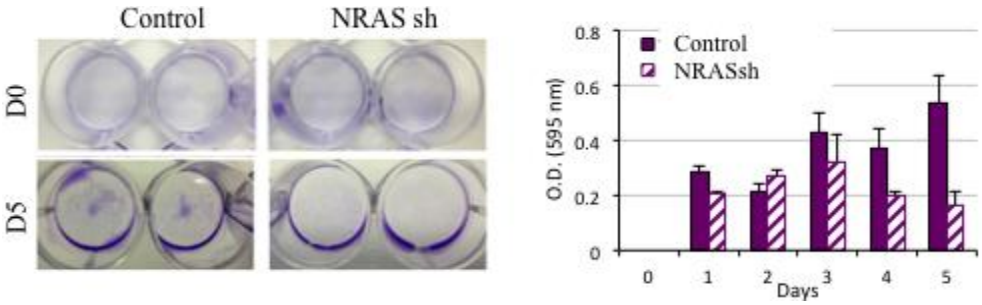


Figure 8. NRAS silencing inhibits MAPK signaling. NRAS mutant melanoma cells were transduced with a non-targeting shRNA or NRAS shRNA. Total cell lysates were analyzed by immunoblotting with the indicated antibodies.

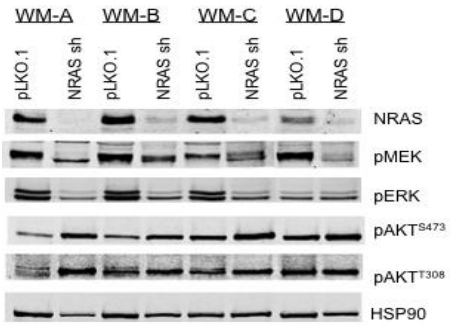


Figure 9. MEK inhibition inhibits proliferation of some NRAS mutant melanoma cells. NRAS mutant melanoma cells were treated with different concentrations (0, 100, or 1000 nM) of the allosteric MEK Inhibitor Trametinib. Total cell lysates were analyzed by immunoblotting for the indicated proteins.

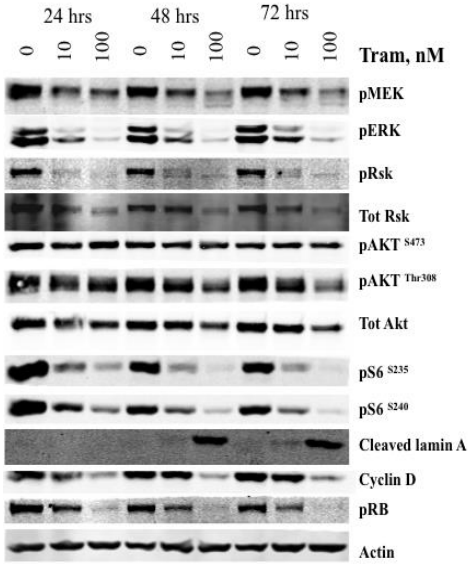
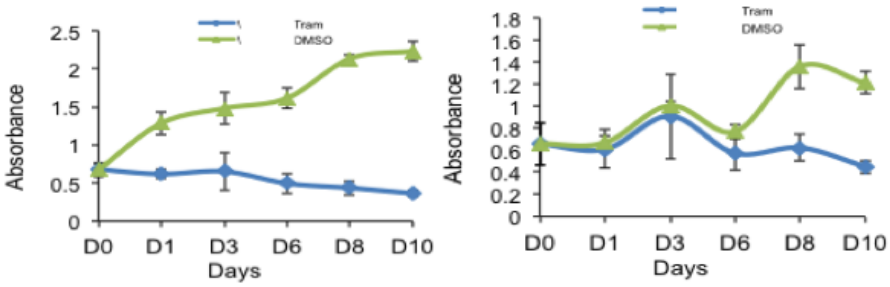


Figure 10. Proliferation of NRAS mutant melanomas. MEK inhibition halts the growth of NRAS mutant melanomas. Two different NRAS mutant cells were treated with 100 nM of Trametinib for different times (0-10 days).



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. None				☐Submitted ☐Accepted ☐Published

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

Yes___X_____ No_____

If yes, please describe your plans:

We will submit a manuscript to a peer-review journal after completion of further studies. The manuscript will describe the biological effects of NRAS depletion, characterization of critical NRAS effectors, and evaluation of targeting candidate effectors on NRAS mutant tumors.

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.

None

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.

None

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.

BIOGRAPHICAL SKETCH

NAME **VILLANUEVA, Jessie**	POSITION TITLE **Assistant Professor**
eRA COMMONS USER NAME (credential, e.g., agency login) **JVILLANUEVA**	

INSTITUTION AND LOCATION	DEGREE	MM/YY	FIELD OF STUDY
Universidad Peruana Cayetano Heredia, Lima, Peru	B.S.	06/94	Biology
University of Miami School of Medicine, Miami, FL	Ph.D.	12/03	Molecular Cell and Developmental Biology

A. PERSONAL STATEMENT

My long-term research goal of is to develop effective therapies for melanoma that result in durable responses. To achieve this objective we are investigating the molecular mechanisms of drug resistance in melanoma. We have demonstrated that BRAF-V600E-mutant melanomas acquire resistance to BRAF inhibitors through reactivation of the MAPK pathway and upregulation of receptor tyrosine kinases (RTKs). Enhanced RTK signaling leads to PI3K activation and increased tumor survival. Targeting simultaneously the MAPK and PI3K pathways using combination therapies can overcome acquired drug resistance. Clinical trials based on these and others' work are planned. We are also investigating sensitivity/resistance to BRAF/MEK/PI3K inhibitors in diverse genetic melanoma subgroups aiming to identify additional mutations or signaling alterations that may modulate response to BRAF/MEK/PI3K inhibitors. Additionally, we have recently identified novel mutations in MEK2 and found that concurrent BRAF amplification and MEK2 mutation confer resistance to the combination on BRAF and MEK inhibitors. A second focus in the laboratory is to investigate the role of oncogenic NRAS in melanoma aiming to identify potential targets for therapy.

My laboratory has extensive expertise in preclinical evaluation of novel therapies and discovery of mechanisms of drug resistance using a wide array of melanoma models, including organotypic tumor and in vivo animal models. This puts us in a unique position to achieve our overall goal of identifying and characterizing novel targets for melanoma therapy

B. 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
2014-present Member, University of Pennsylvania Abramson Cancer
Melanoma and Cutaneous Malignancies Program
2010-2011 Staff Scientist, The Wistar Institute, Philadelphia, PA
2009-2010 Associate Staff Scientist, The Wistar Institute, Philadelphia, PA

Professional Associations:

American Association for Cancer Research (AACR)
The Society for Melanoma Research (SMR)
Institute for Translational Medicine and Therapeutics (ITMAT)

Fellowships and Awards:

2012 V Scholar
2012 AACR Minority Scholar in Cancer Research Award

2004-2005 National Cancer Institute, Research Training Fellowship
1999 Honorable Mention, Eastern Student Research Forum, Miami, FL
1996-1997 Graduate Fellowship, University of Miami School of Medicine

C. SELECTED PEER-REVIEWED PUBLICATIONS (selected from 26 publications)

1. 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. 2013. A modified HSP70 inhibitor shows broad activity as an anticancer agent. Mol Cancer Res 11:219-229. PMCID: PMC3606282.
2. Desai BM*, **Villanueva J***, Nguyen T-T K, Lioni M, Xiao M, Kong J, Krepler C, Vultur A, Flaherty KT, Nathanson KL, Smalley KSM, Herlyn M. *Co-first authors. 2013. The anti-melanoma activity of dinaciclib, a cyclin-dependent kinase inhibitor, is dependent on p53 signaling. PLoS One 8:e59588. PMCID: PMC3601112.
3. 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. 2013. The novel SMAC mimetic Birinapant exhibits potent activity against human melanoma cells. Clin Cancer Res 9:1784-1794. PMCID: PMC3618495.
4. Vultur A, **Villanueva J**, Krepler C, Rajan G, Chen Q, Xiao M, Li L, Gimotty PA, Wilson M, Nathanson KL, Herlyn M. 2013. MEK inhibition affects STAT3 signaling and invasion in human melanoma cell lines. Oncogene Epub ahead of print April 29. PMCID: PMC3769503.
5. 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. 2013. Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B (high) cells. Cancer Cell 23:811-825. PMCID: NIHMS478379.
6. **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. 2013. Concurrent MEK2 mutation and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma. Cell Rep Epub ahead of print Sep 17. pii: S2211-1247(13)00464-6. PMID: 24055054.
7. O'Connell MP, Marchbank K, Webster MR, Valiga AA, Kaur A, Vultur A, Li L, Herlyn M, **Villanueva J**, Liu Q, Yin X, Widura S, Nelson J, Ruiz N, Camilli TC, Indig FE, Flaherty KT, Wargo JA, Frederick DT, Cooper ZA, Nair S, Amaravadi RK, Schuchter LM, Karakousis GC, Xu W, Xu X, Weeraratna AT. Hypoxia induces phenotypic plasticity and therapy resistance in melanoma via the tyrosine kinase receptors ROR1 and ROR2. Cancer Discov. 2013 Oct 8.[Epub ahead of print] (PMID: 24104062)
8. Ma XH, Piao SF, Dey S, McAfee Q, Karakousis G, Villanueva J, Hart LS, Levi S, Hu J, Zhang G, Lazova R,
   Klump V, Pawelek JM, Xu X, Xu W, Schuchter LM, Davies MA, Herlyn M, Winkler J, Koumenis C, Amaravadi RK. Targeting ER stress-induced autophagy overcomes BRAF inhibitor resistance in melanoma. J Clin Invest. 2014 Mar 3;124(3):1406-17. doi: 10.1172/JCI70454. Epub 2014 Feb 24. PMID: 24569374. PMCID: PMC3934165.