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 – 6/30/2014

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

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

5. **Grant SAP Number:** 4100062226

6. **Project Number and Title of Research Project:** 4: Analysis of Markers of Progression and Therapy Resistance in Melanoma

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

8. **Name of Principal Investigator for the Research Project:** Ashani Weeraratna, PhD

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:

   $ 320,114.28

   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
Appleton	Lab Assistant	100%	$2,690
Behera	Postdoctoral Fellow	18%	$3,981
Kaur	Pre-Doctoral Trainee	50%	$1,316
Marchbank	Postdoctoral Fellow	47%	$28,111
O'Connell	Sr. Staff Scientist	69%	$83,044
Weeraratna	Principal Investigator	38%	$102,778

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:

ACS/IRG award to Michael O'Connell: $20,000.

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__X___ No_____

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:
Promotion of tumor invasion and pseudosenescence by the aging microenvironment (R01 CA174746-01A1)	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	June 2013,	$1,000,000 (250,000/yr) Awarded from 4/2014 to 3/2019	$979,000

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:

We will be submitting an RO1 application in February 2015.

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

Our laboratory has shown previously that a switch occurs during melanoma progression, whereby tumors switch from canonical Wnt/beta-catenin signaling, to non-canonical Wnt signaling mediated through Wnt5a. During the course of this project, we have shown that the switch to non-canonical Wnt5a signaling actually causes beta-catenin degradation and inhibition of canonical Wnt signaling. We have also shown that this switch from a canonical to non-canonical Wnt phenotype drives both invasion and therapy resistance.

Our recent data indicate that a switch from canonical to non-canonical Wnt signaling regulates autophagy. Autophagy is a process by which cells can clear degraded proteins, engulfing them via double-membraned vesicles called autophagosomes. Beta-catenin has been shown to suppress autophagy. Consistent with this, in preliminary data for this application, we show that Wnt5A high melanoma cells have higher rates of autophagy than Wnt5A low cells.

By activating the ubiquitin ligase SIAH2, Wnt5A increases ubiquitination in melanoma cells, targeting proteins for degradation. The accumulation of misfolded or degraded proteins can act as a "red flag" to the immune system. Increased autophagy can help to clear these proteins. In melanoma specifically, degraded melanocytic antigens such as MART1 are presented on the surface of melanoma cells and attract cytolytic T-Cells. We have previously shown Wnt5A signals to inhibit the expression and presentation of these antigens. *We hypothesize that, by activating autophagy, Wnt5A will signal to rapidly clear degraded proteins, thereby decreasing the number of peptides presented on the cell surface and decreasing activation of the immune microenvironment.*

Overall, we expect that these experiments will determine how autophagy plays a role in the switch from a radial growth phase melanoma to a vertical growth phase melanoma. We will determine the role of Wnt signaling in guiding the formation of the autophagosome and its implications for the initiation of metastasis, therapy resistance and the immune microenvironment.

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	1			3
Female	4		3	
Unknown				
Total	**5**		**3**	**3**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic	1			
Non-Hispanic	4		3	3
Unknown				
Total	**5**		**3**	**3**

	Undergraduate	Masters	Pre-doc	Post-doc
White	2			2
Black	1		1	
Asian	1		2	1
Other	1			
Unknown				
Total	**5**		**3**	**3**

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

Yes____X_____ No_____

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

Phil Cheng, B.Sc. (Ph.D. candidate), University of Zurich.

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

Yes___X____ No_____

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

This research allowed us to implement and cement partnerships with prestigious collaborators at Harvard, the National Institutes of Health and the University of Pennsylvania. This research gave us insights into how cells may use fates such as senescence to evade therapy, and these data were used to garner funding as part of an RO1 awarded to Dr. Weeraratna as the PI, as well as a multi-million dollar melanoma program project (PO1) which has brought in funds to the Institute to multiple PIs, as well as to help support Core Facilities.

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:

This project resulted in collaborations with the following research partners:
Massachusetts General Hospital/ Harvard/ DFCI - Keith Flaherty, Jennifer Wargo
University of Pennsylvania - Giorgos Karakousis, George Xu, Lynn Schuchter, Ravi Amaravadi
Lehigh Valley Health Networks - Suresh Nair.
National Institute on Aging - Kevin Becker, Luigi Ferucci

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 BRAF gene is mutated in 50% of melanoma patients; and patients treated with BRAF inhibitors (e.g., PLX4720) demonstrate robust clinical responses, at least for a time. Recently, a requirement for β-catenin and the canonical Wnt pathway for the robust response to BRAF inhibitors has been discovered. These data suggest that the Wnt5A pathway, which inhibits canonical Wnt signaling, might render tumor cells resistant to BRAF inhibitors. To address this we have the following specific aims:

Specific Aim 1. *Investigate the role of Wnt5A signaling in resistance to BRAF inhibitors*. Using genetic and pharmacologic approaches, we will manipulate Wnt5a and determine the response to BRAF inhibitors *in vitro*. We have shown that inhibiting ROR2 *in vitro* and *in vivo* will dramatically increase the effects of BRAF inhibitors. We will ask in this aim whether resistance can continue to occur in the absence of Wnt5A/ROR2; and, if so, what mechanisms arise to mediate that resistance.

Specific Aim 2. *Investigate whether Wnt5A induces MAPK signaling via CAMKII.* Our preliminary data indicate calcium/calmodulin-dependent protein kinase II (CAMKII) signaling may play a role in Wnt5A mediated resistance to Vemurafenib. We hypothesize that Wnt5A also contributes to increased or at least sustained mitogen- activated protein kinase (MAPK) signaling, in the presence of BRAF inhibitors, via CAMKII activation. We will ask if MAPK signaling is sustained in resistant cells, and if so if CAMKII is an essential intermediate. We will examine the potential of CAMKII inhibitors as adjuvant therapy to BRAF inhibition.

Overall Progress

As proposed, we made stable cell lines overexpressing the BRAF mutant protein ; however, overexpression of the V600E mutant in the cell lines made the vast majority of the cell lines non-viable, making it impossible to examine the downstream signaling effects (Figure 1). Therefore, we turned our first aim into a more targeted approach of delineating the mechanism of Wnt5A mediated resistance to BRAF inhibitors. These findings emphasize the importance of β -catenin in Wnt5A-mediated resistance to BRAF inhibitors, and also uncovered a novel adaptive stress response initiated by Wnt5A.

Specific Aim 1. Investigate the role of Wnt5A signaling in resistance to BRAF inhibitors.

- We have expanded the clinical correlation of Wnt5A to therapy resistance.
- Demonstrated that β-catenin can predict sensitivity to BRAF inhibitors.
- Demonstrated that Wnt5A drives an adaptive stress response in melanoma cells that allows for resistance to BRAF inhibitors.
- Demonstrated that Wnt5A drives this response through p21.

Specific Aim 2. Investigate whether Wnt5A induces MAPK signaling via CAMKII.

- We have shown that Wnt5A increases MAPK signaling, and that more resistant cells have increased both Wnt5A and PO4-CAMKII.
- We have shown that treatment of resistant cells with a CAMKII inhibitor (KN93) inhibits both Wnt5A signaling and p21 expression.
- We have shown that the inhibition of CAMKII using commercial inhibitors increases the sensitivity of melanoma cells to BRAFi, potentially via the disruption of Wnt5A/ p21

signaling.

Some of this work has been published in the prestigious journal *Cancer Discovery* (Impact Factor 15.9) and some of this work is under consideration at our flagship melanoma journal *Pigment Cell Research* (Impact factor 5.9). Some of this work is also being submitted to the top tier journal, *Cancer Cell* (Impact factor 23.8). Data from this project has served as preliminary data to obtain a graduate student fellowship (Joanna M. Nicolay Foundation), an ACS-IRG award to Michael O'Connell in my laboratory, as well as an RO1 and a PO1. Another RO1 will be submitted based on this data in February 2015.

Detailed Progress:

1. Wnt5A expression correlates with resistance in human tumor samples. We sought to assess whether the expression of Wnt5A could predict patient response to Vemurafenib in a small cohort of patients (n=24). Robust clinical response to Vemurafenib is defined as 30% response or higher. To account for human error in measuring response, we extended this definition of clinical response to include samples of 35% or higher. We were able to identify nine patients with 33% response or lower to Vemurafenib, and 15 patients with 38% or higher response to the drug. Examples of Wnt5A staining in correlation to therapeutic response are shown in Figure 2A. Even in this small cohort of patients, the results were significant: seven out of nine patients who demonstrated less than 33% clinical response to Vemurafenib had positive expression of Wnt5A, from 1-3+ intensity. Only two of the remaining 15 patients (38% response or greater) exhibited any Wnt5A expression (1-2+ intensity) as shown in Figure 2B, giving a statistical significance of p=0.002.

If Wnt5A truly confers clinical resistance to BRAF inhibitors, then we would predict that Wnt5A-positive cells might be selected for patients who relapse (become resistant) while on therapy. To test this, we acquired 12 patient samples that had undergone BRAF inhibitor therapy and for whom we had pre-therapy and post-therapy relapsed lesions. We scored the levels of Wnt5A expression in these samples and found that eight of 12 post-relapse samples had increased Wnt5A positivity compared to pre-therapy lesions (i.e., positivity in a larger percentage of the tumor). Two examples are shown in Figure 3C. In these eight patients, positivity increased from an average of 6% of the tumor cells being positive for Wnt5A pre-therapy, to an average of 52% of the tumor cells being positive post-relapse (p=0.016). In the remaining four patients, one sample increased from 25- 30% positivity (which we did not consider significant and therefore scored this unchanged), one remained the same (5% positivity, pre- and post-), one decreased from 16% positivity to 10% positivity, and one decreased from 5% positivity to no positivity. When considering all 12 tumors together, the overall increase in Wnt5A staining went from 8% positivity pre-therapy to 38% positivity post-relapse, and was significant at p=0.018. Interestingly, in one sample for which we had pre-treatment, on-treatment and post-relapse samples, Wnt5A staining increased from 0 to 80% positivity in the on-treatment sample to 100% positivity (and an increase from 1-2+ intensity) in the post-relapse sample.

2. Wnt5A controls β-catenin expression via SIAH2. Wnt5A regulation of canonical Wnt signaling occurs predominantly via upregulation of Siah2, resulting in a GSK3-β-independent

degradation of β-catenin. Additionally, Siah2 is upregulated in metastatic melanomas, so we hypothesized that Siah2 upregulation may be downstream of Wnt5A in melanomas, just as it is during development.

We have previously shown that Wnt5A overexpression in proliferative phenotype melanoma cells can cause a decrease or dysregulation of β-catenin expression in those cells. Further, β-catenin mRNA expression is downregulated in highly invasive compared to poorly invasive melanoma cell lines, and β -catenin expression can better predict patient outcome. rWnt5A treatment of Wnt5A low UACC1273 cells resulted in a decrease of β -catenin expression (Figure 4A). We then knocked down Siah2 in poorly invasive melanoma cells (Figure 4B,C) and showed that in the presence of Siah2 siRNA, β -catenin levels begin to accumulate (Figure 4D). In the absence of Siah2, Wnt5A can no longer affect β -catenin expression or localization (Figure 4D).

3. β -catenin predicts for sensitivity to BRAF inhibitors. We and others have previously demonstrated that melanoma cells undergo a phenotypic switch from a highly proliferative state to a less proliferative, but highly invasive, state. We have shown that this is guided by changes in Wnt signaling pathways, where signaling switches from a β -catenin mediated pathway to a Wnt5A mediated pathway, which degrades β -catenin and promotes metastasis. We have shown that Wnt5A-mediated degradation of β-catenin not only guides metastasis but also promotes resistance to novel targeted therapies for melanoma, such as the BRAF inhibitor Vemurafenib. This was one of the first demonstrations that the processes of metastasis and therapy resistance are mechanistically linked. We find that β-catenin low cell lines demonstrate a sensitivity to PLX4720 with an IC50 of around 100-300nM, whereas β-catenin high cells have much higher IC50s ranging from 2 μm to well over 10μm. We demonstrate here that in a panel of BRAF inhibitor-sensitive and resistant cell lines (Figure 4A), β- catenin expression is elevated in sensitive cell lines. Further, in a panel of patients, those with good response to BRAF inhibitors (Vemurafenib) had higher levels of β-catenin (Figure 4B,C). Our patient data indicate that the vast majority of β-catenin positivity we see is cytoplasmic, and the reasons for the differences observed in relation to altered cellular localization remain unclear.

4. Wnt5A drives an adaptive stress response to PLX4720 in melanoma. We have previously shown that Wnt5A drives invasion in melanoma. We have also shown that Wnt5A promotes resistance to therapy designed to target the $BRAF^{V600E}$ mutation in melanoma. Here, we show that melanomas characterized by high levels of Wnt5A respond to therapeutic stress by increasing p21 and expressing classical markers of senescence, including positivity for senescence-associated β-galactosidase (SA-β-gal), senescence associated heterochromatic foci (SAHF), H3K9Me chromatin marks, and PML bodies. We find that despite this, these cells retain their ability to migrate and invade. Further, despite the expression of classic markers of senescence like SA-β-gal and SAHF, these Wnt5A-high cells are able to colonize the lungs in *in vivo* tail-vein colony forming assays. This clearly underscores the fact that these markers do not indicate true senescence in these cells, but instead an adaptive stress response that allows the cells to evade therapy and invade**.** Notably, silencing Wnt5A reduces expression of these markers and decreases invasiveness. The combined data point to Wnt5A as a master regulator of an adaptive stress response in melanoma, which may contribute to

therapy resistance. We first sought to determine whether Wnt5A-high cells demonstrated increased senescence in the presence of therapeutic stress. Wnt5A-high cells were treated with 5μM PLX4720 for five days and show a significant increase in SA-β-galactosidase staining (Figure 5A), as compared to Wnt5A low cells. They also show an increase in other markers of senescence, including PML bodies (Figure 5B,C).

To address the possibility that Vemurafenib-treated cells might also be senescent-like but capable of invasion, we treated BRAF-mutant Wnt5A-high and Wnt5A-low cells with the BRAF inhibitor PLX4720 and analyzed their therapeutic and metastatic responses. As is the case with ionizing radiation, many of the "senescent-like" Wnt5A-high cells, but not the Wnt5A-low cells, retain significant invasive ability after five days of PLX4720 (Figure 5D), and this invasiveness increases with increased doses of PLX4720. To ensure that it was the senescent-like cells that were migrating, we treated Wnt5A high and low cells for five days with 5μM of PLX4720. Then, cells were treated with C_{12}FDG, a fluorescent marker for SA-β-galactosidase activity. Time lapse imaging confirmed that Wnt5A high, but not low, cells treated with PLX4720 increase their levels of SA-β-galactosidase as determined by C_{12}FDG, and that these "senescent" cells continue to migrate into the scratch wound (Figure 5E). These combined data indicate that Wnt5A-high cells retain the ability to migrate and invade, even in the presence of markers of senescence.

5. Wnt5A drives this adaptive stress response via p21, which can be ameliorated by CAMKII inhibition. P21 has been shown to drive senescence, so we tested the ability of targeted therapy to increase p21 in Wnt5A high cells. Wnt5A high cells endogenously express more p21 than Wnt5A low cells (Figure 6A). Treatment of Wnt5A low cells with recombinant Wnt5A increases p21 (Figure 6B) and treatment of Wnt5A high cells with siRNA against Wnt5A decreases p21 (Figure 6C). Since PKC has been shown to activate p21, and Wnt5A works though the activation of PKC, we asked whether inhibiting PKC could inhibit p21 expression. Cells were pretreated with the PKC inhibitor GO6983, and then exposed to Wnt5A. In the presence of the PKC inhibitor, Wnt5A could not increase p21 expression (Figure 6D). Increases in expression of Wnt5A and p21 are observed after 5 days of treatment with 1 μM PLX4720 in Wnt5A high cells (Figure 6E), but not in Wnt5A low cells (Figure 6F).

Our previous studies indicated that samples from patients with a poor clinical response to Vemurafenib (less than 30% response by RECIST), or samples from tumors from patients who relapsed after an initial response, expressed high levels of Wnt5A. To determine whether p21 was highly expressed in relapsed samples or samples with a low response rate, we stained nine samples from patients who exhibited a RECIST response of less than 30%, or which were samples of recurrent, relapsed tumors. Of these, seven exhibited high levels of p21 staining. Of four samples with a strong response to Vemurafenib (greater than 30% response), two were negative for p21, one had moderate positivity, and one had weak positivity for p21. Even in this small sample set, this was significant to $p=0.03$ using a two-tailed t-test. Shown as a comparison are samples with >30% response rate, which show little p21 expression as compared to ones with a <30% response rate (Figure 6E).

Next, we examined resistant subclones of melanoma cells that had been exposed to PLX4720.

These subclones demonstrate the ability to sustain MAPK signaling even when treated with PLX4720 (Figure 7A). Since Wnt5A can signal to sustain MAPK activity, we examined the resistant subclones for Wnt5A expression. We find that BRAF-resistant subclones increase their Wnt5A and p21 expression (Figure 7B). These same clones increased their PO4-CAMKII expression (Figure 7C) consistent with our hypothesis that Wnt5A signals to sustain MAPK signaling via CAMKII. Treating Wnt5A high resistant cells with an inhibitor of CAMKII (KN93) decreases Wnt5A and p21 (Figure 7D). We then treated cells with KN93, and asked whether inhibiting these signals increased sensitivity to PLX4720. We find that KN93 does not affect cell viability on its own (Figure 7E), but pre-treatment with KN93 sensitizes cells to PLX4720, decreasing the IC50 almost ten-fold (Figure 7F). These data indicate that the inhibition of CAMKII may be a worthwhile pursuit for increasing the efficiency of targeted therapy.

Internal Collaborators:
Meenhard Herlyn, D.V.M., D.Sc.
Maureen Murphy, Ph.D.
Dario Altieri, M.D.
David Speicher, Ph.D.
Rugang Zhang, Ph.D.
Jessie Villanueva, Ph.D.
Imaging Facility
Flow Facility
Animal Facility

External Collaborators:
Massachusetts General Hospital/ Harvard/ DFCI: Keith Flaherty, Jennifer Wargo
University of Pennsylvania: Giorgos Karakousis, George Xu. Lynn Schuchter, Ravi Amaravadi
Lehigh Valley Health Networks: Suresh Nair.
National Institute on Aging: Kevin Becker, Luigi Ferucci

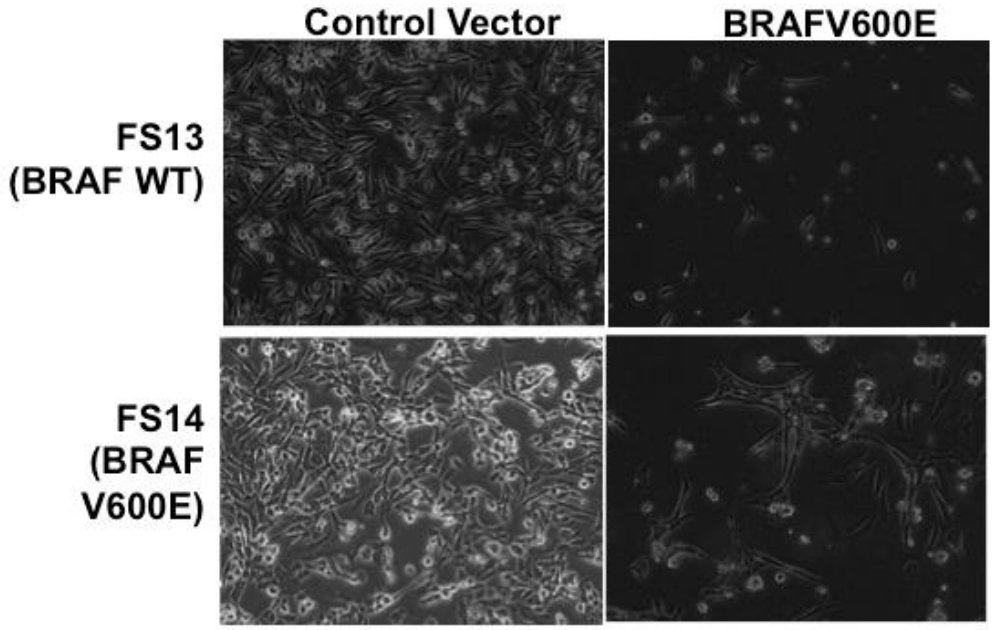

Figure 1. Transfection of BRAFV600E into melanoma cells results in cell death. Two melanoma cell lines, one wild type for BRAF (FS13) , and one already containing BRAFV600E were infected with either a control lentivirus, or one with the BRAFV600E gene. In both cell lines, V600E introduction was detrimental to the health of the cells.

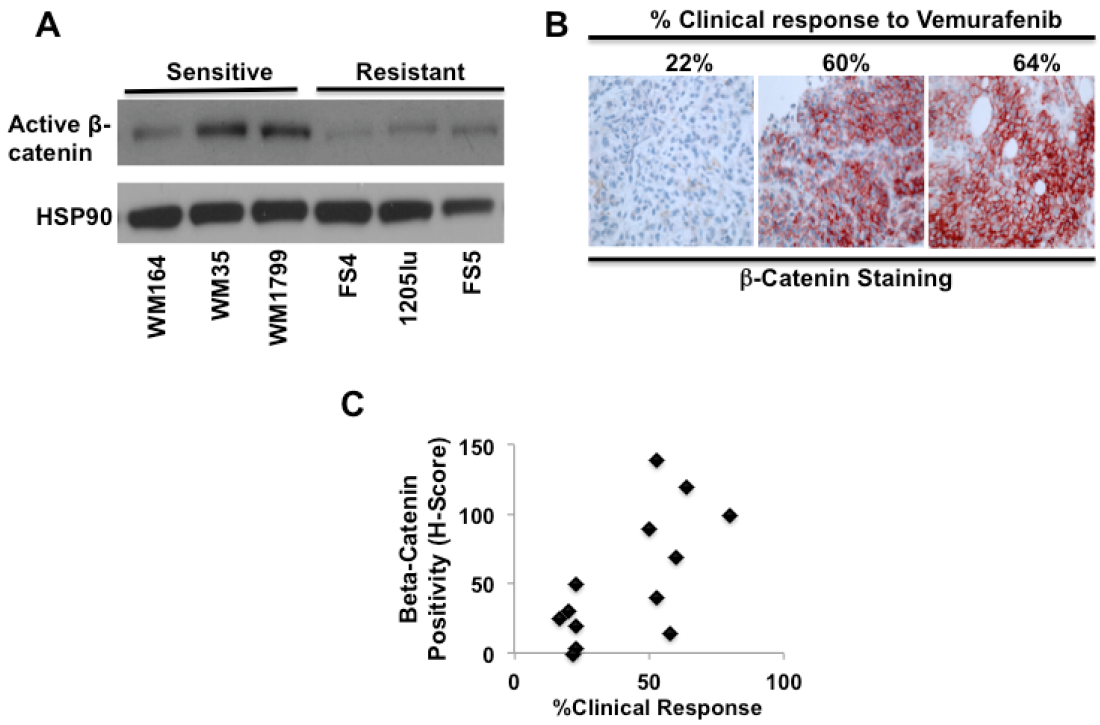

Figure 2. Beta-catenin predicts for sensitivity to BRAF inhibitors. In a panel of BRAF inhibitor-sensitive and resistant cell lines, β- catenin expression is elevated in sensitive cell lines (A). Further, in a panel of patients, those with good response to BRAF inhibitors (Vemurafenib) had higher levels of β-catenin (B,C).

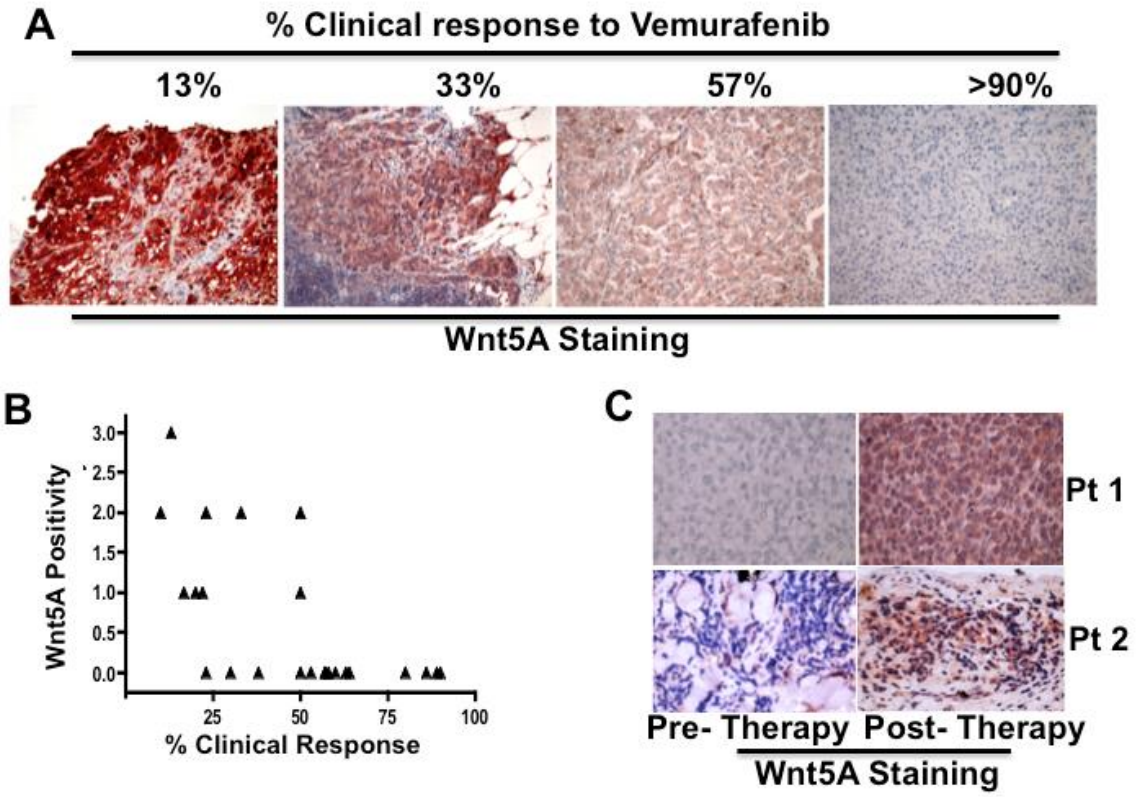

Figure 3. Wnt5A is highly expressed in Vemurafenib resistant cells. Wnt5A is expressed more highly in patients who do not respond to Vemurafenib (less that 30% response by RECIST criteria) as measured by IHC (A) of 23 patients (B). Patients that initially respond, and then relapse also express increased Wnt5A (C).

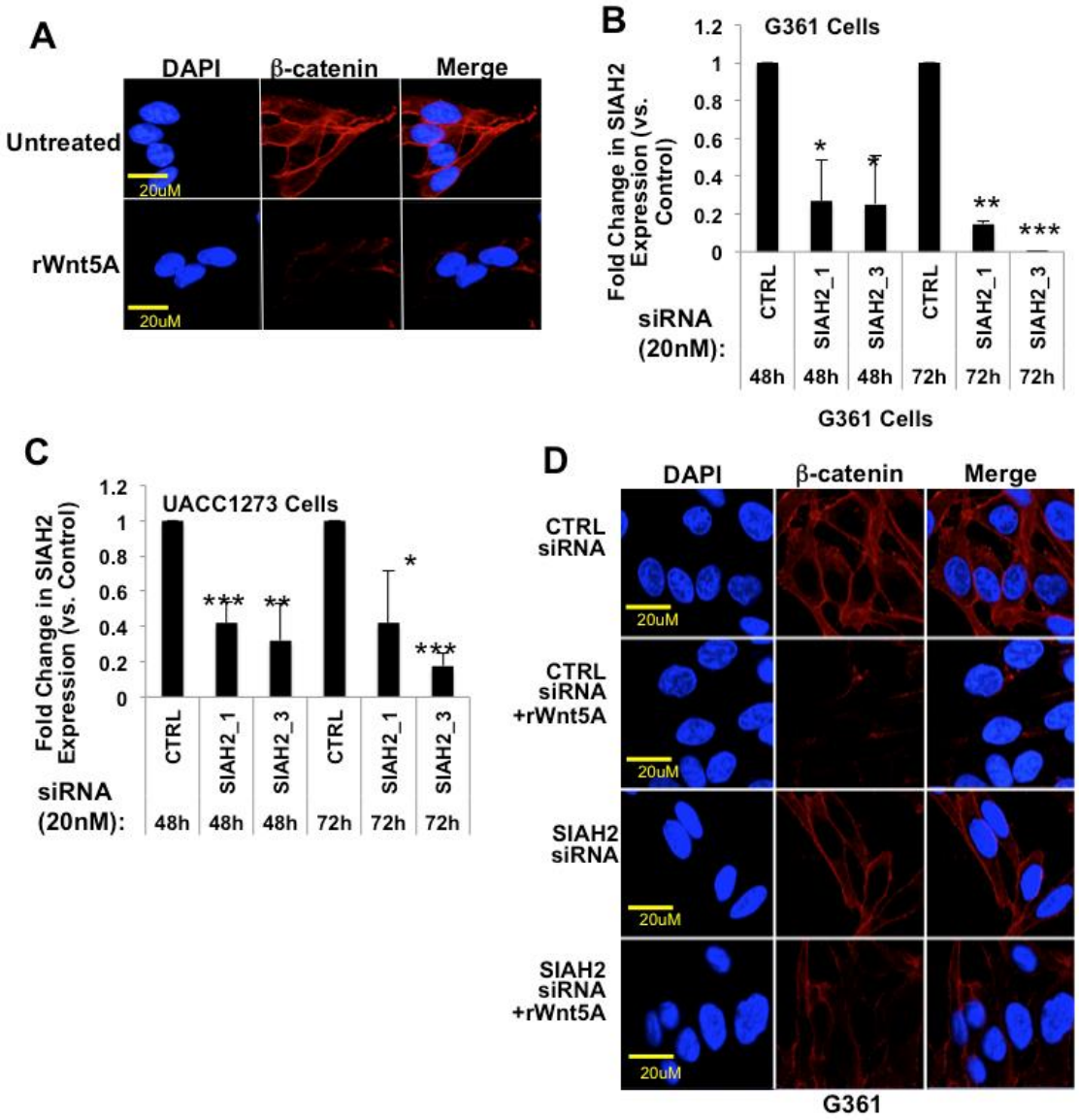

Figure 4. Wnt5A regulation of β—catenin is dependent on SIAH2. Expression of β-catenin in untreated and rWnt5A treated G361 melanoma cells visualized by immunofluorescence (A). Knockdown of SIAH2 in two melanoma cells lines (B,C). Knockdown of SIAH2 prevents Wnt5A degradation of β-catenin (D).

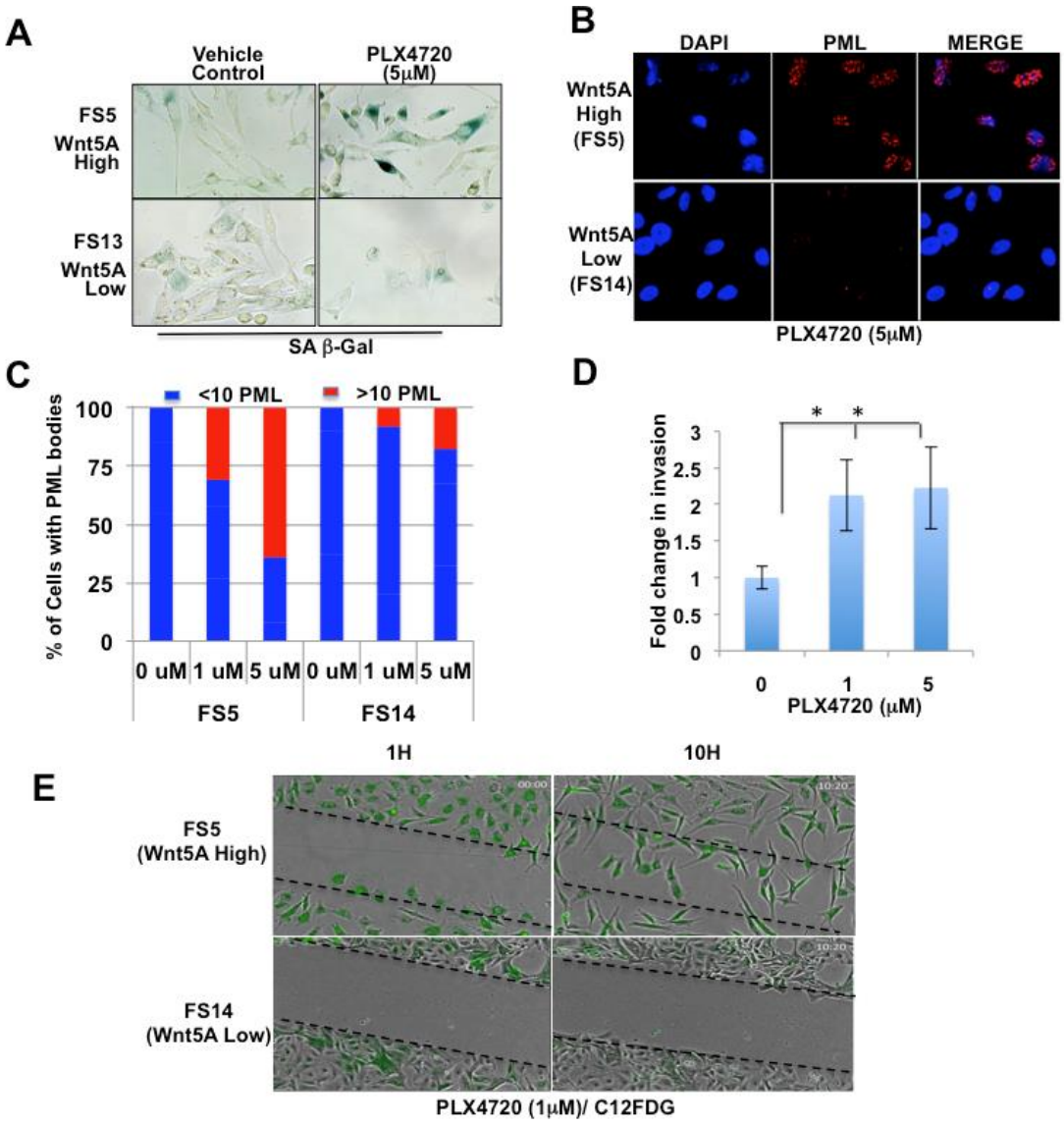

Figure 5. Wnt5A drives a senescent-like stress response in melanoma. Wnt5A-high cells were positive for SA-β-galactosidase whereas Wnt5A-low cells were largely dead at 5 days post-treatment with PLX4720 (A). Immunofluorescence analysis of PML bodies following 5 days of treatment with PLX4720 (B). Quantification of changes in PML bodies in response to PLX4720 treatment (C). Wnt5A high cells remained invasive following treatment with PLX4720 for five days (D). Time-lapse imaging of PLX4720 treated Wnt5A high and low cells labeled with the fluorescent marker of SA-β-galactosidase, C_{12}FDG, and subjected to a wound-healing assay (E).

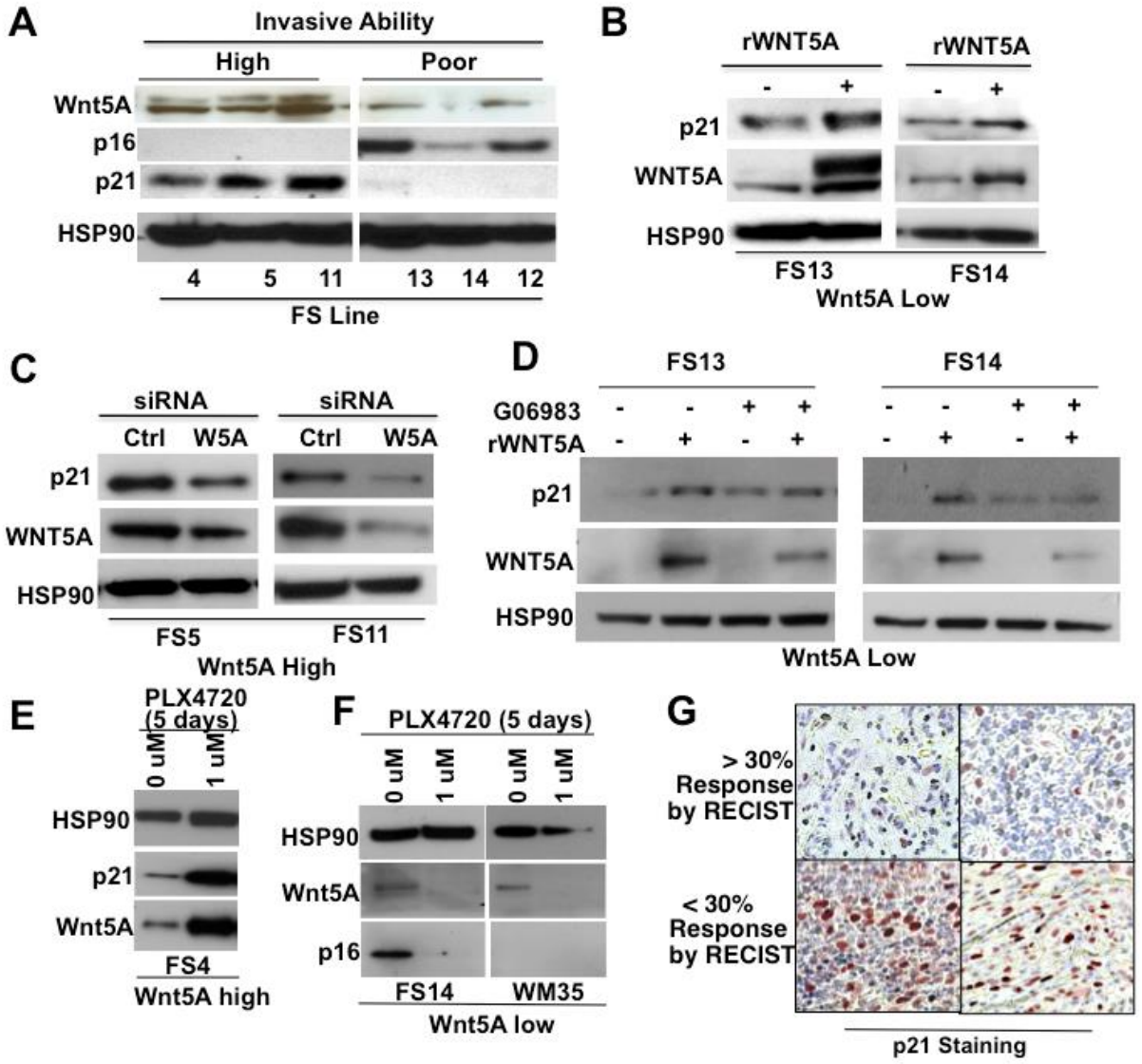

Figure 6. Wnt5A regulates p21 expression in melanoma. (A) Western analysis of cell cycle regulators, p16 and p21 in Wnt5A-high and Wnt5A-low cells. (B) Treatment of Wnt5A-low cells with rWnt5A increases p21 expression and (C) knockdown of Wnt5A in Wnt5A-high cells decreases p21 expression by Western analysis. (D) Inhibition of PKC using GO6983 inhibits the ability of rWnt5A to increase p21. p21 expression remains high in (E). Wnt5A-high melanoma cells following 5 days of treatment with PLX4720, but not in (F) Wnt5A-low melanoma cells. Patient samples with > 30% and <30% response by RECIST were stained for p21 expression by IHC (G).

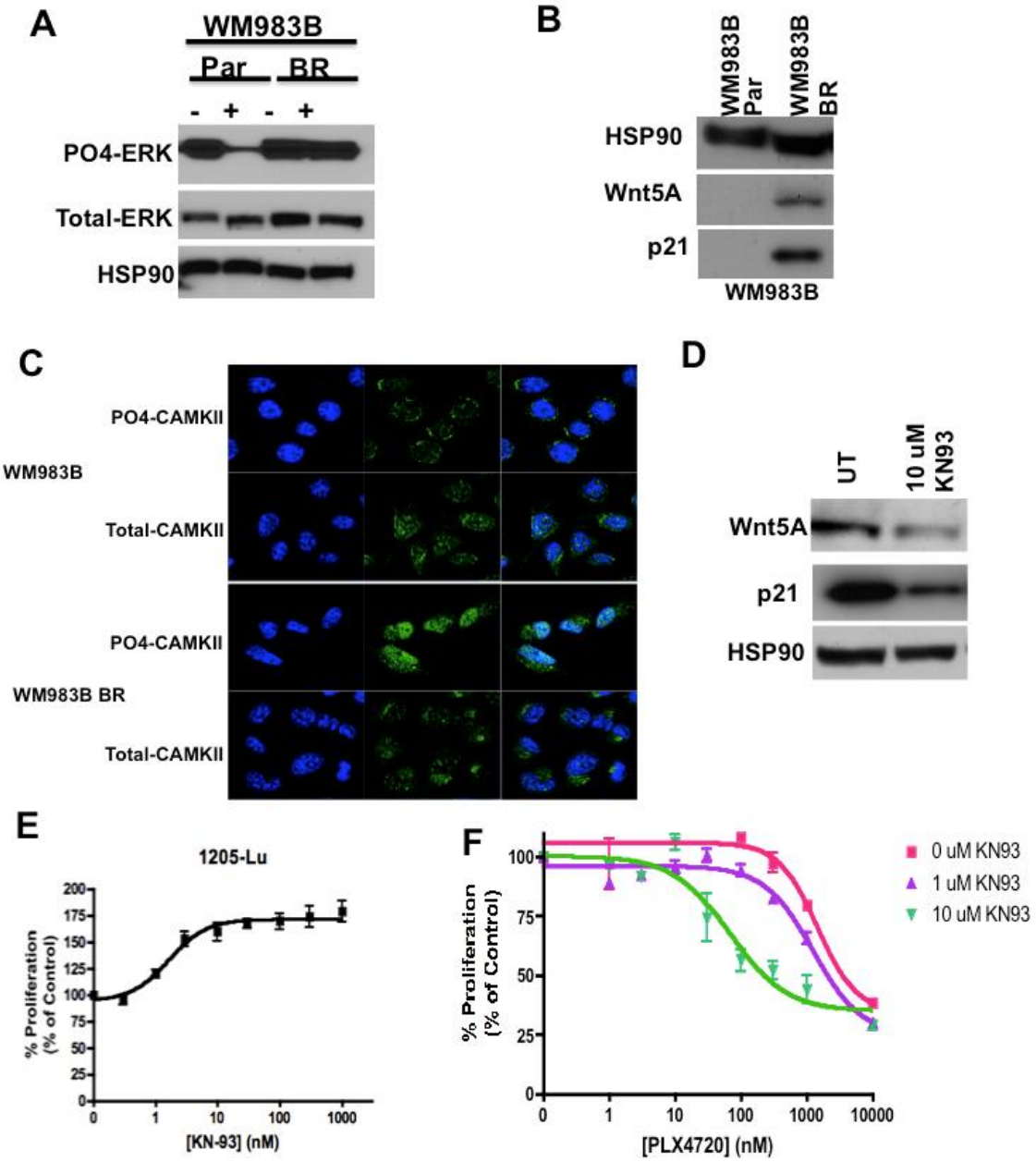

Figure 7. Inhibition of CAMKII inhibits Wnt5A mediated resistance. Resistant subclones sustain MAPK activity even in the presence of inhibitor (A). BRAF resistant subclones increase their Wnt5A and p21 expression (B). These same clones increased their PO4-CAMKII expression (C) Treating Wnt5A high, resistant cells with an inhibitor of CAMKII (KN93) decreases Wnt5A and p21 (D). KN93 treatment alone does not inhibit cell viability (E), but pre-treatment with KN93 sensitizes cells to PLX4720 (F).

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):
Wnt5A promotes an adaptive, senescent-like stress response, while continuing to drive invasion in melanoma cells	Marie R. Webster, Mai Xu, Kathryn A. Kinzler, Jessica Appleton, Amanpreet Kaur, Michael P. O'Connell, Katie Marchbank, Alexander Valiga, Vanessa M. Dang, Michela Perego, Gao Zhang, Ana Slipicevic, Frederick Keeney, Elin Lehrmann, William Wood III, Andrew V. Kossenkov, Flahery KT, Frederick DT, Kevin G. Becker, Meenhard Herlyn, Xu,X, Maureen E. Murphy and Ashani T. Weeraratna.	Pigment Cell and Melanoma Research	August 2014	☒ 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 have two manuscripts that will be submitted to *Cancer Cell* -- one that delineates

Wnt5A control of MAPK, and another that involves the beta-catenin mediated sensitivity to BRAF inhibitors.

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.

The link between the mechanisms that underlie tumor metastasis and therapy resistance is not well defined; however, it is clear that tumors that are more aggressive are likely to be more resistant to therapy. Yet, predicting which tumor is likely to metastasize faster when comparing stage IV tumors is challenging. Our data identify the Wnt5A signaling pathway as a critical mediator of therapy resistance. Thus, the use of Wnt5A as a prognostic marker for both time of progression-free survival as well as therapy response may be of great clinical utility. Data generated throughout the course of this grant indicate that Wnt5A mediates resistance via re-wiring signaling pathways to re-activate MAPK signaling (suppression of beta-catenin, activation of CAMKII). Other data from our laboratory further implicate Wnt5A in resistance to other forms of therapy, and this is mediated by Wnt5A control of ß -catenin. This is supported by a recent study that implicates β-catenin in resistance not only to BRAF inhibitors, but also to mitogen-activated protein kinase (MEK) inhibitors. It should be noted that increases in Wnt5A expression observed post-therapy were observed in melanoma patients who received both BRAF inhibitors and extracellular signal-regulated kinase (ERK) inhibitors as well. Thus, targeting the Wnt5A/ROR2 pathway may not only increase initial sensitivity to BRAF inhibition, but may also provide a useful mechanism for targeting relapsing tumors.

In addition, Wnt5A induces a stress-induced senescent-like response. We show that Wnt5A drives a senescent-like response in melanoma cells exposed to diverse forms of stress. Despite the presence of canonical markers of senescence, these cells retain invasive capacity, and are able colonize distant metastatic sites. These data reinforce the premise that the presence of uniform markers of senescence, like SA-β-gal, chromatin marks, PML

bodies, SAHF and a growth arrest, do not mean the cell is truly senescent. Our data underscore the fact that a better understanding of senescence is needed before we can promote the clinical utility of driving a cell to senescence as a therapeutic endpoint. This has implications not only for cancer biology, but also for aging, revealing the fact that the experimental markers of senescence upon which we currently rely do not always indicate a truly senescent state. Ultimately, driving cells to apoptosis remains the key path to success in the treatment of cancer.

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

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME **WEERARATNA, Ashani T.**	POSITION TITLE **Assistant Professor**		
eRA COMMONS USER NAME **WEERARATNA**			
INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
St. Mary's College of Maryland, St. Mary's City, MD	B.A.	05/91	Biology
George Washington University Medical Center, Washington, DC	M.Phil.	12/97	Molecular and Cellular Oncology
George Washington University Medical Center, Washington, DC	Ph.D.	05/98	Molecular and Cellular Oncology

A. PERSONAL STATEMENT

My laboratory examines links between changes in the tumor microenvironment and melanoma progression. We place a particular emphasis on the Wnt signaling pathway, which comprises a family of proteins that have been shown to have great implications in fetal development as well as cancer. We use a variety of techniques from gene expression profiling to cellular and *in vivo* assays. Over the last decade we have built up both a sound base of technical and observational knowledge, as well as assembled an array of tools to study Wnt signaling in cells of melanocytic origin. Currently we are interested in how the microenvironment guides changes leading to increased metastasis and therapy resistance.

B. POSITIONS AND HONORS

Positions:

1998-2000 Postdoctoral Fellowship, Johns Hopkins Oncology Center, Baltimore, MD
2000-2003 Staff Scientist, National Human Genome Research Institute, NIH, Bethesda, MD
2003-2011 Staff Scientist, Laboratory of Immunology, National Institute on Aging, NIH, Baltimore, MD
2011-Present Assistant Professor, Molecular and Cellular Oncogenesis Program, The Wistar Institute, Philadelphia, PA

C. SELECTED PUBLICATIONS (from a total of 69)

1. Webster MR, Kamat C, Connis N, Zhao M, **Weeraratna AT**, Rudek MA, Hann CL, Freel Meyers CL. 2013. Bisphosphonamidate Clodronate Prodrug Exhibits Selective Cytototxic Activity Against Melanoma Cell Lines. Mol Cancer Ther 2013 Dec 5. Epub ahead of print. PMID: 24310621.
2. 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**. 2013. Hypoxia Induces Phenotypic Plasticity and Therapy Resistance in Melanoma via the Tyrosine Kinase Receptors ROR1 and ROR2. Cancer Discov 3:1378-1393. PMC in Process.
3. O'Connell MP, Fiori JL, Indig FE, Frank BP, Dissanayake SK, French AD, Carter A,

Camilli TC, Earley R, Taub DD, Bernier M, Hewitt SM, **Weeraratna AT**. 2009. The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma. Oncogene 29:34-44. PMCID: PMC2803338.

4. Dissanayake SK, Olkhanud PB, O'Connell MP, Carter A, French AD, Camilli TC, Emeche CD, Hewitt KJ, Rosenthal DT, Leotlela PD, Wade MS, Yang SW, Brant L, Nickoloff BJ, Messina JL, Biragyn A, Hoek KS, Taub DD, Longo DL, Sondak VK, Hewitt SM, **Weeraratna AT**. 2008. Wnt5A regulates expression of tumor associated antigens in melanoma via changes in STAT3 phosphorylation. Cancer Res 68:10205-10214. PMCID: PMC2605679.
5. O'Connell MP, Fiori JL, Baugher KM, Indig FE, French AD, Camilli TC, Frank BP, Hoek KS, Hasskamp J, Elias G, Taub DD, Bernier M, **Weeraratna AT**. 2009. Wnt5A activates the Calpain-mediated cleavage of Filamin A. J Invest Dermatol 129:1782-1789. PMCID: PMC2695838.
6. Dissanayake SK, Wade M, Johnson CE, O'Connell MP, Leotlela PD, French AD, Shah KV, Hewitt KJ, Rosenthal DT, Indig FE, Jiang Y, Nickoloff BJ, Taub DD, Trent JM, Moon RT, Bittner M, **Weeraratna AT**. 2007. The Wnt5A/ PKC pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors, and initiation of an epithelial to mesenchymal transition. J Biol Chem 282:17259-17271. PMCID: PMC2263117.
7. **Weeraratna AT**, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM. 2002. Wnt5a signaling directly affects the motility and invasion of melanoma cells. Cancer Cell 1:279-288. PMID: 12086864.