

AWARD NUMBER: W81XWH-17-1-0501

TITLE: Elucidating Clonal Competition Through Fluorescent Color Coding of Melanoma Cells

PRINCIPAL INVESTIGATOR: Hensin Tsao

CONTRACTING ORGANIZATION: Massachusetts General Hospital  
Boston, MA 02114

REPORT DATE: September 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*  
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

<b>1. REPORT DATE</b> Sept 2018			<b>2. REPORT TYPE</b> ANNUAL		<b>3. DATES COVERED</b> 15 Aug 2017-14 August 2018	
<b>4. TITLE AND SUBTITLE</b> Elucidating Clonal Competition Through Fluorescent Color Coding of Melanoma Cells					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-17-0501	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Hensin Tsao, MD  E-Mail:htsao@mgh.harvard.edu					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Massachusetts General Hospital 55 Fruit Street Boston, MA 02114-2621					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for Public Release; Distribution Unlimited						
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> Tumor evolution fundamentally reflects the expansion and contraction of composite clones. A driving force behind tumor sculpting is intratumoral competition, which facilitates the "stratification" of clones into "winners" and "losers." The <u>purpose</u> of this grant is to understand clonal dynamics as a cornerstone piece of tumor evolution. Our <u>scope</u> is to use fluorescent-labeled cells ("melachroma cells") to observe cellular competition in the context of genetic and epigenetic analysis. In Year 1, we made several important <u>findings</u> . First, we deployed a triple competition experiment and established the presence of a pre-determined "winner" independent of color selection. This suggests that the potential "winner" population may be hard-wired either at a genetic or epigenetic level. Second, we proposed and confirmed that dual oncogene antagonism (i.e. BRAF*+NRAS*) as a potential genetic mechanism for cellular competition; we identified SPRY4 as one mediating pathway the observed antagonism. These findings are currently under review in a manuscript. Finally, using melachroma cells, we determined that there is a reproducible population of cells which is inherently sensitive to MAPK inhibition. Clonal dynamics has been a cornerstone of the tumor evolution theory and our studies to date indicate an intricate interplay between cellular competition and genetic interaction.						
<b>15. SUBJECT TERMS</b> Heterogeneity, cellular competition, oncogene antagonism, melachroma						
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b>	
<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			USAMRMC	
Unclassified	Unclassified	Unclassified	Unclassified	14	<b>19b. TELEPHONE NUMBER</b> (include area code)	

## Table of Contents

	<u>Page</u>
1. Introduction.....	1
2. Keywords.....	1
3. Accomplishments.....	1
4. Impact.....	8
5. Changes/Problems.....	9
6. Products, Inventions, Patent Applications, and/or Licenses .....	9
7. Participants & Other Collaborating Organizations .....	10
8. Special Reporting Requirements .....	11
.....	

1. **INTRODUCTION:** Clonal competition is an inherent process that shapes the heterogeneous landscape of cancer. The major goal of this grant is to visually observe the process of clonal emergence using living fluorescent-coded melanoma cells. The research will encompass the breadth of cell biology, molecular biology and microscopy. Earlier work will focus strictly on cell lines while more advanced work will capitalize the use of animals in tumorigenesis models.
2. **KEYWORDS:** Melanoma, oncogene, drug resistance, subclone, dominance, vital track, emergence
3. **ACCOMPLISHMENTS:**
  - a. What were the major goals of the project?

<b>Specific Aim 1: Understand the hierarchy of clonal stratification</b>	<b>Timeline Target (0-24)</b>
<b>Major Task 1. Determine if there is a “master” organizer</b>	Months
<i>Milestone(s) Achieved: Determine if there is a cell with features of a Master Organizer</i> Subtasks 1, 2 and 3 are currently in progress. Reconstituting the A375melachroma cells for the “drop-by-one” experiment has been more challenging than anticipated given the massive number of subclones. Major Task 1 about 50% complete. Anticipate more data at the end of year 2.	<u>Anticipated target completion date: 24m</u>
<b>Major Task 2. Determine if a single cell can generate a fully stratified population</b>	
<i>Milestone(s) Achieved: Examine the effect of clonal competition in different cell lines</i> Subtasks 1-4 completed. Given the inordinate number of controls, the experiment was performed using 3 distinct clones rather than 15. Also, the intensity of work required us to focus on A375 cells since these cells grow quickly and exhibited much more stable fluorescent colors compared to Meljuso and CHL1 cells. Major Task 2 has been completed and the triple competition experiment findings are outlined below in RESULTS section	<u>Target completion date: 12m</u>
<b>Major Task 3. Observe stratification in vivo</b>	
<i>Milestone(s) Achieved: Determine if clonal specification also occurs in vivo</i> Subtask 1 is complete (protocol under review). Subtasks 2-6 to be completed in Year 2 Major Task 3 is still active.	24
<b>Specific Aim 2: Define the molecular basis of clonal stratification</b>	
<b>Major Task 4. Determine if key melanoma oncogenes confer a “competitive advantage” in vitro</b>	
<i>Milestone(s) Achieved: Determine influence of oncogenic allele on growth and competition</i> Subtasks 1 and 2 complete. Major Task 4 is complete. The paper has currently under review in the journal, ONCOGENE	<u>Target completion date: 12m</u>

<b>Major Task 5. Determine if key melanoma oncogenes confer a growth advantage in vivo</b>	
<i>Milestone(s) Achieved: Determine if oncogene confers growth bias in vivo</i> Subtasks 1-2 in progress. While using Pmel cells in other animal experiments outside of this grant, we noticed that the Pmel immortalized melanocytes exhibited highly erratic tumor penetrance. In order to minimize potentially wasteful animal usage, we will attempt demonstrate competitive growth advantage using 3D systems rather than animals	15
<b>Major Task 6. Identify physiologic pathways that influence clonal dominance</b>	
<i>Milestone(s) Achieved: Establish if there is a unique "winner" or "loser" physiological or molecular profile</i> Subtasks 1-3 are currently underway. Single cell RNA seq (ssRNA-seq) has become the standard for whole transcriptome analysis outside of microarrays. We will explore the use of ssRNA-seq instead of microarrays. We do not anticipate a need to alter the budget. Nevertheless, we have identified one critical gene responsible for "mutual inhibition" (i.e. oncogene antagonism) during competition- the SPRY4. Detailed analyses are listed below and has been incorporated into the ONCOGENE manuscript. Other pathways are currently being analyzed Major Task 6 is about 50% complete.	24
<b>Specific Aim 3: Determine the role of inter-clonal competition in therapeutic resistance</b>	
<b>Major Task 7. Establish the role of resistance mechanisms on clonal competition</b>	
<i>Milestone(s) Achieved: Establish the relative contribution of oncogene vs drug during long term drug selection</i> Subtasks 1-2 and underway. We developed a completely novel "hanging spheroid" assay using fluorescently labeled melanoma cells to observe the emergence of resistance in real time. Major Task 7 is about 50% complete.	24
<b>Major Task 8. Establish the role of resistance mechanisms on clonal competition</b>	
<i>Milestone(s) Achieved: Determine if long-term selection leads to reproducible subclone bias through flow cytometry</i> Subtasks 1-4 have been completed ahead of schedule Major Task 8 is complete. The results suggest that certain subpopulations of cells are uniquely vulnerable to MAPK inhibition and distinct from Adriamycin. See Results	<u>Target completion date: 12m</u>

**b. What was accomplished under these goals?**

**Major Task 1. Determine if there is a "master" organizer**

1) Major activities: Subtasks 1, 2 and 3 are currently in progress. Reconstituting the A375melachroma cells for the "drop-by-one" experiment has been more challenging than anticipated given the massive number of subclones.

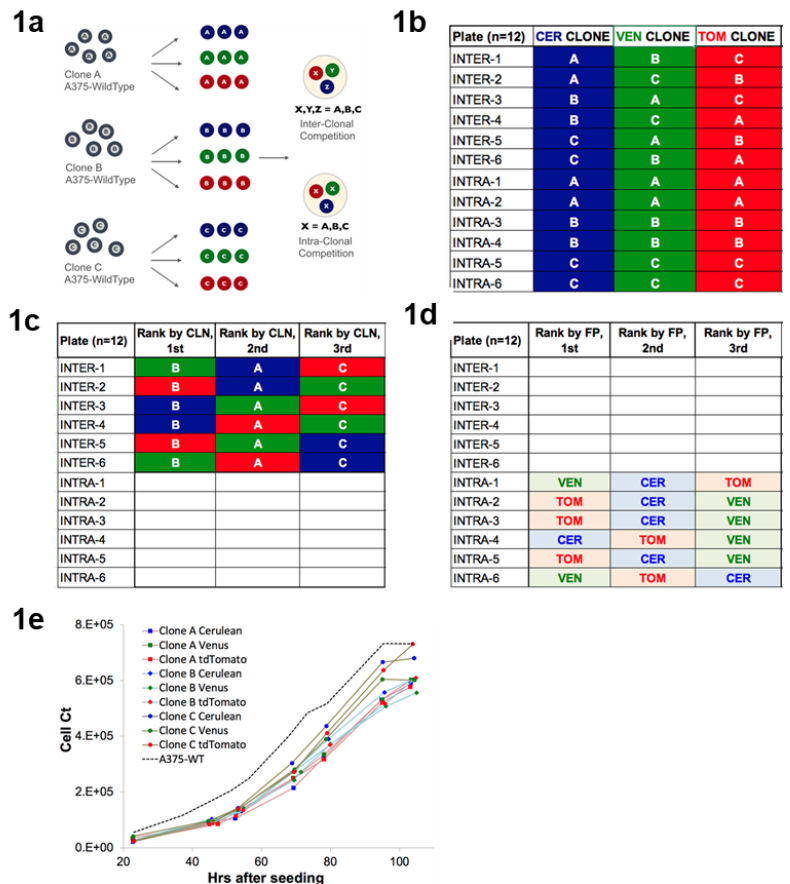
2) Specific objectives and 3) Significant Results: Major Task 1 about 50% complete. Anticipate more data at the end of year 2.

**Major Task 2. Determine if a single cell can generate a fully stratified population.**

1) Major activities: Subtasks 1-4 completed. Given the inordinate number of controls, the experiment was performed using 3 distinct clones rather than 15. Also, the intensity of work required us to focus on A375 cells since these cells grow quickly and exhibited much more stable fluorescent colors compared to Meljuso and CHL1 cells.

2) Specific objectives: To perform a 3-cell, 3-clone, 3-fluorescent protein (FP) “round-robin” interclonal and intracлонаl competition (Figure 1a). In this experiment, we are attempting to determine if a certain subclone always remains the “winner” independent of any influence from fluorescence protein selection.

3) Significant Results: Clonal populations A, B, C were grown from single A375-wt cells. These populations were then transduced with Cerulean (CER; Blue), Venus (VEN; Green) and tdTomato (TOM; Red) respectively, giving rise to 9 different populations. 3-cell interclonal and intracлонаl competitions were then set up using these populations: for interclonal competition, each cell belonged to a different clone (A, B or C), each represented by a different fluorescent protein (FP); for intracлонаl population, each cell was from the same clone. Intracлонаl competitions can reveal a relationship between FP and growth (dis)advantage. Assignment of clones to FP in interclonal (INTER-x) and intracлонаl (INTRA-x) competitions are shown in Figure 1b. Each row had 12 replicates. Each competition took place independently for 12 days. 270 out of 288 competitions yielded sufficient cells for FACS analysis, out of which 256 yielded cell counts > 1E5. The results are highlighted in Figure 1c. Each clone was “ranked” by its frequency in the final population (1st=highest frequency). The ranks were added among the replicates for each row. In the 6 rows of interclonal competitions, presenting all possible clone-FP combinations, Clone B emerged as the most populous clone regardless of the FP it was assigned to (background color), suggesting a growth advantage. Result of intracлонаl competition are shown in Figure 1d. Here, FP, rather than clone, was “ranked” by its frequency. Unlike in Figure 1b, no consistent “winner” emerged in the 6 rows of intracлонаl competition, suggesting that FP is unlikely to confer significant growth advantage. Grow curves of the 9 populations described are shown in figure 1e. Clone B showed no apparent advantage in growth rate compared to Clones A and C, suggesting its growth advantage in the presence of clones A and C was possibly caused by interactions between the clones. *In this experiment, clone B is clearly the “winner” compared to clones A and C regardless of the FP used.*



Major Task 2 has been completed and the triple competition experiment findings suggests that a fully stratified population can result from a single clone B cell.

### Major Task 3. Observe stratification in vivo

1) Major activities: Subtask 1 is complete (protocol under review). Subtasks 2-6 to be completed in Year 2

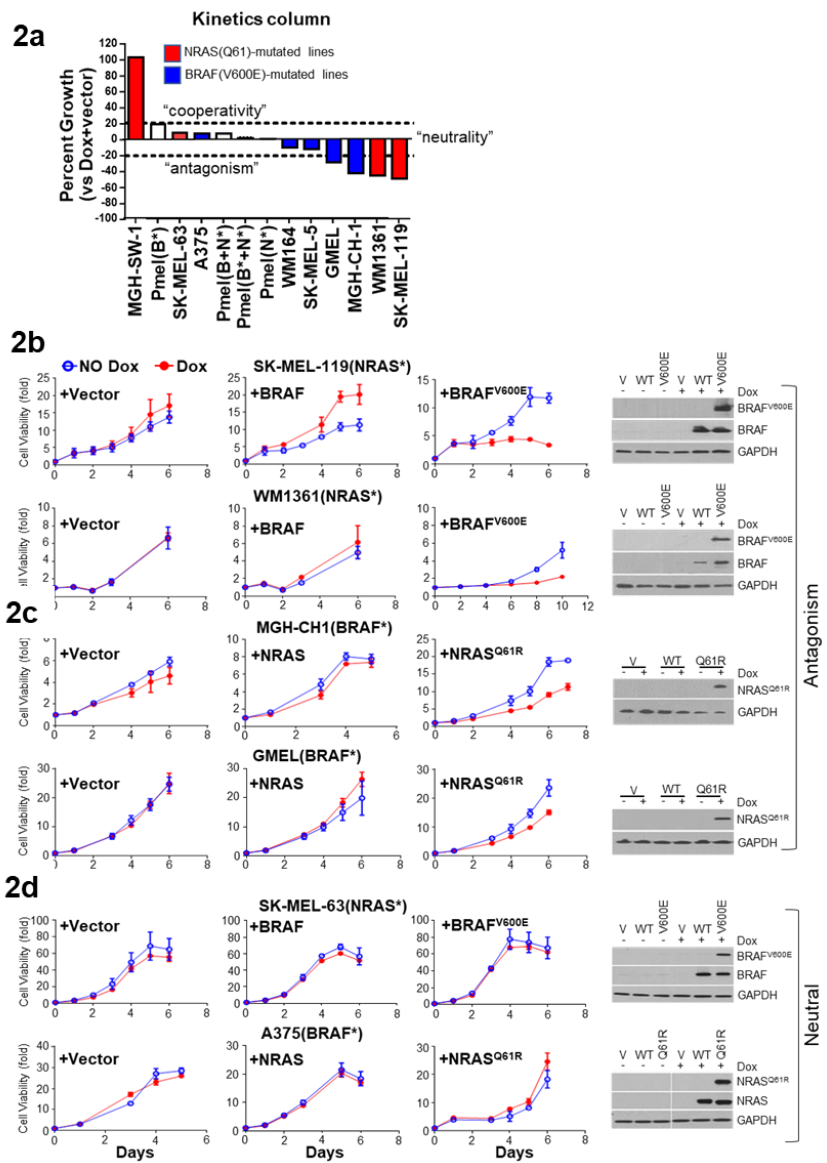
2) Specific objectives and 3) Significant Results: Major Task 3 is still active.

**Major Task 4. Determine if key melanoma oncogenes confer a “competitive advantage” in vitro.**

**1) Major activities:** For Tasks 4 and 6, we studied “competition” using a framework of oncogene exclusion. Within tumors, mutational patterns reflect strong evolutionary forces shaped by clonal competition. While recurrent oncogenic lesions in BRAF (pV600) and N/K/HRAS (pG12/13, p.Q61) predominate within the RAS-MAPK pathway in melanoma, they are rarely identified in conjunction within any single tumor specimen. The biological pressures which govern the emergence and patterning of these activating alleles have not been well characterized. *A priori*, redundancy and antagonism, through growth arrest, apoptosis, senescence or other means, are both possible explanations. Under a redundancy model, the second oncogenic hit would have minimal functional impact and thus exist as a low probability “passenger” oncogene. Alternatively, under a “competition” framework, an additional activating allele would functionally interfere with tumor growth and thus drop out of the final tumor population.

**2) Specific objectives:** Along these lines, we more deeply characterized the mechanism(s) which proscribe the concurrence of BRAF(pV600E) and NRAS(pQ61) mutations in melanoma competitive advantages or disadvantages associated with individual mutations.

**3) Significant results:** We first set out to establish the broader context of competition by examining the impact of dual oncogenes in native NRAS<sup>Q61</sup> and BRAF<sup>V600E</sup> lines. To avoid unwarranted negative selection during the introduction of the “rival” oncogene (i.e. NRAS(Q61) for BRAF(V600E) melanoma lines and BRAF(V600E) for NRAS(Q61) melanoma lines), we used a Tet-On system to synchronize expression of the second allele in a panel of 4 isogenic stable NRAS<sup>Q61R/K</sup> + doxycycline induced Tet-On-BRAF<sup>V600E</sup> lines (designated as “NRAS\*+iBRAF\*”) and 5 BRAF<sup>V600E</sup> + doxycycline induced Tet-On-NRAS<sup>Q61R</sup> lines (designated as “BRAF\*+iNRAS\*”) (Fig. 2a) along with an immortalized primary human melanocyte line (Pmel). The “rival” oncogene was induced with doxycycline (50-100ng/ml) and subjected for 6-day cell viability assays. Using an arbitrary definition of +/-20% above vector for “cooperativity” and “antagonism”, 1 of the 4 (red bars) NRAS\*+iBRAF\* lines exhibited significant cooperativity in growth (MGH-SW-1<sup>NRAS\*</sup>: +102.5%) while the other two demonstrated significant antagonism (SK-MEL-119<sup>NRAS\*</sup>: -49.4% and

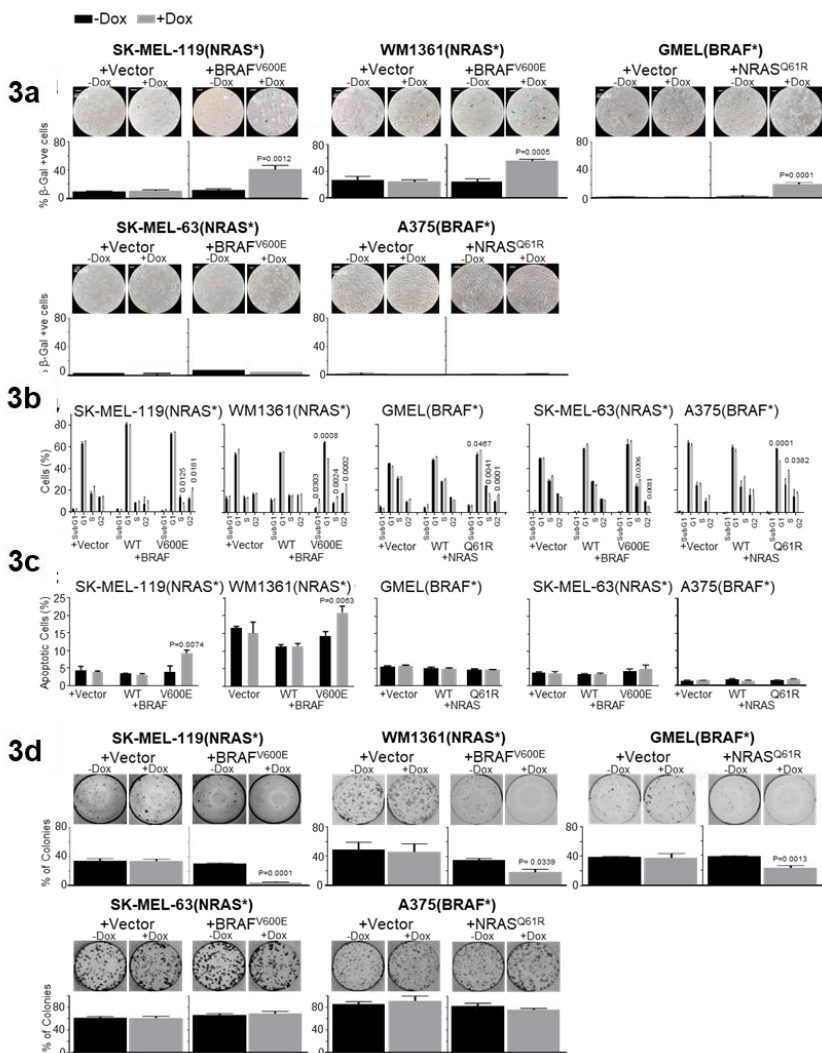


WM1361<sup>NRAS\*</sup>: -45.8%). Among (blue bars) BRAF\*+iNRAS\* lines, interactions were neutral except for GMEL<sup>BRAF\*</sup> and MGH-CH-1<sup>BRAF\*</sup>, which exhibited growth decrements of -29.1% and -42.6%, respectively, with the induction of the exogenous NRAS\* mutation. In the Pmel line (an immortalized melanocyte line with WT BRAF and WT NRAS), we observed better growth with iBRAF<sup>V600E</sup> and combined iBRAF<sup>WT</sup>+iNRAS<sup>Q61R</sup> than with either iBRAF<sup>V600E</sup>+iNRAS<sup>Q61R</sup> or iNRAS<sup>Q61R</sup> alone; all the lines, however, increased <20%. Morphological changes and fluorescent protein expression were validated by fluorescence microscopy and protein expression was confirmed by western blotting at 6<sup>th</sup> day of cell viability. These results indicate that co-expression of BRAF\* and NRAS\* can, at least in a subset of lines, lead to growth arrest and perhaps contribute to the clinical observation of oncogene exclusion.

To isolate the effects of the mutation from the general increases in BRAF or NRAS protein levels, the two most suppressed NRAS<sup>Q61R</sup> lines (SK-MEL-119<sup>NRAS\*</sup> and WM1361<sup>NRAS\*</sup>), two most suppressed BRAF<sup>V600E</sup> cell lines (MGH-CH-1<sup>BRAF\*</sup> and GMEL<sup>BRAF\*</sup>) and two “neutral” BRAF<sup>V600E</sup> and NRAS<sup>Q61R</sup> cell lines (A375<sup>BRAF\*</sup> and SK-MEL-63<sup>NRAS\*</sup>) were selected for further analysis. As shown in Figure 1b, ectopic BRAF<sup>V600E</sup> expression in SK-MEL-119<sup>NRAS\*</sup> and WM1361<sup>NRAS\*</sup> both demonstrated significant growth suppression. Interestingly, forced expression of wild-type BRAF, especially in SK-MEL-119<sup>NRAS\*</sup>, enhanced growth, which is consistent with NRAS<sup>Q61R</sup>'s upstream disposition. Similarly, NRAS<sup>Q61R</sup> induction in MGH-CH-1<sup>BRAF\*</sup> and GMEL<sup>BRAF\*</sup> (Fig. 1c) both confirmed significant growth suppression though ectopic wild type NRAS expression did not appear to alter growth kinetics significantly in these BRAF<sup>V600E</sup> cells.

As expected, the iBRAF<sup>V600E</sup> and iNRAS<sup>Q61R</sup> alleles had no effect on the SK-MEL-63<sup>NRAS\*</sup> and A375<sup>BRAF\*</sup> cell lines, respectively (Fig. 1d). Induced expression of BRAF<sup>V600E</sup> mutant protein in SK-MEL-119<sup>NRAS\*</sup>, WM1361<sup>NRAS\*</sup>, SK-MEL-63<sup>NRAS\*</sup> cells and of NRAS<sup>Q61R</sup> mutant protein in MGH-CH-1<sup>BRAF\*</sup>, GMEL<sup>BRAF\*</sup> and A375<sup>BRAF\*</sup> cells were all confirmed by western blotting at 6<sup>th</sup> day of cell viability (Fig. 2b-d). Figure S3 shows the reduction in cellular density and morphologic changes associated with “rival” oncogene overexpression in antagonistic lines but not in neutral cell lines at day 5.

We next examined the cellular response to oncogene competition. Forced expression of the “rival” oncogene in SK-MEL-119<sup>NRAS\*</sup>, WM1361<sup>NRAS\*</sup> and GMEL<sup>BRAF\*</sup> cells led to steep increases in the percentage of SA-β-gal positive cells (Fig. 3a upper panel). This senescence response was notably absent in the neutral SK-MEL-63<sup>NRAS\*</sup> and A375<sup>BRAF\*</sup> lines (Fig. 3a lower panel). In cell cycle analyses, secondary oncogene induction led to cell cycle arrest at different phases such as G2/M arrest in both SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* (1.5-fold, p<0.01) and GMEL<sup>BRAF\*</sup>+iNRAS\* (1.63-fold, p<0.01)



lines and G2/M arrest in the WM1361<sup>NRAS\*</sup> (1.44-fold, p<0.01) line; there was no significant evidence of arrest in the SK-MEL-63<sup>NRAS\*</sup>+iBRAF\* or A375<sup>BRAF\*</sup>+iNRAS\* cells (Fig. 2b). Though there were no significant changes in subG1 populations, FACS analysis with Annexin-V staining revealed significant increases in the percentage of apoptotic cells in two NRAS<sup>Q61R</sup> lines (SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* and WM1361<sup>NRAS\*</sup>+iBRAF\*) but not in the vulnerable GMEL<sup>BRAF\*</sup>+iNRAS\* line or the neutral SK-MEL-63<sup>NRAS\*</sup>+iBRAF\* and A375<sup>BRAF\*</sup>+iNRAS\* lines (Fig. 3c). Dual oncogene expression also reduced long-term self-renewal capacity (2 weeks) as introduction of the “rival” oncogene suppressed colony formation in the sensitive SK-MEL-119<sup>NRAS\*</sup>+iBRAF\*, WM1361<sup>NRAS\*</sup>+iBRAF\* and GMEL<sup>BRAF\*</sup>+iNRAS\* lines but not the neutral SK-MEL-63<sup>NRAS\*</sup>+iBRAF\* and A375<sup>BRAF\*</sup>+iNRAS\* lines (Fig. 3d). These data suggest that cellular “fitness” in vitro may result from competing signaling cassettes on a genetic level. Biologically, these inputs appear to lead to eventual senescence (“secondary OIS”) may all contribute in part to the observed oncogene exclusion. It also suggests a diverse but possibly coordinated effort to halt cellular expansion. *The results shown suggest that “oncogene competition” may in fact dictate the “competitive edge” and underpin the molecular basis of clonal stratification.*

### Major Task 5. Determine if key melanoma oncogenes confer a growth advantage in vivo

1) Major activities: Subtasks 1-2 in progress.

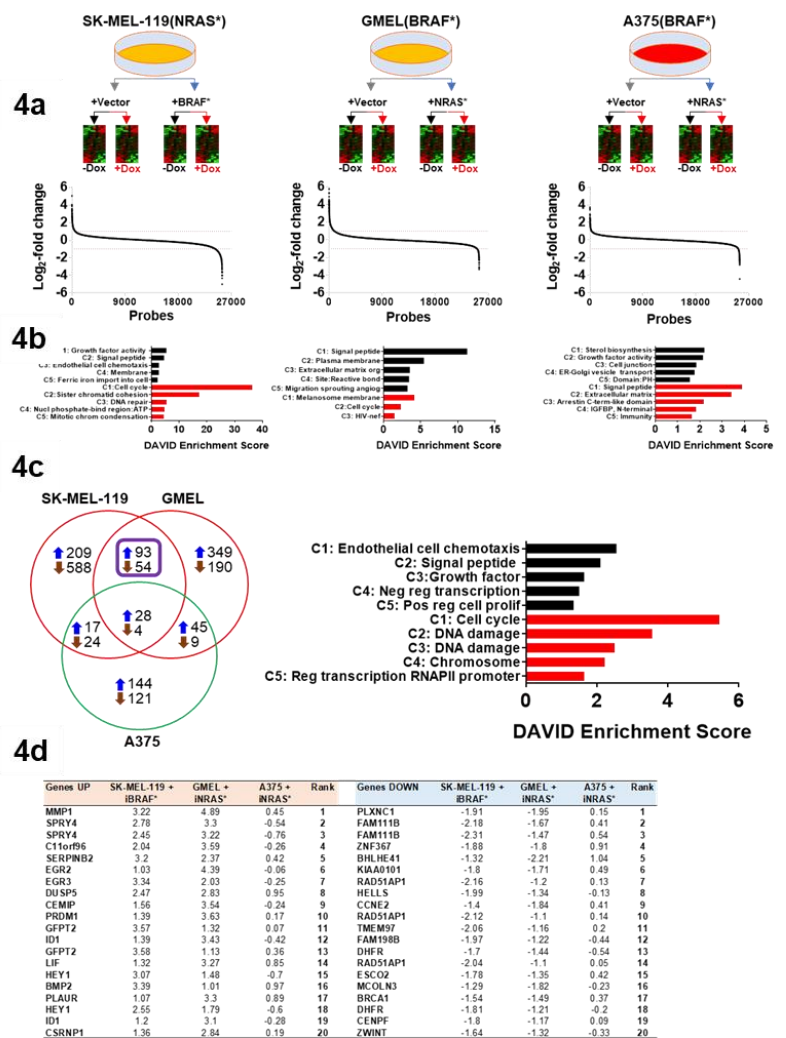
2) Specific objectives and 3) Significant Results: While using Pmel cells in other animal experiments outside of this grant, we noticed that the Pmel immortalized melanocytes exhibited highly erratic tumor penetrance. In order to minimize potentially wasteful animal usage, we will attempt demonstrate competitive growth advantage using 3D systems rather than animals

### Major Task 6. Identify physiologic pathways that influence clonal dominance

1) Major activities: In Major Task 6, we looked for pathways which might suppress growth during competition. We have identified SPRY4 as a mediator and the synthesis of these results are currently under review.

2) Specific objectives: To determine if certain pathways are activated or suppressed during oncogene competition.

3) Significant results: To identify genes involved in mediating oncogene competition, we performed a comparative genome-wide expression (GEX) analysis using a design outlined in Fig 4a. Since the dual oncogenesis occurs in isogenically-matched lines, we first examined expression changes in paired analyses. In the SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* line, 2.20% and 4.43% of the gene probes were upregulated (i.e. increased 2-fold; >1.0 log<sub>2</sub>-fold) and downregulated (i.e. decreased 2-fold; <-1.0 log<sub>2</sub>-fold), respectively, upon induction of exogenous BRAF(V600E). These were 3.44% and 1.39% up-and downregulated, respectively, for GMEL<sup>BRAF\*</sup>+iNRAS\* and 1.24% and 0.89% up-and downregulated, respectively, for



A375<sup>BRAF\*</sup>+iNRAS\*. With BRAF\* overexpression in SK-MEL-119<sup>NRAS\*</sup>+iBRAF\*, the most induced genes were IL1B (5.04 log<sub>2</sub>-fold), MMP1 (4.04 log<sub>2</sub>-fold), IL1A (3.93 log<sub>2</sub>-fold), IL24 (3.84 log<sub>2</sub>-fold) and GFPT2 (3.58 and 3.57 log<sub>2</sub>-fold) while the most suppressed genes were in CXL12 (-4.99, -3.66 and -3.62 log<sub>2</sub>-fold), MGP (-4.31 log<sub>2</sub>-fold), CHRNA1 (-3.95 and -3.58 log<sub>2</sub>-fold), PLPPR4 (-3.7 log<sub>2</sub>-fold) and TRIM22 (-3.55 log<sub>2</sub>-fold). With the introduction of NRAS\* in GMEL<sup>BRAF\*</sup>+iNRAS\*, the most upregulated genes were STC1 (5.75 log<sub>2</sub>-fold), CXCL8 (5.3 log<sub>2</sub>-fold), MMP1 (4.89 log<sub>2</sub>-fold), IGFBP3 (4.55 log<sub>2</sub>-fold) and MB (4.43 log<sub>2</sub>-fold) and the most downregulated genes were TRIM63 (-3.34 log<sub>2</sub>-fold), PHACTRI (-3.18 log<sub>2</sub>-fold), MLANA (-3.07 log<sub>2</sub>-fold), TYRP1 (-2.82 log<sub>2</sub>-fold) and GAGE genes (-2.61 log<sub>2</sub>-fold). In contrast, for the neutral A375<sup>BRAF\*</sup>+iNRAS\* line, GDF15 (3.75, 3.58, 3.45 log<sub>2</sub>-fold), PTPRR (2.95 log<sub>2</sub>-fold), UCA1 (2.86 log<sub>2</sub>-fold), STC1 (2.83 log<sub>2</sub>-fold) and SLC14A1 (2.61 log<sub>2</sub>-fold) exhibited the greatest increase while MGP (-4.4 log<sub>2</sub>-fold), ITGA9 (-2.81 log<sub>2</sub>-fold), SERPINF1 (-2.66 log<sub>2</sub>-fold), A2M (-2.58 log<sub>2</sub>-fold) and ENPP2 (-2.54, -2.48 log<sub>2</sub>-fold) exhibited the most profound decrease in expression levels.

We next subjected the set of all genes that were increased or decreased by at least 2-fold to functional clustering using DAVID (Fig. 4b). Among upregulated genes (i.e. >2-fold), the "SIGNAL PEPTIDE" functional cluster was the highest and second highest annotated cluster in GMEL<sup>BRAF\*</sup>+iNRAS\* (Enrichment score, ES:11.26) and SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* (ES:4.62), respectively. Interestingly, the "SIGNAL PEPTIDE" cluster was the leading annotated set among the most suppressed genes for A375<sup>BRAF\*</sup> (ES: 3.91). The "CELL CYCLE" functional cluster ranked first and second in enrichment, among the set of most suppressed genes (i.e. >2-fold), in the SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* (ES: 36.26) and GMEL<sup>BRAF\*</sup>+iNRAS\* lines (ES: 2.32), respectively; the "CELL CYCLE" cluster were not significantly enriched in the A375<sup>BRAF\*</sup>+iNRAS\* line. One notable cluster is "MELANOSOME MEMBRANE", which was derived from the set of most downregulated genes (i.e. >2-fold) in GMEL<sup>BRAF\*</sup>+iNRAS\* (ES:4.18). To replicate this finding, we used a published list of high impact MITF target genes<sup>8</sup>, MITF targets, as a group, were significantly more suppressed than non-MITF targets; log<sub>2</sub>-fold -0.56±0.03 vs -0.0034±0.0032, P<0.0001, Student T test) in GMEL<sup>BRAF\*</sup> cell lines. In addition, MITF suppression was verified by qPCR in the GMEL<sup>BRAF\*</sup>+iNRAS\* cell lines. Thus, lineage programming appears to be attenuated in the GMEL<sup>BRAF\*</sup>+iNRAS\* line with ectopic NRAS\* expression. Lastly, in only the A375<sup>BRAF\*</sup>+iNRAS\* cells, overexpression of NRAS\* appears to correlate with a functional cluster related to reprogramming of lipid metabolism ("STEROL BIOSYNTHESIS", ES:2.2).

We next sought to identify a shared suppressive physiology by focusing on statistically significant regulated genes in both SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* and GMEL<sup>BRAF\*</sup>+iNRAS\* lines but not the A375<sup>BRAF\*</sup>+iNRAS\* line. As shown in Fig 4c, there were 93 upregulated transcripts (i.e. >2-fold increase) shared between SK-MEL-119(NRAS\*+BRAF\*) and GMEL<sup>BRAF\*</sup>+iNRAS\* upon induction of the rival oncogene while there were 54 downregulated (i.e. >2-fold decrease) transcripts shared by these two antagonized lines. For the set of altered genes shared by the two lines, the "CELL CYCLE" functional cluster showed the greatest enrichment among the most downregulated genes (i.e. >2-fold decrease; ES: 5.46) followed by two DNA damage clusters (ES:3.57 and ES: 2.51). Among the most upregulated genes (i.e. >2-fold increase) shared by SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* and GMEL<sup>BRAF\*</sup>+iNRAS\* but *not* A375<sup>BRAF\*</sup>+iNRAS\*, the "ENDOTHELIAL CELL CHEMOTAXIS" (ES: 2.55) cluster, a "SIGNAL PEPTIDE" (ES: 2.1) cluster and the "GROWTH FACTOR" (ES: 1.64) cluster exhibited the strongest enrichments.

While it is likely that many concurrent pathways have been activated to bring about growth arrest in the SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* and GMEL<sup>BRAF\*</sup>+iNRAS\*, SPRY4 transcripts were among the most upregulated ones in both antagonized SK-MEL-119<sup>NRAS\*</sup>+iBRAF\* and GMEL<sup>BRAF\*</sup>+iNRAS\* lines, but not in the neutral A375<sup>BRAF\*</sup>+iNRAS\* cell line (Fig. 4d Table). *With these results, we provide data for the first time that the SPRY4 pathway may be a crucial physiological determinant of clonal dominance.*

## **Major Task 7. Establish the role of resistance mechanisms on clonal competition**

**1) Major activities:** Subtasks 1-2 and underway.

2) Specific objectives and 3) Significant Results: We developed a completely novel “hanging spheroid” assay using fluorescently labeled melanoma cells to observe the emergence of resistance in real time. This assay is being optimized and we anticipate potentially publishable results in the coming year. Major Task 7 is about 50% complete.

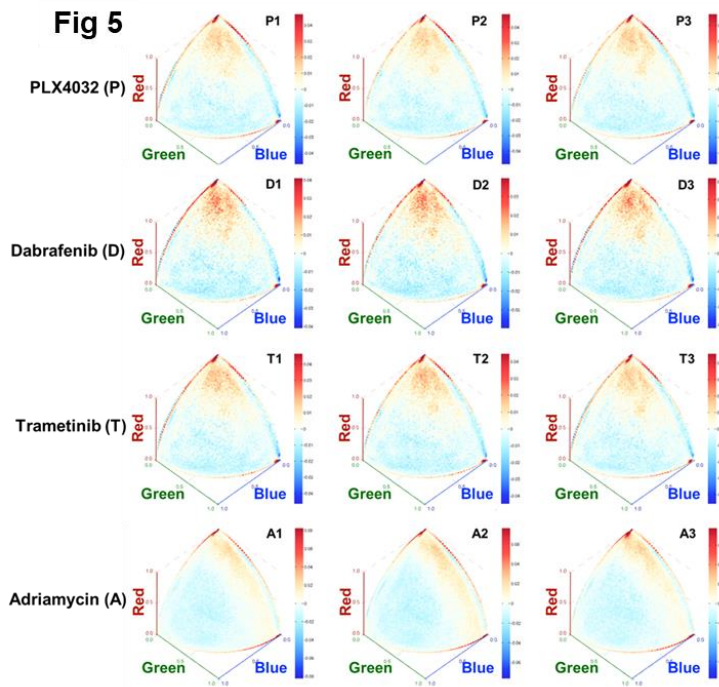
### Major Task 8.

1) Major activities: Subtasks 1-4 have been completed ahead of schedule. Major Task 8 is complete.

2) Specific objectives: To determine if certain subpopulations of cells are uniquely vulnerable to similar classes of inhibitors.

3) Significant Results: In this experiment, A375 MelaChroma cells were subjected to MAPK inhibitors (Plx4032 (P), Dabrafenib(D), Trametinib (T)) and non-MAPK inhibitor (Adriamycin (A)).

Distribution of cell colors are portrayed using the chromaticity grid (Fig 5). Each plot represents the difference in spherical histogram values between the drug-treated and control populations (Treated-Ctrl), in % of total analyzed population. Red represents chromaticity values favored by drug treatment, blue represents those disfavored. >2.4E5 cells were analyzed for each sample, 3 samples for each drug (x1, x2, x3; x=P,D,T,A). Drug dosage was based on IC75: P=2.0uM, D=1.0uM, T=3.2nM, A=0.1uM. Consistent pattern of chromaticity change was observed for each drug. *The results suggest that certain subpopulations of cells are uniquely vulnerable to MAPK inhibition and distinct from Adriamycin.*



### c. What opportunities for training and professional development has the project provided?

Nothing to report. Project not intended to provide training.

### d. How were the results disseminated to communities of interest?

Paper under review at ONCOGENE. Results will be disseminated through publication.

### e. What do you plan to do during the next reporting period to accomplish the goals?

During the next year, we will

1. Obtain IRB and ACURO approval for the animal studies in order to execute the in vivo aims
2. Analyze gene expression data to explore “winner” vs “loser” physiology
3. Optimize a 3D multicolor mixed culture system to observe clonal competition more directly

## 4. IMPACT:

### a. What was the impact on the development of the principal discipline(s) of the project?

The connection between oncogene rivalry and potential clonal competition is a new look on how clonal heterogeneity evolves and how oncogenic mutations become patterned. There was in fact tremendous enthusiasm at ONCOGENE during the initial reviews and we anticipate that our revisions will lead eventually to a publication.

**b. What was the impact on other disciplines?**

Nothing to report outside of cancer biology fields

**c. What was the impact on technology transfer?**

Nothing to report

**d. What was the impact on society beyond science and technology?**

Nothing to report

**5. CHANGES/PROBLEMS:**

**a. Changes in approach and reasons for change**

Nothing to report

**b. Actual or anticipated problems or delays and actions or plans to resolve them**

Cell culturing and pooling took longer to optimize. Delay in animal protocol at MGH. This protocol has now been submitted and is under review.

**c. Changes that had a significant impact on expenditures**

Nothing to report

**d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report

**6. PRODUCTS:**

**a. Journal publications.**

Kumar R, et al...Tsao H. Growth Suppression by Dual BRAF(V600E) and NRAS(Q61) Oncogene Expression is Mediated by SPRY4 in Melanoma, under review; acknowledgement of federal support- YES

**b. Books or other non-periodical, one-time publications.**

Nothing to report

**c. Other publications, conference papers, and presentations.**

**2017** "Rare variant, gene based association study of hereditary melanoma"/Invited Lecturer

"Genetics and Epigenetic Control of Melanoma"/Chair

Triennial International Pigment Cell Conference 2017; Denver, CO

**2017** "Therapeutic Checkmate for melanoma?"/Invited lecture

College of Physicians of Philadelphia, Philadelphia, PA

**2018** "Patient case: melanoma," "High risk hereditary melanoma"/Invited lectures

Orlando Dermatology Aesthetic and Clinical Conference; Miami, FL

**2018** "Top 10 Stories of 2017"/ Invited Lecturer

"Cases from the Pigmented Lesion Clinic"/ Chair and Moderator

Advances in Cosmetic and Medical Dermatology; Maui, HI

**2018** "Journal Watching: Melanoma"/Invited Lecturer

"Update on systemic therapies for melanoma"/Invited Lecturer

"Late Breaking Abstracts 1-4"/ 4-part Forum Director

American Academy of Dermatology; Orlando, FL

**2018** “Update on Melanoma Therapeutics” and “New Insights into High Risk Hereditary Melanoma”/Alfred L. Weiner Memorial Lectures 60th Annual Meeting of the Noah Worcester Dermatological Society, Savannah, GA

**2018** “Therapeutic Checkmate for Melanoma”/ Malkinson Lecturer Chicago Dermatological Society, Chicago, IL

**2018** “Therapeutic checkmate in melanoma”/Keynote speaker; 2018 British Academy of Dermatology meeting; Edinburgh, Scotland

**d. Website(s) or other Internet site(s)**

Nothing to report

**e. Technologies or techniques**

Nothing to report

**f. Inventions, patent applications, and/or licenses**

Nothing to report

**g. Other Products**

Nothing to report

**7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**What individuals have worked on the project?**

Name:	Hensin Tsao
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.20 CM
Contribution to Project:	Dr. Tsao has supervised Dr. Kumar, provided troubleshooting and research design suggestions, helped analyze the data and wrote a significant part of the manuscript
Funding Support:	Air Force Office of Scientific Research, NIH, Melanoma Research Alliance

Name:	Raj Kumar
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 CM
Contribution to Project:	Dr. Kumar performed the experiments at the benchside, acquired the primary data, did the initial analysis of the data, made the first set of

	plans for experimentation, made figures for the manuscript and wrote the first draft of the manuscript.
Funding Support:	Air Force Office of Scientific Research

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

**What other organizations were involved as partners?**

Nothing to report

**8. SPECIAL REPORTING REQUIREMENTS**

Nothing to report