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PRINCIPAL INVESTIGATOR: Hensin Tsao

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114

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**6. AUTHOR(S)**
Hensin Tsao, MD

E-Mail:htsao@mgh.harvard.edu

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Massachusetts General
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55 Fruit Street
Boston, MA 02114-2621

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**14. ABSTRACT**
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 purpose of this grant is to understand clonal dynamics as a cornerstone piece of tumor evolution. Our scope 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 findings. 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.

**15. SUBJECT TERMS**
Heterogeneity, cellular competition, oncogene antagonism, melachroma

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**Page**

1. **INTRODUCTION:** Competition between clones is a selection process that sculpts the composition of cancer. The major goal of this grant is to visually track the process of clonal emergence using living fluorescent-coded melanoma cells. The research encompasses the breadth of cell biology, molecular biology and microscopy. Through our discoveries, we have found that clonal competition may be engendered and engineered by oncogene competition through differential stability.

2. **KEYWORDS:** Melanoma, oncogene, drug resistance, subclone, dominance, vital track, emergence

3. **ACCOMPLISHMENTS:**
   **a. What were the major goals of the project?**

| **Specific Aim 1: Understand the hierarchy of clonal stratification** | **Timeline Target** (0-24) |
|---|---|
| **Major Task 1. Determine if there is a "master" organizer** | Months |
| *Milestone(s) Achieved: Determine if there is a cell with features of a Master Organizer*<br>Major Task 1 completed. | Completed by month 24 |
| **Major Task 2. Determine if a single cell can generate a fully stratified population** | |
| *Milestone(s) Achieved: Examine the effect of clonal competition in different cell lines*<br>Major Task 2 completed | Completed by month 12 |
| **Major Task 3. Observe stratification in vivo** | |
| *Milestone(s) Achieved: Determine if clonal specification also occurs in vivo*<br>Subtasks 1-5 completed. | Completed by month 24 |
| | |
| **Specific Aim 2: Define the molecular basis of clonal stratification** | |
| **Major Task 4. Determine if key melanoma oncogenes confer a "competitive advantage" in vitro** | |
| *Milestone(s) Achieved: Determine influence of oncogenic allele on growth and competition*<br>Major Task 4 completed. | Completed by month 12 |
| **Major Task 5. Determine if key melanoma oncogenes confer a growth advantage in vivo** | |
| *Milestone(s) Achieved: Determine if oncogene confers growth bias in vivo*<br>Major Task 5 completed. | Completed by month 24 |
| **Major Task 6. Identify physiologic pathways that influence clonal dominance** | |
| *Milestone(s) Achieved: Establish if there is a unique "winner" or "loser" physiological or molecular profile*<br>Major Task 6 completed. | Completed by month 24 |
| **Major task NCE#1: To determine whether key melanoma oncogenes have "differential vulnerability" in vitro.** | |

| | |
|---|---|
| *Milestone achieved: determine differential stability of melanoma oncogenes in vitro*<br>This Major Task was initiated during the NCE period and is now complete | Completed by month 36 |
| **Specific Aim 3: Determine the role of inter-clonal competition in therapeutic resistance** | |
| **Major Task 7. Establish the role of resistance mechanisms on clonal competition** | |
| *Milestone(s) Achieved: Establish the relative contribution of oncogene vs drug during long term drug selection*<br>Major Task 7 completed. | Completed by month 24 |
| **Major Task 8. Establish the role of resistance mechanisms on clonal competition** | |
| *Milestone(s) Achieved: Determine if long-term selection leads to reproducible subclone bias through flow cytometry*<br>Major Task 8 completed. | Completed by month 24 |
| **Major task NCE#2: To assess if second oncogene NRASQ61 alters "phenotype" of BRAF mutant cells.** | |
| *Milestone(s) Achieved: Demonstrate that NRAS* mutation modulates the BRAF* dependency*<br>Major Task completed. | Completed by month 36 |

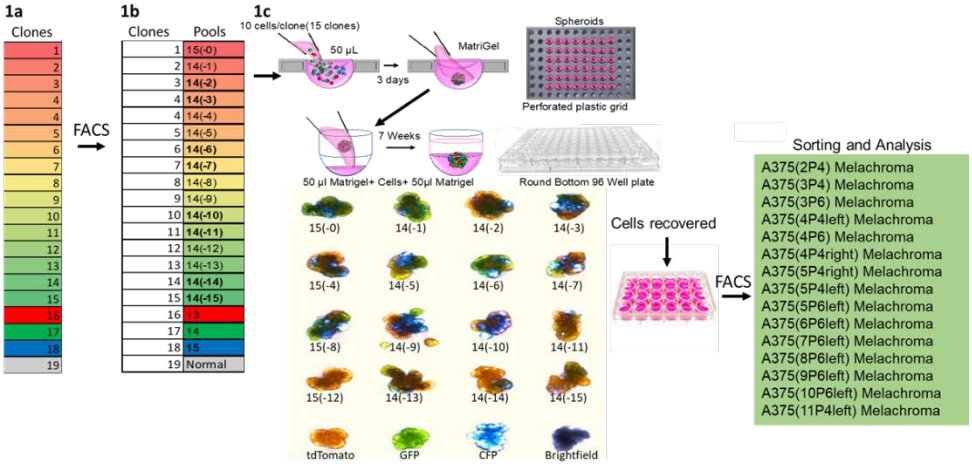
**b. What was accomplished under these goals?**

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

**1) Major activities:** Major Task 1 was completed by end of year 2.

**2) Specific objectives**: The purpose of this task is to investigate whether there are "master" organizers that might be directing the emergence of a dominant and consistent clonal population.

**3) Significant Results**: As shown in Fig 1a, 15 A375$^{melachroma}$ clones were revived and allowed to grow on 2D format for a week. These clones were checked for their fluorescent protein expression with fluorescent microscopy. We created a novel "hanging spheroid" 3D system to image competition in a more faithful system. For inter-clonal competition, the 15 melachroma clones were subjected to fluorescence-activated cell sorting (FACS). The flow cytometer sorts a heterogenous mixture of cell population into single or specific number of one type cells, on the basis of fluorescent characteristics. In drop by one assay, these 15 different A375$^{melachroma}$ clones were pooled in ratio of 1:14 @10 cells/clone of total of 15x10=150 cells omiting one clone out from the inter-clolonal competition(Fig. 1b). The pool of 15 minus one clones were subjected to hanging drop 3D spheroid



melachroma cell culture. The benefit of having the hanging drop culture method is to get non adhearent cell mass called spheroids, is a powerful platform to study tumors, which demonstrate close cancer biology *in-vivo* than traditional 2D culture. In the hanging drop cell culture, 50 µl of 15 or 14 clones @10 cells/clone total 140 to 150 cells per drop were placed or hanged on a grid slot (Fig 1c). The whole grid was placed at 37°C humidified 5%$CO_2$ incubator for 3-5 days and then whole cell mass transferred to round bottom 96 well plate corning 7007 with 50 µl matrigel until the end of the experiment. Images of 3D spheroid culture were taken at regular time intervals by using operetta perkin elmer instrument. The viable cells were recovered using cell recovery solution from spheriods and plated on 24 well plate for growth in DMEM 10% FBS growth medium followed by sorting and analysis. Major Task 1 and the results from spheroid growth and sorting indicate that all cells have the capacity to stratify and compete; in other words, no single drop out led to a depletion of clones. This does suggest that all cells were competent to be in the "winner" pool.

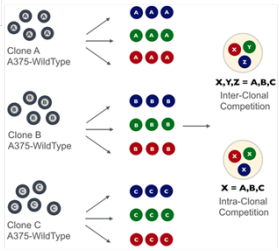
**Aim 2: Major Task 2. Determine if a single cell can generate a fully stratified population.**

**1) Major activities**: Major Task 1 was completed by end of year 1

**2) Specific objectives**: To perform a 3-cell, 3-clone, 3-fluorescent protein (FP) "round-robin" interclonal and intraclonal competition (Figure 2a). 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 intraclonal 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 intraclonal population, each cell was from the same clone. Intraclonal competitions can reveal a relationship between FP and growth (dis)advantage. Assignment of clones to FP in interclonal (INTER-x) and intraclonal (INTRA-x) competitions are shown in Figure 2b. 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 2c. 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 intraclonal competition are shown in Figure 2d. Here, FP, rather than clone, was "ranked" by its frequency. Unlike in Figure 1b, no consistent "winner" emerged in the 6 rows of intraclonal competition, suggesting that FP is unlikely to confer significant growth advantage. Grow curves of the 9 populations described are shown in Figure 2e. 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

2a




2b

| Plate (n=12) | CER CLONE | VEN CLONE | TOM CLONE |
|---|---|---|---|
| INTER-1 | A | B | C |
| INTER-2 | A | C | B |
| INTER-3 | B | A | C |
| INTER-4 | B | C | A |
| INTER-5 | C | A | B |
| INTER-6 | C | B | A |
| INTRA-1 | A | A | A |
| INTRA-2 | A | A | A |
| INTRA-3 | B | B | B |
| INTRA-4 | B | B | B |
| INTRA-5 | C | C | C |
| INTRA-6 | C | C | C |

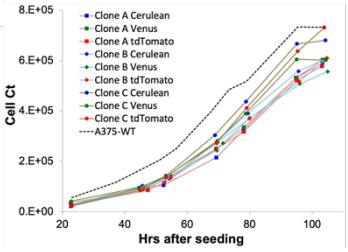
2c

| Plate (n=12) | Rank by CLN, 1st | Rank by CLN, 2nd | Rank by CLN, 3rd |
|---|---|---|---|
| INTER-1 | B | A | C |
| INTER-2 | B | A | C |
| INTER-3 | B | A | C |
| INTER-4 | B | A | C |
| INTER-5 | B | A | C |
| INTER-6 | B | A | C |
| INTRA-1 | | | |
| INTRA-2 | | | |
| INTRA-3 | | | |
| INTRA-4 | | | |
| INTRA-5 | | | |
| INTRA-6 | | | |

2d

| Plate (n=12) | Rank by FP, 1st | Rank by FP, 2nd | Rank by FP, 3rd |
|---|---|---|---|
| INTER-1 | | | |
| INTER-2 | | | |
| INTER-3 | | | |
| INTER-4 | | | |
| INTER-5 | | | |
| INTER-6 | | | |
| INTRA-1 | VEN | CER | TOM |
| INTRA-2 | TOM | CER | VEN |
| INTRA-3 | TOM | CER | VEN |
| INTRA-4 | CER | TOM | VEN |
| INTRA-5 | TOM | CER | VEN |
| INTRA-6 | VEN | TOM | CER |

2e



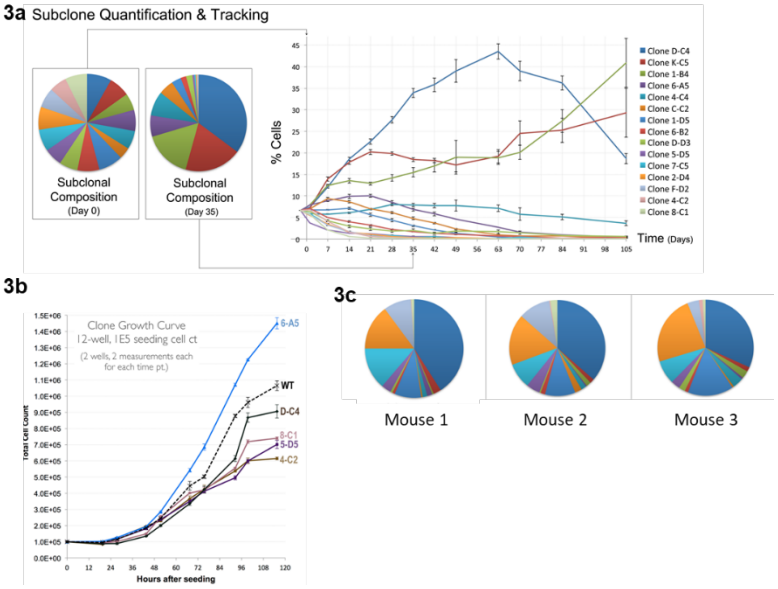
the clones. In this experiment, clone B is clearly the "winner" compared to clones A and C regardless of the FP used. Major Task 2 and the triple competition experiment findings suggests that a fully stratified population can result from a single clone B cell.

**Aim 3: Major Task 3. Observe stratification in vivo**

**1) Major activities:** Major Task 3 was completed by year 2.

**2) Specific objectives**: To observe stratification in vivo

**3) Significant Results**: 15 discrete A375$^{melachroma}$-pool15 cells which were expanded from a single color coded cell were pooled and subjected to growth in vitro (6 replicates) and in vivo (3 NSG mice). As shown in Fig 3a, flow cytometric measurements of proportions of each clone was performed weekly and showed rather consistent patterns. In Fig 3b, the most prevalent subclone ("winner", D-C4), least prevalent subclone ("loser", 4-C2) and several intermediate subclones were subjected to pure subclone growth kinetics. Interestingly, there was modest difference between the "winner" and "loser" subclones in isolation. The fast growing subclone, 6-A5, underwent attrition in the pooled competition. Tumors were isolated from the animals about 40 days after hindlimb injection and subjected to flow cytometry. The fractional composition of the subclones was relatively consistent between the 3 individual mice (Fig 3c) though the pattern between the in vivo and in vitro findings were quite different. Major Task 3 and these results indicate that stratification can be observed in vivo and that the same in vivo "winners" can be recovered in tumors.



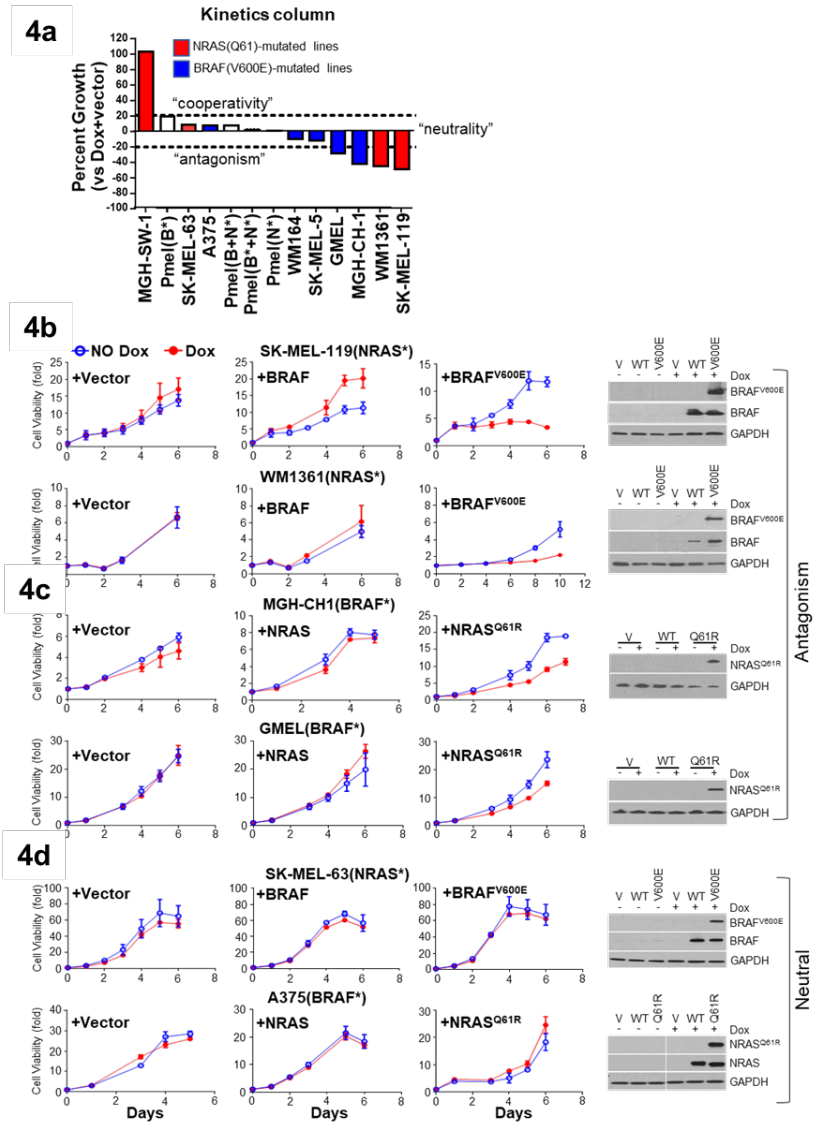

**Aim 2: Major Task 4. Determine if key melanoma oncogenes confer a "competitive advantage" in vitro.**

**1) Major activities:** Major Tasks 4 was completed by year 2 and has been published as part of our manuscript in ONCOGENE (PMID: 30651601).

**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$^{Q61}$ and BRAF$^{V600E}$ 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 isogeneic stable NRAS$^{Q61R/K}$ + doxycycline induced Tet-On-BRAF$^{V600E}$ lines (designated as "NRAS*+iBRAF*") and 5 BRAF$^{V600E}$ + doxycycline induced Tet-On-NRAS$^{Q61R}$ lines (designated as "BRAF*+iNRAS*") (Fig. 4a) along with an immortalized primary human melanocyte line (Pmel). The "rival" oncogene was induced with

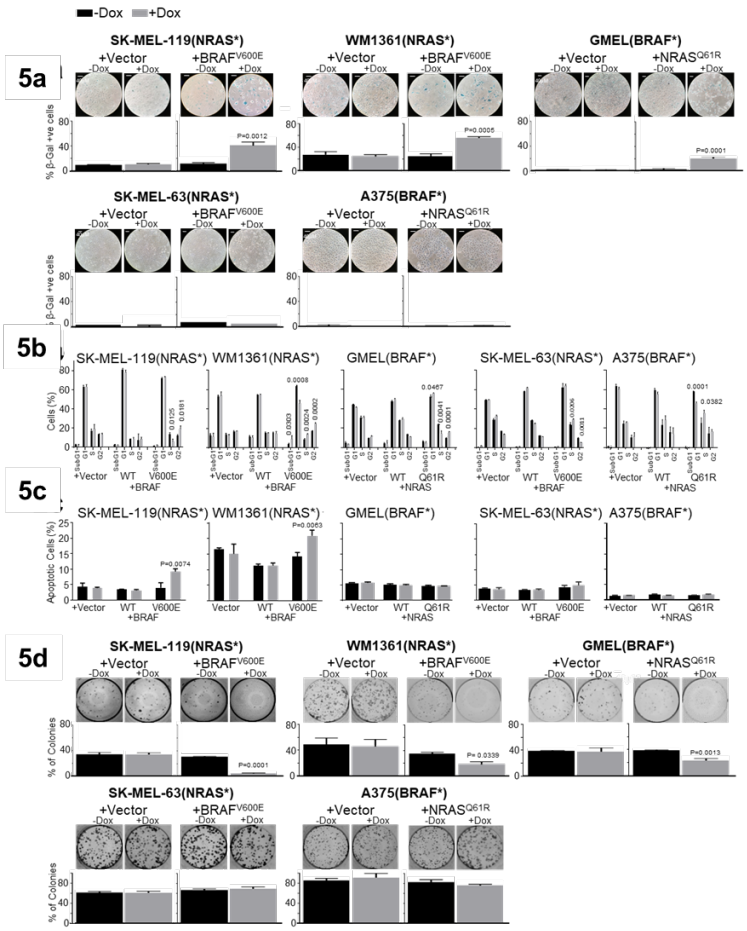
doxycycline (50-100ng/ml) and subjected for 6-day cell viability assays. Using an arbitrary definition of +/-20% above vector for "cooperativity" and "antagonism", 1 of the 4 (red bars) NRAS*+iBRAF* lines exhibited significant cooperativity in growth (MGH-SW-1 $^{NRAS*}$: +102.5%) while the other two demonstrated significant antagonism (SK-MEL-119$^{NRAS*}$: -49.4% and WM1361$^{NRAS*}$: -45.8%). Among (blue bars) BRAF*+iNRAS* lines, interactions were neutral except for GMEL$^{BRAF*}$ and MGH-CH-1$^{BRAF*}$, 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$^{V600E}$ and combined iBRAF$^{WT}$+iNRAS$^{Q61R}$ than with either iBRAF$^{V600E}$+iNRAS$^{Q61R}$ or iNRAS$^{Q61R}$ 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$^{th}$ 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$^{Q61R}$ lines (SK-MEL-119$^{NRAS*}$ and WM1361$^{NRAS*}$), two most suppressed BRAF$^{V600E}$ cell lines (MGH-CH-1$^{BRAF*}$ and GMEL$^{BRAF*}$) and two "neutral" BRAF$^{V600E}$ and NRAS$^{Q61K}$ cell lines (A375$^{BRAF*}$ and SK-MEL-63$^{NRAS*}$) were selected for further analysis. As shown in Figure 4b, ectopic BRAF$^{V600E}$ expression in SK-MEL-119$^{NRAS*}$ and WM1361$^{NRAS*}$ both demonstrated significant growth suppression. Interestingly, forced expression of wild-type BRAF, especially in SK-MEL-119$^{NRAS*}$, enhanced growth, which is consistent with NRAS$^{Q61R}$'s upstream disposition. Similarly, NRAS$^{Q61R}$ induction in MGH-CH-1$^{BRAF*}$ and GMEL$^{BRAF*}$ (Fig. 4c) both confirmed significant growth suppression though ectopic wild type NRAS expression did not appear to alter growth kinetics significantly in these BRAF$^{V600E}$ cells. As expected, the iBRAF$^{V600E}$ and iNRAS$^{Q61R}$ alleles had no effect on the SK-MEL-63$^{NRAS*}$ and A375$^{BRAF*}$ cell lines, respectively (Fig. 4d). Induced expression of BRAF$^{V600E}$ mutant protein in SK-MEL-119$^{NRAS*}$, WM1361$^{NRAS*}$, SK-MEL-63$^{NRAS*}$ cells and of NRAS$^{Q61R}$ mutant protein in MGH-CH-1$^{BRAF*}$, GMEL$^{BRAF*}$ and A375$^{BRAF*}$ cells were all confirmed by western blotting at 6$^{th}$ 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$^{NRAS*}$, WM1361$^{NRAS*}$ and GMEL$^{BRAF*}$ 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$^{NRAS*}$ and A375$^{BRAF*}$ lines (Fig. 5a 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$^{NRAS*}$+iBRAF* (1.5-fold, p<0.01) and GMEL$^{BRAF*}$+iNRAS* (1.63-fold, p<0.01) lines and G2/M arrest in the WM1361$^{NRAS*}$ (1.44-fold, p<0.01) line; there was no significant evidence of arrest in the SK-MEL-63$^{NRAS*}$+iBRAF* or A375$^{BRAF*}$+iNRAS* cells (Fig. 5b). 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$^{Q61R}$ lines (SK-MEL-119$^{NRAS*}$+iBRAF* and WM1361$^{NRAS*}$+iBRAF*) but not in the vulnerable GMEL$^{BRAF*}$+iNRAS* line or the neutral SK-MEL-63$^{NRAS*}$+iBRAF* and A375$^{BRAF*}$+iNRAS* lines (Fig. 5c). 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$^{NRAS*}$+iBRAF*, WM1361$^{NRAS*}$+iBRAF* and GMEL$^{BRAF*}$+iNRAS* lines but not the neutral SK-MEL-63$^{NRAS*}$+iBRAF* and A375$^{BRAF*}$+iNRAS* lines (Fig. 5d). <u>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*</u>.




**Aim 2: Major Task 5. Determine if key melanoma oncogenes confer a growth advantage in vivo**

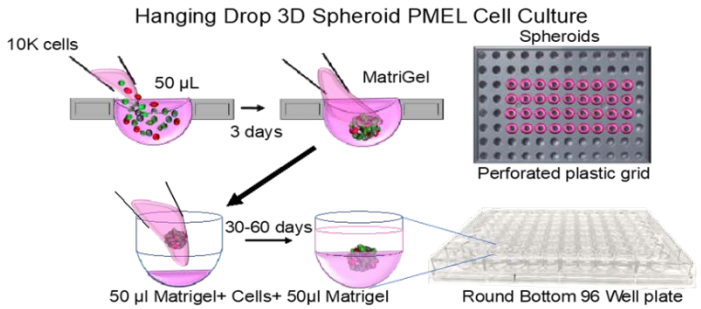
**1) Major activities**: <u>Major Task 5 was completed by end of year 2</u>.

**2) Specific objectives**: To determine if oncogene confers growth bias in 3D spheroid culture instead of animals.

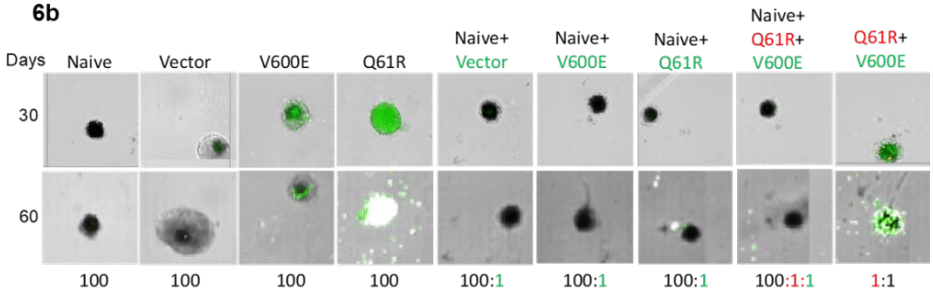
**3) Significant Results:** To confirm a selective advantage among other cells without the oncogenic lesion. In hit and run assay: we ectopically expressed BRAF(V600E) and NRAS(Q61K) alleles, together or independently in Pmel immortalized melanocytes to observe the effect of this change on competition. The Pmel naïve, vector, BRAF (p.V600E) and NRAS (p.Q61R) were mixed in the ratio of 100 or 100:1 naïve:oncogene respectively. For hanging drop culture assay, drops of 50 μl containing 10,000 cells, were hung on a grid slot. The whole grid was placed at 37°C humidified 5% $CO_2$ incubator for 3-5 days and then whole cell mass transferred to round bottom 96 well plate and allowed to form 3D spheroids for 30 days (Fig 6a). Imaging of 3D spheroid culture were performed at regular

time intervals (Fig 6b). As shown in Fig 6b, BRAF(V600E) and NRAS(Q61R) led to oncogenic outgrowh upon induction (green fluorescence). When we altered the ratio to simulate mutant competition and outgrowth, a 1:100 mutation:naïve ratio did not lead to apparent outgrowth of minor BRAF(V600E) or NRAS(Q61R) cells. The most likely explanation is that oncogenic reprogramming of BRAF* or NRAS* cells does not actually lead to a significant enough hyperproliferative state to outcompete the naïve cells. Interestingly, in a "clash of the titans" scenario whereby BRAF* cells were competed against NRAS* cells in a 1:1 ratio, it appears that BRAF* is the strong winner (Fig 6b, far right panel). <u>These results indicate that in a 3D system using immortalized melanocytes, BRAF* cells appears to outcompete NRAS* in a 1:1 competition but neither BRAF* nor NRAS* alone can outcompete untransformed melanocytes in 1:100 mutant:naïve cell competition</u>.

6a




6b



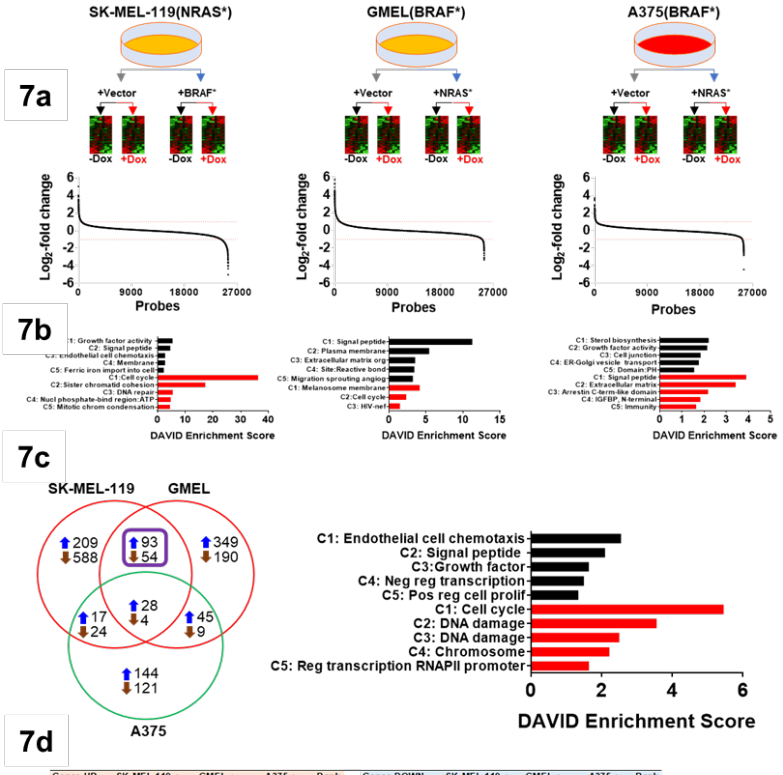

**Aim 2: Major Task 6. Identify physiologic pathways that influence clonal dominance1) Major activities:**

**1) Major activities:** <u>Major Task 6 was completed by end of year 2</u> and has been published as part of our manuscript in ONCOGENE (PMID: 30651601).

**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 7a. Since the dual oncogenesis occurs in isogenically-matched lines, we first examined expression changes in paired analyses. In the SK-MEL-119NRAS*+iBRAF* line, 2.20% and 4.43% of the gene probes were upregulated (i.e. increased 2-fold; >1.0 log2-fold) and downregulated (i.e. decreased 2-fold; <-1.0 log2-fold), respectively, upon induction of exogenous BRAF(V600E). These were 3.44% and 1.39% up-and downregulated, respectively, for GMELBRAF*+iNRAS* and 1.24% and 0.89% up- and downregulated, respectively, for A375BRAF*+iNRAS*. With BRAF* overexpression in SK-MEL-119NRAS*+iBRAF*, the most induced genes were IL1B (5.04 log2-fold), MMP1 (4.04 log2-fold), IL1A (3.93 log2-fold), IL24 (3.84 log2-fold) and GFPT2 (3.58 and 3.57 log2-fold) while the most suppressed genes were in CXL12 (-4.99, -3.66 and -3.62 log2-fold), MGP (-4.31 log2-fold), CHRNA1 (-3.95 and -3.58 log2-fold), PLPPR4 (-3.7 log2-fold) and TRIM22 (-3.55 log2-fold). With the introduction of NRAS* in GMELBRAF*+iNRAS*, the most upregulated genes were STC1 (5.75 log2-fold), CXCL8 (5.3 log2-fold), MMP1 (4.89 log2-fold), IGFBP3 (4.55 log2-fold) and MB (4.43 log2-fold) and the most downregulated genes were TRIM63 (-3.34 log2-fold), PHACTRI (-3.18 log2-fold), MLANA (-3.07 log2-fold), TYRP1 (-2.82 log2-fold) and GAGE genes (-2.61 log2-fold). In contrast, for the neutral A375BRAF*+iNRAS* line, GDF15 (3.75, 3.58, 3.45 log2-fold), PTPRR (2.95 log2-fold), UCA1 (2.86 log2-fold), STC1 (2.83 log2-fold) and SLC14A1 (2.61 log2-fold) exhibited the greatest increase while MGP (-4.4 log2-fold), ITGA9 (--2.81 log2-fold), SERPINF1 (-2.66 log2-fold), A2M (-2.58 log2-fold) and ENPP2 (-2.54, -2.48 log2-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. 7b). Among upregulated genes (i.e. >2-fold), the "SIGNAL PEPTIDE" functional cluster was the highest and second highest annotated cluster in GMELBRAF*+iNRAS* (Enrichment score, ES:11.26) and SK-MEL-119NRAS*+iBRAF* (ES:4.62), respectively. Interestingly, the "SIGNAL PEPTIDE" cluster was the leading annotated set among the most suppressed genes for A375BRAF* (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-119NRAS*+iBRAF* (ES: 36.26) and GMELBRAF*+iNRAS* lines (ES: 2.32), respectively; the "CELL CYCLE" cluster were not significantly enriched in the A375BRAF*+iNRAS* line. One notable cluster is "MELANOSOME MEMBRANE", which was derived from the set of most downregulated genes (i.e. >2-fold) in GMELBRAF*+iNRAS* (ES:4.18). To replicate this finding, we used a published list of high impact MITF target genes8, MITF targets, as a group, were significantly more suppressed than non-MITF targets; log2-fold -0.56±0.03 vs -0.0034±0.0032, P<0.0001, Student T test) in GMELBRAF* cell lines. In addition, MITF suppression was verified by qPCR in the GMELBRAF*+iNRAS* cell lines. Thus, lineage programming appears to be attenuated in the GMELBRAF*+iNRAS* line with ectopic NRAS* expression. Lastly, in only the A375BRAF*+iNRAS* cells, overexpression of NRAS* appears to correlate with a functional cluster related to reprogramming of lipid metabolism ("STEROL BIOSYNTHESIS", ES:2.2).



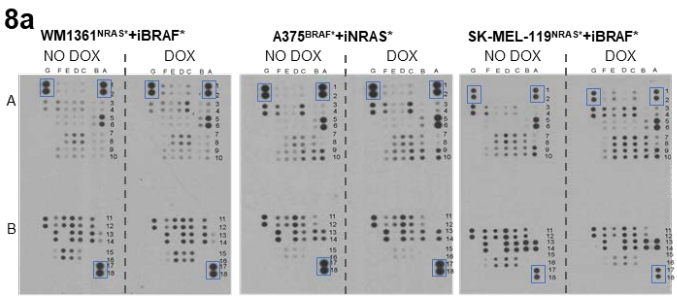

| Genes UP | SK-MEL-119 + iBRAF* | GMEL + iNRAS* | A375 + iNRAS* | Rank | Genes DOWN | SK-MEL-119 + iBRAF* | GMEL + iNRAS* | A375 + iNRAS* | Rank |
|---|---|---|---|---|---|---|---|---|---|
| MMP1 | 3.22 | 4.89 | 0.45 | 1 | PLXNC1 | -1.91 | -1.95 | 0.15 | 1 |
| SPRY4 | 2.78 | 3.3 | -0.54 | 2 | FAM111B | -2.18 | -1.67 | 0.41 | 2 |
| SPRY4 | 2.45 | 3.22 | -0.76 | 3 | FAM111B | -2.31 | -1.47 | 0.54 | 3 |
| C11orf96 | 2.04 | 3.59 | -0.26 | 4 | ZNF367 | -1.88 | -1.8 | 0.91 | 4 |
| SERPINB2 | 3.2 | 2.37 | 0.42 | 5 | BHLHE41 | -1.32 | -2.21 | 1.04 | 5 |
| EGR2 | 1.03 | 4.39 | -0.06 | 6 | KIAA0101 | -1.8 | -1.71 | 0.49 | 6 |
| EGR3 | 3.34 | 2.03 | -0.25 | 7 | RAD51AP1 | -2.16 | -1.2 | 0.13 | 7 |
| DUSP5 | 2.47 | 2.83 | 0.95 | 8 | HELLS | -1.99 | -1.34 | -0.13 | 8 |
| CEMIP | 1.56 | 3.54 | -0.24 | 9 | CCNE2 | -1.4 | -1.84 | 0.41 | 9 |
| PRDM1 | 1.39 | 3.63 | 0.17 | 10 | RAD51AP1 | -2.12 | -1.1 | 0.14 | 10 |
| GFPT2 | 3.57 | 1.32 | 0.07 | 11 | TMEM97 | -2.06 | -1.16 | 0.2 | 11 |
| ID1 | 1.39 | 3.43 | -0.42 | 12 | FAM198B | -1.97 | -1.22 | -0.44 | 12 |
| GFPT2 | 3.58 | 1.13 | 0.36 | 13 | DHFR | -1.7 | -1.44 | -0.54 | 13 |
| LIF | 1.32 | 3.27 | 0.85 | 14 | RAD51AP1 | -2.04 | -1.1 | 0.05 | 14 |
| HEY1 | 3.07 | 1.48 | -0.7 | 15 | ESCO2 | -1.78 | -1.35 | 0.42 | 15 |
| BMP2 | 3.39 | 1.01 | 0.97 | 16 | MCOLN3 | -1.29 | -1.82 | -0.23 | 16 |
| PLAUR | 1.07 | 3.3 | 0.89 | 17 | BRCA1 | -1.54 | -1.49 | 0.37 | 17 |
| HEY1 | 2.55 | 1.79 | -0.6 | 18 | DHFR | -1.81 | -1.21 | -0.2 | 18 |
| ID1 | 1.2 | 3.1 | -0.28 | 19 | CENPF | -1.8 | -1.17 | 0.09 | 19 |
| CSRNP1 | 1.36 | 2.84 | 0.19 | 20 | ZWINT | -1.64 | -1.32 | -0.33 | 20 |

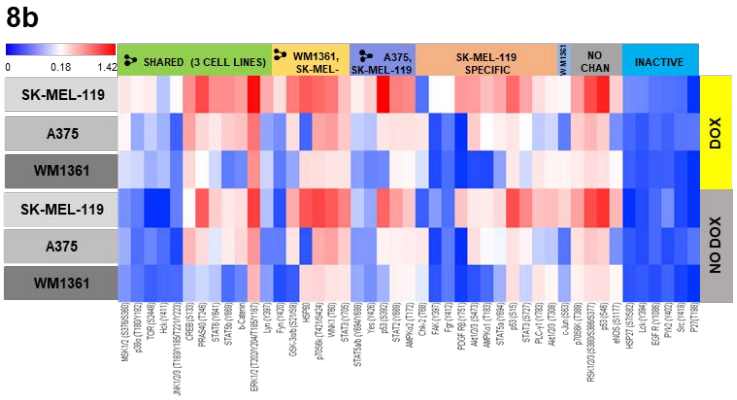
We next sought to identify a shared suppressive physiology by focusing on statistically significant regulated genes in both SK-MEL-119NRAS*+iBRAF* and GMELBRAF*+iNRAS* lines but not the A375BRAF*+iNRAS* line. As shown in Fig 7c, there were 93 upregulated transcripts (i.e. >2-fold increase) shared between SK-MEL-119(NRAS*+BRAF*) and GMELBRAF*+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-119NRAS*+iBRAF* and GMELBRAF*+iNRAS* but not A375BRAF*+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-119NRAS*+iBRAF* and GMELBRAF*+iNRAS*, SPRY4 transcripts were among the most upregulated ones in both antagonized SK-MEL-119NRAS*+iBRAF* and GMELBRAF*+iNRAS* lines,

but not in the neutral A375BRAF*+iNRAS* cell line (Fig. 7d Table). With these results, we provide data for the first time that the SPRY4 pathway may be a crucial physiological determinant of clonal dominance.

We also interrogated an extended panel of phosphokinases using a phosphokinase assay (R&D systems, Inc., Minneapolis, MN). The cell lines; WM1361($^{NRAS*}$), A375$^{BRAF*}$ and SK-MEL-119$^{NRAS*}$ exposed to doxycycline to induce BRAF*, NRAS* and BRAF* respectively, were harvested at 5 day, washed with 1x phosphate-buffered saline and lysed in a RIPA buffer (Boston Bioproducts, Ashland, MA) supplemented with halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL). The protein concentrations of the lysates were measured by BCA protein assay kit (BioRad, Hercules, CA). whole cell lysates of 1x10$^7$ cells/ml were subjected for phosphokinase array and for each cell line, the densitometric values were normalized to reference spots on each immunoblots and compared in fold change with uninduced sample. We identified multiple changes (Fig 8a) including consistent increases in p-MSK1/2 (>2.43 fold), p-p38α (>2.70 fold), p-ERK1/2 (>1.26 fold), pJNK1/2/3 (>2.23 fold), GSK-3α/b (>1.35 fold), and p-CREB (>2.03 fold) suggesting that stress signaling via JNK and p38α pathways may be activated during dual oncogenesis. In the heat map, colored tiles represent shared and cell specific regulation of phosphorylation of protein kinases. Densitometry was performed and normalized to reference spots (blue squares) on each blot (Fig 8b). However, there were no single signaling pathway that predicted antagonism.




**Aim 2: Major task NCE#1: To determine whether key melanoma oncogenes have "differential vulnerability" in vitro.**

**1) Major activities:** In brief, our lab has discovered that both NRAS and BRAF oncogenes are differentially vulnerable to degradation up on translation inhibition, mainly the NRAS* which has shorter lifetime than BRAF* protein. We report to exploit its nature by using protein translation inhibitor(s) such as cycloheximide, puromycin and "omacetaxine" an FDA approved novel compound that blocks protein synthesis through translation elongation inhibition. characterized. Our findings indicate that oncogenes, such as BRAF* and NRAS*, may be differentially labile and that one novel approach would be to pharmacologically leverage this vulnerability for therapeutic gain.

We performed comparative growth analysis using a de novo synthesis of cellular protein inhibition "chase platform". As we know recurrent oncogenic mutations such as BRAF (pV600) and N/K/HRAS (pG12/13, p.Q61) predominate in the RAS-MAPK pathway in melanoma. The biological processes that regulate the stability and half-life of these activating alleles have not been fully understood.

**2) Specific objective:** To determine if BRAF (pV600E) and NRAS (pQ61) mutations affect protein stability and venerability, associated with individual mutations.

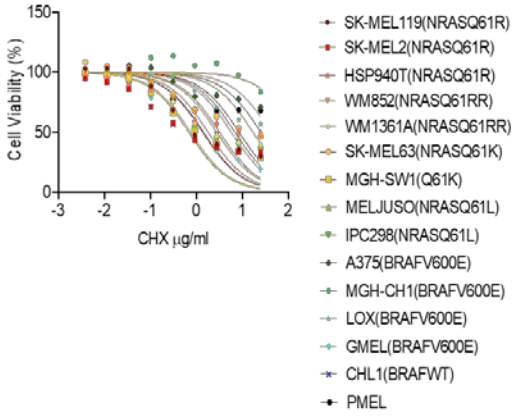
**3) Significant results:** We first set out to analyze the cell growth in context to protein translation inhibition by examining the impact on protein stability of various NRAS and BRAF mutant cell lines. We chose nine NRAS(Q61) cell lines (SK-MEL-119(NRAS$^{Q61R}$), SK-MEL-2(NRAS$^{Q61R}$), HSP940T(NRAS$^{Q61R}$) WM852(NRAS$^{Q61R}$), WM1361A(NRAS$^{Q61R}$), SK-MEL-63(NRAS$^{Q61K}$), MGH-SW1(NRAS$^{Q61K}$), Meljuso(NRAS$^{Q61L}$), and IPCA298(NRAS$^{Q61L}$)), four BRAF(V600E) cell lines

(A375(BRAF*), MGH-CH-1(BRAF*), LOX(BRAF*) and GMEL(BRAF*)), and two $BRAF^{WT}$/$NRAS^{WT}$ cell lines (CHL1 and immortalized primary melanocyte (PMEL)) for this study (Table 9a). Most of the NRAS mutant melanoma cell lines demonstrated significant growth suppression in parallel to NRAS protein depletion with cycloheximide, as compared to BRAF mutant cells. *In-vitro* cell viability assay at 48 hours of 8 µg/ml cycloheximide treatment, mean survival fraction BRAF* versus NRAS* mutant melanoma cells was determined to be considerably higher and cycloheximide antibiotic $IC_{50}$ value of growth inhibition was also found to be significantly more in BRAF* mutant cells as compared to NRAS* mutant cell lines (Fig. 9b,c). Since, in NRAS mutant cells treated with cycloheximide, the change in NRAS expression (Fig 10a) appears to be decoupled from changes in expression of BRAF (Fig 10b), it also suggests higher susceptibility of mutant NRAS to translation inhibition and cellular growth arrest than BRAF mutant cell lines.

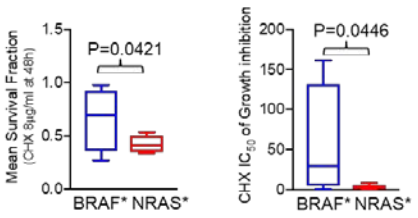
9a

| No. | Table 1 Cell line | BRAF | NRAS | IC50 of CHX µg/ml at 48h |
|---|---|---|---|---|
| 1 | *SK-MEL119* | *WT* | *Q61R* | *1.427* |
| 2 | SK-MEL2 | WT | Q61R | 0.7508 |
| 3 | HSP940T | WT | Q61R | 6.899 |
| 4 | WM852 | WT | Q61RR | 5.754 |
| 5 | WM1361A | WT | Q61RR | 2.787 |
| 6 | SK-MEL63 | WT | Q61K | 8.482 |
| 7 | MGH-SW1 | WT | Q61K | 1.393 |
| 8 | Meljuso | WT | Q61L | 3.184 |
| 9 | IPCA298 | WT | Q61L | 1.802 |
| 10 | *A375* | *V600E* | *WT* | *42.38* |
| 11 | MGH-CH1 | V600E | WT | 161 |
| 12 | LOX | V600E | WT | 17.11 |
| 13 | GMEL | V600E | WT | 0.6849 |
| 14 | CHL1 | WT | WT | 1.376 |
| 15 | PMEL | WT | WT | 25.33 |

9b




9c



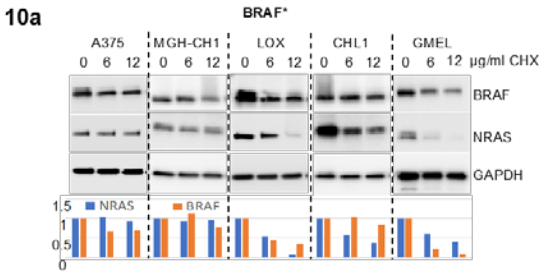

**Aim 3: Major Task 7. Establish the role of resistance mechanisms on clonal competition**

**1) Major activities:** Major Task 7 was completed by end of year 2.

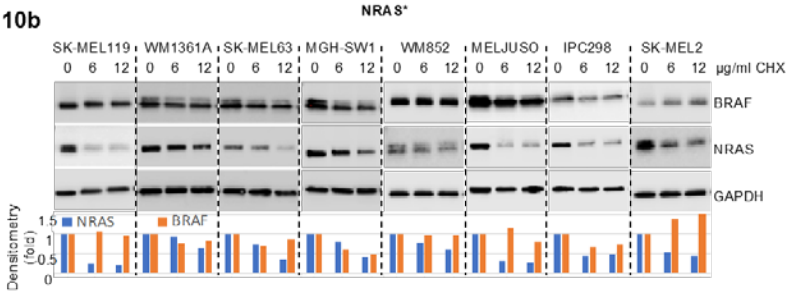
**2) Specific objectives:** To determine whether second oncogene expression affects vemurafenib sensitivity in clonal competition.

**3) Significant Results:** In both BRAF mutant and NRAS mutation inducible the A375$BRAF^{V600E}$+i$NRAS^{Q61}$ and MGH-CH-1$BRAF^{V600E}$+iNRASQ61 lines, we observed resistance to BRAF inhibition (Fig. 11a) suggesting that resistance can be engendered de novo even in the absence of drug. The $NRAS^{Q61R}$ mutation was expressed in A375(BRAFV600E) and MGH-CH-1(BRAFV600E) cell lines by inducing with doxycycline (0, 10, 100,1000 ng/ml) for 24 hours prior treating with vemurafenib and subjected for 6 days cell viability assays using cell-titer-glow reagent. Both A375(BRAF*)+iNRAS* and MGH-CH-1(BRAF*) cell lines showed significant resistance to vemurafenib (0, 0.1, 1.0, 10 µM) in dose dependent manner upon $NRAS^{Q61R}$ induction as compared to vemurafenib that alone showed significant suppression in dose dependent fashion. In 3D spheroid cultures, the A375 melanoma cells stably expressing vector-GFP, CFP, NRAS Q61R and NRASQ61K were subjected for inter clonal competition with all possible combinations in the presence of BRAF inhibitor vemurafenib (Plx4032) for 14 Days. We used 10

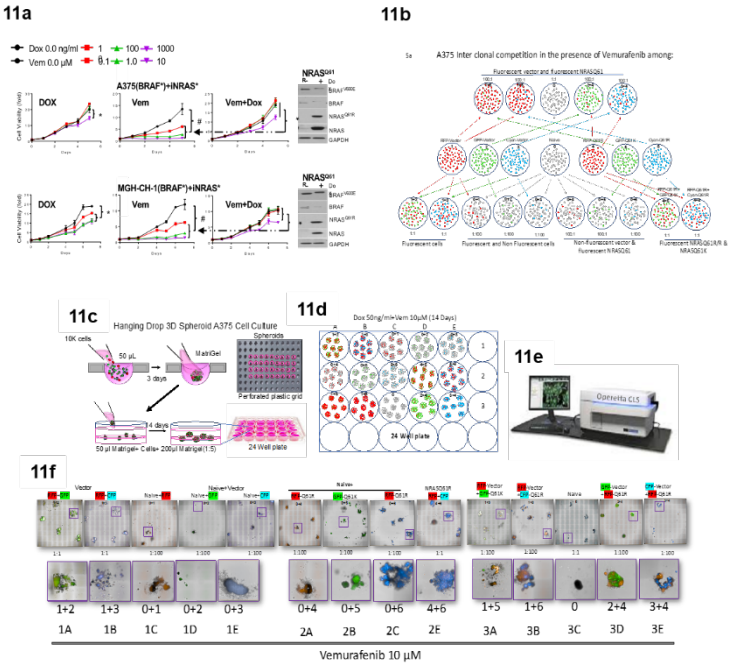
10a




10b



times higher concentration 10µM Plx in 3D culture as compared to 2D culture of 1µM IC50 in view of the concentration gradient in 3D spheroid which is always higher at outside than inside of the spheroids. Also, to rule out the effect of fluorescence on drug resistance, different color combinations were included. The growth of the spheroids in different 14 combination are shown in Figure 11b and the assay is shown diagrammatically in Fig 11c-e. This design addresses possible competition among non-fluorescent vector and fluorescent NRASQ61 cells (2A, 2B, 2C), competition among non-fluorescent and fluorescent vector cells (1C,1D,1E), competition among fluorescent NRASQ61R/R mutation carrying cells (2E), competition among fluorescent vector cells (1A,1B), and competition among fluorescent vector and fluorescent NRASQ61(3A,3B,3C,3D,3E). We observed that A375 BRAF$^{V600E}$+iNRAS$^{Q61}$ mixed either with naïve or fluorescent vector in 1:100 ratio, over the time of 14 days of forced NRASQ61 expression induced by doxycycline (50ng/ml), the A375 BRAF$^{V600E}$+iNRAS$^{Q61}$ clone emerged as a major population and formed many spheroids that continue to grow uninterrupted irrespective of mixture type and ratio in the presence of Plx4032. Hence, second oncogene NRASQ61 confers resistance to Plx4032 treatment as compare to naïve and vector expressing cells Figure 11f (2A2B,2C,2E & 3A,3B,3D,3C).

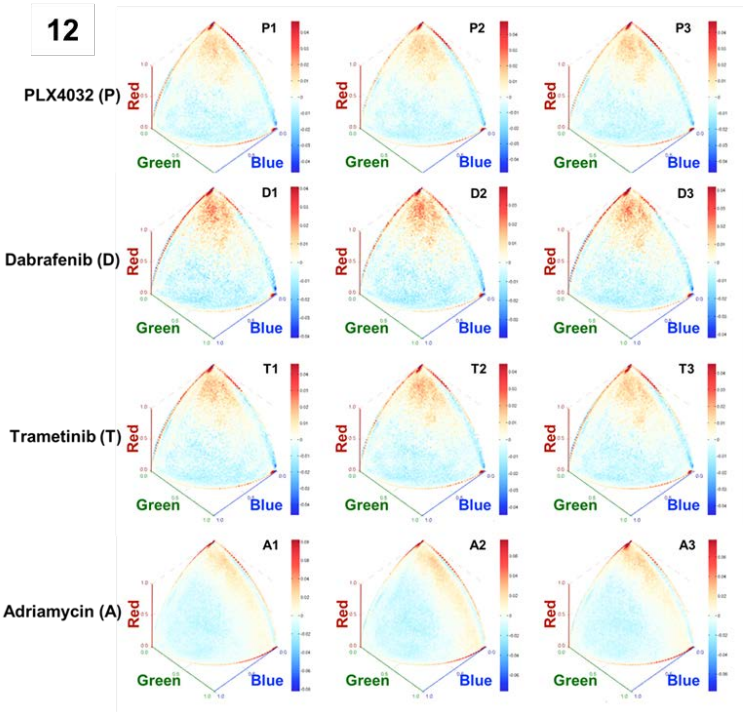



**Aim 3: Major Task 8. Establish the role of resistance mechanisms on clonal competition**

**1) Major activities:** Major Task 8 was completed by end of year 2.

**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 12). 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

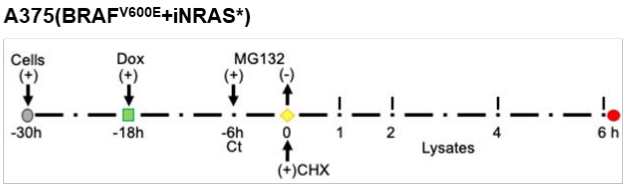


was observed for each drug. The results suggest that certain subpopulations of cells are uniquely vulnerable to MAPK inhibition and distinct from Adriamycin.

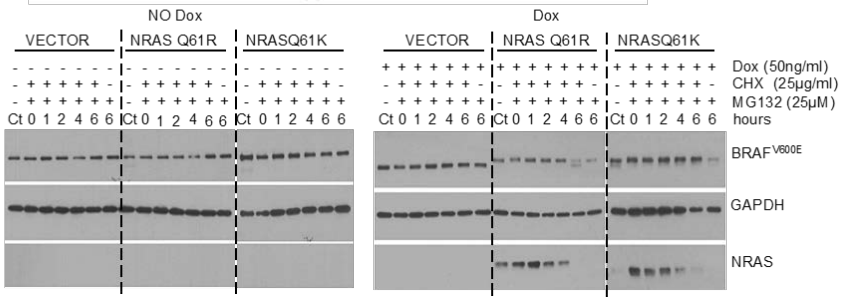
**Aim 3: Major task NCE#2: To assess if second oncogene NRASQ61 alters "phenotype" of BRAF mutant cells.**

**1) Major task:** To assess if second oncogene NRASQ61 alters "phenotype" of BRAF mutant cells. To determine how ectopic expressed NRASQ61R drive the oncogenic potential in NRASQ61 transformed BRAFV600E mutant population and regulate its turnover and drug resistance. A well characterized BRAFV600E mutant A375 melanoma cell line was selected to ectopically express NRASQ61, designated as A375(BRAFV600E+iNRASQ61). The biological processes that regulate BRAFi/MEKi resistance and half-life of NRASQ61 allele have been partially characterized.

13a




13b




**2) Specific objective:** To determine if NRASQ61 depletion is limited to endogenous protein expression upon protein translation inhibition. Whether ectopic expressed NRAS depletion by cycloheximide inhibit NRAS dependent A375(BRAF(V600E)+iNRASQ61) cell growth in vitro?

**3) Significant results:** We used a tet-on system to synchronize expression of the second oncogene in A375 isogenic stable BRAFV600E + doxycycline induced NRASQ61R/K line (designated as "BRAF*+iNRAS*"; Fig 13a) to check whether NRASQ61 depletion is limited to endogenous expression. The NRASQ61) oncogene was induced with doxycycline (50 ng/ml) for 18 hours followed by MG132 (25 µM) treatment. After 6 hours, MG132 was removed to release the proteasome halt and cycloheximide was added to inhibit the new protein synthesis. Whole cell lysates were collected at 0, 1, 2, 4 and 6 hours and resolved on 4-12% SDS-PAGE. In time kinetics, A375(BRAF*+iNRAS*) line showed significant NRASQ61 protein depletion but no significant change was seen at the level of BRAFV600E protein in context to CHX treatment (Fig 13b). Moreover, these results indicate that neither ectopic nor endogenous expression, however post-translation modification accounts for the decrease in NRAS protein. These findings suggest that NRAS* mutation has retransformed A375(BRAFV600E) to drug resistance cell line with a hallmark of NRAS mutant "phenotype" and modulated the genetic BRAF status.

**c. What opportunities for training and professional development has the project provided?**
Nothing to report. Project not intended to provide training beyond the methodology of scientific research as taught one-on-one with the postdocs.

**d. How were the results disseminated to communities of interest?**
Papers:
Kumar R, Njauw CN, Reddy BY, Ji Z, Rajadurai A, Klebanov N, Tsao H. Growth suppression by dual BRAF(V600E) and NRAS(Q61) oncogene expression is mediated by SPRY4 in melanoma. Oncogene. 2019 May;38(18):3504-3520.

Conference abstracts and presentations:
Kumar R, Zhenyu Ji., Tsao H. Downregulation of NRASq61 and its value in the biology of Melanoma. AACR Annual Meeting, 2019 in Shenzhen, China (A0233) 05/2019

Kumar R, Ji Z., Njauw J., Tsao H. Dissecting mutual exclusivity of oncogenic mutations in melanoma. AACR Annual Meeting, 2017 in Washington DC., USA (Late-Breaking Research #307). 04/2017

**e. What do you plan to do during the next reporting period to accomplish the goals?**
N/A. Grant is over.

4. **IMPACT:**

**a. What was the impact on the development of the principal discipline(s) of the project?**
Work funded by this project has confirmed, and extended, the idea that all cancer cells have the potential to stratify and to act as a "master regulator." While these are conceptual in nature, mechanistically, the intracellular rivalry between oncogenic drivers and the involvement of SPRY4 in "oncogene antagonism" is now a novel idea that has been disseminated through publication. During the NCE period, we have discovered that NRAS and BRAF oncogenes have differential stability and therefore represent opportunities for selective targeting. We will continue to follow up on clonal competition in light of "oncogene competition" through additional work.

**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**
None

**b. Actual or anticipated problems or delays and actions or plans to resolve them**
Nothing to report

**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, Njauw CN, Reddy BY, Ji Z, Rajadurai A, Klebanov N, Tsao H. Growth suppression by dual BRAF(V600E) and NRAS(Q61) oncogene expression is mediated by SPRY4 in melanoma. Oncogene. 2019 May;38(18):3504-3520.
Acknowledgement of federal support- YES

**b. Books or other non-periodical, one-time publications.**
Klebanov N, Reddy BY, Tsao H. Chapter 31: Melanoma genomics: is melanoma a single disease entity? In Rigel DS et al, eds, Cancer of the Skin, 3rd edition. London: Elsevier, in press

Shaughnessy M, Klebanov N, Tsao H. Clinical and therapeutic implications of melanoma genomics. J Transl Genet Genom, 2018;2:14

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

National

1. 2017 "Landscape of rare mutations in hereditary melanoma", Melanoma Research Alliance 2017 Meeting; Washington, DC
2. 2017 "Hereditary melanoma: new syndromes and new biology"/Kay Lecturer; Dept of Dermatology, Johns Hopkins School of Medicine; Baltimore, MD
3. 2017 "Melanoma Immunotherapy: checkpoint to checkmate"/Invited Lecturer; Dermatology and Dermatological Society of Connecticut; Monroe, CT
4. 2017 "Rare variant, gene-based association study of hereditary melanoma"/Invited Lecturer "Genetics and Epigenetic Control of Melanoma"/Chair Triennial International Pigment Cell Conference; Denver, CO
5. 2017 "Therapeutic Checkmate for melanoma?"/Invited lecture College of Physicians of Philadelphia, Philadelphia, PA
6. 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
7. 2018 "Therapeutic Checkmate for Melanoma"/ Malkinson Lecturer Chicago Dermatological Society, Chicago, IL
8. 2018 "Update on melanoma"/Keynote Speaker; Montagna Symposium on Biology of the Skin, Salishan Resort, OR
9. 2019 "Molecules and markers in melanoma"/Invited speaker; Department of Dermatology/Weill Cornell Medical Center
10. 2020 "New insights into melanoma tumor syndromes"/Keynote speaker; "Melanoma 2020: checking in on checkpoint blockade"/Invited speaker; "Molecules and genetic testing 101"/Invited speaker 2020 DF Clinical Symposium, Naples, FL

International

1. 2017 "Update on melanoma pathogenesis and treatment"/Keynote Speaker; 12th Korean Skin Cancer Conference/3rd KNUH Melanoma Symposium; Daegu, Korea; "Hereditary melanoma"/Visiting Professor; Dept Dermatology, Yonsei University School of Medicine; Seoul, Korea
2. 2017 "Pro's and con's of UV mutations in melanoma"/Invited Lecturer; "Molecular genetics of melanoma: mutations to medicine"/Plenary Lecturer; "Determinants of response to; immunotherapy"/Invited Lecturer; "MELANOMA 2 - How the new treatments have modified the disease?"/Chair; International Congress of Dermatology 2017/Buenos Aires, Argentina
3. 2018 "Therapeutic checkmate in melanoma"/Keynote speaker; 2018 British Academy of Dermatology meeting; Edinburgh, Scotland
4. 2019 "Molecular genetics overview"/Keynote speaker; 2019 Canadian Melanoma Conference meeting; Banff, Canada
5. 2019 "Melanoma genetics: from mutations to medicine"/Kung Sun Oh Memorial lecture "Rare variant, gene-based association study of hereditary melanoma"/Cutaneous Biology Research Institute; Department of Dermatology/Yonsei University School of Medicine, Seoul, Korea
6. 2019 "Therapeutic Checkmate in melanoma"/Keynote speaker "Melanoma, molecules and medicine"/Invited speaker 45th Annual Meeting of the Taiwanese Dermatological Association, Kaoshiung, Taiwan

**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 |

| | |
|---|---|
| Name: | Ching-Ni Njauw |
| Project Role: | Lab manager/senior technician |
| Researcher Identifier (e.g. ORCID ID): | |

| | |
|---|---|
| Nearest person month worked: | 4.20 CM |
| Contribution to Project: | Ms. Njauw has expertise in cultivating difficult to grow cells lines and was instrumental in reviving some of the melachroma cells and in providing guidance to Dr. Kumar on the best approach to growing cells. In addition, she is the animal colony manager and assisted Dr. Kumar on the animal experiments. |
| Funding Support: | Air Force Office of Scientific Research |
| Name: | Anpuchchelvi Rajadurai |
| Project Role: | Research Specialist |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 12 CM |
| Contribution to Project: | Ms. Rajadurai assisted Dr. Kumar at the benchside in the design and execution of experiments especially with protein blotting and cell sorting. |
| Funding Support: | Air Force Office of Scientific Research |

| | |
|---|---|
| Name: | Yuanyuan Kang |
| Project Role: | Postdoctoral fellow |
| Researcher Identifier (e.g. ORCID ID): | Dr. Kang has genetics and epigenetics expertise. She assisted in analyzing some of the expression microarray analyses that was published in the ONCOGENE manuscript. |
| Nearest person month worked: | 2.40 CM |
| Contribution to Project: | Dr. Kang |
| 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