AWARD NUMBER: W81XWH-19-1-0143

TITLE: Genomic and Immunologic Correlates of Immunotherapy Response and Resistance via Longitudinal Tumor and Extracellular Vesicle (EV) Analysis

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CONTRACTING ORGANIZATION: MASSACHUSETTS GENERAL HOSPITAL
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REPORT DATE: June 2021

TYPE OF REPORT: Annual Technical Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2021	Annual Technical Report	15MAY2020 - 14MAY2021

4. TITLE AND SUBTITLE
Genomic and Immunologic Correlates of Immunotherapy Response and Resistance via Longitudinal Tumor and Extracellular Vesicle (EV) Analysis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0143

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
MASSACHUSETTS GENERAL HOSPITAL
55 FRUIT ST
BOSTON MA 02114-2621

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Personnel: Dr. Boland (PI) is an expert in two of the FY18 PRCRP Topic Areas – melanoma and immunotherapy. Her leadership roles include serving as the Director of the Massachusetts General Hospital (MGH) Melanoma Surgery Program and the Director of the MGH Surgical Oncology Research Laboratories. She is Associate Member of the Broad Institute of MIT and Harvard and an Assistant Professor at Harvard Medical School (HMS). She is firmly committed to a career understanding how to intelligently use a variety of therapies (including targeted therapies, ICI, intra-lesional therapies) with surgery (neoadjuvant or adjuvant) for the highest quality of care for cancer patients. Her goal is to establish clinically-relevant tools to guide clinical decision-making. Dr. Flaherty is the Director of the Termeer Center for Targeted Therapy and Director of Clinical Research at the MGH Cancer Center. He has a track-record of transitioning novel laboratory research into successful clinical trials in melanoma. He has a strong history of mentoring junior faculty with a superb record of success. Career Development: This award will allow the generation of sufficient preliminary data for an NIH R01 type application. The proposal will allow the integration of Dr. Boland's clinical expertise with novel experimental and analytical techniques to address clinically-relevant questions. Background: Immune checkpoint inhibitors (ICI) targeting cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed death receptor 1 (PD-1) and its ligand (PD-L1) have revolutionized cancer therapy across many cancer types. While 40-45% of patients with metastatic melanoma respond to PD-1 blockade1–3, the majority still succumb due to primary, adaptive, or acquired resistance4. A diverse set of resistance associated alterations (RAAs) have been identified in the clinical and pre-clinical settings, but when multiple RAAs co-exist how these pathways interact has not yet been well-characterized. Additionally, non-invasive mechanisms for monitoring tumor and immune changes in parallel have not yet been established. Hypothesis: Our hypothesis is that acquired immune resistance is dependent upon the temporal accumulation of several distinct RAAs and that analysis of EV-derived transcripts can be utilized to predict and/or monitor both tumor RAAs and immune changes in parallel.

15. SUBJECT TERMS: NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	24	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

Page

1. INTRODUCTION:

One of the most promising recent therapeutic avenues has been cancer immunotherapy: boosting the immune system or training it against tumor antigens. However, only a fraction of patients receive durable clinical benefits from these therapies. There is a pressing need to identify factors predictive of clinical response to help make patient-specific treatment choices, understand the mechanisms of both de novo and acquired immunotherapy resistance, and nominate new therapeutic targets for combination therapy. We are undertaking multi-level analysis of longitudinal patient tumor samples in parallel with peripheral blood-derived extracellular vesicle (EV) RNA expression in patients undergoing checkpoint blockade therapy. We hypothesize that blood-based analysis will allow us to interrogate multiple tumor sites simultaneously, offering a more broad-based analysis of the tumor and immune landscape than tumor analysis alone. Additionally, tissue-of-origin analysis from EV transcripts during treatment will allow us to gain a comprehensive understanding of changes in the tumor microenvironment during checkpoint blockade immunotherapy.

2. KEYWORDS:

Tumor genomics, transcriptomics, immunotherapy, biomarker

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1: integrative analysis of serial tumor samples during ICI for genomic/epigenomic analysis with immune profiling.	Months	
Aim 1.1 – Genomic and epigenomic analysis of tumors		
Patient selection – patient must have at least 3 time points for analysis *Pre-treatment, On-treatment, Post-treatment/Recurrence* *Therapy: aPD1 monotherapy or combination aPD1 + aCTLA4* BRAF V600E mutant (n=5) NRAS mutant (n=2) NF1 mutant (n=1) Triple wild type (n=1) Sequencing completed for n=5 BRAF V600E mutant patients, n=2 NRAS patient, n=1 NF1 patient, and n=1 triple wild type patient. The timeline was impacted by COVID-19 delays, but analysis is back on track.	3-6	MGH (Boland Lab)
Exome sequencing n=9 patients with 3 time points n=27 tumor samples total Sequencing completed and phylogenetic analyses underway.	3-6	Broad (Boland)

ATACseq n=9 patients with 3 time points n=27 tumor samples total Large ATACseq dataset available. However, not all samples from WES and RNAseq have undergone ATACseq.	3-9	MGH/Broad (Boland; technical assistance Kellis)
Phylogenetic analysis n=9 patients with 3 time points n=27 tumor samples total Sequencing completed and phylogenetic analyses underway.	9-12	MGH (Boland Lab, MGH computational team)
Aim 1.2 – Immune characterization.		
RNAseq - using same patient samples from Aim 1.1 n=9 patients with 3 time points n=27 tumor samples total Sequencing completed and transcriptional analyses underway.	3-6	MGH
TCR and BCR calling using same patient samples from Aim 1.1 n=9 patients with 3 time points n=27 tumor samples total Sequencing completed and immunologic analyses underway.	6-12	MGH/Broad (Boland and Kellis Labs)
Multiplexed immunofluorescence using same patient samples (Aim 1.1) n=9 patients with 3 time points n=27 tumor samples total Initial pilot project completed and published (Liu D… Boland GM, Nat Med, May 2021). Multiplex imaging underway for the remaining patient samples. The imaging data analysis is non-trivial and required significant optimization for the initial pilot project. The pipelines now exist and will be utilized to move the remaining samples forward in year 3.	6-12	6-12 MGH (Boland and Stott Labs)

Aim 2: utilizes circulating exosomal RNA to identify, monitor tumoral RAAs		
Aim 2.1 – Bulk exosomal analysis.		
Exosome RNA isolation, paired samples to match tumors in Aim 1 n=9 patients with 3 time points n=27 plasma samples total Samples submitted to pair with pilot patient study (Liu D… Boland GM, Nat Med, May 2021). Bulk transcriptomics completed and analysis underway.	6-9	MGH (Boland Lab)
RNAseq n=9 patients with 3 time points n=27 plasma samples total Samples submitted to pair with pilot patient study (Liu D… Boland GM, Nat Med, May 2021). Bulk transcriptomics completed and analysis underway.	9-12	MGH/Broad (Boland)
Comparison with tumor data n=9 patients with 3 time points n=27 tumor samples total Integrative analysis underway for pilot patient study (see above), pairing deep tumor characterization with bulk EV analysis. Once this pilot completed, other samples will be sequenced and analyzed.	12-18	MGH/Broad (Boland and Kellis Labs)
Aim 2.2 – Cell-specific exosome selection.		
Deconvolution of bulk exosome signals into tumor/immune components n=9 patients with 3 time points	12-15	MGH/Broad

n=27 plasma samples total Awaiting sequencing results. Pilot analysis of existing datasets underway.		(Boland and Kellis Labs)
Cell-specific exosome capture Melanoma cell lines: A375 (BRAF/MEKi sensitive and resistant), RPMI 7951, MeWo, SkMel30 Other cell lines: T cells (Jurkat E.61), B cells (RPMI-1788), Megakaryocyte (MEG-01), and Fibroblast (SV40) Experiments are underway utilizing cell line specific exosome capture. This technology was the foundation for an NIH U18 award (PIs: Stott, Boland) utilizing circulating EV for COVID-19 detection and monitoring changes in the immune microenvironment.	15-24	MGH (Boland and Stott Labs)
Focused sequencing Cell line-derived exosomes to confirm selective capture Pilots on cell lines and n=5 patient samples completed.	15-24	MGH (Boland and Stott Labs)
RNAseq n=9 patients with 3 time points n=27 plasma samples total We are awaiting optimization of Aim 2.2 methods prior to running patient samples. Samples from n=5 pilot available, but characterized by ddPCR thus far.	24-36	MGH/Broad (Boland; analysis Boland, Stott, and Kellis Labs)
Aim 2.3 – Modeling of ICI response.		
Predictive modeling using bulk exosomal RNA data n=9 patients with 3 time points n=27 plasma samples total Pending	15-20	MGH/Broad (Boland and Kellis Labs)
Predictive modeling using selected exosomal RNA data Training set from cell lines (Aim 2.2) Patient samples: n=9 patients with 3 time points, n=27 plasma samples total Pending	24-36	MGH/Broad (Boland, Stott, and Kellis Labs)
Aim 3: functionally validate EV-derived transcripts and proteins		
Aim 3.1 – RNA and protein modulation		
In vitro cell culture Melanoma cell lines: A375 (BRAF/MEKi sensitive and resistant) RPMI 7951 MeWo SkMel3	12-24	MGH (Boland Lab)
Candidate overexpression and/or knockdown miRNA miRNA4454 miRNA548A3 miR4472-2 miR4664 Protein PD-L1 HLA-A/B This work has begun and data will be included below.	12-24	MGH (Boland Lab)
In vitro assays – cell proliferation, wound-healing, transwell invasion Melanoma cell lines: A375 (BRAF/MEKi sensitive and resistant)	12-24	MGH (Boland Lab)

RPMI 7951 MeWo SkMel3 Overexpression/knockout of candidates (above) in a subset of the melanoma cell lines, starting with A375 sensitive/resistant cell lines Efforts ongoing.		
Aim 3.2 – Exosome and immune cell interaction		
Normal blood collection, T cell isolation/expansion n=5 health donor collections	12-24	MGH (Boland Lab)
T cell activation n=5 health donor collections	12-24	MGH (Boland Lab)
Exosome treatment Melanoma cell lines: A375 (BRAF/MEKi sensitive and resistant) RPMI 7951 MeWo SkMel3 1. Overexpression/knockout of candidates in a subset of melanoma cell lines, starting with A375 sensitive/resistant cell lines 2. Isolation of exosomes derived from overexpressing/knockout cell lines 3. Comparison of miRNA/protein expression between cell line and exosomes 4. If concordant (i.e. the overexpression or knockout of cellular expression is reflected in changes in exosome expression), exosomes harvested and used to treat non transfected/infected cell lines (parental) to assess for phenotypic changes Efforts ongoing.	12-36	MGH (Boland Lab)
RNA and protein analysis 1. Assess changes in gene/protein expression in exosome treated tumor and immune cells Efforts ongoing. Manuscript in preparation.	24-36	MGH (Boland Lab)

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Aim 1.1 and Aim 1.2: Patients/samples have been identified and submitted for WES, RNAseq, and ATACseq for all patients. The samples for WES/RNAseq are being analyzed. ATACseq samples were submitted to the MIT Core, but we are assessing how many samples from each data set overlap. There may be under-representation of this patient cohort in the ATACseq data. Efforts are ongoing to identify samples that have not yet been submitted for analysis.. The FFPE blocks that pair with the samples submitted for sequencing are available, but have not been cut/sent for imaging yet.

Our initial longitudinal analysis has now been published in Nature Medicine: https://www.nature.com/articles/s41591-021-01331-8 and was used to establish analytical pipelines. The process development for the imaging analysis was significant and was done in collaboration with our collaborator, Peter Sorger. We are now expanding the analysis and will include these samples during year 3.

Aim 2.1: Plasma samples from the patients identified in Aim 1.1/1.2 have been allocated for the experiments in Aim 2.1 and 2.2. Bulk EV have been isolated from n=15 timepoints from patient in Nature Medicine manuscript, RNAseq completed, integrative analysis with tumor sequencing underway.

Aim 2.2: Cell specific Exosome/Extracellular Vesicle (EV) Capture.
We have utilized Ab capture of tumor and immune cell line derived EVs and show promising results quantified by qPCR. We were awarded an NIH U18 grant (PIs: Boland, Stott) to use similar techniques for assessment of SARS CoV-2 and immune EV capture. These efforts are maturing. We ran a pilot of n=5 patient samples and have early signs of successful tumor and immune EV. Expansion to longitudinal samples will occur in year 3 once techniques fully validated.

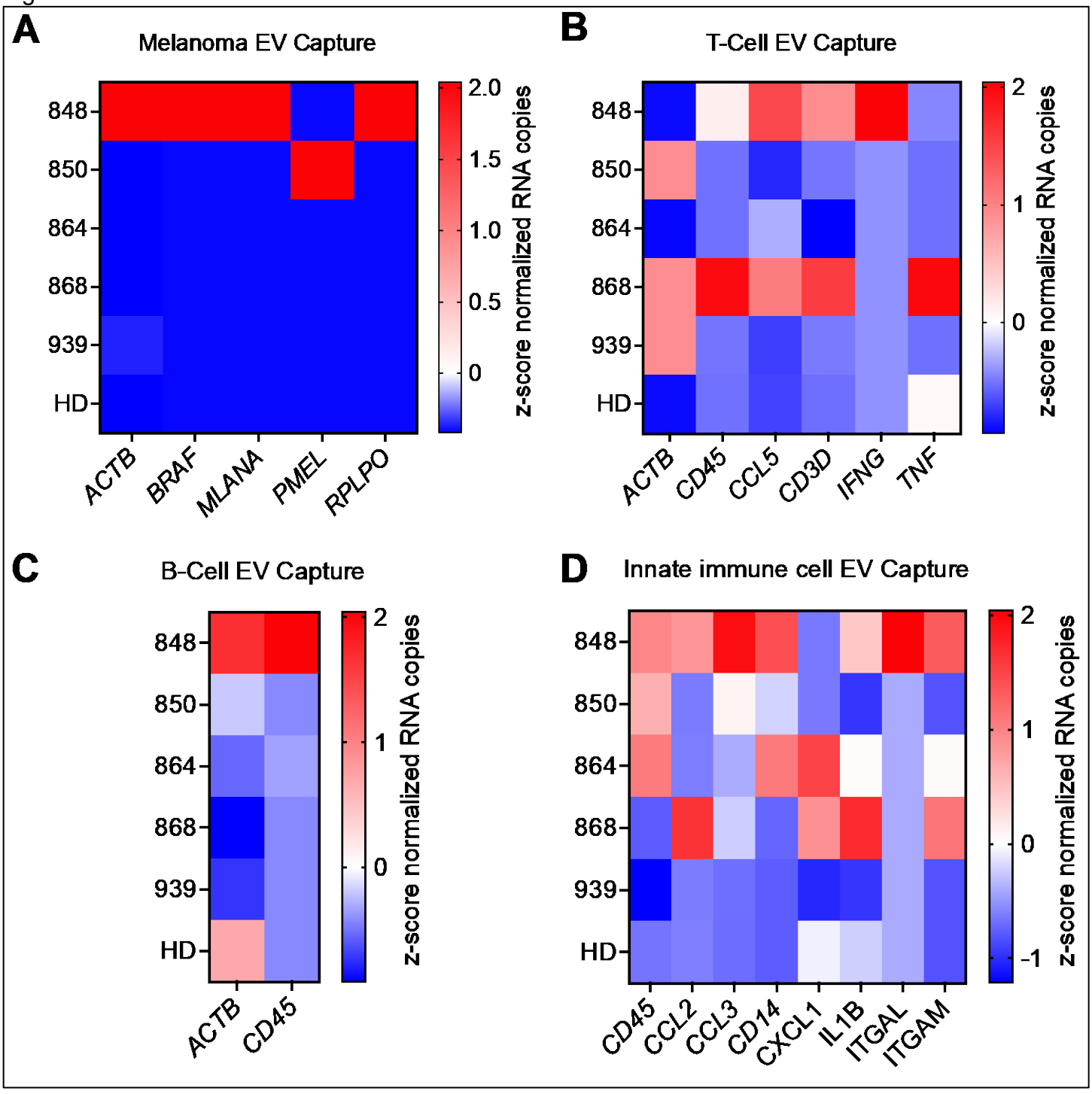
Aim 3.1: Functional validation of EV-derived miRNA and/or PD-L1 and HLA-A/B.
This work has been ongoing and data will be included below. Since the last annual report, we further validated a candidate miRNA found to be implicated in targeted therapy resistance and analyzed EV and cell line RNAseq. Putative targets identified and validated in sequencing data (included below).

Aim 1: integrative analysis of serial tumor samples during ICI for genomic/epigenomic analysis with immune profiling.

Liu D, Lin JR… **Boland GM**. Evolution of delayed resistance to immunotherapy in a melanoma responder. Nature Medicine, May 2021.
https://www.nature.com/articles/s41591-021-01331-8

Aim 2: utilizes circulating exosomal RNA to identify, monitor tumoral RAAs.
We are using the EV-Chip for cell-specific EV capture. Cell line data and pilot patient data below.

Figure 2:



Aim 3: functionally validate EV-derived transcripts and proteins

This Aim builds upon our now-published data suggesting a role for EV transcriptional profiles in predicting/monitoring ICI response and emergence of RAAs.

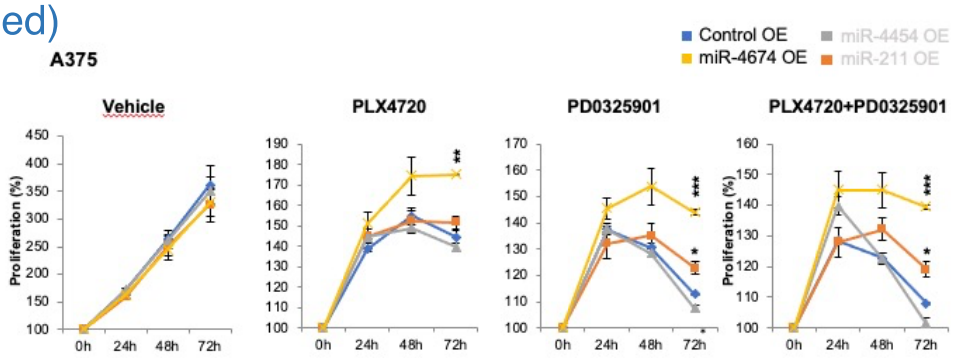
Shi A…. Kellis M, **Boland GM**. Plasma-derived extracellular vesicle analysis and deconvolution enable prediction and tracking of melanoma checkpoint blockade outcome. Sci Adv, Nov 2020.

https://advances.sciencemag.org/content/6/46/eabb3461.abstract

Several miRNA were nominated from this analysis and have been now been validated in vitro. miR-4674 overexpression induces resistance to BRAF- and MEK-inhibition in vitro (Figure 1). EV from miR-4674 OE cells were used to treat non-OE cells and demonstrated increased expression of miR-4674 and increased cell viability during targeted therapy treatment (Figure 2). We next used paired A375-S and A375-Resistant cell lines to examine RNA expression in cells & EV, assessing if putative miR-4674 targets were enriched in EV. We found that FAT3 transcripts (a predicted miR-4674 target) are higher in A375-S EV (Figure 3). We next used the RNAseq data sets from the paper (Shi et al, above) to assess if FAT3 transcripts were higher in ICI responders, finding that pre-treatment FAT3 expression in EV correlated with response to ICI (Figure 4). FAT3 is a modulator of YAP-phosphorylation and may have a tumor suppressor role in melanoma (Figure 5). Longitudinal EV transcriptional signals were assessed and FAT3 persistence was seen in ICI responders (Figure 6).

Figure 1:

Overexpression of miR-4674 induce proliferation and increased viability (data unpublished)



miR-4674 transiently over-expressed in A375 and subjected to proliferation experiments. Viability assays to MAPK inhibitors were performed with miR-4674transfectants for the time points indicated.

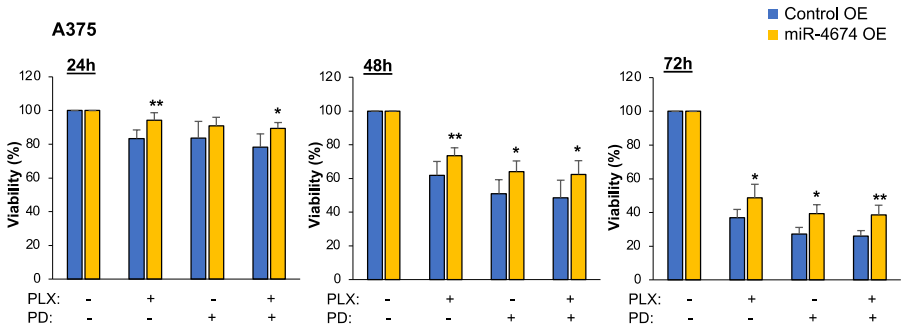
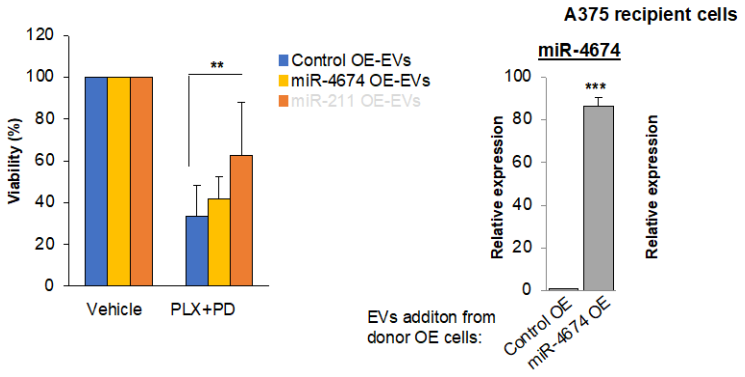


Figure 2:

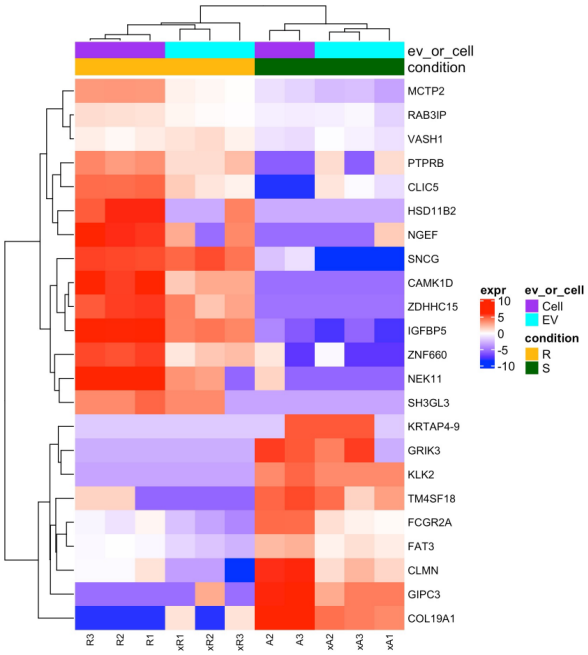
- miR-4674 overexpressing cells were generated
- EV were isolated and were used to treat A375 cells
- Viability assays were performed after 48h of treatment with MAPKi



- miRNA are upregulated in melanoma cell lines in the setting of targeted therapy & provide a survival advantage when delivered to other melanoma cells via cancer-derived EVs

Figure 3:

Using PLX resistant melanoma model and their EV compared to sensitive cell lines and EV, we identify 9 targets genes that were significantly overexpressed in the sensitive group



Are some miR-4674 target-genes significantly overexpressed in the sensitive samples?

Targetscan ∩ **miRDB** ∩ **miRmap** 223

23 genes with p<0.05 (Mann–Whitney–Wilcoxon test),
There is a tumor suppressor gene **FAT3**
FAT3 is overexpressed in sensitive cells and EV
p-value = 0.008 (Wilcoxon rank sum exact test)

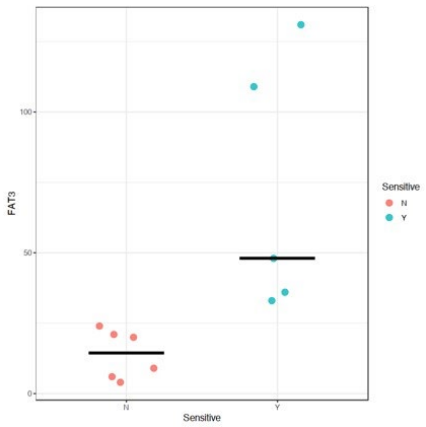


Figure 4:

Are these targets genes important also in the IO treatment setting?
High FAT3 baseline level associated with Response to ICB treatments

We looked at a cohort of RNAseq and Microarray from Exosomes of Metastatic melanoma patient pre-treatment with ICB **71 samples** (platform and batch corrected with ComBat)

	W	p value
GRIK3	685.00	0.28583
KLK2	553.50	0.63565
TM4SF18	585.00	0.92036
FCGR2A	571.00	0.78985
FAT3	772.00	0.03490
CLMN	713.00	0.16146
GIPC3	612.00	0.83689
COL19A1	640.00	0.59226
MIR4674	744.00	0.07651

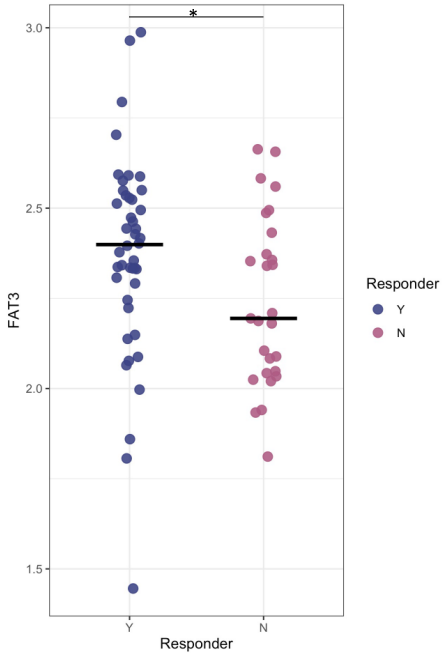
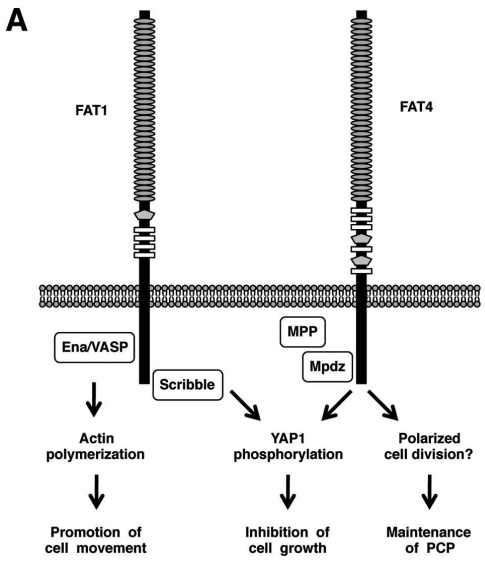


Figure 5:

FAT signaling cascades induce YAP1 phosphorylation which is involved in inhibition of cell growth



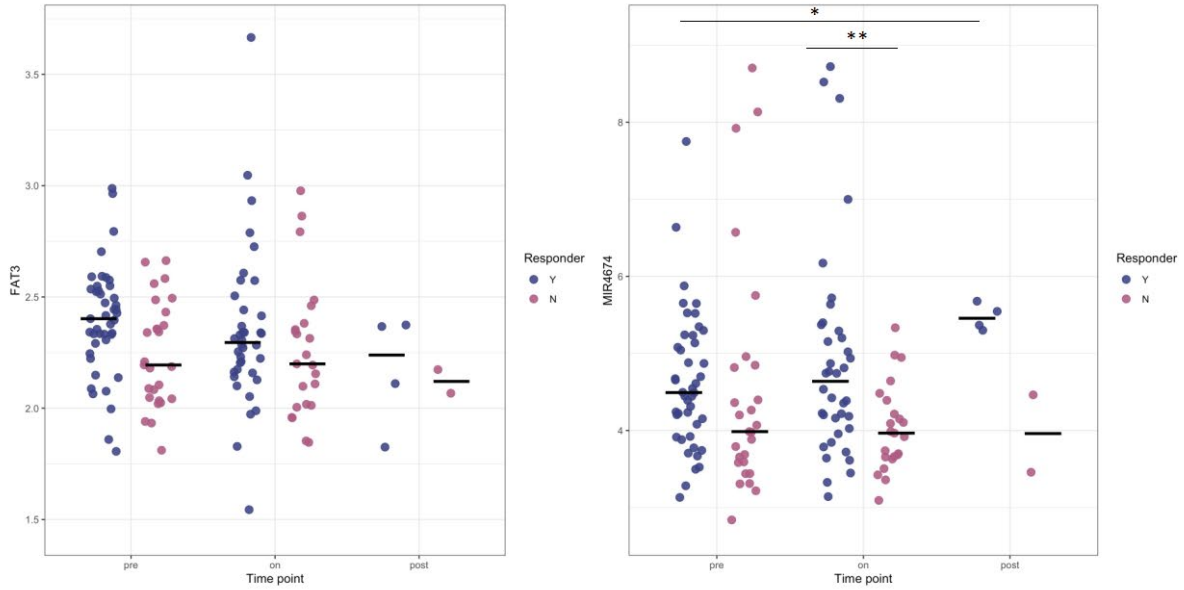
B FAT genes alterations observed in several oncological settings

FAT	Human cancers	Omics alterations and cancer biology
FAT1	Oral cancer	Homozygous deletion & mRNA downregulation Promoter hypermethylation & mRNA downregulation
	Astrocytoma and glioblastoma	Loss of heterozygosity
	Breast cancer	mRNA & protein downregulation in invasive cancer
	Melanoma	Aberrant processing of FAT1 protein
	Leukemia	mRNA upregulation in AML, preB-ALL & T-ALL Poor prognosis in preB-ALL with *FAT1* upregulation
	Pancreatic cancer	Point mutation
FAT2	HNSCC	Point mutation
FAT3	Pancreatic cancer	Point mutation
FAT4	Breast cancer	Promoter hypermethylation & mRNA downregulation
	Lung cancer	Promoter hypermethylation & mRNA downregulation
	Pancreatic cancer	Point mutation
	HNSCC	Point mutation
	HCC	Point mutation
	Melanoma	Point mutation
	Gastric cancer	Loss of heterozygosity & point mutation

Katoh M: Function and cancer genomics of FAT family genes (Review) . Int J Oncol 41: 1913–1918, 2012

Figure 6:

Are the Exosomes level of FAT3 and miRNA4674 affected by the ICB treatment? FAT3 level in EV of responder patient is decreasing with the treatment instead miRNA4674 is significantly increasing



miRNA4674 on treatment is significantly higher in responder patient

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

I co-coordinated a Department of Surgery Grant Writing Program Jan 2020 in conjunction with surgical leaders at Brigham and Women's Hospital.

I completed a course at MGH: "Bridging Academia with Industry" where researchers create teams and work with industry partners/venture capital to build projects and/or ideas with potential for marketing and start-up opportunities.

2020	Characterizing the Hippo/YAP Pathway in Melanoma Immunotherapy Invited Lecturer. Melanoma Research Foundation (MRFBC). Virtual Presentation.
2020	Evidence-Based Management of Melanoma Invited Lecturer. Madigan Army Medical Center, Hospital Grand Rounds. (Virtual).
2020	Evidence-Based Management of Melanoma Invited Lecturer. Oregon-Washington ACS Symposium. Virtual Presentation and Tumor Board.
2020	Multi-Modality of Melanoma Immunotherapy Response and Resistance Invited Lecturer. Immuno-Oncology Summit, Boston, MA (Virtual)
2021	Creating a Career as a Surgeon Scientist Invited Lecturer. Jefferson MD/PhD Mentorship Seminar. Thomas Jefferson University, Philadelphia, PA (Virtual)
2021	Surgical Management in Melanoma: Where are We Now and Where are We Going Invited Lecturer. International Symposium on Melanoma and Other Cutaneous Malignancies. New York, NY (Virtual)
2021	Surgical Management in Melanoma: Where are We Going? Invited Lecturer. American College of Surgeons Cancer Program (Virtual)
2021	Multi-Modality Approach to Predicting and Monitoring Melanoma Immunotherapy Response and Resistance. Invited Lecturer. Molecular Med Tri-Con Conference & Expo (Virtual)
2021	What's Old is New Again: Transformative Change in the Management of Melanoma. Visiting Professor. Department of Surgery Grand Rounds. Johns Hopkins (Virtual)
2021	Protein Biomarkers in Plasma: Revealing Biological Insights into the Tumor Microenvironment. Invited Speaker. Cell and Gene Therapy Insights (Webinar)

International

2020	Blood Based Biomarkers of Immunotherapy Response and Resistance Invited Lecturer. Liquid Biopsy Congress, United Kingdom (Virtual).
2020	Using Proteomics and Multiplex Immunofluorescence to Enhance Immunotherapy. Invited Lecturer. Flow-Cytometry & Multiplexing Tools Symposium, United Kingdom (Virtual).

2020	Multimodality Approach to Predicting and Monitoring Immunotherapy Response and Resistance. Invited Lecturer. Symposium on Biomarkers in Immunotherapy. Bergen, Norway (Virtual)

The projects referenced above were presented in a variety of forums (local, national, international) for discussion and to create opportunities for collaboration.

There were also efforts to target groups of interest (e.g. Liquid Biopsy Congress, UK).

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We now have the majority of data in hand (aside from some imaging data to be generated). The goal for this year is to analyze and integrate the data sets. We have created these types of pipelines and learned how to optimize this process for the Nature Medicine paper, and I'm optimistic we can complete the projects proposed.

I am also focused on publication of generated data to support additional funds for further research.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have now successfully published the first patient data in this series (Liu D, Lin JR… Boland GM, Nat Med May 2021) – creating pipelines to integrate bulk and single-cell resolution sequencing with single-cell resolution imaging. The goal would be to recapitulate this type of longitudinal analysis with more patients and integrate the EV analysis.

Simultaneously, we have improved our cell-specific EV capture and obtained funding for further technology development in the setting of monitoring COVID-19 infection (NIH U18). We will move forward with more patient samples during this final year of funding.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The techniques arising from this work will have impact on a variety of other specialties/disciplines including novel computational techniques (with the Kellis Lab at MIT) and with bioengineering (with the Stott Lab at MGH). The immune EV capture was repurposed in the setting of the COVID-19 pandemic, and Dr. Stott and I obtained an NIH U18 grant.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

While the technological development here may not be patentable, the analytical approaches are amenable to licensing agreements, and we have been working with Partners Innovation on the marketing/dissemination of these novel analytical approaches.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

While we are a few steps away from conversion of this type of approach to a CLIA environment, we aspire to create a reproducible EV platform that could eventually be used clinically.

We are also focused on using these studies for education of the community regarding immunotherapy response and resistance.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

There have been no major changes to the scope of the proposed project. There were some experimental delays to the COVID-19 pandemic, but we are back and functioning at full capacity.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

One major issue in our proposed experimental timeline relates to delays in data generation/analysis due to the COVID-19 pandemic. I doubt this is unique to our project, but I think we are back on track for completion of the proposed work on time.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Not applicable.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Liu D, Lin JR, Robitschek E, Kasumova GG, Heyde A, Shi A, Kraya A, Zhang G, Moll T, Frederick DT. Chen YA, Wang S, Schapiro D, Ho LL, Bi K, Sahu A, Mei S, Miao B, Sharova T, Alvarez-Breckinridge C, Stocking J, Kim T, Fadden R, Lawrence D, Hoang MP, Cahill DP, Malehmir M, Nowak MA, Brastianos PK, Lian CG, Ruppin E, Izar B, Herlyn M, Van Allen E, Nathanson K, Flaherty KT, Sullivan RJ, Kellis M, Sorger PK, **Boland GM**. Evolution of delayed resistance to immunotherapy in a melanoma responder. Nature Medicine. May 2021.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Not applicable.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Previously reported.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Not applicable.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

We continue to optimize cell-specific EV capture approaches and novel EV related computational approaches.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

We are working to identify licensing opportunities for analytical and computational approaches generated through this work.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

We are creating a dataset/database of paired tumor/EV analysis
Our software, modeling approaches are novel to EV based datasets
We are generating and validating novel EV cell-specific selection approaches

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Other Support
Genevieve M. Boland

ACTIVE

Adelson Foundation Fund (PI: Flaherty) 10/01/2014 – 09/31/2021 16.7% effort
Adelson Medical Research Foundation
Combination approaches to overcome resistance to targeted therapy in melanoma
The aim of this project is to collaborate with other funded researchers in the Adelson Program in Cancer Research to understand mechanisms of resistance to combination treatment regimens including signal transduction inhibitors and immunotherapy using tumor biopsy samples and other tissues from patients enrolled on 10 different clinical trials investigating novel melanoma therapeutics.

1R01CA214744-01A1 (PI: Mahmood) 08/01/2017-07/31/2022 5% effort NIH
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Cytotoxic lymphocyte function PET Imaging to predict cancer immunotherapy response
We propose an imaging approach to measure cytotoxic lymphocyte function within a tumor as a new imaging paradigm for tumoral response evaluation to immune modulators.

1 U54 CA225088-01 (Sorger) 03/08/2018-02/28/2023
1% effort
NIH/NCI
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Systems Pharmacology of Therapeutic and Adverse Responses to Immune Checkpoint and Small Molecule Drugs
This award established a Center for Cancer Systems Pharmacology that applies network-level computational models informed by multi-omic phenotyping of patient-derived specimens to understand mechanisms of drug response, resistance and toxicity for targeted small molecule drugs and immune checkpoint inhibitors in melanoma, triple negative breast cancer and brain cancers.

1R01CA229851 (PI: Sullivan, Sharpe) 05/17/2018 – 04/30/2022 5% effort
NIH/NCI (MGH)
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Abbreviated targeted therapy to improve anti-PD-1 inhibitor efficacy in melanoma
This project will determine the effectiveness of abbreviated mitogen activated protein kinase (MAPK)-targeted (MTT) therapy combined with anti-PD-1 therapy, identify patients most likely to benefit, determine the effects of this therapy on the tumor microenvironment and immune memory subsets, and identify novel candidate targets to combine with MTT and anti-PD1, based on an in vivo CRISPR screens in melanoma mouse models.

5U2CCA233195-03 10/01/18 – 10/01/2023 2% effort
NCI
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Human Tumor Atlas Network/U2C Grant – The Cellular Geography of Therapeutic Resistance in Cancer
The goal of this project is to generate a multi-disciplinary effort to characterize and catalogue human tumors under a variety of clinically-relevant conditions.

No Award No. (Boland, Saladi, Liu) 04/01/2019 – 06/30/2021 1% effort

Melanoma Research Foundation Breakthrough Consortium (MRFBC)
MRF1420 K St NW Fl 7 Washington DC 20005-2500
Young Investigator Research Team Award to Advance the Field of Translational Immuno-Oncology
Characterizing the Role of the Hippo Pathway during Melanoma Immunotherapy
The goal of this project is to analyze and characterize the role of Hippo/YAP signaling during treatment of melanoma patients with immunotherapy and identify novel therapeutic combinatorial strategies.

W81XWH1910143 (Boland) 05/15/2019-05/14/2022 15% effort
Department of Defense
DOD 1077 Patchel Street, Fort Detrick, MD, 21702-5024; 301-619-7071
Genomic and immunologic correlates of immunotherapy response and resistance via longitudinal tumor and extracellular vesicle (EV) analysis.
The goal of this project is to analyze longitudinal samples from patients treated with immunotherapy for melanoma to characterize the interplay of genetic, immunologic, and blood-based markers of response and resistance.

2PO1 CA163222-06 (Fisher) 08/06/2019 – 08/06/2022 1% effort

NIH
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
T*argetable epigenetic and transcriptional mechanisms in melanoma that shape the microenvironment.*
The goal of this project is to characterize epigenetic regulators of melanoma metastasis and therapy response.

1R01CA240299-01A1 (Miller) 09/01/2020 – 05/31/2025 5% effort
NIH
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Statistical methods for cancer genomics and cell-free DNA analysis
The main objective of the proposed project is to develop and test a flexible suite of statistical methods for cancer detection and analysis using cfDNA sequencing data at low tumor fractions. Our central hypothesis is that structured probabilistic models of genomic signals of cancer in cfDNA data, along with careful handling of errors and biases, will enable cancer detection and classification with high sensitivity and specificity.

1U18TR003793-01 (Stott, Boland) 11/30/20 - 11/29/22 5% effort
NIH
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Microfluidic isolation and characterization of SARS-CoV-2 and virus-related exosomes
We will repurpose our existing exosome microfluidic isolation technology to measure viral loads in plasma, saliva, and stool. Once validated, we will bring our technology to a clinical pathology lab to collect data for FDA certification.

No Award No. (Perez) 07/01/20-06/30/2021

Completed:

Partners Innovation Development Grant: Boland (PI) 04/01/2019 – 04/01/2020
Deconvolution of circulating exosomal RNA signatures in melanoma immunotherapy
The goal of this project is to utilize artificial intelligence to deconvolve and selectively enrich microvesicles from patient plasma. I am directly responsible for sample acquisition, data collection and analysis

Pending:

NIH R01 RCA257508A
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
PI (Kellis, Boland, Rai) 07/01/2021 – 06/30/2026 10% effort
Single-cell epigenomic-transcriptomic tumor-immune functional dissection of melanoma immunotherapy
The goal of this proposal is to use cutting scRNAseq, snATACseq, and functional perturbation to investigate epigenetic regulation of tumor-immune interactions.

DF/HCC Cancer Immunology Program Immunotherapy Toxicity SPORE
Core project Co-PI (Villani, Elledge, Boland) 08/01/21 – 07/31/2026 0% effort
Decoding Immune Networks, T Cell Clonotypes, and Biomarkers Associated with irAEs

The goal of this project is to identify the phenotype of T ccelllel repertoires in irAEs, characterize the antigen-TCR specificities of irAE TIL, and assess circulating biomarkers for patients at risk for irAE.

Department of Defense Melanoma Research Program Translational Research Award with Collaborator Option
DOD 1077 Patchel Street, Fort Detrick, MD, 21702-5024; 301-619-7071
Targeting Epigenetic Modifiers of Phenotype Switching in Targeted Therapy-Resistant Melanoma
(Sub BU- Wu) 07/01/21-06/30/24 0% effort

Name: *Alvin Shi*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Mr. Shi contributed to EV data analysis under the guidance of Dr. Manolis Kellis*

Funding Support: *NSF Graduate Research Fellowship (Award #2016226995).*

Name: *Marta Diaz Martinez*
Project Role: *Postdoc*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *9*
Contribution to Project: *Dr. Diaz Martinez worked on the in vitro miRNA analysis*
Funding Support: *Alfonso Martin Escudero Foundation Fellowship Award*

Name: *William Michaud*
Project Role: *Staff Scientist*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Dr. Michaud worked on the in vitro EV analysis relating to PD-L1 and HLA-A*
Funding Support: *U54 Systems Pharmacology of Therapeutic and Adverse Responses to Immune Checkpoint and Small Molecular Drugs (50% salary support); 18% from this DoD grant.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Changes to OS 2019 - 2020

1R01CA240299-01A1 (Miller) 09/01/2020 – 05/31/2025
5% effort
NIH
National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892
Statistical methods for cancer genomics and cell-free DNA analysis
The main objective of the proposed project is to develop and test a flexible suite of statistical methods for cancer detection and analysis using cfDNA sequencing data at low tumor fractions. Our central hypothesis is that structured probabilistic models of genomic signals of cancer in cfDNA data, along with careful handling of errors and biases, will enable cancer detection and classification with high sensitivity and specificity.

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Completed:
Partners Innovation Development Grant: Boland (PI) 04/01/2019 – 04/01/2020
Deconvolution of circulating exosomal RNA signatures in melanoma immunotherapy
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Pending:
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Targeting Epigenetic Modifiers of Phenotype Switching in Targeted Therapy-Resistant Melanoma
(Sub BU- Wu) 07/01/21-06/30/24 0% effort

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Stott Laboratory – MGH
Facilities, collaboration
Kellis Laboratory – Massachusetts Institute of Technology
Personnel, collaboration
Broad Institute – sequencing platforms (fee for service)
Liu Laboratory – DFCO
Collaboration

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.