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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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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT: 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.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

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, analysis still underway.	3-6	MGH (Boland Lab)
Exome sequencing n=9 patients with 3 time points n=27 tumor samples total Sequencing completed and analysis 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. Manuscript focused on ATACseq data in preparation, but still not finalized for publication.	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 analysis 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 analysis 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 analysis 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 requires significant optimization for the initial pilot project. Several other individual patient pilot projects underway, but data integration with sequencing data still pending.	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	12-15	MGH/Broad

n=9 patients with 3 time points n=27 plasma samples total Initial data show feasibility of this approach. Have not scaled up to complete patient/time point scale of proposal. However, initial patient pilot studies support utilization of this approach.		(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 utilizing cell line specific exosome capture shows feasibility of this approach. 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 host immune microenvironment. Data presented later.	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. Data included below.	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 Accomplished thus far for initial patient whose tumor data has already been published (Liu D… Boland GM, Nat Med, May 2021). Data included below.	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 Ongoing.	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	12-24	MGH (Boland Lab)

HLA-A/B This work has begun and data will be included below.		
In vitro assays – cell proliferation, wound-healing, transwell invasion Melanoma cell lines: A375 (BRAF/MEKi sensitive and resistant) 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. Current data included below.	12-24	MGH (Boland Lab)
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. Existing data included below.	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 as part of a larger cohort. Individual level longitudinal analysis is planned for after the larger cohort samples have been analyzed.

ATACseq samples were submitted to the MIT Core, and analysis ongoing. There is under-representation of this patient cohort in the ATACseq data. Efforts are ongoing to identify samples that have not yet been submitted for analysis..

FFPE blocks that pair with the samples submitted for sequencing are available, and a small group of them have been sent for pilot studies using multiplex imaging. Due to heavy data analysis requirements, the full set of imaging studies has not been completed.

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. The second phase of samples have been sent with analysis underway. However, the longitudinal paired data

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.

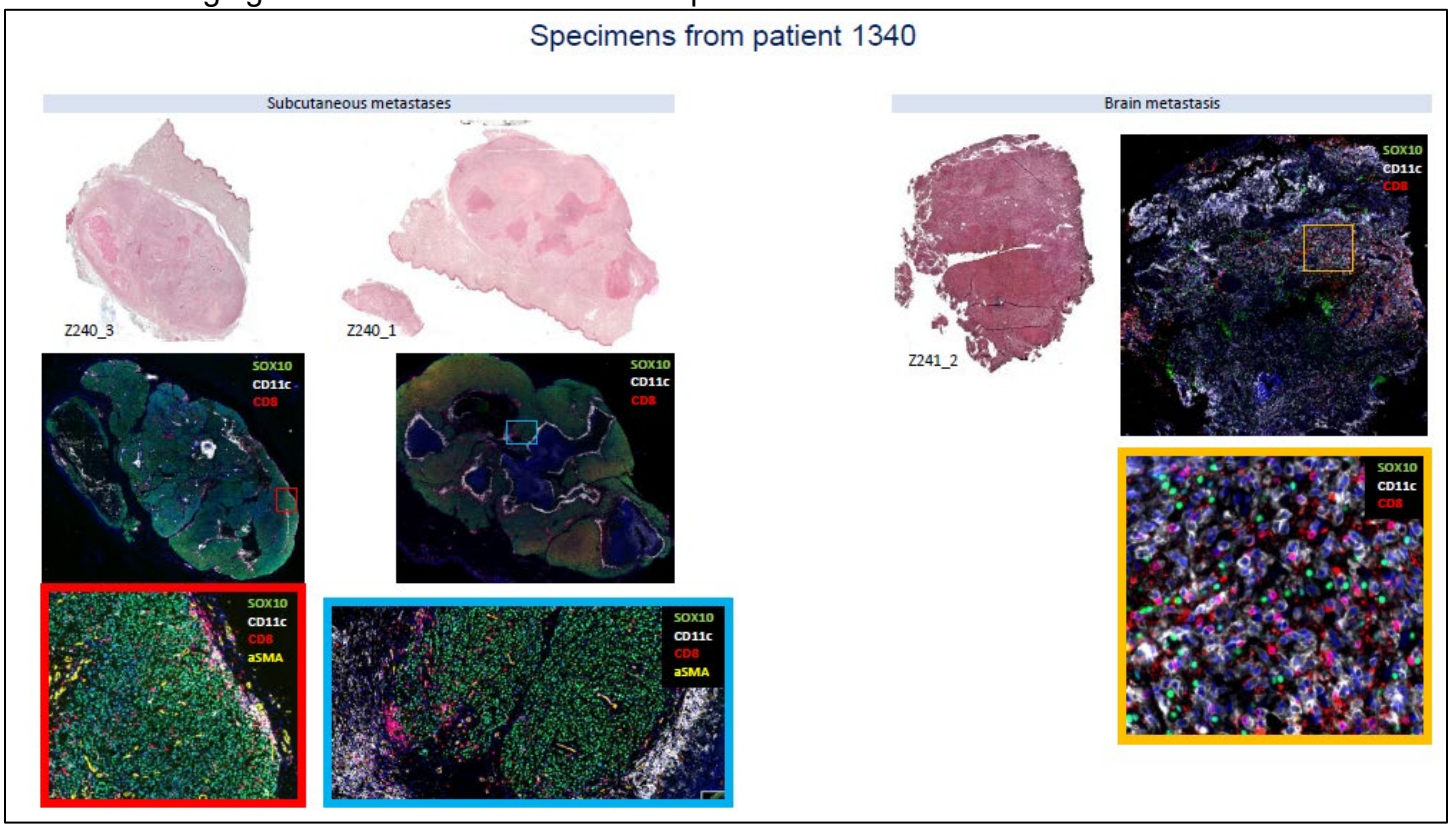
Aim 2.2: Cell specific Exosome/Extracellular Vesicle (EV) Capture.
We have utilized Ab capture of tumor and immune cell line derived EVs and show promising results quantified by qPCR. We were awarded an NIH U18 grant (PIs: Boland, Stott) to use similar techniques for assessment of SARS CoV-2 and immune 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

Additional imaging data included from additional patient:



In addition to the cutaneous melanoma histologies listed in the SOW, we are doing similar longitudinal work for non-cutaneous, mucosal melanoma patients. The workflow arising from the DOD work has been applied to longitudinal analysis of these patient samples.

scRNAseq comparing mucosal melanoma (MM) samples to cutaneous melanoma (CM) samples.

scRNASeq

MM vs CM:

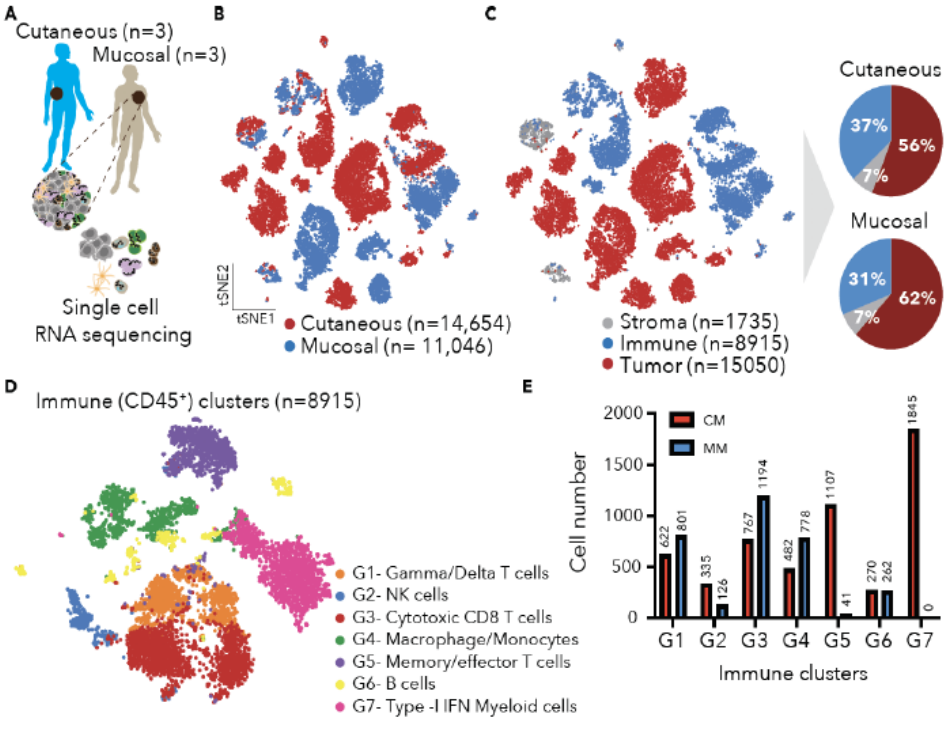
Similar proportion of cell types (tumor, immune, stroma)

Differences in immune cell populations:

- MM has > G4 cluster (macrophage/monocyte)

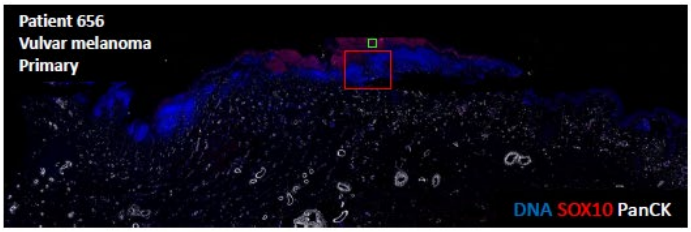
- MM has <<< memory/effector T cells

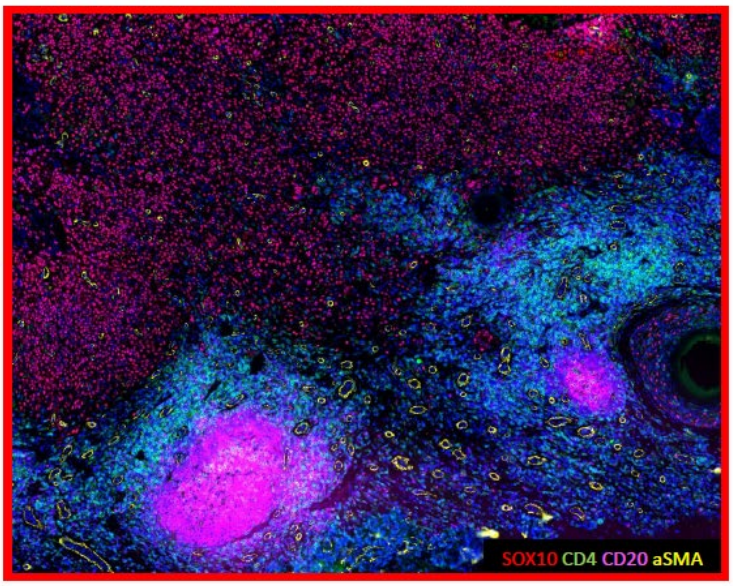
- MM has <<< Type-I IFN myeloid cells



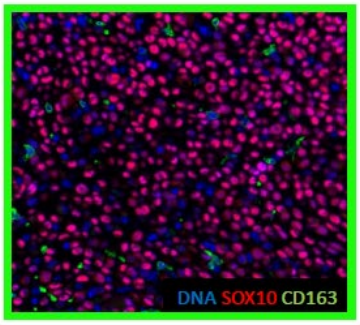
Longitudinal analysis of vulvar melanoma patient (primary tumor, 2 distinct organ-specific metastases)

Vulvar melanoma – primary tumor

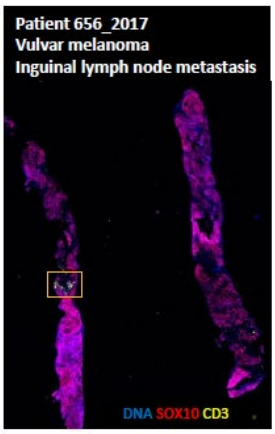


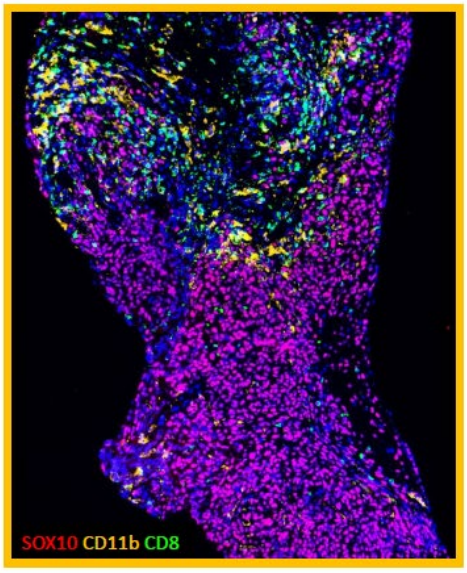


- Primary vulvar lesion
- Abundant CD4+ T cells in stroma
- **Tertiary lymphoid structures**
- Some PDL1+ macrophage infiltration in the tumor, abundant PDL1+ myeloid cells in the stroma

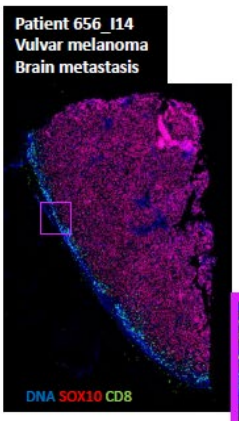


Metastases: site-specific differences

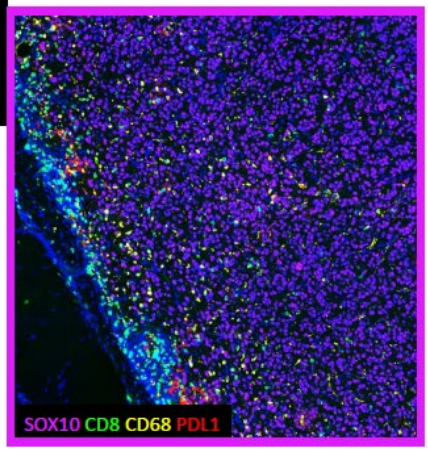




- Inguinal LN biopsy
- Core biopsy
- Macrophages and CD8+ T cells excluded from the tumor



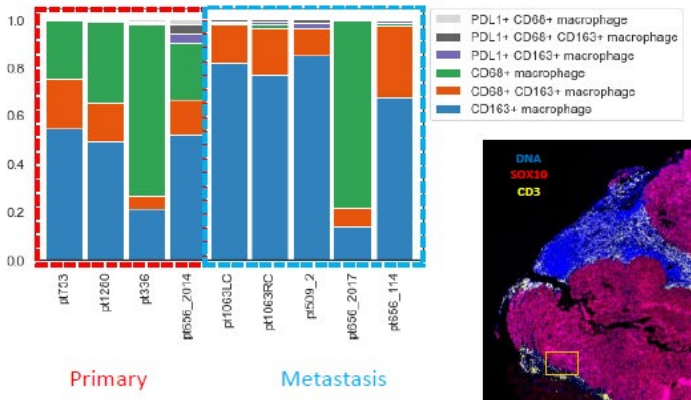
- Some macrophage infiltration
- CD8+ T cells lining the tumor

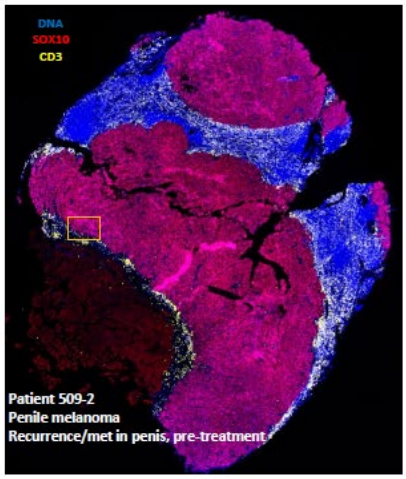


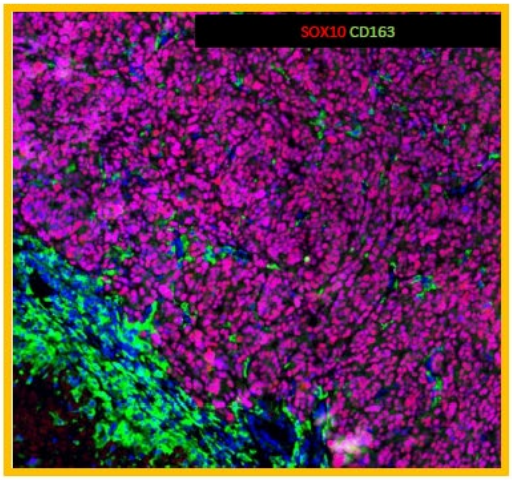
Slide Credit: Sorger Lab (Tuulia Vallius)

Longitudinal imaging within the same patient over time and across primary and metastatic tumors allows assessment of specific immune cell populations.

Myeloid populations



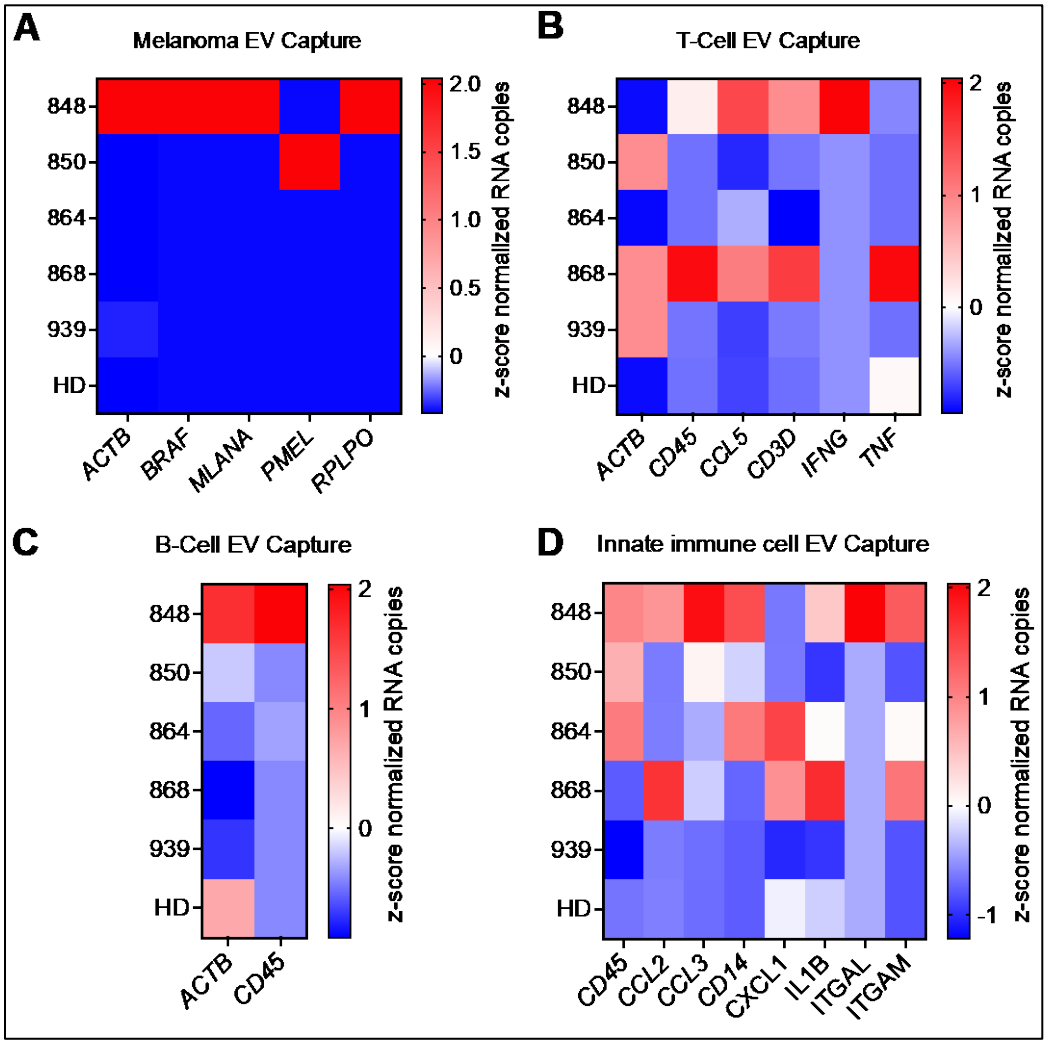




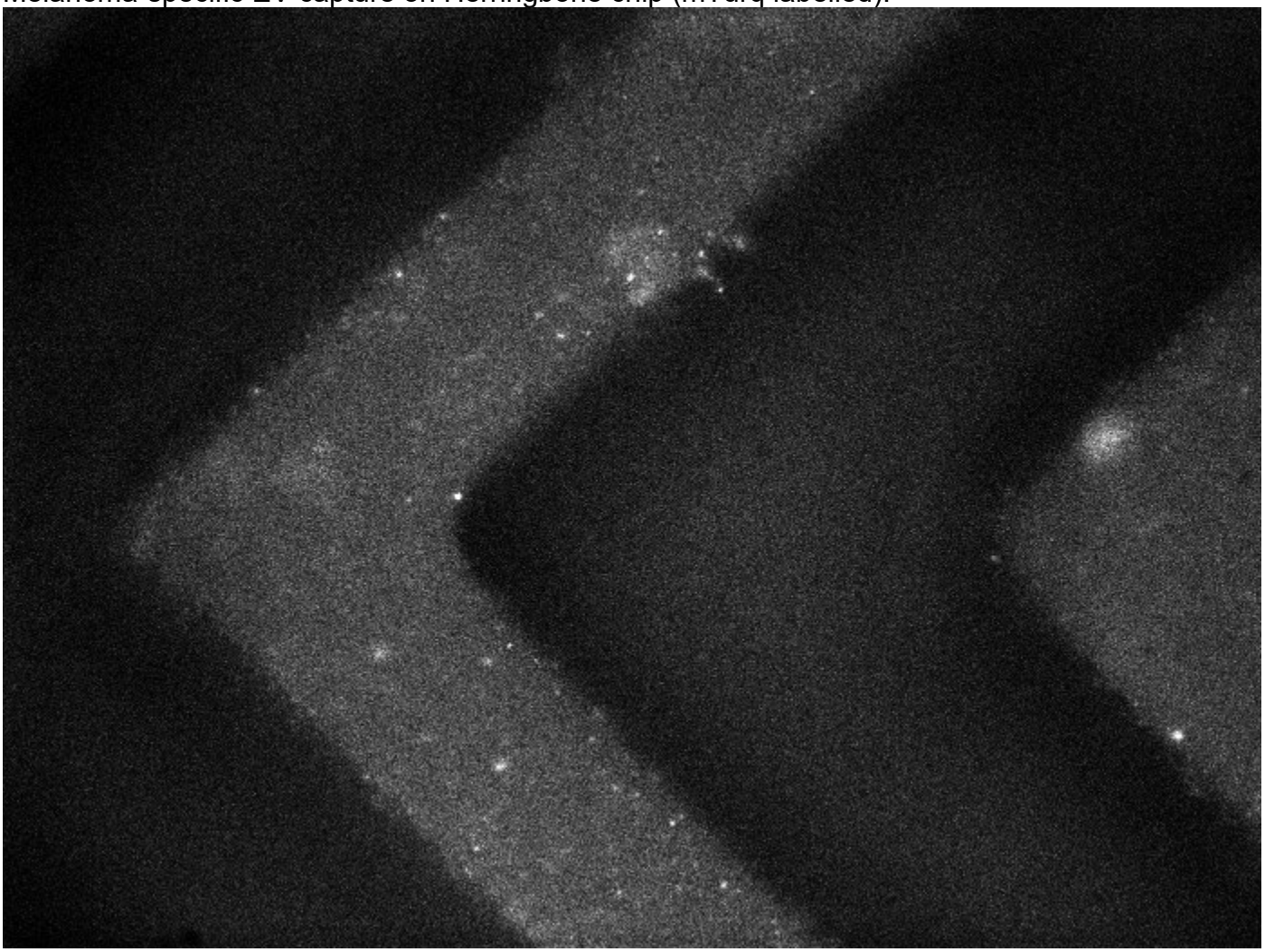
Abundant macrophages lining the tumor, some infiltration into the tumor

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.

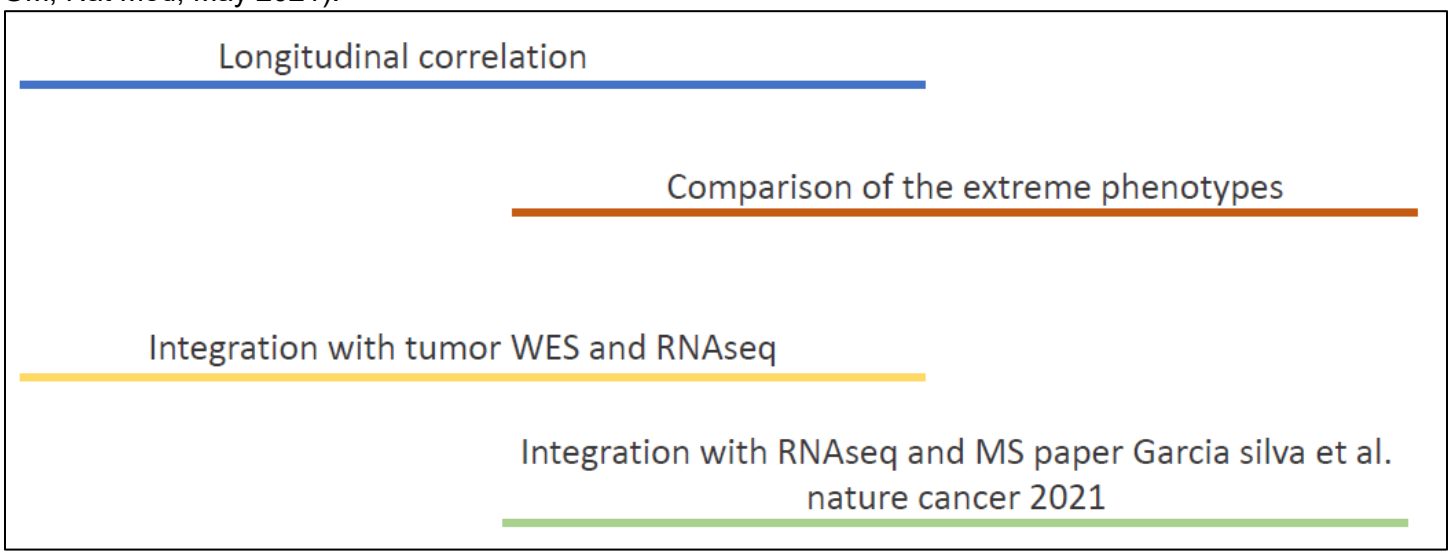
Melanoma and immune-specific EV capture (cell lines and patient samples)



Melanoma-specific EV capture on Herringbone chip (mTurq labelled).

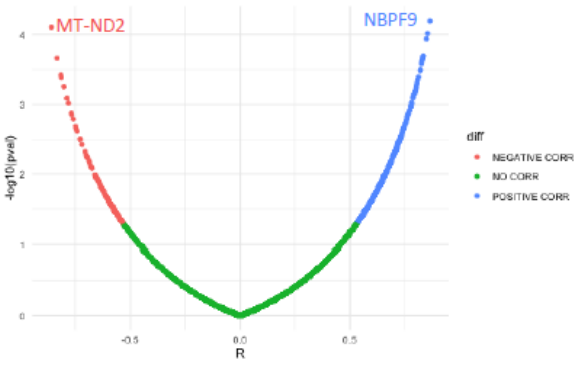


EV specific analysis of bulk EV transcriptomes derived from a highly studied patient (Liu D… Boland GM, Nat Med, May 2021):



Longitudinal changes in EV transcripts. Specifically, negative correlations were seen with B2M, ARID4B, TGFBR2 and positive correlations with AKT2 and NF2 (data below):

Longitudinal changes



1208 coding genes significantly correlated with the time points

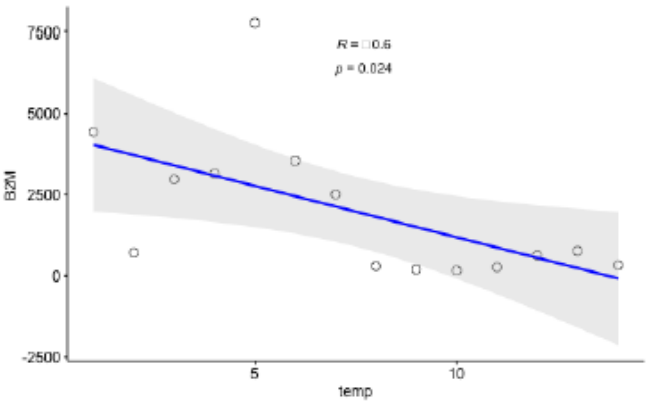
NEGATIVE CORRELATED

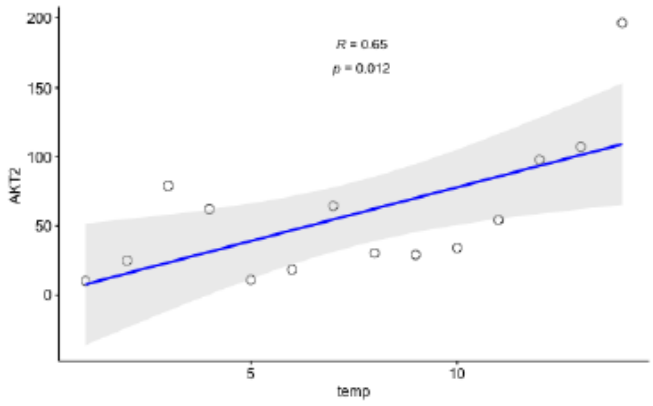
X1	pval	R	diff
ARHGEF28	0.01117412	-0.6540047	NEGATIVE CORR
KTN1	0.01276175	-0.6449313	NEGATIVE CORR
MYH9	0.01451142	-0.6358836	NEGATIVE CORR
MSN	0.01651042	-0.6265143	NEGATIVE CORR
B2M	0.02433117	-0.5965379	NEGATIVE CORR
ARID4B	0.02630303	-0.5901599	NEGATIVE CORR
FLYWCH1	0.02924338	-0.5812835	NEGATIVE CORR
TGFBR2	0.03008625	-0.5788624	NEGATIVE CORR
POLQ	0.03522709	-0.5650956	NEGATIVE CORR
BBC3	0.0366983	-0.5614319	NEGATIVE CORR

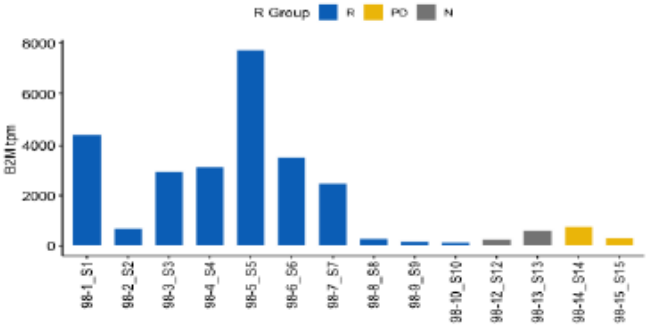
POSITIVE CORRELATED

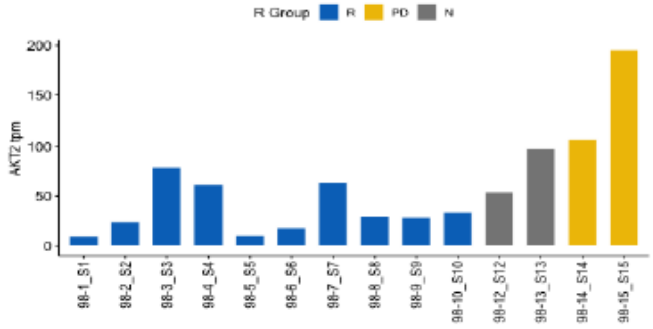
X2	pval	R	diff
WRN	0.003267588	0.7262545	POSITIVE CORR
PHF6	0.004262396	0.7122459	POSITIVE CORR
DCTN1	0.004287609	0.7119258	POSITIVE CORR
BIRC3	0.004414623	0.7103357	POSITIVE CORR
FOXO3	0.004476375	0.7095736	POSITIVE CORR
CREB3L2	0.009190445	0.6968563	POSITIVE CORR
BCL7A	0.009585712	0.6935013	POSITIVE CORR
AKT2	0.01202396	0.6890332	POSITIVE CORR
NF2	0.01212196	0.6884774	POSITIVE CORR
DAZAP1	0.01453698	0.6857578	POSITIVE CORR

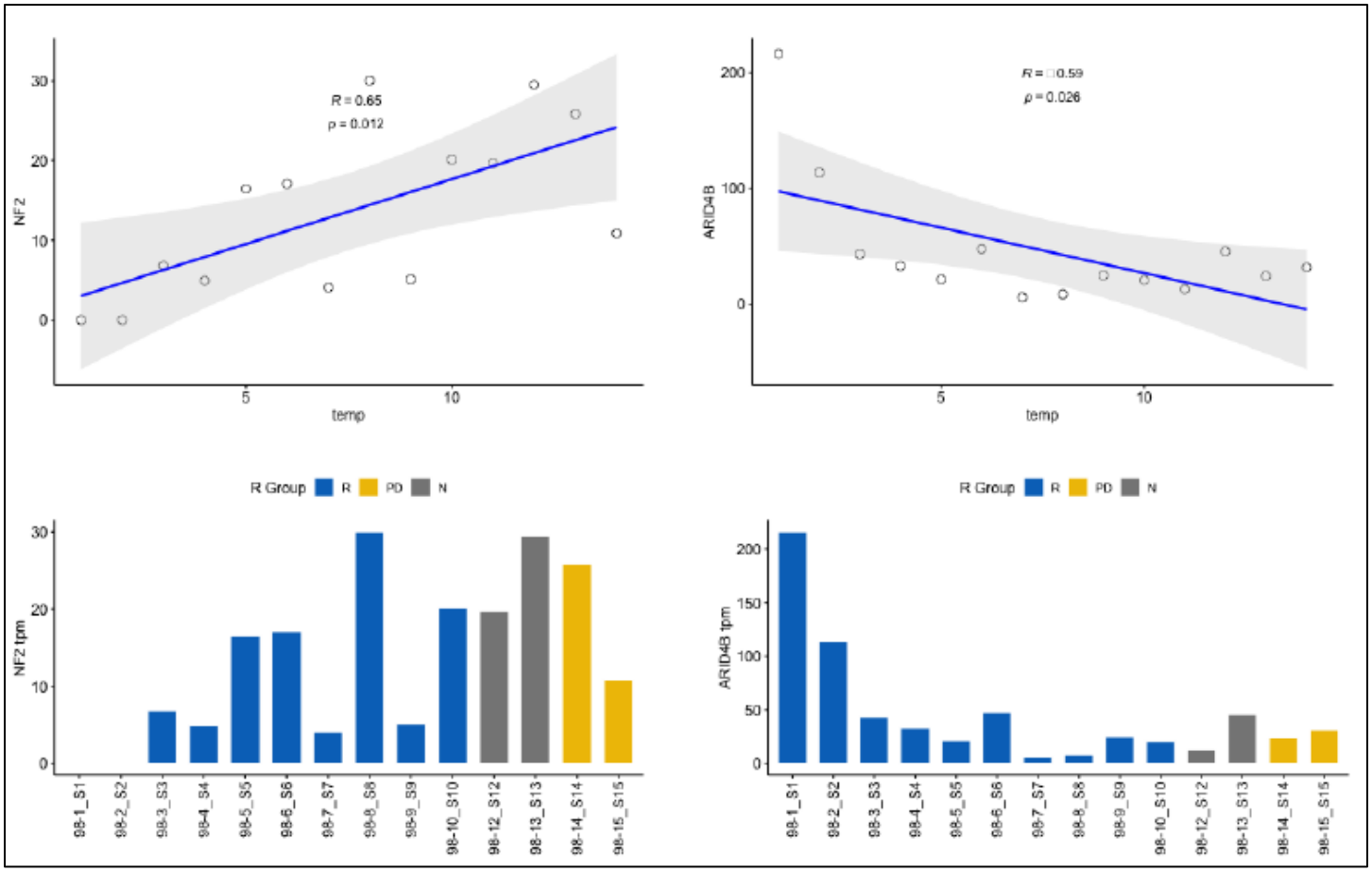
Longitudinal changes







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p = 0.012
NF2
temp
R = □0.59
p = 0.026
ARID4B
temp
R Group R PD N
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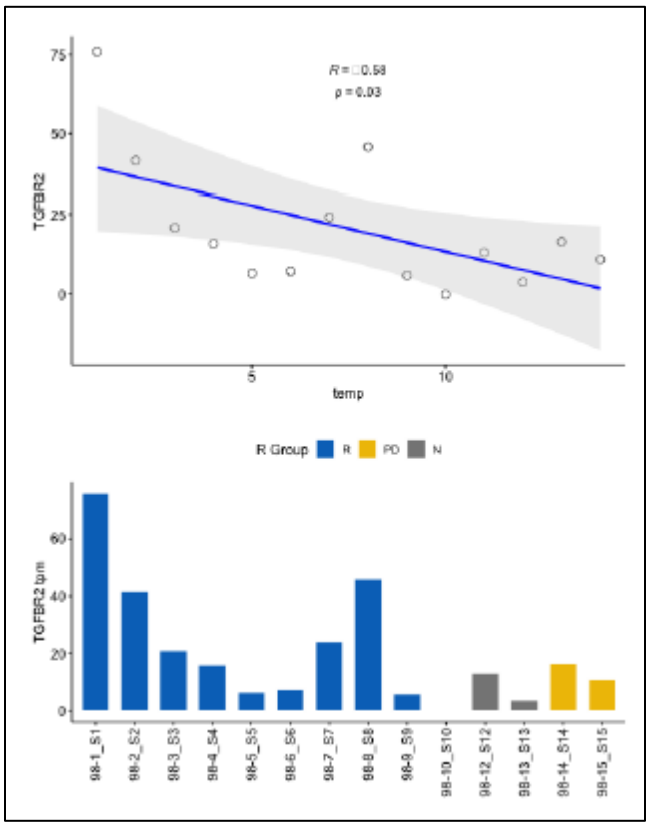
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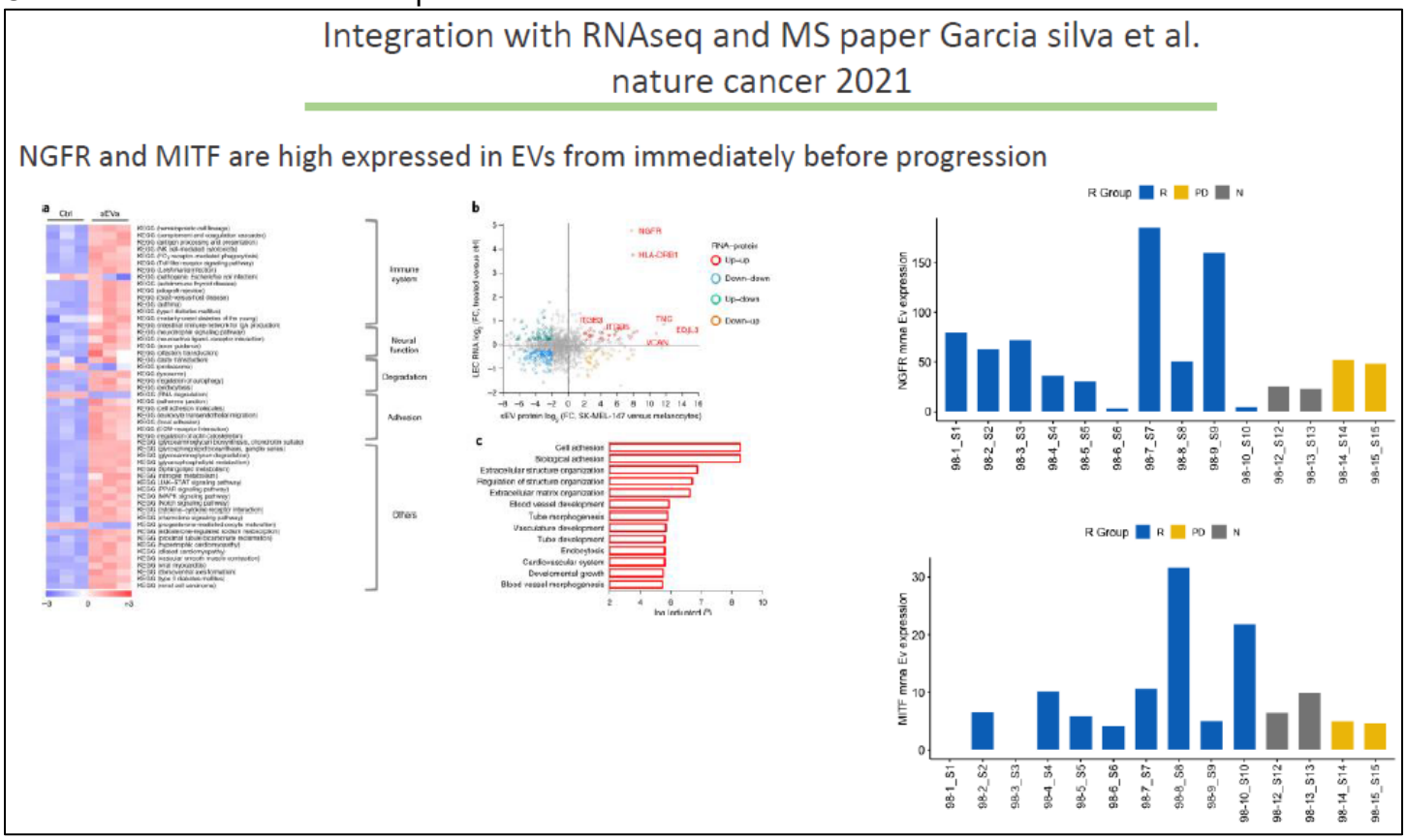
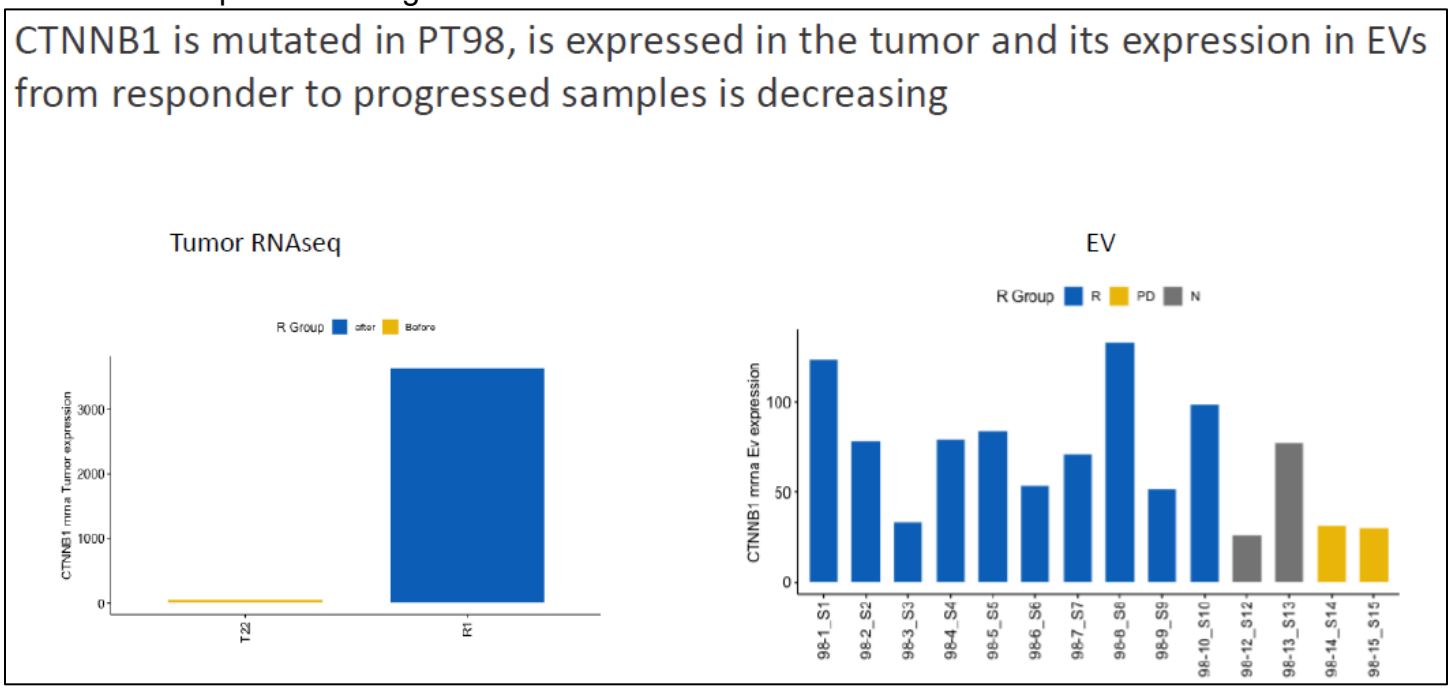
98-10_S10
98-12_S12
98-13_S13
98-14_S14
98-15_S15

Correlation with tumor transcriptomes:



Certain transcriptomes change at the level of tumor and EV and correlate with resistance to ICB:



What is yet to be determined from the bulk data is the cell of origin, which we be addressed more fully via the cell specific EV capture experiments.

Aim 3: functionally validate EV-derived transcripts and proteins

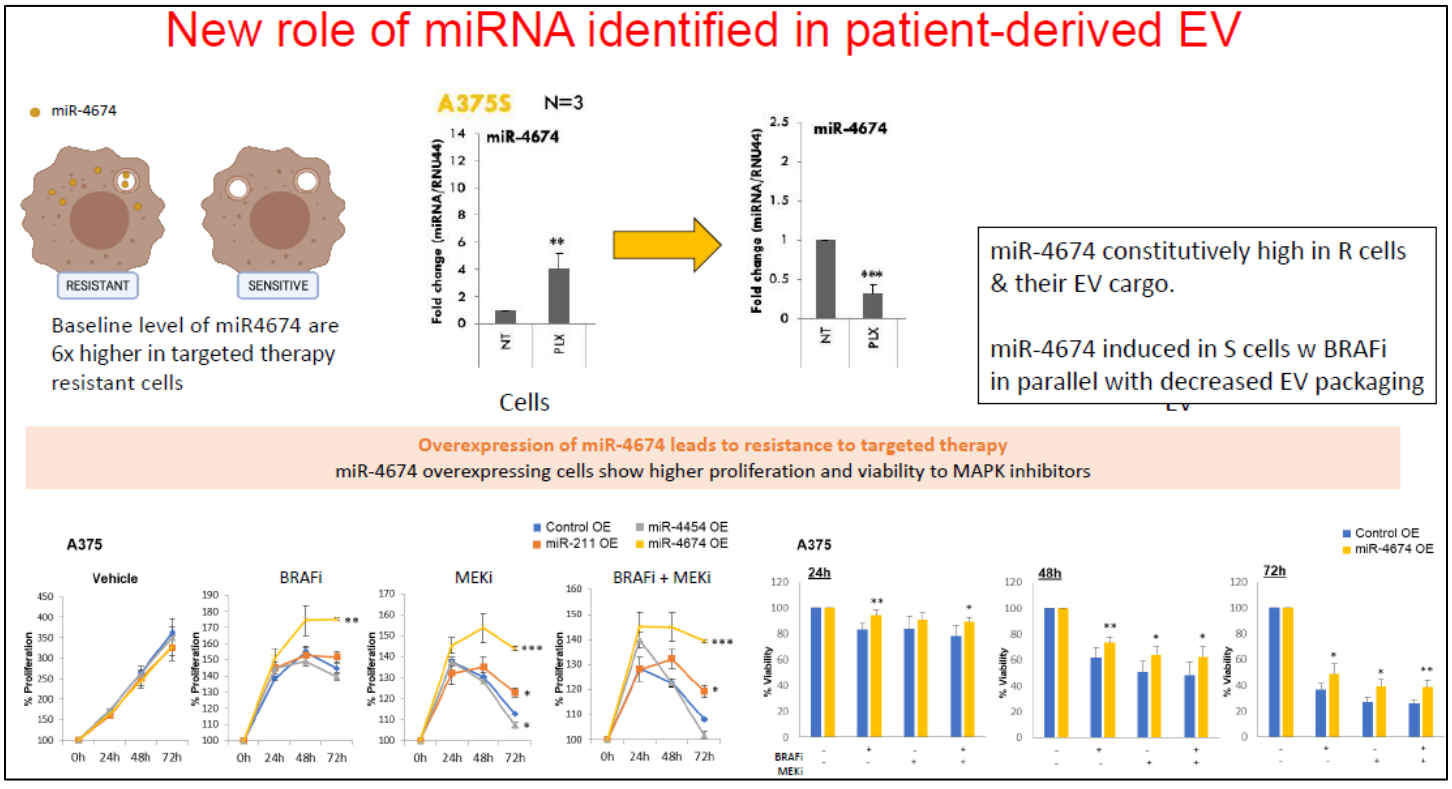
This Aim builds upon our now-published data suggesting a role for EV transcriptional profiles in predicting/monitoring ICI response and emergence of RAAs.

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. Multiple cell lines and miRNA as listed were screened. Negative data not included for the sake of time/space. However, promising results arose from miR-4674. Data from this candidate included below. Manuscript in preparation.

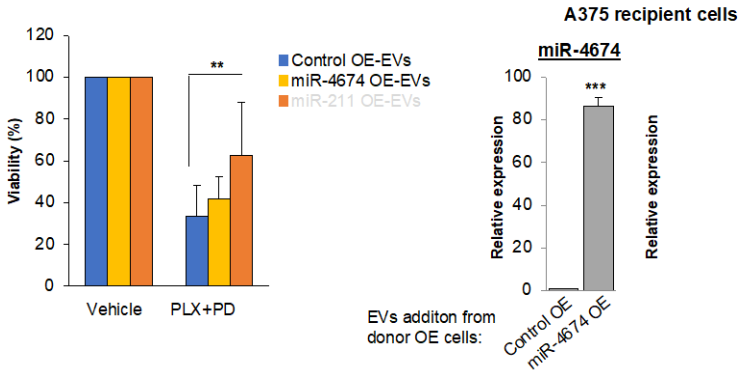
miR-4674 overexpression induces resistance to BRAF- and MEK-inhibition in vitro (Figure 1). EV from miR-4674 OE cells were used to treat non-OE cells and demonstrated increased expression of miR-4674 and increased cell viability during targeted therapy treatment (Figure 2). We next used paired A375-S 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).

In vitro data focused on miR-4674



In vitro characterization of miR-4674 in cells and EV:

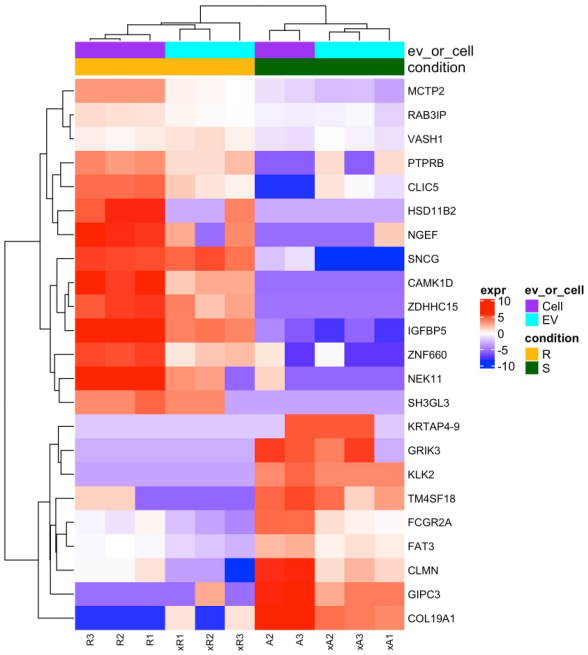
- miR-4674 overexpressing cells were generated
- EV were isolated and were used to treat A375 cells
- Viability assays were performed after 48h of treatment with MAPKi



- 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

Predicted targets of miR-4674:

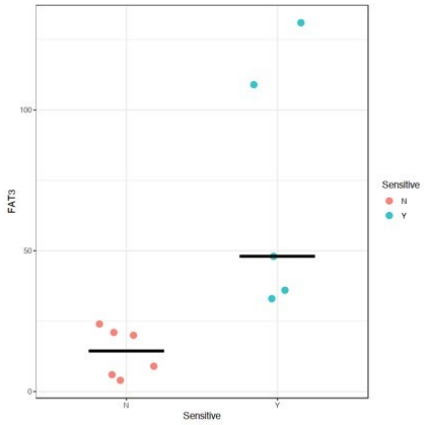
Using PLX resistant melanoma model and their EV compared to sensitive cell lines and EV, we identify 9 targets genes that were significantly overexpressed in the sensitive group



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

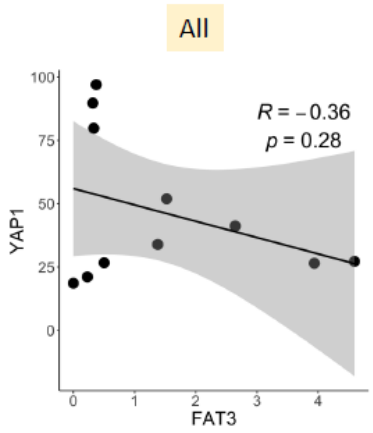
Targetscan ∩ **miRDB** ∩ **miRmap** 223

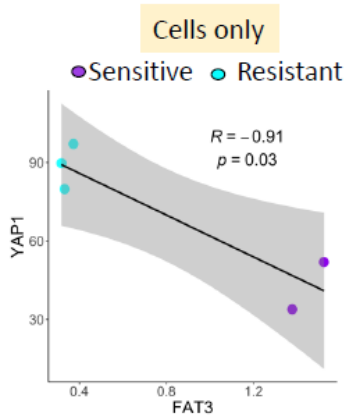
23 genes with p<0.05 (Mann–Whitney–Wilcoxon test),
There is a tumor suppressor gene **FAT3**
FAT3 is overexpressed in sensitive cells and EV
p-value = 0.008 (Wilcoxon rank sum exact test)



How does YAP1 and FAT3 expression relate to targeted therapy responsiveness:

YAP1 and FAT3 are negatively correlated & resistant cells have higher higher YAP1/FAT3 ratio





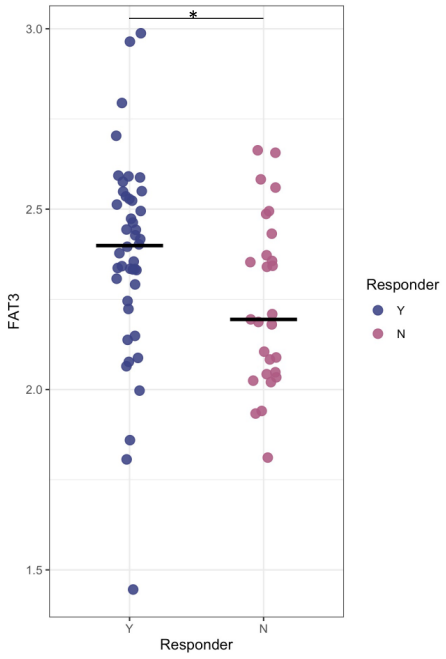
	FAT3	YAP1
XA1.tpm	4.592600	27.2659
XA2.tpm	2.642920	41.2125
XA3.tpm	3.936090	26.5123
A2.tpm	1.378240	33.8999
A3.tpm	1.523420	51.9214
R1.tpm	0.317436	89.7327
R2.tpm	0.372615	97.0265
R3.tpm	0.331405	79.8087
XR1.tpm	0.501158	26.7026
XR2.tpm	0.227260	21.1047
XR3.tpm	0.000000	18.6104

Do the findings in targeted therapy resistance apply to clinical data sets for immune checkpoint blocking agents:

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

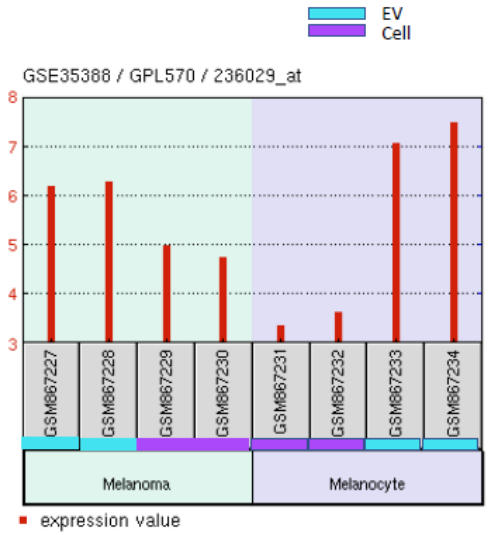
We looked at a cohort of RNAseq and Microarray from Exosomes of Metastatic melanoma patient pre-treatment with ICB **71 samples** (platform and batch corrected with ComBat)

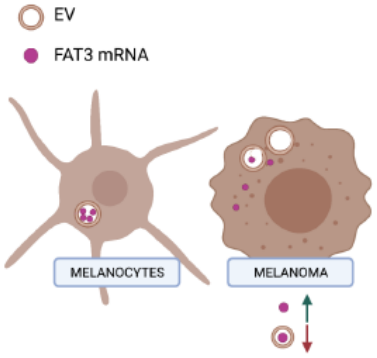
	W	p value
GRIK3	685.00	0.28583
KLK2	553.50	0.63565
TM4SF18	585.00	0.92036
FCGR2A	571.00	0.78985
FAT3	772.00	0.03490
CLMN	713.00	0.16146
GIPC3	612.00	0.83689
COL19A1	640.00	0.59226
MIR4674	744.00	0.07651



Data from published datasets on normal melanocytic EV support the role of FAT3 in cancer:

Normal Melanocyte EVs show high level of FAT3 mRNA



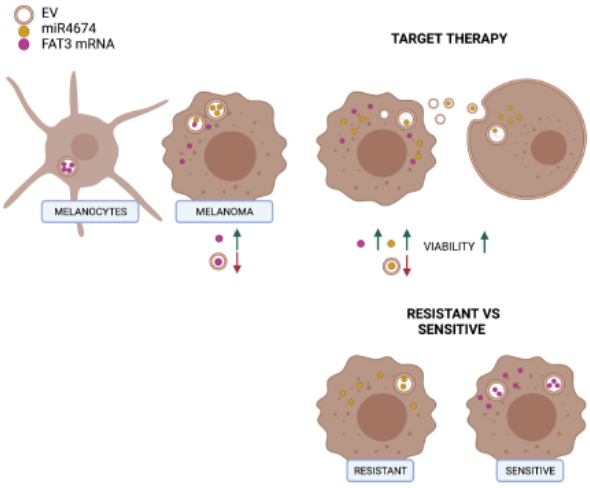


Data from Xiao et al. Identifying mRNA, microRNA and protein profiles of melanoma exosomes. *PLoS One* 2012

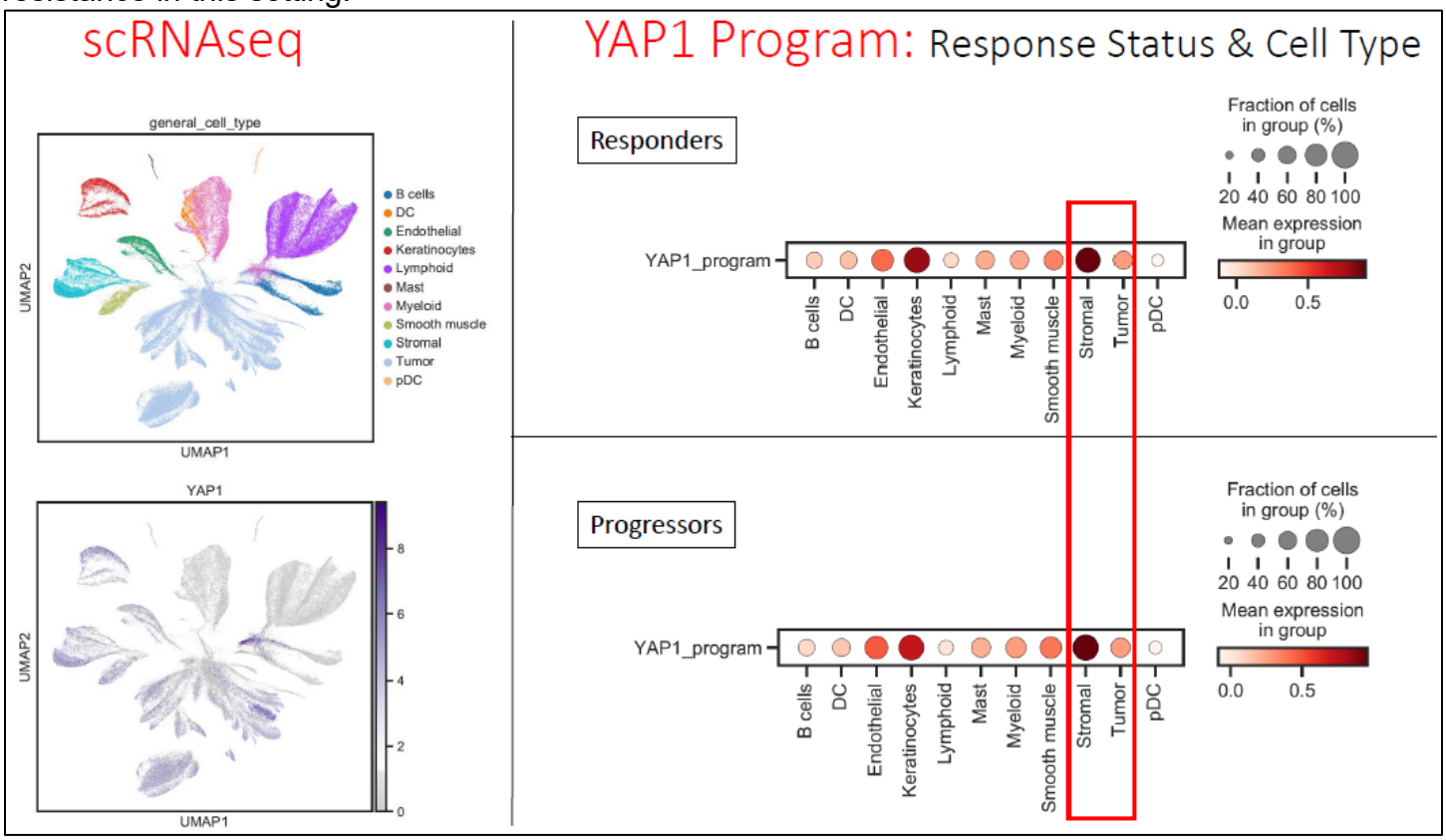
Combined, in vitro work shows that miRNA nominated from patient samples can lead to targeted therapy resistance in vitro. Overexpression of miR-4674 results in decreased FAT3 expression, supporting a potential role in YAP1 mediated resistance:

Summary

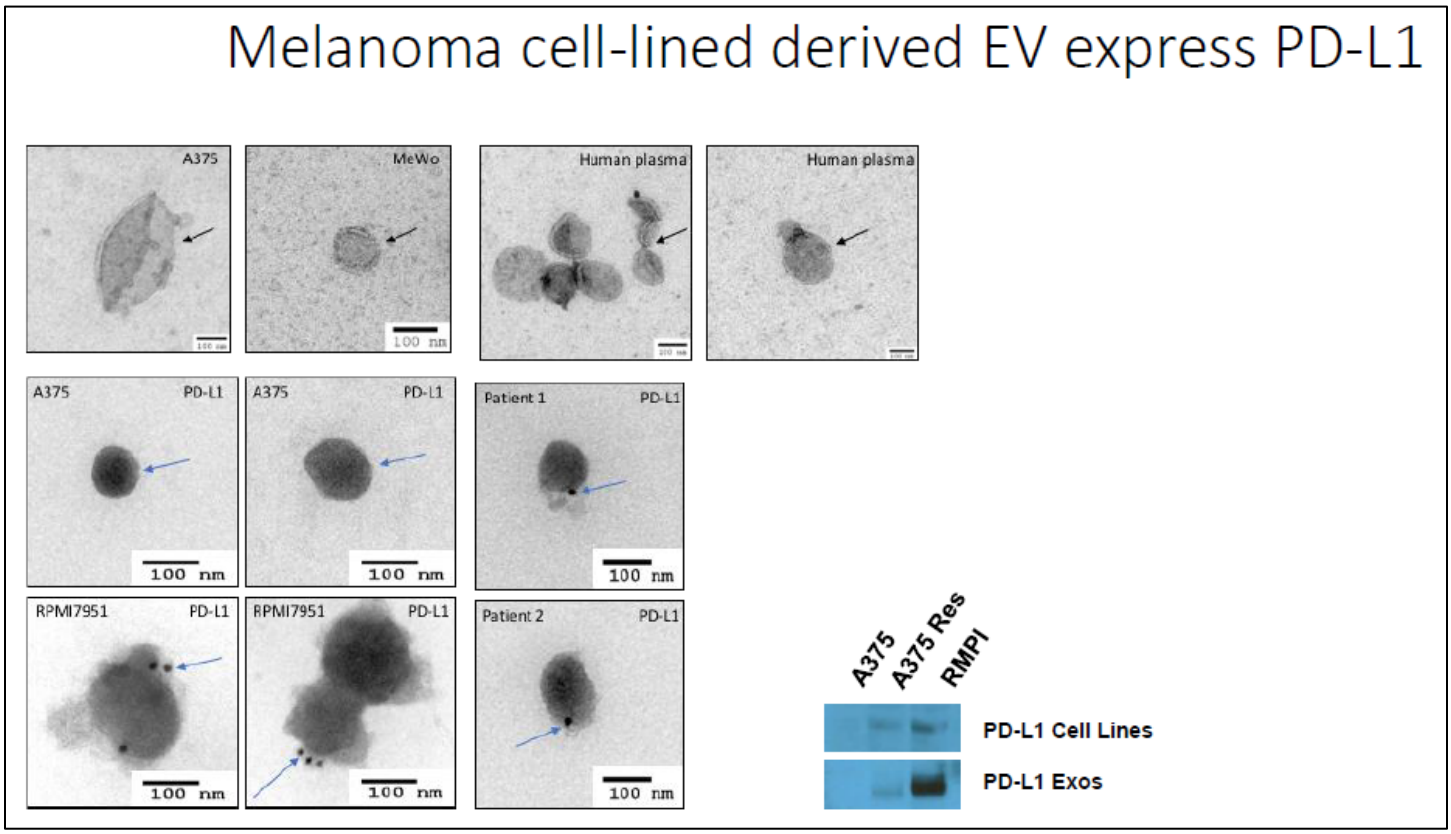
- Screen of patient-derived EV nominated multiple miRNA
- miR-4764 induces TT therapy resistance in vitro
- miR-4764 OE leads to decreased FAT3 expression
- Potential role in YAP1-mediated resistance



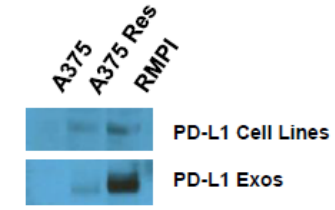
Of note, patient scRNAseq data from ICB treated patients supports the role of YAP1 mediated resistance in this setting:

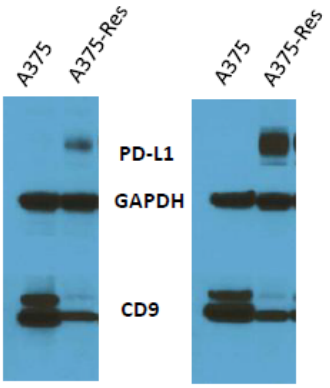


In addition, efforts at EV modulation of immune cells has been ongoing. EV PD-L1 overexpression in cells resulted in higher levels in EV, which could be used to transfer PD-L1 from PD-L1 high cells to PD-L1 low cells in vitro. However, no differences in T cell immune activation seen with tumor-derived EV treatment:



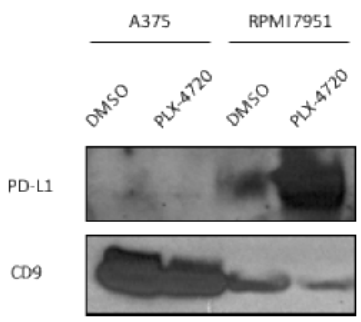
BRAFi increases PD-L1 in cells and EV

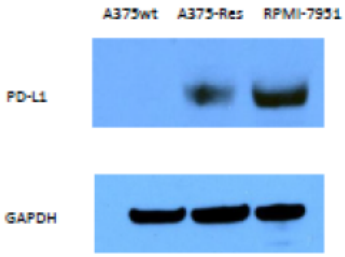


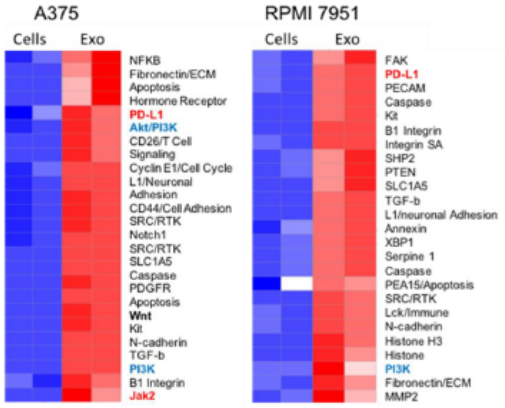


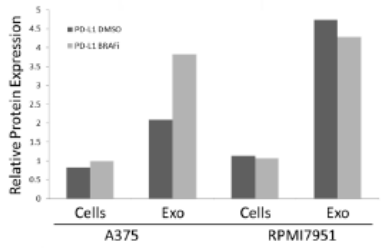
Cell Lines treated with DMSO

Cell Lines treated with PLX 4720 (10um)







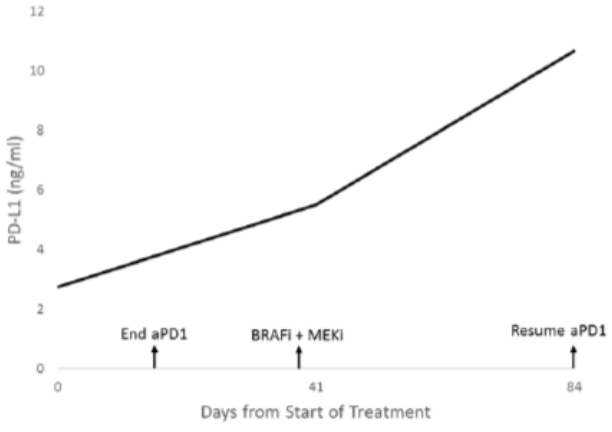


Patient data: Treatment with BRAFi increases EV PD-L1

PD-L1 Elisa Expression

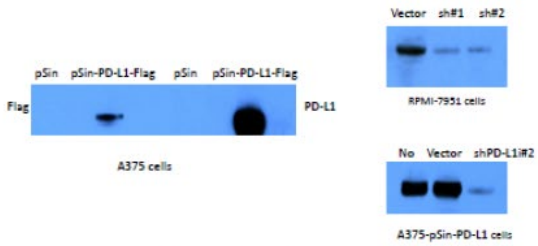
	Average	St Dev
BRAF WT	3.11	2.62
BRAF V600	2.08	1.61

Treatment with BRAFi increases exosomal PD-L1 levels in patients.

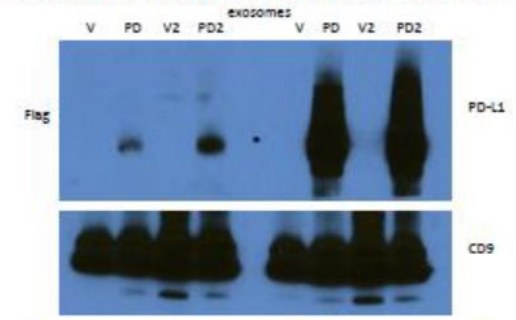


EV OE PD-L1 can transfer PD-L1 from PD-L1 high to PD-L1 low cells

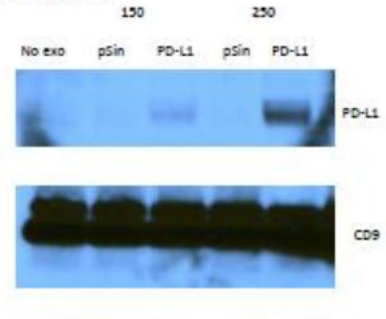
A375 cells transduced with PD-L1-Flag were generated. shRNAi constructs were used to knockdown PD-L1 in RPMI-7951 and A375-PD-L1-Flag cells in short term cultures.



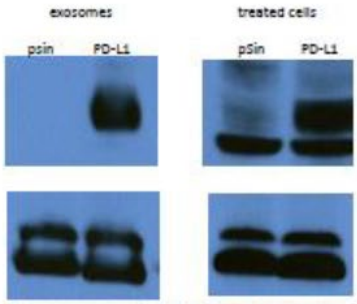
Exosomes from A375-PD-L1 transfer PD-L1 to A375 wt cells in a dose dependent manner



Two different exosome preps from A375-pSin (V) and A375-pSin-PD-L1-Flag

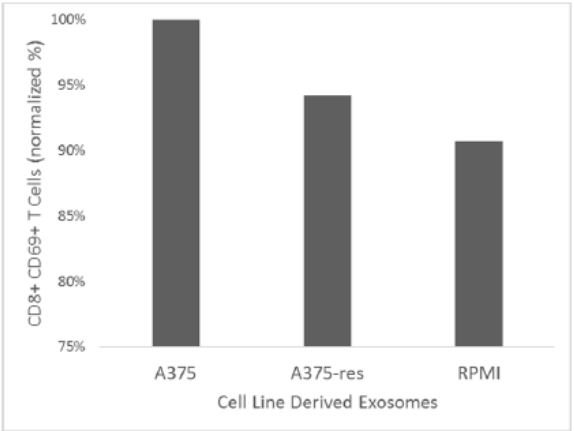


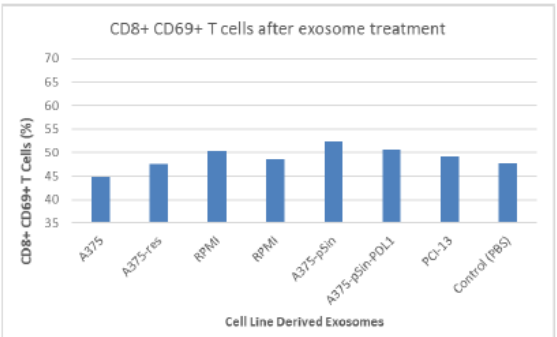
Exosome preps of 150 uL or 250 uL were used to treat A375 cells

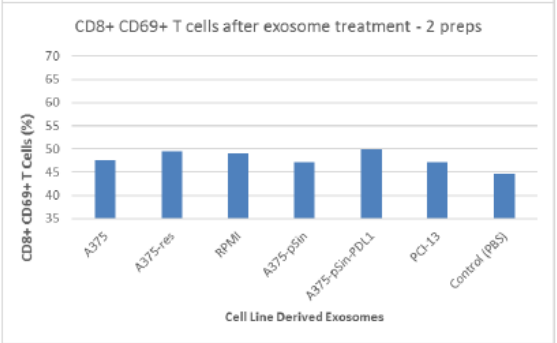


Exosomes from A375-pSin and A375-pSin-PD-L1 cells were isolated and used to treat wild type A375 cells. After 48 hrs cell lysates were tested for PD-L1 levels.

No clear T cell activation with EV exposure from sensitive and resistant cell lines







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.

I was selected for the American College of Surgeons Aspiring Leaders Pilot Program (2021).

The manuscript arising from the DOD supported work (Liu et al… Boland GM. Nat Med 2021) was awarded the MGH Martin Prize in Clinical Medicine for the highest impact publication from the entire Massachusetts General Hospital research efforts in 2021 (awarded in 2022). This award comes with of research support.

Characterizing the Hippo/YAP Pathway in Melanoma Immunotherapy
Invited Lecturer. Melanoma Research Foundation (MRFBC). Virtual Presentation.

Evidence-Based Management of Melanoma
Invited Lecturer. Madigan Army Medical Center, Hospital Grand Rounds. (Virtual).

Evidence-Based Management of Melanoma
Invited Lecturer. Oregon-Washington ACS Symposium. Virtual Presentation and Tumor Board.

Multi-Modality of Melanoma Immunotherapy Response and Resistance
Invited Lecturer. Immuno-Oncology Summit, Boston, MA (Virtual)

Creating a Career as a Surgeon Scientist
Invited Lecturer. Jefferson MD/PhD Mentorship Seminar. Thomas Jefferson University, Philadelphia, PA (Virtual)

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)

Surgical Management in Melanoma: Where are We Going?
Invited Lecturer. American College of Surgeons Cancer Program (Virtual)

Multi-Modality Approach to Predicting and Monitoring Melanoma Immunotherapy Response and Resistance.
Invited Lecturer. Molecular Med Tri-Con Conference & Expo (Virtual)

What's Old is New Again: Transformative Change in the Management of Melanoma.
Visiting Professor. Department of Surgery Grand Rounds. Johns Hopkins (Virtual)

Protein Biomarkers in Plasma: Revealing Biological Insights into the Tumor Microenvironment.
Invited Speaker. Cell and Gene Therapy Insights (Webinar)

Multi-Modality of Melanoma Immunotherapy Response and Resistance
Invited Lecturer. Immuno-Oncology Summit, Boston, MA (Virtual)

Creating a Career as a Surgeon Scientist
Invited Lecturer. Jefferson MD/PhD Mentorship Seminar. Thomas Jefferson University, Philadelphia, PA (Virtual)

Surgical Management in Melanoma: Where are We Now and Where are We Going
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Invited Speaker. Cell and Gene Therapy Insights (Webinar)

Multimodality Approach to Identifying Biomarkers in Melanoma Immunotherapy Response and Resistance.
Invited Speaker. Pharma R&D Week (Virtual)

What's Old is New Again: Using Translational Science to Understand Melanoma Biology.
Invited Lecturer. Univ of Alabama Experimental Therapeutics Seminar Series (Virtual)

Multimodality Analysis in Melanoma Immunotherapy Response and Resistance.
Invited Lecturer. Immuno-Oncology Summit, Boston, MA (Virtual).

Melanoma Therapeutic Response & Resistance: What We've Learned From a Decade of Immunotherapy.
The Surgical Biology Club (Virtual).

A Multidisciplinary Approach to Neoadjuvant, Intralesional, and Cell-Based Therapy.
Co-Chair/Moderator. Society of Immunotherapy of Cancer. Washington, DC.

Surgical Management of Melanoma.
18th Annual International Symposium of Melanoma and Other Cutaneous Malignancies. New York, NY (Virtual).

Management of Patients with Advanced Melanoma.
NCCN Annual Conference 2022 (Virtual).

Learning as We Go: Multidisciplinary Care of Melanoma in the Modern Era.
Visiting Lecturer. Melanoma Medical Oncology Grand Rounds. MD Anderson Cancer Center, Houston, TX (Virtual).

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.

Nothing to Report

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. We have continued these efforts in cutaneous melanoma (CM), but have also expanded to rare melanoma subtypes including mucosal melanoma (MM). We have been awarded a Melanoma Research Alliance Team Science Award to use this foundational data to identify druggable vulnerabilities in MM and assess the biological impact. 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 continue with both bulk and cell-specific EV analysis. We completed bulk EV analysis of the patient from the Nature Medicine manuscript. Additionally, the functional EV work focusing on candidates derived from patient EV samples identified miR-4674 which is previously non-characterized and which appears to induce therapy resistance in vitro. Our functional work suggest this is via modulation of the YAP1 pathway. The final validation experiments are ongoing and the manuscript is in preparation.

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 to apply this to COVID-19.

The techniques arising from this work are now being applied to other, therapy non-responsive melanoma subtypes including mucosal melanoma (MM). Rapid progress would not have been possible without the creation of pipelines for this type of analysis via the DOD support.

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. Additionally, the COVID-19 work arising from the EV techniques supported here have been submitted for patent with Dr. Boland and Dr. Stott as co-inventors.

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.*

The longitudinal tumor analysis (bulk, imaging) and integration have created a high-throughput system that is being applied broadly in melanoma. The goals would be to use this information to create tumor/immune panels with potential clinical impact.

While we are a few steps away from conversion of this type of approach to a CLIA environment, we aspire to create a reproducible imaging, sequencing, and/or 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.

The expansion of the efforts in cutaneous melanoma to additional genotypes is ongoing, but not yet complete. However, the approaches have now been leveraged in mucosal melanoma (MM) which is an unmet clinical need. 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. While not all candidates in the functional data yielded meaningful results, one nominated candidate miR-4674 has shown interesting findings and work is ongoing to characterize this more deeply.

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.

The technology developed here was transitioned to COVID-19 detection. We submitted a patent application for isolation of COVID-19 virus in parallel with cell-specific EV capture.

- **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".

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.

Other Support Changes since 2021
Genevieve M. Boland

ACTIVE

Adelson Foundation Fund (PI: Flaherty) 10/01/2014 – 09/31/2023 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.

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.

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.

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. (Boland, Bar-Peled, Sade-Feldman) 02/01/2022 – 02/01/2023
Jonathan Kraft Translational Team Award
Identification of Novel Druggable Targets in Rare Melanomas

No Award No. (Boland) 04/01/2022 – 04/01/2023
Martin Prize (Clinical Research)

No Award No. (Boland, Hacohen, Bar-Peled) 04/01/2022 – 04/02/2025
Melanoma Research Alliance Team Science Award
Identification and Validation of Novel Druggable Targets in Rare Melanomas
The goal of this program is to identify druggable targets in acral and mucosal melanoma (AM/MM) and identify tumor-specific expression of druggable candidates.

Completed:

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.

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.

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
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.

Pending:

NA

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.*