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
SOMERVILLE, MA

REPORT DATE: June 2020

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 2020	Annual Technical Report	**15MAY2019 - 14MAY2020**

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
SOMERVILLE, MA

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: 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. 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**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	30	**19b. TELEPHONE NUMBER** *(include area code)*

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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. 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 in process for n=5 BRAF V600E mutant patients, n=2 NRAS patient, n=1 NF1 patient, and n=1 triple wild type patient. Therefore, phylogenetic analysis on hold pending data release (delays in processing due to COVID-19).	3-6	MGH (Boland Lab)
Exome sequencing n=9 patients with 3 time points n=27 tumor samples total	3-6	Broad (Boland)

ATACseq n=9 patients with 3 time points n=27 tumor samples total	3-9	MGH/Broad (Boland; technical assistance Kellis)
Phylogenetic analysis n=9 patients with 3 time points n=27 tumor samples total	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	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	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 Sequencing in process for n=5 BRAF V600E mutant patients, n=2 NRAS patient, n=1 NF1 patient, and n=1 triple wild type patient. Therefore, RNAseq & TCR/BCR analyses on hold pending data release (delays in processing due to COVID-19). FFPE block are available on these patients/tumors, but are awaiting submission for imaging once lab activities are resumed post COVID-19. At MGH, we are entering Phase II where a small group of lab members can begin to return to lab.	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	6-9	MGH (Boland Lab)
RNAseq n=9 patients with 3 time points n=27 plasma samples total	9-12	MGH/Broad (Boland)
Comparison with tumor data n=9 patients with 3 time points n=27 tumor samples total Plasma samples are available for analysis. Plans are underway to isolated exosomes for analysis in next 1-2 months.	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 n=27 plasma samples total	12-15	MGH/Broad (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.	15-24	MGH (Boland and Stott Labs)
Focused sequencing Cell line-derived exosomes to confirm selective capture	15-24	MGH (Boland and Stott Labs)

Some preliminary data has been generated via this approach.		
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.	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	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	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) RPMI 7951 MeWo SkMel3 Overexpression/knockout of candidates (above) in a subset of the melanoma cell lines, starting with A375 sensitive/resistant cell lines This work is underway.	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	12-36	MGH (Boland Lab)

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 This work is underway. Data will be included below.		
RNA and protein analysis 1. Assess changes in gene/protein expression in exosome treated tumor and immune cells This work is underway. Data not yet available for analysis.	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 were submitted to the Broad Institute in late Jan/early Feb, but processing has been on hold due to COVID-19. ATACseq samples were submitted to the MIT Core at the same time, but final analysis is also on hold due to COVID-19. As MA enters phase II, labs are now beginning to partially reopen. We are optimistic that data from these samples will be available for analysis over the summer 2020. The FFPE blocks that pair with the samples submitted for sequencing are available, but have not been cut/sent for imaging yet. As the labs reopen to capacity, we will prioritize these samples for multiplex immunofluorescent imaging. We anticipate that this will also be accomplished during the summer 2020.

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. However, exosome isolation and analysis has been held due to both COVID-19 and the goal to optimize isolation procedures and processes in order to maximize the yield of data from each sample. We only have 1 opportunity to successfully run these experiments and want to assure ourselves we can maximize exosome capture and RNA isolation prior to analyzing these samples. QC and technical optimization ongoing.

Aim 2.2: Cell specific Exosome/Extracellular Vesicle (EV) Capture.
We have utilized Ab capture of immune cell line derived EVs and show promising results in capturing CD45 or CD3-specific EVs, quantified by qPCR. We have similar promising data for melanoma/tumoral signals.

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. We did a broad characterization of a variety of candidate miRNA identified in our patient-level EV analysis.

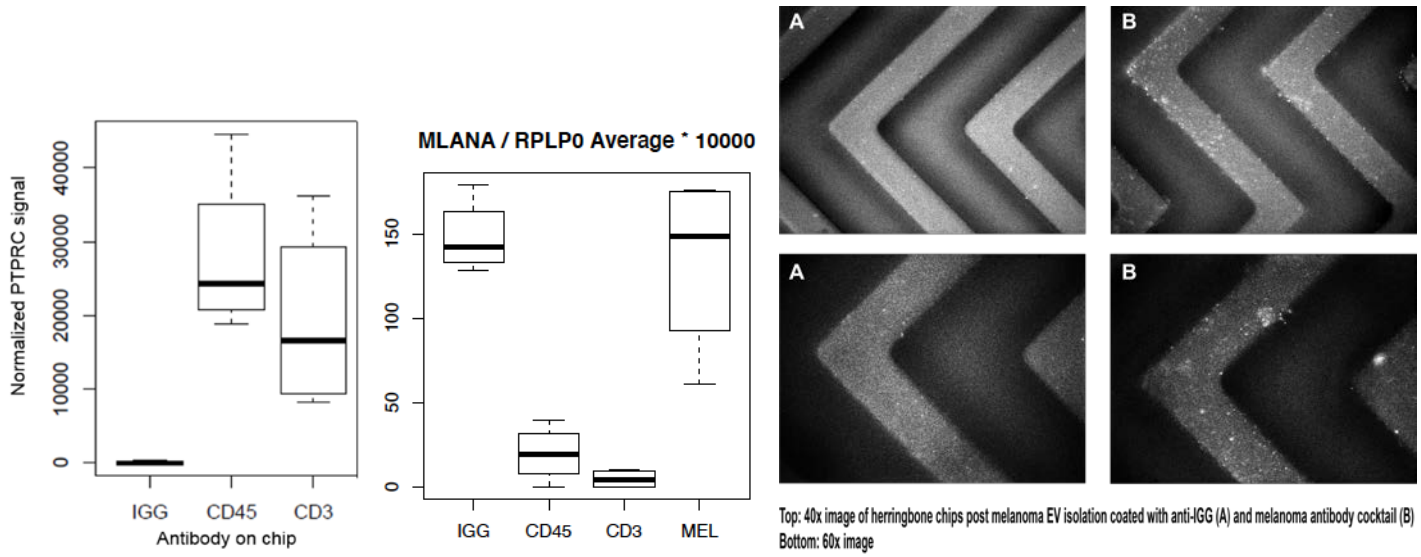


Figure Legend: (left): Ab selection of CD45 and CD3 selectively enrich for those signals, validated by ddPCR. (center): Ab selection of melanoma cell line-derived EVs is enriched as compared to CD45/CD3 as validated by ddPCR. (right): capture of mTurqoise labelled EVs on the herringbone chip showing increased capture (panels B) as compared to IgG control (panels A) – top row is 40x magnification, bottom row is 60x magnification.

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. We did a broad characterization of a variety of candidate miRNA identified in our patient-level EV analysis.
We undertook a broad screen of both well-characterized melanoma miRNA (miR-211-5p and 204-5p) in both cells and EVs in parallel with novel, newly identified miRNA from our melanoma patient EV data (see below). In the end, we narrowed the focus to A375S melanoma cell line for further functional validation and narrowed the panel of miRNA to miR-211-5p (well established in cells) and 2 newly identified miRNA (miR-4454 and miR-4674). Manuscript in preparation.

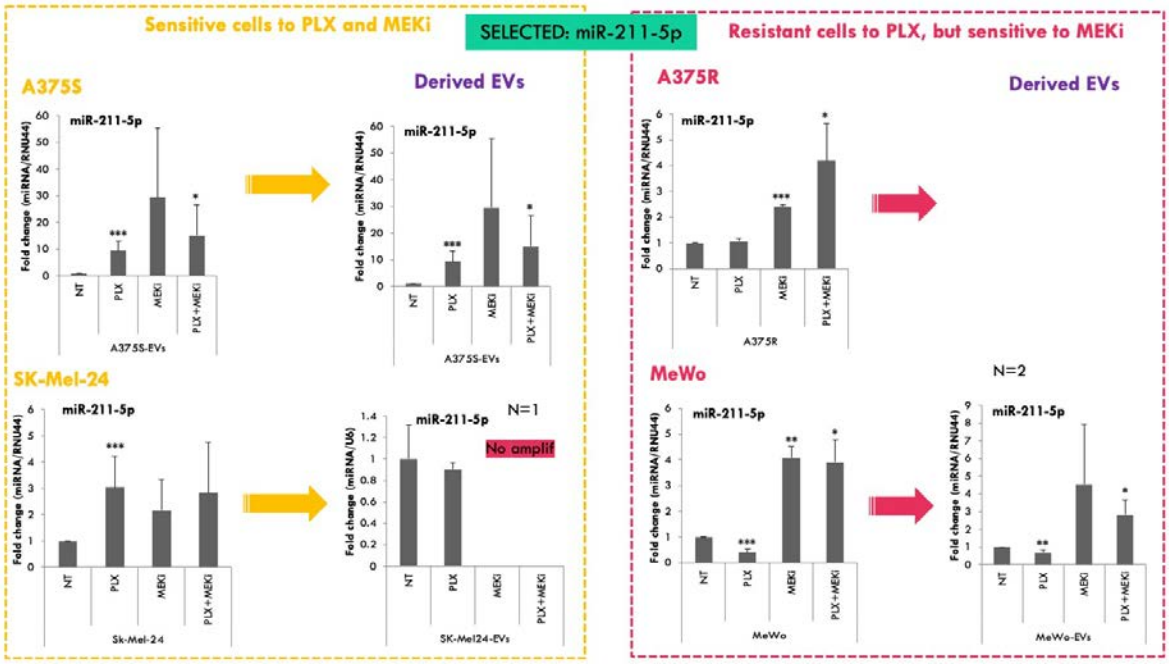


Figure miR-211-5p: We elected to initially characterize miRNA that are well established to play a role in melanoma targeted therapy resistance (in cells), but whose presence/role in extracellular vesicles (EVs) has not yet been characterized. This will allow us to interpret the data arising from the newly identified miRNA of interest, whose roles in melanoma cells (and EVs) have not yet been described. Cell lines described in Aim 3.1 (A375S (sensitive to BRAFi), A375R (induced resistance to BRAFi), SK-Mel-24, and MeWo were used to assess changes in miRNA expression in the untreated and also in the treated state). PLX: BRAF-inhibitor, MEKi: MEK-inhibitor; PLX + MEKi: co-treatment with both BRAFi and MEKi. Left side (yellow box) are cell lines sensitive to BRAFi therapy. Right side (red box) are cell lines resistant to PLX, but sensitive to MEKi. Within each box, the left panel is from cell lines and right panel is data from cell line-derived extracellular vesicles (EVs). *We see increased levels of miR-211-5p in cells, with concomitant increases in miRNA levels in EVs in response to therapy to which the cell line is susceptible.*

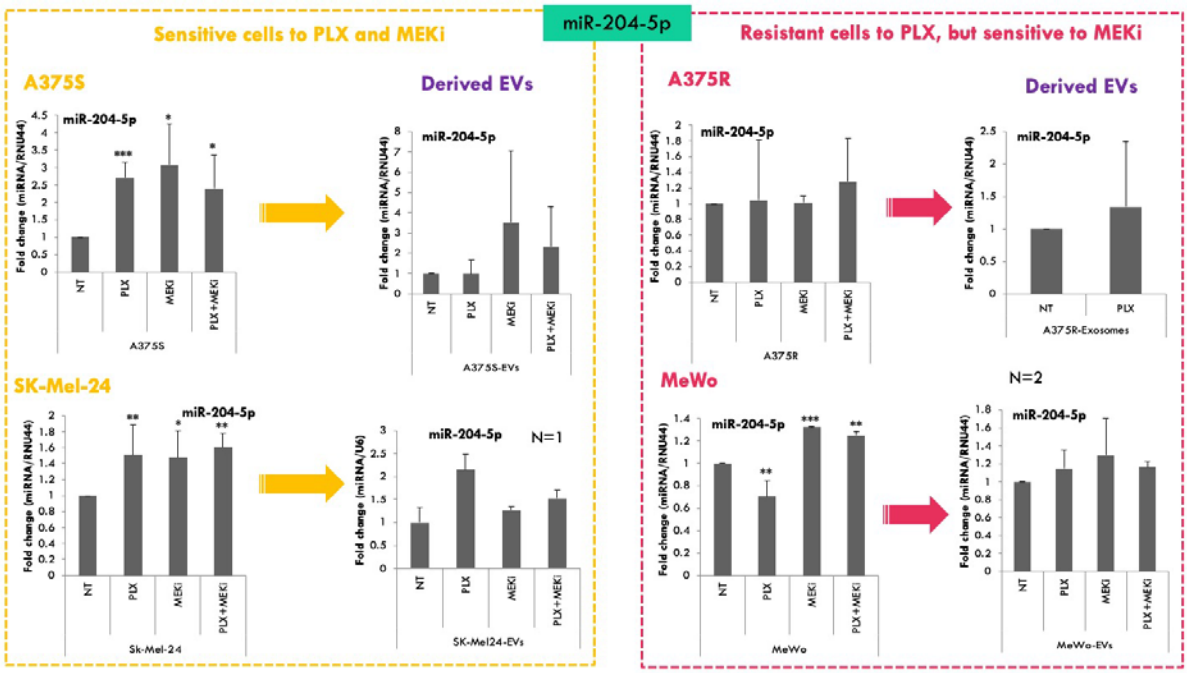


Figure miR-204-5p: We elected to initially characterize miRNA that are well established to play a role in melanoma targeted therapy resistance (in cells), but whose presence/role in extracellular vesicles (EVs) has not yet been characterized. This will allow us to interpret the data arising from the newly identified miRNA of interest, whose roles in melanoma cells (and EVs) have not yet been described. Cell lines described in Aim 3.1 (A375S (sensitive to BRAFi), A375R (induced resistance to BRAFi), SK-Mel-24, and MeWo were used to assess changes in miRNA expression in the untreated and also in the treated state). PLX: BRAF-inhibitor, MEKi: MEK-inhibitor; PLX + MEKi: co-treatment with both BRAFi and MEKi. Left side (yellow box) are cell lines sensitive to BRAFi therapy. Right side (red box) are cell lines resistant to PLX, but sensitive to MEKi. Within each box, the left panel is from cell lines and right panel is data from cell line-derived extracellular vesicles (EVs). *We see increased levels of miR-204-5p in cells, with concomitant increases in miRNA levels in EVs (not statistically significant) in response to therapy to which the cell line is susceptible.*

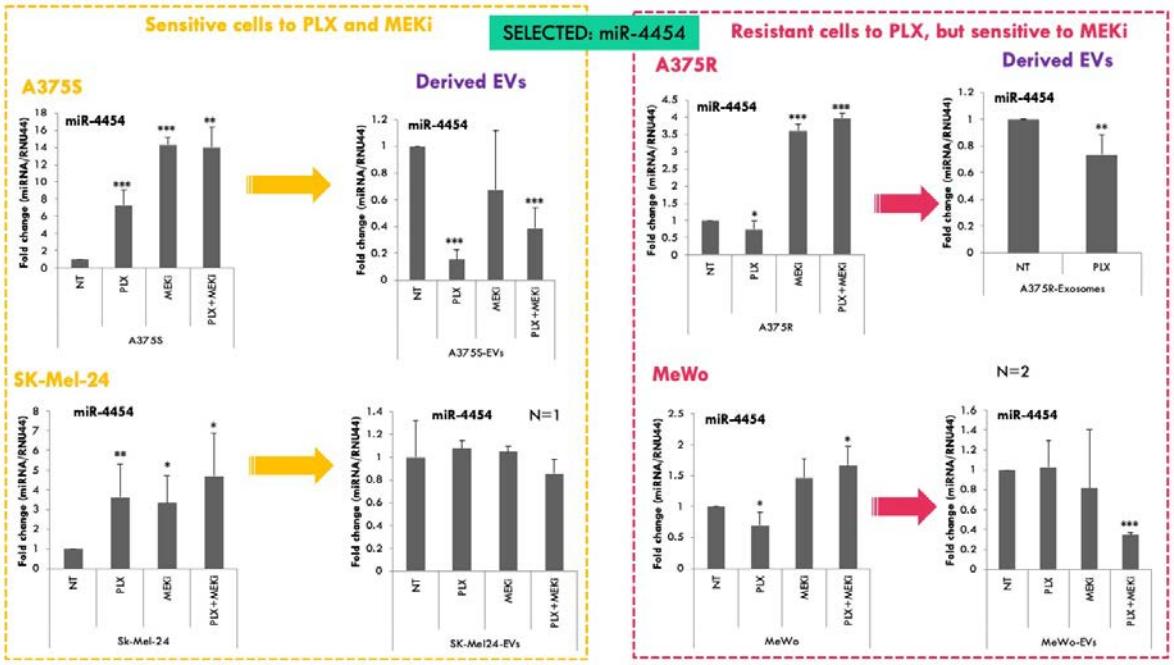


Figure miR-4454: Cell lines described in Aim 3.1 (A375S (sensitive to BRAFi), A375R (induced resistance to BRAFi), SK-Mel-24, and MeWo were used to assess changes in miRNA expression in the untreated and also in the treated state). PLX: BRAF-inhibitor, MEKi: MEK-inhibitor; PLX + MEKi: co-treatment with both BRAFi and MEKi. Left side (yellow box) are cell lines sensitive to BRAFi therapy. Right side (red box) are cell lines resistant to PLX, but sensitive to MEKi. Within each box, the left panel is from cell lines and right panel is data from cell line-derived extracellular vesicles (EVs). *We see increased levels of miR-4454 in cells, but decreased levels in EVs in response to therapy to which the cell line is susceptible.*

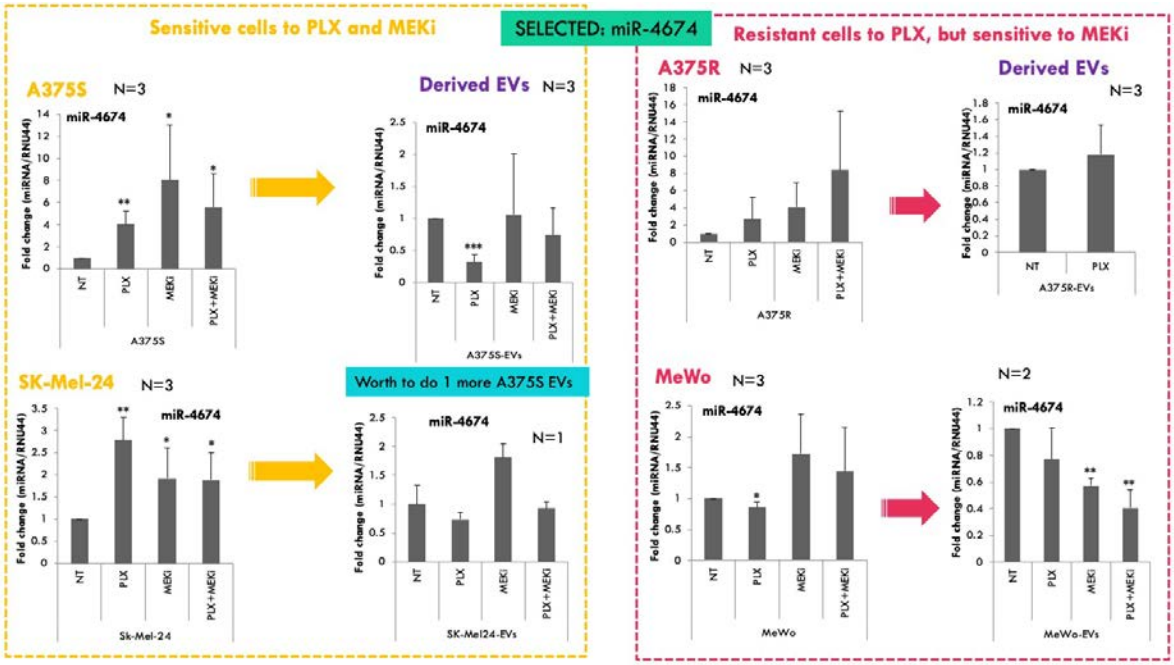


Figure miR-4674: Cell lines described in Aim 3.1 (A375S (sensitive to BRAFi), A375R (induced resistance to BRAFi), SK-Mel-24, and MeWo were used to assess changes in miRNA expression in the untreated and also in the treated state). PLX: BRAF-inhibitor, MEKi: MEK-inhibitor; PLX + MEKi: co-treatment with both BRAFi and MEKi. Left side (yellow box) are cell lines sensitive to BRAFi therapy. Right side (red box) are cell lines resistant to PLX, but sensitive to MEKi. Within each box, the left panel is from cell lines and right panel is data from cell line-derived extracellular vesicles (EVs). *We see*

increased levels of miR-4674 in cells, but decreased levels in EVs in response to therapy to which the cell line is susceptible.

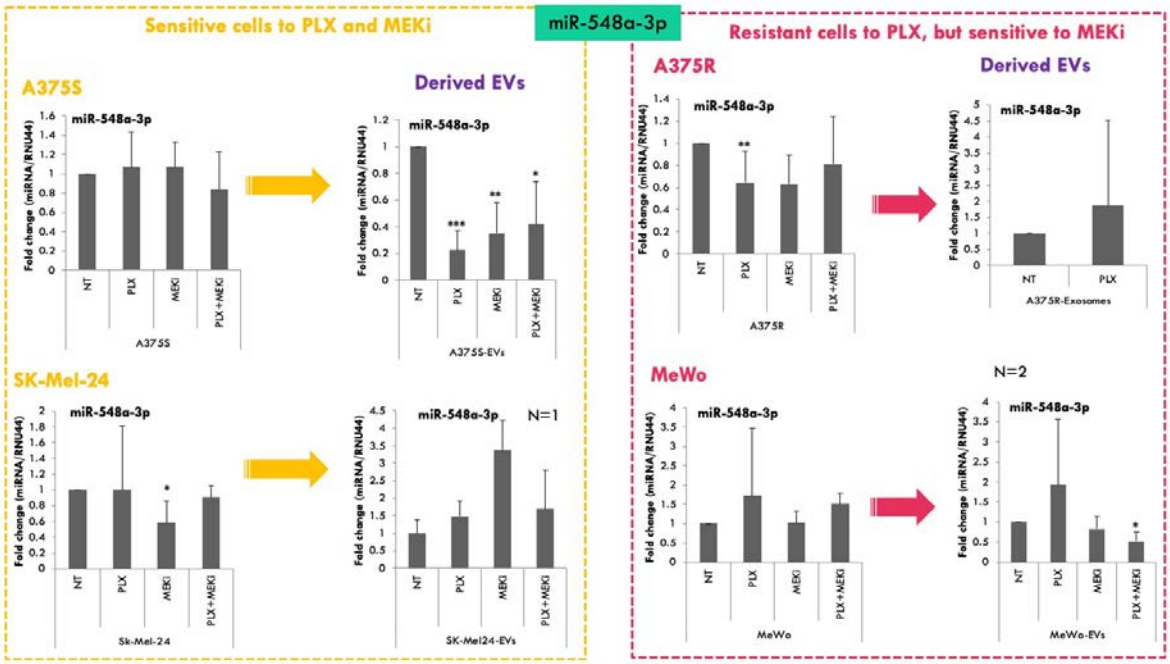


Figure 548-3p: Cell lines described in Aim 3.1 (A375S (sensitive to BRAFi), A375R (induced resistance to BRAFi), SK-Mel-24, and MeWo were used to assess changes in miRNA expression in the untreated and also in the treated state). PLX: BRAF-inhibitor, MEKi: MEK-inhibitor; PLX + MEKi: co-treatment with both BRAFi and MEKi. Left side (yellow box) are cell lines sensitive to BRAFi therapy. Right side (red box) are cell lines resistant to PLX, but sensitive to MEKi. Within each box, the left panel is from cell lines and right panel is data from cell line-derived extracellular vesicles (EVs). *We see no change in levels (or minimal change) of miR-548-3p in cells, but decreased levels in EVs in response to therapy to which the cell line is susceptible.*

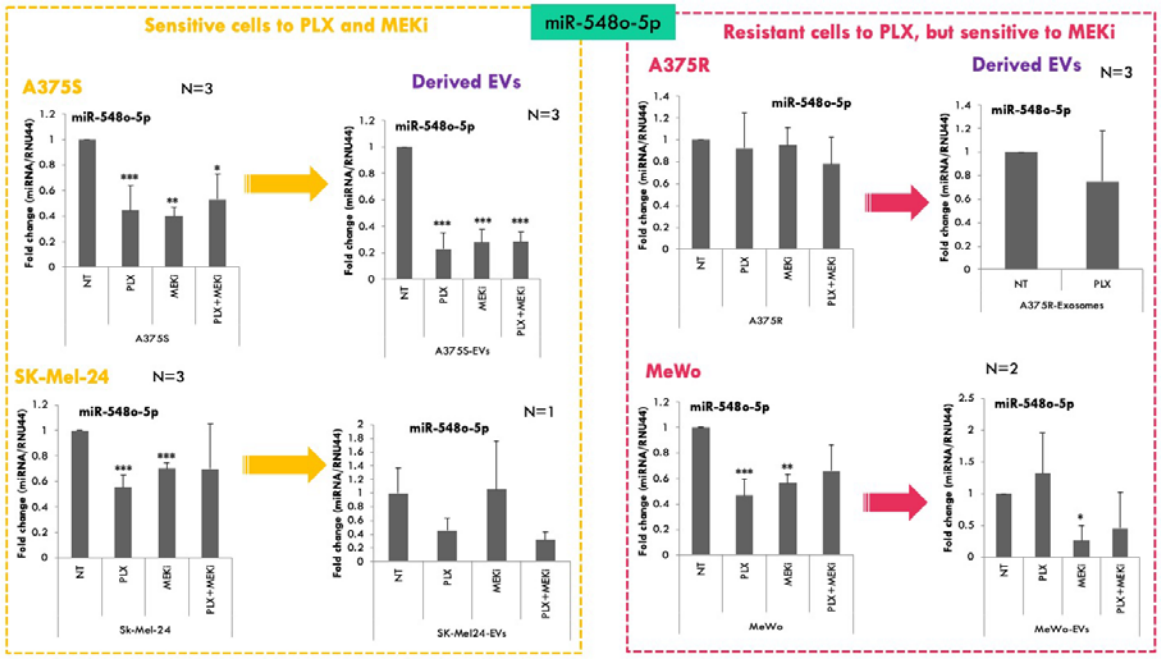
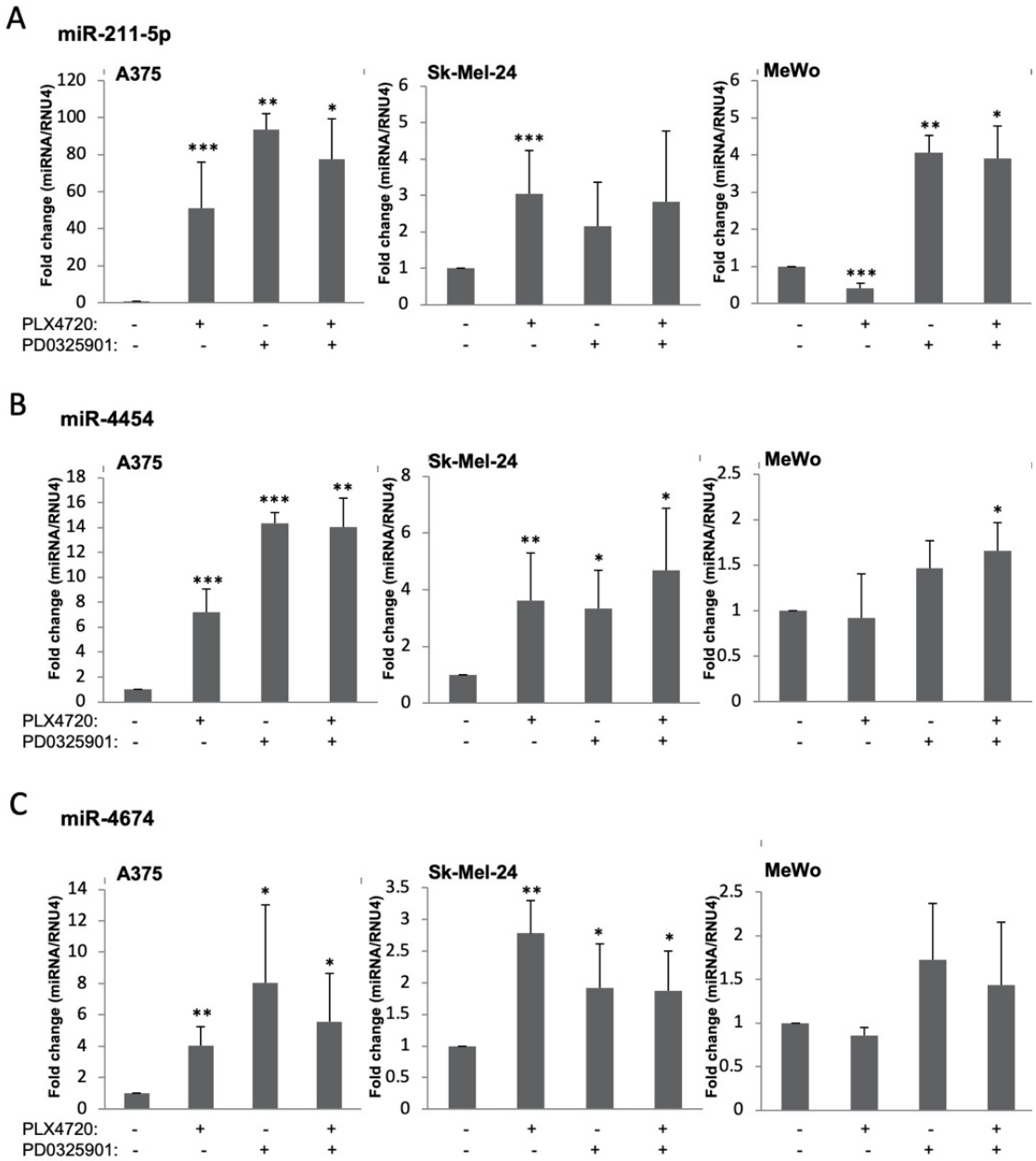


Figure 548o-5p: Cell lines described in Aim 3.1 (A375S (sensitive to BRAFi), A375R (induced resistance to BRAFi), SK-Mel-24, and MeWo were used to assess changes in miRNA expression in the untreated and also in the treated state). PLX: BRAF-inhibitor, MEKi: MEK-inhibitor; PLX + MEKi:

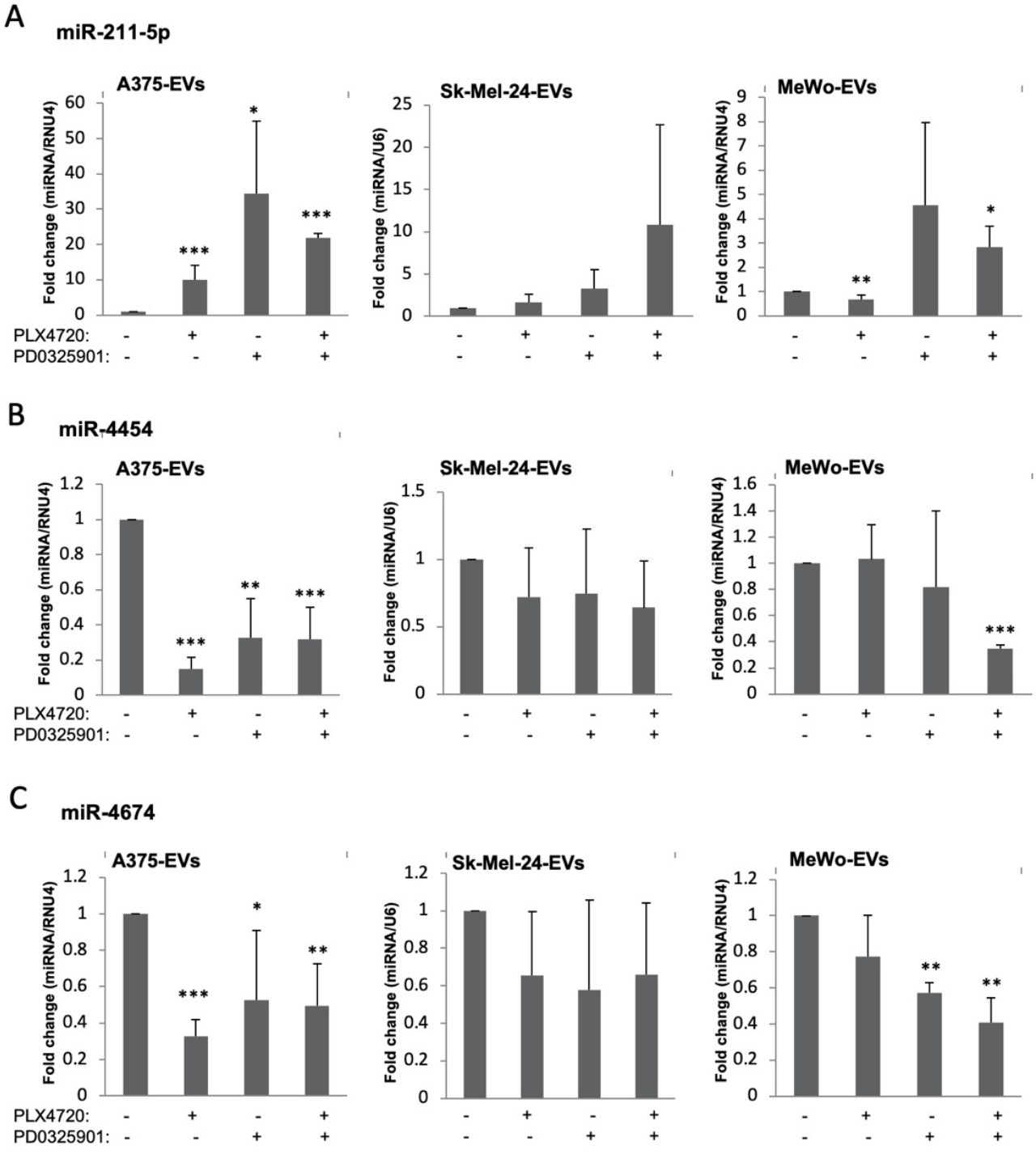
co-treatment with both BRAFi and MEKi. Left side (yellow box) are cell lines sensitive to BRAFi therapy. Right side (red box) are cell lines resistant to PLX, but sensitive to MEKi. Within each box, the left panel is from cell lines and right panel is data from cell line-derived extracellular vesicles (EVs). *We decreased levels of miR-548o-5p in cells, with concomitant decreased levels in EVs in response to therapy to which the cell line is susceptible.*

In a subset of cell lines (A375S, SK-Mel24, and MeWo) and with a more streamlined panel of miRNA (miR-211-5p, miR-4454, and miR-4674) we proceeded with overexpression and inhibition of exosome/extracellular vesicle production via treatment with GSW4869. Data will be presented below.

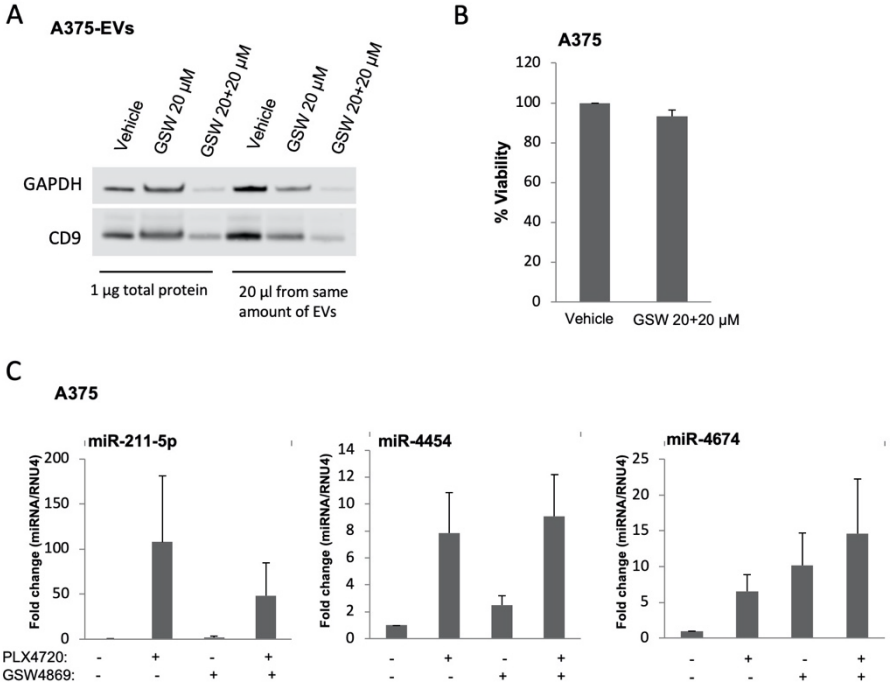
miRNA Expression in Selected Melanoma Cell Lines: Cell lines were treated with BRAF-inhibitor (PLX4720) and MEK-inhibitor (PD0325901) or the combination. It is know that A375 and SK-Mel-24 cells are sensitive to BRAFi and combination BRAFi + MEKi. MeWo cells are not sensitive to BRAFi, but are sensitive to MEKi. *We show that treatment of the cell lines with their respective inhibitor (or combination) results in increased expression of miR-211-5p, miR-4454, and miR-4674 within cells.*

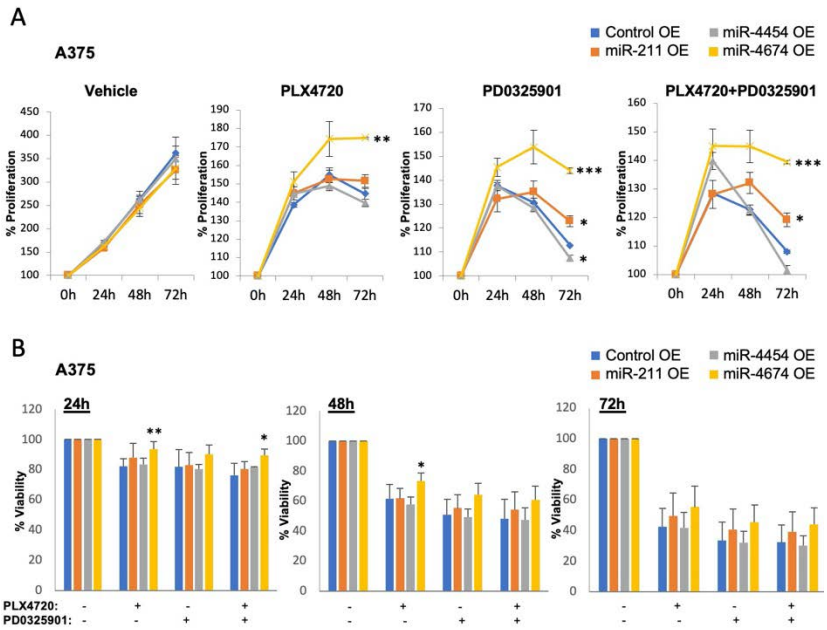


miRNA Expression in Selected Melanoma Cell Line-Derived Extracellular Vesicles (EVs): Cell lines were treated with BRAF-inhibitor (PLX4720) and MEK-inhibitor (PD0325901) or the combination. It is know that A375 and SK-Mel-24 cells are sensitive to BRAFi and combination BRAFi + MEKi. MeWo cells are not sensitive to BRAFi, but are sensitive to MEKi. *We show that treatment of the cell lines with their respective inhibitor (or combination) results in increased expression of miR-211-5p in EVs, but decreased levels of miR-4454 and miR-4674 within EVs derived from these same cell lines.*

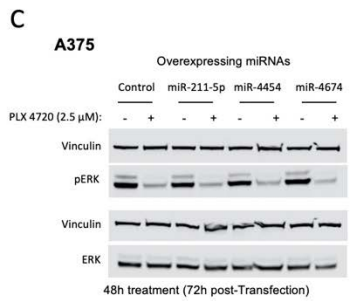


Insight in EVs and miRNAs dynamics. GSW4869 was used to block EVs production at 20 µM added each 24h for 48h. (A) CD9 reduction as detected by western blot was used to determine the optimal concentration of GSW4869. (B) No differences in viability were seen after treatment for 48h with GSW4869. (C) miRNA expression inside the cells is determined by qPCR after blocking EVs production and treatment with MAPK inhibitors for 48h.



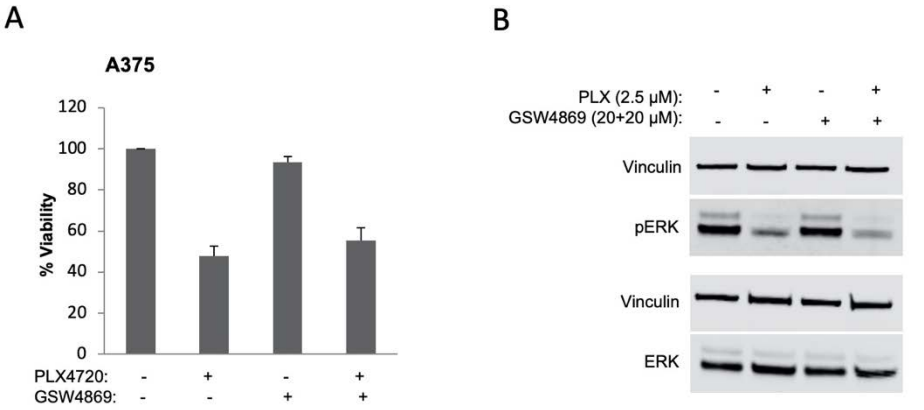


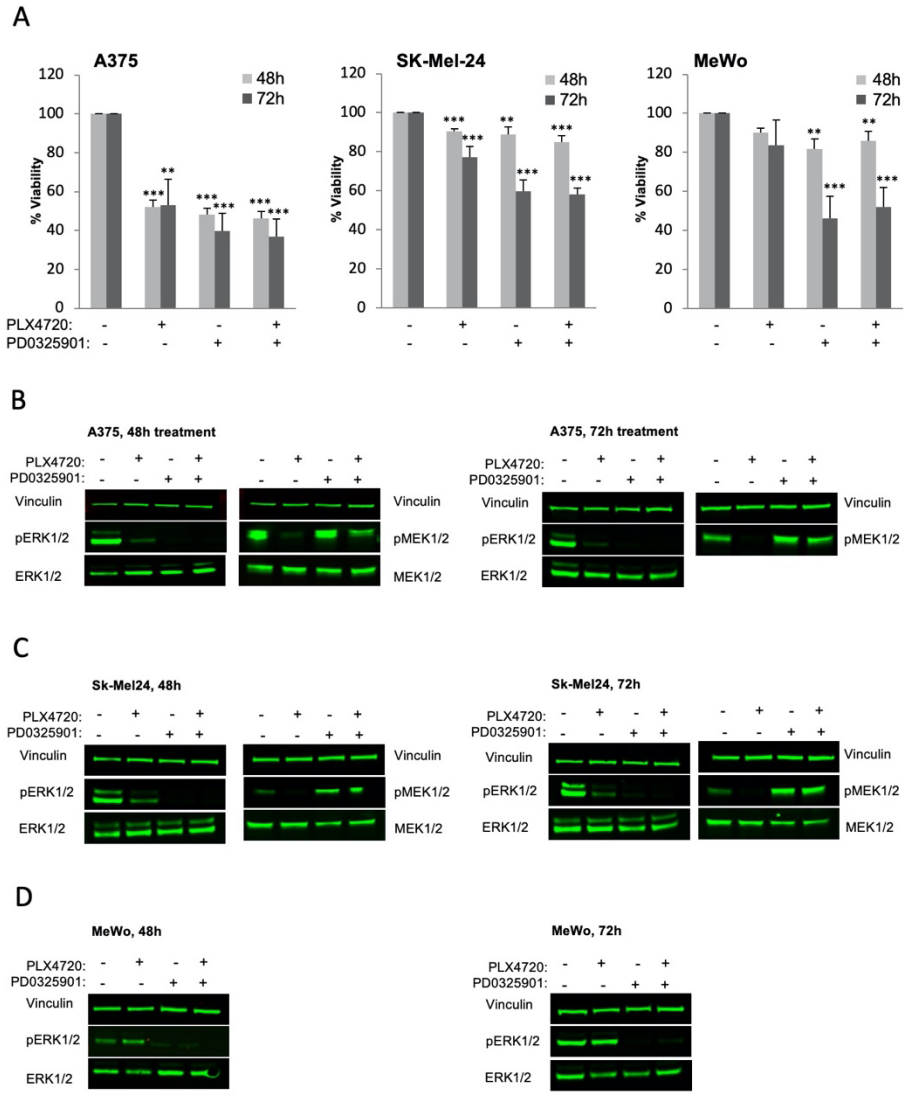
Role of miRNAs in resistance to targeted therapy. miR-211-5p, miR-4454 and miR-4674 were transiently overexpressed in A375 to be subjected to proliferation assays (A) up to 72h and viability assays (B) for the time points indicated in the presence of MAPK inhibitors. (C) Western blot for MAPK pathway changes. *Our results show that overexpression of miR-4674 resulted in increased proliferation and viability, while overexpression of miR-211 resulted in increased proliferation*.



C) Western blot for MAPK pathway 20h??
Inhibition of miRNAs to be included here later.

Characterization of miRNA and EVs role in resistance to targeted therapy. A375 cells were treated with the BRAF inhibitor (PLX4720) or the EV production inhibitor (CSW4869) for 48h and subjected to viability assays (A) and western blot (B) for the antibodies shown. (C) A375 cells were transfected with miR-4674 mimics and treated with PLX4720 and/or GSW4869 to be analyzed in proliferation and viability assays (data not shown, in preparation). *Our results show that treatment with BRAFi resulted in decreased viability with associated changes in p-ERK protein expression (as expected), while treatment with GSW4869, which blocks EV production did not alter these changes in cell viability and/or protein production*.

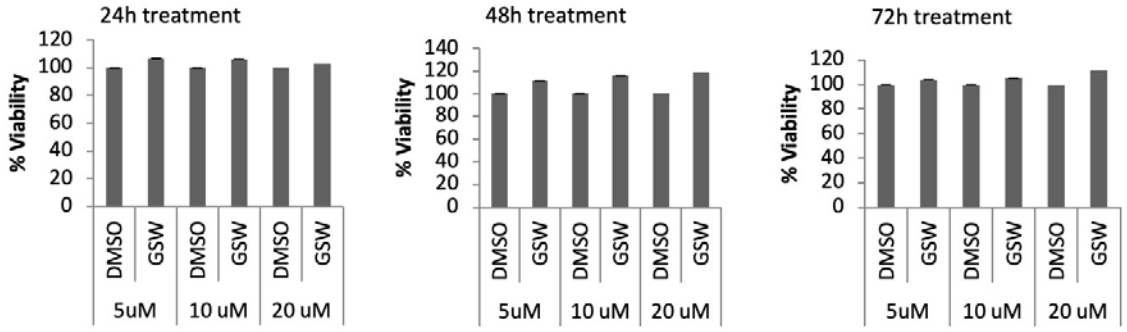




Supplemental Figures: Characterization of the cell lines sensitivity/resistance to targeted therapy. (A) The indicated human melanoma cell lines were tested in viability assays after 48h or 72h treatment with MAPK inhibitors. A375 (B), Sk-Mel-24 (C) and MeWo (D) cell lysates were subjected to immunoblotting to the antibodies shown after cell exposure for 48h (left) or 72h (right) to the BRAF inhibitor, the MEK inhibitor or its combination. *Our results show changes in viability and MAPK signaling expected with treatment with BRAFi and/or MEKi.*

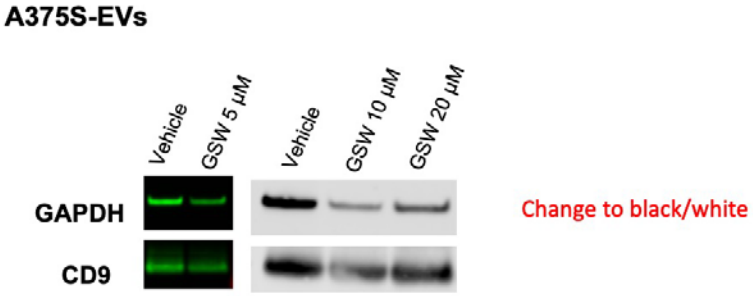
Supplemental Figures: High concentrations of GSW4869 are needed to block EVs production efficiently. (A) A375 were treated with different concentrations of GSW4869 for 24h, 48h and 72h and tested in viability assays. (B) A375 cells were kept for 48h with the indicated GSW4869 doses and their supernatants were subjected to immunoblotting after ultracentrifugation. *Our results show that higher exposure times and concentrations of GSQ4869 are needed to block EV production and do not impact cell viability.*

A



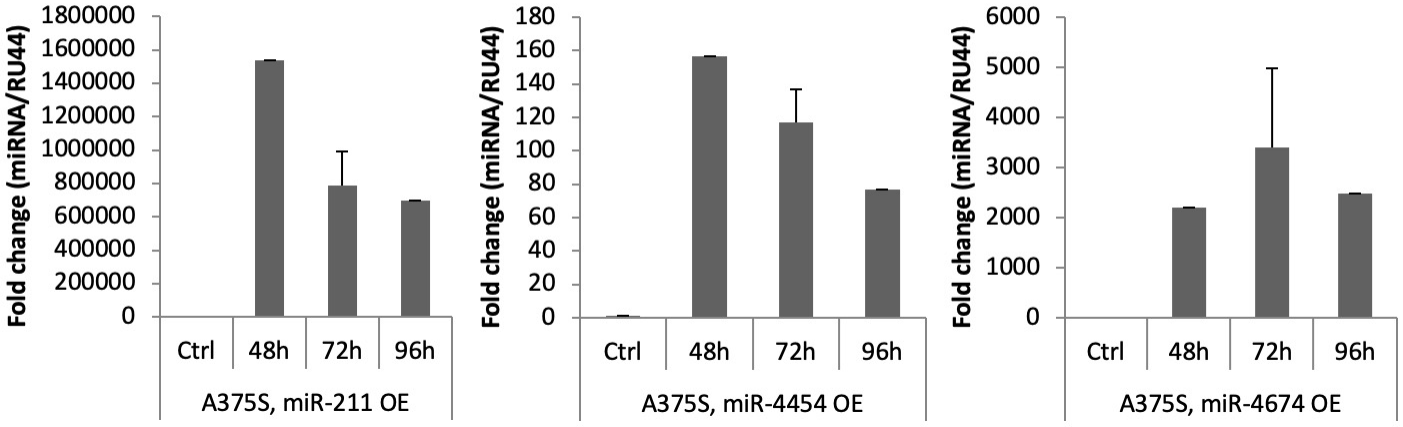
B

Need to do one more viability exp (5, 10, 20 uM)

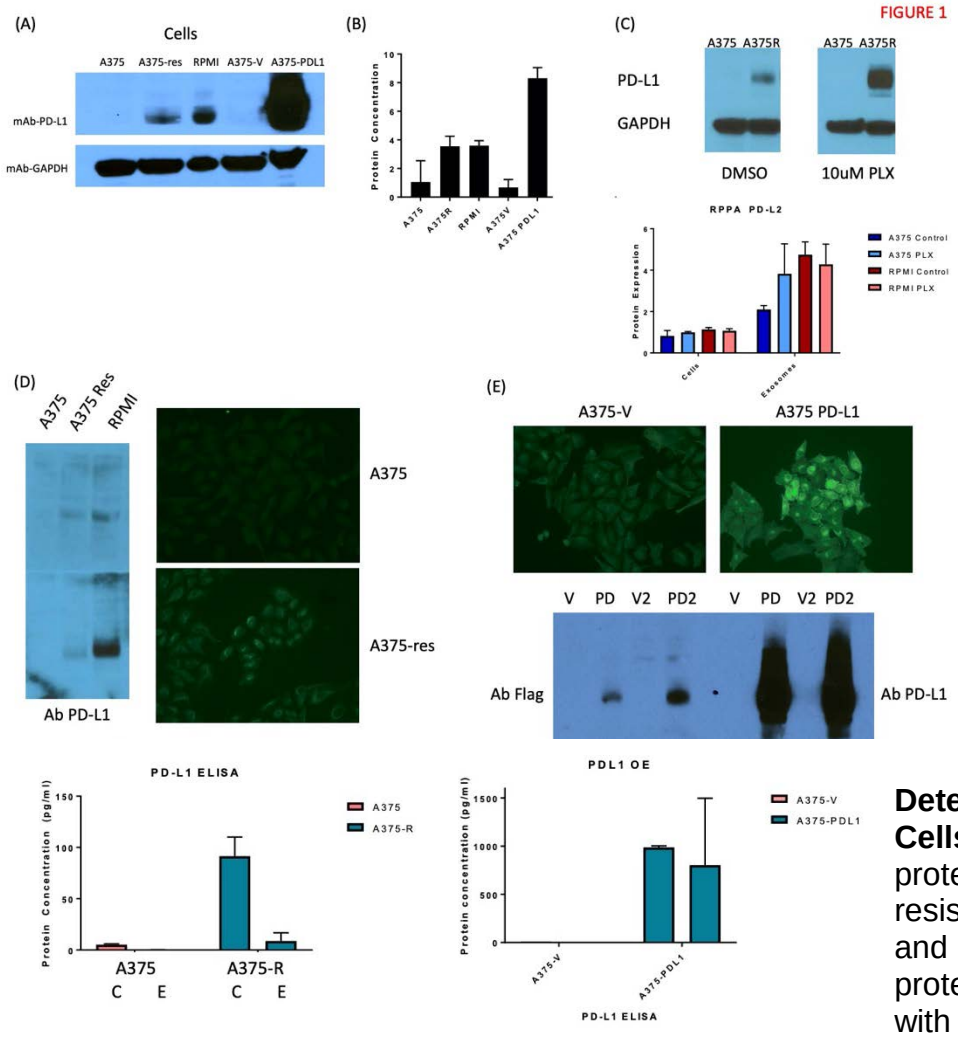


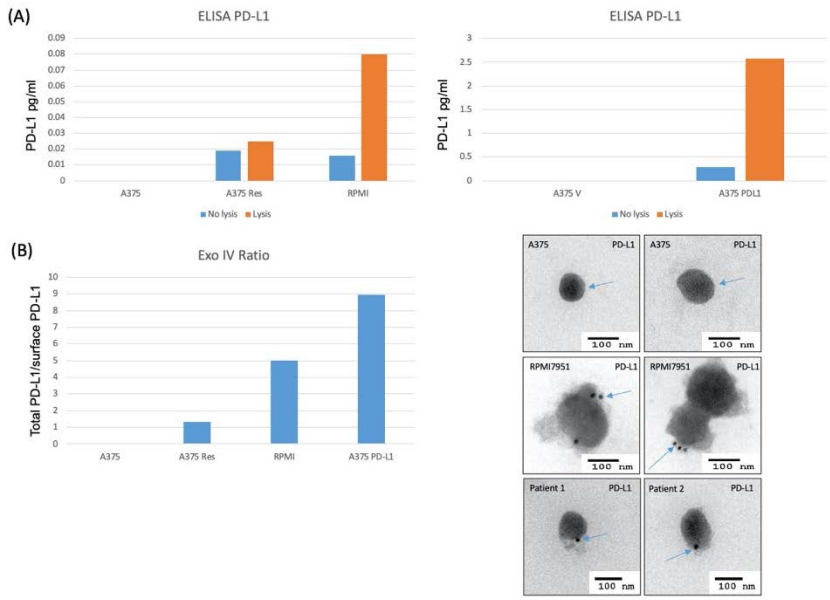
Supplemental Figures: Transfection efficiency. (A) miRNA overexpression levels were detected by qPCR after 48h, 72h and 96h post-transfection. (B) reduction of miRNA expression was shown after 48h, 72h and 96h post-transfection. *Our results show efficient overexpression of our target miRNA at various time points.*

A



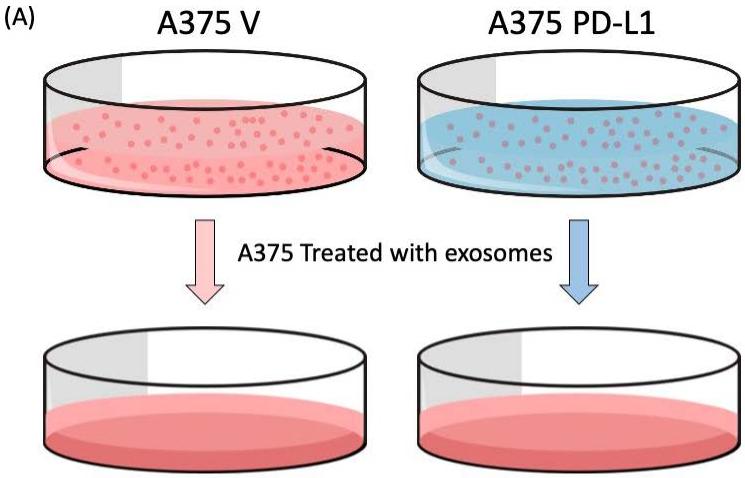
Role of PD-L1 in Melanoma Cells. (A, B) Increased protein expression of PD-L1 in BRAFi resistant cells, and 100 fold increase in PD-L1 protein expression with overexpression (A: Western Blot; B: ELISA). (C) Induced expression of PD-L1 upon treatment with BRAFi (PLX) in BRAFi resistant cells. (D, E) Confirmation via Western Blot and immunoflourescence. *Our results show efficient overexpression of our target miRNA at various time points.*



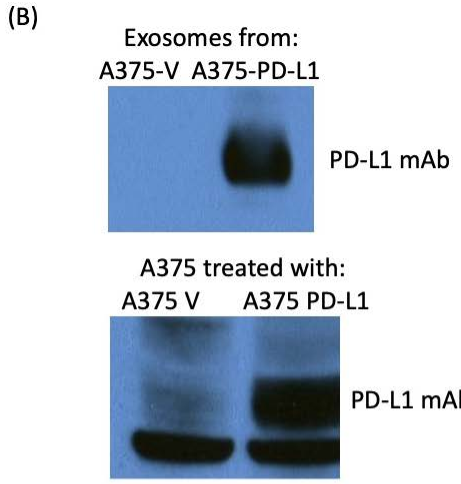


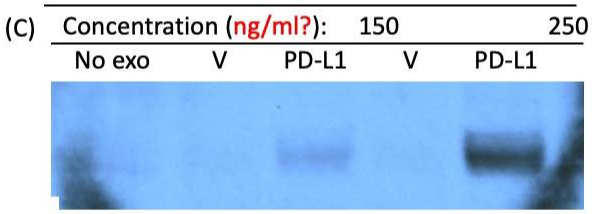
Detection of PD-L1 Protein in Cells/EVs. (Top panels) Increased protein expression of PD-L1 in BRAFi resistant cells and paired EVs (left), and ~100 fold increase in PD-L1 protein expression in cells and EVs with overexpression (right) via ELISA (A) ELISA of both unlysed and lysed proteim extract from EVs demonstrates that there is PD-L1 protein both on the surface (blue – unlysed EVs) and within the EV (orange – lysed EVs) (B) We calculated a ratio of total PD-L1 (lysed)/EV membrane (unlysed) EVs, which is higher in resistant cells and exceptionally high in OE cells (right panel), (left panel) shows EM with immunogold labeling of PD-L1 on surface of EVs. *Our results demonstrate that melanoma cell line-derived EVs have PD-L1 protein both on the surface and within the vesicle and the amount of protein increases in the setting of BRAFi resistance.*

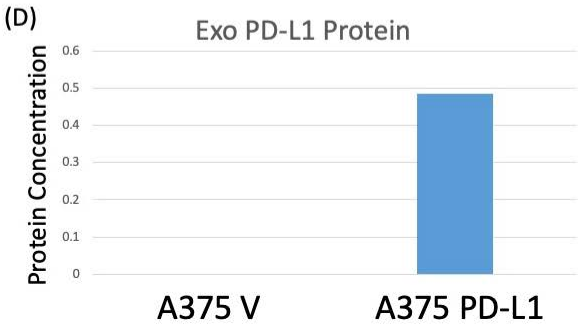
PD-L1 protein can be transferred from "high" cells to "low" cells via EVs. (A) model of experimental structure. A375V (vehicle) cells without overexpression of PD-L1 are grown in parallel with A375 PD-L1 (overexpressing) cells. EVs from each cell line are used to treat the parental (low PD-L1) A375 cells. (B) Western Blot analysis showing overexpression of PD-L1 in OE cells. (C, D) Demonstrates increased PD-L1 protein expression with increasing concentration of PD-L1 high EVs in culture (C: Western Blot; D: ELISA). *Our data demonstrates that PD-L1 protein can be transferred via EVs*.

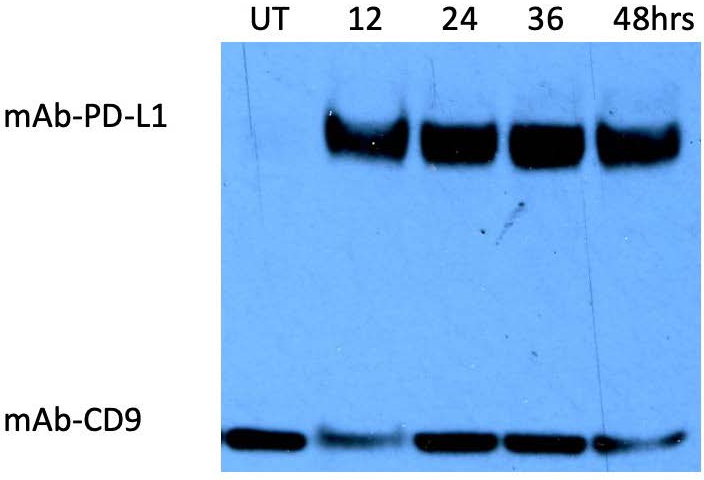


Do we have IF of these cells too?









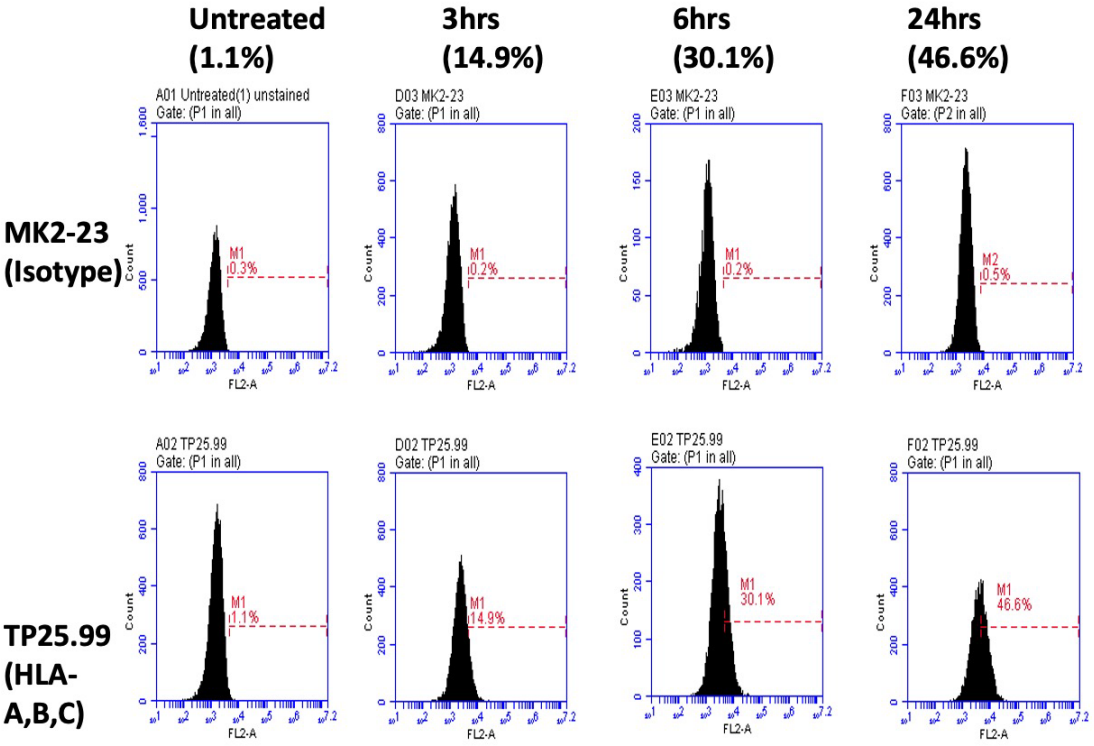
A375 cells treated with exos from a375-PD-L1 cells. Cell extracts harvested at 12, 24, 36, and 48hrs post Treatment.
CD9 is control for exosomal contamination.

This EV-Mediated PD-L1 Transfer is Sustained. Western Blot analysis reveals persistent expression of PD-L1 in recipient (PD-L1 low) cell lines for up to 48 hrs post-treatment. *Our results show the protein transfer is durable for at least up to 2 days*.

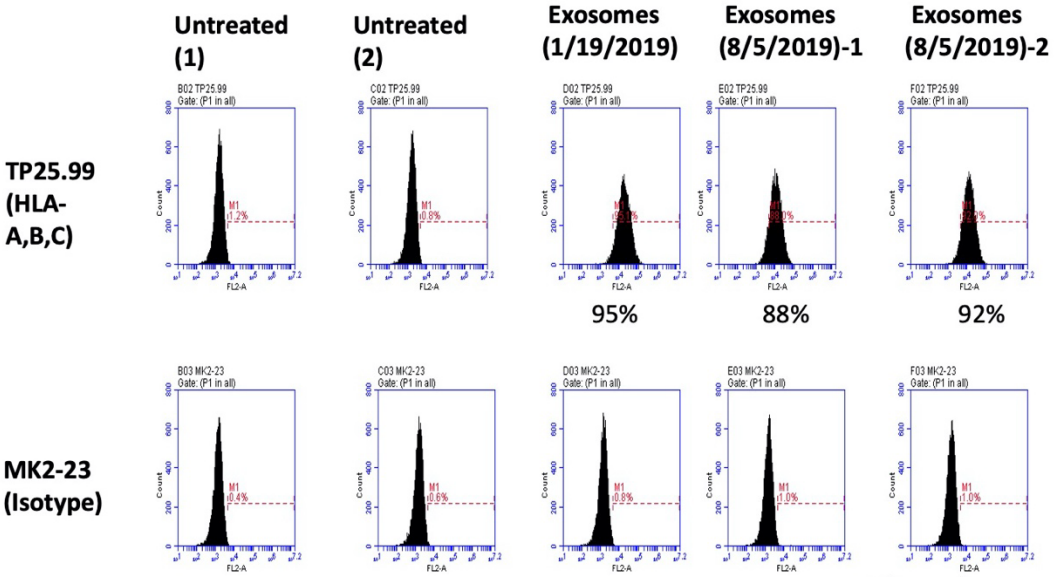
Role of HLA-A in Melanoma Cells and EVs. Both A375 and RPMI7951 cell lines are HLA-A2 positive, while FO-1 cells are HLA(-). We isolated EVs from A375 and RPMI cells and treated FO-1 cells to examine transfer of HLA-A via EVs. *Our results show efficient overexpression of our target miRNA at various time points.*

Role of HLA-A in Melanoma Cells and EVs. EVs from A375 cell lines (HLA-A2 +) were isolated and used to treat FO-1 cells (HLA(-)). HLA-A expression on recipient cells (bottom panel, TP25.99) was used to compared to negative control (upper panel MK2-23). *Our results show increasing expression of HLA-A exclusively on treated FO-1 cells as a function of increasing exposure.*

FO-1 cells treated with 25ug of exosomes for 3, 6 and 24 hours



FO-1 cells treated with several preps of RPMI7951 exosomes for 24hrs



Role of HLA-A in Melanoma EVs. EVs from RPMI7951 cell lines (HLA-A2 +) were isolated and used to treat FO-1 cells (HLA(-)). HLA-A expression on recipient cells (top panel, TP25.99) was used to compared to negative control (lower panel MK2-23). *Our results show increasing expression of HLA-A exclusively on treated FO-1 cells from a different HLA-A(+) cell line.*

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."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

I have completed the Harvard GRASP R01 grant writing course. I also co-coordinated a Department of Surgery Grant Writing Program Jan 2020 in conjunction with surgical leaders at Brigham and Women's Hospital.

I was nominated and selected for 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. Foundational ideas from this work (in tumor and EV analysis) have informed these projects.

I presented related work in a variety of forums:

2019	Utilizing Extracellular Vesicles for Prediction and Monitoring of Immunotherapy Responses in Melanoma. Invited Lecturer. Molecular Med TriCon. San Francisco, CA.
2019	Tumor and Immune Monitoring via Exosomal Analysis in Patients on Targeted Therapy with Anti-PD1 Immunotherapy. Clinical Investigator Award Recipient: Society of Surgical Oncology, San Diego, CA.
2019	Toward the Future of Noninvasive Monitoring of Targeted Therapy. Invited Lecturer/Discussant. American Society of Clinical Oncology, Chicago, IL.
2019	Blood-Based Biomarkers of Response and Immune-Related Adverse Events During Immune Checkpoint Blockade. Invited Lecturer, Immuno-Oncology Summit, Boston, MA.
2019	Characterization of Immune Checkpoint Response and Resistance via Genetic, Transcriptomic, and Epigenetic Analyses. Invited Lecturer, Immuno-Oncology Summit, Boston, MA.
2019	Use of Extracellular Vesicles for Monitoring Cancer. Moderator/Lecturer. American College of Surgeons, San Francisco, CA.
2019	Multimodality Approach to Melanoma Immunotherapy Response and Resistance Invited Lecturer. Sydney Kimmel Cancer Center Grand Rounds, Philadelphia, PA.
2020	Evolution of Immunotherapy Resistance in Melanoma. Invited Lecturer. Carnegie Institute of Embryology, Baltimore, MD.
2019	Longitudinal Tumor Analysis in Melanoma. Invited Lecturer. Melanoma Symposium, Otranto Italy.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

As mentioned above, the preliminary data included here was 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 (ASCO, ACS, Cancer Center Programs). I also moderated a session at the Liquid Biomarkers Gordon Conference (summer 2019) to connect with members of the liquid biopsy community.

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.

I am eager to get the sequencing/analysis portion of the grant back on track due to delays relating to COVID-19. We are also working to optimize cell-specific EV selection. We elected to delay the proposed patient sample EV processing until the QC was complete, but we have been using other patient-derived samples to optimize this process.

1. Analysis of sequencing data when available
2. Cell specific and bulk EV analysis

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 made significant headway in a variety of ways:

1. Generation of integrated data sets (Aim 1). While the analysis of these datasets are delayed, the creation of these longitudinal, integrated data sets is novel and will add insight into changes occurring in tumor and in circulation during immunotherapy.
2. We are developing new techniques for cell-specific EV selection (Aim 2) and we are making progress with validating/refining these techniques in vitro with the goal of applying this technology to patient samples.
3. We have made significant headway (Aim 3) in characterizing various miRNA in targeted therapy response/resistance, new approaches to EV delivery of proteins (PD-L1 and HLA-A), and functional validation of these approaches.

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). I was awarded an MGH Innovation Discovery Grant to develop these cell-selective EV capture and AI-based analytical approaches further.

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.

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.

During in vitro analysis (Aim 3), we did narrow the miRNA list and cell lines for each project based upon the success/lack of success of initial screening experiments (i.e. some miRNA experiments were less consistent or without clear patterns of expression and these candidates were de-prioritized for subsequent experiments).

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 this has limited our ability to stay specifically on track time-wise. We did elect to proceed with Year 2/3 in vitro experiments up front, so we are ahead of schedule in this regard. We anticipate catching up to the proposed timeline once our laboratories are back to full activity (in MA, we are currently in Phase II reopening, which equates to ~ 50% capacity/operations as compared to pre-COVID-19).

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).*

Shi A, Kasumova G, Michaud WA, Cintolo-Gonzalez J, Mehta A, Chien I, Frederick DT, Cohen S, Plana D, Johnson D. Flaherty KT, Sullivan RJ, Kellis M, **Boland GM**. Plasma-derived exosomal analysis and deconvoution enables prediction and tracking of checkpoint blockade response. Science Advances. Revisions, resubmission pending.

2 manuscripts in preparation (re: EV miRNA, PD-L1 and HLA-A transfer).

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.*

2019	Utilizing Extracellular Vesicles for Prediction and Monitoring of Immunotherapy Responses in Melanoma. Invited Lecturer. Molecular Med TriCon. San Francisco, CA.
2019	Tumor and Immune Monitoring via Exosomal Analysis in Patients on Targeted Therapy with Anti-PD1 Immunotherapy. Clinical Investigator Award Recipient: Society of Surgical Oncology, San Diego, CA.
2019	Toward the Future of Noninvasive Monitoring of Targeted Therapy. Invited Lecturer/Discussant. American Society of Clinical Oncology, Chicago, IL.
2019	Blood-Based Biomarkers of Response and Immune-Related Adverse Events During Immune Checkpoint Blockade. Invited Lecturer, Immuno-Oncology Summit, Boston, MA.

2019	Characterization of Immune Checkpoint Response and Resistance via Genetic, Transcriptomic, and Epigenetic Analyses. Invited Lecturer, Immuno-Oncology Summit, Boston, MA.
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2019	Multimodality Approach to Melanoma Immunotherapy Response and Resistance Invited Lecturer. Sydney Kimmel Cancer Center Grand Rounds, Philadelphia, PA.
2020	Evolution of Immunotherapy Resistance in Melanoma. Invited Lecturer. Carnegie Institute of Embryology, Baltimore, MD.
2019	Longitudinal Tumor Analysis in Melanoma. Invited Lecturer. Melanoma Symposium, Otranto Italy.

- **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".

Name: *Genevieve Boland*
Project Role: *Principal Investigator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1.8*
Contribution to Project: *Dr. Boland leads these efforts and coordinates with the various laboratory collaborating groups.*

Funding Support:

Adelson Foundation Fund (Flaherty) 10/01/14 – 09/31/2020 2 calendar
Adelson Medical Research Foundation $309,476
Combination approaches to overcome resistance to targeted therapy in melanoma

1R01 CA193970 (Sullivan and Mehnert) 12/01/2015-11/30/2020 1.2 Cal Mos
NCI/NIH $2,392,801
Promoting Bim-Driven Apoptosis through MAPK inhibition: A therapeutic Strategy in Advanced Melanoma

Melanoma Research Alliance (Mahmood) 05/15/17 – 5/14/22 1 calendar
$125,000
Granzyme B imaging to predict efficacy of immunotherapy in melanoma

1R01CA214744-01A1 (Mahmood) 07/01/17-06/30/22 0.6 cal
NIH $250,000
Cytotoxic lymphocyte function PET Imaging to predict cancer immunotherapy response

1 U54 CA225088-01 (Sorger; Flaherty co-project leader) 2/1/2018-1/31/2023 0.12 c
NIH/NCI $146,548
Systems Pharmacology of Therapeutic and Adverse Responses to ImmuneCheckpoint 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.

Human Tumor Atlas Network/U2C Grant: Boland (Investigator) 10/01/18 – 10/01/2023 0.25 cal
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.

Melanoma Research Foundation Breakthrough Consortium (MRFBC) Young Investigator Research Team Award to Advance the Field of Translational Immuno-Oncology (PI: Boland, Saladi, Liu)
04/01/2019 – 04/01/2021 0.1 cal
$150,000
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.

Department of Defense CDMRP Career Development Award (Boland) 09/01/2019 – 09/01/2022 1.8 cal
$120,000
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.

Partners Innovation Development Grant (Boland, Stott) 03/01/2019 – 03/01/2020 0 cal
$50,000
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.

2PO1 CA163222-06 (Fisher) 08/06/2019 – 08/06/2022 0.1 cal
NIH
Targetable 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.

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.

Name: *Genevieve Boland*
Project Role: *Principal Investigator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1.8*
Contribution to Project: *Dr. Boland leads these efforts and coordinates with the various laboratory collaborating groups.*
New Funding Support:
1 U54 CA225088-01 (Sorger; Flaherty co-project leader) 2/1/2018-1/31/2023 0.12 c
NIH/NCI $146,548
Systems Pharmacology of Therapeutic and Adverse Responses to ImmuneCheckpoint and Small Molecule Drugs

Human Tumor Atlas Network/U2C Grant: Boland (Investigator) 10/01/18 – 10/01/2023 0.25 cal
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.

Melanoma Research Foundation Breakthrough Consortium (MRFBC) Young Investigator Research Team Award to Advance the Field of Translational Immuno-Oncology (PI: Boland, Saladi, Liu)

04/01/2019 – 04/01/2021 0.1 cal
$150,000

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.

Partners Innovation Development Grant (Boland, Stott) 03/01/2019 – 03/01/2020
0 cal $50,000
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.

2PO1 CA163222-06 (Fisher) 08/06/2019 – 08/06/2022 0.1 cal
NIH
Targetable 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.

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)

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.*