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TITLE: Epigenetic Regulation of the Melanoma Microenvironment

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CONTRACTING ORGANIZATION: Icahn School of Medicine at Mount Sinai, New York, NY

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Deregulation of epigenetic states promotes melanoma progression. MacroH2A, a histone variant associated with transcriptional repression, is downregulated in melanoma vs. benign nevi, where it suppresses proliferation and metastatic potential. However, its role as a barrier to tumorigenesis has not been investigated *in vivo*. We found that mice constitutively lacking macroH2A variants exhibit accelerated melanoma growth compared to their wild-type counterparts. MacroH2A-deficient tumors display impaired cytotoxic T cell function and increased monocyte infiltration, consistent with a compromised anti-tumor immune response, as well as upregulation of *Ccl2*, *Cxcl1* and *Il6*, which are myeloid chemo-attractants. Through single-cell transcriptomic profiling of the entire melanoma microenvironment, we identified alterations in the immune cell compartment in macroH2A-deficient tumors as a consequence of the cancer-associated fibroblast population expressing and secreting such myeloid chemo-attractants. Altogether, our data supports a novel tumor suppressor role for macroH2A through repression of pro-inflammatory signaling within the melanoma microenvironment.

15. SUBJECT TERMS
Melanoma, tumor microenvironment, macroH2A, histone variant, cancer-associated fibroblasts, anti-tumor immunity

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1. INTRODUCTION:

Deregulation of epigenetic states has emerged as a critical driver of aberrant transcriptional programs promoting melanoma development and therapeutic resistance. The Bernstein laboratory showed that macroH2A, a histone variant associated with transcriptional repression, is downregulated in melanoma vs. benign nevi and suppresses melanoma cell proliferation and metastatic potential. However, its role as a barrier to tumorigenesis has not been investigated *in vivo*. We have assessed macroH2A function during the entire process of melanoma development in an animal model where macroH2A is either present (WT) or absent (dKO). We found that melanoma growth was significantly enhanced in dKO animals and the immune cells within these macroH2A-deficient tumors, which normally destroy cancer cells, were abnormal. We thus hypothesized that the absence of macroH2A variants promotes melanoma aggressiveness through a defect in immune cell anti-tumor response. This could stem either from melanoma cells eliciting tolerance by the immune system, from functional deficiencies intrinsic to immune cells, and/or from activation of cancer-associated fibroblasts (CAFs) that can promote an immunosuppressive environment. We proposed to understand the link between macroH2A deficiency and immune tolerance *in vivo* by characterizing how macroH2A loss in melanoma cells promotes their escape from immune surveillance, and how macroH2A regulates the anti-tumor activity of immune cells. This proposal has the potential to highlight macroH2A as a prognostic marker for response to immune checkpoint blockade and/or as a regulator of tractable molecular targets for therapy, and overall, to help refine the clinical benefits of immunotherapy for melanoma patients. Moreover, as the histone variant macroH2A has not been implicated in melanoma immunity, this proposal is innovative and timely given that immunotherapy is currently at center stage of melanoma therapeutics.

2. KEYWORDS:

Melanoma, tumor microenvironment, macroH2A, histone variant, cancer-associated fibroblasts, anti-tumor immunity

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Please see attached document (Major Goals)

What was accomplished under these goals?

Please see attached document (Accomplishments)

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

Participation in professional development activities for key personnel Dan Filipescu:

May 2022 – ISMMS Skin Biology and Diseases Resource-based Center Annual Retreat, selected talk

How were the results disseminated to communities of interest?

Nothing to Report.

We plan to continue carrying out the tasks as stated in the Statement of Work.

4. **IMPACT:**

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

We have identified an important role for histone variants in regulating gene expression in cancer-associated fibroblasts (CAFs), a novel finding to our knowledge in any cancer.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

We experienced difficulties with obtaining sufficient numbers of the melanoma-specific macroH2A1.2 cKO mouse strain, whose use is described in Major Task 2, Subtask 7. Founders for the macroH2A1.2$^{wt/wt}$ and macroH2A1.2$^{fl/fl}$ sublines were generated but have a limited lifespan due to leaky Cre transgene activation and development of spontaneous melanomas when reaching breeding age in a majority of mice. Of note, JAX laboratories, which provide the melanoma strain used to derive these mice, now highlights this issue in the strain description: https://www.jax.org/strain/013590 . This resulted in the impossibility to expand the strain and obtain cohorts of mice in sufficient numbers for melanoma induction by 4-HT application in order to compare 12 macroH2A1.2$^{wt/wt}$ and 12 macroH2A1.2$^{fl/fl}$ mice as proposed. We are currently expanding this strain and expect to have sufficient mice in the following year.

Nothing to Report.

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

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

The manuscript supported by this award is currently in revision at *Nature Cell Biology*, and has been deposited in the publicly available domain through the journal submission process at:
https://www.researchsquare.com/article/rs-1578023/v1
Acknowledgement of support: YES

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

Nothing to Report.

Other publications, conference papers and presentations.

Seminar presentation by key personnel Dan Filipescu:

October 2021 - Society for Melanoma Research International Congress (virtual), poster presentation: *MacroH2A limits melanoma progression by inhibiting chemokine expression in cancer-associated fibroblasts*

September 2021 - EMBO Workshop on Physiology and Function of Histone Variants (virtual), selected talk: *MacroH2A limits melanoma progression by inhibiting chemokine expression in cancer-associated fibroblasts*

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	**Dan Filipescu, PhD**
Project Role:	Instructor
Researcher Identifier:	https://orcid.org/0000-0001-6381-2557
Nearest person month worked:	3.4
Contribution to Project:	Dr. Filipescu performed experiments, collected and analyzed data, interpreted results and prepared them for presentations and publication; worked closely with collaborators.
Funding Support:	N/A

Name:	**Emily Bernstein, PhD**
Project Role:	PI
Researcher Identifier:	https://orcid.org/0000-0001-6533-8326
Nearest person month worked:	0.6
Contribution to Project:	Dr. Bernstein oversees research design, data interpretation, manuscript preparation, supervises Drs. Filipescu & Jostes, and coordinates collaborative efforts.
Funding Support:	N/A

Name:	**Sina Jostes, PhD**
Project Role:	Postdoctoral Fellow
Researcher Identifier:	https://orcid.org/0000-0003-3212-6727
Nearest person month worked:	2.6
Contribution to Project:	Dr. Jostes has been instrumental in maintaining melanoma cell lines and assisting with mouse colony maintenance.
Funding Support:	N/A

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

New

937310 (Bernstein) Melanoma Research Alliance
6/1/2022 - 5/31/2024
Primary Place of Performance: Icahn School of Medicine at Mount Sinai
Role: PI
Investigating ARID2 as a suppressor of melanoma metastasis
Aim 1. To investigate the molecular mechanisms underlying ARID2 function as a suppressor of melanoma metastasis.

Year	Calendar Months
2023	0.240
2024	0.240

Overlap: NONE

(Bernstein) Melanoma Research Foundation
9/30/2022 - 8/31/2024
Primary Place of Performance: Icahn School of Medicine at Mount Sinai
Role: PI
Chromatin remodeling alterations and consequences for melanoma metastasis
Aim 1. Interrogate PBAF as a suppressor of melanoma metastasis *in vivo*.
Aim 2. Investigate the role of PBAF in the brain niche *in vivo*.

Year	Calendar Months
2023	0.600
2024	0.600

Overlap: NONE

Completed

619959 (Bernstein) Melanoma Research Alliance
7/1/2020 - 6/30/2022
Primary Place of Performance: Icahn School of Medicine at Mount Sinai
Role: PI
Histone variant regulation of the melanoma microenvironment

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: QUAD CHARTS:

9. APPENDICES:

Major goals

Aim 1. Decipher the immune microenvironment of macroH2A-deficient melanoma at single-cell resolution. Single cell RNA-sequencing (scRNA-seq) provides an unprecedented means to profile the cellular subsets of the melanoma microenvironment. With a focus on the immune cell fraction of the tumor, we will perform scRNA-seq on WT and dKO $BRAF^{V600E}$/PTEN-deficient melanomas. This study will reveal the identity of cells affected by macroH2A loss in the tumor immune microenvironment and provide transcriptional clues to their altered function in anti-tumor activity.

Major Task 1: scRNA-seq of tumor infiltrating immune cells

IACUC approvals; ACURO review and approval
Target project months: 0-3
Status: completed during Year 1

IRB approvals; HRPO review and approval
Target project months: 14-17
Status: completed ahead of schedule during Year 1

Subtask 1: Induce melanomas in macroH2A WT and dKO triallelic murine model (autochthonous, genetically induced model)
Target project months: 6-7
Status: completed during Year 1

Subtask 2: Generate single-cell suspension from three tumors per genotype, isolate populations of interest and process for droplet-based scRNA-seq library generation on Chromium 10X instrument. Sequence libraries on Illumina NextSeq 500 and perform preliminary data processing to generate single cell gene count matrix. Generate cell clusters and annotate them to known cell populations.
Target project months: 8-10
Status: completed during Year 1

Subtask 3: For each identified population, contrast their relative abundance and analyze transcriptional heterogeneity.
Target project months: 10
Status: completed during Year 1

Subtask 4: Run published tools to assess changes in activation state and cell differentiation trajectory upon macroH2A loss.
Target project months: 11
Status: completed during Year 1

Subtask 5: Bioinformatic analysis leveraging known ligand-receptor interactions to identify origin of immunosuppressive signals in dKO tumors.
Target project months: 12
Status: ongoing

Aim 2. Deconvolute macroH2A function in the tumor and immune compartments of the anti-tumor response. Our mouse model lacks macroH2A in all cell types, and thus, impaired anti-tumor immunity could stem from intrinsic changes in tumor cells, or from functional deficiencies of the immune system in these mice. **Aim 2.1**. To clearly delineate the impact of the tumor genotype, we will use macroH2A-deleted melanoma cells (e.g. B16, YUMM, YUMMER) for tumor graft assays in WT immunocompetent hosts and for CD8+ T cell mediated killing assays *in vitro*. Further, we will utilize a novel conditional KO mouse model to ablate macroH2A specifically in melanoma cells. This will allow us to determine whether (and how) melanoma cells deficient for macroH2A induce dysfunction in the immune cell compartment. **Aim 2.2.** To test the functionality of macroH2A-deficient immune cells independently of the tumor genotype, we will assess dKO CD8+ T cell activation *in vitro.* Furthermore, we will generate melanoma grafts in mice with a T cell conditional macroH2A deletion to test whether macroH2A-deficient T cells fail to control melanoma development. **Aim 2.3**. We will investigate the effects of macroH2A loss on therapeutic outcomes in melanoma by administering BRAF inhibitors (efficacy in BRAF/PTEN murine model is dependent on cytotoxic T cells) or checkpoint blockade therapy.

Major Task 2: Determine if macroH2A deficiency has a tumor-intrinsic effect

Subtask 1: Generate B16-F1, B16-OVA and YUMMER1.7 cell lines harboring macroH2A1 and/or -2 deletion by CRISPR/Cas9 methodology.
Target project months: 1
Status: completed during Year 1

Subtask 2: Characterize the proliferation, migration and invasion characteristics of these cell lines in vitro as a consequence of macroH2A loss.
Target project months: 2-3
Status: completed during Year 1

Subtask 3: Generate subcutaneous allografts of macroH2A-proficient and deficient B16-F1 and YUMMER1.7 cells into C57BL/6 WT hosts, measure growth and immunophenotypic impact of macroH2A loss.
Target project months: 4-7
Status: completed during Year 1

Subtask 4: Generate subcutaneous allografts of most promising cell line from Subtask 3 into C57BL/6 WT hosts and deplete differentially represented immune populations identified in Subtasks 3, then measure impact on tumor growth.
Target project months: 8-12
Status: no longer planned

Subtask 5: In vitro T cell killing assay using macroH2A CRISPR B16-OVA cells and OT-I CD8+ T cells.
Target project months: 13-14
Status: completed ahead of time during Year 1

Subtask 6: Generate melanoma-specific macroH2A1.2 cKO mouse.
Target project months: 3-12

Status: completed during Year 1

Subtask 7: Induce melanomas with macroH2A1.2 ablation restricted to tumor compartment, analyze growth and immunophenotype.
Target project months: 13-18
Status: ongoing

Major Task 3: Identify genes responsible for macroH2A-mediated immune escape in melanoma cells

Subtask 1: Prepare RNA-seq libraries from macroH2A-deficient melanoma cell lines demonstrating immune escape and sequence on Illumina NextSeq 500
Target project months: 18-22
Status: planned as stated in SOW with a focus on CAFs

Subtask 2: ChIP for macroH2A, H3K27ac, H3K4me1, H3K4me3, prepare libraries and sequence on Illumina NextSeq 500.
Target project months: 22-26
Status: planned as stated in SOW with a focus on CAFs

Subtask 3: Integrated analysis of RNA-seq and ChIP-seq from cells +/- macroH2A and overlap with macroH2A-enriched domains.
Target project months: 26-32
Status: planned as stated in SOW with a focus on CAFs

Major Task 4: Determine if macroH2A deficiency has an immune cell- intrinsic effect

Subtask 1: Stimulate purified WT and dKO CD8+ splenocytes with CD3 and CD28 antibodies in vitro, followed by analysis of activation markers, cytokine production and proliferation.
Target project months: 13
Status: completed ahead of schedule during Year 1

Subtask 2: Generate T-cell specific macroH2A1.2 cKO mouse.
Target project months: 3-9
Status: completed during Year 1

Subtask 3: Analyze growth and immunophenotype of B16 and YUMM1.7 allografts in mice from Subtask 2
Target project months: 9-12
Status: completed during Year 1

Subtask 4: Generate OT-I TCR-transgenic, T-cell specific macroH2A1.2 cKO mouse.
Target project months: 9-15
Status: no longer planned

Subtask 5: In vitro T cell killing assay using B16-OVA cells and OT-I macroH2A1.2-deleted CD8+ T cells.
Target project months: 16-17
Status: no longer planned

Subtask 6: Generate Thy1.1/1.2 Pmel-1 TCR-transgenic, T-cell specific macroH2A1.2 cKO mouse; Thy1.1 Pmel-1 TCR-transgenic, macroH2A WT mouse; BRAF/PTEN melanomas on C57BL/6 background (Thy1.2).
Target project months: 16-24
Status: no longer planned

Subtask 7: Inject congenically marked macroH2A1.2 KO (Thy1.1/1.2) and WT (Thy1.1) Pmel-1 CD8+ T cells into melanoma-bearing Thy1.2 mice. Compare cytotoxic activity and exhaustion of CD8+ T cells in vivo as function of intrinsic macroH2A levels.
One group of 6 B16-bearing Thy1.2 C57BL/6 mice will be injected with a 1:1 mix of macroH2A1.2 KO (Thy1.1/1.2) and WT (Thy1.1) Pmel-1 CD8+ T cells. Each mouse will provide a data point for both WT and macroH2A1.2 KO conditions.
Target project months: 24-26
Status: no longer planned

Major Task 5: Identify macroH2A-regulated genes responsible for proper T cell function

Subtask 1: Prepare RNA-seq libraries from macroH2A-deficient naïve and activated CD8+ T cells and sequence on Illumina NextSeq 500
Target project months: 18-22
Status: completed

Subtask 2: ChIP for macroH2A, H3K27ac, H3K4me1, H3K4me3, prepare libraries and sequence on Illumina NextSeq 500.
Target project months: 22-26
Status: planned as stated in SOW with a focus on CAFs

Subtask 3: Integrated analysis of RNA-seq and ChIP-seq from cells +/- macroH2A and overlap with macroH2A-enriched domains.
Target project months: 26-32
Status: planned as stated in SOW with a focus on CAFs

Major Task 6: Identify therapeutic implications of macroH2A loss in melanoma

Subtask 1: Immunohistochemistry on archival de-identified human melanoma samples; scoring macroH2A levels and tumor infiltrating immune cells.
Target project months: 18-21
Status: ongoing

Subtask 2: Generate WT and dKO BRAF/PTEN melanomas.
Target project months: 25-28
Status: completed ahead of time

Subtask 3: Treat melanoma-bearing mice with BRAF inhibitors and measure response via tumor growth.
To mitigate risk of attrition, 8 instead of 6 mice will be used per genotype. One group of WT and one group of dKO Braf/Pten/Tyr-Cre mice, both will be treated. Growth kinetics will be compared to existing data from non-treated WT and dKO mice.
Target project months: 27-28
Status: completed ahead of time

Subtask 4: Treat melanoma-bearing mice with checkpoint blockade and measure response via tumor growth and recovery of cytotoxic T cell function.
To mitigate risk of attrition, 8 instead of 6 mice will be used per genotype. One group of WT and one group of dKO Braf/Pten/Tyr-Cre mice, both treated. Growth kinetics will be compared to existing data from non-treated WT and dKO mice.
Target project months: 29-30
Status: planned as stated in SOW

Major Task 7: Publication of data from Aims 1&2

Subtask 1: Prepare a manuscript describing our findings, the first to investigate the contribution of histone variants to melanoma progression and immune response in vivo, for a high-impact journal such as Cancer Cell, Nature Medicine, Nature Cancer, or Cancer Discovery.
Target project months: 33-36
Status: ongoing (currently in revision process)

Accomplishments

Note: All accomplishments during Year 1, detailed in the previous report, are not included here.

Major Task 1: scRNA-seq of tumor infiltrating immune cells

Subtask 5: Bioinformatic analysis leveraging known ligand-receptor interactions to identify origin of immunosuppressive signals in dKO tumors.

In order to perform this analysis we must include a third WT and dKO tumor in the scRNA-seq study. Once this data becomes available in the next funding period, the analysis will be performed. Data produced so far shows cancer-associated fibroblasts (CAFs) are the strongest source of signals to other cell types in the TME, underlining their importance in this model, and the strength of their interactions with neural crest-derived (tumor) and myeloid cells increases even further in the dKO. This analysis will also probe whether dKO cells of the tumor compartment are responsible for signals that hyper-activate dKO CAFs, in contrast to a fully CAF-intrinsic phenotype of hyper-activation in the absence of macroH2A.

Major Task 2: Determine if macroH2A deficiency has a tumor-intrinsic effect

Subtask 4: Generate subcutaneous allografts of most promising cell line from Subtask 3 into C57BL/6 WT hosts and deplete differentially represented immune populations identified in Subtasks 3, then measure impact on tumor growth.

We will not pursue this task, as it was dependent on a positive result in Subtask 3. Subtask 3 did not highlight a difference in growth upon individual macroH2A isoform ablation via CRISPR, nor significant immunophenotypic changes (i.e. infiltrating immune cell frequency or tumor rejection) in these allograft models. Therefore, we lack a functional readout for this subtask and a target cell type to deplete.

Subtask 7: Induce melanomas with macroH2A1.2 ablation restricted to tumor compartment, analyze growth and immunophenotype.

This task was significantly delayed by difficulties with obtaining sufficient numbers of the melanoma-specific macroH2A1.2 cKO mouse strain. Founders for the macroH2A1.2$^{wt/wt}$ and macroH2A1.2$^{fl/fl}$ sublines were generated but have a limited lifespan due to leaky Cre transgene activation and development of spontaneous melanomas when reaching breeding age in a majority of mice. Of note, JAX laboratories, which provide the melanoma strain used to derive these mice, now highlights this issue in the strain description: https://www.jax.org/strain/013590. This resulted in the impossibility to expand the strain and obtain cohorts of mice in sufficient numbers for melanoma induction by 4-HT application in order to compare 12 macroH2A1.2$^{wt/wt}$ and 12 macroH2A1.2$^{fl/fl}$ mice as proposed. We are currently expanding this strain and expect to have sufficient mice in the following year.

Major Task 3: Identify genes responsible for macroH2A-mediated immune escape in melanoma cells

Through multiple approaches defined in previous tasks, we have been unable to identify melanoma-intrinsic properties that indicate immune evasion. However, scRNA-seq of the entire TME and transcriptomic analyses of CAFs isolated from WT and dKO melanomas demonstrated that CAFs are the major source of pro-inflammatory signals in dKO melanomas. This oriented our subsequent analyses away from melanoma cells and towards CAFs, where performing the subtasks detailed in the SOW for Major Task 3 appeared most relevant.

Subtask 1: Prepare RNA-seq libraries from macroH2A-deficient melanoma cell lines demonstrating immune escape and sequence on Illumina NextSeq 500

We successfully performed RNA-seq on CAFs flow-sorted from 4 WT and 4 dKO melanomas based on CD140a (*Pdgfra*) expression, which we found specific for this cell type (**Fig. 1a**). We confirmed the significant upregulation of several proinflammatory cytokines and chemokines such as *Ccl2*, *Cxcl1* and *Il6* cytokines, and the activation of the TNFα-NF-κB inflammatory signaling pathway (**Fig. 1b, c**). Differentially expressed genes assessed in the sorted CAFs overlapped in a statistically significant manner with those identified by scRNA-seq in the combined mesenchymal populations (**Fig. 1d**).

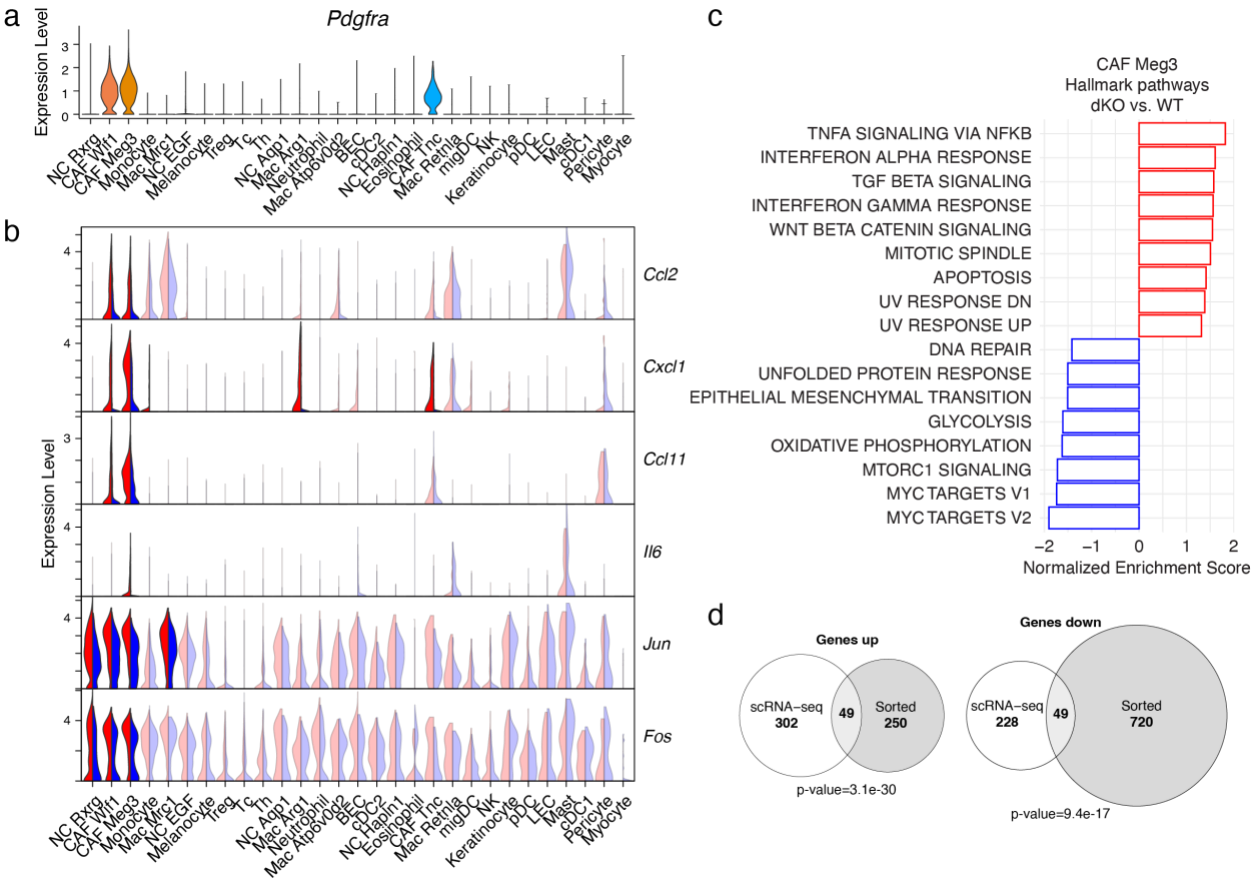

Figure 1. Pro-inflammatory signals in dKO tumors originate from CAFs. a) Violin plot of *Pdgfra* gene expression across cell types/states in the melanoma TME demonstrates its specificity for CAFs. b) Genes of interest with significant upregulation in the dKO in clusters highlighted with bold colors (Wilcoxon rank sum test adjusted P-value < 0.05). c) Significant Hallmark pathways in GSEA analysis of dKO vs. WT performed in the CAF Meg3 cluster. d) Intersections of DEGs across single-cell and bulk RNA-seq modalities in CAFs. DEGs in the scRNA- seq dataset were determined by grouping all CAF clusters as one, prior to contrasting by genotype. P-values of Fisher's exact test shown.

Subtask 2: ChIP for macroH2A, H3K27ac, H3K4me1, H3K4me3, prepare libraries and sequence on Illumina NextSeq 500.

We successfully carried out genome-wide macroH2A1 profiling using Cut&Run (Skene and Henikoff, 2017) in primary WT CAF cultures. We profiled H3K27me3 occupancy by Cut&Run in WT and dKO cells but did not detect major changes upon macroH2A loss. Enhancers are emerging regulators of inflammatory gene transcription (Higashijima and Kanki, 2021), notably in the context of the NF-κB signaling, a pathway that mediates activation of *CCL2*, *CXCL1* and *IL6*-associated (super)enhancers (Bonello et al., 2011; Brown et al., 2014; Liu et al., 2021; Weiterer et al., 2020) and is upregulated in the absence of macroH2A (**Fig. 1b, c**). Moreover, macroH2A suppresses a subset of enhancers (Gaspar-Maia et al., 2021), and FOSL2, whose enrichment at enhancers marks their activation in the context of tumorigenesis (Fontanals-Cirera et al., 2017; Zanconato et al., 2015), is upregulated in dKO CAFs. Therefore, we investigated the epigenetic regulatory landscape of inflammatory genes by performing ChIP-seq for H3K27ac, a mark of active enhancers and promoters. We identified active enhancers in serum-stimulated, cultured CAFs, and found dKO CAFs had increased H3K27ac levels at 4116 and decreased at 2652 enhancers (**Fig. 2a, b**). To determine whether the observed changes in H3K27ac were associated with the upregulation of inflammatory genes in the dKO, we plotted this mark at proximal and distal regulatory elements of the 39 significantly upregulated genes associated with inflammatory processes. We observed increased enrichment at enhancers within 50 kb of the TSS of these genes (**Fig. 2c**) and at promoters (**Fig. 2d**), consistent with increased transcription. Taken together, this suggests that regulatory elements controlling inflammatory gene expression in CAFs are modulated by macroH2A.

Subtask 3: Integrated analysis of RNA-seq and ChIP-seq from cells +/- macroH2A and overlap with macroH2A-enriched domains.

As previously shown (Sun et al., 2018), and consistent with its association with gene repression, genome-wide macroH2A was excluded from the bodies of highly expressed genes and retained at lowly expressed ones, in anti-correlation with H3K27ac levels at promoters. At the 39 upregulated inflammatory genes, macroH2A1 was enriched above a control set of static genes of matched expression levels, both at gene bodies and within 50 kb (**Fig. 2e**). As representative examples, loci such as *Ccl2/Ccl11* (**Fig. 2f**) and *Ptgs2*, the predominant prostaglandin-endoperoxide synthase in CAFs (not shown), had either an upregulated SE or multiple interspersed enhancers as measured by H3K27ac in their vicinity, and within a called macroH2A1 chromatin domain (MCD) (Sun et al., 2018) as identified in WT CAFs. Genome-wide, we found macroH2A1 was enriched at genes with significant changes in expression upon its loss, as opposed to static genes of matched expression levels. These DEGs were more frequently located in MCDs than static genes (**Fig. 2g**). Similarly, enhancers that gained or lost H3K27ac in the dKO displayed higher macroH2A enrichment in the WT (**Fig. 2h**). Of note, this involved both up- and downregulated genes and enhancers, suggesting that macroH2A variants may not act exclusively as repressors, but may also take part in gene or enhancer activation, either directly or indirectly, in line with data from other cellular models (Chen et al., 2014, 2015; Gamble et al., 2010; Lavigne et al., 2015; Recoules et al., 2022). Nevertheless, in the case of inflammatory gene regulation by macroH2A in CAFs, its repressor role appears predominant.

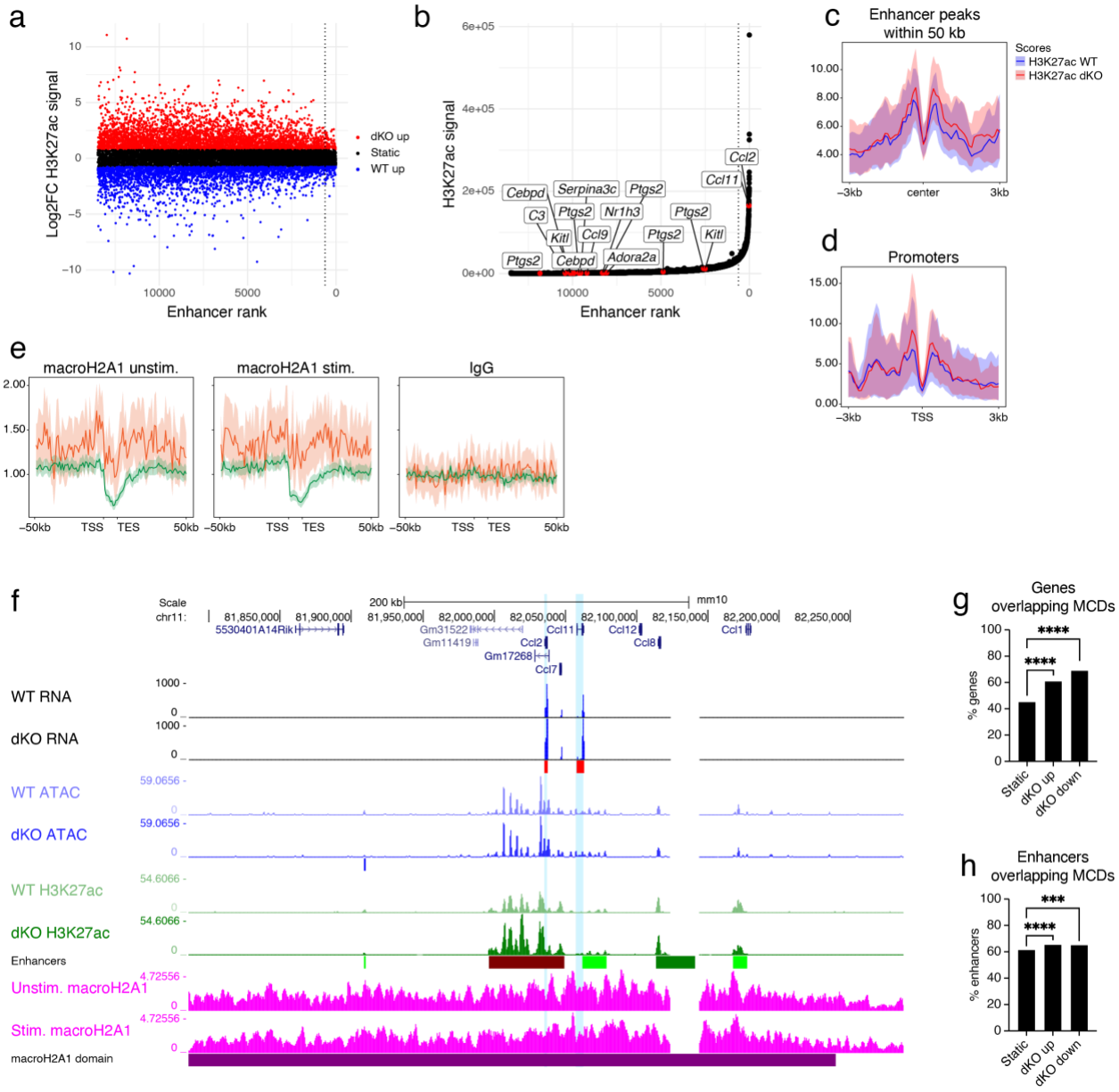

Figure 2. Regulatory elements of inflammatory genes gain H3K27ac in macroH2A- deficient CAFs. a) Changes in H3K27ac signal at all detected enhancers in cultured CAFs depicted by MA plot. Traditional (TE) and superenhancers (SE) are separated by a vertical dashed line. Enhancers with a log2 fold change > 0.75 are shown in color for the respective genotype. b) Hockey plot highlighting TEs and SEs gaining H3K27ac in dKO within 50 kb of upregulated inflammatory genes (red) among all TEs and SEs ranked by H3K27ac signal in cultured CAFs. c) Average profile of H3K27ac ChIP signal in serum-stimulated WT vs. dKO cultured CAFs at ATAC peaks located in enhancers within 50kb of TSS of inflammatory genes upregulated in dKO sorted CAFs, n = 137. d) As in (c), at promoters, n= 39. e) Average profile of macroH2A CUT&RUN signal in cultured WT CAFs before and after serum stimulation, at inflammatory genes upregulated in dKO sorted CAFs and static genes of matched expression levels, $n_{Inflammatory\ up}$ = 39, n_{Static} = 385. f) UCSC browser screenshot centered around the *Ccl2* locus, 200 kb up- and downstream, showing indicated transcriptomic and epigenomic features. Bars under RNA-seq and ATAC-seq tracks indicate significantly up- (red) or downregulated (blue) genes or accessible regions in dKO vs. WT sorted CAFs. Below H3K27ac tracks, bright and dark bars indicate TEs and SEs, respectively; red, blue and green denote gain, loss and no change respectively of H3K27ac level in dKO vs. WT CAFs. Bars under macroH2A CUT&RUN tracks indicate domains of macroH2A1 enrichment, with dark magenta identifying domains concentrating the highest levels of macroH2A1 signal. g) Percentage of overlap between genes differentially up, down or static genes of matched expression levels in dKO vs. WT sorted CAFs and MCDs, $n_{dKO\ up}$ = 357, $n_{dKO\ down}$ = 884, n_{Static} = 3708. h) Percentage of overlap between ATAC peaks located in enhancers that gain, lose or maintain static H3K27ac levels in dKO vs. WT and MCDs, $n_{dKO\ up}$ = 6659, $n_{dKO\ down}$ = 5211, n_{Static} = 18961.

Major Task 4: Determine if macroH2A deficiency has an immune cell-intrinsic effect

Subtask 4: Generate OT-I TCR-transgenic, T-cell specific macroH2A1.2 cKO mouse.
+
Subtask 5: In vitro T cell killing assay using B16-OVA cells and OT-I macroH2A1.2-deleted CD8+ T cells.
+
Subtask 6: Generate Thy1.1/1.2 Pmel-1 TCR-transgenic, T-cell specific macroH2A1.2 cKO mouse; Thy1.1 Pmel-1 TCR-transgenic, macroH2A WT mouse; BRAF/PTEN melanomas on C57BL/6 background (Thy1.2).
+
Subtask 7: Inject congenically marked macroH2A1.2 KO (Thy1.1/1.2) and WT (Thy1.1) Pmel-1 CD8+ T cells into melanoma-bearing Thy1.2 mice. Compare cytotoxic activity and exhaustion of CD8+ T cells in vivo as function of intrinsic macroH2A levels.

We will no longer pursue these tasks due to the negative results obtained in the previous subtasks of Major Task 4. Those results did not demonstrate a role for macroH2A1.2 in the immunophenotype or cell killing ability of CD8+ T cells. This could be either due to other macroH2A isoforms compensating for its absence, or due to the predominant effect of other cell types, namely CAFs, in the immune escape of macroH2A-deficient tumors. In addition, experiments in these proposed subtasks are unable to model the role of CAFs. Altogether, these considerations do not predict a positive result using this complex *in vivo* system.

Major Task 5: Identify macroH2A-regulated genes responsible for proper T cell function

Subtask 1: Prepare RNA-seq libraries from macroH2A-deficient naïve and activated CD8+ T cells and sequence on Illumina NextSeq 500

We observed that macroH2A deficiency caused a limited number of changes in gene expression upon activation of naïve CD8 T cells. Among the 52 genes upregulated in the dKO, several were associated with T cell activation, in line with previous immunophenotypic findings in Major Task 4, Subtask 1. The direction of this difference however does not explain the reduction of T cells in dKO tumors, and suggests the latter is a consequence of other changes in the tumor microenvironment.

Major Task 6: Identify therapeutic implications of macroH2A loss in melanoma

Subtask 1: Immunohistochemistry on archival de-identified human melanoma samples; scoring macroH2A levels and tumor infiltrating immune cells.

We performed this task on an initial batch of 10 human primary melanomas, where a pathologist manually scored macroH2A1 and macroH2A2 levels, lymphocyte infiltration and frequency of CD8 T cells. This cohort had overall very little variation in macroH2A levels, which did not allow us to identify a correlation with CD8 infiltration. This was likely due to the small number of samples analyzed or the age of the tissue blocks, which may have had poor tissue quality. We are currently expanding this analysis to melanoma samples on a commercial tissue microarray. We will stain and count CD8 T cells and monocytes using an automated pipeline available at our institution, to improve the reproducibility of the approach.

Subtask 2: Generate WT and dKO BRAF/PTEN melanomas.
+
Subtask 3: Treat melanoma-bearing mice with BRAF inhibitors and measure response via tumor growth.

In an initial experiment, we treated 6 mice per genotype with BRAF inhibitor from day 25 post-induction until tumors escaped therapy to reach sizes comparable to untreated animals (**Fig. 3a**). Tumor growth was significantly inhibited by BRAFi for a much longer period than anticipated, requiring at least 100 days of continuous drug administration. However, given the high variation in response to drug treatment (CV of 27% in WT, 37% in dKO), the increased average tumor size in the dKO did not reach significance. Power calculation ($\alpha = 0.05$, $1-\beta = 0.8$) estimates a sample size of ~50 per group would be required to reach significance at day 50. This number of mice under treatment is unachievable given the drug cost and experimental effort involved. Furthermore, dKO tumors under BRAFi were on average 31% larger than WT counterparts during the course of the treatment, an increase on par with the 30% higher size of dKO vs. WT seen in the absence of treatment. This suggested the effect of BRAFi is independent of macroH2A levels. Calculation of tumor growth inhibition (%TGI) as (1 - (mean volume of treated tumors)/(mean volume of control tumors)) x 100% (Hather et al., 2014) also showed that BRAFi did not have inferior efficacy in the dKO (**Fig. 3b**). Altogether, this data suggests loss of macroH2A promotes tumor growth through distinct pathways from those targeted by BRAFi.

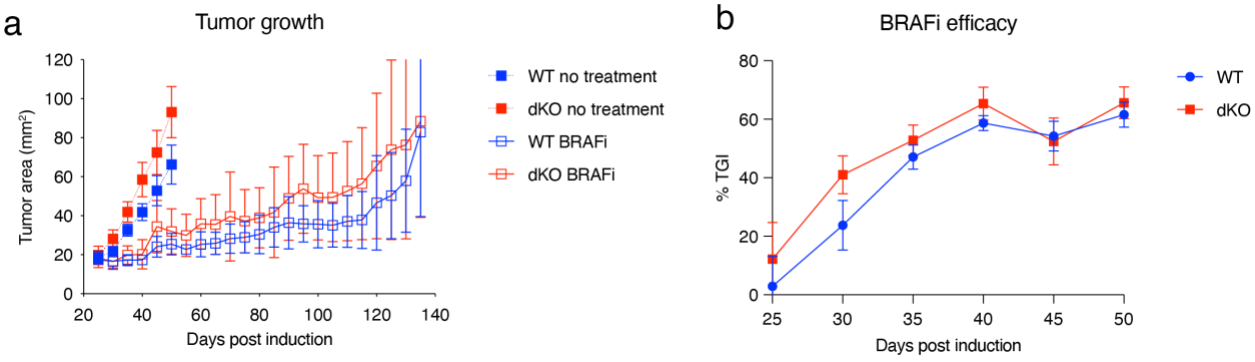

Figure 3. BRAF inhibition delays murine melanoma progression independently of macroH2A. a) Growth kinetics of WT and dKO mouse melanomas under BRAFi, retrospectively compared to untreated mice. Vemurafenib (also known as Zelboraf or PLX4032) was admixed in chow at 1.42 g/kg administered *ad libitum* from 25 days post tumor induction onwards. $n_{WT\ no\ treatment} = 22$, $n_{dKO\ no\ treatment} = 22$, $n_{WT\ BRAFi} = 6$, $n_{dKO\ BRAFi} = 6$. Mean and 95% confidence interval error bars are shown. b) Calculation of BRAFi efficacy as percent tumor growth inhibition (% TGI) at time points shared between no treatment and BRAFi mice. Mean and SEM shown.

Major Task 7: Publication of data from Aims 1&2

Subtask 1: Prepare a manuscript describing our findings, the first to investigate the contribution of histone variants to melanoma progression and immune response in vivo, for a high-impact journal such as Cancer Cell, Nature Medicine, Nature Cancer, or Cancer Discovery.

We submitted a manuscript titled "*MacroH2A restricts melanoma progression via inhibition of inflammatory gene expression in cancer-associated fibroblasts*" to Nature Cell Biology. The manuscript was also published as a pre-print on ResearchSquare (https://www.researchsquare.com/article/rs-1578023/v1). It was received favorably by reviewers and is currently under revision. Reviewers and editors requested a more in-depth characterization

of the molecular mechanisms underlying macroH2A-mediated repression of inflammatory genes, as well as strengthening correlations between macroH2A levels and immune dysfunction in human melanoma.

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