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TITLE: Identification of Glycomic Alterations During Melanoma Metastasis

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14. ABSTRACT We hypothesized that the ability of melanoma cells to abandon the primary site, survive, and grow in distal organs requires changes in glycosylation, some of which are site-specific. Our primary objective is to identify glycomic changes in melanoma that: 1) predict the ability of primary melanoma to metastasize and 2) promote colonization of distal organs, and particularly the brain. In the first year of funding we have obtained a large cohort of primary and metastatic melanomas (to different sites of metastasis) which we will examine for glycan profiling (Aim 1). This analysis is expected to reveal glycan signatures associated with metastasis in general, or to organ-specific tropism. On the other hand, we have conducted in vivo experiments that have provided further evidence of the ability of FUT8 inhibition to interfere with the growth of already established metastasis (Aim2). These findings support the development of FUT8 inhibitors as new therapeutic agents for melanoma treatment.					
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1. INTRODUCTION:

Melanoma is a major burden on the healthcare system with an incidence expected to double every 20 years. Early disease can be cured by surgery, however long-lasting or curative treatments are lacking for patients diagnosed with advanced disease. Despite the recent progress in the treatment of metastatic melanoma, overall survival (OS) is less than 50% at 3 years (3). BRAF inhibitors alone or in combination with MEK inhibitors show remarkable initial efficacy for BRAF-mutant metastatic melanoma patients (4-7), but are plagued by near-universal relapse with drug-resistant disease (8, 9). In contrast, immunotherapies (anti-CTLA4 or anti-PD1/PD-L1 checkpoint inhibitors) can yield strikingly durable responses (10), but are limited by response rates of no more than 60% even in optimal patient populations as selected for clinical trials (11). Moreover, both types of therapy have significant associated toxicities (12), thus identification of biomarkers that improve prognostic models is expected to aid melanoma patient management and selection for therapy. While these therapeutic advances have brought hope of curative treatment to metastatic melanoma patients, more advances are needed to expand that hope to additional patients. Identifying the molecular and cellular mechanisms that drive melanoma progression is critical to our understanding of melanoma biology and is expected to uncover novel therapeutic targets and strategies, and/or useful prognostic markers.

Carbohydrates are often altered in tumors and are involved in immune evasion and cell homing, survival, and anchorage. Despite multiple examples of glycosylation enzymes as clinical drug targets (i.e. glycosyltransferase inhibitor Zavesca-Gaucher's disease (13, 14); glycosidase inhibitors zanamivir (Relenza) and oseltamivir (Tamiflu)-influenza (15), and migalastat-Fabry's disease (13)) and FDA-approved prognostic glycan biomarkers (e.g. CA-19-9, *aka* sialyl Lewis A-pancreatic cancer (16)) currently on the market or in advanced clinical trials, glycosylation is an underexplored area for therapeutic and prognostic development. This is mostly due to our limited understanding of the roles of specific glycans in *clinical melanoma*, and the mechanisms by which glycans are regulated. In recent work, funded by our prior DOD Award, our laboratories established a systems-based approach to identify, at the glycan and glycogene level, specific changes in glycosylation *driving* metastasis in clinical melanoma (*Cancer Cell*, 2017 (1)). Focusing on paired primary:metastasis samples, we pinpointed a set of glycans and corresponding glycogenes altered during tumor progression in patients. We identified core fucosylation, mediated by FUT8, as a driver of metastasis and showed that core fucosylation impairs cleavage of a critical adhesion protein (L1CAM). In our current project, we expanded our previous work to examine: 1) if distinct glycosylation signatures associate with different sites of metastasis. 2) whether specific changes in glycosylation (e.g. core fucosylation) are required for establishment or maintenance of site- specific metastases, and particularly for brain metastasis.

2. KEYWORDS:

Melanoma, metastasis, tropism, glycans, lectin, lectin array, glycogene, FUT8

3. ACCOMPLISHMENTS:

What were the major goals of the project?

We hypothesize that the ability of melanoma cells to abandon the primary site, survive, and grow in distal organs requires changes in glycosylation, some of which are site-specific. Our primary objective is to identify glycomic changes in melanoma that: 1) predict the ability of primary melanoma to metastasize, and 2) promote colonization of distal organs, and particularly the brain. On one hand, our studies might reveal glycan signatures as prognostic biomarkers, which can serve to identify early-staged melanoma patients at high risk of developing metastasis and could benefit from recently approved adjuvant therapies (2). On the other hand, our work will provide proof-of-principle for the development of glycosyltransferase inhibitors for prevention or treatment of melanoma metastasis

What was accomplished under these goals?

1) Major activities

- Define the glycan signatures of melanoma primary and metastatic samples from various distal sites
- Demonstration that FUT8 is required for the establishment of melanoma metastasis.

2) Specific objectives;

- Test if distinct glycosylation signatures associate with different sites of metastasis.
- Assess the therapeutic potential of inhibiting FUT8

3) Significant results

Test if distinct glycosylation signatures associate with different sites of metastasis.

We hypothesized that distinct alterations in glycans are required to establish and maintain metastases at specific secondary sites. We collected FFPE tissue blocks from the NYU Melanoma Research Program BioRepository (IRB protocol #10362). For this study, the following tissues were collected: nevi (n=10), primary melanoma (n=18) and metastatic melanoma to the following secondary sites: lymph node (LN) n=22, subcutaneous (SC) n=22 and brain (Br) n=18. Tumor areas of the tissues were marked by a pathologist to remove contaminating normal tissue that might confound the analysis. Designated tumor areas were macrodissected, deparaffinized, rehydrated and protein was extracted. Protein samples were then labeled with NHS-Cy3 or -Cy5 and analyzed on our lectin microarrays in dual color using standard protocols. Reference was a mixture of 5 primary, 5 metastatic samples and 3 melanoma cell lines. Normalized data was subjected to hierarchical clustering using the Pearson correlation coefficient (R) as the distance metric and average linkage analysis to generate heatmaps. We observed strong discrimination between primary melanomas and various metastatic sites by a select group of lectins (**Figure 1**), $p < 0.05$, student's t-test). Metastatic melanoma showed higher levels of core fucosylated N-glycans (lectins: LcH, PSA), α -2,3 sialic acids (diCBM40), complex N-glycans (PHA-E, PHA-L, Calsepa), oligo-mannose (HHL, NPA, GNA, ConA, H84T) than primary. Lower levels of α -1,2 fucose (SNA-II, TJA-II) and β -GalNAc (BPA, WFA) were observed in metastasis. This data is in line with our previous study where we have examined distinct glycomic changes between paired primary and metastatic melanoma. A careful examination of **Figure 1** shows that metastatic melanoma is more heterogeneous than primary, potentially reflecting secondary site-specific glycan signatures. We analyzed data for 3 main metastatic sites of melanoma: lymph node (LN, n=22) subcutaneous (SC, n=22) and brain (Br, n=18).

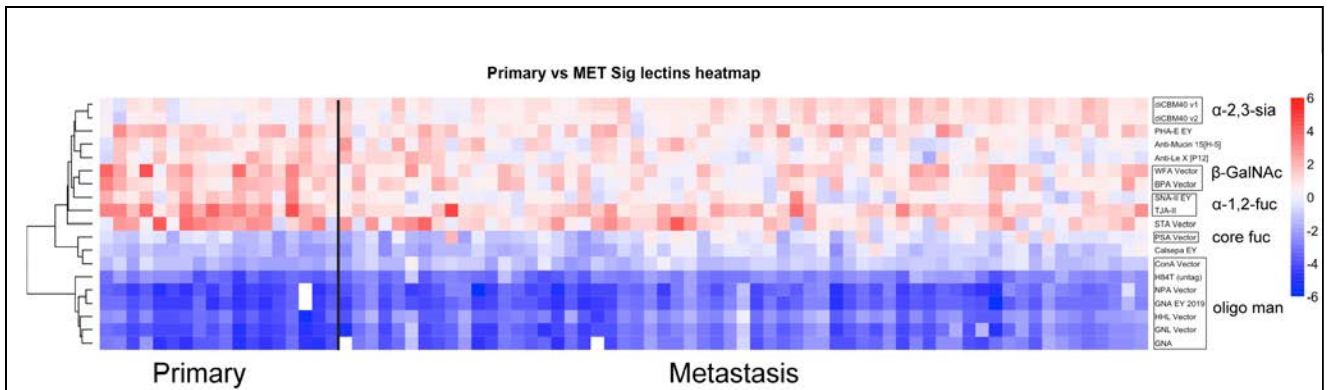
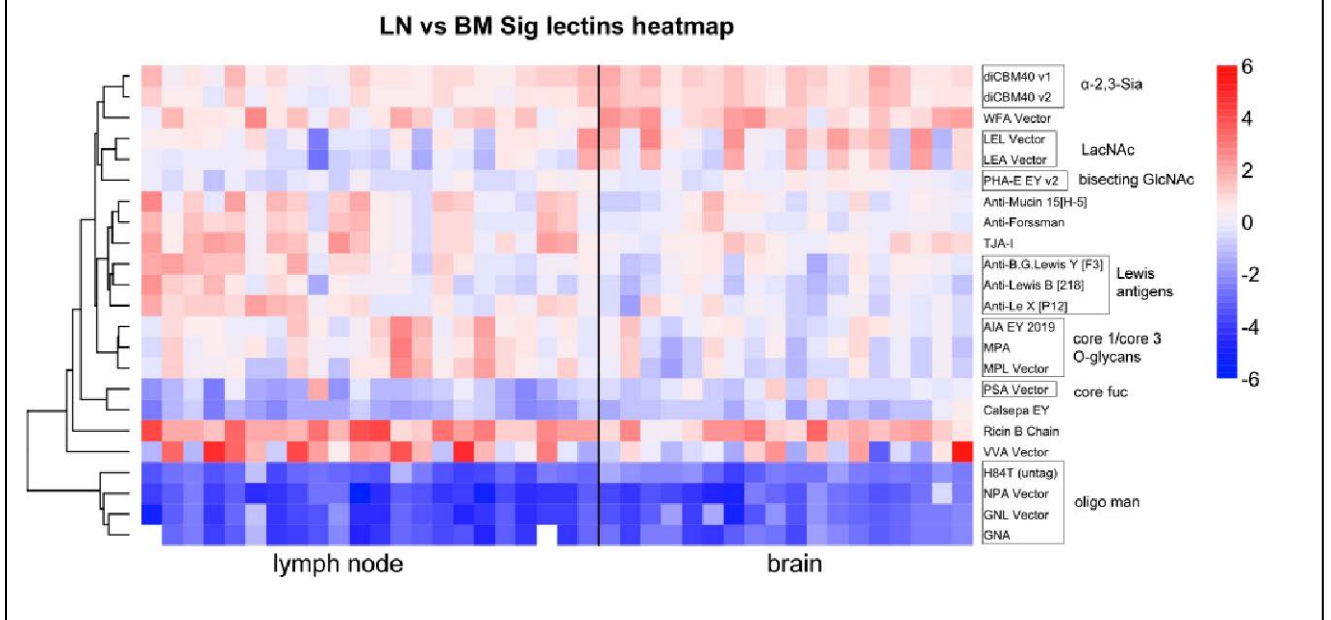
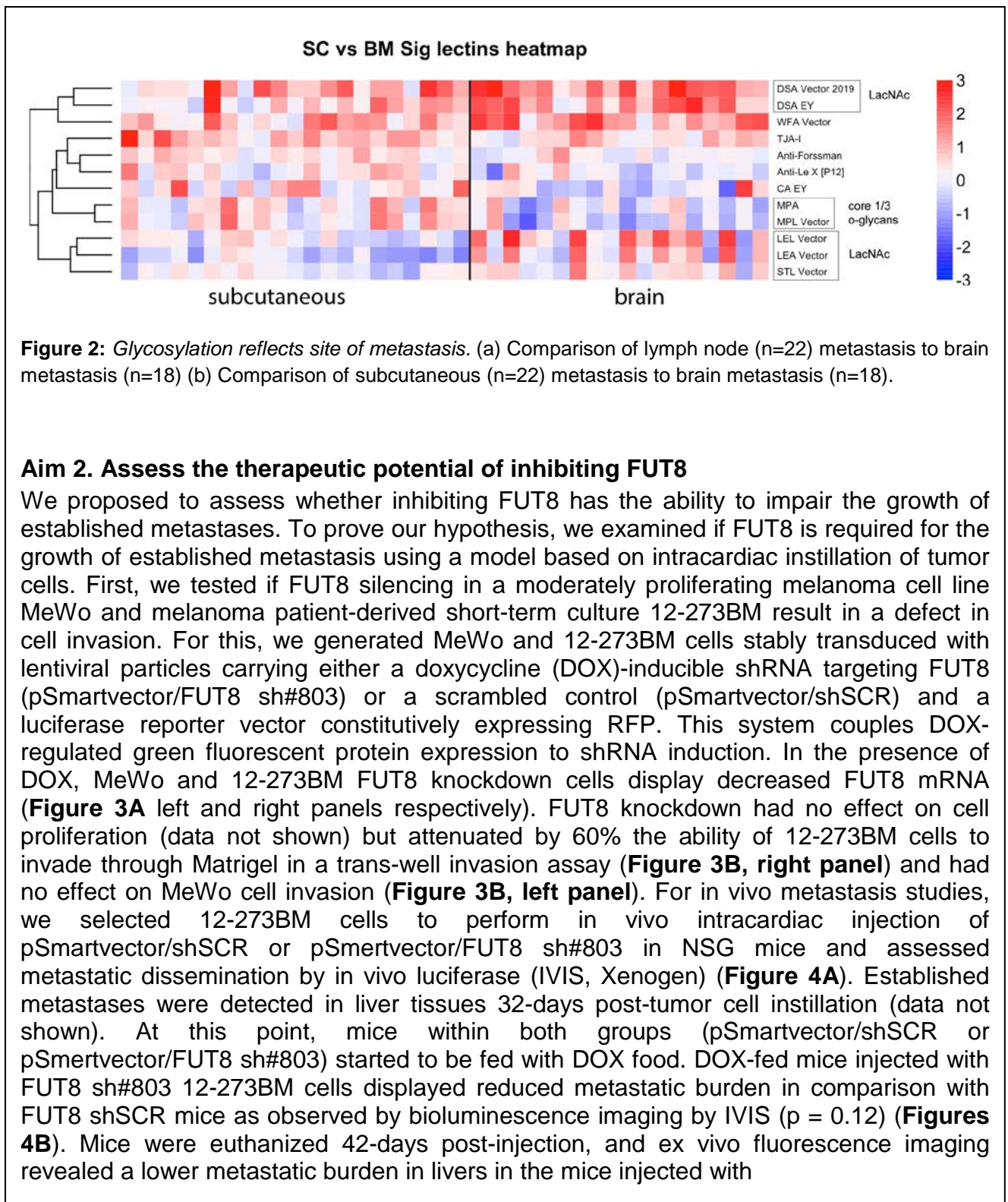


Figure 1: Glycomic profiling of melanoma primary (n=18) and metastatic (n=62) samples.

We observed that compared to LN, both Br and SC melanoma metastasis have significantly higher levels of core fucose (LcH, PSA) and oligo mannose (GNA, HHL, NPA, H84T, **Figure 2 a-b**, $p < 0.05$, student's t-test). Melanoma brain metastasis showed additional distinct glycan signatures in compare to LN metastasis including lower levels of core 1/3 O-linked glycans (AIA, MPA) and lewis antigens (Le^x , Le^y , Le^b) and higher levels of α -2,3-sialic acids (diCBM40), bisecting GlcNAc (PHA-E) and LacNAc (LEA, **Figure 2a** $p < 0.05$, student's t-test) glycans. Brain metastasis also show significantly higher levels of LacNAc (DSA, LEA, STA) and lower levels of core 1/3 O-linked glycans (MPA, **Figure 2b** $p < 0.05$, student's t-test) when compared to subcutaneous melanoma. Our data provides evidence that glycosylation reflects metastatic site selection and could play a mechanistic role in adaptation and growth of melanoma to different organs.





12-273BM FUT8 sh#803 cells (**Figures 4C**). Histological evaluation of liver tissues of mice injected with FUT8 sh#803 12-273BM cells exhibited significantly reduced tumor burden per liver as determined by analyzing total tumor area in a H&E section ($p < 0.05$) (**Figure 5**). All together data indicates FUT8 inhibition as a viable therapeutic strategy against advanced melanoma.

Figure 3:

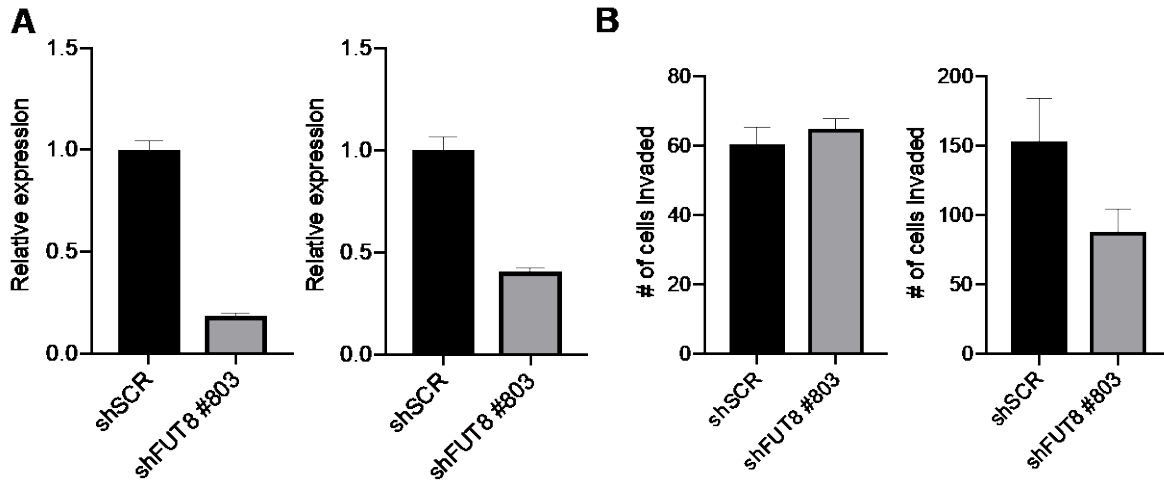


Figure 3: FUT8 silencing reduces 12-273BM melanoma short-term culture invasiveness in vitro (A) Real-time qPCR of FUT8 levels in MeWo (left panel) and 12-273BM (right panel) cells stably transduced with DOX-inducible pSmartvector-shFUT8 #803 or shSCR. qPCR graph shows average relative expression normalized to GAPDH (**B**) Trans-well Matrigel invasion by MeWo (left panel) and 12-273BM (right panel) cells transduced with pSmartvector-shFUT8 #803 or shSCR. Invading cells were quantified by counting the number of MeWo and 12-273BM cells that invaded into the basal side of Matrigel-coated trans-well inserts after 48 and 24 hr respectively.

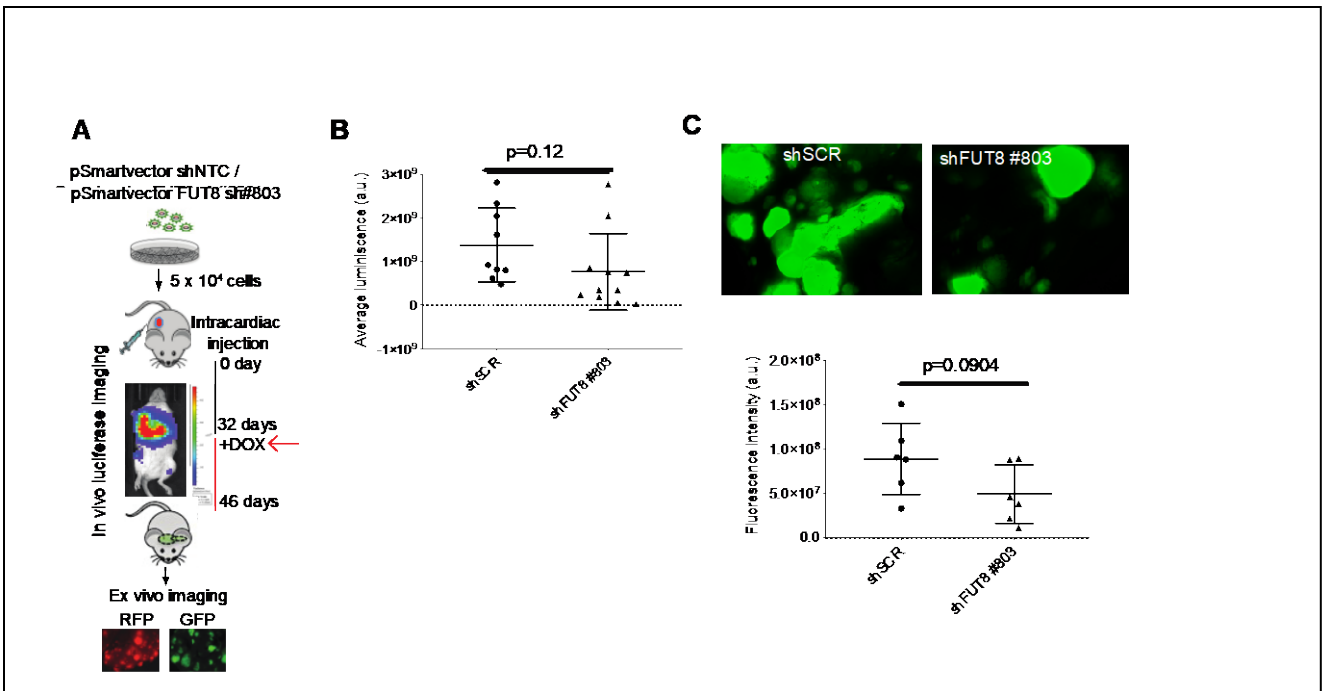


Figure 4: FUT8 Silencing Impairs the Growth of Established Metastasis In Vivo (A) Schematic representation of the in vivo metastasis assay with 12-273BM melanoma short-term culture cells stably transduced with DOX-inducible pSmartvector-shFUT8 #803 or shSCR instilled by intracardiac injection into NSG mice (n = 12 mice per group). **(B)** Average radiance after intracardiac injection of 12-273BM cells at end point of the experiment (42-days post-injection). DOX treatment was started 32-days post-injection. **(C)** Ex vivo fluorescent microscopic images of mouse liver metastases at termination of the experiment.

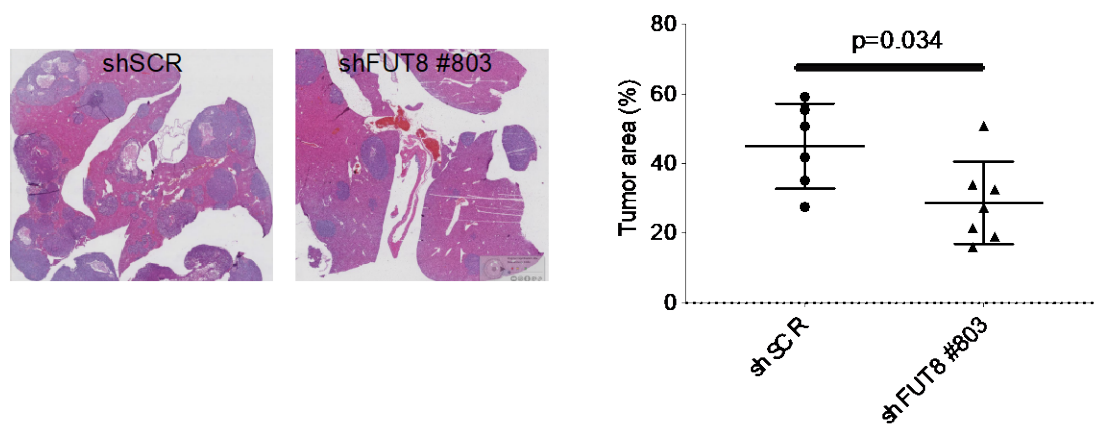


Figure 5: Histological evaluation of tumor burden of liver tissues of mice injected with FUT8 sh#803 12-273BM cells or shSCR.

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

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

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

Skin cancer is the most common type of cancer, and melanoma is its most deadly form. With a relatively early age of onset compared to other cancers, melanoma has a high impact in terms of life years lost. The most devastating complication of melanoma is spread to other organs (i.e. liver, lung, brain), a phenomenon called metastasis. Although promising new therapies have been recently approved, many tumors are not responsive or become resistant after initial responses. Therefore, in order to develop new and more therapies there is an imperative need to understand better how these small tumors (measured at millimeters at diagnosis) become able to reach and adapt to distant organs.

Proteins in the cell membrane often acquire sugar modifications, which shape the interactions between a tumor cell and its environment. For instance, these sugar branches modify the recognition of tumor cells by the immune system. We utilized a technique developed by Dr. Lara K. Mahal and only available at her laboratory. The advantage of this technology is the ability to examine multiple sugars simultaneously in an unbiased and automated manner. We identified sugar changes characteristic of melanoma tissues isolated from various distal organs (lymph node, brain, sub cutaneous), which may be required for adaptation to those organs. We also identified that inhibition of some of the enzymes responsible for those sugar modifications can impair melanoma dissemination.

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:

Changes in approach and reasons for change

Nothing to Report

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

Nothing to Report

Changes that had a significant impact on expenditures

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.

Nothing to Report

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

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

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

<http://hernandolab.com> – Our laboratory website provides general information about this project.

- **Technologies or techniques**

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

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<i>Name:</i>	<i>Eva Hernando</i>
<i>Project Role:</i>	<i>PI</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>-----</i>
<i>Nearest person month worked:</i>	<i>---</i>
 <i>Contribution to Project:</i>	 <i>Oversee the project, procurement of human melanoma samples, designing and interpretation of the in vivo experiments results</i>
<i>Funding Support:</i>	<i>---</i>
 <i>Name:</i>	 <i>Lara Mahal</i>
<i>Project Role:</i>	<i>PI</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>-----</i>
<i>Nearest person month worked:</i>	<i>---</i>
 <i>Contribution to Project:</i>	 <i>Oversee the project, designing and interpretation of the glycomics data analysis</i>
<i>Funding Support:</i>	<i>---</i>

<i>Name:</i>	<i>Praveen Agrawal</i>
<i>Project Role:</i>	<i>Post-doctoral fellow</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>-----</i>
<i>Nearest person month worked:</i>	<i>---</i>
 <i>Contribution to Project:</i>	 <i>Procurement of human melanoma samples for lectin array, and array data analysis. Designing and interpretation of the in vivo experiments results.</i>
<i>Funding Support:</i>	<i>---</i>
 <i>Name:</i>	 <i>Shuhui Chen</i>
<i>Project Role:</i>	<i>Graduate student</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>-----</i>
<i>Nearest person month worked:</i>	<i>---</i>
 <i>Contribution to Project:</i>	 <i>Performing lectin array and glycomics data analysis</i>
<i>Funding Support:</i>	<i>---</i>

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

Nothing to Report

What other organizations were involved as partners?

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: