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TITLE: Characterization and Therapeutic Targeting of a Novel Metastasis-Suppressive Pathway in Colon Cancer

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CONTRACTING ORGANIZATION: University of California, San Francisco

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14. ABSTRACT Cancer metastasis, the spread of cancer to other organs in the body, is the ultimate cause of death in most cancer patients. Cancer cells often hijack regulatory pathways in the cell to achieve dysregulated gene expression of the key components of these pathways. However, as most regulatory programs are yet to be annotated, the systematic discovery of such pathologic regulatory pathways remains elusive. Here, we report, for the first time, the development of a new computational framework, termed RPADA, that helps identify novel regulators that drive human cancers. We have applied PRADA to models of colon cancer metastasis to identify a previously unknown pathway, mediated by the protein RBMS1, as a suppressor of metastatic progression. This study introduces an entirely new approach for studying complex human diseases such as cancer and will benefit veterans, their beneficiaries, and the patient population in general.					
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1. INTRODUCTION:

Cancer metastasis, the spread of cancer to other organs in the body, is the ultimate cause of death in most cancer patients. Cancer cells often hijack regulatory pathways in the cell to achieve dysregulated gene expression of the key components of these pathways. However, as most regulatory programs are yet to be annotated, the systematic discovery of such pathologic regulatory pathways remains elusive. Here, we report, for the first time, the development of a new computational framework, termed RPADA, that helps identify novel regulators that drive human cancers. We have applied PRADA to models of colon cancer metastasis to identify a previously unknown pathway, mediated by the protein RBMS1, as a suppressor of metastatic progression. This study introduces an entirely new approach for studying complex human diseases such as cancer and will benefit veterans, their beneficiaries, and the patient population in general.

2. KEYWORDS:

Colon cancer, cancer progression, metastasis, metastasis suppressor, RBMS1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The project has three Specific Aims, divided into Major Tasks, and subdivided into Subtasks (showing here Specific Aims and Subtasks only; will discuss Subtasks in details below).

Specific Aim 1: **Characterize RBMS1 as a regulator of RNA stability.**

Major Task 1: RBMS1 irCLIP (Months 1-6) – **100% completion**

Major Task 2: Reporter assays (Months 6-16) – **100% completion**

Major Task 3: Identification of the key targets of RBMS1 (Months 6-16) – **100% completion**

Specific Aim 2: **Determine the contribution of RBMS1 to colon cancer metastasis**

Major Task 1: Patient-derived xenograft mouse models of colon cancer liver metastasis (Months 1-12) - **70% completion**

Major Task 2: Xenograft mouse models of additional CRC cell lines (Months 12-24) – **not started**

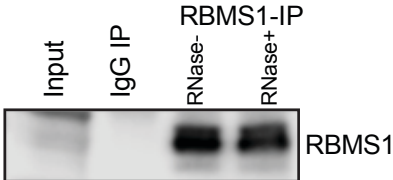
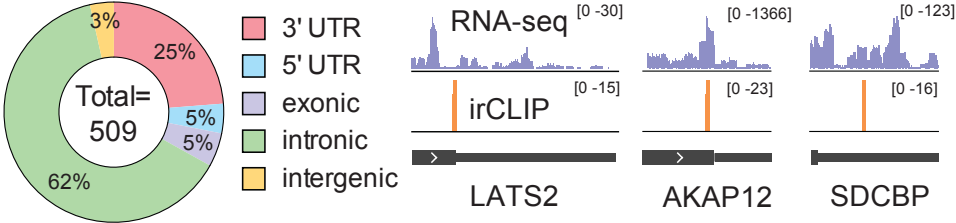
Major Task 3: Quantification of RBMS1 and its targets in clinical samples (Months 12-24) – **not started**

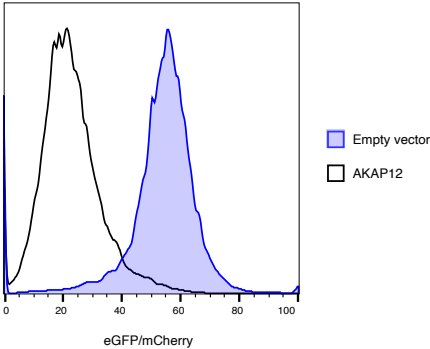
Specific Aim 3: **Clinical relevance of RBMS1 as a therapeutic target**

Major Task 1: Therapeutic re-activation of the RBMS1 pathway (Months 1-12) - **25% completion**

Major Task 2: Pre-clinical studies of HDAC1-mediated induction of the RBMS1 pathway (Months 12-24) – **not started**

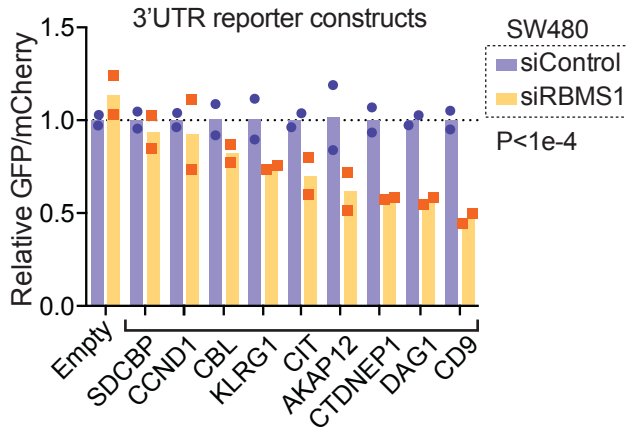
What was accomplished under these goals?

Specific Aim 1: Characterize RBMS1 as a regulator of RNA stability.	Timeline (months)
Major Task 1: RBMS1 irCLIP	
<p>Subtask 1: Validating immunoprecipitation antibodies for endogenous RBMS1 IP.</p>  <p><i>A commercially available antibody (rabbit monoclonal, Abcam, ab150353) worked best to immunoprecipitated RBMS1. In the figure above, SW480 cell lysate was used to IP RBMS1, along normal rabbit IgG control, and analyzed by Western Blot.</i></p>	1-2 Completed
<p>Subtask 2: (Contingency) V5-tagged RBMS1 expressed transgenically in RBMS1 (V5 IP already established).</p> <p><i>Did not need as a commercially available antibody targeting endogenous RBMS1 has worked.</i></p>	2-3 Not needed
<p>Subtask 3: RBMS1 IP followed by high-throughput sequencing (irCLIP).</p> <p><i>The irCLIP libraries were successfully prepared following a published protocol and sequenced. Briefly, RNA was crosslinked to proteins in vivo by exposure to UV, and RBMS1-RNA complexes were immunoprecipitated as described in Subtask 1. Excess RNA bound to RBMS1 was digested with RNase and a fluorescent DNA linker was ligated to the 3' end of the RBMS1-protected RNA fragments. The complexes were resolved on a denaturing PAGE gel and fluorescently labelled complexes migrating above the RBMS1 protein band were extracted. The recovered linker-ligated RNA was then reverse transcribed, PCR amplified, and sequenced on Illumina HiSeq 4000 sequencer.</i></p>	2-4 Completed
<p>Subtask 3: irCLIP analysis: binding site discovery and target identification.</p>  <p><i>The irCLIP data was analyzed using CLIP ToolKit (CTK) package, which resulted in a high-confidence list of 509 RBMS1 binding sites, with a significant enrichment of binding to the last exon/3' UTR (relative to the total length of genomic features), as shown in the figure above. Last exons from LATS2, AKAP12, and SDCBP are also shown as examples of RBMS1 binding patterns.</i></p>	4-6 Completed

Major Task 2: Reporter assays		
<p>Subtask 1: Clone 3' UTRs from targets and control non-targets.</p> <p><i>A bifluorescent bidirectional lentiviral vector, containing a CMV promoter driven expression of GFP with a restriction cloning site in its 3' UTR, and a hPGK promoter driven expression of PuroR-T2A-mCherry (allowing selection of stable integrants as well as detection of control fluorescence signal) was used to clone ~300 bp DNA fragments containing RBMS1-binding sites. Nine targets were chosen, containing RBMS1-binding sites from SCDBP, CCND1, CBL, KLRG1, CIT, AKAP12, CTDNEP1, DAG1, CD9. An empty vector was used as a control.</i></p>	6-8	Completed
<p>Subtask 2: Generate the associated cell lines. Validate cell lines by flow cytometry.</p> <p><i>Vectors constructed in Subtask 1 were stably integrated in the genome of SW480 cells by lentiviral delivery and puromycin selection. The resulting cell lines were analyzed by flow cytometry, along non-infected SW480 cells and cells that were delivered the reporter vector without any sequences cloned downstream of GFP (baseline fluorescence of GFP and mCherry).</i></p>  <p><i>As shown in the figure above, inserting a DNA fragment surrounding RBMS1-binding site from the 3' UTR of AKAP12 downstream of GFP in the reporter vector resulted in significantly lower GFP/mCherry fluorescence ratio compared to empty vector control as determined by flow cytometry.</i></p>	8-10	Completed
<p>Subtask 3: Perform measurements using qPCR.</p> <p><i>The cell lines constructed and validated in Subtask 2 were used for RNA extraction in biological triplicates and quantifying GFP mRNA expression. mCherry mRNA was used as internal control (controlling for transgene copy number). One-tailed U-test was used for testing significance in comparing RBMS1 binding and non-binding mRNA expression.</i></p>	10-12	Completed
<p>Subtask 4: Select functional reporters and generate lines in SW480-RBMS1 knockdown cells.</p> <p><i>To directly prove that RBMS1 binding results in altered mRNA stability, the reporter cell lines constructed in Subtask 2 were used to knockdown RBMS1 using siRNA transfection, along non-targeting control siRNAs.</i></p>	12-14	Completed

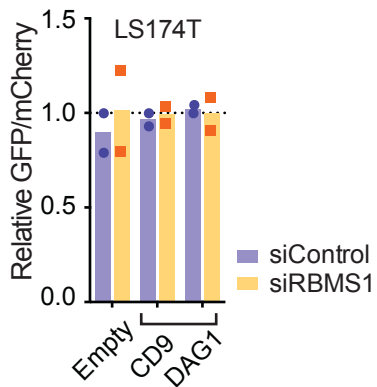
Subtask 5: Repeat measurements for RBMS1-KD cells.

Reporter cell lines generated in Subtask 4 were used for RNA extraction in biological triplicates and quantifying GFP mRNA expression. One-tailed U-test was used for testing significance.



The majority of targets tested caused the downregulation of GFP mRNA expression in siRBMS1-treated cells, as compared to non-targeting siRNA-treated control, shown in the figure above.

The reporters containing DAG1 and CD9 fragments, that showed the highest difference in GFP expression upon RBMS1 knockdown, were also integrated in LS174T cells where RBMS1 expression is endogenously silenced. As hypothesized, the RBMS1 knockdown did not change the expression of GFP mRNA in this condition (see figure below), confirming that in SW480 cells, the reporter expression is specifically controlled by RBMS1.



14-16

Completed

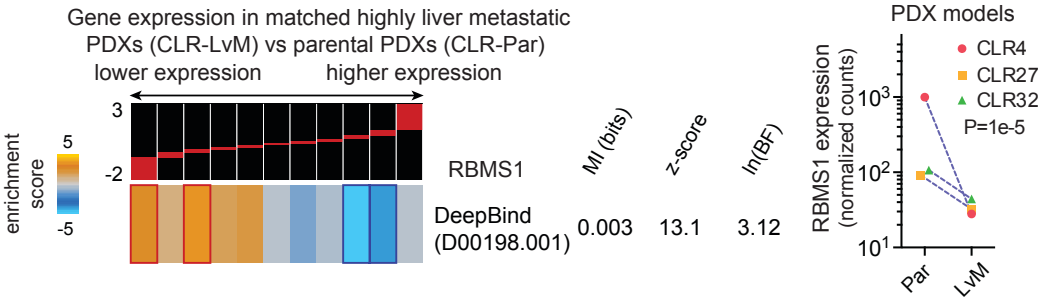
Major Task 3: Identification of the key targets of RBMS1

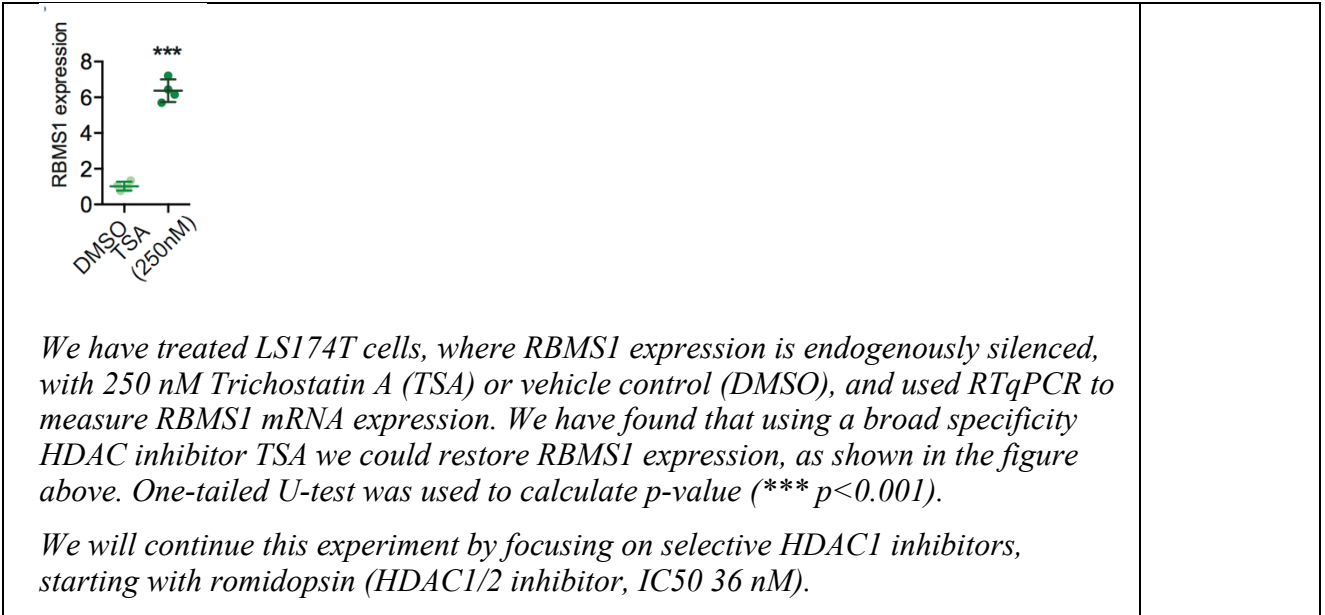
Subtask 1: Integrated analysis to identify a gene signature for RBMS1-mediated dysregulation.

We performed a systematic search of mRNAs that are: (i) destabilized and down-regulated in RBMS1 knock-down cell lines, and (ii) directly bound by RBMS1 based

6-16

Completed

<p><i>on the irCLIP data. This analysis revealed a 80-gene RBMS1 signature set and will form the basis for the future work on this pathway.</i></p>	
<p><i>Milestones Achieved: genome-wide determination of RBMS1 binding sites at nucleotide resolution; functional validation of RBMS1 mediated regulation of its direct targets.</i></p>	<p>16 Completed</p>
<p>Specific Aim 2: Determine the contribution of RBMS1 to colon cancer metastasis</p>	
<p>Major Task 1: Patient-derived xenograft mouse models of colon cancer liver metastasis</p>	
<p>Subtask 1: Obtaining HRPO and ACURO approvals for working with human research material and performing animal experiments. <i>The approvals are still pending, document processing times are significantly longer due to COVID-19 pandemic.</i></p>	<p>1-12 In progress</p>
<p>Subtask 2: Obtaining RNAseq data from PDX samples through collaboration with the Tavazoie lab. <i>The Tavazoie lab has performed RNA sequencing experiments of a small cohort of CRC PDX samples and agreed to share the data for the purposes of this project.</i></p>	<p>1-6 Completed</p>
<p>Subtask 3: Analyzing RNAseq data from PDX samples</p>  <p>Gene expression in matched highly liver metastatic PDXs (CLR-LvM) vs parental PDXs (CLR-Par) lower expression higher expression</p> <p>enrichment score: 5 (red), 0 (black), -5 (blue)</p> <p>RBMS1 MI (bits): 0.003 z-score: 13.1 ln(BF): 3.12 DeepBind (D00198.001)</p> <p>PDX models: CLR4 (red circle), CLR27 (orange square), CLR32 (green triangle) RBMS1 expression (normalized counts): 10¹ to 10³ P=1e-5</p> <p><i>We have analyzed the data obtained in Subtask 2, and we have found that increase in metastatic potential of the PDXs was accompanied by the silencing of RBMS1, as shown in the figure above, right panel. On the left, we have also found that mRNAs predicted to bind RBMS1 showed lower expression in highly metastatic PDXs, as compared to poorly metastatic parental PDXs.</i></p>	<p>6-12 Completed</p>
<p>Specific Aim 3: Clinical relevance of RBMS1 as a therapeutic target</p>	
<p>Major Task 1: Therapeutic re-activation of the RBMS1 pathway</p>	
<p>Subtask 1: Screening for HDAC1 inhibitors that increase RBMS1 expression</p>	<p>1-6 50% completed</p>



Our initial SOW proposed completing Specific Aim 3, Major Task 1 (Therapeutic re-activation of the RBMS1 pathway) during the first year of the project. However, the laboratory schedule was highly modified in March, 2020 due to the COVID-19 pandemic and the suspension of all non-essential laboratory activity at UCSF.

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

During the first year of the project, I discussed the progress of my work with my principal mentor, Dr. Goodarzi, during our weekly meetings. I have also presented the results of this project during several Goodarzi lab meetings. I have also met and discussed this project with my co-mentors, dr. Warren and Dr. Corvera.

I have attended the weekly seminar series organized by the Department of Biochemistry and Biophysics as well as Hellen Diller Family Comprehensive Cancer Center at UCSF. I have attended Quantitative Biology of the Cancer Cell Symposium (UCSF, February 24-25, 2020). When the in-person meetings ceased in March, 2020 due to the COVID-19 pandemic, I have continued attending the seminars online, organized by UCSF and other institutions. I have found particularly useful the series of cancer-related seminars organized by Dana-Farber Cancer Institute. I have additionally attended several workshops organized by the Office of the Career and Professional Development at UCSF (“Developing Your Independent Research Program”, “TRAIN-UP Applied”).

I had the opportunity to work with my colleague Dr. Lisa Fish, a fellow post-doc in the Goodarzi lab, who has consulted me on performing RBMS1 irCLIP, and which helped me to become a skilful user of this technique.

How were the results disseminated to communities of interest?

The results of the project have been published in *Cancer Discovery* journal:

Yu J, Navickas A, Asgharian H, *et al.* RBMS1 Suppresses Colon Cancer Metastasis through Targeted Stabilization of Its mRNA Regulon. *Cancer Discov.* 2020;10(9):1410-1423. doi:10.1158/2159-8290.CD-19-1375

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

I will continue to follow the plan detailed in the SOW of the project, which mainly consists of addressing the clinical side of the role of RBMS1 in colon cancer progression.

I plan to present the results of this project in a scientific meeting, focusing on cancer biology as well as RNA biology and post-transcriptional gene expression control mechanisms.

4. IMPACT:

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

The survival of the vast majority of patients with colon cancer depends on whether cancer cells within their tumors attain the ability to metastasize to distal organs such as the liver. The metastatic cascade is a complex multi-step process, which has been proven difficult to predict, prevent, or treat. Discovering the underlying regulators of cancer metastasis enables therapeutic interventions that specifically target cancer progression and may save thousands of lives every year. Similarly, they provide an avenue for identifying tumors at a higher risk of metastasis. However, systematic discovery of these regulatory pathways is no simple task. While known pathways have been the subject of numerous studies, annotation of new metastatic pathways has lagged behind. Given our dual expertise in computational and experimental cancer biology, we are ideally situated to tackle this complex problem. Over the years, we have developed an integrated platform that cycles between *in silico* predictions and *in vitro/vivo* validations to build previously unknown pathways of cancer progression.

Scientific impact: We have recently added a new computational tool, called PRADA, to our software toolkit, which uses mathematical models of regulatory networks to deduce drivers of cancer progression from whole-transcriptomic measurements of clinical samples. The preliminary results provided here showcase the strength of PRADA in revealing novel regulatory pathways that play a role in human disease. While we have focused our research on colon cancer metastasis, PRADA is general in concept and can be applied to other biological models and human pathologies. As such, it will be of great interest to the scientific community. Our discovery of a novel metastasis-suppressive pathway, mediated by the RNA-binding protein RBMS1, and its role in modulating mRNA stability and gene expression also constitutes an impactful discovery. Together, both the approach and findings presented here are conceptually novel and open an entirely new avenue of research.

What was the impact on other disciplines?

Clinical and translational impact: Given that RBMS1 is almost completely silenced in aggressive cancers, its quantification in tumor biopsies can provide a measure of risk for metastatic relapse. Moreover, restoring RBMS1 expression and the activity of its downstream pathway may provide a therapeutic avenue in adjuvant settings for limiting the rate of relapse. Our mechanistic work on the function of this RBMS1-mediated pathway will also provide additional insights into the biology tumors.

What was the impact on technology transfer?

Should we success in leveraging this pathway as a therapeutic target or prognostic biomarker, we will work with The Clinical & Translational Science Institute (CTSI) at UCSF to rapidly translate our findings to the clinic.

What was the impact on society beyond science and technology?

Our findings on metastasis suppressing role of RBMS1 in colon cancer have the potential to be translated to the clinic and thus improve the quality of life of colon cancer patients.

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

I experienced a delay in obtaining HRPO and ACURO approvals for working with human research material and performing animal experiments, due to increased document processing times at UCSF. I plan to submit the forms for approval as soon as they are available locally.

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.

Yu J*, Navickas A*, Asgharian H*, *et al.* RBMS1 Suppresses Colon Cancer Metastasis through Targeted Stabilization of Its mRNA Regulon. *Cancer Discov.* 2020;10(9):1410-1423. doi:10.1158/2159-8290.CD-19-1375 (*contributed equally)
Published.
Acknowledgment of federal support (yes).

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)**

Nothing to report.

- **Technologies or techniques**

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

We have developed a computational approach, termed PRADA, that identifies RNA-binding proteins underlying gene expression modulations. Applying this approach to a dataset comparing gene expression in poorly and highly metastatic colon cancer cells identified RBMS1 as a suppressor of colon cancer metastasis. The description of PRADA can be found at github.com/goodarzilab/PRADA.

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

Nothing to report.

- **Other Products**

We have generated a high quality RBMS1 irCLIP dataset in SW480 cells that will be valuable for the scientific community studying RNA-protein interactions. The data can be accessed at Gene Expression Omnibus (GSE147749).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Albertas Navickas*

Project Role: *PI*

Researcher Identifier (e.g. ORCID ID): *0000-0003-0016-2643*

Nearest person month worked: *12*

Contribution to Project: *Albertas Navickas supervised the project and performed the experiments described in this report.*

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?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

9. APPENDICES

Nothing to report.