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

PRINCIPAL INVESTIGATOR: Albertas NAVICKAS

CONTRACTING ORGANIZATION: University of California, San Francisco, CA

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Albertas NAVICKAS

E-Mail: albertas.navickas@ucsf.edu

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University of California, San Francisco
600 16th Street,
San Francisco, CA 94158

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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.

15. SUBJECT TERMS
Colon cancer, cancer progression, metastasis, metastasis suppressor, RBMS1

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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) - **100% completion**

Major Task 2: Xenograft mouse models of additional CRC cell lines (Months 12-24) – **100% completion**

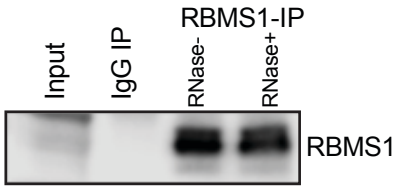
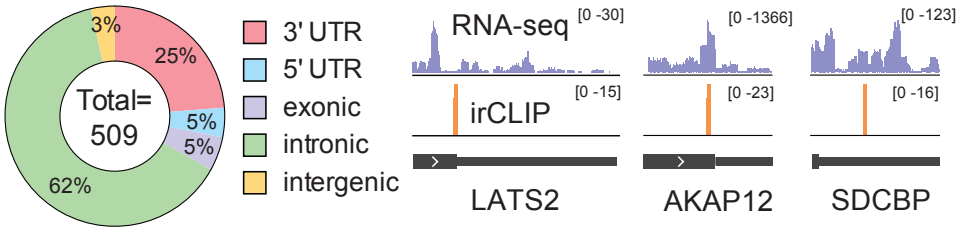
Major Task 3: Quantification of RBMS1 and its targets in clinical samples (Months 12-24) – **100% completion**

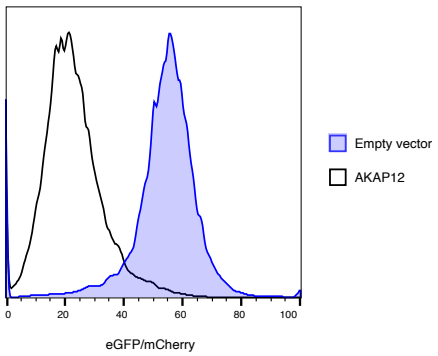
Specific Aim 3: **Clinical relevance of RBMS1 as a therapeutic target**

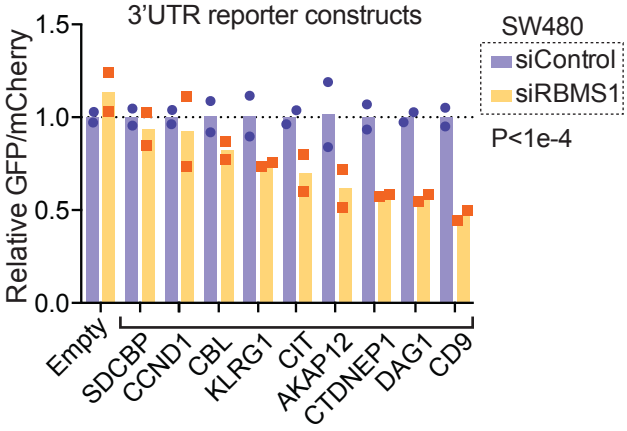
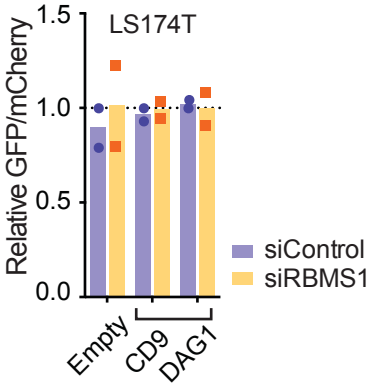
Major Task 1: Therapeutic re-activation of the RBMS1 pathway (Months 1-12) - **100% completion**

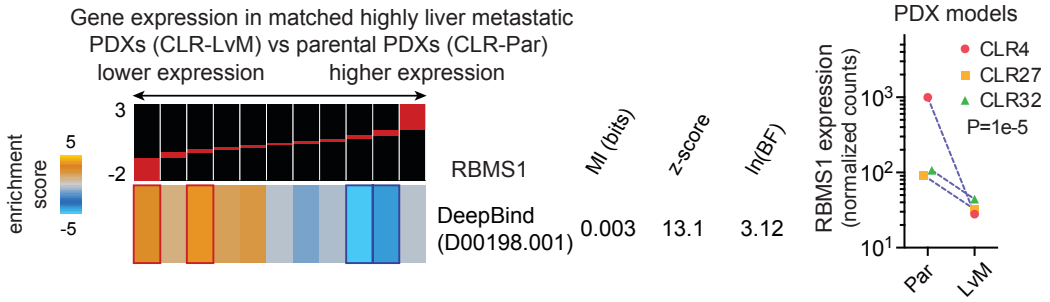
Major Task 2: Pre-clinical studies of HDAC1-mediated induction of the RBMS1 pathway (Months 12-24) – **100% completion**

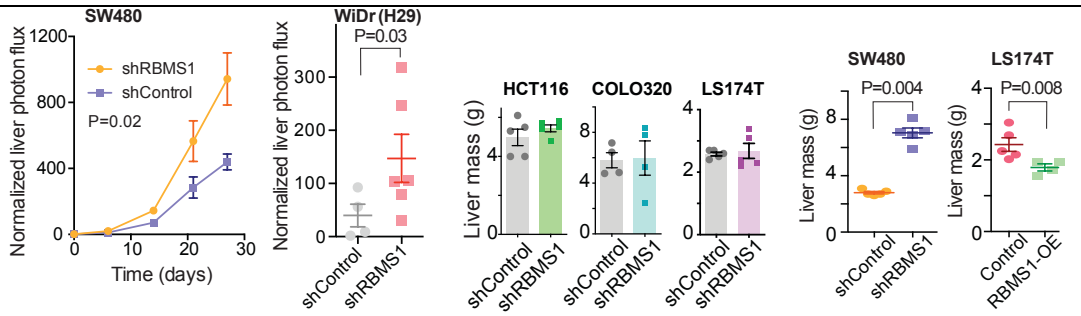
What was accomplished under these goals?

Specific Aim 1: Characterize RBMS1 as a regulator of RNA stability.	**Timeline (months)**
Major Task 1: RBMS1 irCLIP	
Subtask 1: Validating immunoprecipitation antibodies for endogenous RBMS1 IP.  *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.*	1-2 **Completed**
Subtask 2: (Contingency) V5-tagged RBMS1 expressed transgenically in RBMS1 (V5 IP already established). *Did not need as a commercially available antibody targeting endogenous RBMS1 has worked.*	2-3 **Not needed**
Subtask 3: RBMS1 IP followed by high-throughput sequencing (irCLIP). *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.*	2-4 **Completed**
Subtask 3: irCLIP analysis: binding site discovery and target identification.  *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.*	4-6 **Completed**

Major Task 2: Reporter assays	
Subtask 1: Clone 3' UTRs from targets and control non-targets. *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.*	6-8 **Completed**
Subtask 2: Generate the associated cell lines. Validate cell lines by flow cytometry. *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).*  *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.*	8-10 **Completed**
Subtask 3: Perform measurements using qPCR. *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 RMBS1 binding and non-binding mRNA expression.*	10-12 **Completed**
Subtask 4: Select functional reporters and generate lines in SW480-RBMS1 knockdown cells. *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.*	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**

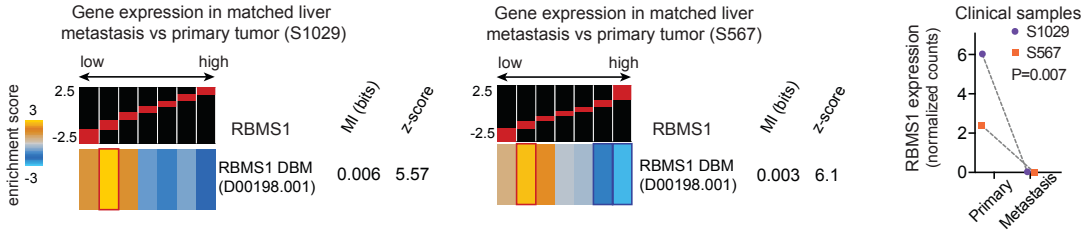
on the irCLIP data. This analysis revealed a 80-gene RBMS1 signature set and will form the basis for the future work on this pathway.	
Milestones Achieved: genome-wide determination of RBMS1 binding sites at nucleotide resolution; functional validation of RBMS1 mediated regulation of its direct targets.	16 **Completed**
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	
Subtask 1: Obtaining HRPO and ACURO approvals for working with human research material and performing animal experiments. *ACURO approval obtained for mouse experiments.* *HRPO approval not required as we did not process human research material.*	12-24 **Completed**
Subtask 2: Obtaining RNAseq data from PDX samples through collaboration with the Tavazoie lab. *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.*	1-6 **Completed**
Subtask 3: Analyzing RNAseq data from PDX samples  *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.*	6-12 **Completed**
Major Task 2: Xenograft mouse models of additional CRC cell lines	
Subtask 1: Compare liver colonization of two models with high and two models with low RBMS1 expression Subtask 2: Compare liver colonization of two models before and after RBMS1 inactivation	12-24 **Completed**



We performed metastatic liver colonization assays with control and RBMS1-depleted or overexpressing cells of different colon cancer genetic backgrounds. We found that in RBMS1-expressing cell lines (SW480, WiDr), RBMS1 knockdown resulted in increased liver metastatic burden, as measured by in vivo luminescence (all colon cancer cells used in this study express luciferase and allow for continuous in vivo imaging). In contrast, in RBMS1-silenced cell lines (HCT116, COLO320, LS174T), RBMS1 knockdown did not change the metastatic liver colonization, as measured by liver mass at the experimental endpoint. In contrast to this, RBMS1 knockdown in SW480 cells led to significantly increased metastasis-bearing liver mass. In agreement to our previous experiments, RBMS1 overexpression in LS174T cells reduced the metastasis-bearing liver mass.

Major Task 3: Quantification of RBMS1 and its targets in clinical samples

Subtask 1: Selection of proper clinical samples for downstream analysis



We obtained RNA samples from two matched CRC primary tumor and metastasis pairs from the Warren and Corvera labs. We performed RNA-seq on these samples, and observed that (i) RBMS1 was silenced in metastases as compared to matched primary tumors, and (ii) the RBMS1 regulon was similarly downregulated in metastases when compared to matched primary tumors.

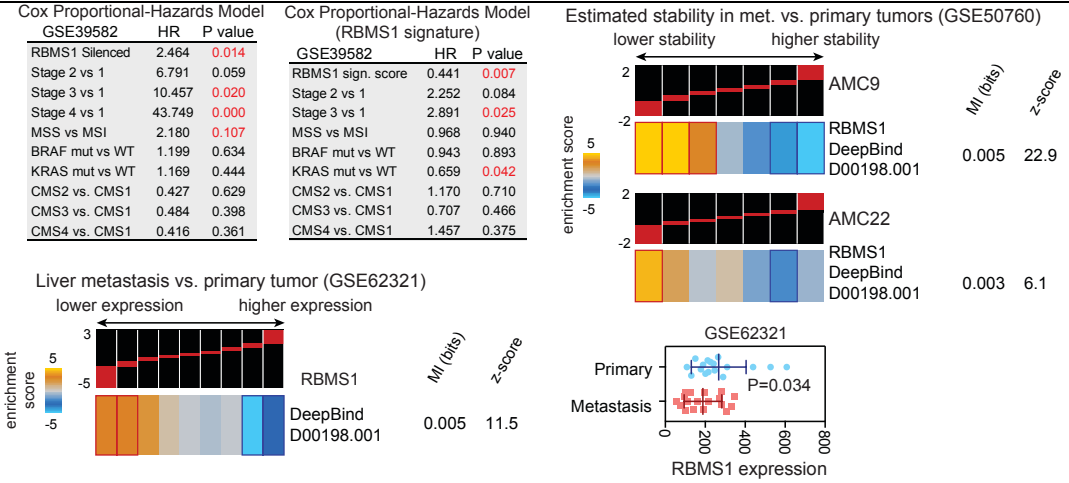
Subtask 2: Multivariate analysis of existing datasets	12-18 **Completed**

Cox Proportional-Hazards Model

GSE39582	HR	P value
RBMS1 Silenced	2.464	0.014
Stage 2 vs 1	6.791	0.059
Stage 3 vs 1	10.457	0.020
Stage 4 vs 1	43.749	0.000
MSS vs MSI	2.180	0.107
BRAF mut vs WT	1.199	0.634
KRAS mut vs WT	1.169	0.444
CMS2 vs. CMS1	0.427	0.629
CMS3 vs. CMS1	0.484	0.398
CMS4 vs. CMS1	0.416	0.361

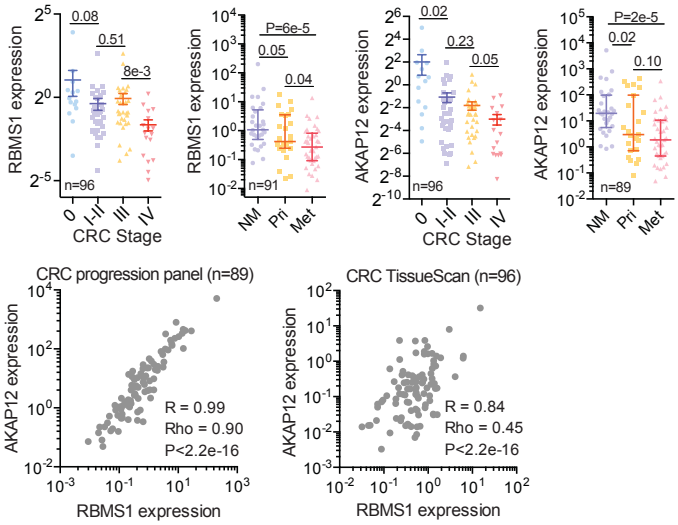
Cox Proportional-Hazards Model (RBMS1 signature)

GSE39582	HR	P value
RBMS1 sign. score	0.441	0.007
Stage 2 vs 1	2.252	0.084
Stage 3 vs 1	2.891	0.025
MSS vs MSI	0.968	0.940
BRAF mut vs WT	0.943	0.893
KRAS mut vs WT	0.659	0.042
CMS2 vs. CMS1	1.170	0.710
CMS3 vs. CMS1	0.707	0.466
CMS4 vs. CMS1	1.457	0.375



We performed multivariate analysis of RBMS1 and its regulon (RBMS1 signature score) expression in CRC clinical samples (GSE39582). We observed that these parameters remained both significant predictors, even when controlled for other known risk factors, such as the stage, microsatellite instability (MSI) or oncogene mutations. We observed in additional clinical datasets that RBMS1 expression was downregulated in metastases when compared to primary tumors (GSE62321), and, accordingly, RBMS1 regulon was also downregulated and destabilized in metastases when compared to primary tumors (GSE62321, GSE50760).

Subtask 3: qPCR measurements in select clinical samples



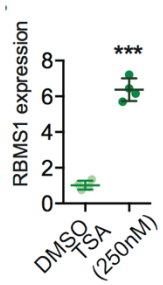
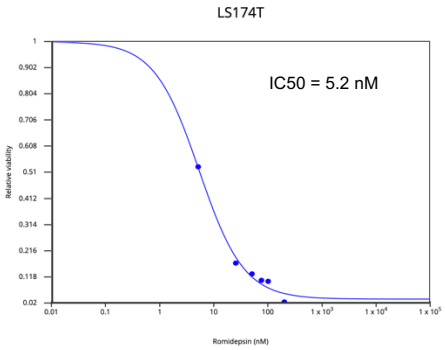
We performed RBMS1 and APAK12 (RBMS1 target) qPCR (relative to HPRT) in 96 CRC stratified on the basis of tumor stage (CRC TissueScan), and in 89 tissue samples (29 normal mucosa (NM), 25 primary colon cancer (Pri), and 37 liver metastases (Met) – CRC progression panel). We found that RBMS1 and AKAP12 expression anticorrelated with CRC stage and progression, while RBMS1 and AKAP12 expression was highly correlated between each other in these datasets.

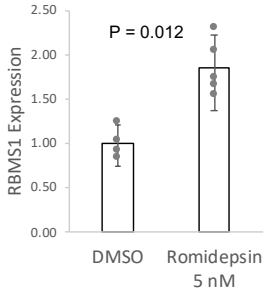
Subtask 4: Analysis of collected data

18-22

Completed

22-24

As illustrated in the data collected for Subtask 1-3, RBMS1 expression was consistently downregulated in metastatic CRC samples, PDXs and cell lines. This correlated with the reduced abundance and stability of RBMS1 target mRNAs. We also showed that knocking down RBMS1 in CRC cell lines that express it contributed to increased metastatic potential in vivo, and, conversely, reconstituting RBMS1 expression in RBMS1-silenced CRC cell lines reduced their metastatic potential.	**Completed**
Milestones Achieved: Clinical relevance of RBMS1 activity	24 **Completed**
Specific Aim 3: Clinical relevance of RBMS1 as a therapeutic target	
Major Task 1: Therapeutic re-activation of the RBMS1 pathway	
Subtask 1: Screening for HDAC1 inhibitors that increase RBMS1 expression Subtask 2: Determining IC50s for inhibitors across multiple lines  *We have treated LS174T cells, where RBMS1 expression is endogenously silenced, with 250 nM Trichostatin A (TSA) or vehicle control (DMSO), and used RTqPCR to measure RBMS1 mRNA expression. We have found that using a broad specificity HDAC inhibitor TSA we could restore RBMS1 expression, as shown in the figure above.*  *To test the effect of Romidepsin, a specific inhibitor of HDAC1/2, we first determined its IC50 for LS174T cells. We treated the cells with 0-200 nM Romidepsin or vehicle (DMSO) control, and determined cell viability by performing*	1-24 **Completed**

a luminescent cell viability assay. We determined that the IC50 concentration for LS174T cells was 5.2 nM.  *We then treated LS174T cells with 5 nM of Romidepsin or vehicle control and measured the RBMS1 expression by RTqPCR. We found that Romidepsin-treated cells showed a modest but significant increase in RBMS1 expression when compared to vehicle-treated cells.*	
Major Task 2: Pre-clinical studies of HDAC1-mediated induction of the RBMS1 pathway	
Subtask 1: Pre-clinical testing of Romidepsin *We did not proceed to mouse experiments with drug-treated LS174T cells. Although efficient in RBMS1 activation, TSA is a broad specificity HDAC inhibitor with limited therapeutic utility. Romidepsin, a specific HDAC1/2 inhibitor, did not show a robust enough RBMS1 activation to test its effect in animal studies.*	12-18 **Completed**
Milestones Achieved: Pre-clinical testing of RBMS1 re-activation	24 **Completed**

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

During the duration of this 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 presented the results of this project in the Bay Area RNA Conference (UCSF, December 4, 2020) where I was selected for an oral presentation.

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.

Commentary: Carter H. Loss of RNA-Binding Protein RBMS1 Promotes a Metastatic Transcriptional Program in Colorectal Cancer. Cancer Discov. 2020;10(9):1261-1262.

I have been selected for an oral presentation at the *Bay Area RNA Conference* (December 4, 2020): RBMS1 Suppresses Colon Cancer Metastasis through Targeted Stabilization of Its mRNA Regulon.

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?

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

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.

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).

Commentary: Carter H. Loss of RNA-Binding Protein RBMS1 Promotes a Metastatic Transcriptional Program in Colorectal Cancer. Cancer Discov. 2020;10(9):1261-1262.

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

Nothing to report.

Other publications, conference papers and presentations.

Bay Area RNA Conference, December 4, 2020
Oral presentation: RBMS1 Suppresses Colon Cancer Metastasis through Targeted Stabilization of Its mRNA Regulon.
Acknowledgment of federal support (yes).

- **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:	*24*
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?

Northing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Award chart.

9. APPENDICES

Award chart.