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14. ABSTRACT

The regulation of mRNA loading onto polysomes is an understudied area of NF1 tumor cell biology and we are poised to take advantage of the model system provided by *Nf1/Arf*-deficient mouse Schwann cells. This will allow us to identify which mRNAs are associated with polysomes to understand the mechanism of how mRNAs are preferentially loaded onto or unloaded from ribosomes in cells lacking *Nf1* and *Arf*. There were two major milestones for this first year of the grant proposal: 1) isolate and maintain murine Schwann cell cultures from our mouse lines, and 2) identify mRNAs whose translation rates are lower or higher in each Schwann cell background. We have successfully been breeding colonies of $Nf1^{fl/fl}$, $Arf^{fl/fl}$, and $Nf1^{fl/fl}/Arf^{fl/fl}$ mice. From these mice, we have harvested embryonic dorsal root neurosphere cells and cultured them into Schwann cells. Ribosome protection and RNA sequencing revealed 27-downregulated mRNAs and 41-upregulated mRNAs in $Nf1^{fl/fl}/Arf^{fl/fl}$ Schwann cells.

15. SUBJECT TERMS
NF1, ARF, tumor suppressors, mRNA, translation

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1. INTRODUCTION

Plexiform neurofibromas (PNs) arising from Schwann cells are a hallmark manifestation of NF1. Benign PNs can transform into highly aggressive MPNSTs, which are the leading cause of mortality in NF1 patients. However, it is unclear what events cause the transition from benign PNs to malignant MPNSTs. Others now provide evidence that the ARF tumor suppressor initially prevents growth and proliferation of *Nf1*-deficient cells. The regulation of mRNA loading onto polysomes is an understudied area of NF1 tumor cell biology and we are poised to take advantage of the model system provided by *Nf1/Arf*-deficient mouse Schwann cells. This will allow us to identify which mRNAs are associated with polysomes to understand the mechanism of how mRNAs are preferentially loaded onto or unloaded from ribosomes in cells lacking *Nf1* and *Arf*. Identifying new proteins involved in both preventing and promoting NF1 will provide novel insights into both the etiology of this debilitating disease and open new possibilities into novel therapeutic targets.

2. KEYWORDS

NF1, ARF, tumor suppressors, mRNA, translation

3. ACCOMPLISHMENTS

Major Goals of the Project

There were two major milestones for this first year of the grant proposal: 1) isolate and maintain murine Schwann cell cultures from our mouse lines, and 2) identify mRNAs whose translation rates are lower or higher in each Schwann cell background.

Goals Accomplished

MAJOR TASK 1: Maintain Schwann cell cultures.

We have successfully been breeding colonies of *Nf1*$^{fl/fl}$, *Arf*$^{fl/fl}$, and *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ mice. From these mice, we have harvested embryonic dorsal root neurosphere cells. These were then cultured in vitro into Schwann cells. We now have three independent lines of Schwann cells representing the three genotypes from above.

MAJOR TASK 2: Determine translation rates in Schwann cells.

Total RNA from each cell background was isolated, depleted of rRNA and sequenced to obtain total RNA transcripts. For ribosome footprinting, cell lysates were incubated with RNase T1 to digest unprotected RNA. Ribosome-bound RNA was pelleted by ultracentrifugation over a sucrose cushion and pelleted RNA was purified and prepared for sequencing. Processed reads were aligned to the mouse genome using TopHat, read counts aligning to coding sequences of genes were determined by HTSeq and differential translation efficiency was determined using DESeq2. Our initial cutoff for significance was a 2-fold change in translation efficiency (up or down). Importantly, our RNA sequencing comparison was also be performed on total mRNA from the same cell backgrounds. This provides us with an internal total cellular mRNA control.

It was apparent from our initial ribosome profiles (**Figure 1**) between the three genotypes that there would be significant difference in the ribosome protected mRNA fragments. Indeed, the polysomes from *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ Schwann cells was significantly elevated compared to either of the other two genotypes.

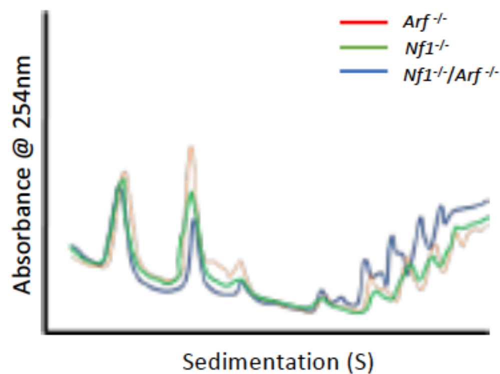

Figure 1. Ribosome profiles from three Schwann cell genotypes. Using the three independent Schwann cell cultures, we performed ribosome fractionation assays. First, cell lysates from each line were separated on sucrose gradients and absorbance of the resulting gradient was monitored at 254nm.

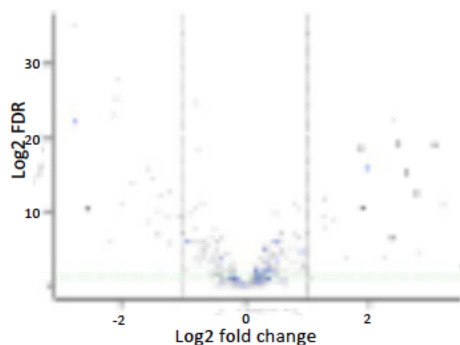

Figure 2. Identification of translationally regulated mRNAs. Polyribosome fractions were isolated and subjected to ribosome footprinting to identify mRNAs that were protected by ribosomes. mRNAs that were translationally regulated (up or down) from *Nf1*$^{fl/fl}$ and *Arf*$^{fl/fl}$ Schwann cells were combined and then compared to mRNA translation from *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ Schwann cells. Each dot represents an individual mRNA. The longitudinal dotted lines represent significance up and down.

Ribosome protection and RNA sequencing revealed that there were a significant number of mRNAs that were either significantly upregulated or downregulated in *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ Schwann cells (Figure 2). We are currently performing a GeneGo^{TM} analysis on these mRNAs to determine if there are any pathways that are significantly regulated in *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ Schwann cells. Regardless of that analysis, we have identified 27-downregulated mRNAs and 41-upregulated mRNAs. We will now move onto the second aim of our proposal in year 2. We will first perform sequence analysis of the 5'- and 3'-untranslated regions of the 30 of the mRNAs we have identified. We then generate luciferase reporters for our top hits to identify which regions of the mRNA are important for translation regulation in *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ Schwann cells. Finally, we will perform RNA immunoprecipitations and mass spectrometry analysis to identify proteins that are bound to these mRNA regions.

<u>Training Opportunities</u>
Nothing to Report

<u>Results Disseminated to the Community</u>
I participated this past year in disseminating our initial findings to three independent groups of large donors to the American Cancer Society. These donors visited my laboratory at Washington University where I discussed the research in this grant proposal and how our results were moving the field of breast cancer research forward. We engaged in a question-and-answer session where the donors queried me on the clinical impact of this work. I anticipate doing this laboratory tour again next year and have already been asked by the American Cancer Society to do so.

Plans for Next Reporting Period

In the final year 2, we will focus exclusively on MAJOR TASKS 2 and 3. Specifically, we will begin experiments aimed at determining the motifs in untranslated regions of each mRNA responsible for altered translation. We will also identify proteins that are bound to altered mRNAs in $Nf1^{fl/fl}/Arf^{fl/fl}$ Schwann cells.

4. IMPACT

Impact on Principal Discipline

Our current work will be incredibly impactful for those studying NF1. We have discovered that $Nf1^{fl/fl}/Arf^{fl/fl}$ Schwann cells exhibit unique ribosome profiles and have uncovered novel mRNAs that are translationally regulated in these cells. This information will be deposited in a public database for RNA sequencing so that other scientists can view the raw data.

Impact on Other Disciplines

Nothing to Report

Impact on Technology Transfer

Nothing to Report

Impact on Society

We have disseminated the data and ideals from this grant proposal to several groups in the St. Louis community including the American Cancer Society. They were encouraged by our progress and excited about the future clinical impact our work might provide.

5. CHANGES/PROBLEMS

Changes in Approach

Nothing to Report

Anticipated Problems or Delays

Nothing to Report

Changes in Human, Animal Biohazards and/or Selective Agents

Nothing to Report

6. PRODUCTS

Publications, Conference Papers and Presentations

Nothing to Report

Internet Sites

Nothing to Report

Technologies or Techniques

Nothing to Report

Inventions, Patents and/or Licenses

Nothing to Report

7. PARTICIPANTS

Individuals That Have Worked on Project

Name:	Jason D. Weber
Project Role:	PI
Nearest person month worked:	0.24
Contribution to Project:	Dr. Weber served as the mentor for Ms. Kuzmicki in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH CA262804; W81XWH-21-1-0476; W81XWH-21-1-0466

Name:	Anna Grobelny
Project Role:	Graduate Student
Nearest person month worked:	6
Contribution to Project:	Ms. Grobelny performed all of the experiments outlined in specific aim 1 for year 1

Changes in Active Other Support for PD/PI

Nothing to Report

Other Organizations Involved as Partners

Nothing to Report