Award Number: W81XWH-21-1-0391

TITLE: Collaborative Regulation of Translational Targets by the ARF and NF1 Tumor Suppressors

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REPORT DATE: October 2023

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE
October 2023

2. REPORT TYPE
Final

3. DATES COVERED
01Jul2021-30Jun2023

4. TITLE AND SUBTITLE

Collaborative Regulation of Translational Targets by the ARF and NF1 Tumor Suppressors

5a. CONTRACT NUMBER
W81XWH-21-1-0391

5b. GRANT NUMBER
NF200089

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 three major milestones for this grant proposal: 1) identify mRNAs whose translation rates are lower or higher in each Schwann cell background, 2) determine motifs in untranslated regions of each mRNA responsible for translation, and 3) identify proteins bound to these mRNAs. Ribosome protection and RNA sequencing revealed 27-downregulated mRNAs and 41-upregulated mRNAs in $Nf1^{fl/fl}/Arf^{fl/fl}$ Schwann cells. Alignment of these mRNAs revealed very few consistent regions or motifs of interest. RNA pulldown experiments revealed numerous proteins, namely a class of RNA helicases, was bound to many of the mRNAs being translated more efficiently.

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

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

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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 three major milestones for this grant proposal: 1) identify mRNAs whose translation rates are lower or higher in each Schwann cell background, 2) determine motifs in untranslated regions of each mRNA responsible for translation, and 3) identify proteins bound to these mRNAs. Ribosome protection and RNA sequencing revealed 27-downregulated mRNAs and 41-upregulated mRNAs in *Nf1*$^{fl/fl}$/*Arf*$^{fl/fl}$ Schwann cells. Alignment of these mRNAs revealed very few consistent regions or motifs of interest. RNA pulldown experiments revealed numerous proteins, namely a class of RNA helicases, was bound to many of the mRNAs being translated more efficiently.

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.

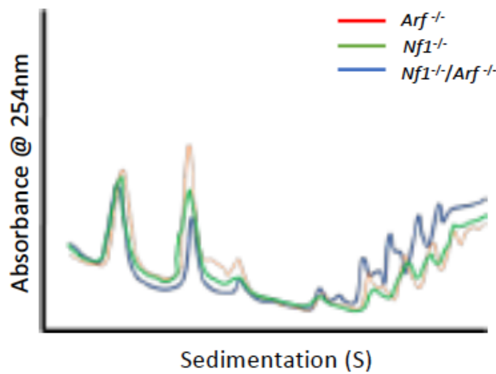

Figure 1. Ribosome profiles from three Schwann cell genotypes. Using the three independent Schwann cell cultures, we performed ribosome fractionation assays. First, cell lysates from each line were separated on sucrose gradients and absorbance of the resulting gradient was monitored at 254nm.

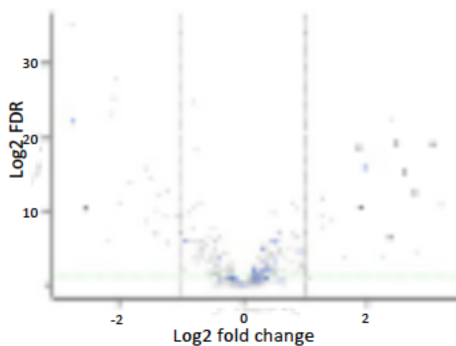

Figure 2. Identification of translationally regulated mRNAs. Polyribosome fractions were isolated and subjected to ribosome footprinting to identify mRNAs that were protected by ribosomes. mRNAs that were translationally regulated (up or down) from *Nf1*$^{fl/fl}$ and *Arf*$^{fl/fl}$ Schwann cells were 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. Finally, we will perform RNA immunoprecipitations and mass spectrometry analysis to identify proteins that are bound to these mRNA regions.

MAJOR TASK 3: Determine motifs in untranslated regions of each mRNA responsible for their translation.

We first performed sequence analysis of the 5'- and 3'-untranslated regions (UTR) of 30 of the mRNAs we have identified. Looking at the peaks that were protected by ribosomes and their location on the mRNA, it became obvious that most protection occurred in the 3'-UTR (**Figure 3**). However, there were two highly significant peaks in the 5'-UTR and within the intronic region of the open reading frame.

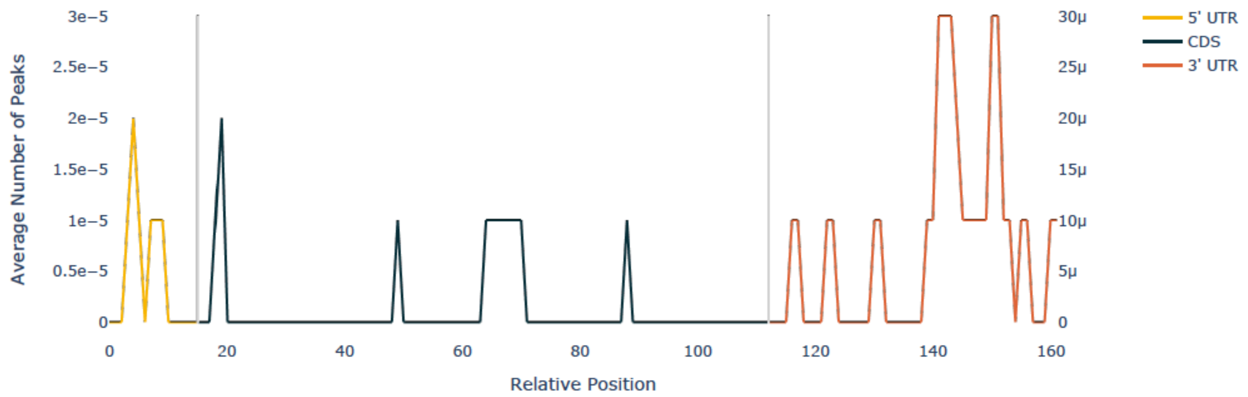

Figure 3. mRNA regions protected by ribosomes. mRNAs isolated from polysomes were analyzed by RNA sequencing to identify regions of mRNA that were protected by ribosomes. These regions were mapped onto each mRNA and depicted as the sum of the top 30 mRNAs upregulated by ARF and NF1 loss.

We then generated 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. We chose to analyze NPM and eEF1a1. We also used GAPDH mRNA as a control for the luciferase assays. As depicted in **Figure 4**, we utilized both the 5'- and 3'-UTRs to determine regions that influenced translational control. We used rapamycin treatment as a positive control to inhibit translation. Clearly, regulation of eEF1a1 is controlled by its 5'-UTR while NPM is controlled by its 3'-UTR (**Figure 4**).

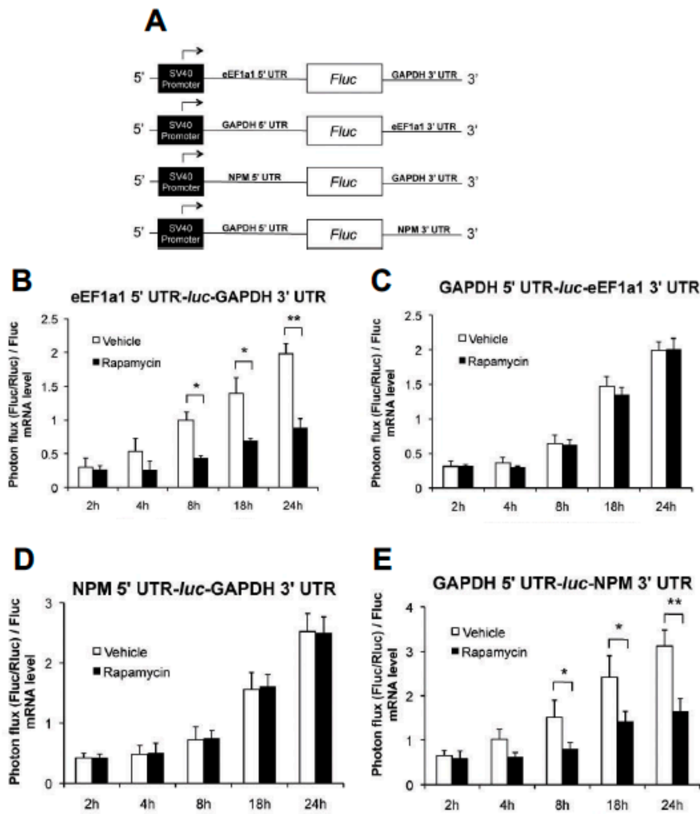

Figure 4. Luciferase-based reporters for translation. A, UTRs for each mRNA were cloned in-frame with firefly luciferase driven by a CMV promoter. B-E, each expression construct was introduced into *Arf/Nf1*-deficient cells and allowed to be translated over 24 hours in the presence or absence of rapamycin.

After performing these experiments for 10 mRNAs, we identified sequences within the 5'- and 3'-UTR that were responsible for the translational regulation. These sequences were combined for all mRNAs identified and the top 5 significant sequences were ranked (**Figure 5**).

Rank	Motif	P-value
1	ACACACGCCUCC	1e-77
2	CUCACACCGG	1e-56
3	UGCGAGAGGUCA	1e-56
4	CUCCCACGCU	1e-45
5	AGGUACACGAGC	1e-44

Figure 5. mRNA sequences. Sequences from mRNA UTRs were aligned across multiple RNAs and the top five sequences were identified and significant P-values.

MAJOR TASK 4: Identify proteins bound to mRNAs.

Numerous studies have shown that UTR binding proteins specifically alter the translation regulation of gene expression. We used a classical biochemical approach to identify unique UTR binding proteins. *Nf1/Arf*-null cells were lysed and whole cell extracts were mixed with biotinylated 5' and 3' UTR mRNAs. RNA pull-down assays were performed using streptavidin beads to precipitate mRNA-protein complexes. Bound proteins were separated by SDS-PAGE and processed for commercial mass spectrometry identification. We were able to use the 3'-UTR of NPM as bait for bound proteins and identified 18 proteins including MDA5, TRIM25, DDX18, and DHX9. We performed immunoblot analysis for these proteins to validate their binding to the NPM 3'-UTR (**Figure 6**).

Figure 6. mRNA interactions. 5'- and 3'- UTR sequences from NPM were used as bait in whole cell lysates of *Arf/Nf1*-null cells. Precipitates were isolated and proteins separated by SDS-PAGE. Proteins were visualized by immunoblot analysis with the indicated antibodies.

Training Opportunities

Nothing to Report

Results Disseminated to the Community

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.

Plans for Next Reporting Period

This grant has concluded the two years of funding.

4. IMPACT

Impact on Principal Discipline

Our current work will be incredibly impactful for those studying NF1. We have discovered that $\textit{Nf1}^{fl/fl}/\textit{Arf}^{fl/fl}$ 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

Jasonweberlab.com

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. Grobelny 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 the experiments outlined in each specific aim

Changes in Active Other Support for PD/PI

Nothing to Report

Other Organizations Involved as Partners

Nothing to Report