AWARD NUMBER: W81XWH-20-1-0377

TITLE: Merlin-ASPP2 Tumor Suppressor Interactions in Mechanosensory Signal Transduction from Schwann Cell Junctions in Neurofibromatosis Type 2

PRINCIPAL INVESTIGATOR: Robert F. Hennigan, Ph.D.

CONTRACTING ORGANIZATION: Cincinnati Children's Hospital Medical Center

REPORT DATE: JUNE 2022

TYPE OF REPORT: Annual Progress Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUNE 2022	Annual	5/15/21 - 5/14/22

4. TITLE AND SUBTITLE
Merlin-ASPP2 Tumor Suppressor Interactions in Mechanosensory Signal Transduction from Schwann Cell Junctions in Neurofibromatosis Type 2

5a. CONTRACT NUMBER
SPR201524

5b. GRANT NUMBER
W81XWH-20-1-0377

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert Hennigan

E-Mail: Robert.Hennigan@cchmc.org

5d. PROJECT NUMBER
NF190083

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Cincinnati Children's
Hospital Medical Center
3333 Burnet Ave.
Cincinnati, OH 45229

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 Neurofibromatosis Type 2 is an inherited disease characterized by bilateral schwannomas that are caused by inactivation of the product of the NF2 tumor suppressor gene, Merlin. We used a powerful new technique, proximity biotinylation, to identify a new merlin binding protein, ASPP2, a tumor suppressor that interacts with a range of oncogenic signal transduction molecules. We hypothesized that merlin-ASPP2 interactions are required to regulate mechano-sensory signal transduction. To test this, we will determine if merlin-ASPP2 interaction is required from merlin function and identify the merlin and ASPP2 binding proteins that connect them with upstream cell junction complexes. Despite significant obstacles imposed by the Covid 19 pandemic, we made substantial progress addressing Aim 1d. We identified a cohort of proteins that are co-proximal to both Merlin and ASPP2 and require Merlin to associate with ASPP2, in both growing and contact inhibited cells. In doing so we validated a more sensitive and powerful proximity biotinlyation technique and generated data that will inform the work moving forward.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

Introduction

We continue to make progress towards achieving the project goals in the last year, although the continuing effects of the COVID-19 pandemic has still had a significant impact on our work. As detailed in last year's progress report, as a consequence of first the quarantine and limits on the number of people in a given space severely restricted that time devoted to benchwork, I chose not to hire a research assistant. During the second year, I attempted to hire a research assistant but due the acute labor shortages impacting the entire institution, I have, so far, failed to do so. However, beginning this summer I will employ a highly experienced PhD level researcher on a temporary basis. Since it will not be necessary to train this individual, this will allow us to "play catch up" and address some of the objectives that we have so far deferred. Additionally, this will also facilitate the training permanent research associate. Despite these challenges, we have made significant progress, including submitting a manuscript for publication. This study details the functional consequences of Merlin dimerization and conformation upon activation by PIP_2. This work grew out of our efforts to implement binding assays using purified recombinant Merlin-NanoLuc fusion proteins described in Specific Aims 1b and 2b. The findings detailed in this paper will significantly affect how we address these aims.

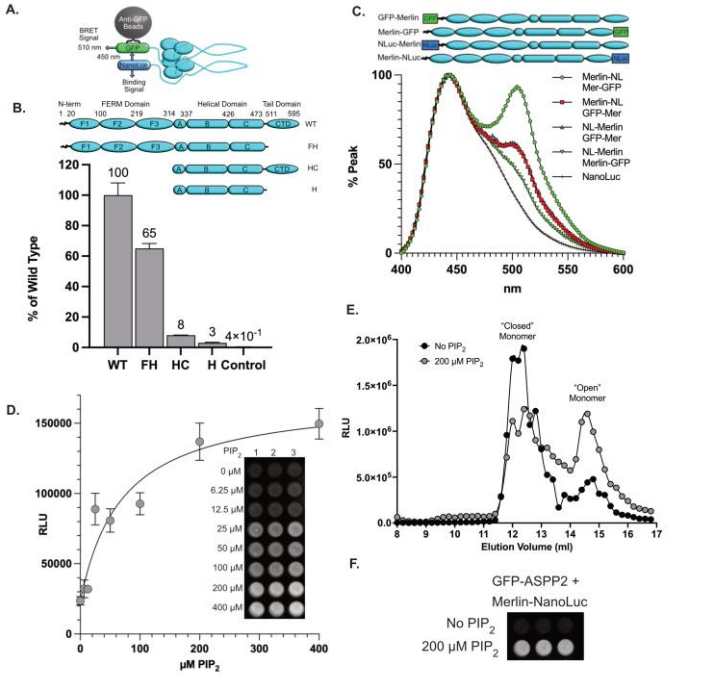
Keywords

Neurofibromatosis Type2, NF2, Merlin, ASPP2, TP53BP2, Mechanosensory Signaling.

Accomplishments

The major accomplishment we can report is the submission of a manuscript that is now under revision. A preprint of this work was accepted by the preprint server, bioRxiv {Hennigan, 2021 #1588}. The key points in this work are summarized in Figure 1. We demonstrated that merlin dimerizes primarily via the N-terminal FERM domain (Figure 1B). Bioluminescence resonance energy transfer (BRET) analysis between NanoLuc and GFP in dimeric Merlin complexes showed that Merlin dimers are orientated such that their C-termini are in close proximity (Figure 1C). Dimerization is increased in the presence of PIP_2 (Figure 1D) which causes a shift to a more open conformation as shown by gel filtration chromatography (Figure 1E), as has been shown in the literature [2]. In short, this work delineates a mechanism by which Merlin is activated in response to PIP_2 signaling. This suggests that the active form of Merlin is a PIP_2 bound dimer. We therefore tested if PIP_2 affected the Merlin-ASPP2 interaction. Indeed, we have found that PIP_2 enhanced this interaction significantly, as indicated by increased Merlin-NanoLuc activity on GFP-ASPP2 bound beads (Fig. 1F). This is consistent with our observation that a non-phosphorylatable mutant of Merlin, S518A, has enhanced ASPP2 binding (Figure 3 from the submission) since S513A also has enhanced dimerization and favors the open conformation [1]. This observation will affect how we accomplish Aim 1b which is intended to defined the minimal merlin binding sequence on ASPP2.

Figure 1.



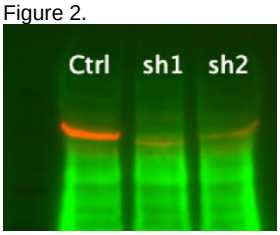
A. A schematic diagram of the Merlin dimerization assay using purified Merlin-GFP bound to and anti-GFP nanobody
B. Dimerization assays using Merlin deletion mutants
C. BRET assays using combinations of N-and Cterminal fusions of Merlin to either GFP or NanoLuc.
D. Incubation of Merlin with PIP_2 increases dimerization
E. Gel filtration chromatography of Merlin-NanoLuc showing two conformers representing closed conformation (right) and open conformation monomer (left). PIP_2 causes a decrease in the proportion of the closed conformation peak with a corresponding increase in the open conformation.
F. GFP-ASPP2 bound beads incubated with purified Merlin-NanoLuc in the presence and absence of 200 μM PIP_2.

Aim 1. Test the hypothesis that the Merlin-ASPP2 interaction is required for Merlin function in mechano-sensory signaling and contact inhibition.

Aim 1A. Using Schwann cells with CRISPR mediated knockdown of *ASPP2* to test if *ASPP2* loss phenocopies one or more Merlin null phenotypes.

We are now evaluating a new set of lentiviral vectors designed to knock down ASPP2 expression. As before we were able to partially know down ASPP2 expression with two new shRNAs that reduce ASPP2 levels to 35% ad 39% of control respectively (Fig. 2). While we will evaluate the growth characteristics of these cells, we suspect this level of knockdown is insufficient for our needs. We are now experimenting with further gRNA targets and combinations of gRNAs that show partial knockdown to achieve a more complete knockdown. We expect to have an answer to this set of experiments by the end of June 2022 and, with personnel available to work on this project, ASPP2 KO cell lines by the end of the summer.

Figure 2.



ASPP2 expression levels in an immunoblot of HSC-1λ cells expressing two different gRNAs targeting ASPP2 (sh1 & sh2). ASPP2 is shown in red and total protein is in green

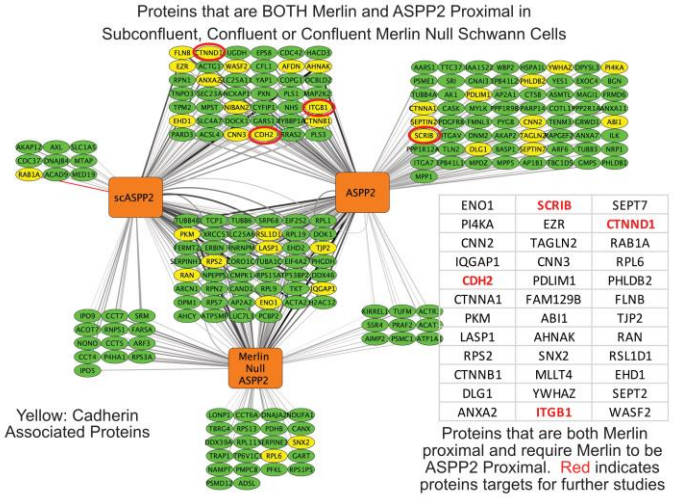
Aim 1B. Refine the identified 206 amino acid sequence from amino acids 129 to 335 on ASPP2 where Merlin binds, and generate ASPP2 mutants deficient for Merlin binding, and test if mutant as well as wild type ASPP2 rescues ASPP2 loss.

We will now incorporate PIP_2 with our assays designed to probe this interaction, bearing in mind that the Merlin face of the complex is likely to be dimeric and difficult to define as a concise peptide sequence. It is now necessary to determine if ASPP2 is also a PIP_2 binding protein and if it also dimerizes.

Aim 1C. Determine if the ASPP2-Merlin complexes interact with other oncoregulatory signaling pathways in response to mechanosensory signaling.

We were not satisfied with the initial suppliers of tunable stiffness ECM substrates needed to perform these experiments. We are tentatively scheduled to evaluate new suppliers and test making out own substrates in the fall.

Figure 3



A proximity map of previously identified merlin proximal proteins tha are also proximal to ASPP2-APEX fusion proteins in subconfluent, confluent and Confluent, Merlin-null iHSC-1λ Schwann cells. Yellow indicates proteins mapped to the adhesion molecules. The red indicates the position of N-cahderin (CDH2), β-integrin, (ITGB1), δ-catenin (CTNND1) and Scribble (SCRIB) and being ASPP2 proximal in the presence of Merlin but not in its absence.

Aim 1D. Use proximity biotinylation to test if the Merlin-ASPP2 interactome is responsive to mechanosensory signaling

As we detailed in last yeas progress report, we have transitioned form the $BirA^{R118G}$ based proximity biotinylation enzyme to the more powerful APEX2 system. The lentiviral expression constructs described in the aim, Merlin-GFP, GFP-ASPP2 and ASPP2-GFP have been constructed and their expression has been validated. The specific pulldown experiments described in the Aim are awaiting successful establishment of the ASPP2-Merlin double KO cells described in **Aim 1a.** As we described in last year's progress report, we established cell lines expressing APEX-ASPP2 and ASPP2-APEX in iHSC-1λ Schwann cells. This experiment revealed a cohort of merlin and ASPP2 co-proximal proteins, thus identifying protein complexes that are potentially sites of the Merlin-APSS2 interaction. This year we expressed the APEX-ASPP2 and ASPP2-APEX fusion proteins in Merlin null iHSC-1λ Schwann cells. A proximity map of these data is shown in Figure 2. This depicts known Merlin proximal proteins (identified previously) that are also ASPP2 proximal in subconfluent or confluent HSC-1λ Schwann cells or Merlin null iHSC-1λ Schwann cells. These data identify a set of Merlin proximal proteins that require Merlin to associate with ASPP2, further clarifying the Merlin-ASPP2 interactome. This subset of proteins includes the cell junctional proteins N-cahderin (CDH2), β-integrin, (ITGB1), δ-catenin (CTNND1) and Scribble (SCRIB). As we discussed in the 2020 progress report, these proteins

will be used in the combined proximity biotinylation and pull-down experiments intended to identify the Merlin and ASPP2 binding proteins that connect to upstream cell junctions described in Aim 2a. The proximity data from the Merlin null cells further validates the choice of these proteins. Progress with these experiments is described below.

Aim 2 Identify the Merlin and ASPP2 binding proteins in upstream cell junction complexes.

Aim 2A. Use a combined immunoaffinity purification and proximity biotinylation strategy to identify proteins that connect Merlin with cell junctions via α-actinin, scribble or ZO-1.

We have established iHSC-1λ and expressing N-cadherin-APEX, β-integrin-APEX, δ-catenin-APEX and Scribble-APEX. We have validated these constructs and are currently performing the proximity labelling and mass spec identification experiments for these cells. Additionally, we decided to express these constructs in Merlin null iHSC-1λ cells to evaluate the effect of Merlin on these complexes, like what we have done with ASPP2 as detailed above. We are currently generating cell lines co-expressing Merlin-GFP with N-cadherin-APEX, β-integrin-APEX, δ-catenin-APEX and Scribble-APEX for the combined co-immunoprecipitation/proximity biotinylation experiments described in the aim. We expect the full dataset from these experiments by the end of August 2022.

Aim 2B. Identify new Merlin binding proteins among candidate interactors.

We deferred the development of the rapid cloning protocols described Aim 2B due to a lack of manpower caused by the pandemic. We have now resumed this development process and expect to have this system functional by the end of the summer. In response to the results of the dimerization study described above we will now use the more dimeric Merlin-S518A-NanoLuc or Merlin-ΔEL-NanoLuc constructs to screen for interactions in the indirect assays described in the Aim. For the direct assay using purified Merlin-NanoLuc probes we will perform the screens in the presence and absence of 200 μM PIP_2.

Impact

Merlin dimerization identifies a basic biochemical mechanism by which Merlin is activated in response to PIP_2 signaling (Fig. 1). This observation requires us to evaluate the Merlin-ASPP2 interaction in the context of PIP_2 dimerized merlin and phospho-S518 merlin. This is exemplified by the enhanced Merlin-ASPP2 binding in the presence of PIP_2 (Fig. 2).

Changes in the ASPP2-Merlin co-proximal proteins revealed by proximity biotinylation in the presence and absence of Merlin highlight sets of proteins that may be critical to Merlin and ASPP2 function. Specifically, these include junctional proteins such as the N-cadherin complex (Figure 2B), multiple endocytic and Rap1 related proteins (Fig. 2C) and oncogenic signaling proteins such as HIPPO and Ras-MAPK (Figure 2D). The proximity biotinylation experiments for N-cadherin-APEX, β-integrin-APEX, δ-catenin-APEX and Scribble-APEX described in Aim 2a that are expected to further clarify the junctional complexes that the Merlin-ASPP2 complex responds to. Together these data will have the effect of focusing our efforts towards identifying Merlin and ASPP2 binding proteins in upstream cell junction complexes described in Aim 2b.

Changes/Problems

In last year's report we described two significant problems that we are still struggling with. First, our efforts to generate ASPP2 null cells remain unsuccessful. Despite testing several new gRNAs, including commercially supplied vectors, we still found that the knockdowns were only partial. We are now attempting to identify effective combinations of gRNAs amongst those that work partially. Secondly, we have had difficulty getting a consistent, reproducible results in experiments using variable stiffness hydrogels to stimulate mechanosensory pathways as described in the preliminary data of the proposal (Fig 4 of the proposal) and used in Aims 1b and 1d. We plan to focus on this issue this fall. We still anticipate that both these issues will be fully resolved this fall and the experiments that rely on the hydrogels completed this year.

Products

None

Participants & Other Collaborating Organizations

None

Special Reporting Requirements

None

Appendices

References

1. Merlin Tumor Suppressor Function is Regulated by PIP_2-Mediated Dimerization
Robert F. Hennigan, Craig S. Thomson, Nancy Ratner
bioRxiv 2021.11.11.468247; doi: https://doi.org/10.1101/2021.11.11.468247

2. Chinthalapudi K, Mandati V, Zheng J, Sharff AJ, Bricogne G, Griffin PR, et al. Lipid binding promotes the open conformation and tumor-suppressive activity of neurofibromin 2. Nature communications. 2018;9(1):1338. Epub 2018/04/08. doi: 10.1038/s41467-018-03648-4. PubMed PMID: 29626191; PubMed Central PMCID: PMCPMC5889391.