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, Cincinnati, OH

REPORT DATE: June 2021

TYPE OF REPORT: Annual

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 2021	Annual	15May2020-14May2021

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

5a. CONTRACT NUMBER

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, Marvland 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
Unclassified					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	7	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

INTRODUCTION

It is a considerable understatement to say that our progress on this project has been adversely impacted by the global Covid-19 pandemic. The challenges posed by the pandemic over the first year of funding have manifested themselves in two major ways. First, in response to local and state mandates, Cincinnati Children's Hospital enforced an eight-week lockdown period in 2020 extending from late March through early June. Non-essential personnel, including basic researchers, were told to stay home. This overlapped the beginning of year 1 funding in May of 2020. Subsequently, after the quarantine was lifted, strict masking and limits on the number of people in a given space were enforced without exception. This effectively limited the amount of time in the lab devoted to benchwork to roughly four hours a day. Secondly, since this limited time precluded the effective training of new personnel, I decided not to fill the technician position funded by the grant at this time. As a consequence, we have underspent our budget for this period to a considerable degree. Because of the limited amount of bench time available under these restrictions, we focused on what we judged to be the most important aspects of the research plan that we can carry out under these circumstances. In spite of the unprecedented obstacles presented by the Covid-19 pandemic, we are excited by the significant progress we made this year.

Keywords

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

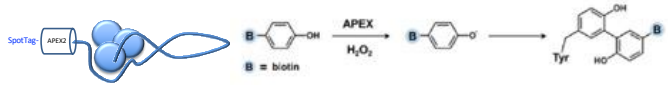
Accomplishments

In response to the limitations imposed by the pandemic we chose to focus our efforts on primarily on Aim 1D, proximity biotinylation experiments to define the merlin-ASPP2 interactome. We did this for several reasons. First, we wanted to maximize the impact of our limited efforts by performing experiments which we felt were more likely to inform the future direction of the project. Secondly, as described in the proposal, we changed our model cell system. We moved on from the immortalized mouse Schwann cells with a floxed *Nf2* alleles that we used in our previous proximity biotinylation studies. These cells had a very high rate of proliferation and a marked tendency to spontaneously transform, thus complicating assays measuring contact inhibition of growth and mechanosensory signaling. Therefore, as described in the proposal, we used iHSC-1λ immortalized human Schwann cells (2) to avoid these problems. To confirm that these two cell types were equivalent, we performed a proximity biotinylation experiment with Merlin in iHSC-1λ to compare with our previous data. Finally, in comparing our model cell systems we also evaluated a different proximity biotinylatyion enzyme, APEX22.

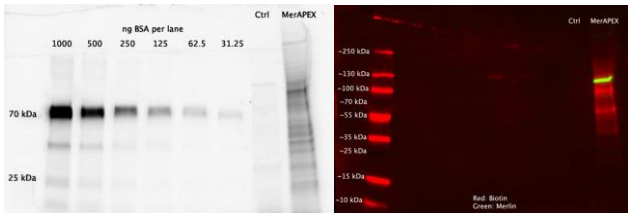
Previous proximity biotinylation experiments utilized constructs expressing Merlin fused in frame to BirA^{R118G}, a mutant of the *E.coli* biotin ligase that allows the active biotin catalytic intermediate to diffuse away from the enzyme, thus biotinylating adjacent proteins (1). Although we had great success with this system, the relatively low specific activity of the mutant enzyme required long incubation times to label a small proportion of endogenous proximal proteins. This required that the experiments were

Figure 1. APEX2 Mediated Proximity Biotinylation

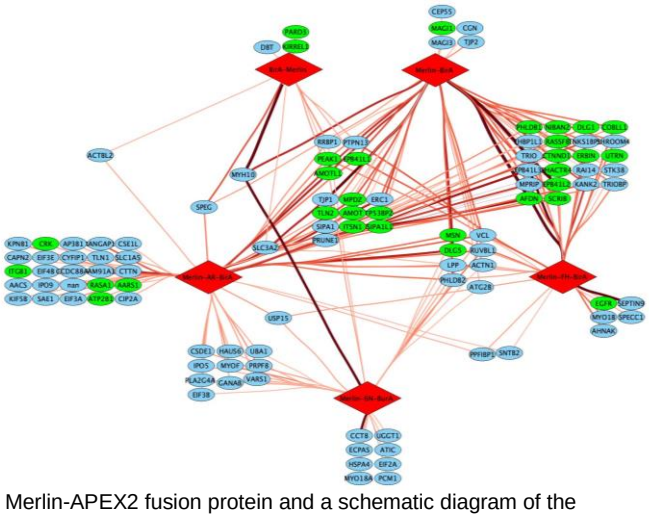
A.



B.



C.



A. Merlin-APEX2 fusion protein and a schematic diagram of the covalent biotinylation of a proximal tyrosine residue.
B. Merlin-APEX2 mediated proximal biotinylation relative to control showing total protein (left) and a western blot (right) probed for merlin (green) and biotin (red).
C. The full Merlin-BirA^{R118G} proximity network depicting the biotinylated proteins (Blue) identified by merlin and merlin mutant-BirA^{R118G} constructs (Red) (1). Proteins also biotinylated by Merlin-APEX2 are shown in green.

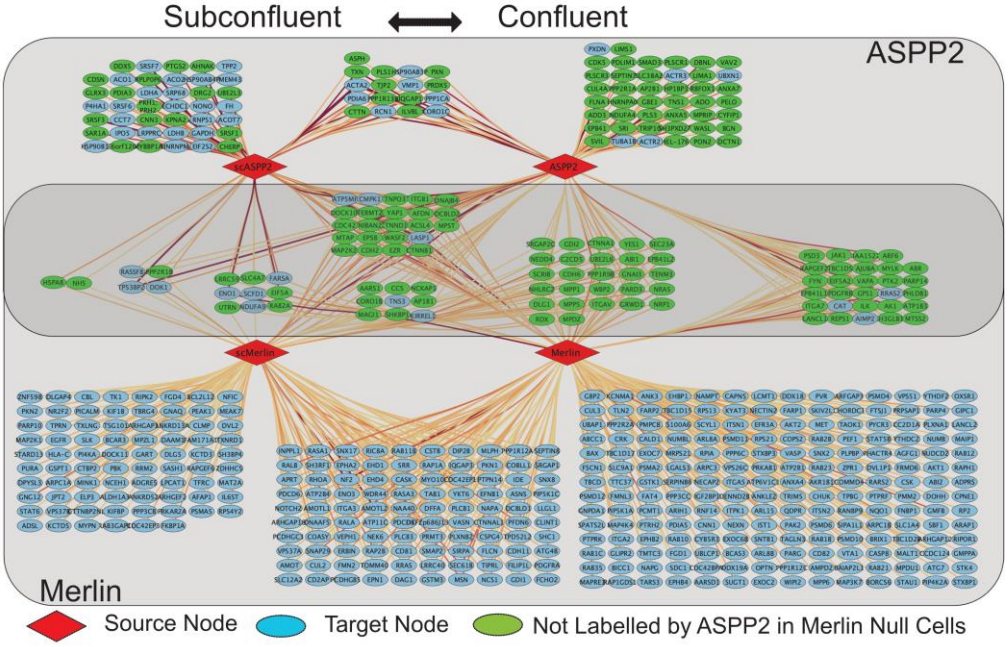
performed at a relatively large scale, making the entire procedure cumbersome. Furthermore, it was apparent that the long incubation time did not effectively label endogenous proteins with a short half-life, failing to detect signaling molecules such as kinases, phosphatases, GTPases, GAP proteins and cell surface receptors. We therefore sought alternatives.

APEX2 is an engineered ascorbate peroxidase derived from soybean (3), it uses hydrogen peroxide (H_2O_2) as an electron source to catalyze the oxidation of biotin-tyramide to produce a highly reactive, short lived radical that will covalently bind to proximal proteins, predominately on tyrosine residues (Figure 1A)(4). As a practical matter, cells infected with lentivirus expressing Merlin-APEX2 are pre incubated with biotin-tyramide, the reaction is initiated by adding hydrogen peroxide for 1 minute, then the reaction is quenched with a cocktail of antioxidants, the cells are harvested, biotinylated proteins are purified with streptavidin and biotinylated proteins are identified via mass spec. This resultes in biotinylation of a range of endogenous proteins (Figure 1B). The one-minute H_2O_2 incubation time, combined with the enhanced catalytic activity associated with the APEX2 enzyme, results in a snapshot of proximal proteins within that one-minute time frame. Therefore, unlike BirA, this labeling technique is not affected by either protein half-life or the kinetics of the Merlin-target interaction. Also, APEX2-based proximity biotinylation, had much greater sensitivity than BirA and yielded superior results. This allowed experiments to be performed with far fewer cells, 5 x 10^6 vs ~10^8. The experiments could now be performed with a single 15 cm TC dish per replicate, rather than multiple, large 5-level flasks. This change alone greatly simplified sample handling and dramatically increased experimental throughput and allowed us to vary experimental conditions in a way that would have been difficult, if not impossible with BirA.

In direct comparison of Merlin-APEX2 to our published Merlin-BirA results (Figure 1C), we found that wild type Merlin-APEX2 identified 385 proteins compared to 53 for Merlin-BirA. These included numerous kinases, phosphatases, small GTPases, GTPase-activating proteins, receptor tyrosine kinases, cadherins and integrins; proteins that were not identified by BirA. Two thirds of the proteins identified by wild type Merlin-BirA were also identified by wild type Merlin-APEX2 (24/53) plus another 6 proteins identified by various mutant Merlin-BirA constructs. Additionally, analysis of both datasets for the functional enrichment of specific pathways as defined by KEGG yielded identical results, identifying proteins functioning in tight junctions, adherens junctions, focal adhesions and the Hippo pathway.

Taken together these results show that the APEX2 system yields the same results as BirA but with far greater power, sensitivity and ease of use. Therefore, we decided to use this technique to address the proximity

Figure 2 Merlin-ASPP2 Proximal Network Map



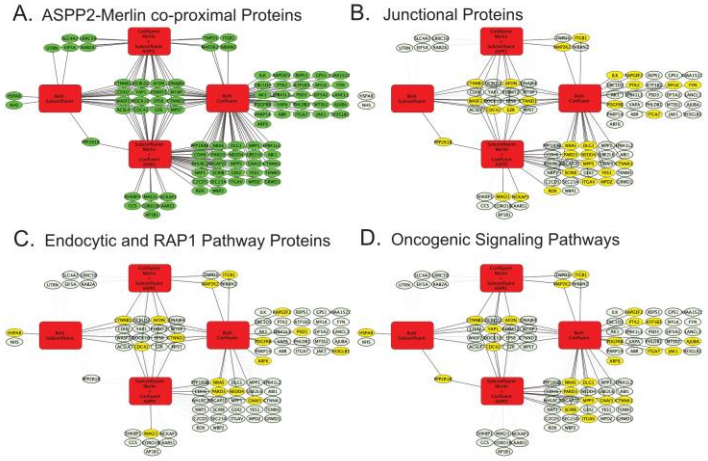
A proximity map depicting biotinylated proteins as blue target nodes, clustered based on their interaction with the red source nodes. Subconfluent ASPP2 (red node top, left) confluent ASPP2 (red node, top, right), subconfluent Merlin (red, node, bottom, left) and confluent Merlin (red, node, bottom, right). The amount of biotinylation is depicted by the amount of red in the connecting edges. The shaded area in the center represents proteins labelled by both ASPP2 and Merlin. The green target nodes identify Merlin-dependent ASPP2 proximal proteins, defined as proteins that are labelled by ASPP2 in Merlin WT but not Merlin null cells, ie co-proximal proteins.

biotinylation aspects of our Aims first to take maximal advantage of our limited bench time. Specifically, were performed some of the experiments described in Aim 1d. We constructed two lentiviral constructs expressing an N-terminal APEX22-ASPP2 and a C-terminal ASPP2-APEX22 plus an additionalo control expressing APEX22 alone. After infection into iHSC-1λ cell, proximity biotinylation experiments were performed at both subconfluent and confluent cell densities. Proximity biotinylation experiments were also performed with Merlin-APEX2 expressing iHSC-1λ cells were also at subconfluent and confluent cell densities. Finally, N-terminal APEX2-ASPP2 and a C-terminal ASPP2-APEX2 were also performed in iHSC-1 λ cells that were made NF2 null using Crispr. Similar experiments with these cell lines testing the response to different substrate stiffnesses as described in the proposal will be performed next.

The results of these experiments were excellent, we identified cohorts of proteins that are proximal to both Merlin and ASPP2 thus identifying molecular complexes where merlin-ASPP2 interactions may occur (Figure 2). Additionally, we identified clear changes in the co-proximal populations at subconfluent, growth-promoting cell densities relative to confluent, growth inhibiting cell densities. This attached functional consequences to these putative complexes. Finally, experiments performed using Merlin null cells expressing ASPP2-APEX2 and APEX2-ASPP2 showed significant changes in the ASPP2 proximal population relative to wild type cells (Figure 3). Specifically, subconfluent ASPP2 identified 97 proteins, 39 of which are also labelled by Merlin, 28 of these are not biotinylated by ASPP2 in the absence of Merlin. In confluent cells, ASPP2 labelled 154 proteins, 88 of which are also labelled by Merlin, 80 of these are not labelled by ASPP2 in the absence of Merlin. Additionally, of the 52 proteins biotinylated by subconfluent ASPP2 but not merlin, 28 still require Merlin. In confluent cells, 61 ASPP2 proximal proteins are not labelled by Merlin but 49 still require Merlin to be labelled by ASPP2.

This experiment identified a total of 89 proteins that may be called co-proximal, ones that are labelled by both Merlin and ASPP2, but require Merlin in order to be biotinylated by ASPP2. Depicting these data as a network identified nine different subsets of ASPP2-Merlin co-proximal proteins, reflecting different combinations of confluent or subconfluent merlin and ASPP2 proximal proteins (Figure 3A). However, the clear trend for both Merlin and ASPP2 individually and for Merlin-ASPP2 co-proximal proteins is a dramatic increase the number of proximal proteins in confluent cells relative to subconfluent cells. Functional enrichment analysis suggests that in confluent cells there is and increased association of ASPP2 and Merlin with known merlin proximal structures such as tight junctions, focal adhesions and adherens junctions (Figure 3B), previously unknown pathways such as Rap1 signaling and endocytosis (Figure 3C) plus numerous oncogenic signaling pathways (Figure 3D).

Figure 3. ASPP2-Merlin Co-Proximal Proteins, Pathway Enrichment



A. A proximity map focused on co-proximal proteins, (shaded area from Figure 3, green target nodes.) The source nodes (red) represent four different conditions, i-left) both ASPP2 and Merlin subconfluent, iii-right) both ASPP2 and Merlin confluent, iii-top) ASPP2 subconfluent and Merlin confluent and iv bottom) ASPP2 confluent and Merlin subconfluent.
B. Yellow highlighted target nodes that map to cell junctions, including tight junctions, adherens junctions, actin cytoskeleton and focal adhesions.
C. Yellow highlighted target nodes that map to endosomes or members of the Rap1 signaling pathway.
D. Yellow highlighted target nodes that map to oncogenic signaling pathways, including the Hippo, Ras, MAPK, cAMP, VEGF and ErbB2 pathways.

Impact

These experiments go a long way towards fulfilling the goal of Aim 1d, to define changes in the ASPP2-Merlin interactome in response to mechanosensory signaling. Further experiments include performing these experiments different ECM stiffnesses and identifying proteins that bind to Merlin and ASPP2 using the screening methods described in the proposal. Additionally, we will test for changes in merlin proximal proteins in the absence of ASPP2 once ASPP2 null iHSC-1λ cells become available. We have also performed this analysis using APEX2 fused to two other known Merlin-binding proteins, Angiomotin and YAP1 (data not shown). The results of these experiments shown significant overlap in the merlin co-proximal populations along with highly informative differences between the ASPP2, Angiomotin and YAP1 interactomes.

Changes/Problems

Together these experiments will inform the choices we will make moving forward as we complete the rest of the Aims. Specifically, the goal of Aim 2 is to identify the Merlin and ASPP2 binding proteins that connect to upstream cell junctions. Aim 2a proposes to use combined proximity biotinylation and pull-down experiments on three junctional proteins that are proximal to but do not bind to merlin, α-actinin, scribble and ZO-1. In response to the new data, we will modify Aim 2a in the following ways. First, given the clear superiority of APEX2 over BirA we will switch to that system for this experiment. Secondly, since the human iHSC-1λ cells are much more stable than the mouse Schwann cell system and we have validated the behavior of merlin in these cells we will proceed with the iHSC-1λ system. Finally, in the human iHSC-1λ cells both Merlin-APEX2 and ASPP2-APEX2 biotinylate scribble but neither α-actinin or ZO-1 are represented in this dataset. In contrast, the adherens junctions are represented by the transmembrane receptor, N-cadherin and their proximal signaling proteins α-catenin, β-catenin and δ-catenin. Similarly, focal adhesions are represented by integrin β1, and multiple integrin α subunits including α2, α3, α5, α7 and α_V. Focal adhesion signaling proteins such as Focal Adhesion Kinase, (FAK), Integrin Linked Kinase (ILK) and Myosin Light Chain Kinase (MLK) are also present. We will therefore perform the proposed proximity biotinylation experiment in Aim 2a using, N-cadherin and integrin β1 in place of α-actinin or ZO-1. N-cadherin and integrin β1 are labelled by both Merlin and ASPP2 in a merlin dependent manner and, being the critical transmembrane receptors for adherens junctions and focal adhesions respectively. We feel these changes to Aim 2a will give us a better chance to achieve to define the direct interactions that connect Merlin and with upstream signaling complexes.

Aside for the restrictions imposed by the pandemic we have experienced two significant problems. First, our initial efforts to generate ASPP2 null cells have not been fully successful. As described in the proposal, we tested several gRNAs targeting the ASPP2 mRNA but due to difficulties getting accurate expression data with the anti-ASPP2 antibodies we had chosen, we found that the knockdowns were only partial. Since this will clearly require extended trouble shooting, we chose to put this part of the project aside until we could devote more time to addressing the problems that we encountered. 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 suspect that this require finding a commercial source with more consistent products or developing the ability to make our own. We anticipate that both these issues will be fully resolved once we have sufficient time to address them after Covid restrictions are lifted.

Products

None

Participants & Other Collaborating Organizations

None

Special Reporting Requirements

None

Appendices

References

1. Hennigan RF, Fletcher JS, Guard S, Ratner N. Proximity biotinylation identifies a set of conformation-specific interactions between Merlin and cell junction proteins. Science signaling. 2019;12(578). Epub 2019/04/25. doi: 10.1126/scisignal.aau8749. PubMed PMID: 31015291.

2. Li H, Chang LJ, Neubauer DR, Muir DF, Wallace MR. Immortalization of human normal and NF1 neurofibroma Schwann cells. Lab Invest. 2016;96(10):1105-15. Epub 2016/09/13. doi: 10.1038/labinvest.2016.88. PubMed PMID: 27617404.

3. Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY. Directed evolution of APEX22 for electron microscopy and proximity labeling. Nat Methods. 2015;12(1):51-4. Epub 2014/11/25. doi: 10.1038/nmeth.3179. PubMed PMID: 25419960; PMCID: PMC4296904.

4. Hung V, Udeshi ND, Lam SS, Loh KH, Cox KJ, Pedram K, Carr SA, Ting AY. Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX22. Nat Protoc. 2016;11(3):456-75. doi: 10.1038/nprot.2016.018. PubMed PMID: 26866790; PMCID: PMC4863649.