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

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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. We have identified Merlin as necessary for ASPP2 localization thus identifying it as a critical binding partner for ASPP2 function. Furthermore, we identified a basic biochemical mechanism by which Merlin is activated in response to PIP_2. We have also greatly expanded our understanding of the Merlin interactome and identified novel binding partners that may represent a novel mechanism of action.

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Introduction

We continued to make progress towards achieving the project goals in the last year, although due to the effects of the COVID-19 pandemic many of the experiments described in the proposal were delayed and are still incomplete. As a result, we have requested a one year no cost extension (NCE) to complete this work. Despite these challenges, we have made significant progress. As detailed below we have published a paper and are now in the final stages of preparing another manuscript. We have generated a significant amount of proximity biotinylation data using the APEX system described in previous reports. The manuscript presents new proximity data for Merlin isoform 1 and isoform 2, in confluent and sub-confluent cells. Additionally, we will present proximity data for ASPP2, Angiomotin, YAP1, N-cadherin, Scribble and Integrin beta 1 in the presence and absence of Merlin. This work takes advantage of the results presented in our recent paper to identify new Merlin binding proteins that are regulated by PIP_2 interaction. We have also submitted an R01 based on these data and will also be submitting another grant to the NF program to follow on from this work.

Keywords
Neurofibromatosis Type2, NF2, Merlin, ASPP2, TP53BP2, PIP2, Sechanosensory Signaling.

Accomplishments

The major accomplishment we can report is the publication of our manuscript **Merlin Tumor Suppressor Function is Regulated by PIP_2-Mediated Dimerization** in the journal PLOS One {Hennigan, 2023 #2914}. 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. We demonstrated that when Merlin binds the signaling phospholipid PIP_2, it shifts to an open conformation that leads to the formation of a dimer that has increased binding affinity for target proteins. We tested if Merlin conformation affected the Merlin-ASPP2 interaction. Indeed, we have found that open conformation merlin mutants significantly enhanced this interaction, as indicated by increased Merlin-NanoLuc activity on GFP-ASPP2 bound beads (Fig. 1). This is consistent with our observation that a non-phosphorylatable mutant of Merlin, S518A, has enhanced ASPP2 binding since S513A also has enhanced dimerization and favors the open conformation.

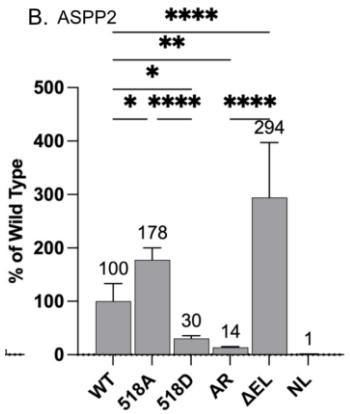
Aim 1. Test the hypothesis that the Merlin-ASPP2 interaction is required for Merlin function in mechano-sensory signaling and contact inhibition.

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

As detailed in previous reports we were able to partially knock down ASPP2 expression with two shRNAs that reduce ASPP2 levels to 35% ad 39% of control respectively. The use of additional gRNAs and combinations of gRNAs that show partial knockdown to failed to achieve more complete knockdown. Furthermore, ASPP2 KO cell lines that we do have did not have significantly different growth characteristics relative to a scrambled sRNA control. We conclude that that ASPP2 knockdown to this level does not phenocopy Merlin loss in these cells.

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.

Figure 1



ASPP2 binding to merlin mutants that are non-phosphorylatable (S518A), phosphomimetic (S518D), closed conformation and (AR) and open conformation (ΔEL). These experiments demonstrate enhanced Merlin binding with hypo-phosphorylated, open conformation Merlin

As described in last year's report we will now determine if ASPP2 is also a PIP_2 binding protein and if it also dimerizes. These experiments are now planned for the fall of this year. However, since we could not identify a phenotype for our ASPP2 KO cells we will not test for phenotypic rescue as described in the aim.

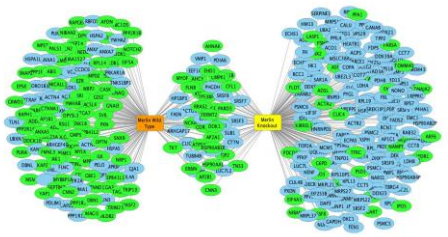
Aim 1C. Determine if the ASPP2-Merlin complexes interact with other oncoregulatory signaling pathways in response to mechanosensory signaling.

We have now generated the GFP and RFP constructs and are in the process of constructing iHSC-1λ cell lines accomplish this Aim.

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

As detailed in last year's report we have not yet found a tunable stiffness ECM substrate to perform large scale proximity biotinylation experiments. As an alternate we performed experiments in confluent vs sub-confluent cells. As will be detailed in the manuscript in preparation, we found significant differences in Merlin proximal proteins in these conditions. However, ASPP2 proximal proteins were not significantly different in sub-confluent vs confluent cells, suggesting that it less responsive to cell density. Significant differences in ASPP2 proximal proteins were seen in Merlin WT and Merlin KO cells (Figure 2, top). Specifically, ASPP2 proximity to N-cadherin complex components, N-cadherin, α-catenin, β-catenin, δ-catenin and Afadin is reduced. As is the PFGF receptor beta and the Hippo pathway effector YAP1 (Figure 2) while the transferrin receptor is increased. These data suggest that merlin functions to locate ASPP2 to these structures, (rather than the other way around). We are designing experiments to follow up on this observation.

Figure 2



Gene	Protein	APEX	Merlin Wild Type	Merlin Knockout	WT vs KO. T-Test p=
TP53BP2	ASPP2	0	102	34	0.001
CDH2	N-cadherin	3	9	2	0.001
CTNNA1	Catenin alpha-1	4	10	0	0.000
CTNNB1	Catenin beta-1	8	39	2	0.000
CTNND1	Catenin delta-1	12	34	8	0.000
AFDN	Afadin	3	12	2	0.005
PDGFRB	PDGF Receptor beta	1	9	0	0.000
YAP1	Transcriptional coactivator YAP1	0	7	0	0.007
TFRC	Transferrin receptor protein 1	2	1	16	0.000

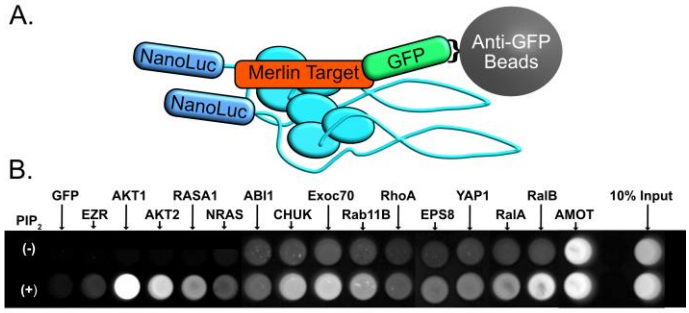
Top. A proximity map of ASPP2 proximal proteins in merlin wild type, (left node) and Merlin knockout cells (right node) showing significant difference in ASPP2 proximal proteins with and without Merlin expression. Merlin proximal proteins are in green
Bottom. A heat map showing changes in the number of peptides mapped from adherens junction (CDH2, CTTNA1, CTTNB1, CTTND1, Afadin), Cell surface receptors (PDGFRb, TFRC) and HIPPO pathway (YAP1). These data suggest that Merlin localized ASPP2 to these structures,

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 control and Merlin knockout iHSC-1λ cells expressing N-cadherin-APEX, β-integrin-APEX, δ-catenin-APEX and Scribble-APEX and have performed proximity biotin experiments on most of them. The remaining experiments are ongoing. We have validated these constructs and have mass spec data for these cells. We are currently analyzing the data we have generated so far. Preliminary analysis suggest that Merlin is necessary to stabilize Integrin β1 and δ-Catenin complexes but not Scribble or N-Cadherin. We are still evaluating candidate binding proteins that may link Merlin to these complexes.

Figure 3. PIP_2 Enhanced Merlin Binding



A. A schematic diagram depicting the Merlin binding assay. Purified Merlin-NL probe is mixed with GFP-fused Merlin target proteins expressed in 293T cells and purified using an anti-GFP nanobody bound to magnetic beads.

B. A composite image of the luminescence from Merlin binding assays performed in the absence (top row) and presence (bottom row) of 200 μM diC8-PIP_2. Left to right: GFP (negative control), Ezrin, AKT1, AKT2, p120 Ras-GAP, N-ras, Abi1, INFk-B kinase (CHUK), Exocyst 70, Rab11B, RhoA, EPS8, YAP1, RalA, RalB, Angiomotin and a 10% input control.

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

Direct binding assays using purified Merlin-NanoLuc probes are now performed the screens in the presence and absence of 200 μM PIP_2. These

experiments identified new sets of direct Merlin binding proteins (Figure 3). These include protein kinases, AKT1, AKT2 and CHUK, small GTPases, RalA, RalB, Rab11a and RhoA and components of the exocyst, including Exoc70, a critical structure responsible for exocytosis (Figure 3). These experiments suggest a regulatory mechanism in which PIP_2 bound, dimeric Merlin binds regulatory kinases and GTPases to regulate exocytic trafficking. To determine the putative targets for Merlin dependent trafficking, we tested for differences in the cell surface proteome of Crispr derived Merlin knockout cells versus controls at confluence, using cell surface biotinylation, affinity purification and mass spectroscopy. We identified several cell surface proteins with higher expression levels on Merlin null cells, relative to controls, at confluence (Table I). These new data suggest the hypothesis that Merlin reregulates the trafficking of a specific set of cell surface proteins by direct interaction with the exocyst components, RalA, RalB, Exoc70 and Rab11. This year we will submit grant application to both the NIH and the DOD to test this hypothesis.

Table I. Candidate Surface Proteins with Impaired Trafficking in Merlin Null Schwann Cells.

Gene	Protein	Scr Control	MerKO	Fold
ICAM1	Intercellular adhesion molecule 1	3	65	21.9
SLC3A2	4F2 cell-surface antigen heavy chain (CD98)	7	120	16.2
IL6ST	Interleukin-6 receptor subunit beta (gp130)	6	43	7.3
KIRREL	Kin of IRRE-like protein 1	3	16	5.3
B4E106	Monocarboxylate transporter 1	15	74	5.0
ITGA6	Integrin alpha-6	3	12	4.0
LGALS1	Galectin-1	31	120	3.9
SLC7A5	Large neutral amino acids transporter small subunit 1	4	14	3.1
CLMP	CXADR-like membrane protein	6	18	3.0
NFASC	Neurofascin	6	18	3.0
SLC39A1	Zinc transporter ZIP10	16	47	2.9

Crispr mediated Merlin knockout and scrambled control Schwann cells were grown to confluence then surface proteins were biotinylated with N- hydroxysuccinimidyl -PEG(12) biotin. After lysis, biotinylated proteins were isolated by streptavidin affinity chromatography and identified by MALDI-TOF mass spectroscopy. Normalized peptide hits are shown with the fold increased in Merlin KO cells.

Impact

We identified a basic biochemical mechanism by which Merlin is activated in response to PIP_2. We have also greatly expanded our understanding of the Merlin interactome and identified novel binding partners that may represent a novel mechanism of action in the regulation of cell surface trafficking via direct interaction between Merlin and components of the exocyst. Following up on this observation we identified cell surface proteins that are overexpressed on the surface of merlin null cells including the amino acid transporter complex SLC3A2, the IL-6 receptor IL6ST and the cell adhesion molecule ICAM2 (Table I). Each of these are potential therapeutic targets that may also be therapeutic targets for Schwannoma. We have tentatively ruled out ASPP2 as a critical binding partner for Merlin function but have identified Merlin as necessary for ASPP2 localization thus identifying Merlin as a critical binding partner for ASPP2 function.

Changes/Problems

The fact that the ASPP2 knockout cells did not phenocopy Merlin loss suggests that the Merlin-ASPP2 interaction is not critical for Merlin function. Proximity biotinylation experiments suggest that Merlin is required for ASPP2 to associate with adherens junctions (Figure 2) and we are designing experiments to test this hypothesis. The lack of an ASPP2 phenotype and the identification of multiple other Merlin binding proteins naturally shifts our focus away the Merlin-ASPP2 interaction.

Our observation that Merlin is activated by PIP_2 required us to change how we perform many of the experiments described in this proposal. Specifically, Specifically Aims 1c, 1d, 2a and 2b use purified Merlin protein in the direct binding assay that we described in Figure 2 of the proposal. The expanded number of potential Merlin interacting proteins identified by the APEX proximity biotinylation experiments and the necessity to evaluate these interactions in the presence and absence of PIP_2 requires a much greater quantity of purified Merlin protein. To address this, we purchased a BioRad NGC Plus Quest 10 chromatography system designed for high resolution separation and isolation of biomolecules. This system allows us to purify Merlin in sufficient quantities to perform the experiments described in the Aims. Furthermore, it seems likely that we will be able to isolate enough Merlin in sufficient quantity and purity to provide our colleague Dr. Kye Stachowski with material for him to perform cryoEM through this association with the Ohio State CryoEM consortium. This is not a stated goal of our proposal but if the opportunity to do this experiment presents itself we intend to pursue it.

Products

None

Participants & Other Collaborating Organizations

None

Special Reporting Requirements

None

Appendices

References