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14. ABSTRACT

Schwannomas are benign peripheral nerve tumors that are initiated by biallelic mutation of the *NF2* tumor suppressor gene, resulting in loss of function of the *NF2* gene product, merlin. Loss of merlin drives tumor formation but there is no consensus on the mechanism of merlin's tumor suppressor function. Merlin has no intrinsic catalytic activity, its function regulated by the proteins with which it interacts. We used proximity biotinylation and direct binding assays to perform a global proteomic analysis that identified 52 merlin-associated proteins. The majority of merlin proximal proteins are components of cell junctional signaling complexes including actin binding proteins and members of the Hippo pathway. This pattern of protein interactions suggests that merlin functions as part of a mechano-sensory signal transduction network. We hypothesize that the loss of merlin destabilizes or impairs assembly of these structures, causing abnormal, cancer causing signals. To test this hypothesis, we will again use proximity biotinylation to look for changes in key cell junction components in the presence or absence of merlin. We expect that the experiments described in this proposal will yield greater insight into consequences of the loss of merlin that may be used to devise therapies to treat this disease.

15. SUBJECT TERMS

Neurofibromatosis Type 2, NF2, Merlin, Cell Junctions

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Table of Contents

Page

Introduction………………………………………………………………………………………….3

Body…………………………………………………………………………………………………...3-6

Key Research Accomplishments………………………………………………………………..5

Reportable Outcomes…………………………………………………………………………6

Conclusion…………………………………………………………………………………… 6

References…………………………………………………………………………………….7-8

Appendices……………………………………………………………………………………. N/A

Introduction

Neurofibromatosis Type 2 is an inherited disease characterized by bilateral schwannomas of the 8th cranial nerve and other tumors of the nervous system, including meningiomas and ependymomas. The tumor suppressor gene responsible for this disorder, NF2, encodes a 70 kDa member of the Ezrin-Radixin-Moesin (ERM) protein called merlin (1, 2). Merlin acts as a context dependent tumor suppressor that controls contact inhibition of growth (3, 4). Merlin has been implicated as a key regulator of multiple cellular functions including signal transduction, ubiquitination and intracellular trafficking (5-7). However, merlin does not possess catalytic activity and the precise mechanism by which merlin mediates these functions is unknown. We used a global proteomic strategy to take a comprehensive census of merlin associated proteins in Schwann cells. This interactome analysis identified merlin as a key component of cell-cell and cell-substrate junctions. We hypothesized that merlin is necessary for the assembly of cell junctions and their signaling complexes. Merlin loss would then result in the activation of oncogenic signal transduction cascades due to aberrant cell junction signaling. Our objective is to test this hypothesis by defining changes in cell junctional complexes and signaling molecules in the presence and absence of merlin.

Body

Specific Aims

Our objective is to test this hypothesis by defining changes in cell junctional complexes and signaling molecules in the presence and absence of merlin. We will carry out the following specific aims.

Aim 1 Test the hypothesis that merlin is necessary for the assembly of normal cell junction signaling complexes.

Rationale: If merlin is necessary for the assembly of cell junction signaling complexes then the composition of these complexes will change upon merlin loss. We will focus on two known cell junction signaling proteins, angiomotin (Amot) and erbin (Erbbip2), that directly bind to merlin and were identified as merlin associated proteins in our proximity biotinylation screen (Fig. 1C) (8-10). Angiomotin localizes to TJs and regulates the Hippo pathway (11, 12) while Erbin localizes to AJs and regulates the Ras-MAPK pathway (13, 14). Both are scaffold proteins that functionally interact with oncogenic signaling proteins (8, 15). We will define the interactome for both angiomotin and erbin in both *Nf2* wild type and CRE infected *Nf2* null Schwann cells at high and low cell densities. Significant changes in the interactomes of these proteins, as defined by proximity biotinylation, will confirm the hypothesis that merlin is required for the assembly of angiomotin and erbin cell junctional signaling complexes.

Aim 2 Test the hypothesis that merlin function is required for Schwann cell junction assembly.

Rationale: If merlin is necessary for the assembly of Schwann cell junctions then the composition of cell junction complexes will change upon merlin loss. To test this, we will generate a set of fusions between $BirA^{R118G}$ and ZO-1 (Tjp1), α-actinin (Actn1) and vinculin (Vcn), proteins that were identified as in our preliminary experiments and are consensus components of TJs, AJs and FAs (Fig. 1C & Fig. 1D). We will use proximity biotinylation to define the composition of these junctional complexes in *Nf2* wild type and null Schwann cells at both high and low cell density. Significant changes will confirm that merlin is required for cell junction assembly.

Aim 3 Identify changes in merlin interactome in the confluent and subconfluent Schwann cells.

Rationale: Merlin is a mediator of contact inhibition of growth; therefore, merlin activity is induced at high cell density relative to low cell density. To identify merlin interactions that correlate with merlin's growth suppressive activity we will perform proximity biotinylation experiments using Merlin-$BirA^{R118G}$ in sparse and dense Schwann cells. Identification of merlin interactions that are specific for high density, growth suppressive conditions will identify specific interacting proteins required for merlin mediated tumor suppression.

As detailed in last year's annual report, we encountered significant difficulties using the PITCh system to generate BirA fusions within endogenous genes. At that time, we could detect the recombination product in a nested PCR assay but were unable to detect any colonies containing the appropriate BirA recombination product. We described several strategies to address this problem.

1. Knock out NF2 in the immortalized human Schwann cell lines iHSC-1λ.
2. Developed a lentiviral expression system to separately evaluate the efficacy of gRNAs.
3. Construct the described BirA fusion proteins in a DOX inducible system.

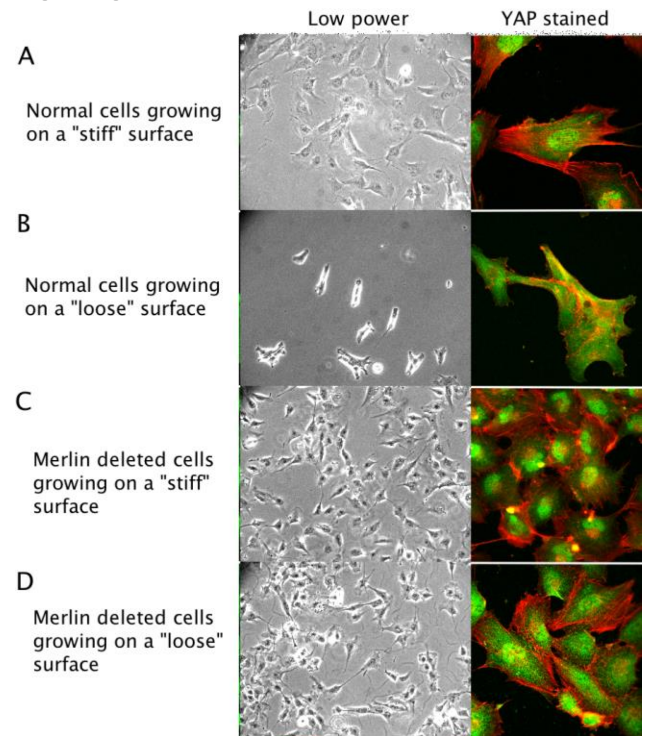
However, we were ultimately unable to use the PITCh system to make BirA fusions with endogenous genes. Since the ultimate objective of this project is to answer questions about NF2 rather than develop technology, once the limitations of the PITCh system became apparent, we explored other techniques to achieve our goals. Specifically, we:

1). Developed a simple assay system to measure the role of merlin in mechanosensing.

2). Construct vectors to express BirA fusion constructs in immortalized human Schwann cell lines.

3). Streamline and the proximity biotinylation/mass spec protocol to enable us to identify specific protein complexes within the overall merlin interactome.

Figure 1 Merlin is Required for Mechanosensory Signaling In Schwann Cells



Normal human and NF2 mutant Schwann cells grown on "rigid" and "flexible" surfaces.
A. iHSC-1λ NF2^{wt} cells on 50 kPa, fibronectin-coated hydrogel, in phase contrast (left) and stained for YAP1 (fright).
B. iHSC-1 λ NF2^{wt} cells on 4 kPa, fibronectin-coated hydrogel.
A. iHSC-1λ NF2^{KO} cells on 50 kPa, fibronectin-coated hydrogel.
B. iHSC-1 λ NF2^{KO} cells on 4 kPa, fibronectin-coated hydrogel.

Mechanosensing Assay

Our proximity biotinylation data suggests that merlin regulates of cell growth in response to mechanical cues, specifically the rigidity, or stiffness, of the surrounding environment. We used the Crispr mediated NF2 knockout cells described in last year's report to test this hypothesis. To determine if Merlin is required for mechanosensory signaling in Schwann cells, we plated immortalized wild type and Merlin knockout human Schwann cells on fibronectin coated polyacrylamide hydrogels with defined elasticity as described by Young's modulus and expressed in kilo Pascals (kPa). Control cells plated on the rigid, 50 kPa substrate had a predominately nuclear YAP localization, consistent with a growth permissive state **(Fig 1A**). Whereas control cells plated on the flexible 4 kPa substrate had a more cytosolic YAP localization **(Fig 1B**) characteristic of a growth suppressive status that is indicative of Hippo pathway activation in response to ECM "stiffness". In contrast, immortalized human Schwan cells with a Crispr mediated knockout of the NF2 gene displayed clear, nuclear localized YAP on either the rigid or flexible substrates **(Fig 1C, D**). This experiment clearly shows that pathway in Schwann cells, Merlin is necessary for mechanosensory activation of the hippo in response to mechanical cues—the elasticity of the ECM microenvironment. This represents a simple assay system to evaluate merlin function in response a specific mechanical que that is more precisely controlled than cell density. We will now perform the proximity biotinylation experiments described in the Aims 1, 2 and 3.

Lentiviral Expression System.

Initially, we had hoped to construct BirA fusions with endogenous genes using the PITCh system, so that they would have normal expression levels to avoid overexpression artifacts. Once we determined that the PITCh system would not work for us, we pivoted to a lentiviral system. We constructed a lentiviral expression system adapted to drive expression from a minimal EF1-a promoter bicistronic with either puromycin or

hygromycin selectable markers (Fig. 2A). In practice, these viral vectors have allowed us to rapidly construct cell lines that express BirA fusion proteins to moderate levels. We are currently using this system in place of PITCh to continue experiments described in Specific Aims that produce a comprehensive interactome of the cell junctional structures that merlin interacts with.

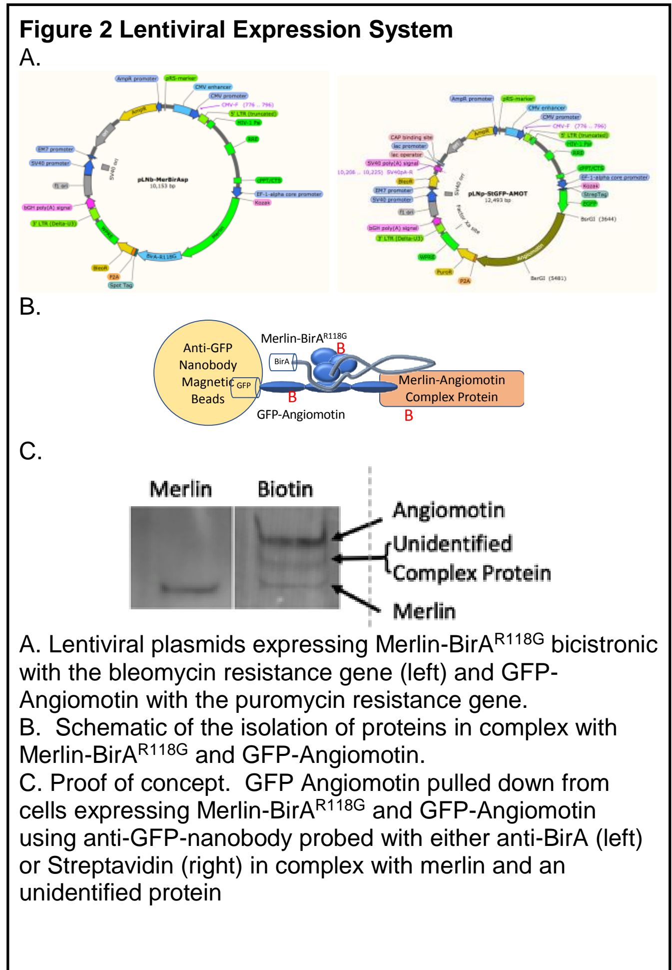


Figure 2 Lentiviral Expression System

A. Lentiviral plasmids expressing Merlin-BirA^{R118G} bicistronic with the bleomycin resistance gene (left) and GFP-Angiomotin with the puromycin resistance gene.
B. Schematic of the isolation of proteins in complex with Merlin-BirA^{R118G} and GFP-Angiomotin.
C. Proof of concept. GFP Angiomotin pulled down from cells expressing Merlin-BirA^{R118G} and GFP-Angiomotin using anti-GFP-nanobody probed with either anti-BirA (left) or Streptavidin (right) in complex with merlin and an unidentified protein

Identification of Subsets of the Merlin Interactome.

One disadvantage of the proximity biotinylation strategy is that the technique does not distinguish between proteins that directly bind to merlin, those that associate with merlin via another molecule or those that are simply in close proximity. As described in last year's report, we designed a rapid, sensitive secondary screen that utilizes nanobody-based pulldown assay and a merlin-luciferase probe and distinguish amongst these possibilities. Furthermore, we realized that this system may be adapted to identify specific subsets of merlin interacting proteins as a way of constructing a more detailed map of the merlin interactome. A proof of concept experiment is shown in Figure 2 B, C. Immortalized Schwann cells were infected such that they co-express Merlin-BirA^{R118G} and a known merlin binding protein fused to GFP. In this example we used angiomotin, one of the target proteins described in Aim 1. Pulldowns using anti-GFP nanobody were probed with streptavidin to identify those proteins that are proximal to merlin and in a complex with angiomotin. As shown in the Fig. C., a biotinylated band in between angiomotin and merlin identifies a protein that is likely to be in a stable tri-molecular complex with merlin and angiomotin. This experimental strategy will be used to supplement the proximity biotinylation data that will be generated in experiments describe in the proposal. Specifically, we are making the BirA fusion constructs described in the Aims 1 and 2; Angiomotin, Erbin, α-actinin, ZO-1 and vinculin, using our new lentiviral expression system. Co-expression of, for example, Erbin-BirA with Merlin-GFP and identification of biotinylated proteins from nanobody precipitated Merlin-GFP complexes will identify subsets of the biotinylated proteins that specifically interact with the Erbin-Merlin complex. This type of experiment will be performed in addition to the proximity biotinlyation experiments described in the original proposal.

Key Research Accomplishments.

- Development of a new biological assay for merlin function focused on the mechanosensory signaling function of cell junctional complexes that will place merlin function in this specific signaling system.
- Construction of new lentiviral reagents to rapidly establish cell lines expressing the BirA fusion proteins described in the specific aims as a replacement for the PITCh system.
- Refinement of the proximity biotinylation procedure to identify specific subsets of protein complexes within the merlin interactomes. These experiments will provide a significantly more detailed understanding of the merlin proteome.
- Implementation of a secondary screen that unambiguously distinguishes between direct merlin binding proteins. This system revealed that merlin binds to cell junctional proteins indirectly via an unknown intermediate. Execution of the experiments described in the proposal will be greatly facilitated by adapting this protocol as a way of fully delineating merlin's relationship to these structures.

Reportable Outcomes

Publications:
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.

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Identification of Novel Therapeutic Targets for NF2 Schwannomas, Cancer Free Kids, $25,000: PI: Robert F. Hennigan

Conclusion

We have made significant progress in developing, testing and implementing improved techniques to facilitate interactome analysis via proximity biotinylation. This includes redesigning the reagents to enable us to directly isolate BirA fusion proteins which will facilitate the identification of proteins directly bound to merlin using newer, nanobody-based affinity reagents that we described in the 2018 progress report. Combining these with the proximity biotinylation techniques we described previously, has enabled us to perform proximity biotinlyation and interaction analysis in a more streamlined and cost-efficient manner that yields quality data about dynamic subsets of merlin interactions as it functions as a tumor suppressor.

This is somewhat of a consolation prize since the PITCh protocol for generating endogenous BirA fusion proteins proved to be impractical and we therefore abandoned this strategy as soon as this became clear. Several of the several steps we took to address the problems we were facing with the PITCh protocol, including adopting the iHSC-1λ cells system and changing to a more efficient lentiviral delivery system, have proved to be essential to the continuing success of this project. We are using these new techniques to perform the experiments on angiomotin and erbin described Aim 1, the cell junction experiments described in Aim 2 and the cell density experiments described in Aim 3. We feel that the justification for performing these experiments is still valid and anticipate that out next set of publications will use data generated from these experiments.

In last year's progress report described a simple, powerful secondary screen that successfully addresses a significant weakness of the BirA proximity biotinylation strategy, distinguishing between direct and indirect interactions. This allowed us to generate the data needed to publish the merlin proximity in a relatively high impact journal, Science Signaling, to prepare a separate publication focusing on the role of merlin dimerization that we stumbled upon using these techniques, to write a small drug study grant that was funded and to submit a new independent investigator award to the DOD.

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