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Identification of Novel Signaling Pathways in NF2

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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 its function.

Neurofibromatosis Type 2, NF2, Merlin, Cell Junctions
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 BirAR118G 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-BirAR118G 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.
Aims 1 and 2

Crispr PITCh

We have made significant progress in constructing the plasmids required to insert BirA<sub>R118G</sub> in frame C-terminal to the proteins described in the proposal, angiomotin, erbin, ZO1, α-actinin and vinculin. We have sequenced the first and last exons for these genes immortalized Nf2<sub>flo</sub> mouse cells. We have constructed and verified by sequencing the pCRIS-PITCh-BirAHygro C-terminal insertion plasmid and the pX330A-Empty-PITCh gene targeting plasmid. We have deferred construction of the pCRIS-PITCh-HygroBirA N-terminal insertion plasmid for reasons that will shortly become clear. We have also constructed and verified by sequencing the gene targeting and C-terminal insertion plasmids for angiomotin using three different gRNA target sites in the last exon are designed to fused BirA<sub>R118G</sub> C-terminal to endogenous angiomotin. We then transfected immortalized Nf2<sub>flo</sub> mouse cells with the either the angiomotin directed plasmids or empty vector controls. The efficiency of gRNAs was evaluated in transient transfections in three ways.

1. PCR amplification of the last exon of the cells followed my T7 mismatch repair assay.
2. PCR amplification of the last exon of the angiomotin targeted and control cells followed by TIDE analysis to detect indels in the sequence trace (16).
3. Nested PCR amplifying the angiomotin last exon in then using primers specific for the BirA-N-terminus and the angiomotin C-terminus designed to detect the recombination product, followed by sequencing of any amplified band to confirm in-frame insertion

The results of these experiments were mixed. We were never able to observe evidence mismatches any of the gRNAs we designed by either T7 assay or TIDE analysis. Sequencing confirmed that recombination resulted in in-frame fusion (Figure 1c). We performed positive control experiments using the original PITCh reagents targeting the FBL gene transfected in human 293T cells. Previously, we had demonstrated that these reagents result in generation of the appropriate endogenous FBL-GFP fusion protein, validating the protocol and the efficiency of the gene specific gRNA. However, we were unable show that this proven gRNA worked in transient transfections, similar to our experience with angiomotin. Since these assays work well when used to evaluate gRNA efficacy in CRISPR mediated knockouts using lentivirus (described below), we therefore reasoned that the T7 mismatch and TIDE analysis were insufficiently sensitive to be used in transient transfection.

Despite our inability to confirm gRNA efficacy, we were able to PCR amplify the angiomotin-BirA recombination product (Figure 1B). Since we could detect the recombination product in a nested PCR assay (Figure 1c) we decided to place the Angiomotin-C-term transfected cells in selection with hygromycin. Within three weeks we selected 24 colonies of hygro resistant cells. However, we were unable to detect any colonies containing the angiomotin-BirA recombination product. We attempted this selection procedure for several other
angiomotin directed gRNAs and also for the PITCH system targeting several other genes, including Nf2 itself. In each case, the appropriate recombination product can be detected by nested PCR, but stable recombinant clones were not found. These data indicate that recombination via CRISPR mediated MMEJ occurs, but that selection fails, suggesting a systemic problem with the PITCH system. The Crispr PITCH system requires antibiotic selection using either puromycin or hygromycin resistance genes expressed downstream of a T2A "self-cleaving" peptide sequence in the fusion protein (Figure 1). The experiments described in this proposal were designed to be performed in an immortalized mouse cell line that has a floxed Nf2 allele. We had known the concentration of puromycin and hygromycin required for selection in these cells was quite high relative to other cell lines in the lab (5 µg/ml vs 0.5 µg/ml puro and 500 µg/ml hygro vs 50 µg/ml)). We suspect that a high concentration of antibiotics requires high levels of the relevant resistance genes for successful selection. Since this system expresses the resistance marker under control of the promoter for the knock in gene, the levels of this protein may be too low to select for the desired recombination event, contributing to our inability to select for knock out clones. The hygromycin resistant colonies we identified were the result of random integration resulting in high enough hygro resistance gene expression for colony formation.

To address this problem, we are investigating several possible solutions.

1. We will change cell system to cells that are more sensitive to puromycin and hygromycin in the hope that lower levels of the hygro and puro resistance genes will enable practical selection.
2. We will use a lentiviral gRNA-Cas9 system (pLentiCRISPRv2puro and pLentiCRISPRv2hygro) to separately evaluate the efficacy of gRNAs in populations where 100% of the cells are expressing the gRNA of interest and the BirA Hygromycin replacement vector.
3. Construct the described BirA fusion proteins in established Dox inducible system (17) as described ibn the pitfalls section of the proposal.

NF2 Null Human Schwann Cell Line

We decided to switch to a different cell system consisting of a human Schwann cell line immortalized with telomerase and CDK4 (18) called iHSC-1λ. These cells are that is sensitive to puro at 0.5 µg/ml and hygro at 50 µg/ml. We generated merlin null cell lines via

![Figure 2 Merlin Null Immortalized Human Schwann Cells (iHSC)]
Crispr using lentivirus expressing Cas9 and gRNAs targeted to sites in the first or second exon of the human NF2 gene. Three gRNA sites were designed using CCTop software and the efficiency of each gRNA was evaluated using T7 endonuclease mismatch repair assays and TIDE sequencing analysis exactly as described in our proposal (Figure 2 a-c). After selection with puromycin (at 0.5 µg/ml) pools of clones (iHSC-1λ-T2, T5 and T9) were lysed and probed for merlin expression. Merlin proteins expression was lost in all three tested gRNAs (Figure 2d). Growth curves demonstrated that the merlin null cell lines had faster growth rates and reached higher densities than the parental iHSC-1λ or control lenti infected cells expressing a scrambled gRNA (iHSC-1λ-SCN), showing that they expressed the merlin null phenotype.

This experiment generated another immortalized merlin deficient Schwann cell in which perform the PITCh assay described in our proposal that is much less resistant to hygromycin. Furthermore, these experiments demonstrate that lentiviral infection is a more efficient way to deliver PITCh reagent of Schwann cells than transient transfection. We are investigating the possibility of adapting the BirA-T2A-Hygro replacement cassette for lentiviral delivery, possibly using integration deficient lentivirus, to increase the probability of selecting for rare recombination events.

**Direct vs Indirect Merlin Binding**

The overall objective of this proposal essentially leverages our experience with BirA<sup>R118G</sup> based proximity biotinylation combined with Crispr mediated knock in experiments to produce a comprehensive interactome of the cell junctional structures that merlin interacts with. However, proximity biotinylation 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. We therefore designed a rapid, sensitive secondary screen that utilizes nanobody-based pulldown assay and a merlin-luciferase probes and distinguish amongst these possibilities (Fig. 3a). We selected a representative set of 10 merlin proximal proteins, including two known merlin binding proteins, angiomotin and Lats1, to further evaluate. The other proteins are α-actinin, afadin, angiomotin, ASPP2, Band 4.1B, Lats1, Myosin IIB, Scribble, Vinculin and ZO-1. The positive control angiomotin had the highest merlin-NanoLuc associated activity. ASPP2 and Lats1 had moderate levels, 30 and 18-fold over control, respectively (Figure 3b). The remaining proteins, afadin, Band 4.1B, Myosin IIB, Scribble, and ZO-1 all had, exceeding the threshold of two-fold over the RFP negative control. In direct interaction assays merlin directly binds to 3 of the proteins we tested, angiomotin, ASPP2 and Lats-1. The dotted line represents the basal luciferase activity in the RFP negative control.

**Figure 3 Merlin Binding Screen**

A. A schematic representation of the merlin interaction screen using a merlin-luciferase reporter construct and RFP- or GFP-based binding partners immunoprecipitated with anti-RFP or anti-GFP nanobodies.
B. Co-transfection experiments for 10 RFP-fused merlin proximal proteins. The dotted line represents the basal luciferase activity in the RFP negative control.
C. Direct interaction with merlin confirms direct, high-affinity interactions with interaction with angiomotin, ASPP2 and Lats 1. The dotted line represents the basal luciferase activity in the RFP negative control.
**Key Research Accomplishments.**
- Construction of PITCh reagents for angiomotin, NF2
- Initial evaluation of PITCh procedure.
- Generation of alternative cell line to solve the selection problems encountered in our initial PITCh experiments.
- Generation of conventional Dox-inducible BirA fusion constructs as a back-up strategy.
- Development 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**
None

**Conclusion**

We have made significant progress in testing the practicality of the PITCh protocol, a method that remains critical for the efficient generation of multiplex proximity biotinylation experiments that is necessary for a full understanding of the role that merlin plays in cell junctional signaling. We have identified a significant roadblock to achieving this goal in our inability to identify stable recombinant clones containing in frame gene of interest-BirA fusions, despite evidence that the recombination event occurs as predicted. We have outlined several steps we hope to ameliorate this problem, including adopting a more antibiotic sensitive cell background and changing to a more efficient lentiviral delivery system. We are currently in the process of evaluating these steps. We also developed a simple, powerful secondary screen that successfully addresses a significant weakness of the BirA proximity biotinylation strategy, distinguishing between direct and indirect interactions. We are confident that we will quickly determine if the PITCh system is viable or aggressively move to the backup strategy involving the generation of Dox-inducible cell lines as described in the original proposal.
References


