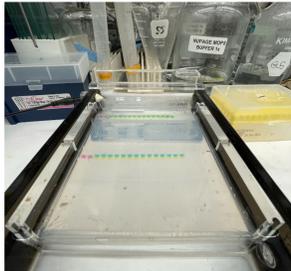


DEVELOPING A PLATFORM TO STUDY THE PATHOGENIC MECHANISMS OF NF1 MISSENSE MUTATIONS



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Neurofibromatosis type 1 (NF1) is an autosomal dominant condition. One of the most serious manifestations of NF1 is the growth of tumors on the nerve sheath. To date, over 3,000 pathogenic mutations have been identified in the NF1 gene, which encodes the protein neurofibromin. Missense mutations (MMs) account for ~38% of these pathogenic mutations and result in an amino acid substitution as a result of a single base pair mutation. The pathogenic mechanisms for many MMs have not yet been determined. We hypothesize that MMs disrupt neurofibromin's protein-protein interactions. To test that, prime editing was used to add a FLAG tag to N-term of NF1 in immortalized human Schwann cells (hSCs). Created FLAG-NF1 hSC will allow the use of immunoprecipitation (IP) and mass spectrometry (MS) to identify MM-dysregulated neurofibromin protein interactors when MMs are modeled. To confirm the successful integration of the FLAG tag into NF1 hSC lines, the potential FLAG-NF1 hSCs were screened via polymerase chain reaction (PCR) for the flag tag insert and then sent for sequencing. Positively identified clones were analyzed via T-vector cloning. Further, sequencing identified NF1 FLAG-tagged homozygous and heterozygous cell lines. Lastly, FLAG-antibody immunoblotting did not detect FLAG-tagged neurofibromin, indicating an inability to use FLAG-IP/MS. Next steps will use different FLAG-antibodies, as well as testing other protein tags. Overall, this project will develop a platform to further study pathogenic NF1 MMs.

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Abstract: Neurofibromatosis type 1 (NF1) is an autosomal dominant condition. One of the most serious manifestations of NF1 is the growth of tumors on the nerve sheath. To date, over 3,000 pathogenic mutations have been identified in the NF1 gene, which encodes the protein neurofibromin. Missense mutations (MMs) account for ~38% of these pathogenic mutations and result in an amino acid substitution as a result of a single base pair mutation. The pathogenic mechanisms for many MMs have not yet been determined. We hypothesize that MMs disrupt neurofibromin's protein-protein interactions. To test that, prime editing was used to add a FLAG tag to N-term of NF1 in immortalized human Schwann cells (hSCs). Created FLAG-NF1 hSC will allow the use of immunoprecipitation (IP) and mass spectrometry (MS) to identify MM-dysregulated neurofibromin protein interactors when MMs are modeled. To confirm the successful integration of the FLAG tag into NF1 hSC lines, the potential FLAG-NF1 hSCs were screened via polymerase chain reaction (PCR) for the flag tag insert and then sent for sequencing. Positively identified clones were analyzed via T-vector cloning. Further, sequencing identified NF1 FLAG-tagged homozygous and heterozygous cell lines. Lastly, FLAG-antibody immunoblotting did not detect FLAG-tagged neurofibromin, indicating an inability to use FLAG-IP/MS. Next steps will use different FLAG-antibodies, as well as testing other protein tags. Overall, this project will develop a platform to further study pathogenic NF1 MMs.

Background and Objectives

Figure 1. NF1 is an autosomal-dominant genetic disorder affecting 1 in ~3,500 newborns with a wide range of clinical manifestations. Despite a variety of clinical trials, Selumetinib, the MEK1/2 inhibitor, is the only FDA-approved therapy for treating NF-associated tumors, demonstrating an unmet need for novel NF1 therapies.

Figure 2. Approximately 38% of NF1 pathogenic mutations are missense mutations. Due to the lack of hotspots and the large size of neurofibromin, it becomes difficult to hypothesize about the pathogenic mechanisms of NF1.

Hypothesis: We speculate that pathogenic NF1 missense mutations affect neurofibromin's interaction with protein partners.

Goal: To develop FLAG-tagged platform to study NF1 pathogenic missense mutations.

Methods

Figure 3. Prime editing is a novel, CRISPR-based gene editing technology that permits gene editing without double-strand breaks. Prime editing uses prime editing guide RNA (pegRNA), reverse transcriptase, and Cas-9 nickase to edit genes.

Figure 4. Using prime editing, we developed a pipeline to insert a FLAG tag into immortalized human Schwann cells (hSCs). We transfect a FLAG pegRNA and the prime editing machinery using polyethylenimine (PEI). Then, neurons were added to select positively transfected cells. Further, we performed a FLAG-screen PCR in the population of prime edited cells. After clone picking, individual clones were sequenced and expanded for Western immunoblotting. Created with BioRender.com.

Results

Figure 5. Sanger sequence of homozygous (H2) and heterozygous (Het) FLAG-NF1 hSCs demonstrates seamless FLAG integration. Sanger sequencing of H2 FLAG-NF1 hSC line demonstrated FLAG tagging in the N-term of neurofibromin. After TA cloning and sanger sequencing of each allele, the Het FLAG-NF1 hSC demonstrated the presence of both an NF1 FLAG-tagged and WT allele. This can be seen in the alignment of the N-term region of neurofibromin.

Figure 6. hSC line SCC FLAG-NF1 H2 and Het display normal neurofibromin levels but no FLAG tag is not detected in immunoblotting. This indicated an inability to pull-down and use for further analyses.

Figure 7. Using endogenously-tagged hSC lines, we will screen pathogenic MMs that affect neurofibromin protein-protein interactors. Looking forward, we will use different FLAG-antibodies and repeat FLAG-antibody. Additionally, we will tag NF1 with different protein tags, such as HA tag, 3xFLAG, and o-Myc tags. Once an hSC FLAG-tagged cell line is established, we will model MMs and determine their effect on neurofibromin protein interactions via mass spectrometry. Overall, this project aims to develop a platform to further study pathogenic NF1 MMs.

References

Acknowledgments