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MODELING NEUROFIBROMATOSIS TYPE II IN ZEBRAFISH

A Thesis Presented to the Faculty of the University Honors Program
Northeastern Illinois University

In Partial Fulfillment of the Requirements
of the NEIU Honors Program
for Graduation with Honors

Hannah Nuszen
December 2020

HONORS SENIOR PROJECT

ACCEPTANCE AND APPROVAL FORM

Hannah Nuszen

Modeling Neurofibromatosis Type II in Zebrafish

This thesis has been reviewed by the faculty of the NEIU Honors Program and is found to be in good order in content, style, and mechanical accuracy. It is accepted in partial fulfillment of the requirements of the NEIU Honors Program and graduation with honors.

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In loving memory of my grandfather, Henry Gutman. A man of few words, many songs, and a great deal of wisdom.

You must know what you know and know what you don't know.

ABSTRACT

Neurofibromatosis Type 2 (NF2) is a rare genetic disorder, classified as a neurocutaneous syndrome inherited through an autosomal dominant genetic mutation or deletion of the *NF2* gene in humans. The tumor suppressor gene NF2 encodes the cytoskeletal protein MERLIN to promote apoptosis and inhibit abnormal cellular proliferation. Mutations in *NF2* lead to a deficiency of functional MERLIN in humans and can result in hearing loss, disability, and premature death. These symptoms are due to uncontrollable growth of schwann cells generating bilateral benign tumors (schwannomas) within the nerve sheath of cranial nerve VIII. Here we seek to understand the function of MERLIN by studying homologous proteins in zebrafish

(*D. rerio*). To begin our analysis of MERLIN function, we determined that NF2 has been duplicated in the zebrafish genome to yield two paralogs, *NF2a* and *NF2b*. Compared to human MERLIN, the proteins encoded by NF2a and NF2b have retained 79% and 72% amino acid identity, respectively, including high conservation of the signaling "blue box" motif. Our data also shows that *NF2a* and *NF2b* are expressed at different times during embryonic development, suggesting non-redundant functions. Lastly, we used a catalytically inactive variant of Cas9 to knock down transcription of *nf2a* and/or *nf2b* in zebrafish. Following knockdown, zebrafish displayed evidence of cellular over proliferation in hematopoietic cells. Using immunofluorescence, we also observed fewer apoptotic cells in the brain of knockdown zebrafish when compared to wildtype siblings. Thus, our data suggests that *nf2a* and *nf2b* promotes cell survival in the brain and the blood island.

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INTRODUCTION

Background

Neurofibromatosis commonly refers to three genetically dominant disorders. These three disorders are neurofibromatosis 1 (NF1), neurofibromatosis 2 (NF2), and schwannomatosis. All three present in the clinic as benign peripheral nerve sheath tumors (PNSTs). While PNSTs are relatively uncommon, making up just 8.6% of all primary brain and central nervous system tumors reported in 2011–2015, the tumors that arise from these disorders can result in loss of nerve function and/or neuropathic pain (Brandt et al., 2019). While the specific genetic variants causing these disorders are well defined, the molecular mechanisms in which they drive tumorigenesis remain unclear and represent a key area of research.

Of the three dominant disorders, NF2 is relatively rare (1 in 33,000) and has a known genetic component caused by mutations in the *NF2* gene which encodes the protein MERLIN. The molecular function of MERLIN and its contribution to tumorigenesis is not completely understood. Previous studies have shown MERLIN is a membrane-cytoskeleton scaffolding protein, linking actin filaments to the cell membrane (Liu, 2020). In this way, MERLIN acts as a tumor suppressor protein and plays a pivotal role in several other cancers by regulating cell proliferation and cell migration (Morrow & Shevde, 2012).

Clinical Description

Neurofibromatosis type 2 (NF2) is characterized by the development of schwannomas on the vestibular branch of both eighth cranial nerves (Blakeley et al., 2012). Most NF2 patients develop bilateral vestibular schwannomas that are associated with other cranial nerves and spinal nerve roots, while some have also attained cranial and spinal meningiomas and, less frequently, intraspinal ependymomas. Although NF2 causes slow growing tumors that are benign, the disorder diminishes a patient's quality of life through effecting hearing, mobility, balance, and possibly causing facial disfigurement. Depending on the location and quantity of these noncancerous tumors, NF2 can also lead to early mortality with the average age of death in people diagnosed with NF2 being 36 years (Blakeley et al., 2012).

Epidemiology

The first case of NF2 was discovered by Wishart in 1822 in which he provided the first clear description of the disorder of Michael Blair, age 22, that suffered from tumors of the dura matter, brain, and auditory nerves (Ruggieri et al., 2016). The prevalence of NF1 is known to affect 1 in every 3,000 people, whereas NF2 affects 1 in 33,000 people worldwide (Nation Institute of Medicine, 2020). Earlier diagnosis and better survival through improved medical treatments has caused NF2 prevalence to rise through the established clinical diagnostic criteria as seen in Table 1 (Evans, 2009).

Table 1: Diagnostic criteria for NF2 based on the National Institute of Health (NIH) criteria. NF2 can be diagnosed when a pathogenic mutation in the NF2 gene is identified or when the following criteria are fulfilled.[1]

Main Criteria	**Additional Criteria**
Bilateral vestibular schwannomas (VS) or family history of NF2 *plus*	Unilateral VS *plus* any two of the following: meningioma, glioma, neurofibroma, schwannoma, posterior subcapsular lenticular opacities
1) Unilateral VS or	or
2) Any two of the following: meningioma, glioma, neurofibroma, schwannoma, posterior subcapsular lenticular opacities	Multiple meningioma (two or more) *plus* unilateral VS or any two of the following: glioma, neurofibroma, schwannoma, and cataract

Etiopathogenesis

The NF2 gene is made up of 17 coding exons that translate to the 595 amino acid cell membrane-related protein MERLIN. Tumors that arise from the NF2 gene are caused by the loss of MERLIN in Schwann and meningeal cells. Schwann cells are derived from the neural crest and produce the myelin sheath around neuronal axons within the peripheral nervous system (PNS). Schwann cells are responsible for maintaining and regenerating motor and sensory neurons of the PNS in addition to the cells mainly being required for myelinating and supplying nutrients to individual axons of the PNS neurons (Manoukian et al., 2020). The genome of NF2 patients have shown a wide range of mutations in NF2

[1] Table 1is referred from Neurofibromatosis type 2 (Nf2): A clinical and molecular review (Evans, 2012)

frameshift, missense, or splice-site mutations (Evans, 2009). The phenotypes arising from these genetic mutations are dependent on how the mutation occurred and whether the protein product retains some functionality, gains a new function, or is completely missing from the cell. Frameshift mutations are most common and are found to lead to higher risk of mortality and more severe cases than other mutations (Evans, 2009).

NF2 is caused by a mutation on Chromosome 22 that in approximately half of cases have been due to inheriting an abnormal NF2 allele from a parent. Two mechanisms exist in which a patient who is heterozygous for an abnormal NF2 allele can develop NF2. Firstly, the normal allele may be lost through a loss of heterozygosity (LOH) event later in development. Second, a *de novo* mutation of NF2 may occur and take place after fertilization, resulting in a mosaic expression of two cell lines. Somatic mutations, by definition, cannot be passed on to offspring. However, somatic mosaicism may prevent the molecular diagnosis from being established unless tumor tissue is analyzed.

The occurrence of autosomal dominant inheritance of a gain of function NF2 allele has been found to have an earlier onset age (~18 yrs.) through the maternal gene being affected while a later onset age (~24 yrs.) through paternal gene being affected (Evans 1998). The expression of mutant NF2 alleles in such cases have been found to be controlled by endogenous regulatory sequences resulting in a loss of function rather than a dominant oncogenic effect (McClatchey & Giovannini, 2005).

Pathology

Schwannomas are benign nerve sheath tumors composed of Schwann cells that grow around the peripheral nervous system. In NF2, Schwannomas are usually slow growing and have a predisposition for the superior vestibular branch of the VIII cranial nerve (Muranen, 2005). Schwannomas are identified as areas of spindle-shaped cells with intertwining fascicles that may contain blood vessels (Fig. 1a). Schwannomas are differentiated from sporadic tumors by having a more lobular structure that could only be sporadically transformed to malignant peripheral nerve sheath tumors after radiation treatment (McClatchey & Giovannini, 2005).

Another common tumor found in NF2 is meningiomas, which forms on membranes that cover the spinal cords and brain (Fig. 1b). These tumors originate in the meninges from arachnoid cap cells and are often slow growing and benign. Meningiomas that are less than 2 cm in diameter are amongst the more conventional intracranial tumors. Meningiomas compress brain parenchyma and can invade and distort adjacent bones, which can lead to subtle symptoms that are easily mistaken for other health conditions (McClatchey & Giovannini, 2005).

Neurofibroma, ependymomas, and gliomas are additional types of benign tumors caused by the condition of NF2. Neurofibroma is a small proportion of a nerve related tumor which is composed of schwann cells, fibroblasts, and mast cells. The tumor mainly occurs in the skin which causes identifiable soft bumps. Neurofibromas often press against nerves, as well as grows within them. Ependymomas (Fig. 1c) and gliomas (Fig. 1d) are tumors that originate in the central nervous system and rarely metastasize. The primary location for these tumors is in the cervical spine and brainstem but may spread

throughout the cerebrospinal fluid to other parts of the body. Although neurofibroma, ependymomas, and gliomas are noncancerous in NF2, malignant progression may still occur (Gronholm et al., 2005).

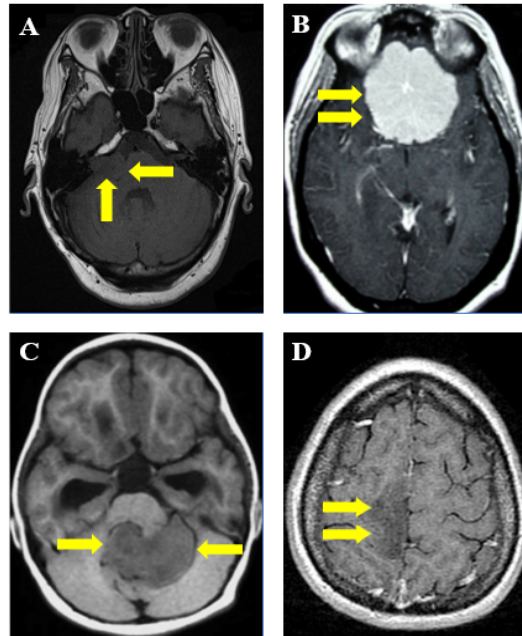

Figure 1: **MRI images of tumor characterization**. T1 weighted MRI without contrast characterizing (a) schwannoma on the right cerebellopontine angle, arising from the vestibular nerve branch of the eighth cranial nerve,[2] (b) axial sequence of large midline frontal meningioma with a spoke-wheel pattern,[3] (c) ependymoma located in the fourth ventricle,[4] (d) post contrast-enhanced T1-weighted axial of low-grade glioma.[5]

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[2] Figure 1a is referred from Department of Radiology of RIPAS Hospital (Bickle, 2012)

[3] Figure 1b is referred from MedScape (Islam et al., 2016)

[4] Figure 1c is referred from MedScape (Bruce et al., 2018)

[5] Figure 1d is referred from Medscape (Jain, 2012)

Contact Inhibition and Cell Adhesion

Normal cells have the ability to survive and proliferate by the direction of environmental signals through intercellular and matrix adhesions. Normal cells undergo growth arrest when in contact with adjacent cells and form intercellular junctions or when they are detached from the extracellular matrix (McClatchey & Yap, 2012). This process is known as contact inhibition which is a regulatory mechanism that functions to keep cells growing into a one cell thick layer. If a cell has plenty of available substrate, it replicates rapidly and moves freely. This process continues until the cells occupy the entire space. At this point, normal cells will stop replicating. In contrast, neoplastic cells evade contact inhibition of proliferation which leads to a disruption of tissue organization that is a defined event in cancer (Cooper, 2000). Cancer can proliferate and avoid contact inhibition by cancer cells inhibiting apoptosis within the cell cycle and downregulating mitogen signaling pathways, regardless of external factors.

Reports have been made that Merlin can control contact-dependent inhibition of proliferation and that a key consequence of Merlin deficiency is a failure of the cell to undergo contact-dependent inhibition of proliferation (Muranen et al., 2005). Merlin have been found to regulate contact-dependent inhibition of proliferation through interaction with the hyaluronic acid receptor CD44 in some cell types (Cooper & Giancotti, 2014). Due to the basis of contact-dependent inhibition of proliferation being poorly understood, the study of Merlin may provide novel insight to better establish that defective cell communication can contribute to both tumor initiation and metastasis (Evans, 2009). By doing so, this would provide an explanation for the tumorigenic and metastatic consequences of Nf2 deficiency in humans.

Molecular Pathways

The *NF2* tumor suppressor gene encodes the FERM (**4**.1 protein/**E**zrin/**R**adixin/**M**oesin) domain protein Merlin. Merlin is composed of a globular amino-terminal FERM domain followed by an α-helical coiled-coil domain and a carboxy-terminal domain (Cooper & Giancotti, 2014). The protein Merlin interacts with a number of proteins that are localized at or underneath the plasma membrane such as with ERMs and the intracellular domain of CD44. In addition, Merlin interacts with membrane-associated proteins and regulates the formation of membrane domains. Merlin's organization of intercellular contacts is essential to normal function in contact inhibition whereas in Merlin-deficient tissue, Merlin's organization is disrupted as seen with Neurofibromatosis Type 2. The importance of Merlin's organization is supported in *Drosophila* by the observation that nascent Merlin is recruited to the plasma membrane (LaJeunesse et al., 1998). NF2 mutations may be caused by a missense mutation that is thought to occur and be distributed throughout the FERM domain and C-terminal tail of the MERLIN protein (Pearson et al., 2000). Many questions remain about the precise mechanism of Merlin and whether Merlin executes multiple independent functions or controls a specific combination of pathways. By answering these key questions, this will allow a better understanding of how critical Merlin is in tumor suppression.

ERM Proteins

The ERM proteins (**E**zrin/**R**adixin/**M**oesin) and the protein Merlin of the NF2 gene are members of the band 4.1 superfamily and are both FERM-containing proteins (Michie et al., 2019). Although evidence indicates ERM and Merlin are closely related, their functions differ in that Merlin regulates contact inhibition and is an integral part of

cell-cell junctions, while ERM proteins assist in the formation and maintenance of specialized plasma membrane vesicle structures (Michie et al., 2019). However, Merlin and the ERM proteins do share similar functions such as linking membrane proteins with the cortical cytoskeleton and both being involved in numerous signaling pathways with highly important functions in the PNS and CNS (McClatchey & Fehon, 2009). In addition, these proteins also share nearly identical tertiary structures, but differentiate in how Merlin and ERM interacts with other proteins and how they mediate control over pathways (Michie et al., 2019). The regulation of Merlin and ERM proteins also differ through ERM proteins being negatively regulated by an intramolecular interaction between their N and C termini, while Merlin is conformationally regulated (Ramesh 2004). Merlin continues to show differentiation from the ERMs by Merlin going beyond the linking membranes to the actin cytoskeleton and assembling protein complexes at the membrane. Merlin also functions at the membrane and in the nucleus while ERM are mainly restricted to the membranes and the cytoplasm (McClatchey & Fehon, 2009). What is most interesting about Merlin and this protein's relation to ERM proteins is that although Merlin is lacking in tumors of NF2 patients, ERM proteins are still expressed (Ramesh, 2004).

Actin remodeling

A critical aspect of Merlin function is the proteins association with the actin cytoskeleton. Although Merlin is similar to ERMs, Merlin localizes to specific regions of cortical actin remodeling, Merlin does not have an actin-binding domain at the C terminus, but instead may bind actin directly via an N-terminal domain. However, Merlin’s interaction with the actin cytoskeleton may occur indirectly through the

cytoskeletal proteins βII spectrin, paxillin or even the ERM proteins themselves (Evans 2009). Loss of Merlin function has resulted in changes in the morphology of the actin cytoskeleton similarly to the loss of ERM function leading to dramatic alterations in the cortical actin cytoskeleton. Although the altered RhoGTPase activity could be an indirect consequence of these phenotypes, it has been suggested that Merlin may also directly control actin cytoskeleton remodeling (Kros, 2001). Furthermore, it has been reported that Merlin and the ERMs can directly interact with and inhibit the function of N-WASP that normally controls activation of the actin nucleator Arp2/3 which nucleates actin subunits and drives actin filament branching in specialized cortical domains.

Rac-PAK and mTORC1 Signaling

Through Merlin's role in binding F-actin and stabilizing actin filaments, it has been speculated that the protein suppresses mitogenic signaling at the cell cortex to mediate contact inhibition. Active Merlin is known to suppress Rac-PAK signaling and restrain activation of the mTORC1 pathway. The p21-activated kinases (PAKs) acts as a modifier of the cell surface and intracellular signaling cascades that function both in the

cytoplasm and the nucleus (Brandt et al., 2019). Due to the loss of Merlin, aberrant PAK1 activity has been found in NF2 patients that lead to elevated levels of Rac, which is a GTP-binding protein that is required for normal cell proliferation and migration. Studies have suggested that PAK plays a key role in regulating Schwann cells due to loss of PAK activity in NF2 tumors not reducing two signaling proteins, Erk and Akt, in which were initially thought to mediate PAK function (Ye &Field 2012).

It has also been reported that the loss of Merlin activates mammalian target of rapamycin complex 1 (mTORC1) signaling pathway independently of Akt and Erk signaling in tumor cells (James et al., 2009). NF2 has been identified as a novel negative regulator responsible for the deregulation of mTORC1, which causes abnormal growth and proliferation of NF2-linked tumors. Although it is known that NF2 interacts directly with PAK1 and mTORC1, the molecular mechanism connecting the loss of merlin to mTORC1 activation, as well as whether PAK is inhibited by Merlin or Merlin is inhibited by PAK continues to remain unclear. Figure 2 illustrates the complexities of the intracellular pathway regulated by Merlin.

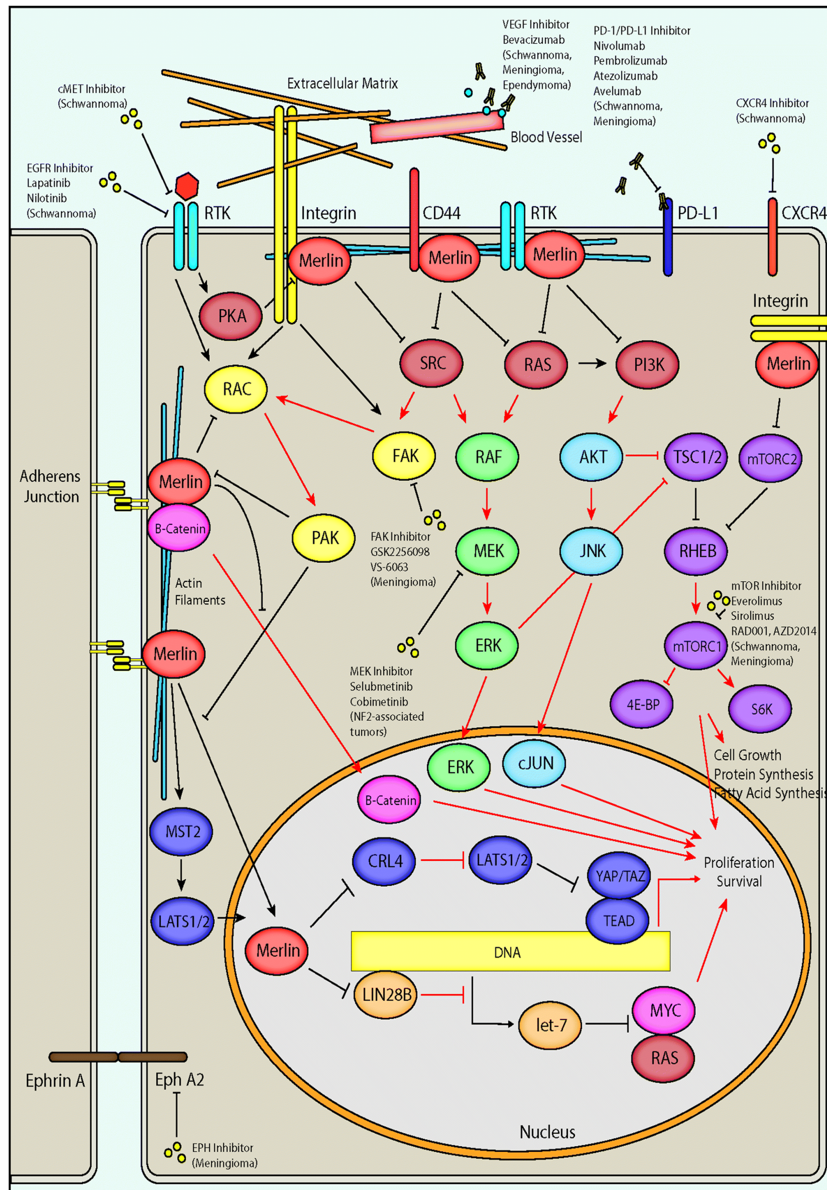

Figure 2: Schematic representation of NF2/Merlin. Depiction of major Intracellular pathways regulated by the protein product of the NF2 gene, Merlin. Growth-Factor and Membrane-Receptor Signaling. [6]

[6] Figure 2 is referred from An Update on the CNS Manifestations of Neurofibromatosis Type 2 (Coy et al., 2020)

Research suggests that Merlin may regulate receptor tyrosine kinase activity and perhaps trafficking. Merlin has also been reported to interact with several proteins with established roles in growth-factor receptor signals, in which Merlin can form a ternary complex with protein dubbed Magicin and Grb2, an adaptor that coordinates receptor tyrosine kinase and Ras signaling, and with EBP50/NHE-RF1, which can lead to the interaction with Erbin. Merlin has been also speculated to inhibit EGFR internalization and signaling upon cell to cell contact (Evans, 2009). Merlin's role in later stages of endocytosis suggests the proteins ability to interact with Hepatocyte Growth-Factor Receptor Substrate (HRS) that controls lysosomal trafficking of membrane receptors. The localization of Merlin to vesicular structures has been reported to be localized to lipid rafts, which further supports a role in membrane receptor trafficking. The study of Merlin could, therefore, provide a novel insight into growth-factor receptor signaling and trafficking in normal cells, as well as further understanding of growth-factor receptor deregulation in tumors.

MAIN STUDY

NF2 Management

Currently, there is no cure for NF2, and therefore, repeated surgeries and radiation treatments have been carried out in order to aim to localize tumor control. However, such tactics have often led patients with damaged nerves and CNS structures to have greatly diminished NF2 patients' quality of life. Clinical trials continue to be underway so that new therapies may be integrated (Blakeley et al., 2012). Of all present-day treatments available, stereotactic radiosurgery has been the most popular amongst NF2 patients due to the therapy being able to treat functional abnormalities and small tumors of the brain with great precision in fewer high-dose treatments than traditional therapies. Symptoms of the NF2 disorder, such as vision impairment and hearing loss due to the location of the tumor, have been mitigated through determining NF2 patient candidates for cochlear nerve implant (CNI) or auditory brainstem implant (ABI) for hearing restoration and surgery to correct cataracts and retinal abnormalities for vision restoration. Other complaints of NF2 patients consist of tinnitus, dizziness, facial numbness, balance problems, and seizures is best managed at a specialty clinic with an initial screening and annual follow-up evaluations.

Studying NF2 in Zebrafish

Identification of gene homologs and the functional characterization of their encoded proteins have been identified in many metazoans, including invertebrates, fish, and birds. Zebrafish (*Danio rerio*) have long been used for the identification of genes required for early vertebrate development and have emerged as a model organism for the study of various cancers (Kimmel, 1989). Zebrafish has been an advantageous model for

cancer research as they lack a fully developed immune system during the early larvae stages. The absence of a matured immune system allows transplantation of tumor cells without host rejection while also providing investigators the ability to test the effect of various drugs on tumor growth (Amsterdam et al., 2004). In addition, approximately 70% of the genes present in humans are represented in the zebrafish genome. Therefore, genetic manipulation of genes associated with proto-oncogenes or tumor suppressors have yielded important knowledge toward our understanding of human cancers.

To gain a better understanding of the Merlin protein, the *NF2* gene and its role in Neurofibromatosis 2, we began a systematic study of NF2 homologs in zebrafish. This project details the identification of 2 merlin paralogs, their temporal expression patterns and the effects of knocking down these genes during embryonic development.

Specific Aims

The objective of this research is to generate mutant organisms and characterize their phenotypes associated with silencing the NF2 gene. Specifically, our research focused on the following aims:

Aim 1: Determine the expression patterns of the NF2 gene in zebrafish to identify when Merlin is activated.

Approach: Design NF2a and NF2b primers to detect the NF2 gene during the embryonic stages of zebrafish development through use of Gel Electrophoresis.

Aim 2: Characterize Merlin's tumor suppressive function by knocking down theNF2 gene in zebrafish.

Approach: Design custom oligos for NF2a and NF2b to synthesize gRNA. The gRNA will be microinjected with dCas9 into zebrafish embryos. At 24 h, antibody stains will be used to quantify the apoptotic cells within the forebrain through use of the confocal microscope.

METHODS

Identification of NF2 Homologs in Zebrafish (*D. rerio*)

Sequences for the *NF2* gene in humans was identified using the *ensembl* genome browser (ensembl.org) and compared to transcripts present in the *ensembl* zebrafish database to identify 2 paralogs named *NF2a* and *NF2b*.

Multisequence Alignment of Human NF2, Nf2a and Nf2b

Amino acid sequences for human NF2, zebrafish NF2a, and zebrafish NF2b were downloaded and compared using the web-based sequence alignment tool Multialin (Corpret 1988).

Analysis of Zebrafish Gene Expression *in silico*

To predict when *nf2a* and *nf2b* are expressed, I searched for these genes on the Expression Atlas housed at the EMBL_EBI (Expression Atlas, 2020). These data were derived from transcriptomic profiling (RNAseq) of poly-adenylated mRNA expressed in zebrafish from 1-cell to 5 days post fertilization (White et al., 2017).

Analysis of *nf2a* and *nf2b* Gene Expression using RT-PCR

To determine when *nf2a* and *nf2b* are expressed, NF2a and NF2b primers were designed using the online primer design tool, primer3. RNA was extracted and purified from Zebrafish (D. rerio) embryos raised at 28°C. RNA was collected at the embryonic stages of blastula 30% epiboly, pharyngula prim-5 (24 h), hatching long-pec (48 h), and larval protruding-mouth (72 h). Ten embryos were collected from each embryonic stage and placed in 100ul of Trizol and stored at -20°C. RNA extraction and purification were performed following the standard protocol provided by Invitrogen and stored in -80°C.

(Invitrogen) cDNA was obtained through conducting Real-Time Reverse Transcription (RT) using QuantiTect Reverse transcriptase Qiagen kit (Qiagen, 2013-2017). Polymerase chain reaction (PCR) technique was performed using the Thermal Cycler and Q5 High-Fidelity 2X Master Mix kit (BioLabs inc.). The amplified PCR products were then analyzed by a 1.5% agarose Electrophoresis Gel using TAE buffer in order to determine gene expression patterns of NF2a and NF2b.

Knockdown of *nf2a* and *nf2b* using Enzyme-dead Cas9 Nuclease

gRNA Synthesis: A custom oligo was designed for *NF2a* and *NF2b* and then compared with the constant oligo to produce 117 base pairs. An annealing and an extending reaction were then conducted using the thermal cycler. DNA was obtained and purified using IBI- Gel/PCR DNA Fragments Extraction Kit and then run on a 2% agarose gel to verify product obtained. Using mMESSAGE mMACHINE T7 Transcription Kit, an In Vitro Transcription reaction was conducted and then incubated at 37℃ overnight. gRNA was then purified, pelleted and then run on a 2% agarose gel to verify product obtained.

Microinjection of gRNA and mRNA encoding dCas9 (catalytically inactive dCas9, a generous gift from Didier Stanier, University of Cologne, Germany) (Rossi et al., 2015). The gRNA was then microinjected along with mRNA encoding dCas9 into the yolk of the embryos during the one to two cell stage. Embryos were incubated at 28℃ for 24 hours post fertilization (h), dechorionated, and then stored in 4% PFA overnight.

Antibody Staining for Caspase-3 to Label Apoptotic Cells

The 4% PFA was later replaced with methanol to permeabilize the cells. PBST (Phosphate buffered saline with Tween-20) and PDT (Phosphate buffered saline with DSMO and Trition X) were used to conduct washes prior and post addition of Alpha Tubulin, DAPI, and Rabbit Anti-active Caspase along with their conjugated antibodies. A blocking buffer was used to prevent nonspecific binding of the membrane. The 24 h embryos were then analyzed using the confocal microscope to measure the apoptotic cells within the forebrain.

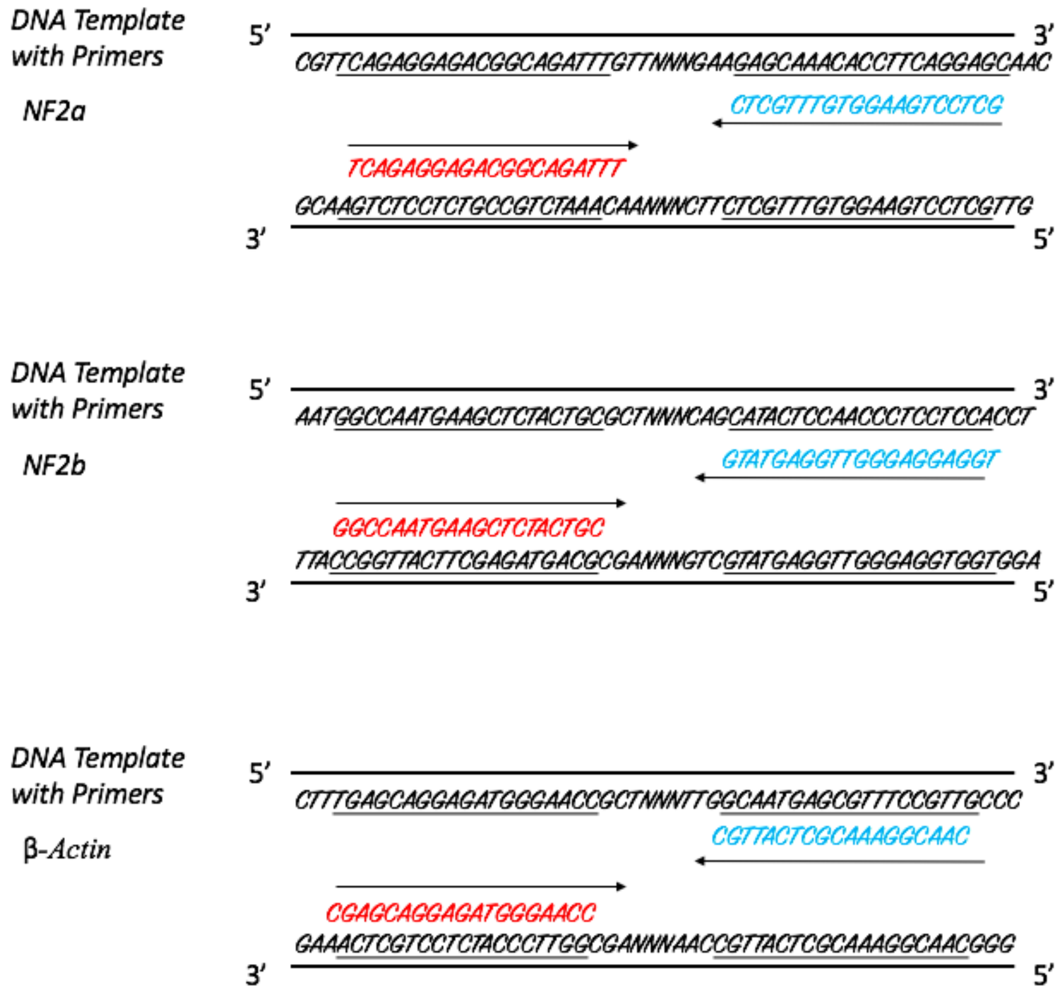

Figure 3: **Amplified and duplicated segments of DNA Template with *NF2a*, *NF2b*, and β-*Actin* primers.** Forward and Reverse Primers of *NF2a* and *NF2b* were designed to create copies of targeted DNA sequence.

RESULTS

Comparison of Human and Zebrafish NF2 Homologs

The zebrafish genome encodes two homologs of NF2/Merlin, named Nf2a and Nf2b. A comparison of the amino acid sequences of human and zebrafish Merlin show that the gene is highly conserved being 46% similar to Human (Shabardina et al., 2019). A closer look at the amino acid sequence also shows that the Zebrafish paralogs maintain the highly conserved “blue box” motif (174-YQMTxxMWEE-194) known to be required for NF2 signaling. Suggesting that these proteins may retain a similar function to human Merlin.

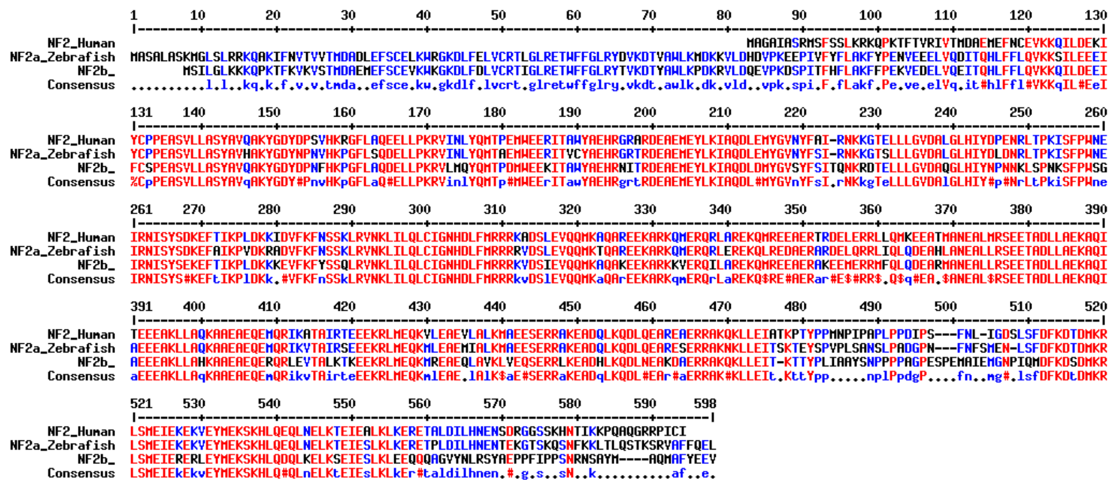

Figure 4: Comparison of NF2 in Humans to Nf2a and Nf2b in Zebrafish. Red amino acids are identical, blue are similar, and black are non-conserved residues. The percent identity of Zebrafish *NF2a* and the Human NF2 gene is 79%. The percent identity of Zebrafish *NF2b* and the Human NF2 gene is 71%.[7]

[7] Figure 4 is referred from Multiple Sequence Alignment with Hierarchical Clustering (Corpet, 2000)

NF2a and NF2b have Different Temporal Expression Patterns as Predicted *in silico*

To begin the study of NF2 in zebrafish, we wanted to know when the gene is expressed during embryonic development. To do this, we found a publicly available database that allowed me to browse all the mRNAs sequenced (RNAseq) in zebrafish at different stages of embryonic development. A search for *nf2a* showed that the gene was expressed from the zygotic (1-cell) stage through the gastrula 50% epiboly stage and then no longer expressed from the gastrula 75% epiboly stage through 5 dpf. *nf2b*, on the other hand, showed no expression during the zygotic stage, but then showed expression from the cleavage 2-cell stage through 5 dpf.

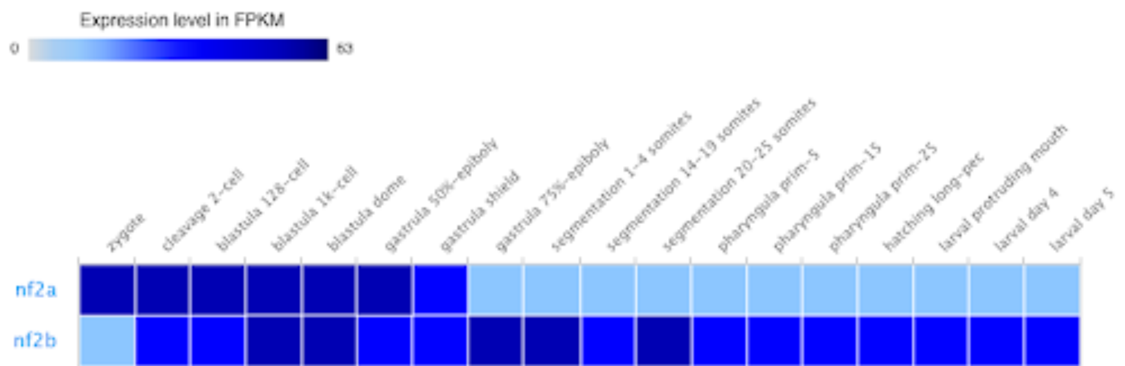

Figure 5: In-silico gene expression analysis of *nf2a* and *nf2b* through the first 5 days of development. Reverse-transcription PCR of *Nf2a and Nf2b* conflict with predicted expression patterns.[8]

[8] Figure 5 is referred from Expression Atlas (2020)

To verify these results we obtained *in* silico, we designed NF2a and NF2b primers for reverse-transcription PCR and extracted RNA from four different embryonic stages of development. Our results disagreed with the predicted expression as indicated NF2a was not expressed during 30% epiboly and 1 dpf, but then was expressed during 2 dpf and 5 dpf. The results for NF2b indicated expression during 30% epiboly, no expression during 1 dpf, and then expression during 2 dpf and 5 dpf. We concluded from these results that nf2a is not expressed in the oocytes and that the development of NF2a is under exclusive control of the zygotic genome starting before 2 dpf, but not before 24 h. We have also determined that nf2b is expressed at 30% epiboly, turns off during the transitions at 1 dpf when the maternal transcripts are cleared. It once again turns on at 2 dpf and 5 dpf when the zygotic genome itself gives rise to nf2b. The identified NF2a and NF2b gene expression patterns differed from the in-silico gene expression analysis reported in Zebrafish development seen in Figure 4. Data obtained indicated that NF2a is expressed at the hatching long-pec stage (48 h) and larval protruding-mouth stage (72 h), while *in silico* showed that zebrafish in these embryonic stages had little to no expression (Fig. 5). Furthermore, NF2a obtained at blastula 30% epiboly (4.66 h) did not show gene expression, where in silico did show gene expression at blastula dome (4.33 h) and blastula 50% epiboly (5.25 h) (Fig. 5 & 6). No expression of NF2 at the pharyngula prim-5 stage (24 h) of development was found, which corresponded with the in-silico gene expression analysis reported (Fig. 5 & 6).

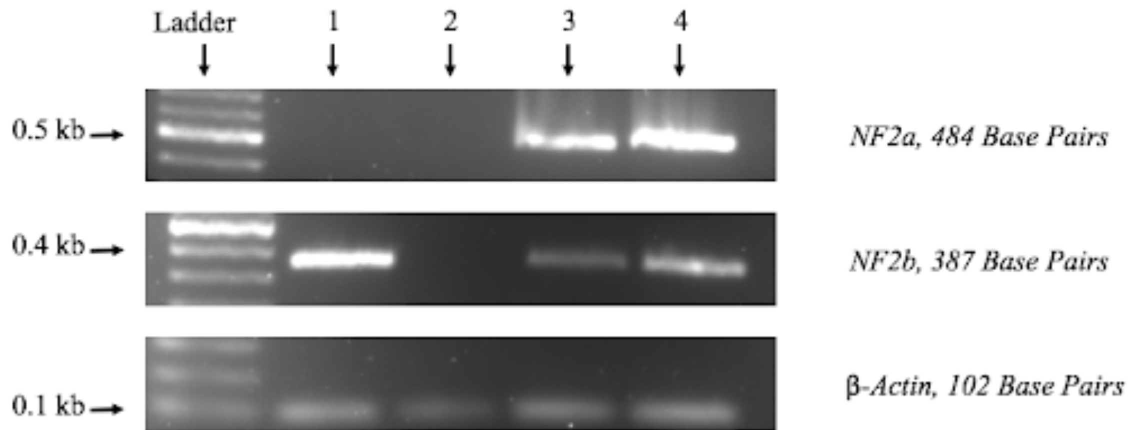

Figure 6: NF2 Gene Expression Pattern. 1.5% Agarose gel electrophoresis analysis of DNA of NF2 gene and β-*Actin* obtain from PCR products. 1.0-10.0kb of DNA ladder, Lane 1 is blastula 30% epiboly/ 4.66 h, Lane 2 is pharyngula prim-5/ 24 h, Lane 3 is hatching long-pec/ 48 h, and Lane 4 is larval protruding mouth/ 72 h. First column is NF2a, second column is NF2b, third column is the positive control/ β-*Actin*.

Knockdown of *nf2a* and *nf2b* Causes Excessive Proliferation of Hematopoietic Cells in Zebrafish 'Blood Islands'

To better understand the function of nf2a and nf2b we used a modified protocol of CRISPR/Cas9 to knockdown *nf2a* and *nf2b*. Following knockdown, we observed 24 h zebrafish at 40X through use of the stereo microscope. Our results indicated that microinjecting gRNA and mRNA encoding an enzyme-dead Cas9 nuclease transcriptionally repressed Merlin1a and Merlin1b that lead to diverse morphological phenotypes including increased cellular proliferation in the posterior blood island, bulging "tumors" in the notochord, in addition to gene expression patterns of NF2a and NF2b being observed, phenotypes of dCas9 injected *nf2a and nf2b* were also identified at 24 h (Fig. 6). Abnormalities detected in nf2a injected (Fig. 6B & C) in comparison to

uninjected zebrafish embryo (Fig. 6A), included severe curvature of the notochord and increased cellular proliferation in the posterior blood island. For Figure 6B, potential tumors on caudal fin were also identified. While injected nf2a embryos had more severe phenotypes, nf2b injected embryos had more moderate curvature of the notochord. These data suggest that Nf2a and Nf2b may be important for inhibiting excessive proliferation in these hematopoetic cells.

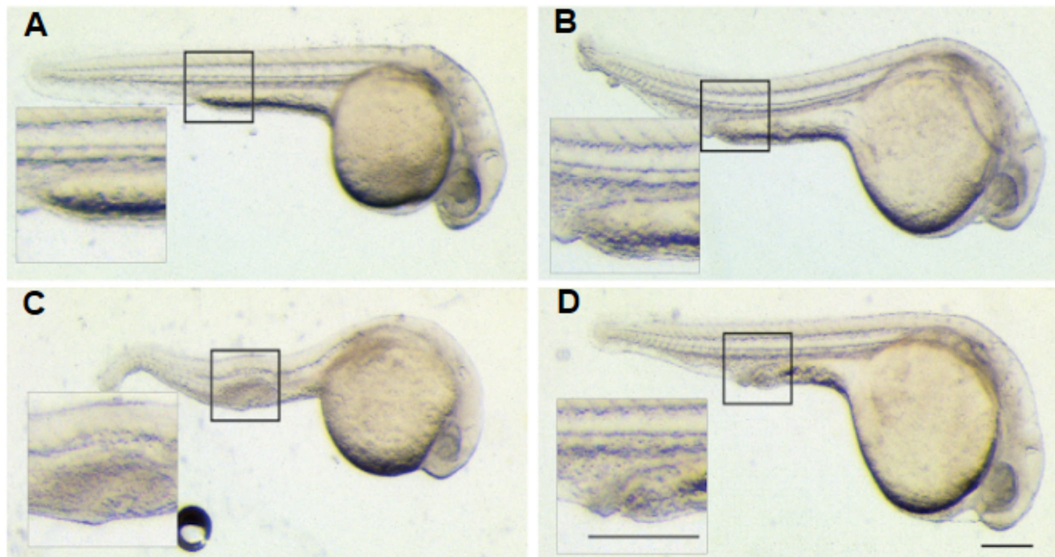

Figure 7: **Cellular proliferation in the posterior blood island at 24 h embryos produced by microinjections of *nf2a* and *nf2b*.** Severe curvature phenotype of the notochord in *nf2a* injected (B and C) and moderate curvature phenotype of the notochord in *nf2b* injected (D) are shown relative to an uninjected control embryo (A).

To further identify probable cause of phenotypes identified (Fig. 7), apoptotic cells of 84 zebrafish embryos at 24 h were observed and counted through use of the confocal microscope (Fig 8). Apoptotic cells were observed in the forebrain and compared to control, nf2a injected, and nf2b injected through use of ANOVA (Fig. 9). The ANOVA model indicated a significant difference for microinjected embryos at 24 h with nf2a and/or nf2b showing less apoptotic cells than control.

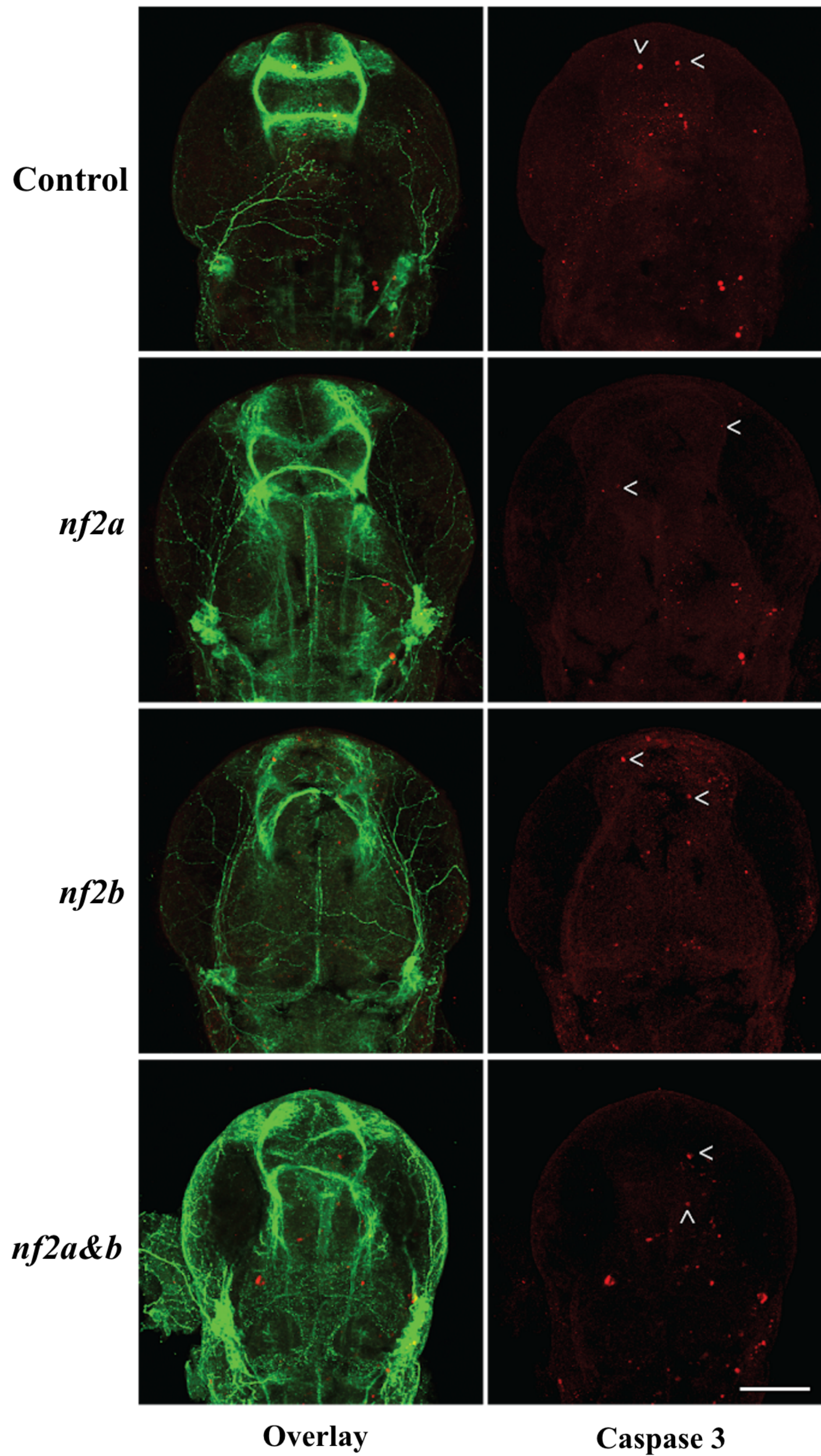

Figure 8: Confocal microscopy analysis of apoptosis in zebrafish forebrain at 24 h.

Arrowheads indicate apoptotic cells.

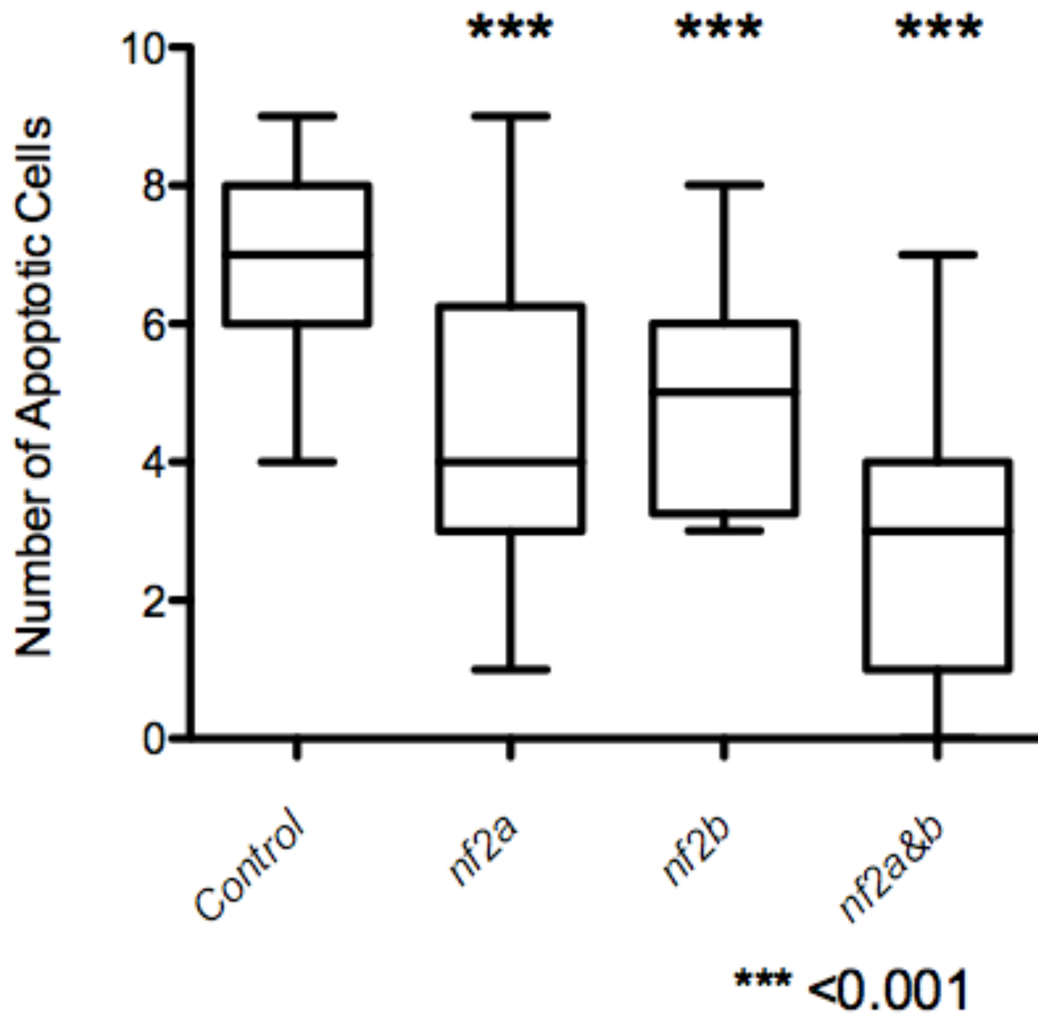

Figure 9: ANOVA model of Apoptotic cells in the forebrain. Apoptotic cells in the forebrain at 24 h in comparison to the uninjected control embryos. ANOVA model indicates significant difference for microinjected embryos at 24 h ($p<0.001$).

DISCUSSION

A deficiency of Merlin leads to the NF2 genetic disorder that can result in hearing loss, disability, and premature death because of uncontrollable growth of benign tumors within the nervous system. Although surgeries reduce tumor mass have been the most sought out treatment for managing NF2, these therapeutic techniques do not eradicate the full effects of NF2, nor provide a long-term cure. The tumor suppressor gene NF2 is known to prevent abnormal cell division through encoding MERLIN to promote apoptosis and preventing cellular proliferation. Through our research we have determined that *NF2a* is not expressed in the zebrafish oocytes and that the development of *NF2a* is under exclusive control of the zygotic genome starting before hatching long-pec/48 h, but not before pharyngula prim-5/24 h (Fig. 6). We have also determined that *NF2b* is expressed at blastula 30% epiboly/ 4.66 h, turns off during the transitions at 1dpf when the maternal transcripts are cleared (Fig. 6). It once again turns on at hatching long-pec/48 h and larval protruding-mouth/72 h when the zygotic genome itself gives rise to *NF2b* (Fig. 6). Using publicly available dataset, *in silico*, we found that nf2a is maternally deposited and highly expressed through the first 6 h of development, while nf2b is expressed immediately after fertilization and maintained through 5 days of development (Fig. 4).

Genetic knockdowns of nf2a and nf2b led to varying morphological phenotypes including substantial proliferation of hematopoietic cells in 24 h zebrafish (Fig. 7). Researchers backcrossed generations of C57/B6 background crossed with C57B6 Mx1 Cre transgenic mice and found that NF2 knockout mice may exhibit two distinct

phenotypes affecting hematopoietic cells (Larsson 2008). This research supports NF2's crucial role in regulating stem cells within the hematopoietic compartment.

Apoptotic cells were observed in the forebrain of zebrafish at 24 h (Fig. 8). The statistical analysis that was used for quantification of apoptotic cells was the ANOVA model. ANOVA demonstrated statistical significance for microinjected embryos at 24 h with nf2a and/or nf2b indicating a decrease in the amount of apoptosis localized within the forebrain of zebrafish (Fig. 9). Previous research has shown that inhibiting endogenous NF2 by siRNA knockdown rescued INS-1E cells from high glucose- and high-glucose/palmitate-induced apoptosis, as demonstrated by decreased caspase-3 levels (Yuan et al., 2016). Therefore, by silencing NF2 β-cells were protected from high-glucose/palmitate-induced apoptosis in isolated human islets (Yuan et al., 2016).

Phenotypic characteristics of NF2 have been well documented in the literature. In zebrafish l, the phenotypic characteristics included curvature of the notochord, increase of cell proliferation in the blood islands, and bulging "tumors" in the notochord (Fig. 7). With our results we noticed a significant discrepancy between nf2a and nf2b phenotypic characteristics. The characteristics of nf2a were seen as more severe as opposed to nf2b. We postulate that the reason behind the discrepancy we see between nf2a and nf2b is that, nf2a is expressed earlier on in the embryological development stages of zebrafish which can induce more severe phenotypes given the fact that early on in the embryological development important structures are being created.

We hope to further our research by pinpointing exactly when NF2a and NF2b are expressed during embryological stages of zebrafish development along with quantifying absolute levels of RNA transcripts based on quantitative analysis. Our future aspiration

also includes conducting a whole-mount in situ hybridization to determine where NF2a/NF2b are expressed and generating nf2a and nf2b knockdown lines to assess the effects of lifelong loss of the NF2 gene in zebrafish. Our long-term goal is to establish a zebrafish model of Neurofibromatosis Type 2 so that we may further our understanding of the protein merlin that will lead to a potentially better alternative cure to reduce tumorigenesis and prevent the escalation of NF2-related tumors.

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