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It is entitled:
Signaling Pathways Controlling CNS Myelin Compaction in Gain of Function Rasopathies

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Signaling Pathways Controlling CNS Myelin Compaction in Gain of Function RASopathies

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Doctorate of Philosophy

University of Cincinnati, College of Medicine
2015
ABSTRACT

Titus-Mitchell, Haley E. Ph.D., Neuroscience Graduate Program, University of Cincinnati, College of Medicine, 2015. Signaling Pathways Controlling CNS Myelin Compaction in Gain of Function RASopathies.

This dissertation describes 2 major studies on gain of function Rasopathy mouse models of Neurofibromatosis Type 1 (NF1) and Costello syndrome. In human Rasopathies, Ras-GTP pathway activation compromises brain function. To study myelin decompaction and aberrant cognitive behavior \textit{in vivo} we characterized two novel mouse models for CNS myelin disruption in NF1 and Costello syndrome; iPlpCre;Nf1flox and iPlpCre;HRasG12V. In murine models, loss of Nf1 or hyperactive HRas GTPase causes myelin decompaction. We show that brain myelin decompaction occurs within one month of Nf1 loss or hyperactive HRas in oligodendrocytes. As the feedback loops in NF1 and Costello Syndrome were largely unknown, we set out to define the role of Ras and downstream effectors in myelin compaction. We identified Notch and nitric oxide signaling pathways downstream of Ras GTPase and cross talk, after Nf1 loss or hyper activation of HRas, in oligodendrocytes and identified therapeutic targets for myelin repair. We ran pre-clinical therapeutic trials for improvement in myelin compaction and behavior. Constitutive activation of Notch signaling in oligodendrocytes partially recapitulates the myelin phenotype and loss of canonical Notch signaling through Rbpj loss, or treatment with gamma secretase inhibitor, partially rescues decompaction. In Nf1 and HRas mutants, mature oligodendrocytes exclusively increase levels of nitric oxide (NO) and inhibition of nitric oxide synthase (NOS) partially rescues decompaction. Inhibition of NOS completely rescues decompaction in Nf1;Rbpj mutants, indicating that Notch and NO pathways contribute to myelin decompaction. Therefore, Rbpj-dependent and NO signaling pathways are potential
therapeutic targets in NF1 patients. All cells are thought to be heterozygous for NF1 in NF1 patients. We show hemizygous Nf1 loss in oligodendrocytes causes myelin decompaction which worsens over time, and correlates with hyperactive behavior. In this setting, inhibiting Notch or NOS completely rescues myelin decompaction and behavioral abnormalities. All cells are thought to be heterozygous for HRas mutation in Costello syndrome patients. Targeting NO and MEK signaling fully rescues myelin thickness and compaction in HRas mutants. Thus, data suggest that in hemizygous HRas mutants, direct inhibition of Notch is not required for correction of signaling that controls myelin thickness. However, our findings suggest it is likely that pathway cross talk/feedback is targeting Notch signaling, directly or indirectly. Therefore, MEK and NOS pathways are potential therapeutic targets in Costello patients.

Despite similarities in hyperactivation of RasGTPases and downstream signaling, such as MEK/ERK, single inhibitor treatment with L-NAME or MEKi in HRas mutants diminished Notch signaling, whereas Notch signaling was elevated in Nf1 mutants. The differences in rescue of compaction and myelin thickness within and between Nf1 and HRas mutants were due, at least in part, to level of Notch signaling. Future studies could further explore pathway cross talk, including positive and negative feedback loops in relation to the level of signaling pathway activity.
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADAM</td>
<td>A Disintegrin And Metalloprotease</td>
</tr>
<tr>
<td>AF6</td>
<td>ALL-1 Fusion partner in chromosome 6</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin repeats</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOP</td>
<td>Anterior Open transcription factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus Callosum</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF-1, Su (H), Lag1</td>
</tr>
<tr>
<td>Dil</td>
<td>Delta-like ligand</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta/ Serrate/ Lag2</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular Domain</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular single regulated kinases</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine Exchange Factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Gial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Gist</td>
<td>Gliothaldehyde</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor Receptor Bound Protein 2</td>
</tr>
<tr>
<td>GSI</td>
<td>Gamma Secretase Inhibitor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Hes</td>
<td>Hairy and Enhancer of split</td>
</tr>
<tr>
<td>Ibα1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>MAM</td>
<td>Mastermind</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MKP3</td>
<td>Map Kinase Phosphatase 3</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis</td>
</tr>
<tr>
<td>NF2</td>
<td>Neurofibromin gene</td>
</tr>
<tr>
<td>NG2</td>
<td>neuron/ glia - type 2 antigen</td>
</tr>
<tr>
<td>NIDC</td>
<td>Notch Intracellular Domain</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte Progenitor Cell</td>
</tr>
<tr>
<td>PDE4</td>
<td>Phosphodiesterase 4</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet Derived Growth Factor Receptor alpha</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PLCε</td>
<td>Phospholipase Cε</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid Protein</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>qRTPCR</td>
<td>quantitative Reverse Transcription PCR</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma (kinase)</td>
</tr>
<tr>
<td>RALGDS</td>
<td>RAL guanine nucleotide dissociation stimulator</td>
</tr>
<tr>
<td>RAM</td>
<td>RBPJ Associated Molecule</td>
</tr>
<tr>
<td>RBPJκ</td>
<td>Recombination Signal Binding Protein 1 for J-kappa</td>
</tr>
<tr>
<td>RIN1</td>
<td>RAS and RAB interactor 1</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2 domain</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3 domain</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
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<tr>
<td>TIAM1</td>
<td>T-lymphoma invasion and metastasis-inducing protein 1</td>
</tr>
<tr>
<td>TMICD</td>
<td>Transmembrane intracellular domain</td>
</tr>
<tr>
<td>TxA</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>UBO</td>
<td>Unidentified Bright Object</td>
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</table>
Acknowledgements

I would like to express my appreciation for my mentor, Dr. Nancy Ratner. She is an incredibly strong female scientist role model. I have learned a great deal, not only in scientific techniques and experimental design, but in how to think more critically, write with clarity, and present to a wide variety of audiences. I deeply appreciate the unwavering encouragement for professional development and growth in and outside of the lab. I would like to thank my committee members; Dr. Kenneth Campbell (chair), Dr. Rhett Kovall, Dr. Masato Nakafuku, and Dr. Ronald Waclaw for their feedback and advice throughout my dissertation project.

I would like to thank the current and past members of the Ratner lab for lively discussion of data and for technical support. I would specifically like to thank my team of mentees; Sadiq Silbak, Madeleine Bogard, and Amanda Klotter. They were truly invaluable and I could not have processed all of my data without them. I would like to thank Tilat Rizvi for helping train me as a member of the CNS project in the lab, for her technical assistance, and for her feedback on the project. I would like to thank Chandra Moon and Bethany Bresnen for being the best lab manager and administrative assistant at CCHMC. I would like to thank the Neuroscience Graduate Program for investing in my education and specifically the current and past director for allowing me to serve as an ambassador to the program. I would like to thank Deb Cummins and Sharon Weber for all they have done as coordinator of the program.

Lastly, I would like to thank my entire family and my friends who have supported me unconditionally. I dedicate this dissertation work to my mother, Ms. Terri Titus. My life goal is to cure Multiple Sclerosis and my hope is that this research on myelin compaction will provide new avenues for therapies to treat neurodegenerative diseases.
Chapter 1

Introduction
It is estimated that 7% of the American population is afflicted with a brain disorder due to inherent disease or injury (Borlongan et al., 2013). Oligodendrocytes are the myelinating glia of the CNS that wrap multiple axons with a myelin sheath, made up of lipids and proteins, as insulation for proper axonal conduction (Nave, 2010; Baumann and Pham-Dinh, 2001). Myelination is the formation of a myelin sheath by the oligodendrocyte around a nerve fiber to enhance conductance of the neuronal axon. Dysmyelination, defective structure of myelin around an axon, can slow axonal conductance which interrupts normal signaling between cells and axonal degeneration can diminish communication entirely. Demyelination, including paranodal destruction and lateral migration of sodium channels, can lead to progressive axonal degeneration (Miron et al., 2011).

Interestingly, changes that occur in brain ultrastructure during development and in aging can share molecular mechanisms that may underlie dysfunction. There are several intrinsic and extrinsic factors that influence oligodendrocyte differentiation. Intrinsic regulation includes transcriptional regulation, chromatin remodeling, and post transcriptional control through microRNA (Emery, 2010). Extracellular regulation involves ligands, secreted molecules, and neuronal activity (Emery, 2010). It is important to understand the balance of inhibitors and growth factors to properly target different periods in oligodendrocyte differentiation (Fancy et al., 2010; Zhang et al., 2011). Signaling pathways controlling myelin formation and stability are of great interest as learning and motor skills are linked to changes in myelin (Fields, 2011; McKenzie et al., 2014).

In human Rasopathies, mutations in Ras-MAPK pathway genes compromise brain function (Rauen, 2013). Our lab studies the role of oligodendrocytes and myelin in gain of function RASopathies, including Neurofibromatosis type 1 (NF1) and Costello syndrome,
which myelination changes have been implicated and cognitive deficits exist. Gain of function Rasopathies are characterized by an increase in Raf/MEK/ERK pathway activity downstream of Ras GTPase (Gottfried et al., 2010). Within the Ras superfamily of proteins there are classical Ras proteins (H, N, and K) and non-classical Ras proteins (R-Ras 1, 2, 3) (Cox and Der, 2010). The classical Ras proteins are ubiquitously expressed and highly homologous yet have functional differences (Grewal et al., 2011). Ras GTPases relay signaling from cell surface receptors (Figure 1.1). Receptor tyrosine kinases are high affinity cell surface receptors which bind ligand (i.e. growth factors, cytokines, and hormones) in the extracellular domain and form dimers that auto phosphorylate. The phosphorylation of specific tyrosine residues creates binding sites for Src Homology 2 (SH2) domain in which the SH2 domain of the growth factor receptor bound protein 2 (GRB2) can bind (Rajalingam et al., 2007). The Src Homology 3 (SH3) domain on the N-terminal of GRB2 can then bind to a guanine exchange factor (GEF), such as son of sevenless (SOS), which promotes the activation of Ras GTPase through the exchange of GDP for GTP (Bos et al., 2007). Ras GTPases relay signals from the RTK to downstream effectors, including; Raf, PI3K, PLCɛ, AF6, RALGDS, TIAM1, RIN1 that influence cell proliferation, survival, and growth (Ratner and Miller, 2015; Vigil et al., 2010). Raf/MEK/ERK signaling downstream of Ras is highly conserved across species; C. elegans, drosophila, and in mammals (Cox and Der, 2010). Of the known effectors downstream of Ras, the Raf/MEK/ERK pathway has shown the most therapeutic potential in gain of function Rasopathies, such as NF1 (Ratner and Miller, 2015). Ras GAPs, such as neurofibromin, trigger GTP hydrolysis and reset the Ras GTPase by converting Ras-GTP (active) to Ras-GDP (inactive) (Bos et al., 2007) (Figure 1.1). The contribution of each GAP in Ras inactivation is determined by cell type expression patterns and activity (Grewal et al., 2011).
Neurofibromatosis type 1 (NF1) is a gain of function Rasopathy caused by a mutation of the *NF1* gene on chromosome 17q11 and is prevalent, affecting about 1 in 3000 individuals (Cohen and Shuper, 2010); half of the cases are autosomal dominant and half are sporadic. The *NF1* tumor suppressor gene product, neurofibromin, contains a Ras GTPase activating protein-related domain (Ras-GRD) and functions as a GTPase activating protein (GAP) that triggers GTP hydrolysis and thus converts Ras-GTP (active) to Ras-GDP (inactive) (Buchberg et al., 1990; Trovo-Marqui and Tajara, 2006). Neurofibromin acts as an “off signal” and when diminished or lost results in sustained Ras GTPase signaling. Neurofibromin has also been shown to increase cyclic adenosine monophosphate (cAMP) in astrocytes (Dasgupta et al., 2003). Therefore, loss of *NF1* may lead to low levels of cAMP in patients.

The major feature of NF1 is the formation of Schwann cell tumors (i.e. neurofibromas) in the periphery. Additionally, patients present with café-au-lait spots, freckling, osseous dysplasia, iris Lisch nodules, and optic pathway glioma. Learning and developmental disorders are present in a majority of NF1 patients (Acosta et al., 2012) which significantly impact quality of life. There are several CNS manifestations of NF1, including macrocephaly and an increase in the size of white matter tracts in the brain that have been associated with behavioral deficits (Pride et al., 2010). Additionally, T2 hyper-intensities on MRI known as unidentified bright objects (UBOs) may be representative of dys/demyelination (Margariti et al., 2007). In NF1 patients, changes in diffuse tensor imaging (DTI) correlate with T2 hyper intense regions (van Engelen et al., 2008). *NF1* is highly expressed in rodent and human oligodendrocytes (Daston et al., 1992). In NF1 patients it is believe that most to all of the cells in the brain are heterozygous for *NF1* and changes in brain structure are global (van Engelen et al., 2008; Williams et al., 2009). Glial cells are thought to play a major role in the manifestations of NF1 in the central
nervous system (CNS). In murine models of Neurofibromatosis Type 1 at 12 months, ultrastructure analysis through electron microscopy revealed decompaction of myelin, including intra period line splitting, in white matter tracts of the brain as well as in the optic nerve (Mayes et al., 2013). The oligodendrocyte cell lineage is targeted for protection and remyelination of damaged, degenerating axons. The brain and spinal cord from murine models of NF1 have increased glial cell lineage proliferation; including astrocytes and oligodendrocyte precursor cells (OPCs) (Bennett et al., 2003; Hegedus et al., 2007).

Costello syndrome is a rare gain of function Rasopathy caused by a heterozygous activating mutation of HRAS on chromosome 11p15.5 that leads to constitutive HRas GTPase activity (Gripp et al., 2010). Interestingly, the most commonly mutated positions in Costello Syndrome, amino acids 12 and 13, are also the most commonly mutated in oncogenic Ras (Bos, 1989). The most prevalent mutation, G12S, is present in 80% of patients and the second most common mutation is G12A (Seeburg et al., 1984). A subset of Costello patients have the severe V12-HRas mutation, which we modeled. Costello syndrome patients have characteristic dysmorphic craniofacial features, short stature, and failure to thrive, as well as congenital heart defects and ophthalmologic abnormalities (Rauen, 2013). The brain in Costello syndrome has not been studied in great detail, but, macrocephaly, ventriculomegaly, dysmyelination, and variable cognitive delay a have been reported (Gripp et al., 2010; Rauen, 2013). Our lab developed CNPaseHRasG12V mice, in which HRas is hyper active in glial cells with CNP from E12.5 on. Of note, these mice have enlargement of white matter tracts (Mayes et al., 2013) consistent with the patient phenotype in Costello syndrome and in NF1, where loss of a RasGAP leads to an increase in active (-GTP bound) Ras (Pride et al., 2010).
Aberrant signaling to Ras effectors results from mutations in Ras proteins themselves as in Costello syndrome (caused by mutations in HRas), or inactivation of Ras-GAPs as in NF1 (caused by mutations in NF1) (Gysin et al., 2011). Gain of function Rasopathies are characterized by an increase in Raf/MEK/ERK pathway activity (Gottfried et al., 2010). MEK is essential for gliogenesis, of astrocyte and oligodendroglia precursors, and levels of MEK1/2 regulate gliogenesis in the developing cortex (Li et al., 2012). Ras-GTP activates MAPK-ERK signaling (Vigil et al., 2010), which is critical for oligodendrocyte function. MAPK-ERK activation drives OPC specification and proliferation and timing of differentiation (Fyffe-Maricich et al., 2011; Li et al., 2012; Guardiola-Diaz et al., 2012). ERK contributes to myelin thickness, as extra myelin wraps are observed in oligodendrocytes expressing activated ERK and ERK1/2 control myelin thickness during repair (Fyffe-Maricich et al., 2013; Ishii et al., 2013). Previous studies have targeted ERK signaling at developmental time points in Nf1 mutant mice and successfully rescued enlarged white matter tract size with MEK inhibitors (Wang et al., 2012). Myelin relies on regulated levels of Ras-GTP, as adult induced Nf1 loss or embryonic onset of hyper-activation of H-Ras-GTP in oligodendrocytes cause myelin decompaction in one year old mice (Mayes et al., 2013).

The focus of our study is on the myelin producing oligodendrocyte. Oligodendrocyte progenitor cell (OPC) differentiation occurs through a process of initial intrinsic inhibition of myelin gene expression, followed by inhibition of myelin gene repressors, and finally myelin gene expression (Emery, 2010). During mammalian nervous system development Notch pathway activation initially promotes gliogenesis through the canonical Notch pathway (Figure 1.2), in which Delta/Serrate/Lag-2 (DSL) Ligands (i.e. Jagged 1 & 2 and delta-like ligands 1 & 4) and Notch Receptors (1-4) bind on adjacent cells for intercellular communication (Fortini, 2009).
This trans binding promotes a conformational change and the Notch receptor extracellular domain (ECD) undergoes proteolytic cleavage by a disintegrin and metalloprotease (ADAM), then Notch intracellular domain (NICD) is cleaved by gamma secretase (Kopan, 2010). The NICD is then able to translocate to the nucleus where the RAM (RBPJ associated molecule) and ANK (ankyrin repeats) domains bind to the nuclear effector CBF-1, Su (H), Lag1 (CSL) DNA binding protein; recombination signal binding protein 1 for J-kappa (RBPJκ) in vertebrates (Kopan, 2010). The co-activator Mastermind (MAM) forms the CSL-NICD-MAM complex resulting in activation of transcription for Notch target genes hairy and enhancer of split (Hes); specifically Hes1 and Hes5 in OPCs (Borggrefe and Liefke, 2012; Nicolay et al., 2007), (Figure 1.2). Hes transcription factors are dimers that bind to the DNA and repress gene expression involved in differentiation and maturation (Kageyama et al., 2007; Fischer and Gessler, 2007). Therefore, canonical Notch pathway signaling initially promotes glial cell fate yet inhibits maturation of OPCs (Grandbarbe et al., 2003; Liu et al., 2006; Park and Appel, 2003; Zhao et al., 2010). The non-canonical Notch pathway involves the interaction of the Notch receptors on the oligodendrocyte with the F3/contactin1 and NB3/Contactin6 ligands on the neuronal axon (Hu et al., 2003). This intracellular communication results in the translocation of NICD to the nucleus where it binds with Deltex to promote maturation and myelination through myelin gene expression (Laursen and Ffrench-Constant, 2007).

Aberrant Notch signaling occurs in disease, including neurodegenerative disease (Jurynczyk and Selmaj, 2010) and Ras-driven tumors (Mazur et al., 2010a; Baumgart et al., 2014). It is well known that gamma secretase inhibitors prevent NICD domain cleavage and therefore prevent Notch pathway activation. Given the importance of the Notch pathway in stem cell regulation, neurogenesis/gliogenesis, and regulation of myelination it is likely that targeting
this pathway may rescue aberrant oligodendrocyte lineage cell behavior in gain of function Rasopathies. Previous studies found that administration of the gamma secretase inhibitor (GSI) MW167 in vitro leads to a decrease in Hes1 mRNA in qRTPCR analysis as well as a decrease in NICD for Notch 1 in wild type murine oligodendrocyte cells (Jurynczyk et al., 2008). Administration of the GSI MK-003 in vitro leads to a decrease in Notch pathway activity measured through levels of Hes1 and Hes5 mRNA in qRTPCR analysis with a subsequent decrease in proliferation and an increase in apoptotic cell death in human T-cell leukemia cell lines (Tammam et al., 2009). Inhibition of canonical Notch signaling increased oligodendrocyte ensheathment of axons (Watkins et al., 2008).

Remyelination can be a spontaneous process in response to damage and inflammation; the OPCs migrate, differentiate into mature oligodendrocytes, and myelinate (Miron et al., 2011). In neurodegenerative disorders there can be a road block to remyelination. For example, in Multiple Sclerosis (MS) an up regulation of DSL ligand Jagged1 on astrocytes and subsequent canonical Notch pathway activity in early stage oligodendrocytes prevents maturation into myelinating oligodendrocytes (Jurynczyk and Selmaj, 2010). Previous studies treated mice with adult induced experimental autoimmune encephalomyelitis (EAE) with GSI MW-167 (Sigma) through intracranial injections. EAE is an inflammatory demyelinating disease and the GSI treatment resulted in a decrease in axonal damage and an increase in remyelination measured through toluidine blue stain pathological analysis as well as a decrease in the clinical score (0 = no signs, 5 = death), therefore increased clinical recovery, measured through behavioral analysis at 3 weeks post treatment (Jurynczyk et al., 2008). Notch signaling inhibitors are currently being tested therapeutically; including gamma secretase inhibitors which prevent the final cleavage step of the Notch receptor intracellular domain (Lathia et al., 2008). As GSI MK-003 crosses the
blood brain barrier it was an ideal candidate for pre-clinical trials in our murine gain of function Rasopathy models.

During development there is a “love-hate relationship” between the Notch and the Ras GTPase pathway that can result in Notch pathway competition or cooperation with the Receptor tyrosine kinase (RTK)/Ras GTPase pathway (Sundaram, 2005). Ras-GTP acts up- or down-stream of Notch pathway signaling (Sundaram, 2005). In the fly, 65% of RTK/Ras targets are responsive to Notch pathway signaling (Hurlbut et al., 2009). The Notch pathway antagonizes the RTK/Ras pathway, possibly providing output specificity, through regulation of RTK ligands, RTK’s (i.e. EGFR), and intracellular antagonists to RTK signal transduction (Hurlbut et al., 2007). The RTK/Ras pathway alters the Notch pathway through regulation of DSL ligands (i.e. Delta in fly), Notch receptor endocytosis as well as localization, and target gene expression through regulation of co-repressors (Hurlbut et al., 2007). The Notch and RTK/Ras pathway integration involves reciprocal transcriptional regulation. Several signaling components are responsive to both the Notch and RTK/Ras pathways, singly activated or simultaneously activated, and are considered nodal points of integration of the pathways (Hurlbut et al., 2009). In fly these include: sprouty which has an inhibitory role on the RTK/Ras pathway through inhibition of GEF activity and of the downstream effector Raf, of Map kinase phosphatase 3 (MKP3) which inhibits MAPK, GTPase activating protein 1 (GAP1) which drives Ras to the GDP bound inactive form, Anterior Open (AOP) transcription factor that inhibits RTK/Ras target genes, and fringe which is involved in post transcriptional modification of the Notch receptor (Hurlbut et al., 2009). These changes in gene expression can lead to a decrease in RTK/Ras signaling and an increase in Notch pathway activity. However, change in either the RTK/Ras or Notch pathway activity can shift the outcome based on the new integration of
signaling. For example, in a gain of function Rasopathy the RTK/Ras activity is increased, therefore it is likely that regulation of the Notch pathway is altered.

The Notch pathway is important to cell differentiation and has been linked to both normal stem cells and glioma cancer stem cell biology (Baron 2003; Z. Wang et al. 2008). It has also been shown to activate the Nestin promoter in gliomas (Shih and Holland, 2006). *In vivo* studies of NICD expression in mice showed an induction of Nestin expression and an expansion of the SVZ via cooperation with K Ras (Shih and Holland, 2006). As the feedback loops in NF1 and Costello Syndrome were largely unknown, we set out to define the role of Ras and Notch signaling in myelin compaction.

Additionally, reactive oxygen species (ROS) were of interest in CNS dysfunction in gain of function Rasopathies. Reactive oxygen species include hydrogen peroxide (H$_2$O$_2$), superoxide O$_2^-$, and nitric oxide (NO). Three mammalian nitric oxide synthases (NOS 1, 2, & 3/ n, i, & e) produce NO from L-arginine, oxygen and NADPH. Inside target cells, NO activates guanylate cyclase and oxidizes, ADP ribosylates, or nitrosylates proteins, DNA and lipids. Previous studies in the Ratner lab have implicated nitric oxide signaling in *Nf1* mutant brain (Mayes et al., 2013). Hyperactive behavior in mice has been noted in other systems with elevated levels of reactive oxygen species leading to oxidative stress (Chen et al., 2012; Dumont et al., 2011). NF1 and Rasopathy patients often present with hyperactivity (Acosta et al., 2012) and in adult ADHD patients studies show increased reactive oxygen and impaired oxidative balance (Selek et al., 2012). Thus, reactive oxygen may also play a role in Rasopathy patient’s hyperactive behavior.

Injured oligodendrocytes express nitric oxide synthases, iNOS/NOS2 (Boullerne and Benjamins, 2006) and/or nNOS/NOS1 (Yao et al., 2010; Yao et al., 2012) producing nitric oxide (NO). Nitric oxide, from iNOS, down regulates the expression of myelin genes (i.e. *PLP* and
MOG) preceding the death of oligodendrocytes in human mixed glial culture in vitro (Jana and Pahan, 2013). In LPS lesions of demyelination and axonal degeneration, there is an increase in iNOS and total reactive oxygen species (ROS) and a subsequent accumulation of mitochondria (di Penta et al., 2013). Additionally, nitric oxide regulates stem cell differentiation and promotes change in cell fate, from neurons to glia, during migration from the SVZ due to chromatin modification (Bergsland et al., 2014) (Lameu et al., 2012). nNOS exposure of OPC’s, induced damage as early as 3 days and was present in the corpus callosum at 2 weeks. Damage, in the form of demyelination, is mediated through mitochondria dysfunction in oligodendrocytes (Natarajan et al., 2013). nNOS mediates mitochondrial injury in LPS stimulated oligodendrocytes and subsequent decrease in mitochondrial membrane potential leading to decreased survival of oligodendrocyte precursor cells (Yao et al., 2012). Previous studies in the Ratner lab have shown an increase in all three NOS proteins in white matter tracts after adult induced Nf1 loss or E12.5 HRasG12V expression in oligodendrocytes. The NOS inhibitor L-NAME significantly improves the myelin decompaction phenotype driven by embryonic onset of hyper-activation of HRas in oligodendrocytes as well as a behavioral phenotype (i.e. increased response to startle) (Mayes et al., 2013).

Low basal levels of nitric oxide protects oligodendrocytes through inhibition of peroxynitrite-induced ERK phosphorylation, however, elevated levels of NO are detrimental as NO reacts with superoxide to form toxic peroxynitrite (Li et al., 2011). Ras-GTP and p-ERK are elevated in hemizygous and homozygous Nf1 mutant glial precursors (Bennett et al., 2003). Thus, p-ERK may influence NO/peroxynitrite levels and cellular outcomes. Several lines of evidence have suggested key roles for reactive oxygen and nitrogen as upstream regulators and downstream effectors of Ras signaling (Ferro et al., 2012) as previous studies have shown Ras
signaling, through the downstream effectors MEK/ERK, upstream of ROS generation (Heimfarth et al., 2013).

Previous studies in the Ratner lab show that oligodendrocyte specific increases in Ras signaling, due to adult induced Nf1 loss or hyperactive HRas signaling from E12.5 on, cause changes in tight and gap junction proteins within myelin. Claudins control paracellular transport selectivity and are regulated by environmental cues, such as NO (Angelow et al., 2008). Reduction in the tight junction protein claudin-11 and mis-localization of the gap junction protein Cxn32 occurred in oligodendrocytes in Nf1 and HRas mutants underlying myelin decompaction and was reversed by anti-oxidant treatment (Mayes et al., 2013).

Cross talk of nitric oxide and Notch signaling has been well characterized for the cells of the perivascular niche. Previous studies support the importance of NO signaling and Notch pathway activation in the maintenance of the cancer stem cell phenotype in the perivascular niche during gliomagenesis. Perivascular nitric oxide activates Notch signaling and promotes stem-like character in PDGF-induced glioma cells. This is evidence that NO can act upstream of Notch. In PDGF induced glioma cells; exposure to the NO donor (GSNO) resulted in an increase in Notch intracellular domain (NICD) required for transcription of downstream target genes (Charles et al., 2010). Notch signaling is required for NO enhancement of the phenotype. Suppression of NO activity \textit{in vivo} decreases Notch signaling/phenotype and increases survival (Charles et al., 2010). However, cross talk of NO and Notch signaling in the oligodendrocyte cell lineage is not well understood. We set out to further define the role of NO signaling in myelin compaction.

As described, the role of Notch and NO downstream of Ras GTPase and the cross talk between Notch-NO-Ras in CNS myelin compaction in gain of function Rasopathies warranted
further investigation. **The central hypothesis** of my thesis was that aberrant CNS glial cell proliferation, differentiation, and/or myelination in gain of function Rasopathies, such as NF1 and Costello syndrome, results from altered activation of the Notch pathway downstream of Ras in the oligodendrocyte cell lineage. **The underlying goal** of my research was to define Notch and NO downstream of Ras GTPase and the cross talk between Notch-NO-Ras in CNS myelin compaction and to identify and test therapeutic targets for CNS abnormalities.
Figure 1.1 The Ras GTPase Pathway

Receptor tyrosine kinase (RTK), Growth factor receptor bound protein 2 (GRB2), Guanosine diphosphate (GDP), Guanosine triphosphate (GTP), Guanine exchange factor (GEF), Son of sevenless (SOS), GTPase activating protein (GAP), Neurofibromin (NF1).
Figure 1.2 Canonical Notch Pathway Signaling

Notch extra cellular domain (Notch ECD), A disintegrin and metalloprotease (ADAM), Transmembrane intracellular domain (TMICD), Notch intracellular domain (NICD), Mastermind (MAM), Recombination signal binding protein 1 for J-kappa (RBPJ), Repressor (Rep.).
Chapter 2

Materials and Methods
2.1 Cellular and Molecular Analysis

- **Gene Expression**: Ninety percent confluent embryonic day 12.5 murine spinal cord mixed glial precursor cultures were prepared from wild type and *Nf1*⁻/⁻ mutant embryos, and maintained in serum-free medium with N2 and B27 supplements on laminin coated dishes at 37°C, then stimulated for 5 min. with FGF2 as described (Bennett et al., 2003). Total RNA was isolated from passage 2-3 cells using Trizol reagent (Invitrogen, San Diego, CA) followed by phenol/chloroform extraction and ethanol precipitation. RNA integrity was verified with an Agilent Bio analyzer 2100, and cDNA synthesized (Superscript cDNA synthesis kit, Invitrogen, San Diego, CA) from 10μg RNA using an oligo(dT) primer. Biotinylated double stranded product was hybridized to Affymetrix MOE430A Gene Chip mouse oligonucleotide arrays. Microarray gene expression data was processed using Genespring 6.1 (Silicon Genetics, Redwood City, CA). To identify deregulated transcripts, genes that were ≥2 fold up or down regulated in *Nf1*⁻/⁻ cultures as compared to wild type were identified by Student’s *t*-test. (p<0.05). Twelve genes were identified, of which *Hes5* showed the most up-regulation.

- **qRT-PCR**: qRT-PCR was carried out on glial progenitor RNA from E12.5 spinal cord cultures (Bennett et al., 2003) in triplicate using primers for *DII-1* R2409 =CCATAGTGCAATGGGAACAAC/ L2229 = CAAGGATATAGCCCGATGA, *Dll-3* R= CTACAGCGGTCCACCCTCTT, L919 =ATTCTACGCGGCTTCGATGTG, and *Hes5* R=AGAGGGTGGGCCCTGATTA, L= AGGATGAGCTCGTTCCCTCTG. Mouse *Gapdh* primers were used for normalization, and the delta-delta Ct method used for quantification.
Western Blot: E12.5 murine spinal cord cultures treated with FGF2 for 5 min. (Bennett et al., 2003) were lysed in RIPA. Cell sample lysates were sonicated and clarified through centrifugation. Protein (50-100 µg) was separated on SDS-polyacrylamide gradient gels (ISC BioExpress) through electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were probed with rabbit anti-Notch1 antibody (Santa Cruz C20R; sc-6014-R) at 1:200. Anti-Ras10 (Upstate Biotechnology) was used as a loading control. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were used to detect signal with Enhanced Chemiluminescence (ECL) Plus developing system (Amersham Biosciences).

2.2 Murine Models and Analysis

- **Mouse husbandry:** Cincinnati Children's Hospital Research Foundation animal care and use committee approved all animal use. Mice were housed in a temperature- and humidity-controlled vivarium on a 12 hour light-dark cycle with free access to food and water.

- **Mouse Strains:** All mice were maintained on a C57Bl/6 background. Male Gt(Rosa)26Sor\(^{tm1(Notch1)DAM/J}\) (RosaNICD) mice were mated to female tamoxifen-inducible B6.Cg-Tg(Plp1-Cre/ERT)3Pop/J (iPlpCre) mice for both male and female iPlpCre;RosaNICD offspring. Male Nf1\(^{tm1Par/J}\) (Nf1flox) mice were mated to female iPlpCre mice. For behavioral testing, only female mice were used, and these were on a (n=12) backcross to C57BL/6. For other experiments, mice were maintained on largely C57BL/6 background. Tg(Hes5-EGFP)1Gsat/Mmmh (Hes5GFP) mice were used to report recent canonical Notch signaling. Female iPlpCre;Nf1fl/+ breeders were mated to
male Hes5GFP,Nf1flox mice to generate female Hes5GFP,iPlpCre;Nf1flox offspring. Female iPlpCre;Nf1fl/+ breeders were mated to male Rbpjfllox,Nf1flox mice to generate both male and female iPlpCre;Nf1flox,Rbpjfllox offspring. Female iPlpCre;HRasG12V/+ breeders were mated to male Hes5GFP,HRasG12V mice to generate female Hes5GFP,iPlpCre;HRasG12V offspring. Female iPlpCre;HRasG12V/+ breeders were mated to male Rbpjfllox,HRasG12V mice to generate both male and female iPlpCre;HRasG12V,Rbpjfllox offspring. Mice were genotyped by PCR as described for each strain (Brannan et al., 1994; Chen et al., 2009; Doerflinger et al., 2003; Gong et al., 2003; Han et al., 2002; Murtaugh et al., 2003; Zhu et al., 2002). Mice were euthanized when they became paralyzed, failed to groom, had obvious weight loss, or developed tumor masses. iPlpCre;Nf1fl/fl mice, older than 6 months required euthanasia if they developed reactive hyperplasia of hematopoietic cells or peripheral neurofibromas (Mayes et al., 2011).

- **Adult Tamoxifen Injections:** 100ul tamoxifen (1mg/100ul of sunflower seed oil) was administered at 8-10 weeks of age I.P., twice daily for 3 consecutive days, to drive recombination in the inducible PlpCreER\(^T\).

- **Behavior:** Behavioral assessments were completed on iPlpCre; Nf1 fl/+ (breeders 12\(^{th}\) generation backcrossed onto C57BL/6) mice. Acoustic startle response, with prepulse inhibition, was assessed as described with modification (Vorhees et al., 2011). White noise background of 70 dB, prepulses of 73, 77, and 82 dB, and a mixed frequency startle stimulus of 120 dB were used. Animals received 100 trials per day with equal numbers of trials using a Latin square design that was duplicated. Animals were tested on two consecutive days and the data from the second day were analyzed, since animals that are
tested on a single day for the acoustic startle response tend to have greater variability that likely masks group differences.

- **Pre-Clinical Therapeutics:** Gamma secretase inhibitor (GSI; MRK-003 from Merck) was made fresh weekly and dosed at 300 mg/kg in 0.5% methocel by oral gavage (Sparey et al., 2005; Lewis et al., 2007; Tammam et al., 2009). Maximum drug concentrations are present 4 hours post treatment and the drug is measurable up to 72 hours post treatment (Chu et al., 2013). For pathology, we dosed mice once weekly for 4 weeks, and sacrificed then 6 hours after the last dose (n=5 doses). For flow analysis, we dosed mice once weekly for 1 week, and sacrificed mice 6 hours after the last dose (n=2 doses). MEK inhibitor (PD0325901 from Pfizer) was made fresh weekly and dosed at 1.5 mg/kg in .5% methocel/.2% Tween 80 by oral gavage. For pathology, we dosed mice every day for 3 weeks. For flow analysis, we dosed mice every day for 8 days. Fresh solution of L-NG-Nitroarginine Methyl Ester (L-NAME) at 0.4mg/kg (100μM in 1X PBS) was administered daily. For pathology and flow analysis, mice were injected intraperitoneally (IP) daily with 100μL for 7 days and sacrificed 6 hours after the last dose.

### 2.3 Tissue Preparation, Imaging, and Analysis

- **Tissue processing:** We administered BrdU I.P. (50 mg/kg body weight) 3X at 2 hour intervals. Two hours later, we anesthetized mice and perfused with 0.9% saline followed by ice cold 4% paraformaldehyde. Tissues were removed and post-fixed in 4% paraformaldehyde overnight with transfer to 20% sucrose for frozen sectioning.

- **Antibodies:** For western blotting, a rabbit anti-Notch1 antibody (Santa Cruz C20R; sc-6014-R) was used at 1:200 and anti-Ras10 (Upstate Biotechnology) was used as a
loading control. Cryostat sections were air dried and processed for immunohistochemistry, using markers for GFP (GFP 1:2,000; Millipore), glial progenitor cells and immature oligodendrocytes (NG2 1:500, Millipore #AB5320; PDGFRα 1:200, Santa Cruz #SC338), oligodendrocytes (Olig2 1:500, Millipore #AB9610; CC1 1:2,000, Calbiochem), astrocytes (GFAP 1:500, Millipore #MAB360 or 1:1,000 DAKO), microglia (Iba1 1:2,000, Wako #019-19741), and neurons (NeuN 1:500, Chemicon #MAB377). Anti-BrdU (1:200, Abcam #6326) was used to determine entry into S-phase. Markers for fluorescent secondary antibodies (Chicken Alexa 488 and Rabbit or Mouse Alexa 594; Invitrogen, Carisbad, CA) were used at 1:800. Fluoromount G was used to mount fluorescent sections.

- **Imaging Analysis:** Fluorescent images were captured on a Zeiss Axiovert 200M microscope using a 40x Plan-NEO FLUAR objective with filters (405, 488, 565) and ImageJ software or a Nikon C2 Confocal using a 20X objective (2X Zoom for 40X) with lasers (405, 488, 561) and Nikon Elements Software. Counting was performed on 300-600 cells/area/animal in 3-5 animals/genotype using an ImageJ micromanager plugin and Imaris Software. In Imaris, quantification with “Dots” marked objects based on size to exclude cellular processes. For NG2+ cells, additional disqualification used morphology to exclude blood vessel pericytes.

- **Electron Microscopy:** We perfused mice with 4% paraformaldehyde and 2.5% glutaradehyde, post–fixed in the same fixative overnight, and then the corpus callosum was transferred to 0.175M cacodylate buffer, osmicated, dehydrated and embedded in Embed 812 (Ladd Research Industries, Burlington, VT). Ultrathin sections were stained
in uranyl acetate and lead citrate and then photographed on a Hitachi Model H-7600 microscope.

- **Myelin Analysis:** To quantify severity of decompaction, we first rated each myelin sheath as compact or decompact, and calculated the percent of decompact fibers out of total myelinated fibers. A decompact fiber was defined as a fiber with disruption of the myelin sheath (i.e. splitting of myelin lamellae). In a second analysis, we quantified severity of decompaction, rating decompact myelinated fibers as having 1 (25%), 2 (25-50% disrupted), 3 (50-75% disrupted), or all 4 (>75% disrupted) quadrants of the sheath disrupted. We measured g-ratios of all myelinated fibers from electron micrographs (Figure 2.1), by dividing the diameter of each axon by the fiber diameter (diameter of the axon together with its myelin sheath) in 300-500 axons/animal (n=3–5 mice/genotype), using ImageJ software. When a fiber was identified as compact or 4 quadrants decompact, 1 measurement was collected for g-ratio. When a fiber was identified as 2 quadrants or 1 quadrant decompact, 2 or 4 measurements were collected for g-ratio. Quantification was carried out by blinded individuals (SHS, MB).

- **Flow Cytometry:** Adult murine brains were processed for flow cytometry as described (Robinson et al., 2014). Brains were perfused with 30 ml PBS and then dissected, roughly chopped, and incubated in 1 ml Accutase (Millipore) for 30 min at 37°C. After addition of 10% fetal bovine serum (FBS) in Hank’s balanced salt solution (2 ml), the brains were manually triturated with a transfer pipette, transferred to cell strainers (100 mm; BD), and gently processed through the filter using 3 ml syringe plungers. Cell suspensions were centrifuged (3 min 3 480 g), washed with 10 ml buffer (DPBS, 2% FBS, 2 mM EDTA), and recentrifuged. To purify cells from myelin debris, cells were
resuspended in 40% Percoll (Amersham) HBSS and centrifuged at 650 g for 25 min at room temperature. The myelin top layer was aspirated and mononuclear cells were resuspended in buffer. Fc receptors were blocked by incubation with anti-mouse CD16/32 (1 mg/sample; eBioscience) on ice for 10 min. After washing in buffer and incubation in primary antibodies for 1 hr at 4°C, the cells were washed in buffer and resuspended in 200 ml buffer for analysis. Flow cytometry was performed using FacsDiva software for acquisition on an LSR II flow cytometer using UV (355nm), violet (405 nm), blue (488 nm), red (640 nm), and yellow-green (561 nm) lasers. Antibodies recognizing glial progenitors (PDGFRα 1:50; Millipore), immature oligodendrocytes (O4 1:100; Millipore), mature oligodendrocytes (GalC 1:100; Millipore), astrocytes (GLAST 1:10; Miltenyi Biotec), endothelial cells (ICAM-1/CD54 1:200; Biolegend), and blood/microglia (CD45 1:100; BD Biosciences) were used. DCF-DA FITC (1:200; Invitrogen) and Cell ROX Deep Orange or Deep Red (1:1,000; Invitrogen) stained for total reactive oxygen species (ROS). ENZO life sciences kits were used to detect specifically Nitric Oxide (ENZ-51013-200) and Super Oxide / Reactive Oxygen Species (ENZ-51010). Importantly, a positive control (L-arginine, a nitric oxide synthase substrate) and a negative control (C-PTIO, a nitric oxide scavenger) validated staining specificity for nitric oxide (Kalyanaraman et al., 2012). An inducer (pyocyanin) and inhibitor (n-acetyl cysteine) were used for the super oxide/ reactive oxygen species positive and negative controls for compensation. We used live/dead cell stain (1:1,000; Invitrogen) and Annexin V (1:20, Life technologies) to detect apoptosis. For staining pERK 1/2 (1:50, BD Biosciences), cells were fixed and permeabilized. Analysis was performed using FlowJo10 software. Compensation was completed using positive and
negative controls as well as fluorescent minus one (FMO) samples. Initial analysis was performed to gate on single (Forward Scatter - FSC vs. Side Scatter - SSC), then on live (Dead Cell Stain Negative), and then on CNS resident (CD45-) cells, excluding microglia (CD45\text{low}) and hematopoietic cells (CD45\text{high}). Further analysis on live CNS resident cells through gating was performed to quantify glial progenitors (PDGFRα+, O4-), immature OL’s (O4+, GalC-), mature OL’s (O4+, GalC+), and myelinating OL’s (O4-, GalC+) (Figure 2.2). To quantify reactive species, geometric means were quantified for all CNS resident cells and for each of the subsets of the glial cell lineage per sample (n=3/genotype/experiment).

2.4 Statistics

- **Statistical Analysis:** Comparison between two groups (i.e. genotypes, treatments) was done via Student’s t tests using a significance cutoff of p < 0.05. For comparison of three or more groups, one-way ANOVA followed by Tukey post hoc tests were performed using a significance cutoff of p < 0.05. For behavior, acoustic startle was analyzed by mixed linear ANOVA with factors of genotype and trial. Further comparisons averaged across interval, day, and trial for genotype differences were compared by (two-tailed) t-test for independent samples. Kaplan Meier survival curves were created using GraphPad Prism software and Log-rank Mantel-Cox Tests.
Figure 2.1 Axon and myelin sheath, g-ratio analysis. Schematic representation of a cross section of an axon (white) wrapped with myelin (grey). Measurements required to calculate g-ratio include diameter of the axon (d) and diameter of the axon + myelin (D).
Figure 2.2 Oligodendrocyte lineage cell development. As an early oligodendrocyte progenitor cell (OPC) matures into a myelinating oligodendrocyte, the proteins produced by the cell change in a characteristic pattern. By investigating many proteins simultaneously in individual cells, we can distinguish oligodendrocyte lineage cells at different stages of maturation. *Original cartoon, courtesy of Dr. Andrew Robinson of Northwestern University.*
Chapter 3

*Nf1* loss in Oligodendrocytes Induces Myelin Decompaction

through Notch Signaling and Nitric Oxide
3.2 Introduction

Oligodendrocytes and neurons cooperate for proper central nervous system (CNS) function. Oligodendrocyte precursor cells (OPCs) differentiate and mature into myelinating oligodendrocytes that make compact myelin around axons, enabling salutatory conduction and increased conduction velocity (Franklin and Gallo, 2014). Defective myelin sheaths alter neuronal function, while destruction of myelin sheaths causes progressive axonal degeneration (Miron et al., 2011). Signaling pathways controlling myelin formation and stability are of great interest as learning and motor skills are linked to changes in myelin (Fields, 2011; McKenzie et al., 2014).

Ras-GTP activates MAPK-ERK signaling (Vigil et al., 2010), which is critical for oligodendrocyte function. MAPK-ERK activation drives OPC specification and proliferation (Fyffe-Maricich et al., 2011; Li et al., 2012; Guardiola-Diaz et al., 2012), and extra myelin wraps are observed in oligodendrocytes expressing activated ERK (Ishii et al., 2013). In human Rasopathies, mutations in Ras-MAPK pathway genes compromise brain function (Rauen, 2013). The Rasopathy Neurofibromatosis type 1 (NF1) results from NF1 gene mutation/loss, resulting in elevated Ras-GTP due to absence of the NF1 protein, neurofibromin, which negatively regulates Ras (Vigil et al., 2010; Donovan et al., 2002). NF1 patients have learning deficits, delayed acquisition of motor skills, enlarged brain white matter tracts, and/or MRI T2 hyper-intensities that may reflect demyelination or dysmyelination (Gripp et al., 2010; Hyman et al., 2007; DiPaolo et al., 1995). Myelin relies on regulated levels of Ras-GTP, as Nf1 loss or hyper-activation of H-Ras-GTP in oligodendrocytes cause myelin decompaction in year-old mice (Mayes et al., 2013). Intriguingly, in addition to Ras-induced oncogenic stress, perinatal hypoxia
results in myelin decompaction (Jablonska et al., 2012). Injured oligodendrocytes can express iNOS/NOS2 (Boullerne and Benjamins, 2006) and/or nNOS/NOS1 (Yao et al., 2010; Yao et al., 2012) producing nitric oxide (NO), and the NOS inhibitor L-NAME significantly improves the H-RasG12V driven decompaction phenotype (Mayes et al., 2013). Here we asked whether the Notch pathway also contributes to pathological myelin decompaction subsequent to oligodendrocyte Nf1 loss.

In canonical Notch signaling, Notch receptors are activated by ligands (Dll-1,2,4 and/or Jagged-1/-2), causing Notch proteolysis that releases the Notch intracellular domain (NICD), which translocates to the nucleus. A complex of NICD with mastermind (MAM) and recombining binding protein suppressor of hairless (RBPJ) activates gene transcription (Borggrefe and Liefke, 2012). Canonical Notch signaling promotes OPC specification (Grandbarbe et al., 2003; Park and Appel, 2003), but subsequently inhibits OPC differentiation to myelinating oligodendrocytes, likely by inhibiting myelin gene expression via Hes5 (Liu et al., 2006; Zhao et al., 2010). Blocking canonical Notch increased oligodendrocyte ensheathment of axons (Watkins et al., 2008). Possible roles of Notch signaling, via Hes5 and/or other Hes/Hey Notch effectors, on myelin structure remain unstudied.

Aberrant Notch signaling occurs in neurodegenerative disease (Jurynczyk and Selmaj, 2010) and Ras-driven tumors (Mazur et al., 2010a; Baumgart et al., 2014). Notch signaling inhibitors, including gamma secretase inhibitors which prevent Notch cleavage, are being tested therapeutically (Lathia et al., 2008). Ras-GTP can act up- and/or down-stream of Notch pathway signaling (Sundaram, 2005). Here we show that Nf1 loss in oligodendrocytes causes myelin decompaction within a month and identify nitric oxide and canonical Notch signaling as potential therapeutic targets in NF1 patients.
3.3 Results

*Nf1* loss increases canonical Notch pathway activity in oligodendrocyte lineage cells.

To identify signaling pathways downstream of *Nf1* that might affect oligodendrocyte development, we initially carried out gene expression analysis using glial progenitor cultures (wild type; n=3 and *Nf1*-/-; n=2) cultures containing Nestin+, A2B5+, E-NCAM+ and NG2+ progenitors as described (Bennett et al., 2003). Twelve genes were differentially expressed in *Nf1*-/- cultures (p<0.05). Of note, expression of *Hes5*, a target of the canonical Notch signaling pathway, was significantly increased in *Nf1*-/- cells versus wild type cells (Figure 3.1A). We confirmed expression of *Hes5*, a direct transcriptional target of the NICD/MAM/RBPJ complex (canonical Notch signaling), and other mRNAs encoding Notch pathway members, in these glial progenitors using quantitative RT-PCR. Expression of *Hes5*, *delta like ligand 1* (*Dll1*), and *delta like ligand 3* (*Dll3*) significantly increased in mutants (Figure 3.1A). In western blots there was an increase in activated Notch1 (Weijzen et al., 2002) in *Nf1*-/- as compared to wild type cultures (Figure 3.1B).

To test if Notch signaling is relevant to the myelin decompaction phenotype elicited by loss of *Nf1* we used the proteolipid protein CreER\(^T\) (iPlpCre) murine model. In this model, after tamoxifen exposure, 35% of mature oligodendrocytes (100% Olig2+, a 75-80% Olig2+,CC1+) in the corpus callosum show recombination (Mayes et al., 2013). Essentially no NG2+ cells show recombination, 1 or 4 days or 8 weeks post-tamoxifen, as measured in iPlpCre;CMV-ß-actin-loxP-EGFP or YFP mice (Mayes et al., 2013). To test whether loss of *Nf1* leads to canonical Notch pathway activation in white matter, we analyzed iPlpCre;Nf1fl/fl mice that expressed a transgenic *Hes5GFP* reporter allele. In these mice, eGFP expression reflects recent Hes5-driven
Figure 3.1. *Nf1* loss or constitutive activation of Notch signaling causes myelin decompaction within one month.

(A) Fold change from microarray analysis of gene expression (Hes5, p<0.0001; Dll1, p=0.0008, Dll3, p=0.0006) and qRTPCR analysis of gene transcription (Hes5, p<0.0001; Dll1, p<0.0001, Dll3, p<0.0001) of *Nf1* mutant and wild type glial progenitors (*** : p<.001, **** : p<.0001).

(B) Western blot of Notch 1 and Ras10 (loading control) in Nf1-/- and wild type glial progenitor cultures. An increase in activated (97kb) Notch 1.

(C) Timeline of adult tamoxifen inducible recombination murine model and analysis.

(D) Quantification of fold change with standard deviation (SD) compared to wild type of Hes5GFP+, recent canonical Notch pathway signaling, in the oligodendrocyte cell lineage in the forebrain as measured by immunofluorescence of unfixed cells. Increase in *Nf1* mutant as compared to wild type O4+;GalC+ mature oligodendrocytes (p=0.0016, ** ; p<0.01). There was a not a significant change in *Nf1* mutant as compared to wild type PDGFRα+ (p=0.07), O4+;GalC- (p=0.08), or O4-;GalC+ (p=0.3087) oligodendrocyte lineage cells (n=3/genotype).

(E) Quantification of average with standard error of the mean (SEM) of Hes5GFP+, recent canonical Notch pathway signaling, in the oligodendrocyte cell lineage (NG2+, PDGFRα+ and CC1+) in the corpus callosum as measured by immunofluorescence of fixed cryostat sections. Increase in *Nf1* mutant as compared to wild type (n=5/genotype, p=0.02, * ; p<0.05).

(F) Quantification of average (with SEM) of g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of *Nf1* mutant and in NICD mutant as compared to wild type mice (**** ; p<0.0001). No significant difference in g-ratio between *Nf1* and NICD mutants (n= 5,4/genotype, p=0.4545).

(G) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Increase in percent decompaction and severity of decompaction.
in \textit{NfI} (p=0.003) and \textit{NICD} (p=0.007) mutants as compared to wild type mice. There was no significant difference in percent decompaction between \textit{NfI} and \textit{NICD} mutants (n= 5,4/genotype, p=0.3525), however, \textit{NfI} mutants had fibers with 4 quadrants (>75%) decompaction.

(H) Electron micrographs of corpus callosum cross sections at the mid line (top: 30,000X / bottom: 50,000X). Left: Wild Type, Middle: \textit{NfI} mutant, Right: \textit{NICD} mutant. White arrows: areas of myelin decompaction. Red Boxes: Myelin Lamellae (Solid, magnified).
canonical Notch pathway signaling. This allele is not under control of the Cre driver, so that canonical Notch signaling is detectable in all cells where Hes5 is expressed.

We dissociated live cells from adult brain, and then analyzed Hes5GFP+ cells by flow cytometry. Numbers of Hes5GFP+;O4+;GalC+ pre-myelinating oligodendrocytes increased significantly 1 month after tamoxifen treatment (3.14 fold; p=0.002) in Nf1 mutant versus wild type forebrain, while numbers of Hes5GFP+;O4-;GalC+ (mature myelinating oligodendrocytes) in the forebrain were unaltered (p=0.309) (Figure 3.1D). Hes5GFP+;PDGFRα+ (progenitors) and O4+;GalC- (immature oligodendrocytes) cells were also not significantly different Nf1 mutant versus wild type forebrain, although they increased by 1.62 fold (p=0.07) and 2.06 fold (p=0.08) in mutant mice (Figure 3.1D). Thus, Hes5 expression increases in Nf1 pre-myelinating forebrain oligodendrocytes not mature oligodendrocytes. We note that Hes5-independent Notch effectors (i.e. Hes or Hey proteins) may transduce Notch signals in mature oligodendrocytes.

To determine if Hes5GFP+ oligodendrocyte lineage cells increase in mutant white matter, we analyzed the corpus callosum in tissue sections. There is a 43% increase in Hes5GFP+ total oligodendrocyte lineage cells (NG2+, PDGFRα+, and CC1+ cells; p=0.02; Figure 1E), accounted for by an increase in NG2+;Hes5GFP+ progenitors (p=0.021). Several studies have identified recombination in subset of SVZ progenitors, but not white matter NG2 cells as long as 8 weeks after tamoxifen in adult mice (Koenning et al., 2012; Mayes et al., 2013). Effects on NG2 cells may be non-cell autonomous, possibly in response to aberrant mutant oligodendrocytes. There was no significant difference between the percent of Hes5GFP+;PDGFRα+ cells or Hes5GFP+;CC1+ cells in mutants. Taken together with the flow cytometry data, we identified a selective increase in Hes5+ progenitors and immature oligodendrocytes. Increased Hes5 in NG2+ cells may result from response to aberrant mutant
oligodendrocytes. Despite alterations in progenitor/immature oligodendrocyte number, these data do not exclude Notch effects in more mature oligodendrocytes, independent of Hes5.

**Nf1 loss in oligodendrocytes causes myelin decompaction within one month.**

White matter tracts examined 6-12 months after induction of Nf1 loss in iPlpCre;Nf1fl/fl or Nf1fl/+ mice showed decompaction of myelin sheaths at intraperiod lines, the closely apposed outer oligodendrocyte membranes in compact myelin (Mayes et al., 2013). We asked if myelin decompaction is present shortly after adult-induced loss of Nf1 in oligodendrocytes, when mature oligodendrocytes are the main cell type affected by iPlpCre. We examined brains of 3 month old iPlpCre;Nf1fl/fl mice exposed to tamoxifen at 2 months of age, and wild type iPlpCre littermates (Figure 3.1C) using electron microscopy (Figure 3.1H). Unbiased counting of myelinated axons in the corpus callosum revealed a significant decrease in g-ratio (axon diameter/fiber diameter), indicating an increase of myelin thickness, in Nf1 mutant compared to wild type mice (Figure 3.1F). Increased myelin thickness was due to decompaction of the myelin lamellae (Figure 3.1H, red boxes). Both the total number of fibers with decompaction and the severity of decompaction, as measured by quadrants around the axon with decompacted myelin, were increased in Nf1 mutant compared to wild type mice (Figure 3.1G). Thus, the myelin decompaction characteristic of the iPlpCre;Nf1fl/fl model is present by 1 month after tamoxifen administration.

**Constitutive Notch signaling in oligodendrocytes causes myelin decompaction.**

Given that the number of Hes5GFP+;O4+;GalC+ pre-myelinating oligodendrocytes but not Hes5+ mature oligodendrocytes in the forebrain significantly increased in Nf1 mutants (Figure
1D), we directly tested if constitutive Notch activation in mature oligodendrocytes disrupts myelin compaction. We mated RosaNICD mice, which constitutively activate Notch signaling after recombination, with iPlpCre mice (Figure 3.1C). Using electron microscopy, we examined brains of iPlpCre;RosaNICD mice 1 month after tamoxifen administration (Figure 3.1H). Analysis of myelinated axons in the corpus callosum revealed a significant decrease in g-ratio indicating an increase in myelin thickness of NICD mutant compared to wild type mice (Figure 3.1F). The increase in thickness in NICD mutants was due to decompaction of myelin lamellae (Figure 3.1G,H red boxes). Additionally, there was an increase in the total number of fibers with decompaction and the severity of decompaction in NICD mutants compared to wild type mice. However, the phenotype was not as severe in the Nf1 mutant, in that fewer quadrants of individual myelin sheaths were affected (Figure 3.1G).

**Concurrent loss of Rbpj reduces myelin decompaction in Nf1 mutants.**

Recombining binding protein suppressor of hairless (RBPJ) is a transcriptional co-factor required for canonical Notch signaling. To test if Notch signaling is necessary for the Nf1 mutant phenotype, we analyzed iPlpCre:Nf1fl/fl,Rbpjfl/fl mice at 3 months of age, 1 month after tamoxifen administration (Figure 3.2E). To verify that canonical Notch pathway signaling is inhibited in vivo, we crossed iPlpCre;Rbpjfl/fl mice with Hes5GFP reporter mice. Using flow cytometry of forebrain cells at one month post tamoxifen induced recombination, canonical Notch pathway activity, as measured by numbers of Hes5GFP+ cells, decreased by 45% (p=0.016) in Hes5GFP,iPlpCre;Rbpjfl/fl compared to wild type mice. Analysis of myelinated axons in the corpus callosum using electron microscopy (Figure 3.2A,B) revealed a significant increase in g-ratios, indicating a partial correction of aberrant myelin thickening, in
**Figure 3.2. Concurrent loss of *Rbpj* improves myelin compaction in *Nf1* mutants.**

(A) Electron micrographs of corpus callosum cross sections at the mid line of *iPlpCre;Nf1fl/fl* mice (left: 30,000X / right: 50,000X). White arrows: areas of myelin decompaction.

(B) Electron micrographs of corpus callosum cross sections at the mid line of *iPlpCre;Nf1fl/fl;Rbpjfl/fl* mice (left: 30,000X / right: 50,000X).

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of *iPlpCre;Nf1fl/fl* and in *iPlpCre;Nf1fl/fl,Rbpjfl/fl* as compared to wild type mice (**** ; p<0.0001). Significant increase in g-ratio in *iPlpCre;Nf1fl/fl,Rbpjfl/fl* as compared to *iPlpCre;Nf1fl/fl* mice (n= 5,4/genotype, p=0.001, ** ; p<0.01).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). There was no significant difference in percent decompaction in *iPlpCre;Nf1fl/fl,Rbpjfl/fl* as compared to *iPlpCre;Nf1fl/fl* mice (n= 5,4/genotype, p=0.242), however, there was a decrease in fibers with 4 quadrants (>75%) decompaction.

(E) Intracellular depiction of *iPlpCre;Nf1fl/fl,Rbpjfl/fl* murine model after adult tamoxifen induced recombination.
*iPlpCre;Nflfl/fl,Rbpjfl/fl* mice compared to *iPlpCre;Nflfl/fl* mice (Figure 3.2C). This was due to a partial improvement in myelin compaction (Figure 3.2A,B) and a decrease in severity of decompaction in *PlpCre;Nflfl/fl,Rbpjfl/fl* compared to *PlpCre;Nflfl/fl* mice (Figure 3.2D). We conclude that RBPJ-dependent Notch signaling acts downstream of *Nfl* in oligodendrocytes to regulate myelin compaction.

**Hemizygous loss of *Rbpj* is sufficient to improve myelin compaction in homozygous *Nfl* mutants and loss of *Rbpj* is detrimental in wild type mice.**

Notably, adult-induced homozygous loss of RBPJ-dependent Notch signaling in wild type mice 1 month after tamoxifen, significantly increased myelin thickness and severity of myelin decompaction (Figure 3.3A,B). These data indicate that a constitutive imbalance of Notch activation, hypo- or hyper- activation, can cause defects in myelin compaction.

*iPlpCre;Nflfl/fl* mice show a severe chronic disrupted myelin compaction phenotype. Interestingly, hemizygous loss of *Rbpj* rescued myelin compaction at 6 months, but not at 1 month after tamoxifen (Figure 3.3C,D), suggesting that *Nfl* loss-driven decompaction becomes increasingly dependent on Notch signaling. These data further support that RBPJ-dependent Notch signaling acts downstream of *Nfl* in oligodendrocytes to regulate myelin compaction.
**Figure 3.3.** Hemizygous loss of *Rbpj* is sufficient to improve myelin compaction in homozygous *Nf1* mutants and loss of *Rbpj* is detrimental in wild type mice.

(A) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of *iPlpCre*Rbpjfl/+ and *iPlpCre*Rbpjfl/fl mutants as compared to wild type mice (n=3/genotype, ****; p<0.0001).

(B) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). There was a significant difference in % decompaction in of *iPlpCre*Rbpjfl/+ and *iPlpCre*Rbpjfl/fl mutants as compared to wild type (n=3/genotype, p=0.004, p=0.006).

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of *iPlpCre*Nf1fl/fl,Rbpjfl/+ as compared to *iPlpCre*Nf1fl/fl mice at 3 months (p=0.022, *; p<0.05). Increase in g-ratio in *iPlpCre*Nf1fl/fl,Rbpjfl/+ as compared to *iPlpCre*Nf1fl/fl mice at 8 Months (n= 5/genotype, ****; p<0.0001).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). There was no significant difference in percent decompaction in *iPlpCre*Nf1fl/fl,Rbpjfl/+ as compared to *iPlpCre*Nf1fl/fl mice at 3 months (n= 5/genotype, p=0.836), however, there was a decrease difference in percent decompaction (p=0.004; **; p<0.01) in *iPlpCre*Nf1fl/fl,Rbpjfl/+ as compared to *iPlpCre*Nf1fl/fl mice at 8 months.
**Gamma secretase inhibitor (MRK-003) partially rescues severity of myelin decompaction in Nf1 mutants.**

Gamma secretase inhibitors (GSI) prevent cleavage of the Notch intracellular domain and therefore prevent downstream transcription of target genes (Figure 3.4). To test if decompaction can be acutely improved by inhibiting Notch signaling, we administered GSI MRK-003, at 3 months of age when myelin decompaction is evident (Figure 3.5A). MRK-003 crosses the blood brain barrier and blocks canonical Notch signaling as measured by total Hes5 mRNA (Chu et al., 2013). One month of weekly MRK-003 exposure in iPlpCre;Nf1fl/fl mice significantly decreased canonical Notch pathway activity, as measured by the number of Hes5GFP+ cells in the corpus callosum (Figure 3.5B). We also measured Hes5GFP+ cells by flow cytometry. Reductions in Hes5GFP+ cells in the forebrain were noted at 6 hours (50%, p=0.028), 12 hours (63%, p=0.002), and 18 hours (45%, p=0.008) post treatment with GSI MRK-003. Prolonged efficacy correlates with the finding that MRK-003 can be detected in blood up to 72 hours after adult mouse dosing (Chu et al., 2013). Using electron microscopy, we examined brains of iPlpCre;Nf1fl/fl mice. Analysis of the corpus callosum revealed a significant increase in g-ratio, indicating a decrease in myelin thickness, in MRK-003 treated mice compared to vehicle-treated iPlpCre;Nf1fl/fl mice (Figure 3.5C,D). We identified both a decrease in the total number of fibers with decompaction and a decrease in the severity of decompaction in MRK-003 treated mice compared to vehicle-treated iPlpCre;Nf1fl/fl mice (Figure 3.5E). Importantly, wild type mice treated with MRK-003 did not show altered myelin thickness or myelin decompaction compared to vehicle treated controls (Figure 3.5C-E). These results support the conclusion that pharmacological inhibition of Notch signaling improves myelin decompaction caused by Nf1 loss, even after the phenotype has manifested in vivo.
Figure 3.4. Mechanism of Gamma Secretase Inhibitor

Notch extra cellular domain (Notch ECD), A disintegrin and metalloprotease (ADAM), Transmembane intracellular domain (TMICD), Gamma secretase inhibitor (GSI), Notch intracellular domain (NICD), Mastermind (MAM), Recombination signal binding protein 1 for J-kappa (RBPJ), Repressor (Rep.)
**A**

GSI MRK-003 Weekly Administration

- Birth
- 2 Mo Tamoxifen
- 3 Mo GSI
- 4 Mo GSI
- Analysis

**B**

Corpus Callosum Notch Activity

- Hes5 GFP+ cells / 40X field
- Vehicle
- GSI

**C**

- Wild Type
- Vehicle
- GSI

- PigCre: Nf1 fli1
- Vehicle
- GSI

**D**

Corpus Callosum Myelin Thickness

- g-ratio (Axon Diameter / Fiber Diameter)
- WT Veh
- WT GSI
- Nf1 Veh
- Nf1 GSI

**E**

Corpus Callosum Myelin Decompaction

- % Decompacted Fibers
- WT Veh
- WT GSI
- Nf1 Veh
- Nf1 GSI

*ns*

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**Figure 3.5. Gamma secretase inhibitor (MRK-003) partially rescues severity of myelin decompaction in Nf1 mutants.**

(A) Timeline of adult tamoxifen inducible recombination murine model, GSI MRK-003 administration, and analysis.

(B) Quantification of average (with SEM) of Hes5GFP+, recent canonical Notch pathway signaling, in the corpus callosum as measured by immunofluorescence of fixed cryostat sections. Decrease in GSI MRK-003 as compared to vehicle treated mice (n=3/genotype, p=0.031, *; p<0.05).

(C) Electron micrographs of corpus callosum cross sections at the mid line (60,000X). [White arrows: areas of myelin decompaction, Top Row: Wild Type, Bottom Row: Nf1 mutant, Left Column: Vehicle, Right Column: GSI MRK-003].

(D) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of GSI MRK-003 as compared to vehicle treated in Nf1 mutant mice (n=5/genotype, ****; p<0.0001). No significant difference in g-ratio between GSI MRK-003 compared to vehicle treated in wild type mice (n=5/genotype, p=0.169).

(E) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in percent decompaction in GSI MRK-003 as compared to vehicle treated in Nf1 mutant mice (n=5/genotype, p=0.042). There was no significant difference in percent decompaction between GSI MRK-003 and vehicle treated wild type mice (n=5/genotype, p=0.4005).
**Nf1** loss in the oligodendrocyte lineage increases progenitors/immature oligodendrocytes and GSI MRK-003 promotes differentiation and maturation of the aberrant progenitors.

These studies, carried out early after tamoxifen administration, strongly support a role for Notch signaling and Nf1 in oligodendrocytes, but do not exclude relevant effects on OPCs. Indeed, when Nf1 is hemizygoously or homozygoously deleted in glial progenitors in *vitro*, numbers of cells increase (Bennett et al., 2003). OPC numbers are also expanded *in vivo* after Nf1 loss (Bennett et al., 2003; Hegedus et al., 2007; Wang et al., 2012). Flow cytometry of the forebrain enabled identification of an increase in PDGFRα+ progenitors (p=0.05), immature O4+;GalC- (p=0.033), and pre-myelinating O4+;GalC+ oligodendrocytes (p=0.023) in Nf1 mutants (**Figure 3.6A**); consistent with the increase in Hes5+;O4+;GalC+ cells (**Figure 3.1D**). There was no change in mature myelinating OL numbers (O4-/GalC+) as a percent of CNS resident cells in Nf1 mutant mice (p=0.45; **Figure 3.6A**).

Immunohistochemistry in *iPlpCre;Nf1fl/fl* corpus callosum confirmed an increase in PDGFRα+ white matter progenitors/immature oligodendrocytes and no change in CC1+ mature oligodendrocytes (**Figure 3.6B**). To test whether we rescue aberrant populations of oligodendrocyte lineage cells in the corpus callosum, we analyzed *iPlpCre;Nf1fl/fl* mice treated with GSI MRK-003. Immunohistochemistry showed a significant decrease in progenitors (PDGFRα+) and an increase in mature CC1+ oligodendrocytes after GSI (**Figure 3.6B**). As expected based on prior findings, in which blocking Notch pharmacologically matured OPCs (Jurynczyk et al., 2008), treatment of wild type mice with MRK-003 also increased mature CC1+ oligodendrocytes. Gamma secretase has substrates in addition to Notch receptors, which could also contribute to effects observed. However, taken together with effects of Rbpj loss, the data support a role for Notch signaling downstream of Nf1 loss.
Figure 3.6. \textit{Nf1} loss in the oligodendrocyte lineage increases progenitors/ immature oligodendrocytes and GSI MRK-003 promotes differentiation and maturation of the aberrant progenitors.

(A) Quantification of average (with SEM) of the oligodendrocyte cell lineage in the forebrain at 3 months of age, as measured by flow cytometry of stained live CNS resident cells (excluding microglia). Increase in the oligodendrocyte cell lineage in \textit{Nf1} mutant as compared to wild type mice (* : p<0.05); PDGFR\(\alpha\)+O4- (p=0.05), O4+, GalC- (p=0.033), and O4+, GalC+ (p=0.023). No significant difference in O4-GalC+ cells in \textit{Nf1} mutant as compared to wild type mice (p=0.4501) (n=3/genotype).

(B) Quantification of average (with SEM) of the oligodendrocyte cell lineage (PDGFR\(\alpha\)+ and CC1+) in the corpus callosum at 4 months as measured by immunofluorescence of fixed cryostat sections. Increase in PDGFR\(\alpha\)+ cells in \textit{Nf1} mutant as compared to wild type vehicle treated mice (n=3,3/genotype, p=0.029, * ; p<0.05). There was no significant difference in CC1+ cells in \textit{Nf1} mutant as compared to wild type vehicle treated mice (n=3,3/genotype, p=0.474). Decrease in PDGFR\(\alpha\)+ cells in GSI-MRK-003 as compared to vehicle treated in both \textit{Nf1} mutant (n=5,3/genotype, p=0.0006) and wild type (n=3,3/genotype, p=0.005) mice. Increase in CC1+ cells in GSI-MRK-003 as compared to vehicle treated in both \textit{Nf1} mutant (n=5,3/genotype, p=0.05) and wild type (n=3,3/genotype, p=0.007) mice.
**Nf1 loss in the oligodendrocyte cell lineage results in intrinsic generation of reactive oxygen species (ROS).**

Pharmacological inhibition of Notch signaling with MRK-003 only partially rescued myelin decompaction caused by Nf1 loss, suggesting that additional signaling pathways contribute to decompaction. We dissociated cells from forebrain and optic nerve of iPp;Nf1fl/+;eGFP mice (6 months post tamoxifen injection) and examined the fluorescent reactive oxygen species (ROS) reporter DCF-DA using flow cytometry. DCF-DA and Cell ROX detect nitric oxide (NO), superoxide (O2-), and reactive nitrogen species (RNS), collectively called ROS. There was no significant shift in DCF-DA fluorescence intensity (p=0.74) or total live DCF-DA+ CNS resident cells (p=0.51), although an increase was observed in some individual mutant mice (Figure 3.7A). Importantly, a significant increase (p = 0.05) in DCF-DA fluorescence intensity was present in sorted GalC+ oligodendrocytes after hemizygous Nf1 loss as compared to controls (Figure 3.7B). In contrast, PDGFRα+ progenitors, GFAP+ astrocytes, and ICAM1+ endothelial cells within the mutant brains showed similar ROS compared to wild type cells. Overall, the data indicate that Nf1 loss results in ROS accumulation in oligodendrocytes.

**Increased reactive oxygen species are detected in hemizygous Nf1 mutants in mutant and wild type oligodendrocytes.**

In iPpCre;Nf1fl/+;eGFP mice, eGFP expression is Cre-driven, thus sorted eGFP+ cells are assumed to have an Nf1 deletion and eGFP-negative cells to be wild type. Strikingly, there was a significant increase in Cell ROX Orange ROS reporter fluorescence intensity in both eGFP+ recombined (p=0.01) and eGFP-negative (p=0.001) GalC+ oligodendrocytes after Nf1 loss. The eGFP- population was larger (p=0.005) than eGFP+ recombined cells, likely reflecting the larger
Figure 3.7: *Nf1* loss in the oligodendrocyte cell lineage results in intrinsic generation of reactive oxygen species (ROS).

(A) Histograms of fluorescence intensity of DCF-DA (Left) and dot plot visualization of flow cytometry analysis of live CNS resident cells gated on DCF-DA+ cells for wild type (Middle, Red box) and *iPlpCre;Nf1fl/+* (Right, Blue box) mice.

(B) Histograms of fluorescence intensity of DCF-DA for wild type (Red) and *iPlpCre;Nf1fl/+* (Blue). An increase is visualized as a shift to the right (arrow) of the *Nf1* mutant (Blue curve) compared to wild type (Red curve) in GalC+ oligodendrocytes. There was no significant increase in fluorescence intensity oligodendrocyte progenitors (PDGFRα), astrocytes (GFAP+), and endothelial cells (ICAM-1+).
**Figure 3.8: Increased reactive oxygen species are detected in hemizygous Nf1 mutants in mutant and wild type oligodendrocytes.**

(A) Dot plot visualization of flow cytometry analysis of gated total live CNS resident cells for GalC+ oligodendrocytes and eGFP in *iPlpCre;Nf1fl/+* (Blue) (*n*=3, *n*=1 per genotype represented).

(B) Histograms of fluorescence intensity of Cell-ROX for wild type (Red) and *iPlpCre;Nf1fl/+* (Blue). An increase is visualized as a shift to the right (arrow) of the hemizygous Nf1 mutant (Blue curve) compared to wild type (Red curve) in both eGFP+ and eGFP- GalC+ oligodendrocytes.
number of total gated cells. (Figure 3.8) Overall, the data indicate that Nf1 loss results in ROS accumulation in mutant oligodendrocytes. ROS also accumulates in other oligodendrocytes, possibly those coupled directly by gap junctions. ROS may diffuse from oligodendrocytes to affect other cell types, although is likely to diffuse only short distances before being reduced by contact with proteins and lipids (Mayes et al., 2013).

*Nf1* loss in the oligodendrocyte cell lineage results in intrinsic generation of reactive oxygen species (ROS); specifically nitric oxide and superoxide.

We confirmed an increase in the number of CNS resident cells with increased ROS 10 months post tamoxifen in *iPlpCre*Nf1fl/fl mice versus wild type (168%, p=0.043) in flow cytometry using CellROX staining. To define the specific species of reactive oxygen present, we tested whether nitric oxide (NO) and/or superoxide (O2-) account for the increase in ROS at 10 months post tamoxifen in *iPlpCre*Nf1fl/fl forebrain. As previously described, there was no change in total ROS in PDGFRα+,GalC- cells in *iPlpCre*Nf1fl/+ forebrain. There was also no significant change in the percent of PDGFRα+,GalC- progenitors that were nitric oxide+ (p=0.684) or superoxide+ (p=0.456), nor a significant change in fluorescence intensity for nitric oxide (p=0.15) or superoxide (p=0.149) in *iPlpCre*Nf1fl/fl forebrain.

We focused on cells in which Nf1 recombination occurs. Gates on immature and mature oligodendrocytes are shown in Figure 3.9A. PDGFRα+,GalC+ immature oligodendrocytes showed an increase in the percent of cells that are O2-positive (p=0.007) and an increase in O2-fluorescence intensity (p=0.018) (Figure 3.9B). The presence of two peaks indicates that O2-increases in a subpopulation of immature oligodendrocytes (Figure 3.9B, black arrow). There was no significant change in the percent of nitric oxide+ cells (p=0.189) nor a significant change
in nitric oxide fluorescence intensity (p=0.113) in these cells (Figure 3B). In PDGFRα−,GalC+
Figure 3.9. 

*Nf1* loss in the oligodendrocyte cell lineage results in intrinsic generation of reactive oxygen species (ROS); specifically nitric oxide and superoxide.

(A) Dot plot visualization of flow cytometry analysis of gated total live CNS resident cells for PDGFRα+GalC+ immature oligodendrocytes and PDGFRα-GalC+ mature oligodendrocytes in wild type (Red) and *Nf1* mutant (Blue) (*n*=3, *n*=1 per genotype represented).

(B) Dot plot visualization of flow cytometry analysis of gated PDGFRα+GalC+ immature oligodendrocytes for Nitric Oxide (NO) positive cells (Left column) and Superoxide (SO) positive cells (Right column) in wild type (Red, 1st Row) and *Nf1* mutant (Blue, 2nd Row) (*n*=3, *n*=1 per genotype represented). Histograms of fluorescence intensity (4th Row) for NO (left column) and O$_2^-$ (right column) (*n*=3, *n*=1 per genotype represented). Increase in the percent O$_2^-$ positive cells (3rd Row; *p*=0.05) and an increase in fluorescence intensity (5th Row; GeoM, *p*=0.0186) in a subset of the population is visualized as two peaks and the peak to the right is increased (arrow) in *Nf1* mutant (Blue curve) compared to wild type (Red curve). Neither percent of NO positive cells (3rd Row; *p*=0.113) or fluorescence intensity (5th Row; GeoM, *p*=0.189) were significantly changed.

(C) Dot plot visualization of flow cytometry analysis of gated PDGFRα-GalC+ mature oligodendrocytes for Nitric Oxide (NO) positive cells (Left column) and Superoxide (SO) positive cells (Right column) in wild type (Red, 1st Row) and *Nf1* mutant (Blue, 2nd Row) (*n*=3, *n*=1 per genotype represented). Histograms of fluorescence intensity (4th Row) for NO (left column) and O$_2^-$ (right column) (*n*=3, *n*=1 per genotype represented). Increase in the percent of NO positive cells (3rd Row; *p*=0.043) and is visualized as a shift to the right (arrow) of the *Nf1* mutant (Blue curve) compared to wild type (Red curve), yet not a significant change in
fluorescence intensity (5\textsuperscript{th} Row; GeoM, p=0.529). Neither percent of O\textsubscript{2}- positive cells (3\textsuperscript{rd} Row; p=0.195) nor fluorescence intensity (5\textsuperscript{th} Row; GeoM, p=0.486) were significantly changed.
mature oligodendrocytes there was no significant change in percent of O$_2$-positive cells (p=0.195) nor in O$_2$- fluorescence intensity (p=0.486) (Figure 3.9C). These cells instead showed a significant increase in the percent of nitric oxide+ cells (p=0.043); a shift to the right in fluorescence intensity indicates that most cells increase NO (Figure 3.9C), although the mean intensity did not change (p=0.529).

**Canonical Notch pathway activity and elevated superoxide are present in apoptotic cells.**

Further analysis revealed that canonical Notch activity measured by Hes5GFP and elevated NO or O$_2$- are present in different cells. Indeed, nearly all viable (live/dead stain negative) CNS-resident Hes5GFP+ cells lacked NO staining (Figure 3.10A,B). Confirming that Notch activation is not upstream of NO generation in oligodendrocytes, mice with constitutive Notch signaling (iPlpCre;RosaNICD) did not significantly alter percent of NO+ or O$_2$-positive cells (p=0.256; p=0.077) or NO or O$_2$- fluorescence intensity (p=0.353; p=0.183).

A small number of wild type and Nf1 mutant Hes5GFP+ cells show elevated O$_2$- (Figure 3.10A,B). Half of the Hes5GFP+;O$_2$-positive cells were Annexin V+, e.g. undergoing apoptotic cell death (Figure 3.10C). Nearly all of Hes5GFP+;O$_2$-positive apoptotic cells were PDGFRα+;GalC+ immature oligodendrocytes (Figure 3.10D). Dying cells are present in wild type and mutant brain, with no significant change in the percent of the Hes5GFP+; O$_2$- cells (p=0.376) or of Hes5GFP+;O$_2$-;Annexin V+ cells (p=0.641), suggesting that killing of immature oligodendrocytes is a normal ongoing process, possibly to prune excessive oligodendrocytes. The number of these dying cells increased in Nf1 mutants by 4-fold, correlated with an overall increase in Hes5GFP+ cells (p=0.033) (Figure 3.10A). Viable Hes5GFP+;O$_2$- cells were progenitors and immature/mature oligodendrocytes; these may ultimately die or differentiate.
Figure 3.10. Canonical Notch pathway activity and elevated superoxide are present in apoptotic cells.

(A) Notch Pathway Activity: Dot plot visualization (Left) of flow cytometry analysis of gated total Hes5GFP+ CNS resident cells (n=3, n=1 per genotype represented). 4 fold increase in Hes5GFP+ cells in Nf1 mutant (Blue, Bottom) compared to wild type (Red, Top) (n=3 per genotype, p=0.034).

(B) Reactive Oxygen Species: Dot plot visualization (Arrow 1) of flow cytometry analysis of Hes5GFP+ CNS resident cells, gated for reactive oxygen species with nitric oxide and superoxide in Nf1 mutant (Blue, Bottom) and wild type (Red, Top) (n=3, n=1 per genotype represented). There was no significant change in the percent of the Hes5GFP+ population that are O$_2^-$ (p=0.376).

(C) Apoptosis: Dot plot visualization (Arrow 2) of flow cytometry analysis of superoxide+ Hes5GFP+ CNS resident cells, gated Annexin V+ apoptotic cells in Nf1 mutant (Blue, Bottom) and wild type (Red, Top) (n=3, n=1 per genotype represented). There was no significant change in the percent of Hes5GFP+;O$_2^-$ that are Annexin V+ (p=0.641).

(D) Oligodendrocyte Cell Lineage: Dot plot visualization (Arrow 3, Right) of flow cytometry analysis of Annexin V+, superoxide+ Hes5GFP+ CNS resident cells, gated for the oligodendrocyte cell lineage with PDGFRα and GalC in Nf1 mutant (Blue, Bottom) and wild type (Red, Top) (n=3, n=1 per genotype represented).
**Inhibition of nitric oxide synthase with L-NAME partially rescues the Nf1 mutant phenotype.**

One month after tamoxifen, there was a 69% increase in PDGFRα+ROS+ cells and a 57.8% increase in GalC+ROS+ cells in Nf1 mutants (**Figure 3.11A**). To define the role of NO in the myelin decompaction phenotype, we examined brains of *iPlpCre;Nf1fl/fl* mice exposed to tamoxifen at 2 months of age and treated at 3 months of age for 7 days with L-NAME, a specific NOS inhibitor (**Figure 3.11B**). Analysis of myelinated axons in the corpus callosum revealed a significant increase in g-ratio and partial rescue of myelin compaction in L-NAME treated *iPlpCre;Nf1fl/fl* mice, due to decreased fibers showing myelin decompaction and a decrease in the severity of decompaction (**Figure 3.11C-E**). Thus, brief pharmacological inhibition of NO production improves myelin decompaction caused by Nf1 loss. Although L-NAME significantly increased the total number of fibers with compact myelin, full rescue of the Nf1 decompaction phenotype was not achieved.

**Inhibition of NOS with L-NAME completely rescues the Nf1 mutant phenotype in mice with Rbpj loss.**

As neither inhibition of nitric oxide production nor Notch pathway inhibition completely rescued the Nf1 phenotype, we tested if inhibition of both pathways can completely rescue myelin decompaction. Using electron microscopy, we examined brains of *iPlpCre;Rbpjfl/fl,Nf1fl/fl* mice exposed to tamoxifen at 2 months of age and treated with vehicle or L-NAME beginning at 3 months of age for 7 days (**Figure 3.11B**). G-ratio increased in L-NAME treated compared to vehicle treated *iPlpCre;Rbpjfl/fl,Nf1fl/fl* mice (**Figure 3.11C,D**). The decrease in myelin thickness correlated with rescue of myelin compaction and a decrease in number of fibers with
Figure 3.11. Inhibition of both nitric oxide and Notch signaling completely rescues myelin compaction in Nf1 mutants.

(A) Quantification of fold change with standard error of the mean (SEM) in Nf1 mutant compared to wild type of ROS+ cells in the forebrain as measured by immunofluorescence of unfixed cells. Increase in Nf1 mutant (n=3) as compared to wild type (n=3) in PDGFRα+;ROS+ cells (p=0.005; **, p<0.01) and GalC+;ROS+ cells (p=0.009; **, p<0.01).

(B) Timeline of adult tamoxifen inducible recombination murine model, L-NAME administration, and analysis.

(C) Electron micrographs of corpus callosum cross sections at the mid line (20,000X). White arrows: areas of myelin decompaction. Top Row: iPlpCre;Nf1fl/fl, Bottom Row: iPlpCre;Nf1fl/fl,Rbpjfl/fl, Left Column: Vehicle, Right Column: L-NAME.

(D) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of L-NAME (n=3) as compared to vehicle (n=5) treated in iPlpCre;Nf1fl/fl mice (**** ; p<0.0001). Increase in g-ratio of L-NAME (n=3) as compared to vehicle (n=4) treated in iPlpCre;Nf1fl/fl,Rbpjfl/fl (**** ; p<0.0001); resulting in a full rescue of the Nf1 mutant decompaction phenotype. There was a significant difference between iPlpCre;Nf1fl/fl,Rbpjfl/fl treated with L-NAME and wild type (**** ; p<0.0001).

(E) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in percent decompaction in L-NAME (n=3) as compared to vehicle (n=5) treated in iPlpCre;Nf1fl/fl mice (p=0.034). Decrease in percent decompaction in L-NAME (n=3) as compared to vehicle (n=4) treated in iPlpCre;Nf1fl/fl,Rbpjfl/fl (p=0.006); resulting in a full rescue of the Nf1 mutant decompaction phenotype.
decompaction (Figure 3.11C,E). Thus, inhibition of nitric oxide production, together with loss of RBPJ-dependent Notch signaling in oligodendrocytes, completely rescues myelin decompaction in Nf1 mutants. The additive effect of dual inhibition suggests that Notch signaling and the production of ROS may be functionally independent. Confirming that Notch activation is not upstream of reactive oxygen species generation in oligodendrocytes using flow cytometry analysis, mice with loss of Rbpj (iPlpCre;Nf1fl/fl,Rbpjfl/fl) did not show altered percent of ROS+ cells (Figure 3.18).

**Heterozygous Nf1 loss in oligodendrocytes causes progressive myelin decompaction**

In NF1 patients, it is believed that heterozygous mutation in NFI underlies patient brain phenotypes (apart from tumors which require somatic mutation of the second allele). We tested if myelin decompaction is present after hemizygous loss of Nf1. We examined brains of 3 month old iPlpCre:Nf1fl/+ mice and wild type littermates exposed to tamoxifen at 2 months of age using electron microscopy (Figure 3.12A). There was no significant difference in myelin thickness quantified as g-ratio. However, both the total number of fibers with decompaction and the severity of decompaction were increased in Nf1 mutant versus wild type mice (Figure 3.12D). Myelin decompaction was more severe after 6 months of hemizygous loss of Nf1 in oligodendrocytes (Figure 3.12B). There was a significant decrease in g-ratio in corpus callosum (Figure 3.12C). Total number of fibers with decompaction and severity of decompaction were increased in Nf1 mutant compared to wild type mice (Figure 3.12D). There was no significant difference in the percent of decompacted fibers in iPlpCre:Nf1fl/+ corpus callosum 6 months as compared to 1 month post tamoxifen. However, there was an increase in fibers with 4 quadrants (>75%) decompaction (Figure 8D). Thus, hemizygous Nf1 loss in oligodendrocytes causes progressive myelin decompaction.
**Figure 3.12. Heterozygous Nf1 loss in oligodendrocytes causes progressive myelin decompaction.**

(A) Electron micrographs of corpus callosum cross sections at the mid line of iPlpCre;Nf1fl/+ mice at 1 month post tamoxifen (left: 30,000X / right: 60,000X). White arrows: areas of myelin decompaction.

(B) Electron micrographs of corpus callosum cross sections at the mid line of iPlpCre;Nf1fl/+ mice at 6 months post tamoxifen (left: 30,000X / right: 60,000X). White arrows: areas of myelin decompaction.

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of Nf1 mutant 6 months post adult induced recombination as compared to wild type and Nf1 mutant 1 month post adult induced recombination mice (n=3/genotype, **** ; p<0.0001). No significant difference in g-ratio between Nf1 mutant 1 month post adult induced recombination as compared to wild type mice (n=3/genotype, p=0.074).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Increase in percent decompaction in Nf1 mutant at 1 month and 6 month post tamoxifen as compared to wild type mice (n=3/genotype, p=0.029, p=0.022). There was no significant difference in percent decompaction in iPlpCre;Nf1fl/+ 6 months post as compared to 1 month post tamoxifen (n=3/genotype, p=0.736), however, there was an increase in fibers with 4 quadrants (>75%) decompaction.
In contrast, there is no significant change in g-ratio or severity of decompaction in $iPlpCre;Nf1fl/fl$ corpus callosum 6 months as compared to 1 month post tamoxifen (Figure 3.13A,B). Again, confirming the myelin decompaction is dose dependent.

**Myelin decompaction precedes progenitor recruitment in Nf1 hemizygotes.**

Previous data of the forebrain, using flow cytometry analysis, show an increase in progenitors and oligodendrocytes $iPlpCre;Nf1fl/fl$ mice at 1 month post tamoxifen. Here we show that there is no significant increase the forebrain, using flow cytometry analysis, in oligodendrocyte lineage cells of $iPlpCre;Nf1fl/+ $ mice at 1 month post tamoxifen (Figure 3.14A). Nor is there an increase in Hes5GFP+ cells of the oligodendrocyte lineage of $iPlpCre;Nf1fl/+ $ mice compared to wild type at 1 month post tamoxifen (Figure 3.14B). Previous data of corpus callosum, using immunohistochemistry on fixed sections, show an increase in progenitors in $iPlpCre;Nf1fl/fl$ mice at 1 month post tamoxifen. Here we show that there is a significant increase in progenitors of $iPlpCre;Nf1fl/+ $ mice at 6 months, yet not at 1 month post tamoxifen (Figure 3.14C,E), concurrent with an increase in Hes5GFP+ progenitors at 6 months that is absent at 1 month post tamoxifen (Figure 3.14D,F). These data suggest that recruitment of progenitors, due to Nf1 loss in mature oligodendrocytes, is dose and time dependent. There is an increase in Hes5GFP+NG2+ cells in $iPlpCre;Nf1fl/fl$ mice at 1 month post tamoxifen that is still present at 6 months post tamoxifen. Importantly this demonstrates that myelin decompaction is present in hemizygous Nf1 mutants prior to oligodendrocyte progenitor recruitment.
Figure 3.13. Severity of myelin decompaction is dose dependent on Nf1 loss.

(A) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of heterozygous Nf1 mutant 6 months as compared to 1 month post adult induced recombination mice (n=3/genotype, **** ; p<0.0001). No significant difference in g-ratio between homozygous Nf1 mutant 6 months as compared to 1 month post adult induced recombination mice (n=3/genotype, p=0.412).

(B) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). There was no significant difference in percent decompaction in iP/C;Nf1fl/+ 6 months post as compared to 1 month post tamoxifen (n=3/genotype, p=0.736), however, there was an increase in fibers with 4 quadrants (>75%) decompaction. There was no significant difference in percent decompaction in iP/C;Nf1fl/fl 6 months post as compared to 1 month post tamoxifen (n=3/genotype, p=0.0697), however, there was an increase in fibers with 4 quadrants (>75%) decompaction.
Figure 3.14. Myelin decompaction precedes progenitor recruitment in Nf1 hemizygotes.

(A) Quantification of average (with SEM) of the oligodendrocyte cell lineage in the forebrain, as measured by flow cytometry of stained live CNS resident cells (excluding microglia). Increase in the oligodendrocyte cell lineage in iPlpCre;Nf1fl/fl mutant as compared to wild type mice (* : p<0.05); PDGFRα+O4- (p=0.05), O4+, GalC- (p=0.0329), and O4+, GalC+ (p=0.0228), yet no change in iPlpCre;Nf1fl/+.

(B) Quantification of fold change with standard deviation (SD) compared to wild type of Hes5GFP+, recent canonical Notch pathway signaling, in the oligodendrocyte cell lineage in the forebrain as measured by immunofluorescence of unfixed cells. Increase in iPlpCre;Nf1fl/fl as compared to wild type O4+ GalC+ mature oligodendrocytes (p=0.0016, ** ; p<0.005), yet not change in iPlpCre;Nf1fl/+.

(C-E) Quantification of average (with SEM) of the oligodendrocyte cell lineage (NG2+, Olig2+, and CC1+) in the corpus callosum as measured by immunofluorescence of fixed cryostat sections.

(D) Increase in NG2+Hes5GFP+ cells in iPlpCre;Nf1fl/fl mice as compared to wild type (p=0.021, * ; p<0.05), yet no change in iPlpCre;Nf1fl/+ (p=0.14).
difference in Olig2+Hes5GFP+ or CC1+Hes5GFP+ cells in Nf1 mutants as compared to wild type.

(E) Increase in NG2+ cells in iPlpCre;Nf1fl/+ mice at 8 months as compared to 3 months (p=0.006, ** ; p<0.01). The high levels of NG2+ cells in the iPlpCre;Nf1fl/fl did not significantly increase at 8 months compared to 3 months (p=0.208). There was no significant difference in NG2+ cell number between iPlpCre;Nf1fl/+ and fl/fl at 8 months (p=0.937).

(F) There was a significant increase in Hes5GFP+NG2+ cells in iPlpCre;Nf1fl/+ mice compared to wild type at 8 months (p=0.0094), yet not at 3 months (p=.243). There was a significant difference between iPlpCre;Nf1fl/+ and fl/fl at 3 months (p=0.05) and 8 months (p=0.048).
Loss of Rbpj is sufficient to rescue myelin compaction and Gamma secretase inhibitor (MRK-003) rescues severity of myelin decompaction and aberrant behavior in PlpCre; Nf1 fl/+ mutants.

Rbpj loss rescued myelin compaction at 6 months, but not one month, after tamoxifen (Figure 3.15 A,B). This rescue suggests that decompaction becomes progressively dependent on Notch signaling. Pharmacological inhibition of Notch signaling improves myelin decompaction caused by homozygous Nf1 loss in vivo. To test if decompaction caused by hemizygous loss of Nf1 is improved by inhibiting Notch signaling, we administered GSI MRK-003 weekly beginning 10 months post tamoxifen administration, after myelin decompaction is evident (Figure 3.16A). Analysis of the corpus callosum revealed a full rescue, quantified as a significant increase in g-ratio, in MRK-003 treated mice iPlpCre;Nf1fl/+ mice (Figure 3.16B,C). The percent of fibers with decompaction and the severity of decompaction decreased in MRK-003 treated mice (Figure 3.16B,D).

Aberrant white matter is associated with behavioral alterations (Fields, 2011; Liu et al., 2012; McKenzie et al., 2014; Mayes et al., 2013; Makinodan et al., 2012). We used mice on a pure C57Bl/6 background for behavioral testing. iPlpCre;Nf1fl/+ mice (12 months old), exposed to tamoxifen at 2 mo. of age, had an increased acoustic startle response at baseline versus littermate controls (Figure 3.16E Left). The same mice were immediately treated with MRK-003 for four weeks, after which the increased response to startle was absent; there was no longer a significant difference between mutants and littermate controls (Figure 3.16E right). These results support the conclusion that pharmacological inhibition of Notch signaling fully rescues myelin decompaction and the aberrant acoustic startle response caused by hemizygous Nf1 loss in oligodendrocytes, even after the phenotype has manifested in vivo.
A  
Corpus Callosum Myelin Thickness

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<th>Nf1 fl/+ (3 Mo)</th>
<th>Nf1 fl/+ (8 Mo)</th>
<th>Rbpj fl/fl (8 Mo)</th>
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B  
Corpus Callosum Myelin Decompaction

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<td>50-75%</td>
<td>25-50%</td>
<td>&lt;25%</td>
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Quadrants:
- >75%
- 50-75%
- 25-50%
- <25%
Loss of *Rbpj* is sufficient to rescue myelin compaction in *iPlpCre;Nf1fl/+* mutants.

(A) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of *iPlpCre;Nf1fl/+,*Rbpjfl/fl* as compared to *iPlpCre;Nf1fl/+* mice at 3 months (n=4,3/genotype, **** ; p<0.001). Increase in g-ratio in *iPlpCre;Nf1fl/+,Rbpjfl/fl* as compared to *iPlpCre;Nf1fl/+* mice at 8 Months (n=3/genotype, **** ; p<0.0001).

(B) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). There was no significant difference in percent decompaction (n=4,3/genotype, p=0.911) in *iPlpCre;Nf1fl/+,Rbpjfl/fl* as compared to *iPlpCre;Nf1fl/+* mice at 3 months, however, there was a decrease difference in percent decompaction (n=3/genotype, p=0.03; * ; p<0.05) in *iPlpCre;Nf1fl/+,*Rbpjfl/fl* as compared to *iPlpCre;Nf1fl/+* mice at 8 months.
Figure 3.16. Gamma secretase inhibitor (MRK-003) rescues severity of myelin decompaction and aberrant behavior in PlpCre; Nf1 fl/+ mutants.

(A) Timeline of adult tamoxifen inducible recombination murine model, GSI MRK-003 administration, and analysis at 12 months.

(B) Electron micrographs of PlpCre; Nf1 fl/+ corpus callosum cross sections at the mid line (30,000X). White arrows: areas of myelin decompaction. Left Column: Vehicle, Right Column: GSI MRK-003.

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of GSI MRK-003 as compared to vehicle treated in Nf1 mutant mice (n=4,3/genotype, ****; p<0.0001).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in percent decompaction in GSI MRK-003 as compared to vehicle treated in Nf1 mutant mice (n=4,3/genotype, p=0.0063).

(E) Quantification of the acoustic startle response (measured as Vmax (mV)). Left: The Nf1 mutant (n=19) mice had an increased Vmax, to the acoustic startle stimulus following the 73 dB, 77 dB, and 82 dB prepulse stimulus as compared with wild type (n=21) mice; genotype x prepulse, F(4,152)=3.05 (p=0.05). Right: The heightened startle response is diminished after treatment with GSI MRK-003, since no significant difference in Vmax was observed between the Nf1 mutant (n=19) compared with wild type (n=21) mice; genotype x prepulse, F(4,152)=2.73 (p=0.077).
**L-NAME rescues severity of myelin decompaction and aberrant behavior in PlpCre: Nf1fl/+ mutants.**

There was no significant change in ROS+ oligodendrocyte lineage cells in the Nf1 hemizygous mutants 1 month post tamoxifen (**Figure 3.17A**). However, there was a 64.2% increase in PDGFRα+ROS+ cells and a 26.4% increase in GalC+ROS+ cells in Nf1 mutants 6 months post tamoxifen (**Figure 3.17A**). These data suggest an increase in ROS over time and that myelin decompaction is present in hemizygous Nf1 mutants prior to an increase in reactive oxygen species.

Data support the conclusion that pharmacological inhibition of NO production improves myelin decompaction caused by homozygous Nf1 loss. To test if decompaction caused by hemizygous loss of Nf1 is improved by inhibition of NO production, we administered L-NAME for a week beginning 6 month post tamoxifen administration. Treatment with L-NAME for 7 days significantly increased g-ratio in iPlpCre:Nf1fl/+ mice (**Figure 3.17C**) and myelin decompaction was fully rescued (**Figure 3.17B,D**).

When a prepulse stimulus was present, the acoustic startle response of iPlpCre:Nf1fl/+ mice exposed to tamoxifen at 2 months of age was heightened, regardless of the prepulse intensity, compared with littermate controls. Beginning at 8 months of age animals were treated with L-NAME for 7 days (n=17 WT and 17 Nf1). We identified a heightened response to startle in the presence of a prepulse stimulus in the iPlpCre:Nf1fl/+ mice versus littermate controls (**Figure 3.17E, left**). The heightened response to startle was diminished in L-NAME treated iPlpCre:Nf1fl/+ mice and there was no significant difference as compared with littermate controls (**Figure 3.17E, right**). Thus, pharmacological inhibition of NO production, like GSI, fully rescues myelin decompaction and behavioral alteration caused by hemizygous Nf1 loss in
Figure 3.17. L-NAME rescues severity of myelin decompaction and aberrant behavior in PlpCre; Nf1 fl/+ mutants.

(A) Quantification of fold change with standard error of the mean (SEM) in Nf1 mutant compared to wild type of ROS+ cells in the forebrain as measured by immunofluorescence of unfixed cells. An increase in Nf1 mutant as compared to wild type in PDGFRα+;ROS+ cells (p=0.019; *, p<0.05) and GalC+;ROS+ cells (p=0.026; *, p<0.05) at 6 months post tamoxifen. No significant difference at 1 month post tamoxifen.

(B) Electron micrographs of 8 month old PlpCre; Nf1 fl/+ mice corpus callosum cross sections at the mid line (30,000X). White arrows: areas of myelin decompaction. Left Column: Vehicle, Right Column: L-NAME.

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of L-NAME as compared to vehicle treated in Nf1 mutant mice (n=4,3/genotype, ****; p<0.0001).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in percent decompaction in L-NAME as compared to vehicle treated in Nf1 mutant mice (n=4,3/genotype, p=0.004).

(E) Quantification of the acoustic startle response (Vmax (mV)). Left: The Nf1 mutant mice had an increase in the Vmax response to the uninhibited startle stimulus at 70dB, but not following any of the prepulse stimuli, as compared with wild type mice (n=17/genotype); genotype x prepulse, F(4,128)=6.31, p<0.01. Right: The heightened startle response of the Nf1 mutant mice is diminished after treatment with L-NAME, no significant difference in Vmax compared with wild type mice (n=17/genotype); genotype x prepulse, F(4,128)=1.41, p=0.25.
oligodendrocytes, even after the phenotypes have manifested in vivo. Again, suggesting the phenotype is dose dependent on loss of Nf1, as dual inhibition is required with complete loss of Nf1 in oligodendrocytes (Figure 3.18).
A

<table>
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<td>Stays High</td>
</tr>
<tr>
<td><strong>iPlpCre;Nf1f/f + L-NAME</strong></td>
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Loss of Nf1 leads to increased Notch and NO. Inhibiting NO production leads to increased Notch.

B

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<th>iPlpCre;RosaNICD &amp; L-NAME</th>
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<td>NOS inhibition (L-NAME)</td>
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C

**iPlpCre-Nf1 flox**

- NO
- Notch
- Myelin Decompaction

**iPlpCre-Nf1 flox**

- L-NAME
- GSI
- Rbpj mutant
- NO
- Notch
- Myelin Decompaction
Figure 3.18 Summary of Notch and nitric oxide signaling in *NICD* and *Nf1* mutant models with treatments compared to vehicle control.

(A) Table of signaling pathway activity level in oligodendrocytes, as measured by flow cytometry of the forebrain, in *NICD* and *Nf1* mutant models with treatments compared to vehicle control.

(B) Table: Summary of myelin compaction and myelin thickness (g-ratio) in *NICD* and *Nf1* mutant models with treatments compared to vehicle control.

(C) Depiction of signaling in *iP1pCre;Nf1flo* murine model after tamoxifen induced recombination.
Using genetic models, we found that Nf1 loss in oligodendrocytes causes rapid myelin decompaction in adult mice, which is partially mediated by canonical Notch signaling. In Nf1-null oligodendrocytes, simultaneously targeting canonical Notch signaling with loss of Rbpj and NOS with L-NAME completely rescued decompaction, indicating that both pathways contribute to the pathological myelin decompaction. In contrast, hemizygous Nf1 loss causes more gradual myelin decompaction, and decompaction in Nf1 hemizygous oligodendrocytes is completely rescued by either treatment. We conclude that canonical Notch signaling and NO act downstream of Nf1 loss, contributing to myelin decompaction in a dose-and-time dependent manner.

Our data are consistent with the finding that in Ras transformed cells and in CNS tumors, Notch1 can be activated, showing that Ras-GTP acts upstream of Notch signaling (Brennan et al., 2009). The Notch pathway can also feedback to antagonize Ras signaling (Hurlbut et al., 2007), which may help explain the absence of brain tumors in iPlpCre;Nf1fl/fl mice (Mayes et al., 2013). It will be of interest to delineate feedback loops through which the Notch and Ras pathways interact in Nf1 mutant oligodendrocytes, as signaling pathways (apart from those identified here) that maintain myelin compaction remain largely unknown. Of note, constitutive expression of p-ERK in oligodendrocytes results in extra myelin wraps, not decompaction (Ishii et al., 2013). Altered pathway integration, levels of p-ERK, and/or pathways downstream of Ras-GTP independent of MEK-ERK signaling may dictate specific responses.

The Notch and Ras pathways are integrated through reciprocal transcriptional regulation (Hurlbut et al., 2009). We found that when Nf1 is lost transcription of Dll1, a ligand, and Hes5, a target gene, increase in glial progenitors in vitro. This is consistent with Ras signaling affecting...
the Notch pathway through regulation of DSL ligands and target gene expression (Hurlbut et al., 2007). Hes5GFP reporter activity also increased in progenitors and immature oligodendrocytes in vivo, but not in mature myelinating oligodendrocytes. Mature oligodendrocytes may use other Hes or Hey proteins, or other effectors, to drive Notch pathway signaling. Given that constitutive Notch signaling (through increased NICD in mature oligodendrocytes) drives decompaction, and that loss of Rbpj in mature oligodendrocytes partially rescues decompaction, we suggest that the effects on myelin compaction and hyperactive behavior are cell autonomous in mature oligodendrocytes.

Notch signaling can fine tune oligodendrocyte maturation, wrapping, and myelination (Wang et al., 1998; Watkins et al., 2008). Decompaction in Nf1 and NICD mutants shows that compaction is also regulated, directly or indirectly, through Notch signaling. Improved myelin compaction in GSI-treated Nf1 mutants supports the conclusion that Notch pathway activation regulates compaction. However, permanent adult-induced loss of RBPJ-dependent Notch signaling in wild type mice resulted in myelin decompaction, but GSI did not. Differences may be explained by permanent genetic alteration of a co-activator/co-repressor in mature oligodendrocytes versus temporary treatment that decreases aberrant Notch activity in mature oligodendrocytes, and may additionally block decompaction-promoting substrates. L-NAME in wild type mice also causes decompaction (Mayes et al., 2013). As both hypo- and hyper-activation of Notch activation and NO cause defects in myelin compaction in the wild type setting, Nf1 loss appears to uncover normal signaling pathways that regulate oligodendrocyte myelin compaction and require balance amongst levels of Ras, Notch and NO.

We do not believe that maturation of immature oligodendrocytes is necessary to improve myelin compaction in Nf1 mutants. First, loss of Rbpj in mature oligodendrocytes did not
promote differentiation of aberrant progenitors, yet rescues decompaction. Second, constitutive activation of Notch signaling in mature oligodendrocytes drove decompaction within a month. Third, the myelin we observe after GSI or L-NAME treatment is thick, normal myelin, not the thin myelin of re-myelination (Franklin and Gallo, 2014). A single week of L-NAME exposure rescued decompaction. This rapid effect strongly supports the idea that rescue of mature myelinating oligodendrocytes account for the decompaction phenotype.

Why are oligodendrocyte progenitors increased in Nf1 mutants? We speculate that stressed Nf1 mutant oligodendrocytes recruit OPCs. Demyelinated lesions also recruit NG2 cells for repair (Franklin and Gallo, 2014; Jurynczyk et al., 2008), and NOS1 increases in injured oligodendrocytes (Yao et al., 2010; Yao et al., 2012). During vascular remodeling, Notch pathway activation drives growth factor secretion, and secreted growth factors affect neighboring cells (Blanco and Gerhardt, 2013). Nitric oxide, and/or other factors produced by Nf1 oligodendrocytes that affect surrounding progenitors, might increase proliferation and prevent differentiation. Nitric oxide, like Notch, plays a role in cell differentiation (Snyder et al., 2012). NO suppresses MOG and PLP transcription and decreases translation of myelin MBP, MOG, PLP, and CNP mRNAs, possibly explaining why NG2 cells remain immature after oligodendrocyte injury (Jana and Pahan, 2013).

Oligodendrocyte progenitors increase in Nf1 mutants in corpus callosum sections, while Nf1 progenitors and immature oligodendrocytes increase in mutant forebrain. This may reflect differences between grey and white matter and/or more progenitor maturation in grey matter. Oligodendrocyte lineage cells are generated at higher levels in adult brain grey versus white matter (Kang et al., 2010). Of the increased immature oligodendrocytes in Nf1 mutant brains, a subset (Hes5+;O2-positive) die, likely as they are not needed for repair. Similarly, in the
demyelinating disease Multiple Sclerosis, Notch pathway activity in OPCs prevents maturation into myelinating oligodendrocytes (Jurynczyk and Selmaj, 2010). In mice, Notch1 activation increases OPC number and blocks differentiation via Hes5 (Liu et al., 2006). In our study, reducing Notch signaling using GSI decreased numbers of aberrant Nf1 progenitors and increased mature oligodendrocytes. There is premature oligodendrocyte differentiation in Notch1+/- mice (Givogri et al., 2002), while complete loss of Notch1 causes premature OPC differentiation and death of mature oligodendrocytes (Genoud et al., 2002). These findings reinforce the idea that levels of Notch signaling differentially affect oligodendrocytes.

Myelin decompaction was progressive in Nf1 hemizygous mice, and importantly was present before increases in oligodendrocyte progenitors were detectable and before NO increased significantly in oligodendrocytes. The rescue of the early hemizygous phenotype in mice by either L-NAME or GSI suggests that low level of cellular dysfunction may be treatable by single agents, which may be relevant to NF1 patients. Different levels of Notch signaling can dictate cell specific phenotypes, and when we define the key Notch effector(s) in oligodendrocytes, it will be of interest to define their levels in hemizygous and homozygously deleted cells, and over time.

Targeting canonical Notch signaling and simultaneously targeting NOS was necessary to completely rescue decompaction when Nf1 is completely lost in oligodendrocytes. Consistent with independence of Notch and NO signaling, constitutive activation of Notch signaling did not change the amount of oligodendrocyte ROS (NO or O2•−). However, the relationship between these two pathways may be complex, and our data do not exclude pathway interactions. NO at low (normal signaling) levels protects oligodendrocytes through inhibition of peroxynitrite-induced ERK phosphorylation, while NO at high levels reacts with superoxide to form toxic
peroxynitrite (Li et al., 2011). Given that hemizygous and homozygous \( Nf1 \) mutant glial precursors show elevated Ras-GTP and p-ERK (Bennett et al., 2003), p-ERK may influence NO/peroxynitrite levels and cellular outcomes. NO can also act upstream of Notch. For example, glioma cells exposed to an NO donor increase NICD expression and transcription of NICD target genes (Charles et al., 2010). We show that inhibition of NOS, with L-NAME, can rescue myelin decompaction resulting from hyper activation of Notch signaling in oligodendrocytes in \( iPlpCre;RosaNICD \) mice. The relationship between NO and Notch downstream of \( Nf1 \) is of great interest for further investigation.

In summary, we identify a pathway in which, downstream of \( Nf1 \) loss in oligodendrocytes, aberrant Notch pathway signaling disrupts myelin compaction. We find that canonical Notch signaling, as measured by Hes5GFP, is not upstream of ROS in \( Nf1 \) mutants and may be independent of nitric oxide in the \( Nf1 \) mutant. We identify gamma secretase inhibition and nitric oxide synthase inhibition as therapies that individually partially rescue the \( Nf1 \) homozygote mutant phenotype. We find that dual inhibition of Notch signaling, through subsequent loss of \( Rbpj \), and inhibition of NO production completely rescues the \( Nf1 \) homozygous mutant phenotype. The phenotype is less severe and is progressive in the \( Nf1 \) hemizygous, in which single agent therapy inhibiting either Notch signaling or NO production fully rescues decompaction. The relevance to patient phenotypes is therefore high as it is likely that many brain cells in NF1 patients are heterozygous for \( Nf1 \) mutation/loss. Thus, Notch and NO pathways are potential therapeutic targets in NF1 patients. Our studies link patient Rasopathy mutations to altered myelin compaction and strongly support recently described links between changes in myelin and behavior (Fields, 2011; Liu et al., 2012; McKenzie et al., 2014; Mayes et al., 2013; Makinodan et al., 2012).
A majority of this chapter is from:

_Nf1_ loss in Oligodendrocytes Induces Myelin Decompaction through Notch Signaling and Nitric Oxide_

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**Author Contributions:** H.T.M. performed animal work and experiments, analyzed data, and drafted the manuscript. M.B. grew cells for microarray and performed the western blot. T.R. performed qRT-PCR and PDGFRα stain on sections and assisted with perfusions. G.C. prepared sections for electron microscopy. S.H.S. and M.B. quantified myelin thickness (g-ratios) and myelin decompaction/severity from electron micrographs. M.W. and C. V. analyzed behavior. N.R. designed the project, aided in data analysis and interpretation, and edited the paper and figures.

**Acknowledgments:** We thank Shyra J. Miller, Nisha Schuler (Cincinnati), and Debra A. Mayes (Wright State University, Dayton OH) for contributions to Figure 1, Amanda Klotter for technical assistance, and Andrew Robinson and Stephen D. Miller (Northwestern, Chicago IL) for providing methods for Flow Cytometry prior to publication. We are indebted to Brian Popko (University of Chicago, Chicago, IL), Luis F. Parada (UTSW), and Tasuku Honjo (Kyoto-U) for providing mouse lines, Marie Dominique Fillipi and Monica DeLay for assistance with Flow, and Merck for providing MRK-003. This work was supported by a grant from the DOD program on Neurofibromatosis, an award from the Cincinnati Children’s Hospital, and NIH R01 NS091037 (to NR). The Cincinnati Children’s Hospital Research Foundation, Flow Cytometry and Pathology Cores provided support for these studies (NIH P30 DK0909710551).
Chapter 4

Hyper activation of HRas in Oligodendrocytes Induces Myelin Decompaction through Notch Signaling, Nitric Oxide, and MEK/ERK Signaling
4.1 Introduction

Rasopathies are genetic disorders in which patients carry heterozygous activating mutations in Ras-MAPK signaling pathway genes. Costello syndrome is a rare gain of function Rasopathy caused by mutation of the *HRAS* gene on chromosome 11p15.5, which results in facial dysmorphism, growth retardation, gastrointestinal, skin and musculoskeletal anomalies, cardiovascular abnormalities, and tumor predisposition (Rauen, 2013). Most Costello patients show *HRASG12S* or *HRASG12A* mutation, and rare patients with a severe form of Costello syndrome the *HRASG12V* mutation. All of these mutations increase cellular GTP-bound H-RAS, thereby activating pathways downstream of activated Ras, including MEK/ERK1, 2 (Gripp et al., 2010).

Frequent mental retardation, macrocephaly and variable cognitive delay have been reported in Costello patients (Gripp et al., 2010; Rauen, 2013). Myelin formed by oligodendrocytes wraps axons, speeding impulse conduction, and MRI studies suggest that brain dysmyelination might occur in Costello patients (Tidyman and Rauen, 2008). *CNPase;HRasG12V* mice, in which HRas-GTP is expressed in the oligodendrocyte lineage starting at embryonic day 12.5, show myelin decompaction. *CNPase;HRasG12V* mutants showed hyperactive behavior and increased response to startle, consistent with proper brain function requiring normal compact myelin.

During development, Ras-GTP activated MEK-ERK signaling is critical for OPC specification, proliferation, and timing of differentiation (Fyffe-Maricich et al., 2011; Li et al., 2012; Guardiola-Diaz et al., 2012). ERK signaling contributes to myelin thickness during repair (Fyffe-Maricich et al., 2013) and extra myelin wraps are observed in oligodendrocytes expressing activated ERK (Ishii et al., 2013). As *Ras-G12V* in oligodendrocytes caused myelin
decompaction, but direct ERK activation increased myelin lamellae, we tested whether acute, adult, expression of $H-RasG12V$ in its endogenous locus affects myelin thickness and/or compaction.

Ras signaling through MEK/ERK can increase reactive oxygen/nitrogen species (Heimfarth et al., 2013). Intriguingly, blockade of nitric oxide synthase (NOS) rescued aberrant behavior and myelin decompaction in CNPase;HRasG12V mice (Mayes et al., 2013). Notch pathway signaling can also act up- and/or down-stream of Ras-GTP (Mazur et al., 2010a; Baumgart et al., 2014; Sundaram, 2005). For example, expression of the Notch receptor effector NICD in brain induced Nestin expression, and expanded the SVZ in cooperation with K-Ras (Shih and Holland, 2006), and increased p-ERK and caused myelin decompaction in oligodendrocytes (Titus-Mitchell et al., submitted). Deleting the Rasopathy gene Nf1 with removal of the Notch effector Rbpj partially rescued myelin decompaction observed in this setting (Titus-Mitchell et al., submitted). It was unclear whether Notch, nitric oxide, and/or MEK signaling contribute to pathological myelin following expression of HRasG12V in oligodendrocytes.

In summary, myelin decompaction occurs within a month of oligodendrocyte HRasG12V/+ mutation, which can be partially rescued by inhibition of canonical Notch signaling, NOS or MEK. Targeting canonical Notch and NO signaling fully rescues myelin decompaction, yet only partially rescues myelin thickness. Importantly, targeting NO and MEK signaling in combination rescues myelin compaction and myelin thickness. Thus, targeting NO and MEK signaling in Costello syndrome patients may improve aspects of brain function.
4.2 Results:

**Constitutive HRas activation in oligodendrocytes causes myelin decompaction.**

In rare Costello Syndrome patients, a severe G12V mutation in HRAS underlies patient phenotypes. We tested if myelin decompaction is present shortly after adult-induced hyper activation of HRas in oligodendrocytes, using iPlpCre. Using this driver, after tamoxifen exposure, 35% of mature oligodendrocytes (100% Olig2+, a 75-80% Olig2+,CC1+) in the corpus callosum show recombination (Mayes et al., 2013). We crossed iPlpCre mice to mice containing two HRas genes under the regulatory control of the HRas endogenous gene promoter. The upstream wild type HRas is flanked by loxP sites, so that the mutant allele is not expressed unless the WT copy is excised (Chen et al., 2009). We examined brains of 3 month old iPlpCre;HRasG12V/+ and iPlpCre;HRasG12V/G12V mice exposed to tamoxifen at 2 months of age, and wild type iPlpCre mice littermates (Figure 4.1A) using electron microscopy (Figure 4.1B,C,D). We quantified myelin thickness by g-ratio analysis, by quantification of total axons, and showing myelin decompaction as the number of quadrants with decompaction (e.g. x <25, 25-50, 50-75, or 75-100% decompaction) for each decompact axon. Unbiased counting of myelinated axons in the corpus callosum revealed a significant decrease in g-ratio, indicating an increase of myelin thickness, in HRas mutants compared to wild type mice (Figure 4.1E). The increase in thickness in HRas mutants was due to decompaction of the myelin lamellae (Figure 4.1F). Both the total number of fibers with decompaction and the severity of decompaction, as measured by quadrants around the axon with decompacted myelin, were increased in HRas mutants compared to wild type mice (Figure 4.1F). Thus, the myelin decompaction characteristic of the iPlpCre; HRasG12V/+ and iPlpCre;HRasG12V/G12V mice is present by 1 month after tamoxifen administration. Hemizygous and homozygous HRasG12V mutations had
A

Adult Induced CreER\textsuperscript{T} Recombination

Birth  2 Mo  3 Mo  Tamoxifen  Analysis

B

C

D

E

Corpus Callosum Myelin Thickness

F

Corpus Callosum Myelin Decompaction

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94
Figure 4.1. Constitutive *HRas* activation in oligodendrocytes causes myelin decompaction.

(A) Timeline of adult tamoxifen inducible recombination murine model and analysis.

(B-D) Electron micrographs of corpus callosum cross sections at the mid line of *iPlpCre*, *iPlpCre;HRasG12V/+*, and *iPlpCre;HRasG12V/G12V* mice at 1 month post tamoxifen (left: 30,000X / right: 50,000X). White arrows: areas of myelin decompaction.

(E) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of *HRas* hemizygous and homozygous mutants 1 month post adult induced recombination as compared to wild type (n=3/genotype, **** p<0.0001). No significant difference in g-ratio between *HRas* hemizygous and homozygous mutants (p=0.451).

(F) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Increase in % decompaction in *HRas* hemizygous and homozygous mutants 1 month post adult induced recombination as compared to wild type (n=3/genotype, p=0.003, p=0.0006; ** p<0.01, *** p<0.001). There was no significant difference in % decompaction between *HRas* hemizygous and homozygous mutants (n=3/genotype, p=0.38).
the same phenotype; there was no difference in g-ratio or a significant difference in the percent of myelinated fibers with decompaction. We focused on \textit{iPlpCre; HRasG12V/+} mice to best model Costello syndrome patients.

**Constitutive HRas activation in oligodendrocytes causes progressive myelin decompaction**

We tested if myelin decompaction is more severe after 6 months of hyper activation of \textit{HRas} in oligodendrocytes. In brains of 8 month old \textit{iPlpCre;HRasG12V/+} and \textit{iPlpCre;HRasG12V/G12V} mice, exposed to tamoxifen at 2 months of age, unbiased counting of myelinated axons in the corpus callosum revealed a significant decrease in g-ratio in \textit{HRas} mutants at 8 months compared to 3 months (Figure 4.2A). In contrast, the number of fibers with decompaction and severity of decompaction were not significantly changed in \textit{HRas} mutants at 8 months compared to 3 months (Figure 4.2B). This data demonstrates a progressive myelin decompaction phenotype in \textit{HRas} mutants over time due to an increase in myelin wraps.

**Concurrent loss of Rbpj reduces myelin de-compaction and Gamma secretase inhibitor (MRK-003) partially rescues severity of myelin decompaction in HRas mutants.**

We tested if Notch signaling is an effector of Ras signaling pathway in \textit{iPlpCre;HRasG12V/+} mice, using a mutant of recombining binding protein suppressor of hairless (RBPJ), the transcriptional co-factor required for canonical Notch signaling or by administration of a gamma secretase inhibitor (GSI). Recombining binding protein suppressor of hairless (RBPJ) is a transcriptional co-factor required for canonical Notch signaling. To test if Notch signaling is necessary for the \textit{HRas} mutant phenotype, we analyzed \textit{iPlpCre;HRasG12V/+;Rbpjfl/fl} mice at 3
A  Corpus Callosum Myelin Thickness

![](Image of graph showing g-ratio (Axon Diameter/Fiber Diameter) for WT and iPipCre; HRasG12V/+ and iPipCre; HRasG12V/G12V groups at 3 and 8 months.

B  Corpus Callosum Myelin Decomposition

![](Image of graph showing percentage of decomposed fibers for WT and iPipCre; HRasG12V/+ and iPipCre; HRasG12V/G12V groups at 3 and 8 months.)
Figure 4.2. Constitutive \( HRas \) activation in oligodendrocytes causes progressive myelin decompaction

(A) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of \( HRas \) mutants 1 month as compared to 6 months post adult induced recombination (\( n=3/\)genotype, ****; \( p<0.0001 \)). No significant difference in g-ratio between \( HRas \) hemizygous and homozygous mutants at 6 months post tamoxifen (\( n=3/\)genotype, \( p=0.097 \)).

(B) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Chronic severe decompaction, as no significant change in % decompaction is evident in hemizygous or homozygous \( HRas \) mutants 1 month as compared to 6 months post adult induced recombination (\( n=3/\)genotype, \( p=0.962, p=0.791 \)) and no significant difference in % decompaction between \( HRas \) hemizygous and homozygous mutants at 1 month and 6 months post tamoxifen (\( n=3/\)genotype, \( p=0.385, p=0.41 \)).
months of age, 1 month after tamoxifen administration (Figure 4.3A). Analysis of myelinated axons in the corpus callosum using electron microscopy (Figure 4.3C) revealed a decrease in the percent of myelinated fibers with decompaction and a decrease in the severity of decompaction in iPlpCre;HRasG12V/+,Rbpjfl/fl compared to iPlpCre;HRasG12V/+ mice (Figure 4.3G). Despite these changes, there was not a significant change in myelin thickness in iPlpCre;HRasG12V/+,Rbpjfl/fl mice compared to iPlpCre;HRasG12V/+ mice due to the number of myelin wraps (Figure 4.3E). With this partial rescue in myelin ultrastructure, we conclude that RBPJ-dependent Notch signaling acts downstream of HRas in oligodendrocytes to regulate myelin compaction.

Gamma secretase inhibitor (GSI) prevents cleavage of the Notch intracellular domain and therefore prevents downstream transcription of target genes. To test if decompaction can be acutely improved by inhibiting Notch signaling, we administered GSI MRK-003 weekly beginning 1 month post tamoxifen administration, when myelin decompaction is evident (Figure 4.3B). Using electron microscopy, we examined brains of iPlpCre;HRasG12V/+ mice exposed to tamoxifen at 2 mo. of age and treated with vehicle or MRK-003 beginning at 3 mo. of age (Figure 4.3D). Analysis of the corpus callosum revealed a significant increase in g-ratio, indicating a decrease in myelin thickness, in MRK-003 treated mice compared to vehicle-treated iPlpCre;HRasG12V/+ mice (Figure 4.3F). We identified both a decrease in the total number of fibers with decompaction and in the severity of decompaction in MRK-003 treated mice compared to vehicle-treated iPlpCre;HRasG12V/+ mice (Figure 4.3H). These results support the conclusion that pharmacological inhibition of Notch signaling can improve myelin decompaction caused by hyper activation of HRas, even after the phenotype has manifested in vivo.
Figure 4.3. Concurrent loss of Rbpj reduces myelin de-compaction and Gamma secretase inhibitor (MRK-003) partially rescues severity of myelin decompaction in HRas mutants.

(A) Timeline of adult tamoxifen inducible recombination murine model and analysis.

(B) Timeline of adult tamoxifen inducible recombination murine model, GSI MRK-003 administration, and analysis.

(C) Electron micrographs of corpus callosum cross sections at the mid line of iPlpCre;HRasG12V/+ mice (Left) and iPlpCre; HRasG12V/+,Rbpjfl/fl mice (Right) (magnification: 30,000X). White arrows: areas of myelin decompaction.

(D) Electron micrographs of corpus callosum cross sections at the mid line of Vehicle (Left) and GSI MRK-003 (Right) treated iPlpCre; HRasG12V/+ mice (magnification: 30,000X). White arrows: areas of myelin decompaction.

(E) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of iPlpCre;HRasG12V/+ and iPlpCre; HRasG12V/+,Rbpjfl/fl as compared to wild type mice (**** ; p<0.001). There was no significant difference between iPlpCre;HRasG12V/+ and iPlpCre; HRasG12V/+,Rbpjfl/fl (n=3,5/genotype, p=0.556)

(F) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of GSI MRK-003 as compared to vehicle treated in HRas mutant mice (n=4/genotype, **** ; p<0.0001 ).

(G) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). There was a significant decrease in % decompaction in iPlpCre;HRasG12V/+,Rbpjfl/fl mutant as compared to iPlpCre;HRasG12V/+ mutant mice (n=3,5/genotype, p=0.05).
(H) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in % decompaction in GSI MRK-003 as compared to vehicle treated in HRas mutant mice (p=0.05). There was no significant difference in % decompaction between GSI MRK-003 treated HRas mice and vehicle treated wild type mice (n=4/genotype, p=0.259).
As in Rbpj mutants, we observed both a decrease in the total number of fibers with decompaction and in the severity of decompaction in MRK-003 treated mice compared to vehicle-treated iPlpCre;HRasG12V/+ mice (Figure 4.3H). Thus, pharmacological inhibition of Notch signaling improves myelin decompaction caused by HRasG12V. Discordance between effects of GSI and Rbpj loss may result from off target effects of the drug.

**Hyper activation of HRas in oligodendrocytes leads to an increase in the number of oligodendrocyte lineage cells.**

In the forebrain of HRas mutants 1 month post tamoxifen, there is not a significant change in the number of PDGFRα+, O4+, or GalC+ cells of the oligodendrocyte cell lineage as compared to wild type (Figure 4.4A). However, flow cytometry enabled identification of a significant increase in the number of O4+;GalC−;Hes5GFP+ immature oligodendrocytes in HRas mutants as compared to wild type (Figure 4.4B), suggesting prolonged Notch pathway activation. Immunohistochemistry of fixed brain sections confirmed an increase in the oligodendrocyte cell lineage, Olig2+ lineage cells and CC1+ oligodendrocytes, in the corpus callosum of hemizygous and homozygous HRas mutants 1 month after tamoxifen (Figure 4.4C). These data indicate an increase in the oligodendrocyte cell lineage in the corpus callosum without a significant change in the entire forebrain. The increase in Hes5GFP+ oligodendrocytes in the entire forebrain may contribute to the changes in the oligodendrocyte cell lineage noted in the corpus callosum. These studies, carried out early after tamoxifen administration, strongly support a role for Notch signaling and HRas in oligodendrocytes but do not exclude relevant effects on OPCs. There is an increase in NG2+ progenitors at 3 months in homozygous, but no hemizygous, HRas mutants (Figure 4.4C). There is an increase in NG2+ cells present at 8 months yet not at 3 months in
**Figure 4.4. Hyper activation of HRas increases the oligodendrocyte cell lineage and Gamma secretase inhibitor (MRK-003) promotes pruning of aberrant mature oligodendrocytes.**

(A) Quantification of average (with SEM) of the oligodendrocyte cell lineage in the forebrain, as measured by flow cytometry of stained live CNS resident cells (excluding microglia). No change in the oligodendrocyte cell lineage in HRas mutants as compared to wild type mice (n=3/genotype).

(B) Quantification of fold change with standard error of the mean (SEM) compared to wild type of Hes5GFP+, recent canonical Notch pathway signaling, in the oligodendrocyte cell lineage in the forebrain as measured by immunofluorescence of unfixed cells. Increase in HRas mutants as compared to wild type O4+ GalC- immature oligodendrocytes (n=3/genotype, ANOVA p=0.034, *; p<0.05). There was a not a significant change in HRas mutants as compared to wild type PDGFRα+ (ANOVA p=0.158), O4+ GalC+ (ANOVA p=0.888), or O4- GalC+ (ANOVA p=0.353) oligodendrocyte lineage cells.

(C-E) Quantification of average (with SEM) of the oligodendrocyte cell lineage in the corpus callosum as measured by immunofluorescence of fixed cryostat sections.

(C) Increase in NG2+ oligodendrocyte progenitors, Olig2+ the oligodendrocyte cell lineage, and CC1+ mature oligodendrocytes in HRas hemizygous and homozygous mutants as compared to wild type (n=3/genotype, ANOVA p=0.015 , p=0.0009 , p=0.002 ) (* ; p<0.05, **; p<0.01).

(D) Increase in NG2+ oligodendrocyte progenitors in HRas hemizygous mutants as compared to wild type at 3 months and 8 months (p=0.022, p=0.0041). No significant change in Hes5GFP+NG2+ oligodendrocyte progenitors in HRas hemizygous mutants as compared to wild type at 3 months and 8 months (n=3/genotype, ANOVA 0.425).
(E) Increase in CC1+ cells in GSI-MRK-003 as compared to vehicle treated in wild type (n=3/genotype, p=0.0071) mice. Decrease in CC1+ cells in GSI-MRK-003 as compared to vehicle treated in HRas mutants (n=3/genotype, p=0.01).
hemizygous \textit{HRas} mutants (\textbf{Figure 4.4D}). These data suggest a dose response of \textit{HRas} activation in the timing of progenitor recruitment. Notably, decompaction in hemizygous \textit{HRas} mutants occurs prior to progenitor recruitment.

\textbf{Gamma secretase inhibitor (MRK-003) promotes pruning of aberrant mature oligodendrocytes in \textit{HRas} mutants.}

To test whether we can rescue aberrant populations of oligodendrocyte lineage cells in the corpus callosum, we analyzed \textit{iPlpCre;HRasG12V/+} mice sacrificed at 4 months of age that had been exposed to tamoxifen at 2 months of age and treated with vehicle or MRK-003 weekly beginning at 3 months of age. Using immunohistochemistry, we examined the corpus callosum and found a significant decrease in aberrant oligodendrocytes (CC1+) (\textbf{Figure 4.4E}). However, aberrant progenitors (PDGFRα+) remained increased relative to wild type. Taken together with effects of \textit{Rbpj} loss, the data support a role for Notch signaling downstream of hyper activation of HRas in oligodendrocytes.

\textbf{Hyper-activation of Notch causes myelin decompaction within one month.}

Given that the number of Hes5GFP+;O4+;GalC- oligodendrocytes in the forebrain significantly increased in \textit{HRas} mutant versus wild type mice, we tested if aberrant Notch activation in the mature oligodendrocytes is sufficient to disrupt myelin compaction. As previously shown, there is a significant decrease in g-ratio and an increase in the total number of fibers with decompaction in \textit{iPlpCre;RosaNICD} mutants compared to wild type mice. This mimicked the \textit{HRas} phenotype, as there was no significant difference in g-ratio or percent of fibers with
decompaction in *NICD* as compared to hemizygous and homozygous *HRas* mutants (Figure 4.5A,B).

**Myelin decompaction resulting from Hyperactivation of Notch can be rescued by inhibition of NOS with L-NAME.**

As noted, constitutive imbalance of Notch activation can cause defects in myelin compaction. One week of treatment with NOS inhibitor L-NAME (Figure 4.6A), rescued hyper-activation of Notch pathway activity in oligodendrocytes (*iPlpCre;RosaNICD*) as measured by a decrease in myelin thickness and a decrease in percent of myelinated fibers with decompaction (Figure 4.6B,C,D). Thus, these data suggest a role for Nitric Oxide signaling in functional regulation of hyperactive Notch signaling in oligodendrocytes that contributes to myelin compaction.

**Inhibition of nitric oxide synthase with L-NAME partially rescues, and in combination with Inhibition of Notch signaling completely rescues, decompaction in the hemizygous *HRas* mutant.**

The finding that genetic or pharmacological inhibition of Notch signaling only partially rescues myelin decompaction in *iPlpCre;HRasG12V/+* mutants suggests that additional signaling pathways contribute to this phenotype. We combined dissociated cells from forebrain and optic nerve of *iPlpCre;HRasG12V/+* mice and examined nitric oxide (NO) in living brain cells using flow cytometry in conjunction with a dye that emits red fluorescence when NOS forms NO. Mutant GalC+ cells, but not PDGFRα+ progenitors or immature oligodendrocytes, showed an increase in NO (Figure 4.7A). To define the role of NO in the myelin decompaction phenotype, we examined brains of *iPlpCre;HRasG12V/+ mice* exposed to tamoxifen at 2 months of age and treated at 3 months of age for 7 days with L-NAME, a specific NOS inhibitor. Analysis of
Figure 4.5. Hyper activation of HRas or hyper activation of Notch causes myelin decompaction within one month.

(A) Quantification of average (with SEM) of g-ratio (axon diameter/fiber diameter). Decrease in g-ratio of hemizygous and homozygous HRas mutants and in the NICD mutant as compared to wild type mice (n=3,3,4/genotype, ANOVA, ****; p<0.0001). No significant difference in g-ratio between HRas mutants and NICD mice (post hoc Tukey tests not significant).

(B) Quantification of percent of decompacted fibers and the severity of decompaction. No significant difference in % decompaction and severity of decompaction in hemizygous or homozygous HRas mutants as compared to NICD mice (n=3,4/genotype, p=0.541, p=0.403).
Figure 4.6. Myelin decompaction resulting from Hyper Activation of Notch can be rescued by inhibition of NOS with L-NAME.

(A) Timeline of adult tamoxifen inducible recombination murine model, L-NAME administration, and analysis.

(B) Electron micrographs of corpus callosum cross sections at the mid line (20,000X). White arrows: areas of myelin decompaction. \textit{iPlpCre;RosaNICD} treated with vehicle (Right) or (L-NAME).

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of L-NAME as compared to vehicle treated in \textit{iPlpCre;RosaNICD} (**** ; p<0.0001).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in % decompaction in L-NAME as compared to vehicle treated in \textit{iPlpCre;RosaNICD} (p=0.021). No significant difference in \textit{NICD} + L-NAME vs. wild type (p=0.426).
Figure 4.7. Inhibition of nitric oxide synthase with L-NAME partially rescues, and in combination with inhibition of Notch signaling completely rescues, decompaction in the hemizygous HRas mutant.

(A) Quantification of fold change with standard error of the mean (SEM) in HRas mutant compared to wild type of NO+ cells in the forebrain as measured by immunofluorescence of unfixed cells. An increase in HRas mutant as compared to wild type in GalC++;NO+ cells (n=3/genotype, p=0.042; *, p<0.05) at 1 month post tamoxifen. No significant difference in PDGFRα++;ROS+ cells (n=3/genotype, p=0.225) and at 1 month post tamoxifen.

(B) Electron micrographs of corpus callosum cross sections at the mid line of Vehicle (Left) and L-NAME (Right) treated iPlpCre;HRasG12V/+ mice (Right) (magnification: 30,000X). White arrows: areas of myelin decompaction.

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of L-NAME as compared to vehicle treated in iPlpCre;HRasG12V/+ mice (n=4,3/genotype, ****; p<0.0001).

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in % decompaction in L-NAME as compared to vehicle treated in iPlpCre;HRasG12V/+ mice (n=4,3/genotype, p=0.009). The was no difference in % decompaction between L-NAME treated iPlpCre;HRasG12V and wild type mice (p=0.246).

(E) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of L-NAME as compared to vehicle treated in iPlpCre;HRasG12V/+ mice (n=4,3/genotype, ****; p<0.0001). Increase in g-ratio of L-NAME as compared to vehicle treated in iPlpCre;HRasG12V+,Rbpjfl/fl (n=5,3/genotype, ****; p<0.0001); resulting in a partial rescue of the HRas mutant myelin thickness. No significant difference between iPlpCre;HRasG12V/+
and *iPlpCre;HRasG12V/+;Rbpjfl/fl* when treated with vehicle (p=0.056) or L-NAME (p=0.1018).

(F) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in % decompaction in L-NAME as compared to vehicle treated in *iPlpCre;HRasG12V/+* mice (n=4,3/genotype, p=0.009). Decrease in % decompaction in L-NAME as compared to vehicle treated in *iPlpCre;HRasG12V/+;Rbpjfl/fl* (n=5,3/genotype, p=0.041); resulting in a full rescue of the *HRas* mutant decompaction phenotype. There was no difference in % decompaction between L-NAME treated *iPlpCre;HRasG12V/+;Rbpjfl/fl* and wild type mice (p=0.348).
myelinated axons in the corpus callosum revealed a significant increase in g-ratio, indicating a
decrease in myelin thickness/partial rescue of myelin compaction in L-NAME treated
iPlpCre;HRasG12V/+ (Figure 4.7B,C) which was due to decreased fibers showing myelin
decomposition and a decrease in the severity of decompaction (Figure 4.7B,D). Thus, brief
pharmacological inhibition of NO production improves myelin decompaction caused by
HRasG12V. Although L-NAME significantly increased the total number of fibers with compact
myelin, full rescue of the HRas decompaction phenotype was not achieved.

**Inhibition of NOS with L-NAME in mice with Rbpj loss rescues decompaction, yet not
myelin thickness, in hemizygous HRas mutants.**

As neither inhibition of nitric oxide production nor Notch pathway inhibition completely rescued
the hemizygous HRas phenotype, we tested if inhibition of both Notch signaling and nitric oxide
production might completely rescue myelin decompaction. Using electron microscopy, we
examined brains of iPlpCre;Rbpjfl/fl,HRasG12V/+ mice exposed to tamoxifen at 2 months of
age and treated with vehicle or L-NAME beginning at 3 months of age. Analysis of myelinated
axons in the corpus callosum revealed a partial rescue of myelin thickness, as measured by an
increase in g-ratio, in L-NAME treated compared to vehicle treated
iPlpCre;Rbpjfl/fl,HRasG12V/+ mice (Figure 4.7E). There was a full rescue of decompaction, as
measured by a decrease in the total number of fibers with decompaction, in L-NAME treated
compared to vehicle treated iPlpCre;Rbpjfl/fl,HRasG12V/+ (Figure 4.7F). Thus brief blockade
of nitric oxide production, in combination with loss of RBPJ-dependent Notch signaling in
oligodendrocytes, rescues myelin decompaction in hemizygous HRas mutants. The additive
effect of dual inhibition suggests that the Notch signaling and production of NO are functionally
independent. However, even this combination treatment was not sufficient to fully rescue myelin thickness.

**Inhibition of Notch signaling rescues myelin decompaction and dual inhibition of nitric oxide/Notch signaling completely rescued decompaction and myelin thickness in the homozygous HRas mutant.**

Data shown demonstrate that loss of Rbpj or exposure to GSI in HRasG12V/+ mutants partially rescues myelin decompaction. GSI treatment also increased g-ratio and decreased myelin thickness in iPlpCre;HRasG12V/G12V mice, compared to vehicle treated mice (Figure 4.8A). There was a full rescue of decompaction, shown as a decrease in percent of fibers with decompaction in iPlpCre;HRasG12V/G12V treated with GSI as compared to vehicle treated mice (Figure 4.8B). These data suggest that dependence on Notch signaling is dose dependent in HRas mutants, as homozygous HRas decompaction was fully rescued with GSI treatment, whereas hemizygous HRas mice (mimicking patients) are only partially rescued (Figure 4.8A,B). We also tested how loss of Rbpj affects homozygous HRas mutant myelin decompaction. Homozygous loss of Rbpj increased g-ratio in iPlpCre;HRasG12V/G12V mice (Figure 4.8C). Notably, there was a dose dependent decrease in severity of decompaction between iPlpCre;HRasG12V/G12V,Rbpjfl/+ and Rbpjfl/fl mutants as well as decrease in percent of fibers with decompaction in iPlpCre;HRasG12V/G12V,Rbpjfl/fl as compared to iPlpCre;HRasG12V/G12V mice (Figure 4.8D). Thus, RBPJ-dependent Notch signaling acts downstream of HRas in oligodendrocytes to regulate myelin compaction, and the effects of Rbpj loss are more profound in mice with two mutant HRas alleles than in those with one mutant allele, indicating that Ras signaling strength affects dependence on the Notch pathway.
A. Corpus Callosum Myelin Thickness

B. Corpus Callosum Myelin Decompaction

C. Corpus Callosum Myelin Thickness

D. Corpus Callosum Myelin Decompaction

E. Corpus Callosum Myelin Thickness

F. Corpus Callosum Myelin Decompaction
Figure 4.8. Inhibition of Notch signaling rescues myelin decompaction and dual inhibition of nitric oxide/ Notch signaling completely rescued decompaction and myelin thickness in the homozygous H\textit{Ras} mutant.

(A) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Full rescue to wild type levels (p=0.75), increase in g-ratio of GSI MRK-003 as compared to vehicle treated in homozygous H\textit{Ras} mutant mice (n=3/genotype, **** ; p<0.0001).

(B) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Full rescue, decrease in % decompaction in GSI MRK-003 as compared to vehicle treated in homozygous H\textit{Ras} mutant mice (n=3/genotype, p=0.002). There was a significant decrease in % decompaction in GSI MRK-003 treated homozygous H\textit{Ras} mutants as compared to vehicle treated wild type mice (p=0.023).

(C) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/+} and \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/fl} as compared to \textit{iPlpCre;HRasG12V/G12V} mice (n=4,3,3/genotype, **** ; p<0.0001 ). No difference in g-ratio between \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/+} and \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/fl} (n=4,3/genotype, p=0.124)

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Full rescue, significant decrease in % decompaction in \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/fl} as compared to \textit{iPlpCre;HRasG12V/G12V} mice (n=3/genotype, p=0.0065). There was a decrease in severity of decompaction in \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/+} as compared to \textit{iPlpCre;HRasG12V/G12V} mice, yet there was not a significant decrease in % decompaction (n=4,3/genotype, p=0.171). Dose dependent difference in % decompaction between \textit{iPlpCre;HRasG12V/G12V,Rbpjfl/+} and
iPlpCre;HRasG12V/G12V,Rbpjfl/fl (n=4,3 genotype, p=0.013). (E) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of L-NAME as compared to vehicle treated in iPlpCre;HRasG12V/G12V,Rbpjfl/fl (n=3/genotype, **** ; p<0.0001); resulting in a full rescue of the HRas mutant myelin thickness.

(F) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). As % decompaction is fully rescued in iPlpCre;HRasG12V/G12V,Rbpjfl/fl as compared to iPlpCre;HRasG12V/G12V mice (n=3/genotype, p=0.007), there was no significant difference in % decompaction in L-NAME as compared to vehicle treated in iPlpCre;HRasG12V/G12V,Rbpjfl/fl (p=0.500). There was no significant difference between L-NAME treated iPlpCre;HRasG12V/G12V,Rbpjfl/fl mutants and wild type mice (p=0.06).
We also tested if effects of inhibition of the combination of Notch signaling and nitric oxide production differ with Ras dosage. We examined brains of \( iPlpCre;Rbpj^{fl/fl},HRas^{G12V/G12V} \) mice exposed to tamoxifen at 2 months of age and treated with vehicle or L-NAME beginning at 3 months of age. Analysis of myelinated axons in the corpus callosum revealed a full rescue of myelin thickness, as measured by an increase in g-ratio, in L-NAME treated compared to vehicle treated \( iPlpCre;Rbpj^{fl/fl},HRas^{G12V/G12V} \) mice (Figure 4.8E). As loss of \( Rbpj \) was sufficient to fully rescue decompaction in homozygous \( HRas \) mutants, as expected there was no change in decompaction with L-NAME treatment (Figure 4.8F). Thus inhibition of nitric oxide production, together with loss of RBPJ-dependent Notch signaling in oligodendrocytes, completely rescues myelin thickness and decompaction in homozygous \( HRas \) mutants.

**Inhibition of MEK with PD-0325901 partially rescues, and inhibition of both MEK / NO signaling fully rescues, myelin decompaction in hemizygous HRas mutant mice**

Given that a single mutant \( HRas \) mutation is found in patients with Costello Syndrome, we decided to evaluate additional downstream effectors. As noted above, Notch signaling activates MEK/ERK signaling in oligodendrocytes. MEK is also a major effector pathway downstream of Ras-GTP. Therefore, we tested effects of blocking MEK/ERK signaling \textit{in vivo}. We used the MEK inhibitor (MEKi; PD0325901), which is known to cross the blood brain barrier. We examined brains of \( iPlpCre;HRas^{G12V/+} \) mice exposed to tamoxifen at 2 months of age and treated with vehicle or MEKi daily beginning at 3 months of age for 21 days (Figure 4.9A). Analysis of myelinated axons in the corpus callosum revealed a partial rescue of myelin thickness, as measured by an increase in g-ratio, in MEKi treated compared to vehicle treated
**Figure 4.9. Inhibition of MEK with PD-0325901 partially rescues, and inhibition of both MEK / NO signaling fully rescues, myelin decompaction in hemizygous HRas mutant mice.**

(A) Timeline of adult tamoxifen inducible recombination murine model, MEKi PD-0325901 daily administration, and analysis.

(B) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of MEKi as compared to vehicle treated in *iPlpCre;HRasG12V/+* (n=3/group, **** ; p<0.0001).

(C) Electron micrographs of corpus callosum cross sections at the mid line of Vehicle (Left) and MEKi (Right) treated *iPlpCre; HRasG12V/+* mice (magnification: 30,000X). White arrows: areas of myelin decompaction.

(D) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in % decompaction and severity of decompaction in MEKi as compared to vehicle treated in *iPlpCre;HRasG12V/+* (n=3/group, p=0.037).

(E) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Full rescue, a significant decrease in % decompaction of MEKi + L-NAME as compared to single agent treatment (L-NAME or MEKi) treated in *iPlpCre;HRasG12V/+* (n=3/group, p=0.032, p=0.025). There was no significant difference between *iPlpCre;HRasG12V/+* treated with MEKi + L-NAME and wild type (n=3/group, p=0.155).

(F) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of MEKi + L-NAME as compared to single treatment (L-NAME or MEKi) in *iPlpCre;HRasG12V/+* (n=3/group, **** ; p<0.0001); resulting in a full rescue of the HRas
mutant myelin thickness. There was a significant difference between MEKi + L-NAME treated
iPlpCre;HRasG12V/+ as compared to wild type mice (n=3/group, p=0.003).
iPlpCre; HRasG12V/+ mice (Figure 4.9B,C). There was a significant decrease in the number of fibers with decompaction and severity of decompaction in MEKi treated compared to wild type iPlpCre;HRasG12V/+ mice (Figure 4.9D), yet rescue was not complete. We targeted both MEK and NOS signaling. The combination of inhibition of NOS, with L-NAME, and MEKi was sufficient to rescue decompaction and myelin thickness (Figure 4.9E,F). Flow cytometry analysis revealed that treatment with MEKi rescues hyperactive Notch signaling (Figure 4.12A). Therefore, it is likely that this combination targets NO, MEK, and Notch signaling. Taken together, these data show a role for Notch and NO and MEK in myelin compaction and myelin thickness in hemizygous HRas mutants. Thus, NO and MEK pathways are potential synergistic therapeutic targets in Costello syndrome patients.

**Inhibition of both MEK / Notch signaling partially rescues hemizygous HRas mutants**

inhibition of MEK can fully rescue myelin compaction in NICD mutants.

Additionally, we tested if inhibition of MEK, together with blockade of Notch signaling, may completely rescue myelin thickness. The combination of loss of Notch signaling through Rbpj loss and MEKi was sufficient to fully rescue decompaction, but failed to fully rescue myelin thickness (Figure 4.10A,B).

As previously described, adult induced expression of NICD in oligodendrocytes causes myelin decompaction at 1 month post tamoxifen. Using flow cytometry, we measured an increase in pERK, a downstream effector of Ras/Raf/MEK signaling, in NICD mutant PDGFRα+ and GalC+ oligodendrocytes as compared to wild type (Figure 4.10C), consistent with findings that in development and disease Notch and Ras GTPase can act up and down
**Corpus Callosum Myelin Decompaition**

(A) Analysis of myelin decompaition percentages across different genotypes and treatments:

- **Quadrants**
  - >75%
  - 50-75%
  - 25-50%
  - <25%

- **WT**
- **HRas G12V+/+**
- **HRas G12V/+/Rbpj fl/fl**
- **Vehicle**
- **+ Meki**

(B) Myelin thickness analysis across different genotypes and treatments:

- **g-ratio** (axon diameter/fiber D)
- **WT**
- **HRas G12V+/+**
- **HRas G12V/+/Rbpj fl/fl**
- **Vehicle**
- **+ Meki**

**Flow of Forebrain Cells, pERK**

(C) Bar graph showing fold change in pERK levels:

- **WT**
- **NICD**
- **PDGFR+**
- **GalC+**

**Vehicle**

- **Meki PD 0325901**

**Corpus Callosum Myelin Thickness**

(D) Image of tissue sections with noted `PipCre; ROSA NICD**

**Corpus Callosum Myelin Decompaition**

(E) G-ratio analysis across different genotypes and treatments:

- **Wild Type**
- **PipCre; ROSA NICD**
- **PipCre; ROSA NICD Meki**

(F) Percentage of decompaition fibers across different genotypes and treatments:

- **Quadrants**
  - >75%
  - 50-75%
  - 25-50%
  - <25%

- **Wild Type**
- **PipCre; ROSA NICD**
- **PipCre; ROSA NICD Meki**
Figure 4.10. Inhibition of both MEK / Notch signaling partially rescues hemizygous HRas mutants inhibition of MEK can fully rescue myelin compaction in NICD mutants.

(A) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Decrease in % decompaction in MEKi as compared to vehicle treated in iPlpCre;HRasG12V/+;Rbpjfl/fl (n=3/group, p=0.05); resulting in a full rescue of the HRas mutant decompaction phenotype. Full rescue, decrease in % decompaction in iPlpCre;HRasG12V/+;Rbpjfl/fl as compared to iPlpCre;HRasG12V/+ (n=3/group, p=0.016) treated with MEKi. There was no difference in % decompaction between MEKi treated iPlpCre;HRasG12V/+;Rbpjfl/fl and wild type mice (n=3/group, p=0.452).

(B) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of MEKi as compared to vehicle treated in iPlpCre;HRasG12V/+;Rbpjfl/fl (n=3/group, ****; p<0.0001); resulting in a partial rescue of the HRas mutant myelin thickness. No significant difference between iPlpCre;HRasG12V/+ and iPlpCre;HRasG12V/+;Rbpjfl/fl when treated with vehicle (n=3/group, p=0.056) or MEKi (n=3/group, p=0.211).

(C) Quantification of fold change with standard error of the mean (SEM) in iPlpCre;RosaNICD mutant compared to wild type of pERK+ cells in the forebrain as measured by immunofluorescence of unfixed cells. An increase in NICD mutant as compared to wild type in PDGFRα+,pERK+ and in GalC+;pERK+ cells at 1 month post tamoxifen (n=3/genotype).

(D) Electron micrographs of corpus callosum cross sections at the mid line of Vehicle (Left) and MEKi PD-0325901 (Right) treated iPlpCre;RosaNICD mice (magnification: 20,000X). White arrows: areas of myelin decompaction.

(E) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter). Increase in g-ratio of MEKi as compared to vehicle treated in iPlpCre;RosaNICD mice (n=3/group, ****;
p<0.0001). There was no significant difference between \textit{iPlpCre;RosaNICD} treated with MEKi as compared to wild type (n=3/group, p=0.0865).

(F) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction). Full rescue, decrease in % decompaction in MEKi as compared to vehicle treated \textit{iPlpCre;RosaNICD} mice (n=3/group, p=0.0005). There was no difference in % decompaction between MEKi treated \textit{iPlpCre;RosaNICD} and wild type mice (n=3/group, p=0.0512).
stream of one another (Sundaram, 2005). We then treated \textit{iPlpCre;RosaNICD} mice, at 3 months old, 1 month after tamoxifen administration, with the MEK inhibitor (MEKi) PD-0325901 daily for 21 days. Myelin decompaction was fully rescued (Figure 4.10D,E,F). Thus, Notch signaling increases p-ERK signaling, and inhibition of ERK signaling through targeting MEK rescues the \textit{NICD} phenotype. Together these data confirm cross talk between MEK/ERK signaling and Notch signaling pathways.

\textbf{Inhibition of MEK fully rescues hemizygous \textit{Nf1} mutants, and inhibition of MEK / Notch or MEK / NO signaling fully rescues myelin compaction in homozygous \textit{Nf1} mutants.}

As cross talk between Notch-MEK/ERK-NO signaling was uncovered in the \textit{HRasG12V} murine model of Costello Syndrome, we performed further analysis on the murine model of NF1. We tested the hypothesis that different levels of Ras GTPase activity and subsequent downstream signaling could lead to changes in response of therapeutically targeting MEK/ERK signaling in gain of function Rasopathies. As the phenotype in hemizygote \textit{Nf1} mutants is less severe (Chapter 3), it is not surprising that MEKi alone was sufficient to fully rescue myelin compaction, as seen with single agent therapy of GSI MRK-003 and L-NAME (Figure 4.11A,B). Inhibition of MEK alone was not sufficient to fully rescue myelin compaction or myelin thickness in homozygous \textit{Nf1} mutants; similar to our findings in \textit{HRas} mutants (Figure 4.11A,B). To further define the role downstream effectors of Ras and pathway cross talk, we tested if inhibition of MEK and Notch or NOS might completely rescue myelin thickness.

The combination of loss of Notch signaling, with \textit{Rbpj} loss, and MEKi is sufficient to rescue decompaction and near full rescue of myelin thickness (Figure 4.11E,F). Similar to \textit{HRas} mutants, loss of Notch signaling leads to a decrease in MEK/ERK signaling, as measured
Figure 4.11. Inhibition of MEK fully rescues hemizygous Nf1 mutants, and inhibition of MEK / Notch or MEK / NO signaling fully rescues myelin compaction in homozygous Nf1 mutants.

(A,C,E) Quantification of percent of decompacted fibers and the severity of decompaction (measured as quadrants of decompaction).

(B,D,F) Quantification of average (with SEM) g-ratio (axon diameter/fiber diameter).

(A) Full rescue, a significant decrease in % decompaction of MEKi as compared to vehicle treated in iPlpCre:Nf1fl/+ (n=3/group, p=0.05). Partial rescue, a significant decrease in % decompaction of MEKi as compared to vehicle treated in iPlpCre:Nf1fl/fl (n=3/group, p=0.05).

(B) Increase in g-ratio of MEKi as compared to vehicle treated in iPlpCre:Nf1fl/+ (n=3/group, ****; p<0.0001); resulting in a full rescue. Increase in g-ratio of MEKi as compared to vehicle treated in iPlpCre:Nf1fl/fl (n=3/group, ****; p<0.0001); resulting in a partial rescue.

(C) Full rescue, a significant decrease in % decompaction of MEKi + L-NAME as compared to single agent treatment (MEKi or L-NAME) treated in iPlpCre:Nf1fl/fl (n=3/group, p=0.02, p=0.04). There is no significant difference between iPlpCre:Nf1fl/fl treated with MEKi + L-NAME and wild type (n=3/group, p=0.155).

(D) Near full rescue, increase in g-ratio of MEKi + L-NAME as compared to single treatment (L-NAME or MEKi) in iPlpCre:Nf1fl/fl (n=3/group, p=0.027, *; p=<0.05, ****; p<0.0001). Significant difference between MEKi + L-NAME treated iPlpCre:Nf1fl/fl mutants as compared to wild type mice (p=0.046).

(E) Full rescue, decrease in % decompaction in MEKi as compared to vehicle treated in iPlpCre:Nf1fl/fl,Rbpjfl/fl (n=3/group, p=0.007). Full rescue, decrease in % decompaction between iPlpCre:Nf1fl/fl,Rbpjfl/fl and iPlpCre:Nf1fl/fl treated with MEKi (n=3/group, p=0.014).
There is no significant difference between \textit{iPlpCre;Nf1fl/fl;Rbpjfl/fl} treated with MEKi and wild type (n=3/group, \(p=0.207\)).

(F) Near full rescue, increase in g-ratio of MEKi as compared to vehicle treated in \textit{iPlpCre;Nf1fl/fl,Rbpjfl/fl} (n=3/group, ****; \(p<0.0001\)). Significant difference between MEKi treated \textit{iPlpCre;Nf1fl/fl,Rbpjfl/fl} mutants as compared to wild type mice (p=0.042).
by pERK. However, flow cytometry analysis revealed that nitric oxide remains high in iPlpCre:Nf1fl/fl;Rbpjfl/fl mice as compared to iPlpCre:Nf1fl/fl mice (Table, Figure 4.12A). Therefore, we targeted both MEK and NO signaling. The combination of inhibition of NOS, with L-NAME, and MEKi is sufficient to rescue decompaction and near full rescue of myelin thickness (Figure 4.12C,D). In contrast to HRas mutants, treatment with MEKi can increase Notch signaling. As shown previously, dual inhibition of Notch signaling with Rbpj loss and NO signaling with L-NAME fully rescued myelin compaction and thickness in Nf1 mutants. Furthermore, targeting NO and Notch signaling may indirectly suppress MEK/ERK signaling. Taken together, these data show a role for Notch, NO, and MEK in myelin compaction in homozygous Nf1 mutants. Thus, Notch, NO and MEK pathways are potential therapeutic targets in NF1 patients.
### Signaling Pathway Activity Level (in Oligodendrocytes)

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<thead>
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<th>Conditions</th>
<th>Notch (HesGFP)</th>
<th>Raf/Mek/Erk (pERK)</th>
<th>Nitric Oxide</th>
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<tr>
<td>iPipCre;RosaNICD</td>
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<td>Increased (vs. WT)</td>
<td>Normal</td>
</tr>
<tr>
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<td>Decreased (vs. HRas)</td>
<td>Stays High</td>
</tr>
<tr>
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<td>Stays High</td>
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</tr>
<tr>
<td>iPipCre;HRasG12V/+/L-NAME</td>
<td>Decreased (vs. HRas)</td>
<td>Increased (vs. HRas)</td>
<td>X</td>
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### Signaling Pathway Activity Level (in Oligodendrocytes)

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<th>Conditions</th>
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<th>Raf/Mek/Erk (pERK)</th>
<th>Nitric Oxide</th>
</tr>
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<td>Normal</td>
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<tr>
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<tr>
<td>iPipCre;NTf/ff/ff,Meki</td>
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<td>Stays High</td>
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<tr>
<td>iPipCre;NTf/ff/ff,L-NAME</td>
<td>Increased (vs. NTf)</td>
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### iPipCre;RosaNICD & Meki & L-NAME

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<th>Full Rescue Compaction, Partial Rescue Thickness</th>
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</tr>
</thead>
</table>

| NOS Inhibition (L-NAME) | Partial Rescue Compaction, Partial Rescue Thickness | Full Rescue Compaction, Partial Rescue Thickness | Full Rescue Compaction, Partial Rescue Thickness | Full Rescue Compaction, Partial Rescue Thickness |

### iPipCre;NTf/ff/ff & Rbpj/loss & Meki & L-NAME

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<th>Notch Signaling (Rbpj loss)</th>
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<th>&amp; Meki</th>
<th>&amp; L-NAME</th>
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</thead>
</table>

| NOS Inhibition (L-NAME) | Partial Rescue Compaction, Partial Rescue Thickness | Full Rescue Compaction, Partial Rescue Thickness | Full Rescue Compaction, Partial Rescue Thickness | Full Rescue Compaction, Partial Rescue Thickness |

### iPipCre;HrasG12V

**Diagram C**

- **MEKI**
- **L-NAME**
- **P-ERK**
- **Rbpj mutant**
- **NO**

### iPipCre;Nf1floX

**Diagram D**

- **MEKI**
- **GSI**
- **P-ERK**
- **Rbpj mutant**
- **NO**

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**Figure 4.12. Summary of Notch, nitric oxide, and MEK/ERK signaling in NICD, HRas, and Nf1 mutant models with treatments compared to vehicle control.**

(A) Table of signaling pathway activity level in oligodendrocytes, as measured by flow cytometry of the forebrain, in NICD, HRas, and Nf1 mutant models with treatments compared to vehicle control.

(B) Table: Summary of myelin compaction and myelin thickness (g-ratio) in NICD, HRas, and Nf1 mutant models with treatments compared to vehicle control.

(C) Depiction of intracellular signaling in iPlpCre;HRasG12V mice after adult tamoxifen induced recombination and target sites for pathway inhibition.

(D) Depiction of intracellular signaling in iPlpCre;Nf1flox mice after adult tamoxifen induced recombination and target sites for pathway inhibition.
4.3 Discussion

Using genetic models, we found that HRasG12V mutation in oligodendrocytes causes rapid myelin thickening and decompaction in adult mice, which is partially mediated by canonical Notch signaling. In homozygous HRas mutant oligodendrocytes, simultaneously targeting canonical Notch signaling with loss of Rbpj and NOS with L-NAME completely rescued myelin thickness in addition to myelin compaction, indicating that both pathways contribute to the pathological myelin disruption. Single agent treatment of hemizygous HRas mutants with GSI, L-NAME, or MEKi partially rescued myelin compaction, indicating that all three pathways contribute to the pathological myelin disruption. Simultaneously targeting canonical Notch signaling with loss of Rbpj and NOS with L-NAME or MEK completely rescued myelin decompaction, yet only partially rescued myelin thickness in hemizygous HRas mutants. We conclude that canonical Notch signaling and NO act downstream of hyperactive HRas, contributing to myelin decompaction in a dose dependent manner. Notably, simultaneous targeting of NOS with L-NAME and MEK completely rescued myelin thickness in addition to myelin compaction. As targeting NO and MEK signaling fully rescue myelin thickness and compaction, data suggest that in hemizygous HRas mutants, direct inhibition of Notch is not required for correction of signaling that controls myelin thickness. However, it is likely that pathway cross talk/feedback is targeting Notch signaling, directly or indirectly.

Ras-GTP can act upstream of Notch signaling and the Notch pathway can feedback to antagonize the Ras pathway (Brennan et al., 2009). Reciprocal transcriptional regulation integrates the Notch and Ras pathways (Hurlbut et al., 2009). Ras is a frequently mutated oncogene in human cancer and has been studied in great detail. Several recent studies have identified a role for Notch signaling in tumor formation and progression. Interestingly, the type
of Notch receptor activity varies based on the cell type and the tumor microenvironment even with the same genetic mutation (Baumgart et al., 2014; Court et al., 2013; Mazur et al., 2010b). For example, in a murine model of lung cancer driven by KRasG12D mutation, deficiency in Notch 1 decreases MAPK activity due to a decrease in Hes1 transcription, subsequent decrease in repression of DUSP1, and therefore an increase in suppression of ERK1/2, whereas deficiency of Notch 2 increases MAPK activity due to an increase in β-Catenin and a subsequent increase in Hes1 (Baumgart et al., 2014).

Hes5GFP reporter activity increased in immature oligodendrocytes in HRas mutants, but not in mature myelinating oligodendrocytes. Notch signaling, in mature oligodendrocytes, may be driven by Hes or Hey proteins, or other effectors. Given that constitutive Notch signaling (through increased NICD in mature oligodendrocytes) drives decompaction, and that loss of Rbpj in mature oligodendrocytes partially rescues decompaction, we suggest that the effects on myelin compaction are cell autonomous in mature oligodendrocytes. When we define the key Notch effector(s) in mature oligodendrocytes, it will be of interest to define their levels in hemizygous and homozygously HRas mutant cells, and over time.

Oligodendrocyte maturation, wrapping, and myelination are regulated through Notch signaling (Wang et al., 1998; Watkins et al., 2008). Decompaction in HRas and NICD mutants shows that compaction is also regulated, directly or indirectly, through Notch signaling. Improved myelin compaction in GSI-treated hemizygous and homozygous HRas mutants supports the conclusion that Notch pathway activation regulates myelin compaction. Myelin decompaction and thickness were further rescued in GSI treated hemizygous HRas mutants as compared to those with Rbpj loss. This could be due to effects from additional GSI substrates and/or non-cell autonomous effects.
In the \textit{HRas} mutant, Notch signaling is not independently regulating myelin thickness. As in homozygous \textit{HRas} mutants with loss of \textit{Rbpj}, myelin decompaction is fully rescued whereas myelin thickness is only partially rescued. Increased myelin thickness is observed with constitutive expression of p-ERK in oligodendrocytes, yet not decompaction (Ishii et al., 2013). Altered pathway integration, levels of p-ERK, and/or pathways downstream of Ras-GTP independent of MEK-ERK signaling may dictate specific responses. It will be of interest to delineate feedback loops through which the Notch and Ras pathways interact in \textit{HRas} mutant oligodendrocytes, as signaling pathways (apart from those identified here) that maintain myelin compaction remain largely unknown.

Injured oligodendrocytes increase NOS1 (Yao et al., 2010; Yao et al., 2012). Nitric oxide, and/or other factors produced by \textit{HRas} mutant oligodendrocytes that affect surrounding progenitors, might increase proliferation. Nitric oxide, like Notch, plays a role in cell differentiation/maturation (Snyder et al., 2012) as it suppresses myelin gene transcription and translation of myelin proteins (Jana and Pahan, 2013). In our study, there is an increase in Nitric oxide only in mature oligodendrocytes and treatment with L-NAME partially rescues the myelin thickness and myelin decompaction phenotype. Hyper activation of \textit{HRas} appears to uncover normal signaling pathways that regulate oligodendrocyte myelin compaction and require balance amongst levels of Ras, Notch and NO.

Why is there an increase in oligodendrocyte lineage cells in \textit{HRas} mutants? We speculate that stressed \textit{HRas} mutant oligodendrocytes recruit oligodendrocyte progenitors. The timing and number of progenitors recruited is dose dependent on \textit{HRas} mutation as there were more progenitors recruited in homozygous as compared to hemizygous \textit{HRas} mutants at 1 month post tamoxifen. Notably, there is an increase in Hes5GFP+O4+GalC- immature oligodendrocytes in
the forebrain of hemizygous and homozygous HRas mutants compared to wild type. Notch activity within the immature oligodendrocyte population, in the demyelinating disease such as Multiple Sclerosis, can promote proliferation and prevent maturation/myelination (Jurynczyk et al., 2008; Jurynczyk and Selmaj, 2010; Franklin and Gallo, 2014). The entire oligodendrocyte cell lineage (NG2+, Olig2+, and CC1+) increases in HRas mutants in corpus callosum sections despite no overall change in oligodendrocyte cell number in the mutant forebrain. This may reflect differences between grey and white matter. In our study, reducing Notch signaling using GSI decreased numbers of aberrant HRas oligodendrocytes. This is consistent with previous studies, in which loss of Notch1 causes premature differentiation and death of mature oligodendrocytes (Genoud et al., 2002).

In HRas mutants, mature myelinating oligodendrocytes account for the decompaction phenotype. Constitutive activation of Notch signaling in mature oligodendrocytes drove decompaction within a month. The myelin we observe after GSI or L-NAME treatment is not the thin myelin of re-myelination (Franklin and Gallo, 2014). A single week of L-NAME exposure partially rescued decompaction. This rapid effect strongly supports the idea that mature myelinating oligodendrocytes underlie the decompaction phenotype.

Targeting canonical Notch signaling and simultaneously targeting NOS was sufficient to completely rescue decompaction in hemizygous HRas mutant oligodendrocytes, and partially rescue myelin thickness. The relationship between these two pathways may be complex, and our data do not exclude pathway interactions. Low (normal) levels of NO in oligodendrocytes can be protective through inhibition of peroxynitrite-induced ERK phosphorylation. At high levels, nitric oxide can be detrimental through reaction with superoxide to form toxic peroxynitrite (Li et al., 2011). MEK/ERK signaling (i.e. pERK) may influence levels of NO/peroxynitrite levels.
For example, Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) is required for gliogenesis and plays a role in myelin sheet formation (Massa et al., 2000; Massa et al., 2004; Wishcamper et al., 2001). Deficiency in SHP-1 leads to a decrease in MAPK/ERK signaling and an increase in reactive oxygen species levels (Gruber et al., 2015). Additionally, previous data shows that myelin decompaction caused by hyper activation of Notch signaling in oligodendrocytes can be rescued by L-NAME. This cross talk may underlie full rescue of myelin thickness and compaction in hemizygous HRas mutant mice treated with NOS inhibitor, L-NAME, and MEKi.

In summary, we identify a pathway in which, downstream of hyper active HRas in oligodendrocytes, aberrant Notch pathway signaling disrupts myelin thickness and myelin compaction. The homozygous HRas phenotype is more susceptible to inhibition of Notch signaling, in which simultaneously targeting Notch signaling or NO production fully rescues myelin thickness in addition to myelin compaction. Both nitric oxide and aberrant Notch signaling contribute to myelin disruption in HRas mutants. We identify gamma secretase inhibition, nitric oxide synthase inhibition, and MEK inhibition as therapies that individually partially rescue the hemizygous HRas mutant decompaction phenotype. We find that dual inhibition of Notch signaling, through subsequent loss of Rbpj, and inhibition of NO production or inhibition of MEK completely rescues the hemizygous HRas mutant decompaction phenotype and partially rescues myelin thickness. Importantly, simultaneously targeting of NOS with L-NAME and MEK completely rescued myelin thickness in addition to myelin compaction, and it is likely that this combination is targeting NO, MEK, and Notch signaling. Taken together, these data show a role for Notch and NO and MEK in myelin compaction and myelin thickness in hemizygous HRas mutants. The relevance to patient phenotypes is high as it is likely that many
brain cells in Costello syndrome patients are heterozygous for \textit{HRasG12V} mutation. The relationship between NO and Notch and ERK downstream of \textit{HRas} is of great interest for further investigation; especially in relation to myelin thickness as direct inhibition of Notch is not required for rescue in hemizygous \textit{HRas} mice.

Previously we showed that dual inhibition of Notch signaling, with loss of \textit{Rbpj}, and NOS, with L-NAME, completely rescued myelin compaction and thickness in homozygous \textit{Nf1} mutants. Additionally, we show that simultaneously targeting MEK signaling and with loss of \textit{Rbpj} or NOS with L-NAME completely rescued myelin decompaction and almost completely rescued myelin thickness in homozygous \textit{Nf1} mutant mice. Of note, analysis revealed that inhibition of Notch signaling lead to a decrease in MEK/ERK signaling. Therefore, inhibition of both Notch and NO signaling may also suppress MEK/ERK signaling directly or indirectly. Thus, Notch, NO, and MEK pathways are potential therapeutic targets in NF1 patients.

Why is the use of dual inhibitors more effective on one Rasopathy murine model over another? In both \textit{HRas} and \textit{Nf1} mutant mice, nitric oxide levels remained high; there was no significant change, after \textit{Rbpj} loss. In both \textit{HRas} and \textit{Nf1} mutant mice, treatment with MEKi led to an increase in nitric oxide in oligodendrocytes. The difference between the two models is evident in Notch signaling levels. In \textit{HRas} mutant mice, treatment with either L-NAME or MEKi suppressed Notch signaling, as measured through \textit{Hes5GFP} reporter. In contrast, treatment with either L-NAME or MEKi elevated Notch signaling in \textit{Nf1} mutants. Therefore, it is not surprising that dual inhibition of NO and MEK is the most effective combination in \textit{HRas} mutants as this likely targets the NO, MEK, and Notch signaling pathways. Alternatively, in \textit{Nf1} mutants, dual inhibition of Notch and nitric oxide is the most effective combination. Inhibition of both Notch and NO signaling may also suppress MEK/ERK signaling directly or
indirectly and therefore dual inhibition of Notch and NO likely targets Notch-MEK/ERK-NO signaling. Further study of pathway cross talk is of great interest and future studies should include measuring the level of MEK/ERK signaling after NOS inhibition with L-NAME in the Nf1 mutants. This may clarify the ability of signaling downstream of Ras to be regulated in a system with an increased level of Ras GTPase activity (Nf1 loss) as compared to a constitutively active HRas GTPase system (HRas mutants). The balance of Notch-MEK/ERK-NO signaling is critical and appears to be regulated differentially in each disease state as each system likely has developed feedback loops based on the strength of the initial/sustained signaling downstream of Ras GTPase.
A majority of this chapter is from:

_H-RasG12V_ expression in oligodendrocytes induces myelin decompaction through the interplay of Notch, NOS, and MEK Signaling_

Titus-Mitchell, H.E., Rizvi, T., Silbak S.H., Bogard, M., Ciraolo, G., Fagin, J., and Ratner, N

**Author Contributions:** H.T.M. performed animal work and experiments, analyzed data, and drafted the manuscript. T.R. performed PDGFRα stain on sections and assisted with perfusions. G.C. prepared sections for electron microscopy. S.H.S. and M.B. quantified myelin thickness (g-ratios) and myelin decompaction/severity from electron micrographs. J.F. provided HRasG12V mice. N.R. designed the project, aided in data analysis and interpretation, and edited the paper and figures.

**Acknowledgments:** We thank Amanda Klotter for technical assistance, and Andrew Robinson and Stephen D. Miller (Northwestern, Chicago IL) for providing methods for Flow Cytometry prior to publication. We are indebted to Brian Popko (University of Chicago, Chicago, IL) and Tasuku Honjo (Kyoto-U) for providing mouse lines, Marie Dominique Fillipi and Monica DeLay for assistance with Flow, and Merck for providing MRK-003. This work was supported by a grant from the DOD program on Neurofibromatosis, an award from the Cincinnati Children’s Hospital, and NIH R01 NS091037 (to NR). The Cincinnati Children’s Hospital Research Foundation, Flow Cytometry and Pathology Cores provided support for these studies (NIH P30 DK0909710551).
Chapter 5

General Discussion
My dissertation describes 2 major studies on gain of function Rasopathy mouse models of NF1 and Costello syndrome. To study myelin decompaction and aberrant cognitive behavior in vivo we characterized two novel mouse models for CNS myelin disruption in NF1 and Costello syndrome; iPlpCre;Nf1flox and iPlpCre;HRasG12V. We showed the time line of myelin disruption and changes of the oligodendrocyte cell lineage. As the feedback loops in NF1 and Costello Syndrome were largely unknown, we set out to define the role of Ras and downstream effectors in myelin compaction. We identified Notch and NOS pathways downstream of Ras GTPase and cross talk, after Nf1 loss or hyper activation of HRas, in oligodendrocytes and identified therapeutic targets for myelin repair. We ran pre-clinical therapeutic trials for improvement in myelin compaction and behavior.

In Chapter 3 we show that myelin decompaction occurs within one month of Nf1 loss in oligodendrocytes. Constitutive activation of Notch signaling in oligodendrocytes partially recapitulates this phenotype and loss of canonical Notch signaling through Rbpj loss, or treatment with gamma secretase inhibitor, partially rescues decompaction. In Nf1 mutants, mature oligodendrocytes exclusively increase levels of nitric oxide (NO) and inhibition of nitric oxide synthase (NOS) partially rescues decompaction. Inhibition of NOS completely rescues decompaction in Nf1;Rbpj mutants, indicating that Notch and NO pathways contribute to myelin decompaction. Hemizygous Nf1 loss in oligodendrocytes causes myelin decompaction which worsens over time, and correlates with hyperactive behavior. In this setting, inhibiting Notch or NOS completely rescues decompaction and behavioral abnormality. Therefore, Rbpj-dependent Notch and NOS pathways are potential therapeutic targets in NF1 patients. Our studies link patient Rasopathy mutations to altered myelin compaction and strongly support recently
described links between changes in myelin and behavior (Fields, 2011; Liu et al., 2012; McKenzie et al., 2014; Mayes et al., 2013; Makinodan et al., 2012).

In Chapter 4 we show that brain myelin decompaction occurs within one month of \(HRasG12V\) mutation in oligodendrocytes. Constitutive activation of Notch signaling in oligodendrocytes partially recapitulates this phenotype and loss of canonical Notch signaling through \(Rbpj\) loss, or treatment with gamma secretase inhibitor, partially rescues decompaction. In \(HRas\) mutants, mature oligodendrocytes exclusively increase levels of nitric oxide (NO) and inhibition of nitric oxide synthase (NOS) partially rescues decompaction. Inhibition of NOS completely rescues decompaction in \(HRasG12V/+;Rbpjfl/fl\) mutants, indicating that Notch and NO pathways contribute to myelin decompaction, yet only partially rescues myelin thickness. Dysmyelination in homozygous \(HRas\) mutants is more dependent on Notch signaling, as loss of Notch signaling through \(Rbpj\) loss, or treatment with gamma secretase inhibitor, fully rescued myelin decompaction yet only partially rescued myelin thickness. Additionally, inhibition of NOS completely rescues myelin decompaction and thickness in \(HRasG12V/G12V;Rbpjfl/fl\) mutants. We conclude that canonical Notch signaling and NO act downstream of hyper active \(HRas\), contributing to myelin decompaction in a dose dependent manner. Simultaneously targeting canonical Notch signaling with loss of \(Rbpj\) and NOS with L-NAME or MEK completely rescued myelin decompaction yet only partially rescued myelin thickness in hemizygous \(HRas\) mutant mice. In contrast, targeting of both NOS with L-NAME and MEK completely rescued myelin thickness in addition to myelin compaction. As targeting NO and MEK signaling fully rescue myelin thickness and compaction, data suggest that in hemizygous \(HRas\) mutants, direct inhibition of Notch is not required for correction of signaling that controls myelin thickness. However, it is likely that pathway cross talk/feedback is targeting Notch
signaling, directly or indirectly. Therefore, MEK and NOS are potential therapeutic targets in Costello syndrome patients as they are heterozygous for HRas mutation.

Notch, NO, and MEK pathways are potential therapeutic targets in NF1 patients as we show that simultaneously targeting MEK signaling and with loss of Rbpj or NOS with L-NAME completely rescued myelin decompaction and almost completely rescued myelin thickness in homozygous Nf1 mutant mice. Notably, the differences in rescue of compaction and myelin thickness within and between Nf1 and HRas mutants were due, at least in part, to level of Notch signaling. Single inhibitor treatment, of MEK/ERK signaling with MEKi or NO signaling with L-NAME, in HRas mutants diminished Notch signaling, whereas Notch signaling was elevated in Nf1 mutants. Additionally, differences in rescue of compaction and myelin thickness within and between Nf1 and HRas mutants were due, at least in part, to levels of MEK/ERK signal and regulation. Loss of Nf1 allows for indirect MEK/ERK pathway suppression through feedback mechanisms whereas in the HRas mutant MEK/ERK needed to be targeted directly. Further study of pathway cross talk is of great interest and future studies should include measuring the level of MEK/ERK signaling after NOS inhibition with L-NAME in the Nf1 mutants. This may clarify the ability of signaling downstream of Ras to be regulated in a system with an increased level of Ras GTPase activity (Nf1 loss) as compared to a constitutively hyperactive system (HRas mutants).

There are pros and cons to every model system. Our goal was to make our model as relevant to the patient disease course while staying within the parameters to test our hypothesis. The hypothesis was that aberrant CNS glial cell proliferation, differentiation, and/or myelination in gain of function Rasopathies, such as NF1 and Costello syndrome, results from altered activation of the Notch pathway downstream of Ras in the oligodendrocyte cell lineage. To
target the myelinating glia we chose an *iPlpCre* driver. However, NF1 patients have a heterozygous mutation of *NF1* in all cell types; 50% of cases are autosomal dominant and 50% of cases are sporadic. Studies are ongoing in the Ratner lab to characterize the role of Notch, NO, and MEK/ERK signaling in *Nf1*+/− mice, in which every cell in the mouse is heterozygous for *Nf1* as in patients. Data presented here show a dose and time dependent increase in severity of the phenotype in *iPlpCre;Nf1fl/+* mice. The phenotype is less severe in heterozygous loss of *Nf1*, in *Nf1*+/− mice, as compared to targeted adult induced hemizygous loss in glia in *iPlpCre;Nf1fl/+* (Mayes et al., 2013). Our data suggests that the *Nf1*+/− microenvironment in the brain may be neuroprotective as compared to selective loss in the oligodendrocyte cell lineage, as there is a lower detectable level of total ROS present (Rizvi, Titus, Mayes et al., in preparation). As treatment with L-NAME or MEKi were detrimental to wild type mice, timing of therapy in patients is critical. As presentation of clinical symptoms is unique in each patient and the disease is progressive, treatment after clinical presentation of brain abnormalities/dysfunction (i.e. T2 hyper intensities, optic nerve enlargement, and/or cognitive dysfunction) is recommended. Future clinical trials could include patients with CNS manifestations as a control arm in comparison to patients with peripheral nervous system (PNS) manifestations of the disease, such as benign neurofibroma(s). As clinical trials are ongoing for MEKi PD-0325901 for NF1 neurofibroma tumor burden, these data may provide support for promising combination trials to treat both PNS and CNS manifestations of the disease.

Costello syndrome patients have a heterozygous mutation in *HRas* in all cells; the most common mutation is G12S found in 80% of patients, the second most common is G12A. We chose to model a severe mutation, *HRasG12V*. The *HRas* mutant phenotype was severe at early onset and continued to progress. For patients with the *HRasG12V* mutation, presenting with
clinical signs of the disease, immediate treatment may be the most beneficial. It would be of interest, in relation to the most common patient genotype, to verify successful pre-clinical therapeutics from trials with the $HRasG12V$ murine model in a $HRasG12S$ murine model.

Using the $iPlpCre$, in Chapter 3 and 4 it is evident that aberrant cell autonomous signaling in the oligodendrocyte cell lineage is leading to myelin disruption and that rescue of myelin compaction is most likely from mature oligodendrocytes compacting existing myelin. We have also shown that hyper activation of Notch signaling can cause decompaction and that loss of $Rbpj$ can rescue, partially or completely, myelin compaction. Therefore, it was surprising that $Nf1$ and $HRas$ mutants had no significant change in Hes5 in mature oligodendrocytes, as measured by the GFP reporter. This is possibly due to other downstream target genes of Notch being activated. Identifying the downstream Notch effector will allow proper understanding of possible pathway interactions between Notch, NO, and Ras/MEK. Future studies could include qRTPCR of white matter tracts in $Nf1$ and $HRas$ mutants. Additional analysis, using single cell RNA sequencing, could be performed on flow sorted oligodendrocytes from $Nf1$ and $HRas$ mutants with a reporter for recombination in PLP+ cells, such as eGFP or tomato red. For intricate biochemical discovery of mechanism an in vivo model is expensive and time intensive. In vitro model systems, such as myelinating co-cultures, should be considered.

The lab has previously shown that protein levels of NOS1/2/3 are upregulated in $HRas$ mutants ($CNPase;HRasG12V/+)$ and even more elevated in $Nf1$ mutants ($iPlpCre;Nf1flox$). We confirmed elevated levels of NO in the mature oligodendrocytes using flow cytometry. Further analysis could include using mass spectroscopy to identify nitrosylated proteins. Future studies could explore the necessity and sufficiency of NOS 1, 2, and/or 3 in oligodendrocytes on the myelin decompaction phenotype in vivo.
Notch signaling, nitric oxide signaling, and MEK/ERK signaling have all been shown to effect myelin gene expression and myelin protein levels. In further deciphering the pathway cross talk, it would be of interest to look at levels of myelin gene expression and transcription after single and combination treatment with inhibitors of Notch signaling (i.e. GSI MRK-003), NOS (i.e. L-NAME), and MEK/ERK signaling (MEKi PD-0325901).

There are limitations using 2D electron micrographs for quantification of myelin characteristics. A standard measurement in the field for myelin thickness in cross section is g-ratio, diameter of the axon divided by the diameter of the entire fiber. An unmyelinated axon has a g-ratio of 1 and an average axon has a g-ratio near 0.7. Through blinded data analysis we quantified percent decompact fibers out of total myelinated fibers. To further this analysis, severity of myelin decompaction noted as the number of quadrants with decompaction, was quantified. In the Nf1 and NICD mutants, an increase in g-ratio was an increase in myelin thickness due to decompaction, not a change in the number of myelin wraps. In contrast, HRas mutants have an increase number of wraps and decompaction that contribute to the lower g-ratio. Therefore, when comparing and contrasting mutant models, terminology such as myelin thickness is more difficult to clarify, as fully rescued decompaction in a HRas mutant may have a lower g-ratio due to the sustained increased number of myelin wraps in a compact fiber. In HRas mutants we can’t exclude the possibility of myelin remodeling, in which newly generated oligodendrocytes fill existing “gaps” on previously myelinated axon segments; resulting in shorter internodes. New samples can be blocked and cut for analysis of internode number and length. In the adult brain, an increase in number and a decrease in length of internodes are indicative of new myelin (Young et al., 2013; Wang and Young, 2014). Myelin remodeling could explain the “overshoot” of g-ratio correction in hemizygous HRas mutants treated with
both L-NAME and MEKi as compared to wild type. Future measurements for further analysis of all fibers could include percent unmyelinated out of total to identify disease severity and to identify *de novo* myelination in response to treatment(s). Analysis could not be completed on 2D EM cross sections for small and medium fibers as cellular processes were not easily excluded based on available inclusion criteria.

Overall, these studies in this dissertation provided greater understanding on the role of Notch, NO, and MEK/ERK signaling in oligodendrocytes on myelin compaction, and hopefully this knowledge may be applied towards discovering effective treatments for diseases with dysmyelination. As clinical trials are ongoing for GSI MRK-003 for neurodegenerative disease (i.e. Alzheimer’s disease) and MEKi PD-0325901 for cancer (i.e. NF1 tumor burden), these data may provide support for promising combination trials.
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