Oligodendrocyte Nf1 Controls Aberrant Notch Activation and Regulates Myelin Structure and Behavior

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Oligodendrocyte *Nf1* Controls Aberrant Notch Activation and Regulates Myelin Structure and Behavior

**Graphical Abstract**

**Highlights**

- Experimental oligodendrocyte *Nf1* inactivation causes myelin and behavioral defects
- Hyper-active Notch in oligodendrocytes is necessary and sufficient to disrupt myelin
- Pharmacological inhibition of Notch normalizes myelin and behavior in *Nf1* mutants
- *Nf1*+/- mouse and patient brains suggest Notch hyper-activation in NF1 pathogenesis

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**In Brief**

López-Juárez et al. find that loss of the RAS-GTP regulator *Nf1* in oligodendrocytes leads to myelin and behavioral defects mediated by hyperactive Notch and upstream pathways. Pharmacological inhibition of Notch signaling rescues aberrant behavior in *Nf1* mutant mice and may improve neurological manifestations in neurofibromatosis type 1 patients.

**Accession Numbers**

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**Oligodendrocyte Nf1 Controls Aberrant Notch Activation and Regulates Myelin Structure and Behavior**

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INTRODUCTION

The RASopathy neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant genetic disorders. In NF1 patients, neurological issues may result from damaged myelin, and mice with a neurofibromin gene (Nf1) mutation show white matter (WM) defects including myelin decompaction. Using mouse genetics, we find that altered Nf1 gene-dose in mature oligodendrocytes results in progressive myelin defects and behavioral abnormalities mediated by aberrant Notch activation. Blocking Notch, upstream mitogen-activated protein kinase (MAPK), or nitric oxide signaling rescues myelin defects in hemizygous Nf1 mutants, and pharmacological gamma secretase inhibition rescues aberrant behavior with no effects in wild-type (WT) mice. Concomitant pathway inhibition rescues myelin abnormalities in homoygous mutants. Notch activation is also observed in Nf1+/− mouse brains, and cells containing active Notch are increased in NF1 patient WM. We thus identify Notch as an Nf1 effector regulating myelin structure and behavior in a RASopathy and suggest that inhibition of Notch signaling may be a therapeutic strategy for NF1.

SUMMARY

The RASopathy neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant genetic disorders. NF1 gene mutation, alone or with subsequent loss of the previously normal somatic allele, can lead to a variety of conditions in NF1 patients, ranging from aesthetic issues such as epidermal hyperpigmentation, to disabling bone malformations and aggressive life-threatening tumors (Ratner and Miller, 2015). NF1-associated neurological abnormalities include learning deficits, delayed acquisition of motor skills, and attention-deficit disorder, with or without hyperactivity; autism manifestations may also be present (Acosta et al., 2006; Garg et al., 2015). Cognitive dysfunction is the most common complication affecting the quality of life of children and adolescents with NF1 (Hyman et al., 2005), many of whom require neuropsychological assessment for educational planning (Acosta et al., 2012).

Significant advances have been made toward understanding how NF1 mutation impacts neurons, and treatments to ameliorate neuronal abnormalities have been proposed. In animal models, activity of hippocampal interneurons (Cui et al., 2008) and dopaminergic neurons (Diggs-Andrews et al., 2013) are affected by Nf1 mutation. Notably, correlated abnormal behaviors are rescued by treatment with statins (Li et al., 2005) and dopamine re-uptake inhibitors (Brown et al., 2010), respectively. Nevertheless, mixed results in clinical studies (Bearden et al., 2015), have precluded definitive recommendation of the use of statins in NF1 patients. Therefore, better understanding of molecular mechanisms underlying NF1 neurological issues is crucial to establish successful treatment regimens.

In addition to neuronal defects, 60%–70% of children with NF1 show white matter (WM) abnormalities, including enlarged brain WM tracts, T2 hyperintensities, and altered fractional anisotropy and diffusivity on diffusion tensor imaging (DTI) (Karsen et al., 2012; North, 2000). Myelin produced by mature oligodendrocytes (mOLs) increases nerve impulse velocity; thus, normal brain function requires normal myelin and oligodendrocyte function (Franklin and Gallo, 2014). Indeed, learning and motor skill acquisition correlate with changes in WM and myelin (McKenzie et al., 2014). Nonetheless, research on effects of Nf1 loss in mOLs is limited. We reported nitric oxide (NO)-mediated myelin decompaction >1 year after Nf1 inactivation, correlating with decreased tight junction (TJ) and GAP-junction (GJ) proteins (Mayes et al., 2013). However, what signaling pathways cause these phenotypes and whether loss of Nf1 in oligodendrocytes causes behavioral changes is unknown.

NF1 is a RAS GTPase-activating protein, so that loss of Nf1 results in increased RAS-mitogen-activated protein kinase (MAPK) activity, delayed acquisition of motor skills, and attention-deficit disorder, with or without hyperactivity; autism manifestations may also be present (Acosta et al., 2006; Garg et al., 2015). Cognitive dysfunction is the most common complication affecting the quality of life of children and adolescents with NF1 (Hyman et al., 2005), many of whom require neuropsychological assessment for educational planning (Acosta et al., 2012).

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Figure 1. Progressive Myelin Decompaction Regulated by MAPK Signaling in pNf1 Mutants

(A and B) Electron micrographs of the corpus callosum (CC) of WT, pNf1+/+, and pNf1f/f mutants show regions of myelin decompaction (A, arrows; B, purple) at the indicated months (mo) post-tamoxifen treatment. Insets: regions from dotted rectangles (50,000 x). Number of myelin wraps (triangles) in WT and pNf1f/f mutants are shown in (B).

(C) The percent of decompacted fibers among total fibers and number of quadrants with decompaction (color code) in the short-term (ST; 1 month) and long-term (LT; 6–10 months) post-tamoxifen is shown. Significantly increased decompaction is found in pNf1f/f mice at ST (n = 5 mice, **p < 0.01), and in pNf1f/+ mice at LT (n = 3 mice, *p < 0.05), as compared to WT (ST-LT, n = 6 mice). Moderate (1–3 quadrants), but not significant (ns), decompaction is observed in pNf1f/+ mice at ST.
signaling (Ratner and Miller, 2015). RAS and Notch pathways can cooperate or antagonize each other in a context-dependent manner. Although Notch acts downstream of NF1 during differentiation of neural stem cells (Chen et al., 2015), no links between these pathways have been described in mOL. Here, we show genetic and pharmacological evidence indicating that Notch signaling controls progressive and NF1 gene-dependent myelin defects in the corpus callosum (CC) of PipCreER;Nf1flox/flox animals. Abnormal behavior in hemizygous mutants is rescued by pharmacological inhibition of Notch. Mechanistically, aberrant Notch activation and myelin defects are rescued by inhibition of NO signaling, revealing NO as a crucial link between RAS and Notch pathways. Increased activation of Notch is also found in Nf1+/− mice and in WM of NF1 patients, suggesting that abnormal Notch signaling is a feature of NF1.

RESULTS

**Nf1 Loss in mOLs Causes Nf1 Gene-Dose-Dependent Progressive Myelin Decompaction**

We previously reported that myelin decompaction occurs in the optic nerve (ON) 6–12 months after tamoxifen-induced deletion of Nf1 in Pip1-expressing mOLs (Mayes et al., 2013). To study whether Nf1 loss-dependent myelin defects occur in the brain and their progression, we analyzed the CC of adult tamoxifen-treated homozygous (PipCreER;Nf1flox/flox, henceforth pNf1f) and hemizygous (PipCreER;Nf1flox/+; henceforth pNf1f+) Nf1 mutant mice and wild-type (WT) animals (Nf1flox or PipCreERT2), using electron microscopy (Figures 1A and 1B). Unbiased counting of myelinated axons showed no significant differences in g-ratio of control animals between 1 and 8 months after tamoxifen treatment (Figures S1A and S1C). Significantly decreased g-ratio in Nf1 hemizygous mutants, as compared to WTs, was detected at 1 month with a further decrease 6 months post-tamoxifen, indicating progressive changes in fiber structure. In contrast, in homozygous Nf1 mutants, decreased g-ratio occurred within 1 month post-tamoxifen, and showed slight but significant recovery by 6 months post-tamoxifen (Figures S1A and S1C). At this time point, the density of EGFP reporter-positive recombined cells did not differ between WT and Nf1 mutants (Figures S1F and S1G). Although Nf1 may recombine with different efficiency than EGFP, this result suggests that the trend toward recovery is not due to a depletion of mutant cells. Both parameters determining g-ratio were affected by Nf1 loss in mOLs; pNf1f mutants showed increased myelin thickness due to decompaction at intraperiod lines (number of myelin lamellae remained unchanged, Figures 1B and S1B) and decreased axon diameter (Figure S1E). The latter is likely secondary to disturbed myelin integrity (Cole et al., 1994; Colello et al., 1994), because neurons with axons in the CC do not express the PipCre allele (Koenning et al., 2012; Mayes et al., 2013).

Regions of decompacted myelin were evident in hemizygous and homozygous pNf1f mutants 1 month post-tamoxifen, albeit to different extents (Figures 1A, insets, and 1B). Decompacted fibers among total fibers were quantified. Additionally, severity of decompaction was reflected by the number of quadrants around each axon showing decompaction (Figure 1C, color code). Control animals show baseline myelin “decompaction” (Figure 1C, 1–2 quadrants) in the short- (ST, 1 month) and long-term (LT, 6–9 months) post-tamoxifen treatment, likely indicating cross-sections through paranodal loops and Schmidt-Lantermann incisures. One month post-tamoxifen, pNf1f/+ mutants show decompaction in 1–3 quadrants, but not significant total decompaction, while pNf1f+/- show significantly increased total decompacted fibers in 1–4 quadrants, as compared to WTs. Confirming a progressive phenotype, by 6–8 months post-tamoxifen significantly increased decompaction was also detected in hemizygous mice (Figure 1C). Thus, myelin decompaction in the CC is Nf1 gene-dose-dependent and progressive.

**Nf1 Regulates Myelin Compaction through RAS-MAPK Pathway Activation**

To define the mechanisms responsible for myelin decompaction in the CC of Nf1 mutants, we first analyzed RAS/MAPK pathway activation, which results in phosphorylation and activation of ERK1/2 (pERK). The WT CC showed pERK immunostaining in rare, mainly CC1− cells (Figure 1D). Consistent with activation of MAPK signaling in Nf1 mutants, increased faint pERK signals were observed in CC1+ mOLs of pNf1f/+ mice and strong pERK signals in pNf1f−/− mice at time points when severe decompaction is observable. Quantitative analysis confirmed significantly increased pERK+;CC1+ mOLs in pNf1f−/− mice 1 month post-tamoxifen, as compared to controls, while a trend toward increase was detected in pNf1f+/- mice (Figure 1E). Other signal pathways could cause myelin decompaction in pNf1f+/- mutants, and/or modestly increased pERK could cause decompaction in the long-term; if so, decompaction should be sensitive to inhibition of MEK, the ERK kinase. At 9 months post-tamoxifen when myelin decompaction is detectable, we treated Nf1 hemizygous mutants for 21 days with PD0325901, a brain penetrant MEK inhibitor (MEKi, 1.5 mg/kg). At this therapeutically relevant dose (Barrett et al., 2008), MEKi fully rescued myelin decompaction (Figure 1C) and g-ratio (Figure S1D) in pNf1f+/- mice. In fact, myelin was more compacted than WTs, suggesting that basal MAPK signaling contributes to the maintenance of normal compact myelin structure. In homozygous Nf1 mutants, MEKi improved the severity of decompaction (quadrants) and significantly increased g-ratio, but failed to fully rescue these

(n = 3 mice). MEKi treatment rescues decompaction in pNf1f+/- mutants (n = 3 mice, *p < 0.05) but does not change decompaction in pNf1f−/− mutants (n = 3 mice). Data are presented as the mean ± SEM. One-way ANOVA; p = 0.0005, and Tukey’s multiple comparisons test.

(D) Immunostaining of pERK (red) and CC1 (white) in the CC of the indicated genotypes. Magnifications of squared regions (left) depict a pERK+;CC1− cell in WT (nuclear signal, arrowhead), pERK+(faint);CC1+ mOLs (yellow arrows) in pNf1f+/-, and pERK+(strong);CC1+ mOLs in pNf1f−/− mice. LV, lateral ventricle. Scale bar, 10 μm.

(E) Significantly increased pERK+;CC1+/total CC1+ mOLs are found in pNf1f−/− mice as compared to WT (n = 4 mice/genotype, t test; **p = 0.0053, data are the mean ± SEM). See also Figures S1 and S2.
parameters (Figures 1C and S1D), perhaps because the treatment did not abolish abnormal MAPK signaling. In support of this hypothesis, while MEKi significantly decreased pERK*/CC1* mOLS, they remained significantly increased in pNf1f/f mice as compared to controls (Figures S2A and S2B). Thus, MAPK signaling contributes to the aberrant myelin compaction in Nf1 mutants, but a clinically relevant dose of MEKi is insufficient to rescue cells with complete Nf1 loss of function.

**Nf1 Loss Increases Notch Pathway Activity in Glial Cells In Vitro and In Vivo**

As in a previous study (Mayes et al., 2013), WT and mutants showed similar MBP expression around CC1* cells (Figure S2C). We aimed to identify other molecules/pathways downstream ERK activation that influence cell signaling and myelin defects following Nf1 loss. We postulated that transcriptional changes might underlie Nf1-loss effects, as MAPK signaling regulates gene expression (Ishii et al., 2014). Using microarray gene expression analysis of glia-enriched cultures (Bennett et al., 2003), we identified genes with significantly altered expression in Nf1+/− as compared to WT cultures. Interestingly, Hes5, a direct transcriptional target of canonical Notch signaling, was the most upregulated gene in Nf1 mutants, and other Notch-related genes (Dll1 andDll3) were also significantly increased, as validated by qRT-PCR (Figure 2A). Upon ligand binding, a cleaved Notch intracellular domain (NICD) is generated, correlating with Notch activation. NICD detection by western blot increased in Nf1+/− as compared to WT cultures (Figure 2B). To verify these results in vivo in an OL-enriched population, we performed RNA sequencing (RNA-seq) transcriptionome analysis using WT and pNf1f/f optic nerves, 1 month post-tamoxifen. We identified significant increases in transcripts encoding the Notch targets Hes5, Cntnap2, and Ctnbp2 (Meier-Stiegen et al., 2010). Transcripts encoding Notch ligands also increased (Dll1, Dlk2, Dner, and Ctn1) or decreased (Thbs1, Postn, and Jag1). Transcripts encoding GJ and TJ proteins expressed in the oligodendrocyte-lineage, including Claudin 11, were also significantly decreased (Figure 2C). To test whether loss of Nf1 in mOLS increases Notch activation in vivo, we analyzed Nf1 mutants carrying the transgene HesGSFP, a reporter of canonical Notch pathway activation that is not expressed significantly in normal mOLS. WT animals and Nf1 mutants contained, as expected, HesGSFP*;GFAP+ astrocytes (Figure S3A) and rare HesGSFP*;CC1* mOLS in the CC (Figure 2D, insets). Although HesGSFP* mOLS were significantly increased in pNf1f/f animals as compared to WT animals (Figures 2E and 2F), they accounted for only 0.41% ± 0.20% of CC1* cells in the CC, and HesGSFP signal did not overlap with pERK*/CC1* mOLS (Figure S3B).

Hes5 is one of many Notch effectors. To test whether other Notch effectors are likely to be activated in mOLS after loss of Nf1, we analyzed NICD immunoreactivity using a specific antibody (aNOTCH). WT mice showed aNOTCH cytoplasmic signals in rare CC1* mOLS. Significantly increased numbers of aNOTCH* (nuclear);CC1* mOLS were detected in pNf1f/f animals (Figures 2D and 2F), corroborating Nf1 loss-driven, abnormal, Notch activation. Interestingly, aNOTCH signals were detected in recombinant and non-recombinant cells (Figure S3C), supporting the idea that cell autonomous and non-cell-autonomous effects occur downstream of Nf1 mutation in mOLS (Mayes et al., 2013).

**Activation of Notch in mOLS Reduces Claudin 11 Expression and Causes Myelin Decompaction**

We investigated whether abnormal Notch activation in mOLS is sufficient to disrupt myelin compaction. We genetically activated Notch signaling in mOLS with the tamoxifen-inducible RosaNICO allele driven by PlpCreER (pNICO). One month post-tamoxifen treatment, myelin decompaction (Figure 2G) and decreased g-ratio (Figure S4A) were found in pNICO animals, as compared to WTs. Decreased Claudin 11 correlates with myelin decompaction in pNf1 mutants (Mayes et al., 2013), and WB analysis showed a significant decrease of this TJ protein in the forebrain of pNf1 mice (Figure S4B). Thus, genetic activation of Notch in mOLS mimics phenotypes caused by Nf1 loss.

**Notch Genetic Inhibition Rescues Myelin Decompaction in Nf1 Mutants**

RBPJk is a transcriptional co-factor required for canonical Notch signaling. To test whether increased canonical Notch signaling...
regulates phenotypes in Nf1 mutants, we genetically inactivated Notch signaling in mOLs using PtgCreER;Nf1f/+ (or Nf1f/ f);Rbpjf/+ mice, in which Rbpj and Nf1 are inactivated upon tamoxifen treatment. Unexpectedly, homozygous Rbpj inactivation in mOLs (pRbpjf/+ ) caused myelin decompaction 1 month post-tamoxifen treatment (Figure 2H). Thus, imbalance in Notch signaling and/or compensatory responses affect myelin compaction in this genetic model. Importantly, hemizygous Nf1 mutants with deletion of Rbpj (pNf1f/+;Rbpjf/+ ) showed full rescue of myelin decompaction (Figure 2H) and g-ratio (Figure S4C) 6–8 months post-tamoxifen. Inactivation of Rbpj in Nf1 homozygotes rescued g-ratio but did not significantly impact myelin decompaction 1 month post-tamoxifen; however, additional treatment of pNf1f/+;Rbpjf/+ mice with MEKi fully rescued compaction to WT levels (Figure 2H). Overall, these data indicate that Notch signaling is crucial for the maintenance of myelin structure in the WT setting and in the context of Nf1 loss.

**Gamma Secretase Inhibition Rescues Myelin Defects and Aberrant Behavior in Nf1 Mutants**

To test whether Nf1 loss-driven phenotypes can be rescued by pharmacological inhibition of Notch we used the blood-brain barrier-permeable gamma secretase inhibitor (GSI) MRK-003 (Chu et al., 2013). GSI administration to WT animals (weekly for 3 weeks) significantly decreased the number of Hes5GFP+ cells in the CC, confirming drug efficacy (Figure 3A). In contrast, GSI treatment had no effect on myelin compaction in WT animals. In parallel, GSI treatment rescued decompaction (Figure 3B) and g-ratio (Figure S4D) in pNf1f/+ animals 6–10 months post-tamoxifen. In fact, myelin compaction increased over WT levels, similar to the effect of MEKi (Figure 1C). In Nf1 homozygous mutants GSI significantly increased g-ratio (Figure S4D), however, no significant changes in myelin decompaction were observed. These data suggest that GSI treatment normalizes the unbalanced Notch signaling that promotes decompaction in Nf1 mutants.

Nf1 patients display mutation/loss of 1 Nf1 allele and present locomotor, cognitive and attention issues. We therefore tested whether behavior is altered in hemizygous Nf1 mutants. We evaluated sensory gating deficits in Nf1 hemizygous mutant mice (C57BL/6 genetic background), by analyzing the prepulse inhibition of startle response (Vorhees et al., 2011), 9 months post-tamoxifen treatment. Significantly heightened response to startle at 73–82 dB, was found in pNf1f/+ mutants as compared to controls (Figure 3C). Remarkably, all increased startle responses (73–82 dB) were blocked by treatment with GSI for 3 weeks, as compared to littermate WTs (Figure 3D). Thus, hemizygous Nf1 mutation in mOLs causes myelin structural defects and behavioral abnormalities that can be rescued by pharmacological inhibition of Notch signaling.

**Notch Activation and Myelin Decompaction Are Due to Increased Nitric Oxide Signaling in Nf1 Mutants**

Nf1 loss in oligodendrocytes was reported to result in increased reactive oxygen/nitrogen species in the forebrain, and treatment with the antioxidant N-acetyl cysteine rescued cell-autonomous and non-cell-autonomous defects in the ON of pNf1 mutants (Mayes et al., 2013). To test whether mOL NO contributes to CC myelin abnormalities, Nf1 mutants were treated with a specific inhibitor of NO synthases L-NAME (N-nitroarginine methyl ester S-NAME) and Aberrant Behavior in Nf1 Mutants Rescues Decompaction and Aberrant Behavior (A) Number of Hes5GFP+ cells in the CC of vehicle-treated (n = 5 mice) or gamma secretase inhibitor (GSI)-treated (n = 12 mice) WT mice (t test, *p = 0.0001). (B) The percent of decompacted fibers and quadrants with decompaction does not change after GSI treatment in WT animals (n = 6 vehicle-treated, n = 4 GSI-treated animals, t test, ns, p = 0.4516) and pNf1f/+ mutants (n = 5 mice/genotype, ns, p = 0.2768). Decompaction is rescued in pNf1f/+ mice treated with GSI (n = 4 mice, t test, **p = 0.0019) as compared to vehicle-treated pNf1f/+ (n = 3). ST, 1 month post-tamoxifen; LT, 6–10 months post-tamoxifen.

(C and D) Evaluation of the acoustic startle response. (C) pNf1f/+ mutant mice (n = 21 mice) present increased V_{max} to the acoustic startle response following the 73 dB, 77 dB, and 82 dB pre-pulse stimuli, as compared to WT mice (n = 19 mice, two-way ANOVA, F[4,152] = 3.05, *p < 0.05). (D) The heightened startle response in pNf1f/+ mutants is abolished after treatment with GSI MRK-003, as no significant difference in V_{max} is observed between WT (n = 21 mice) and pNf1f/+ mice (n = 19 mice, two-way ANOVA, F[4,152] = 2.78).

Error bars show ± SEM. See also Figure S4.
ester, 0.4 mg/kg) for 7 days, starting after severe decompaction is detectable. L-NAME treatment fully rescued myelin compaction (Figure 4A) and g-ratio (Figure S4F) in pNf1f/+ mutants. In pNf1f/+ mice, treatment significantly increased g-ratio, but did not rescue compaction to WT levels. Notably, full rescue of decompaction in the homozygous mutants required inhibiting both Notch and NO signaling (Rbpj deletion and L-NAME treatment) or MAPK and NO signaling (MEKi and L-NAME treatment) (Figures 4A and S4F).

We therefore tested whether elevated NO levels drive Notch activation (Charles et al., 2010; Jeon et al., 2014) in Nf1 mutants or whether Notch activation is an independent Nf1-driven signal in mOLs. L-NAME treatment for 7 days rescued the abnormally increased number of aNotch+/CC1+/CC1+ mOLs in the CC of pNf1f/+ mutants, as compared to L-NAME-treated WT mice (Figures 4B and 4C). Thus, abnormally increased Notch signaling in mOL is reduced by NOS inhibition. Flow cytometry analysis of forebrain showed that neither NO nor superoxide signals changed in Hes5GFP+ cells of pNf1f/+ mutants, versus Hes5+ cells in WT animals (Figure S4E). Demonstrating that Notch does not drive NO, overexpression of NICD in mOLs (PipCreER;RosaNICD) did not alter numbers of NO+GalC+ cells, and NO+ cells remained unchanged in Nf1 mutants with genetic inactivation of canonical Notch signaling as compared to pNf1f/+ mutants (Figures 4D and 4E). A linear MAPK/NO/Notch pathway downstream Nf1 loss might explain why single agent treatments fully rescue myelin decompaction in hemizygous pNf1f/+ mice. However, combined inhibition of MAPK, NO, and Notch signaling (Figures 2H and 4A) is necessary to normalize effects in homozygous mutants plausibly because of a more robust pathway activation; durable feedback loops may also explain this finding.

**Nf1 Loss and Oligodendrocyte Numbers**

Nf1 loss in mOLs was found to increase numbers of Olig2+ cells in the CC 4 days after tamoxifen treatment (Mayes et al., 2013). To test if this increase is durable, we quantified CC Olig2+ cells 1 month post-tamoxifen. Olig2+ cells were not significantly increased in pNf1f/+ mutants versus WT (Figures S6A and S6B). As Olig2 labels all oligodendrocyte lineage
cells, we identified by flow cytometry-specific forebrain cell populations using the markers PDGFRα, O4, and GalC. mOLs (PDGFRα+GalC+ or O4+GalC+) did not show altered numbers in pNf1 mutants. Therefore, overall changes in numbers of oligodendrocytes are unlikely to account for our findings. We note that maturing OLs (PDGFRα+GalC+) were significantly increased in pNf1 mutants, as in the Notch-activity reporter mouse Nf1+/-;Hes5GFP, numbers of Hes5GFP+ cells in CC were significantly increased, as compared to WT animals (Figures 5A and 5B). (C) Immunodetection of NICD (anOTCH) with a specific antibody (Cell Signaling, cs2421) in the subcortical WM (scWM) of NF1 patient brains (n = 2) and normal human brains (n = 2). Representative anOTCH nuclear signals are shown with red arrows (insets). Scale bars, 50 μm. (D) Quantification of total anOTCH+ cells per 20x field suggests increased Notch activity in the scWM of NF1 patients, as compared to normal brain (n = 2 NF1 patients, n = 2 normal brains, 2 technical replicates/condition, t test, **p = 0.0042). (E) Quantification of anOTCH+ cells as percent of total cells per 20x field supports increased Notch activity in the scWM of NF1 patients, as compared to normal brain (n = 2 NF1 patients, n = 2 normal brains, 2 technical replicates/condition, t test, ***p = 0.0014). Error and significance of the graphs reflect only experimental variability. Error bars show ± SEM. See also Figure S5.

**NF1+/- Mice Show Increased Notch Activity, and NF1 Patients Show Hyperactivated Notch**

To determine whether control of myelin compaction through Notch signaling might be relevant to NF1 patients, we first evaluated Notch activation in the brain of Nf1+/- mice, which mimic NF1 patients genotype with germline mutation in one Nf1 allele in all brain cells. Notch activation was elevated in Nf1+/- mutants, as in the Notch-activity reporter mouse Nf1+/-;Hes5GFP, numbers of Hes5GFP+ cells in CC were significantly increased, as compared to WT animals (Figures 5A and 5B).
Finally, we used two antibodies to detect cells with activated Notch* (αNOTCH or NICD) in brain sections from previously described adult NF1 patients (Nordlund et al., 1995). Both antibodies (ab88295 and cs2421) detected higher number of NICD* cells in the subcortical WM of NF1 patients (n = 2), as compared to normal adult brain (n = 2) (Figures 5C and S5A). Quantification of two technical replicates (reflecting experimental variability, not the variability of the biological process), showed significantly increased number of αNOTCH* cells (Figure 5D). To address the issue of precisely matching brain regions in the limited specimens available, we normalized the percentage of αNOTCH* cells to the total number of cells per field and found similar results (Figure 5E). Overall, our data are consistent with the idea that abnormal Notch signaling downstream of NF1 loss and NO increase contribute to NF1 patient pathology.

**DISCUSSION**

The study of WM abnormalities that correlate with motor, sensory, or cognitive changes in pathological situations may help to identify mechanisms relevant to normal brain function. We focused on brain pathology in NF1, a common genetic disorder in which patients show WM disorganization on brain imaging, and neurological deficits. We show that Notch signaling, along with MAPK and NO, are aberrantly activated after NF1 loss in mature oligodendrocytes, causing myelin decompaction and changing animal behavior.

Patient mutations in genes throughout the RAS-MAPK pathway (Rasopathy genes) compromise brain function (Rauen, 2013). NF1 is a well-studied Rasopathy gene that acts as a regulator of RAS-MAPK signaling (Donovan et al., 2002; Ratner and Miller, 2015). Increasing evidence confirms a role for MAPK activation in NF1 brain. For example, abnormally enlarged CC resulting from NF1 loss is rescued by perinatal MEK inhibition (Wang et al., 2012). In oligodendrocyte progenitors with NF1 mutation, basal and FGF-stimulated RAS-GTP are increased, proliferation increases, and differentiation is aberrant, although myelination occurs (Bennett et al., 2003). In contrast to these developmental studies, our analyses ensue after deletion of NF1 in mature cells. Our results are encouraging as they support the idea that alterations in NF1 brain may be susceptible both to prevention and to therapy.

Myelin decompaction resulting from NF1 loss in oligodendrocytes accounts for pathological increases in myelin thickness, as numbers of myelin lamellae did not significantly change. This result differs from studies in which the RAS effector proteins ERK1/2 regulate myelin thickness by increasing numbers of myelin wraps (Ishii et al., 2013), as assessed using gain and loss-of-function models. In our model, after NF1 loss, RAS, MEK, and ERK proteins are present at normal levels; therefore, extrinsic stimuli activate transient signaling through MEK/ERK. The transience of the RAS signal after NF1 loss likely accounts for the discrepancy with studies of constitutive activation or complete loss of ERK. Other RAS effector pathways activated by loss of NF1 may also contribute to decompaction after homozygous NF1 loss, although this seems less likely given that MEK inhibition alone blocks decompaction after hemizygous NF1 loss.

Increased Notch signaling following NF1 loss was demonstrated by microarray/RNA-seq analyses and corroborated by in vivo detection of NICD in CC^+ mOLs. Cell-autonomous roles of oligodendrocyte Notch in controlling myelin compaction are supported by our genetic gain- (NICD) and loss-of-function (Rbpj) models. Furthermore, although GSI can have effects on other substrates, our finding that GSI rescues aberrant myelin decompaction is consistent with our detection of active nuclear NICD in oligodendrocytes and the genetic data indicating that Notch blockade via Rbpj loss reverses effects of NF1 loss. The correlation between rescued decompaction and GSI rescue of abnormal startle response in pNF1 mutants suggests that myelin compaction is relevant to normal behavior. Importantly, GSI treatment did not affect WT myelin structure or behavior in our study. It will be of great interest to define a direct link between abnormal activation of Notch with decompaction and behavior in animal models and whether increased activation of Notch in NF1 patients is linked to their neurological issues.

Notch, via Hes5, regulates many processes in immature oligodendrocyte lineage cells (Liu et al., 2006; Watkins et al., 2008). We found that Hes5 transcript is increased in a NF1 mutant glial precursor-enriched population in vitro, and NICD and Hes1/5 increases were recently reported downstream of NF1 inactivation in neural stem cells (Chen et al., 2015). However, it is likely that Notch effector-driven cell-autonomous effects on mOL do not require Hes5, in that CC^+Hes5^− are rare (albeit increased) in NF1 mutants and do not show detectable ERK activation. It remains possible that in NF1 mutants, Hes5^+ cells and/or an increased population of O4^+GalC^ maturing oligodendrocytes, contribute to abnormal myelin compaction. However, the broad expression of mOL NotchICD in the absence of Hes5 strongly suggests the pathological relevance of alternate Notch effector proteins in this setting.

We previously reported that NF1 mutation in oligodendrocytes causes small but significant increase in NO levels in the forebrain (Mayes et al., 2013). Here, we extend the results to CC oligodendrocytes and demonstrate that increased NO is upstream of Notch-mediated myelin decompaction. In oligodendrocytes, NO is protective at low levels, while at high levels causes ERK1/2-mediated toxicity (Li et al., 2011) and downregulation of myelin genes preceding cell death (Jana and Pahan, 2013). Also at low concentrations, NO induces cGMP-mediated morphological changes (Garthwaite et al., 2015). As no increased mOL cell death was found in pNF1 mice (Mayes et al., 2013), it is probable that NO does not accumulate to lethal levels after NF1 loss. Yet, myelin structural defects develop in an NF1 gene-dose-dependent manner. We propose that the slow progression of myelin decompaction in hemizygous NF1 mutants is caused by modest increased MAPK and downstream signals that causes cumulative damage, while biallelic loss of NF1 causes higher levels of pathway activation and more rapid decompaction. Other NF1 gene-dose-dependent phenotypes have been reported. For example, NF1 hemizygous oligodendrocyte precursors are expanded in vitro, to a lesser extent than NF1 homozygous mutant cells (Bennett et al., 2003). Only NF1^+/− sympathetic neurons develop neurites independent of growth factor (Vogel et al., 1995), but NF1^−/− mice show defects...
in Morris water maze tests and dopamine-based learning (Anastasaki et al., 2015).

It is well known that RAS-GTP signaling can act up or downstream of Notch and have a cooperative or antagonistic outcomes (Sundaram, 2005). In fact, multilayer differentiation of neural stem cells is controlled by Nf1 via RAF/MEK pathway and Notch activation (Chen et al., 2015), however, the link between these signal pathways is unclear. We find that increased active Notch is rescued by L-NAMe treatment, suggesting that NO is a mediator between RAS-Notch signaling in Nf1 mutants.

Thus, a linear MAPK/NO/Notch pathway might drive myelin decompaction and behavioral phenotypes in hemizygous mutants (Figure 6A). In support of this idea, single agent treatment with MEKi (Figure 1C), GSI (Figure 3B), or L-NAMe (Figure 4A) fully rescue myelin decompaction, and GSI alone rescues behavioral abnormalities. Similar pathway interactions have been reported in PDGF-induced gliomas, in which NO/cGMP/PKG (Charles et al., 2010) or NO/ID4/Bagged1 (Jeon et al., 2014) signaling drive Notch activation and tumor growth.

A linear MAPK/NO/Notch pathway, however, does not easily explain why blocking each pathway alone does not rescue decompaction in homozygous mutants. A parsimonious explanation is that the more robust activation of RAS in pNf1flf mutants requires longer treatments with single drugs, higher doses, or combinations of drugs, to fully diminish the effects of RAS over activation (Figure 6B). Alternatively, increased MAPK, NO, and Notch signaling might cause durable transcriptional or posttranscriptional changes that feedback and potentiate the effects of the entire pathway. In support of this idea, NOS1-3 proteins are increased in pNf1 homozygous mutants (Mayes et al., 2013). Given that oligodendrocytes express NOS1 (Yao et al., 2012) and possibly NOS2 (Boullerne and Ben-Aziz, 2006), transcriptional regulation of these and other genes involved in the pathway is conceivable. The specific Notch effector(s) controlling myelin compaction remain unknown. Myelin proteins regulating compaction, including Claudin 11, might be involved. We found that genetic activation of Notch decreases Claudin 11. Of note, mice with loss of the Ptp1 and Cldn11 show impaired motor behavior and myelin decompaction, similar to those found in pNf1 mutants (Chow et al., 2005). Regulation of Cldn11 by Notch has not been reported, and in silico analysis do not show binding sites for CSL/Rbpj (http://genome.ucsc.edu). However, a binding site for PU.1, a transcription factor regulated by Notch (Chen et al., 2008) and downregulated in pNf1 mutants, is upstream of Cldn11. Additional downregulation of TJ/GJ genes (Tjp2, Gjb6, Gjb2) may also contribute to abnormal myelin and behavior (Figure 6).

Most myelinated fibers in Nf1 mutants showed decompaction, even though deletion of Nf1 occurs in a smaller number of mOLs (Koenning et al., 2012; Mayes et al., 2013). This result suggests that a diffusible messenger signals to non-recombinant cells. Diverse paracrine roles of NO have been widely reported in the brain (Calabrese et al., 2007), thus, NO could mediate effects to neighbor cells in pNf1 mutants (Figure 6). In fact, several non-cell-autonomous defects are detected after deletion of Nf1 in mOLs (Mayes et al., 2013).

Summary, activation of Notch signaling, downstream of MAPK and NO, results in myelin decompaction and behavioral changes in Nf1 hemizygous mutants. It is believed that most cells in NF1 patient brains contain one normal NF1 allele and one mutant/lost allele of the NF1 gene. However, there are brain regions with especially poor organization that may represent regions of biallelic NF1 loss, based on DTT (Ferraz-Filho et al., 2012). If biallelic NF1 mutations are present in oligodendrocytes in NF1 patient brains and cause WM alteration (Ferraz-Filho et al., 2012; Karlsgodt et al., 2012), drug combinations might be needed therapeutically. Importantly, our findings that NO/Notch signaling is perturbed in Nf1 mice, and that NICD increases in NF1 patients raise the intriguing possibility that the pathways we identified in mouse mOLs will be relevant to NF1 patients. Based on these studies, drugs that block the NF1 loss-dependent increased NO and subsequent Notch activation should be considered to treat disease manifestations in NF1.

**EXPERIMENTAL PROCEDURES**

**Mouse Strains**

All mouse studies were approved by the Cincinnati Children’s Hospital Research Foundation Institutional Animal Care and Use Review Committee (IACUC). The generation and genotyping of mice carrying Nf1fl/fl[Nf1tm1Par/J] (Zhu et al., 2002), Ptp1Cre [B6.Cg-Tg(Ptp1-CreERT)3Pop/J] (Doerflinger et al., 2003), Hes5-GFP [Tg[Hes5GFP][Sat/Mmn][Gong et al., 2003], Rbpjfl/fl [RBP-Jf/f] (Han et al., 2002), Rosa26Rosa126Sortm1[Notch1]DMJ/J (Murtaugh et al., 2003), and Nf1fl/fl [Bannan et al., 1994] alleles, are described in previous studies. Mice were maintained on the C57BL/6 background. For behavioral studies, only adult female mice were used. In all other studies, we used adult mice of both sexes. Mice were housed in a temperature- and humidity-controlled vivarium on a 12 hr light-dark cycle with free access to food and water.

**Pre-clinical Therapeutics**

Gamma secretase inhibitor (GSI,MK-003; Merck) was made fresh weekly and dosed at 300 mg/kg in 0.5% methocel by oral gavage (Lewis et al., 2007; Spary et al., 2009). For pathology, we dosed mice once weekly for 4 weeks and sacrificed 6 hr after the last dose (n = 5 doses). For flow analysis, we dosed mice once weekly for 1 week and sacrificed mice 6 hr after the last dose (n = 2 doses). Fresh solution of NAMe (8 g/L-arginine methyl ester, 100 μM in 1x PBS; Sigma-Aldrich) was administered daily at 0.4 mg/kg. For pathology and flow analysis, mice were injected intraperitoneally (i.p.) daily for 7 days and sacrificed 6 hr after the last dose. MEK inhibitor (PD0325901; Pfizer) was made fresh weekly and dosed at 1.5 mg/kg/day in 0.5% methocel/0.2% Tween 80, by oral gavage. For pathology, we dosed mice every day for 3 weeks. For flow analysis, we dosed mice every day for 7 days and sacrificed 6 hr after the last dose.

**Behavior**

Behavioral assessments were completed on pNf1+/− mice (F12, backcrossed onto C57BL/6) mice. Acoustic startle response, with prepulse inhibition, was assessed as described (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 2 consecutive days and the data from the second day were analyzed, because animals that are tested on a single day for the acoustic startle response tend to have greater variability that can mask group differences.

**Human Brain Tissues**

Brain specimens were described previously (Nordlund et al., 1995). Briefly, NF1 brains were obtained from patients diagnosed with NF1 based on NIH guidelines. None of these patients showed any evidence of neurological disease other than NF1. Control/normal brains were obtained from adult patients with no neurological diseases reported, although a history of depression was
Figure 6. Model of Nf1 Loss-Driven Myelin Decompaction

Increased MAPK/NO/Notch signaling may mediate myelin decompaction (gray-dotted arrows and insets), by downregulation of TJ/GJ proteins involved in myelin compaction such as Claudin 11 (Cldn11), ZO-2 (Tjp2), and Connexin 26 (Gjb2) in pNf1 mutants.

(A) Decreased Nf1 (blue) results in increased RAS-GTP in mature oligodendrocytes, leading to hyperactive MAPK signaling (RTK → RAS → pMEK → pERK). Production of NO increases in response to hyperactive MAPK pathway (NOS, dashed arrow). Subsequently, NO promotes directly or indirectly (dotted line) Notch cleavage and translocation of NICD to the nucleus, where it regulates gene expression. Furthermore, diffusion of NO from Nf1 mutant oligodendrocytes (white arrows) might affect nearby oligodendrocytes increasing the number of decompacted fibers (bottom). Either pharmacological treatment alone (MEKi, L-NAME or GSI) rescues decompaction (purple lines).

(B) Absent Nf1 (red) results in accumulation of RAS-GTP and stronger induction (thick arrows) of MAPK signaling and NO production. It is possible that long-lasting transcriptional and/or post-transcriptional changes (dotted lines with question marks) in molecules controlling NO levels (for example NOS1-2) might contribute to increases in NO. Subsequently, NO promotes Notch activation and regulation of myelin genes. Combination of inhibitors (MEKi and L-NAME, purple fused lines) rescues decompaction, while single agent treatments (for example GSI, fading purple arrow) improves severity but does not rescue decompaction. Note that genetic inactivation of Notch (Figure 5) combined with MEKi or L-NAME, also rescues compaction in this setting.
reported in one. All brains were harvested within 8 hr after death. Brains were sliced and placed in 4% paraformaldehyde/0.1 M phosphate buffer for 72 hr and prepared for paraffin embedding and storage.

### Statistical Analysis

The minimal number of animals per statistically significant experiment was three. Comparison between two groups used Student’s *t* test with a significance cutoff of *p* < 0.05. Comparison of three or more groups used the one-way ANOVA, followed by Tukey post hoc test with a significance cutoff of *p* < 0.05. For unbiased counting of myelinated axons, mice were randomized and treated in a blinded manner. For behavior, acoustic startle was analyzed by mixed linear two-way 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.

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A.L.-J. and H.E.T. conducted most experiments and analyzed data. J.W.P. conducted animal work and drug dosing. M.R.B. performed microarray and western blot. T.A.R. assisted with perfusions. G.C. prepared blocks and sections for electron microscopy. S.H.S. assisted with statistical analyses. S.H.S. conceived and directed the project, contributed to data analysis and interpretation, and edited the manuscript.

### AUTHOR CONTRIBUTIONS

A.L.-J. and H.E.T. conducted most experiments and analyzed data. J.W.P. conducted animal work and drug dosing. M.R.B. performed microarray and western blot. T.A.R. assisted with perfusions. G.C. prepared blocks and sections for electron microscopy. S.H.S. assisted with statistical analyses. S.H.S. conceived and directed the project, contributed to data analysis and interpretation, and edited the manuscript.

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Supplemental Information

Oligodendrocyte *Nf1* Controls Aberrant Notch Activation and Regulates Myelin Structure and Behavior

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SUPPLEMENTAL INFORMATION

- 6 SUPPLEMENTAL FIGURES
- SUPPLEMENTAL EXPERIMENTAL PROCEDURE
Figure S1 (related to Figure 1). Progressive Decrease in G-Ratio is Regulated by MAPK Signaling in pNf1 Mutants

Scattered plots (A) and bar graph (B) indicating g-ratio in the CC of WT, pNf1f/+ and pNf1f/f mice, at the indicated months (mo) after tamoxifen treatment. g-ratio does not significantly change in WT animals between 1 and 8 mo (n=3, 4/time point); however, it is decreased in pNf1f/+ at 1 mo (n=3, ***P<0.001) and 6 mo (n=3, ****P<0.0001), and in pNf1f/f at 1 mo (n=4, ***P<0.001) and 6 mo (n=3, ****P<0.0001), as compared to WT animals. g-ratio is further decreased in pNf1f/+ mutants from 1 to 6 mo (n=3/time point, ****P<0.0001), and presents a slight but significant recovery in pNf1f/f from 1 to 6 mo (n=4,3/time point, *P<0.01). Two-way ANOVA P<0.0001/Tukey Post Hoc. (B) Number of myelin wraps does not significantly change in pNf1f/+ (6 mo) or pNf1f/f (1-8 mo), as compared to WT animals (n=6,3/genotype, one-way ANOVA). (D) g-ratio in pNf1f/+ is rescued to WT levels following MEKi treatment, but remains significantly decreased in pNf1f/f (n=3, P<0.001). (E) Axon diameter does not significantly change in WT animals between 1 and 8 mo (n=3,4/time point), but decreases in pNf1f/+ at 1 mo (n=3, ****P<0.0001) and 6 mo (n=3, ****P<0.0001), and in pNf1f/f at 1 mo (n=4, ***P<0.001) and 6 mo (n=3, **P<0.01), as compared to WT animals. Axon diameter presents significant recovery in pNf1f/f from 1 to 6 mo (n=4,3/time point, **P<0.01). Two-way ANOVA/Tukey Post Hoc. (F) Immunostaining of EGFP (green) in the brain of PlpCreER;ccEGFP mice indicates the number of recombinant cells in WT and pNf1f/f mutants at 6 months post-tamoxifen (6mo). DAPI staining (blue) indicates nuclei of cells. (G) Quantification of EGFP+ cells in pNf1f/f and WT animals normalized to area unit (left, 100k pixels) or total cells (right, DAPI+ cells), indicates no changes in the density of recombinant cells (t test, 3 technical replicates, left; P=0.32, Right; P=0.23). All data are presented as the mean ± s.e.m except in A (SD). Scale bar=100 um.
Figure S2 (related to Figure 1). Effects of MEKi on pERK and levels of MBP in Nf1 mutants.

(A) Immunofluorescence of pERK (red cells, yellow arrows in magnifications) and CC1 (white) in vehicle- and MEKi-treated WT and pNf1f/f mice, 1 month post-tamoxifen treatment. (B) Quantification of the percent of pERK⁺;CC1⁺ mOLs among total CC1⁺ cells indicates that the abnormal increased ERK activation in pNf1f/f mutants (data from figure 1D) is significantly reduced by MEKi treatment (n=4,3/genotype, t-test, *P=0.0474). MEKi-treated pNf1f/f mutants show, however, still significantly increased pERK⁺;CC1⁺ mOLs, as compared to MEKi-treated WT animals (n=3/genotype, t-test, *P=0.0147). (C) Immunostaining showing similar expression levels of MBP (red) around CC1⁺ mOLs (white, arrows), in the CC of WT and pNf1f/f mutants. 1 mo; 1 month post-tamoxifen. Right: magnification of squared area on the left. LV; lateral ventricle. Scale bar; 100um.
**Figure S3 (related to Figure 2). Identity of Cells With Increased Notch Signaling in Nf1 mutants.**

(A) Immunofluorescence of Hes5GFP (green) and GFAP (red) in coronal section of Nf1<sup>+/−</sup>;Hes5GFP mouse. Hes5<sup>+</sup> cells correspond to GFAP<sup>+</sup> astrocytes in the cortex (magnification 2, yellow arrows). Hes5<sup>+</sup> cells correspond to GFAP<sup>+</sup> astrocytes and unidentified GFAP<sup>−</sup> cells (white arrows, magnification 1) in the CC. (B) Immunostaining of pERK (red) and Hes5GFP (green) in the CC of WT (top) and pNf1<sup>+/−</sup> (bottom) animals, 3 months (3mo) after tamoxifen treatment. Hes5GFP signals do not overlap with pERK in WT or pNf1<sup>+/−</sup> mutants. Instead, pERK signals are detected in CC1<sup>+</sup> mOLs (white, arrows) in pNf1<sup>+/−</sup> mutants. See also Figure 1. Scale bar= 10 um. (C) Immunostaining of aNOTCH (red), the reporter of recombination EGFP (green) and CC1 (white) in WT or Nf1 mutants carrying the reporter allele pEGFP (Plp1CreER;ccEGFP), 6 months after tamoxifen treatment. Magnification of the squared region in the CC of the pNf1<sup>+/−</sup> mutant (bottom), shows aNOTCH signals in EGFP<sup>+</sup> recombinant (arrow heads) as well as in EGFP<sup>−</sup> non-recombinant (arrows) CC1<sup>+</sup> mOLs. DAPI staining (blue) depicts cell nuclei. Scale bar; 5 um. In all panels: CC; corpus callosum, Ctx; brain cortex, LV; lateral ventricle.
Figure S4 (related to Figures 2, 3 and 4). Myelin-Related Phenotypes in Nf1 Mutants Are Mimicked By Notch Activation and Rescued By Inhibition Of Notch, MAPK and Nitric Oxide signal pathways.

(A) Quantification of myelinated axons indicates significantly decreased g-ratio in pNICD (PlpCreER;RosaNICD) mice, as compared to WT mice (n=4, 5 mice/genotype, ****P<0.0001) 1 mo post-tamoxifen treatment. (B) WB analysis of Claudin 11 and actin (loading control) from forebrain lysates of WT or pNICD mice, 1 month post-tamoxifen (1mo). Densitometric analysis of Claudin 11 normalized to actin signals (bottom) indicates significantly decreased Claudin 11 levels in pNf1 mutants (n=3 animals/genotype, t test, ***P=0.0005). (C) Genetic inactivation of Notch signaling significantly increased g-ratio in pNf1f/f;Rbpj f/f double mutants, as compared to pNf1f/f mutants (n=5,4/genotype, ***P<0.0001); however, g-ratio is still decreased when compared to WT animals (n=4, 6 mice/genotype, ****P<0.0001). The g-ratio of myelinated axons of pNf1f/+;Rbpj f/f double mutants increased, as compared to pNf1f/+ mutants (6 mo, n=3/genotype, ****P<0.0001), and was not significantly different from WT animals (n=3, 6 mice/genotype). One-way ANOVA, P<0.0001, Tukey post hoc test. (D) Evaluation of CC myelinated axons in pNf1 and WT mice indicates that GSI treatment results in: no effects on g-ratio of WT mice as compared to vehicle-treated animals (1 mo, n=5/genotype, one-way ANOVA); increased g-ratio in pNf1f/f mutants as compared to vehicle-treated pNf1f/f mutants (1 mo, n=5/genotype, one-way ANOVA, ****P<0.0001); and increased g-ratio in pNf1f/+ mutants, as compared to vehicle-treated pNf1f/+ animals (6 mo, n=4,3/genotype, t-test, ****P<0.0001). (E) Dot plot visualization of flow cytometry analysis showing Hes5GFP+ forebrain cells in WT (red) and pNf1f/f (blue) mice. Although Hes5GFP+ cells increase in pNf1 mutants, as compared to WT (n=3/genotype, t-test, *P=0.0335), both genotypes show negligible Hes5GFP+;NO+ or Hes5+;superoxide+ subpopulations (arrows). ns: no significant changes. (F) Quantification of myelinated fibers indicates that abnormally decreased g-ratio in pNf1f/+ mutants is rescued by L-NAME treatment for 7 days, as compared to vehicle-treated pNf1f/+ mutants (n=4,3/genotype, ****P<0.0001). Abnormally low g-ratio in pNf1f/f mutants is increased after treatment with L-NAME, as compared to vehicle-treated pNf1f/f mutants, (****n=3,5/genotype, P<0.0001), and fully rescued by concomitant treatment with L-NAME and MEKi, as compared to WT animals (****n=6, 3/genotype, P<0.0001). One-way ANOVA P<0.0001, post hoc Tukey test).
Figure S5 (related to Figure 5). Increased Active Notch in NF1 Patients
(A) Immunodetection of activated Notch (aNOTCH) with a specific antibody (abcam ab8925) in the subcortical WM (SCWM) of NF1-patient brains (n=2) and normal human brains (n=2). Representative aNOTCH signals are shown with red arrows in magnification of squared areas. Scale bars 50 µm.
Figure S6 (related to Figure 2). *Nf1* loss and Oligodendrocyte Numbers

(A) Immunostaining of Olig2+ (red) and Hes5GFP+ (green) cells in the CC of WT (top) and *pNf1 f/f* (bottom) mice, at 1 month (1mo) post-tamoxifen treatment. Increased Hes5GFP+ cells (bottom, right) are shown as a reference of the mutant phenotype. Scale bar=200um. CC; corpus callosum, LV; lateral ventricle. (B) Quantification of Olig2+ cells indicates no significant changes in *pNf1* mutants, yet a trend toward increase in *pNf1 f/f* animals (n= 3 animals/genotype, t test; WT vs *pNf1 f/f*; P value= 0.18, WT vs *pNf1 f/+* P value= 0.48). (C) Dot-plot visualization of flow cytometry analysis of the forebrain of WT (red) and *pNf1* mutants (blue) mice, showing PDGFRα+ and GalC+ populations. The percent of PDGFRα+GalC+ mOLs do not differ between WT and *pNf1* mutants (bottom-right gates), but PDGFRα+GalC+ and PDGFRα+GalC- (top gates) maturing OLs are increased. (D) Relative number (fold change of WT) of forebrain Hes5GFP+ cells immunoreactive for PDGFRα, O4 and GalC, from flow cytometry analysis. The population of PDGFRα+GalC+ maturing OLs are significantly increased in *pNf1 f/f* animals (** P=0.001).
SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Induced recombination in mice: For tamoxifen-induced recombination, adult mice (8–10 weeks old) were injected intraperitoneally with tamoxifen (75 mg/kg of body weight, in sunflower seed oil; Sigma-Aldrich), twice daily for 4 consecutive days.

Mouse tissue for histology. Mice were anesthetized and perfused with 1X PBS followed by ice cold 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde overnight and sectioned using a vibratome (Leica) or transferred to 20% sucrose for subsequent frozen sectioning.

Immunostaining and image analysis: Cryostat, vibratome floating, or paraffin-embedded sections (human samples) were processed for immunodetection of proteins using specific antibodies for GFP (Millipore or Nacalai Tesque), CC1 (Calbiochem) NICD (aNOTCH, Abcam and Cell signaling), GFAP (Invitrogen) and pERK (Cell Signaling), Olig2 (Millipore) or MBP (Santa Cruz). Fluorophore-conjugated (Alexa 488, Alexa 568, Alexa 647; Invitrogen) or HRP-conjugated (paraffin-embedded samples) secondary antibodies were used to detect the antigen-antibody complexes. Using the appropriate laser excitation wavelength, images were captured on a Nikon C2 confocal microscope. Manual and automated cell counting were performed using ImageJ and ITCN plugin, respectively.

Electron Microscopy: Wild type and experimental littermate mice were processed at the same time to minimize myelin structure artifacts derived from processing. Animals were perfused with 4% paraformaldehyde/ 2.5% glutaraldehyde, 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). Ultrathin sections were stained in uranyl acetate and lead citrate, and then imaged on a Hitachi Model H-7600 transmission electron microscope.

Myelin Analysis: To quantify decompaction, myelin sheaths were rated as compact or decompacted, defined by fibers with disruption of the myelin sheath (i.e. splitting of myelin lamellae). Severity of decompaction was evaluated, rating decompacted myelinated fibers with 1 (25%), 2 (25–50% disrupted), 3 (50–75% disrupted), or all 4 (>75% disrupted) quadrants of the sheath disrupted. The g-ratio of myelinated fibers was obtained by dividing the diameter of each axon by the fiber diameter (300-500 axons/animal, n=3–5 mice/genotype/condition), using ImageJ software. When a fiber was identified as compact or 4 quadrants decompacted, 1 measurement was collected for g-ratio. When a fiber was identified as 2 quadrants or 1 quadrant decompacted, 2 or 4 measurements were collected for g-ratio. Quantification was carried out by blinded individuals.

Flow Cytometry: Mouse brains were processed for flow cytometry as previously described (Robinson et al., 2014). Briefly, forebrains were dissociated using Accutase (Sigma-Aldrich) cell dissociation cocktail. ENZO Life Sciences kits were used to detect Nitric Oxide (ENZ-51013-200) and Superoxide / Reactive Oxygen Species (ENZ-51010). A positive control (L arginine, a nitric oxide synthase substrate) and a negative control (C, a nitric oxide scavenger) validated staining specificity for nitric oxide. An inducer (pyocyanin) and inhibitor (n-acetyl cysteine) were used for superoxide/reactive oxygen species positive and negative controls. Live/dead cell stain positive control (L arginine, a nitric oxide synthase substrate) and a negative control (C) were used for superoxide/reactive oxygen species positive and negative controls. Live CNS resident cells were gated to quantify Hes5-GFP+ oligodendrocytes. To evaluate reactive oxygen species, geometric means were quantified for all CNS resident cells (n=3 forebrains/ genotype/experiment).

Gene Expression Analysis: 1) Microarray analysis: 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 minutes with FGF2 as described (Bennett et al., 2003). Total RNA was isolated from passage 2-3 cells using Trizol reagent (Invitrogen) 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) from 10ug 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). 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). 2) RNAseq analysis: Total RNA was isolated from optic nerves using Trizol reagent (Invitrogen) followed by chloroform extraction and purification by RNeasy kit (Qiagen). Integrity of RNA was evaluated by Agilent Bioanalyzer 2100 (RIN>8.5) and cDNA was amplified by Ovation system v2. RNA sequencing (35-40 million sequencing reads) was performed using paired-end BP flow cell. RNA-Seq data was mapped to the reference genome mm10, and fold change and variance were calculated using DESeq2 package in R.

RT-PCR: qRT-PCR was carried out on glial progenitor RNA from E12.5 spinal cord cultures in triplicate after FGF2 stimulation using primers for DII-1 R2409 =CCATAGTGCAATGGGAACAAC/ L2229 = CAAGGATATAGCCCGATGA, DII-3 R= CTACAGCGGTCCACCCTCTT, L919 =ATTCTACGGCTTGGATGTG, and Hes5 R=AGAGGGTGGCCCTGATTA, L= AGGATGAGCTCGTCTCTCTG. 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 minutes (Bennett et al., 2003), or forebrain tissue, were lysed in RIPA buffer. 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 (Santa Cruz C20R; sc-6014-R) at 1:200, anti-Ras10 (Upstate Biotechnology) and anti-Claudin-11 (Invitrogen) at 1:1,000. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were used to detect signal with Enhanced Chemiluminescence (ECL) Plus developing system (Amersham Biosciences).

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