Regulation of Peripheral Myelination through Transcriptional Buffering of Egr2 by an Antisense Long Non-coding RNA

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Regulation of Peripheral Myelination through Transcriptional Buffering of Egr2 by an Antisense Long Non-coding RNA

Graphical Abstract

Highlights

- Expression of Egr2 in peripheral nerves is regulated by a long non-coding RNA
- Egr2-AS-RNA gradually recruits an epigenetic silencing complex on the Egr2 promoter
- Egr2-AS-RNA regulates nascent transcription of Egr2
- Expression of Egr2-AS-RNA is regulated by Erk1/2 signaling to YY1

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In Brief
Martinez-Moreno et al. report a role for a long non-coding RNA antisense to the promoter of Egr2, Egr2-AS-RNA, during the response to peripheral nerve injury. Inhibition of Egr2-AS-RNA following sciatic nerve injury reverts EGR2-mediated gene expression and delays demyelination.
Regulation of Peripheral Myelination through Transcriptional Buffering of Egr2 by an Antisense Long Non-coding RNA

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SUMMARY

Precise regulation of Egr2 transcription is fundamentally important to the control of peripheral myelination. Here, we describe a long non-coding RNA antisense to the promoter of Egr2 (Egr2-AS-RNA). During peripheral nerve injury, the expression of Egr2-AS-RNA is increased and correlates with decreased Egr2 transcript and protein levels. Ectopic expression of Egr2-AS-RNA in dorsal root ganglion (DRG) cultures inhibits the expression of Egr2 mRNA and induces demyelination. In vivo inhibition of Egr2-AS-RNA using oligonucleotide GapMers released from a biodegradable hydrogel following sciatic nerve injury reverses the EGR2-mediated gene expression profile and significantly delays demyelination. Egr2-AS-RNA gradually recruits H3K27ME3, AGO1, AGO2, and EZH2 on the Egr2 promoter following sciatic nerve injury. Furthermore, expression of Egr2-AS-RNA is regulated through ERK1/2 signaling to YY1, while loss of Ser184 of YY1 regulates binding to Egr2-AS-RNA. In conclusion, we describe functional exploration of an antisense long non-coding RNA in peripheral nervous system (PNS) biology.

INTRODUCTION

In the vertebrate peripheral nervous system (PNS), Schwann cells (SCs) produce the myelin sheath, the specialized membrane structure that allows rapid nerve conduction. In recent years, significant progress has been made in the identification of key transcriptional regulators of myelination. Evidence generated in the mouse suggests that the transcription factor EGR2 plays the role of a central regulator in this process: (1) EGR2 is activated in SCs after axonal contact, before myelination (Murphy et al., 1996; Topilko et al., 1994); (2) Egr2 null or hypomorphic mutations result in blockade of SCs at the promyelinating stage, after the establishment of a 1:1 ratio with the axons, rendering them unable to proceed with the myelination process (Le et al., 2005; Topilko et al., 1994); (3) forced Egr2 expression in SCs results in the activation of genes encoding structural myelin proteins and enzymes involved in lipid synthesis (Nagarajan et al., 2001); and (4) downregulation of EGR2 expression after peripheral nerve injury results in demyelination (Ghislain et al., 2002; Zorick et al., 1996). In addition to mouse studies, the association of various dominant or recessive Egr2 mutations with several types of human peripheral neuropathies supports the crucial role of Egr2 in the control of SC myelination (Bellone et al., 1999; Timmerman et al., 1999; Warner et al., 1998).

Intracellular signaling pathways activated by both membrane-bound and soluble neuregulins regulate the expression of EGR2 in SCs (Murphy et al., 1996; Svaren and Meijer, 2008; Tavergia et al., 2005). Activation of the MEK-ERK1/2 cascade by neuregulin is responsible for activation of the YY1 transcription factor, which binds to the Egr2 promoter and regulates Egr2 expression (He et al., 2010). Ablation of ERK/12 signaling in sciatic nerves leads to profound inhibition of EGR2 expression and severe hypomyelination (Newbern et al., 2011).

The aforementioned studies reflect the “classical” paradigm of transcriptional regulation, where signaling intermediates activate transcription factors, which in turn bind specific DNA motifs located on promoters to regulate the expression of target genes. However, the role of epigenetic mechanisms (here taken to mean mechanisms such as histone modifications and non-coding RNAs (ncRNAs) that alter gene expression without changing the DNA sequence) orchestrated by ncRNAs that regulate transcription (Hawkins and Morris, 2008) have not been studied. In human cells, there are two independent mechanisms that confer transcriptional gene silencing (TGS): (1) a microRNA (miRNA)-directed mechanism and (2) a long-antisense RNA mechanism (Morris, 2009). Both short- (miRNA)- and long- (antisense)-RNA-mediated TGS in human cells involves the interaction of RNA with promoter regions (Kim et al., 2008; Klase et al., 2007; Omoto and Fujii, 2005; Tan et al., 2009).

Here, we describe a long ncRNA (IncRNA) antisense to the proximal promoter of Egr2. Egr2-AS-RNA shows increased expression during acute peripheral nerve injury. Expression of Egr2-AS-RNA regulates the levels of Egr2 in SCs, and in vivo...
inhibition of Egr2-AS-RNA results in rescue of the EGR2-mediated gene expression profile and delay of demyelination following peripheral nerve injury. Egr2-AS-RNA gradually recruits an epigenetic remodeling complex on the Egr2 promoter, while expression of Egr2-AS-RNA is regulated by ERK1/2 signaling to YY1, which binds to Egr2-AS-RNA in the context of chromatin. Finally, YY1 mediates the interaction between Egr2-AS-RNA and the chromatin remodeling factor ESH2, while loss of Ser184 of YY1 induces direct binding of YY1 to Egr2-AS-RNA.

RESULTS

Discovery of an Antisense IncRNA at the 5’ UTR of Egr2

We have recently shown that miR-709 induces TGS of Egr2 by binding to the myelin-specific element (MSE) region of the Egr2 promoter (Adilakshmi et al., 2012). These data generated a hypothesis regarding the possible transcriptional regulation of the proximal promoter of Egr2 by antisense RNA. To determine whether an antisense IncRNA is present at the 5’ UTR of Egr2, we employed a modified rapid amplification of cDNA ends (5’-RACE) protocol to amplify the antisense strand. The resulting antisense product was cloned, and the sequence is presented in Figure S1A. Attempts to extend the RACE amplification further upstream using primer walking did not reveal any results (data not shown), which suggests that either the antisense RNA is ~1,000 nt long or that it is partially amplified with our RACE protocol. Next, we performed a homology search using the rat antisense sequence against the mouse genome to identify the degree of homology between rat and mouse. This search showed 92% homology (Figure S1B), which allowed us to design mouse-specific primers and perform strand-specific RT-PCR to amplify the antisense product from total RNA isolated from mouse sciatic nerves. To determine whether this AS-RNA had been identified before, we performed a BLAST search using LNCipedia (Volders et al., 2013, 2015) and IncRNAdb (Amaral et al., 2011; Quek et al., 2015). The search returned no matches, which suggests that this RNA is a previously undescribed long non-coding antisense RNA that we subsequently refer to as Egr2-AS-RNA. Finally, to independently validate the expression of Egr2-AS-RNA, we examined publicly available RNA-sequencing (RNA-seq) data from three mouse sciatic nerves to identify RNA-seq reads that map to the identified AS-RNA transcript (Poitelon et al., 2016). We found that the number of reads per kilobase per million (RPKM) of Egr2-AS-RNA ranges between 1.6 and 2.5 and is on average 5 times more than the mouse-specific qPCR as described before (Lu and Tsourkas, 2009). The Ct values obtained from the amplification of Egr2-AS-RNA were then projected on the standard curve to determine the concentration of Egr2-AS-RNA per 100 ng total RNA, which was used as template in all qPCR reactions. This analysis showed that the average concentration of Egr2-AS-RNA in non-injured sciatic nerves was 200 pg/100 ng RNA, while, in injured nerves, the average concentration fluctuated between 350 and 650 pg/100 ng RNA, depending on the time interval following nerve injury (Figure 1E). The low expression levels of Egr2-AS-RNA are within the range of expression of IncRNAs in eukaryotic cells (Mortazavi et al., 2008; Palazzo and Lee, 2015; Rasmköld et al., 2009). The fact that expression levels increase by an average of 4-fold following sciatic nerve injury implies specific functionality of Egr2-AS-RNA in injured peripheral nerves.

Ectopic Expression of Egr2-AS-RNA Results in Demyelination and Inhibition of Egr2 mRNA Expression

Since increased Egr2-AS-RNA expression correlates with reduced Egr2 mRNA levels after sciatic nerve injury, we sought to demonstrate that Egr2-AS-RNA could induce silencing of
transcript expression. We generated a lentivirus expressing Egr2-AS-RNA to infect mouse dorsal root ganglion (DRG) explant cultures 14 days after the addition of ascorbic acid to ensure the presence of myelinated axons. We demonstrate that overexpression of Egr2-AS-RNA (Figure 2E) results in statistically significant inhibition of Egr2 mRNA expression as detected by qPCR (Figure 2A). In addition, ectopic expression of Egr2-AS-RNA results in statistically significant inhibition of EGR2 protein expression (Figure 2B). To examine the effect of the ectopic expression of Egr2-AS-RNA on myelination, we infected myelinated mouse DRG cultures with pLenti-AS-RNA or pLenti-control. To show the specificity of the effect of Egr2-AS-RNA on myelination, we incubated the pLenti-AS-RNA-infected cultures with a scrambled oligonucleotide GapMer (scrambled complementary strand of the AS-RNA) or an Egr2-AS-RNA GapMer (complementary to the Egr2-AS-RNA sequence). One week after infection and treatment with the GapMers, we stained the cultures with myelin basic protein (MBP) and neurofilament (NF) antibodies to detect myelin internodes and integrity of the underlying axons, respectively (Figure 2C). Infection with the pLenti-AS-RNA induces significant expression of Egr2-AS-RNA, which is not affected by the addition of scrambled GapMers, while Egr2-AS-RNA GapMers induce a significant reduction in the amount of Egr2-AS-RNA in the cultures (Figure 2E). Moreover, addition of Egr2-AS-RNA GapMers rescues the demyelination phenotype observed in cultures infected with pLenti-AS-RNA, while scrambled GapMers have no effect (Figures 2D and 2F). There was no difference in total cell numbers between the cultures (Figure S3C).

Inhibition of Egr2-AS-RNA Expression Using Oligonucleotide GapMers Results in Delay of Demyelination following Peripheral Nerve Injury

We developed an in situ, non-swelling, biodegradable hydrogel (O’Shea et al., 2015) loaded with oligonucleotide GapMers (20-mers) against Egr2-AS-RNA. The GapMer-infused hydrogel...
was applied to the sciatic nerve at the time of transection. We designed five GapMers targeting different areas of the Egr2-AS-RNA and a scrambled GapMer for control. Four GapMers induced significant inhibition of Egr2-AS-RNA expression as compared to transected nerves that received hydrogel only or hydrogel plus scrambled GapMers (Figure 3A). Inhibition of Egr2-AS-RNA expression with each of these GapMers resulted in statistically significant increase in the expression of Egr2 mRNA following peripheral nerve injury (Figure 3B). Using electron microscopy, we discovered that addition of the hydrogel plus GapMer at the time of sciatic nerve transection delays the injury-induced demyelination at days 2, 5, and 7 after nerve injury compared to injured nerves alone or injured nerves treated with hydrogel plus scrambled GapMer (Figure 3C). Subtherapeutic concentrations of GapMer remained within the hydrogel beyond 7 days, consistent with in vitro release results for biomacromolecules of a similar molecular weight (O’Shea et al., 2015), and consequently, the study was not extended further. Inhibition of Egr2-AS-RNA expression using GapMers results in a significant reduction in the percentage of demyelinated fibers and an increase in the percentage of myelinated fibers 2, 5, and 7 days following complete sciatic nerve transection.
Figure 3. In Vivo Inhibition of Egr2-AS-RNA Expression Results in Delayed Demyelination after Sciatic Nerve Injury

(A) Effect of five separate oligonucleotide GapMers complementary to five different sequence motifs of Egr2-AS-RNA on the expression of Egr2-AS-RNA in mouse sciatic nerves. Quantification was performed with qPCR, combining RNA from 3 separate mice per individual GapMer. Four GapMers induced significant inhibition of AS-RNA expression (one-way ANOVA \( [5,13] = 5.846, p = 0.0111 \) followed by a post hoc Dunnett’s test \([*p < 0.05]\)) as compared to transected nerves that received hydrogel only (lesion) or hydrogel plus scrambled GapMers.

(B) Inhibition of AS-RNA with each of these GapMers results in significant increase in the expression of Egr2 mRNA as compared to non-treated or scrambled-GapMer-treated injured sciatic nerves (one-way ANOVA \( [F(5,7) = 7.175, p = 0.0111] \) followed by post hoc Dunnett test \([*p < 0.05]\), 3 sciatic nerves per condition).

(C) Nerves that received hydrogel only or hydrogel plus scrambled GapMers show varying degrees of demyelination and axonal damage at 2, 5, and 7 days after sciatic nerve transection. Animals treated with hydrogel plus GapMer appear to have less demyelination and axonal degeneration, and the endoneural space appears more compact without extensive collagen depositions, which is more evident at day 7 as compared to animals treated with hydrogel only or hydrogel plus scrambled GapMers.

(D) Quantification of the myelinated and demyelinated fibers as a percentage of the total number of fibers in non-treated, hydrogel-plus-scrambled-GapMer-treated, and hydrogel-plus-GapMer-treated nerves following sciatic nerve injury. Inhibition of Egr2-AS-RNA expression using specific oligonucleotide GapMers results in significant reduction in the percentage of demyelinated fibers and an increase in the percentage of myelinated fibers 2, 5, and 7 days following complete sciatic nerve transection \((p < 0.05, \text{two-tailed Student’s } t \text{ test of unpaired samples})\).

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requires the presence of an RNA-DNA hybrid (Figure 5B). Next, after treatment with RNase H, which means that the interaction of Egr2-AS-RNA in the complex. This revealed that 48 hr after Egr2-AS-RNA expression is due to a direct effect on transcription, we performed nuclear run-on assays in SCs infected with Lenti-GAPMERS induces a 3-fold increase in Egr2 mRNA at 6, 24, and 48 hr following sciatic nerve injury. We show that 6 hr post-injury, only AGO1 and AGO2 are present on the Egr2 promoter, and inhibition of Egr2-AS-RNA expression inhibits their recruitment on the promoter (Figure 5D). At 48 hr post-injury EZH2, AGO1, AGO2, and H3K27me3 are all recruited to the Egr2 promoter, and this depends on the presence of the Egr2-AS-RNA, since inhibition of its expression results in inhibition of EZH2, AGO1, AGO2, and H3K27me3 binding to the Egr2 promoter (Figure 5E). To discover whether the gradual recruitment of the repressive complex by Egr2-AS-RNA mediates transcriptional repression of Egr2 mRNA, we compared the ChIP results with the expression of Egr2 mRNA at 6, 24, and 48 hr following sciatic nerve injury. We show that 6 hr post-injury, where Egr2-AS-RNA recruits AGO1 and AGO2 on the Egr2 promoter (Figure 5C), Egr2 transcription is repressed and inhibition of Egr2-AS-RNA with specific GapMers induces a significant (38-fold) increase in Egr2 expression compared to injured nerves treated with scrambled GapMers (Figure 5F, 6 hr). At 24 hr post-injury, the Egr2-AS-RNA mediated recruitment of AGO2 and H3K27me3 to the Egr2 promoter (Figure 5D) does not correlate with Egr2 transcriptional repression, since levels of the Egr2 transcript are equal between injured nerves treated with AS-RNA GapMers and those treated with scrambled GapMers (Figure 5F, 24 hr). Finally, at 48 hr post-injury, the Egr2-AS-RNA-mediated recruitment of EZH2, AGO1, AGO2, and H3K27me3 to the Egr2 promoter (Figure 5E) correlates with a modest but significant transcriptional repression of Egr2, since inhibition of Egr2-AS-RNA expression with GapMers induces a 3-fold increase in Egr2 transcript levels compared to injured nerves treated with scrambled GapMers (Figure 5F, 48 hr).

Expression of Egr2-AS-RNA Is Regulated by ERK1/2 Signaling
Since Egr2-AS-RNA has a direct effect on the expression of Egr2 transcript and protein levels (Figures 1, 2, 3, 4, and S1), we hypothesized that neuregulin-mediated ERK1/2 signaling could affect the expression of Egr2-AS-RNA in SCs, since it has also been shown to affect the expression levels of Egr2 (Newbern et al., 2011). Inhibition of neuregulin-induced ERK1/2 activation using UO126 in SCs results in significant inhibition of ERK1/2 phosphorylation, significant reduction of EGR2 protein levels (Figures 6A and 6B), significant upregulation of Egr2-AS-RNA expression, and inhibition of Egr2 transcript expression.

In Vivo Inhibition of Egr2-AS-RNA Expression Rescues the EGR2-Mediated Gene Expression Profile following Peripheral Nerve Injury
As shown above (Figure 1C), peripheral nerve injury results in a significant increase in Egr2-AS-RNA expression up to 12 hr post-injury. To determine how Egr2-AS-RNA affects the expression of EGR2-regulated transcripts following sciatic nerve injury, we treated sciatic nerves with hydrogel plus GapMer to inhibit the expression of Egr2-AS-RNA at the time of sciatic nerve transection and up to 5 days post-injury. This showed that inhibition of Egr2-AS-RNA expression in fully transected nerves rescues the inhibition of Egr2 transcript expression (Figure 4A) and either rescues or delays the inhibition of EGR2-regulated genes that encode structural myelin proteins or transcription factors (Figure 4A). To determine whether the delayed inhibition of gene expression results in delayed downregulation of PMP22 and MPZ myelin protein expression, we analyzed sciatic nerve lysates treated with hydrogel plus GapMer or hydrogel plus scrambled GapMer and untreated injured nerves 2, 5, and 7 days post-injury. We show that inhibition of Egr2-AS-RNA expression results in significant preservation of PMP22 and MPZ protein expression 7 days following complete sciatic nerve transection (Figures 4B and 4C). The delayed inhibition of structural myelin proteins correlates with the delay in demyelination observed after inhibition of Egr2-AS-RNA expression (Figures 3C–3E).

Egr2-AS-RNA Inhibits Nascent Transcription of Egr2 and Mediates Gradual Recruitment of a Chromatin Remodeling Complex on the Egr2 Promoter
In order to distinguish whether the effect of Egr2-AS-RNA on Egr2 expression is due to a direct effect on transcription, we performed nuclear run-on assays in SCs infected with Lenti-GAPMERS or Lenti-control. We show that expression of Egr2-AS-RNA results in statistically significant inhibition of nascent transcription of Egr2 (Figure 5A).

To determine whether Egr2-AS-RNA participates in the formation of a chromatin remodeling complex on the Egr2 promoter, we performed chromatin immunoprecipitation (ChiP) using sciatic nerve chromatin with antibodies against EZH2, AGO1, AGO2, or tri-methylated histone 3 (H3K27Me3) followed by qPCR to detect the presence of the Egr2 promoter and Egr2-AS-RNA in the complex. This revealed that 48 hr after sciatic nerve injury, EZH2, AGO1, AGO2, and H3K27me3 are localized on the Egr2 promoter, and this interaction is inhibited after treatment with RNase H, which means that the interaction requires the presence of an RNA-DNA hybrid (Figure 5B). Next, we asked whether Egr2-AS-RNA is the RNA species responsible for the recruitment of the remodeling complex on the Egr2 promoter following sciatic nerve injury. We performed ChIP using chromatin from injured sciatic nerves 6, 24, and 48 hr after injury. To identify the effect of the Egr2-AS-RNA on the recruitment of EZH2, AGO1, AGO2, and H3K27me3 on the Egr2 promoter, we inhibited the expression of Egr2-AS-RNA with the addition of hydrogel plus GapMer, while control nerves were treated with hydrogel plus scrambled GapMer.

We show that 6 hr post-injury, only AGO1 and AGO2 are present on the Egr2 promoter, and inhibition of Egr2-AS-RNA expression inhibits their recruitment on the promoter (Figure 5D). At 48 hr post-injury EZH2, AGO1, AGO2, and H3K27me3 are present, and inhibition of Egr2-AS-RNA expression abolishes their recruitment on the Egr2 promoter (Figure 5E). Since Egr2-AS-RNA has a direct effect on the expression of Egr2 promoter, we performed ChIP using chromatin from injured sciatic nerves 6, 24, and 48 hr after injury. To identify the effect of the Egr2-AS-RNA on the recruitment of EZH2, AGO1, AGO2, and H3K27me3 on the Egr2 promoter, we inhibited the expression of Egr2-AS-RNA with the addition of hydrogel plus GapMer, while control nerves were treated with hydrogel plus scrambled GapMer.

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(E) Quantification of the total number of myelinated axons in 15 random semithin sections from an area extending 0.5 mm to 5 mm distal to the sciatic nerve transection. Animals treated with hydrogel plus AS-RNA GapMers have a significantly higher number of myelinated fibers 2 and 5 days following complete sciatic nerve transection than untreated animals or animals treated with hydrogel plus scrambled GapMers (*p < 0.05, two-tailed Student’s t test of unpaired samples).
Figure 4. The Egr2-AS-RNA Regulates Egr2 Gene and Egr2 Target Gene Expression during the Nerve Injury Response

(A) Volcano plots of log2 fold change (FC) for EGR2-regulated genes in non-injured versus injured sciatic nerves and injured nerves versus injured nerves treated with hydrogel plus GapMers. The x axis shows the log2 of the FC between the conditions. The vertical central line represents no difference in expression, and the area between the two equidistant lines on both sides of the central line includes genes showing a non-significant change of expression (black dots). On the left side of the lines, the genes with a negative FC are depicted (decreased expression, blue dots), while on the right side of the lines, the genes with a positive FC (increased expression) are shown (yellow dots). The y axis shows the −log of the p value, which means that genes with low p value (more significant) appear toward the top of the plot. The horizontal line divides the significant results (p < 0.005, above) from the non-significant (below).

(B) Representative results from three independent experiments using two sciatic nerve isolates 2, 5, and 7 days post-injury showing expression of PMP22 and MPZ. Actin was used as a loading control.

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(Figure 6C). Our data suggest that neuregulin-mediated ERK1/2 signaling is a negative regulator of Egr2-AS-RNA expression in SCs. Recently, it was shown that loss of axonal contact causes SCs to induce NRG1 type I expression through a mitogen-activated protein kinase (MAPK)-dependent pathway (Stassart et al., 2013). We sought to determine whether NRG1 type III or type I has a distinct role in the expression of Egr2-AS-RNA. We found that stimulation of SCs with NRG1 type III is the main signal that exerts an acute negative regulation of the expression of Egr2-AS-RNA (Figure S4). NRG1 type I did not have any effect after 3 hr but caused a gradual inhibition of Egr2-AS-RNA expression at 6 hr and 24 hr. Our data could explain the acute increase in the expression of Egr2-AS-RNA immediately after nerve injury (Figure 1C), while the gradual inhibition of Egr2-AS-RNA expression 24 hr post-injury (Figure 1C) may reflect gradual inhibition by NRG1 type I as SCs induce its expression.

**NRG1-Induced ERK1/2 Signaling Leads to YY1-Mediated Regulation of Egr2-AS-RNA**

Recently, it was shown that the transcription factor YY1 is part of the ERK1/2 signaling pathway responsible for the upregulation of Egr2 in response to NRG1 in SCs (He et al., 2010). We examined whether the increase in Egr2-AS-RNA expression after inhibition of ERK1/2 signaling (Figure 6C) depends on YY1 and found that stimulation of SCs with NRG1 type III is the main signal that exerts an acute negative regulation of the expression of Egr2-AS-RNA (Figure S4). NRG1 type I did not have any effect after 3 hr but caused a gradual inhibition of Egr2-AS-RNA expression at 6 hr and 24 hr. Our data could explain the acute increase in the expression of Egr2-AS-RNA immediately after nerve injury (Figure 1C), while the gradual inhibition of Egr2-AS-RNA expression 24 hr post-injury (Figure 1C) may reflect gradual inhibition by NRG1 type I as SCs induce its expression.

YY1 Regulates Binding of Egr2-AS-RNA to EZH2

Since inhibition of ERK1/2 signaling in SCs increases binding of YY1 to Egr2-AS-RNA (Figure 6F), we tested whether YY1 affects the functional interactions of Egr2-AS-RNA with chromatin remodeling complexes. It was previously determined that EED and EZH2 are core components of a multi-subunit histone methyltransferase complex, PRC2, with specificity for lysine 27 (H3K27) of histone H3 (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002). We hypothesized that Egr2-AS-RNA interacts physically with protein components of the PRC2 and that YY1 mediates this interaction. We silenced total YY1 expression in SCs using small interfering RNAs (siRNAs) targeting four separate areas of the YY1 sequence (Figure 6G). Then, we performed RIP with ChIP-validated antibodies against EZH2, AGO1, AGO2, and H3K27me3 using RNA from YY1 siRNA or non-targeting siRNA-transfected SCs. In control SCs (non-targeting siRNA transfected), the Egr2-AS-RNA binds and precipitates exclusively with EZH2 (Figure 6H). Following YY1 knockdown in YY1-siRNA-transfected SCs, we detected complete loss of binding of Egr2-AS-RNA to EZH2 and increased binding of Egr2-AS-RNA to AGO1 (Figure 6H). This may affect turnover of Egr2-AS-RNA, since AGO proteins have been previously implicated in ncRNA turnover (Yoon et al., 2015), or it may indicate a “switch” in Egr2-AS-RNA’s function from RPC2-mediated chromatin remodeling to AGO-mediated transcriptional silencing (Janowski et al., 2009). Finally, we show that inhibition of YY1 inhibits Egr2 mRNA expression and induces Egr2-AS-RNA expression (Figure 6B).

The Phosphorylation State of YY1 Regulates Binding to Egr2-AS-RNA

Recently, it was shown that NRG1-mediated MEK-ERK1/2 signaling induces phosphorylation of YY1 at serine 118 (Ser118), Ser184, and Ser247, and this phosphorylation has a key role in regulating Egr2 transcription (He et al., 2010). We hypothesized that the binding of YY1 to Egr2-AS-RNA may be regulated by the state of phosphorylation of YY1 serine residues. We generated several Ser-Ala mutations at positions 118, 184, and 247 and a double mutation at positions 118 and 184. To determine the effect of the loss of each serine on the binding of YY1 to Egr2-AS-RNA, we performed RIP followed by qPCR detection of Egr2-AS-RNA. We show that loss of Ser118 results in significant inhibition of the binding of YY1 to Egr2-AS-RNA compared to non-mutated YY1. However, loss of Ser184 induces a significant increase in binding of YY1 to Egr2-AS-RNA, while Ser247 has no effect compared to the non-mutated construct (Figure 6I). Finally, the double mutation of Ser118 and Ser184 results in increased binding of YY1 to Egr2-AS-RNA compared to the non-mutated protein.
suggesting that Ser184 is the dominant regulatory site (Figure 6I).

**DISCUSSION**

Studies on genetically modified mice (Decker et al., 2006; Le et al., 2005; Topilko et al., 1994) and identification of the mutations associated with peripheral neuropathies (Bellone et al., 1999; Timmerman et al., 1999; Warner et al., 1998) have implicated EGR2 as a central regulator of peripheral myelination (Svaren and Meijer, 2008). During myelination, various SC genes are dynamically regulated, and the majority of these genes are targets of EGR2 transcriptional control (D’Antonio et al., 2006; Jang et al., 2010; Nagarajan et al., 2001; Verheijen et al., 2003). Recently, the transcription factor YY1 has been implicated as a molecular link between extracellular signals and the regulation of EGR2 expression (He et al., 2010). Although the importance of trans-acting proteins (e.g., transcription factors) has been...
Figure 6. Role of NRG1-ERK1/2 Signaling and YY1 in the Regulation of Egr2-AS-RNA Expression

(A) Inhibition of NRG1-induced Erk1/2 phosphorylation in SCs using UO126 results in inhibition of phopsho-ERK1/2 expression and loss of EGR2 expression. Total ERK1/2 and actin were used as loading controls.

(B) Densitometric quantification of inhibition of ERK1/2 phosphorylation and EGR2 expression following incubation of SCs with UO126. Results are presented as mean ± SD from three independent experiments using two separate protein isolations per experiment per condition (p < 0.0001, t = 8.99, df = 14 for p-ERK1/2 and p = 0.0054, t = 4.24, df = 6 for EGR2).

(C) qPCR for Egr2-AS-RNA and Egr2 mRNA following inhibition of ERK1/2 signaling with UO126 in SCs shows significant increase of Egr2-AS-RNA levels and inhibition of Egr2 mRNA expression (n = 3 independent experiments; unpaired two-tailed Student’s t test; p = 0.044, t = 2.44, df = 7 for Egr2-AS-RNA and p = 0.017, t = 4.32, df = 5 for Egr2).

(D) Schematic showing the position of the S1 site upstream of the Egr2 transcription start site (TSS) and flanking the 5' end of AS-RNA. This site (~100 nt) contains a YY1-binding motif and a TATA box (red sequence) in both sense and antisense orientations. MSE stands for myelin-specific element, which is located at the 3' UTR of the Egr2 gene.

(legend continued on next page)
established, the existence of an epigenetic circuit that allows SCs to regulate the expression of EGR2 in response to injury and during myelination has not been previously described. The discovery that the majority of eukaryotic genomes are transcribed (ENCODE Project Consortium et al., 2007) and that many of the resulting transcripts are developmentally regulated (Mercer et al., 2008) but do not encode proteins (Simon et al., 2011) has steered our attention toward the role of IncRNA in the regulation of Egr2 transcription.

We discovered a cis-acting IncRNA antisense to the promoter of Egr2. Since ectopic expression of Egr2-AS-RNA inhibits the expression of Egr2 mRNA, we asked whether Egr2-AS-RNA exerts reversible regulation of Egr2 expression during peripheral nerve injury. During the acute phase of the nerve injury response, the expression of EGR2 is inhibited, and demyelination ensues (Guertin et al., 2005; Parkinson et al., 2008). We determined that Egr2-AS-RNA mediates the inhibition of Egr2 mRNA expression, while inhibition of Egr2-AS-RNA expression results in delayed demyelination, even after complete nerve transection. In addition, inhibition of Egr2-AS-RNA expression restores Egr2 transcript expression levels and rescues the EGR2-regulated gene expression profile in injured nerves. These data raised a series of questions regarding how the expression of Egr2-AS-RNA is regulated and how Egr2-AS-RNA is integrated within the pathways that control the nerve injury response. It has been shown that c-Jun is an essential transcription factor for the reprogramming of mature myelinating SCs to de-differentiated SCs after nerve injury (Arthur-Farraj et al., 2012), c-Jun inhibits Egr2-mediated myelin gene expression (Parkinson et al., 2008) and is a negative regulator of myelination, which suggests a possible interplay between Egr2-AS-RNA expression and c-Jun during the acute nerve injury response.

The expression of EGR2 depends on NRG1-mediated ERK1/2 signaling to YY1 during peripheral myelination (He et al., 2010; Newbern et al., 2011). We identified a portion of the Egr2 promoter adjacent to the TSS that fulfills the criteria for a bidirectional promoter containing a TATA box and a YY1-binding motif (Smale and Kadonaga, 2003). Although this bidirectional promoter affects expression of Egr2-AS-RNA, we cannot target it to generate mice lacking expression of Egr2-AS-RNA, since this approach will also affect expression of Egr2 mRNA through elimination of the TATA box and several transcription factor (TF)-binding sites (Rangnekar et al., 1990). YY1 has been previously implicated in the transcriptional activation of Xist during the initiation and maintenance of X inactivation through direct activation of the Xist promoter (Makhlouf et al., 2014). It has also been shown that YY1 is an RNA-binding protein that binds Xist as an adaptor protein between the IncRNA and chromatin targets (Jeon and Lee, 2011). Here, we discovered that YY1 mediates the binding of Egr2-AS-RNA to EZH2, which is the core component of the PRC2 chromatin remodeling complex. How this function of YY1 is regulated and the biological significance of this function during the nerve injury response is unknown. It is possible that YY1 functions as a molecular scaffold that coordinates targeting of Egr2-AS-RNA to PRC2 and chromatin, thereby coupling expression of Egr2-AS-RNA with transcriptional repression.

The various functions of YY1 can be modulated by post-translational modifications, including phosphorylation (Rizkallah and Hurt, 2009). We describe here a YY1 phospho-switch mechanism (Figure 6J) that regulates binding of YY1 to Egr2 mRNA or Erg2-AS-RNA. We identified Ser184 as the regulatory site that induces binding of YY1 to Egr2-AS-RNA. It has been shown that Aurora B kinase phosphorylates Ser184 of YY1 during G2/M transition of the cell cycle and that protein phosphatase 1 (PP1) rapidly dephosphorylates YY1 at Ser184 (Kassardjian et al., 2012). It is possible that dephosphorylation of YY1 following peripheral nerve injury is cell-cycle dependent as SCs dedifferentiate and that PP1 plays a role in this process. The speed at which a cell responds to an extracellular cue by activating a set of genes and repressing another is of pivotal importance to the fate of that cell. However, this aspect of gene regulation is often not appreciated. Instead, the absolute levels of expression are generally seen as the hallmarks of a
regulated gene (Uhler et al., 2007). Here, we have shown that an antisense RNA transcript that associates with Egr2 promoter in cis regulates the levels of Egr2 transcription in response to extracellular signals. We propose that Egr2-AS-RNA confers transcriptional buffering to maintain the proper levels of Egr2 transcription. Given that non-coding AS-RNAs are often expressed in a tissue- or time-dependent manner, the mechanism of Egr2-AS-RNA regulation of Egr2 transcription involves chromatin remodeling and affects the rate of Egr2 induction rather than the steady-state levels of gene expression. In fact, we have shown that Egr2-AS-RNA gradually recruits a chromatin remodeling complex on the Egr2 promoter, and its role in chromatin plasticity and transcriptional silencing of Egr2 is instructive, since inhibition of Egr2-AS-RNA results in the dissociation of the remodeling complex from the Egr2 promoter.

We have identified an antisense RNA that is induced after nerve injury and regulates the transcription of Egr2 as part of an NRG1-ERK1/2-YY1 signaling axis. This functional evaluation of an antisense IncRNA in SC biology will likely have a major impact on our understanding of the transcriptional regulation of peripheral myelination.

EXPERIMENTAL PROCEDURES

5′-RACE

For 5′-RACE, we used the RLM RACE kit from Ambion, with certain modifications. Total RNA was treated with calf intestine alkaline phosphatase (CIP) to remove free 5′ phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA, and contaminating genomic DNA. The cap structure found on intact 5′ ends of mRNA is not affected by CIP. The RNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5′ monophosphate. A 45-base RNA adaptor oligonucleotide provided by Ambion was ligated to the RNA population using T4 RNA ligase. The adaptor cannot ligate to dephosphorylated RNA, because these molecules lack the 5′ phosphate necessary for ligation. During the ligation reaction, the majority of the full-length, decapped mRNA acquires the adaptor sequence as its 5′ end. We then used random sense decamers that bind to the antisense strand and a primer antisense to the 5′ adaptor in order to amplify the AS-RNA.

Computational Verification of the Egr2-AS-RNA Expression

RNA-seq raw reads from mouse sciatic nerves in samples SRR3222412, SRR3222413, and SRR3222414 were downloaded from the GEO databases (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM2086001). Reads were aligned to the mm10 assembly of the mouse genome with gsnap (Wu and Nacu, 2010). Read mapping to genes was done based on the RefSeq exons, as defined in file http://hgdownload.cse.ucsc.edu/goldenPath/mm10/database/refGene.txt.gz. The coordinates of the AS-RNA on chr10 were added to the exon file. Read summarization was done with featureCounts (Liao et al., 2014), and raw read counts were standardized to the RPKM measure (Mortazavi et al., 2008) using reads with a mapping quality of 20 or better. To estimate the coordinates of the mature RNA, we first looked at the sequenced reads around the appropriate genomic location using the:mieuplex routine of SAMtools (Li et al., 2009), and then we visualized the reads using the Integrative Genomics Viewer (Robinson et al., 2011) as confirmation.

Chromatin Isolation from Sciatic Nerves

2-month-old mice were subjected to sciatic nerve transection. 48 hr after injury, the animals were euthanized, and both nerves (distal part of the lesioned nerve and a 0.5 mm fraction of the contralateral healthy nerve) were isolated. Nerves (5 per condition) were then crosslinked with 1% paraformaldehyde (PFA) in PBS and neutralized with glycine (0.3 M final concentration). Nerves were centrifuged at 2,000 relative centrifugal force (rcf) for 5 min and washed twice with cold PBS plus protease and nuclease inhibitors. Immunoprecipitation lysis buffer was added to the nerves (EpiTecht ChIP OneDay Kit; QIAGEN, Venlo, Netherlands), and the nerves were homogenized mechanically for 15 s with a Pro 200 homogenizer (Proscientific Inc.). Next, the samples were sonicated on ice using the Misonix Sonicator 3000 (Fisher Scientific) for 9 cycles of 30 s on and 90 s off at 80% power to shear the chromatin between 100 and 1,000 bp. For ChIPs using SCs, we used components of the EpiTecht ChIP OneDay Kit. Chromatin was sonicated to an average length of 1–2 kb on ice and centrifuged. The supernatant was used for RNA or DNA ChIP.

DNA ChIP

Lysates were incubated overnight at 4 °C on rotation with the ChIP-verified antibodies EzH2, Ago1, and Ago2 (Cell Signaling, Danvers, MA, USA) at a 1:50 dilution or H3K27 (Millipore, Billerica, MA, USA) at a dilution of 1:25 or without antibody as a control. Chromatin was then precipitated, and DNA was extracted (EpiTecht ChIP OneDay Kit). Recovered material from the input sample and all the ChIP samples per condition were used to perform qPCR of the Egr2 AS-RNA (for primer sequences, see Supplemental Experimental Procedures). Relative enrichment for each experimental sample was calculated as a percentage of the input. For negative control ChIP, we used a non-targeting isotype-matched immunoglobulin G (IgG), and the values in all experiments ranged between 0% and 0.002% of the input sample. These values were used to normalize the data obtained with the target-specific antibodies. For all qPCRs reported in the paper, we performed a no-reverse transcription (RT) control amplification to verify the absence of genomic DNA contamination.

RIP

To perform RIP, we used the magnetic ChIP kit (RNA ChIP-IT; Active Motif, Carlsbad, CA, USA). The antibodies used, analysis, and plotting were the same as those described for DNA ChIP.

Statistical Analysis

To determine statistical significance among the means of three or more independent groups, we used one-way ANOVA. The homogeneity of variances was confirmed with Brown and Forsythe test, and the significance between specific groups was calculated with a post hoc Dunnett test. This analysis was performed for the data in figures 1C, 1D, 3A, 3B, 4C, and 6I. For the rest of the data, we used an unpaired two-tailed t test. To verify Gaussian distribution of the data before applying the t test, we performed the D’Agostino and Pearson and Shapiro-Wilk normality tests. Statistical analysis was performed using GraphPad Prism.

Animal Use and Care

8-week-old male and female C57/B6 WT mice (gender does not affect peripheral nerve injury response) were obtained from The Jackson Laboratory and maintained according to the NIH Guide for the Care and Use of Laboratory Animals. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the Weis Center for Research, Geisinger Clinic.

Western Blots

The full scans of all western blots presented in the paper are included in Figure S6.

Methods for sciatic nerve injury, lentivirus production, mouse DRG explant and use, and RNA purification for miRNA arrays, immunocytochemistry protocols, RT-PCR and qPCR, western blotting, preparation of the hydrogel, in situ hybridization (ISH), nuclear run-on assay, PCR array, electron microscopy, siRNA transfections, and mutagenesis are included in detail in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.07.068.
AUTHOR CONTRIBUTIONS


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