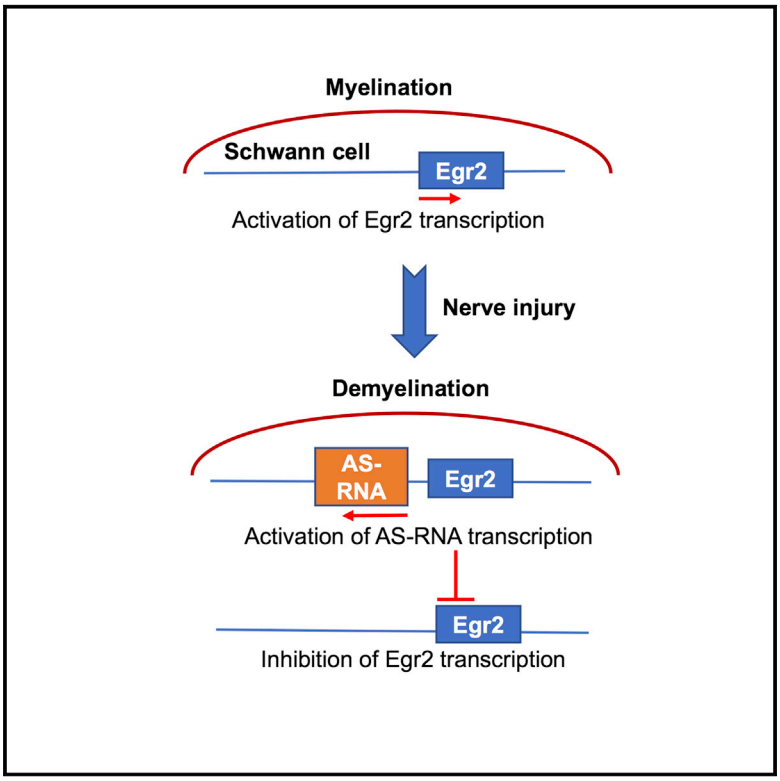
Article

Cell Reports

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

Authors

Margot Martinez-Moreno, Timothy Mark O'Shea, John P. Zepecki, Alexander Olaru, Jennifer K. Ness, Robert Langer, Nikos Tapinos

Correspondence

nikos_tapinos@brown.edu

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.

Martinez-Moreno et al., 2017, Cell Reports *20*, 1950–1963
August 22, 2017
http://dx.doi.org/10.1016/j.celrep.2017.07.068

OPEN ACCESS
CellPress

Cell Reports
Article

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

Margot Martinez-Moreno,[1,4] Timothy Mark O'Shea,[2,3] John P. Zepecki,[1,4] Alexander Olaru,[1] Jennifer K. Ness,[1] Robert Langer,[2,3] and Nikos Tapinos[1,4,5,*]

[1]Molecular Neuroscience and Neuro-Oncology Laboratory, Geisinger Clinic, Danville, PA 17822, USA
[2]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[3]Harvard–Massachusetts Institute of Technology Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[4]Present address: Department of Neurosurgery, Brown University, Providence, RI 02903, USA
[5]Lead Contact
*Correspondence: nikos_tapinos@brown.edu
http://dx.doi.org/10.1016/j.celrep.2017.07.068

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 reverts 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; Taveggia 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 *Erk1/2*$^{CKO(Dhh)}$ 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 (lncRNA) 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 lncRNA 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 lncRNA 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 lncRNAdb (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 median number of RPKM over the entire ~26,000 RefSeq transcripts (Table S1A) in sciatic nerves. In addition, the average relative abundance of Egr2-AS-RNA in the three sciatic nerves was 43 times lower than the expression levels of the *Egr2* transcript (Table S1B). Then, we estimated the coordinates of mature Egr2-AS-RNA and identified that the mature Egr2-AS-RNA sequence spans a 470-nt region (chromosome 10 [chr10], 67537250–67537720) with almost perfect sequence fidelity (Figures S2A and S2B). The discrepancy between the length of mature AS-RNA (470 nt) and the sequence detected by the RACE protocol (~1,000 nt) can be attributed to the random decamers used for the amplification of the RACE product and the possibility that part of Egr2-AS-RNA could be spliced after 5′ end capping.

Egr2-AS-RNA Is Expressed in Mouse Sciatic Nerves and Is Significantly Increased following Nerve Injury

To determine whether Egr2-AS-RNA is expressed during postnatal development of the mouse sciatic nerve, we performed strand-specific qPCR using RNA isolated from postnatal day 1 (P1), P5, P7, and 3-month-old mouse sciatic nerves. This showed that Egr2-AS-RNA is expressed throughout these time intervals, with the highest expression in P1 sciatic nerves (Figure 1A). To detect the expression and cell specificity of Egr2-AS-RNA in mouse sciatic nerves, we performed multiplex fluorescence in situ hybridization targeting Egr2-AS-RNA in combination with mouse S100b, which was used as an SC-specific marker. We detected a signal specific for Egr2-AS-RNA in the cytoplasm (Figure 1B, arrowheads) and the nucleus (Figure 1B, arrow) of S100b-positive SCs.

To examine whether Egr2-AS-RNA plays a role in the regulation of *Egr2* expression after peripheral nerve injury, we performed strand-specific qPCR to detect the expression of Egr2-AS-RNA and *Egr2* mRNA 6 hr, 12 hr, 24 hr, 2 days, 5 days, and 7 days following mouse sciatic nerve transection. We discovered that the expression of Egr2-AS-RNA exhibits a statistically significant increase 6 and 12 hr after sciatic nerve injury (Figure 1C). The expression of *Egr2* mRNA shows statistically significant downregulation at 12 and 24 hr (Figure 1D) and rebounds at days 2, 5, and 7, when expression of Egr2-AS-RNA is minimal. Finally, EGR2 protein expression in sciatic nerve lysates is significantly reduced by 48 hr after injury (Figures S3A and S3B).

Quantification of Absolute Levels of Egr2-AS-RNA in Mouse Sciatic Nerves

In order to determine the absolute abundance of Egr2-AS-RNA in mouse sciatic nerves, we prepared limiting dilutions (10 ng to 1 pg) of a known amount of Egr2-AS-RNA and generated a standard amplification curve of the dilutions using 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 lncRNAs in eukaryotic cells (Mortazavi et al., 2008; Palazzo and Lee, 2015; Ramsköld 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

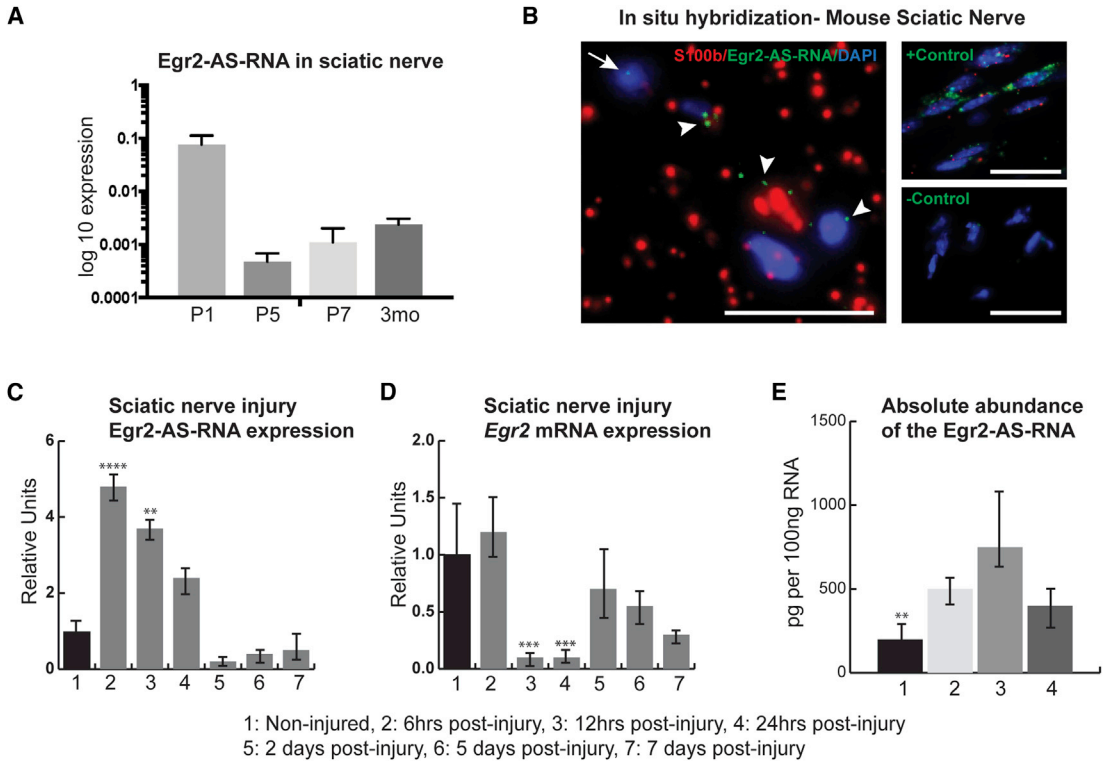


Figure 1. An *Egr2* Antisense RNA Transcript Is Expressed during Sciatic Nerve Development and Is Upregulated after Peripheral Nerve Injury

(A) Expression of Egr2-AS-RNA was detected by strand-specific qPCR in P1, P5, P7, and 3-month-old mouse sciatic nerves.

(B) In situ hybridization in mouse sciatic nerves shows the expression of Egr2-AS-RNA (green signal) in the cytoplasm (arrowheads) and nucleus (arrow) of S100b-positive (red signal) SCs. Positive control shows expression of cyclophilin B (PPIB) (green) and POLR2A (RNA polymerase II polypeptide) (red). Negative control shows no signal using a bacterial dapB probe. Scale bar, 100 μm.

(C) Expression of Egr2-AS-RNA in non-injured sciatic nerves and in sciatic nerves 6 hr, 12 hr, 24 hr, 2 days, 5 days, and 7 days following sciatic nerve injury. The experiments were repeated three times, and significance was calculated with a one-way ANOVA ($F(6,22) = 22.19$, $p < 0.0001$) followed by a post hoc Dunnett's test (**$p < 0.005$, ****$p < 0.00005$).

(D) Expression of *Egr2* mRNA in non-injured sciatic nerves and in sciatic nerves 6 hr, 12 hr, 24 hr, 2 days, 5 days, and 7 days following sciatic nerve injury. The experiments were repeated three times, and the results are presented as mean ± SD. Significance was calculated with a one-way ANOVA ($F(6,15) = 9.458$, $p < 0.0001$) followed by a post hoc Dunnett's test (***$p < 0.0005$).

(E) Absolute abundance of Egr2-AS-RNA is significantly lower in control sciatic nerves than in injured nerves as determined by a one-way ANOVA ($F = 6.935$, $p = 0.0161$) using sciatic nerves from 3 mice per condition.

Egr2 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

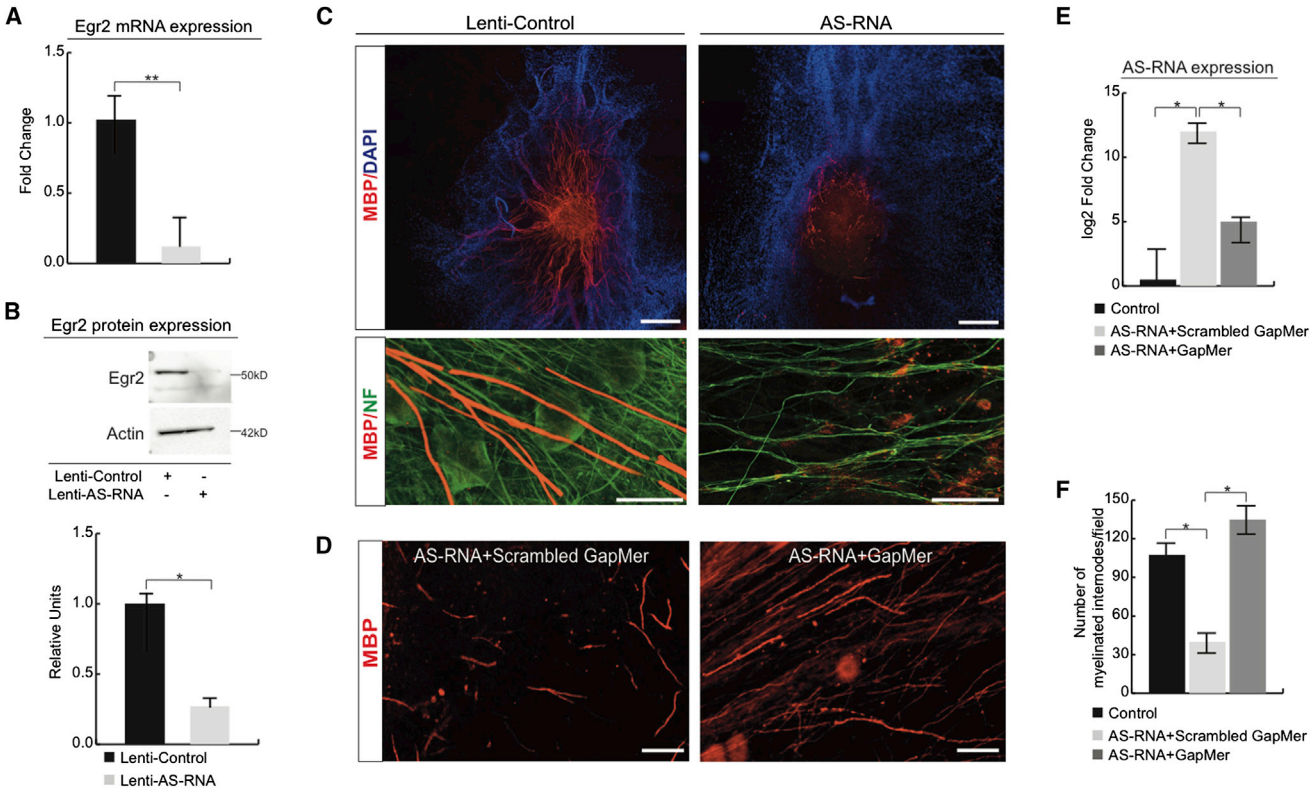


Figure 2. Ectopic Expression of Egr2-AS-RNA Inhibits the Expression of *Egr2* mRNA and Induces Demyelination

(A) Lenti-AS-RNA-infected cultures exhibit a significant decrease in the expression of the *Egr2* mRNA as compared to Lenti-control infected cultures. The experiment was repeated three times, and significance was calculated with a two-tailed unpaired Student's t test (**p = 0.0005, t = 5.571, df = 8). Results are presented as mean ± SD.

(B) Representative western blot showing expression of EGR2 after infection of DRG cultures with Lenti-control or Lenti-AS-RNA. Actin was used as loading control. Densitometric analysis shows that ectopic expression of Egr2-AS-RNA results in a statistically significant decrease in EGR2. The results are presented as mean ± SD from n = 5 per condition. Significance was calculated with a two-tailed unpaired Student's t test (t = 4.722, df = 8, *p = 0.015).

(C) Representative images of Lenti-control- and Lenti-AS-RNA-infected DRG cultures showing extensive demyelination in the cultures infected with Lenti-AS-RNA. Myelinated internodes were stained with MBP (red), and nuclei were stained with DAPI (blue) (top). The lower left panel shows a higher magnification of the myelin internodes (stained red with MBP) and axons (stained green with neurofilament) in Lenti-control-infected cultures. In Lenti-AS-RNA-infected cultures, we detected myelin debris (stained red with MBP), while the axons retain their integrity expressing NF (green). Scale bars, 100 μm.

(D) Rescue of AS-RNA induced demyelination with oligonucleotide inhibitors of Egr2-AS-RNA (GapMers).

(E) Lenti-AS-RNA-infected cultures exhibit significant increase in the expression of AS-RNA as compared to Lenti-control infected cultures, and the expression of AS-RNA is not affected by the addition of scrambled GapMers (two-tailed, unpaired Student's t test, p = 0.0135, t = 3.458, df = 6). Treatment of Lenti-AS-RNA-infected DRG cultures with Egr2-AS-RNA-specific GapMers results in significant inhibition of Egr2-AS-RNA expression as compared to Lenti-AS-RNA-infected cultures treated with scrambled GapMers (two-tailed, unpaired Student's t test, p = 0.0001, t = 14.04, df = 6).

(F) Myelin internodes from ten 10× fields per culture from 4 cultures per condition were measured, and the results are presented as mean ± SD. Significance was calculated with a two-tailed unpaired Student's t test. Lenti-AS-RNA-infected cultures treated with a control (scrambled) GapMer have significantly fewer myelinated internodes than Lenti-control-infected cultures (p = 0.0007, t = 5.352, df = 8). An Egr2-AS-RNA-specific GapMer rescues the Lenti-AS-RNA-infected cultures from AS-RNA-induced demyelination (p < 0.0001, t = 7.949, df = 70).

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 results 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

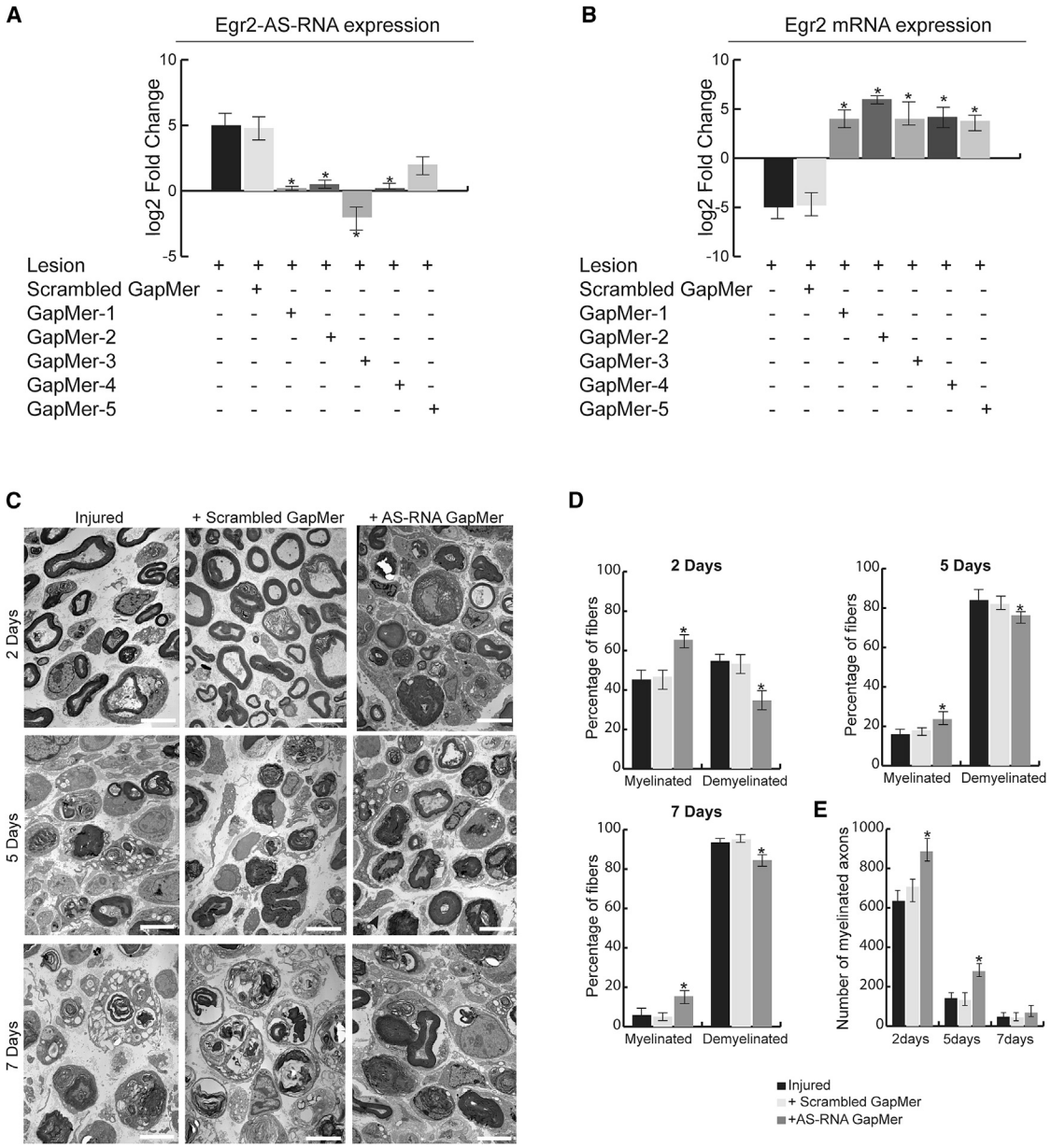


Figure 3. In Vivo Inhibition of Egr2-AS-RNA Expression Results in Delayed Demyelination after Sciatic Nerve Injury

(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, two-tailed Student's t test of unpaired samples).

(legend continued on next page)

(Figure 3D). A statistically significant increase in the total number of myelinated fibers 5 and 7 days following complete sciatic nerve transection was also observed (Figure 3E).

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 an acute and 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-AS-RNA 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 5C). At 24 hr post-injury, AGO2 and H3K27me3 are present, and inhibition of Egr2-AS-RNA expression abolishes their recruitment on the *Egr2* 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

(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).

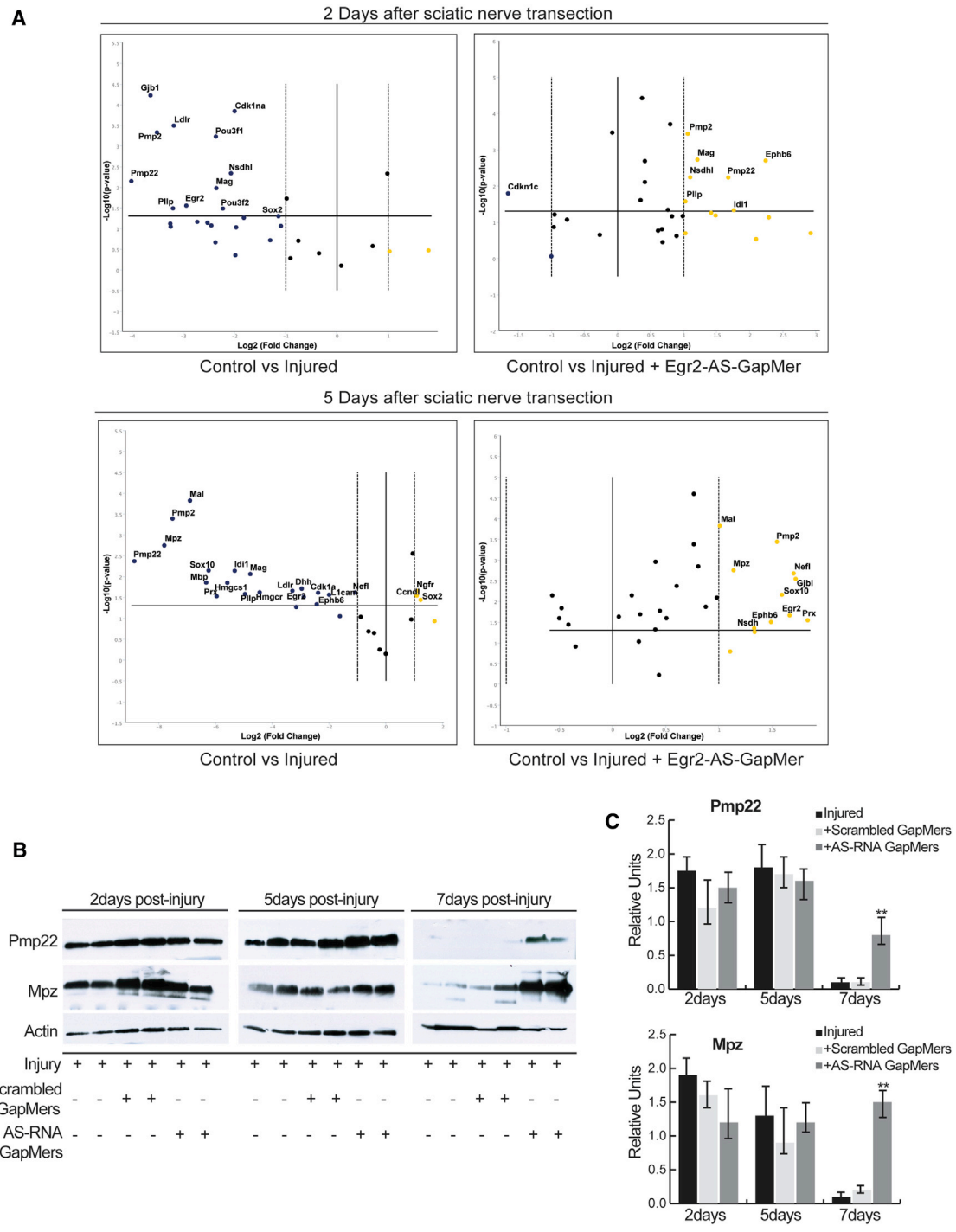


Figure 4. The Egr2-AS-RNA Regulates *Egr2* Gene and *Egr2* Target Gene Expression during the Nerve Injury Response

(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.

(legend continued on next page)

(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 modulation of Egr2-AS-RNA. We identified a 100-nt-long region of the *Egr2* promoter upstream of the transcription start site (TSS) flanking the 5′ end of Egr2-AS-RNA (Figure 6D) that contains a previously described YY1-binding motif on the antisense strand (He et al., 2010) and could possibly regulate the expression of Egr2-AS-RNA (S1 region; Figure S5A). To determine whether YY1 directly associates with this region in living cells, we performed ChIP. We then tested the recruitment of YY1 to the S1 region of the *Egr2* promoter and to a separate region (S2) located further upstream of the *Egr2* promoter (between nucleotides −723 and −647, related to the TSS of *Egr2*) that contains a conserved YY1-binding motif. YY1 was recruited to the S2 region of the *Egr2* promoter only in SCs incubated in the presence of NRG1, which agrees with a previous study showing that YY1 binds to the *Egr2* promoter (He et al., 2010). Inhibition of ERK1/2 signaling with UO126 results in partial but significant inhibition of YY1's interaction with the *Egr2* promoter (Figure 6E). Next, we examined whether YY1 associates with the S1 and S2 regions of single-stranded RNA using RNA immunoprecipitation (RIP). Inhibition of ERK1/2 signaling with UO126 results in a significantly increased association of YY1 with the S1 and S2 regions of Egr2-AS-RNA (Figure 6F). Our data indicate that NRG1-ERK1/2 signaling increases YY1 binding to the *Egr2* promoter and activates *Egr2* transcription while repressing the expression of Egr2-AS-RNA. Inhibition of ERK1/2 signaling results in derepression of Egr2-AS-RNA expression (Figure 6C) through increased binding of YY1 to the S1 and S2 regions of Egr2-AS-RNA (Figure 6F) and inhibition of *Egr2* expression (Figure 6C).

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., 2006). Finally, we show that inhibition of YY1 inhibits *Egr2* mRNA expression and induces Egr2-AS-RNA expression (Figure S5B).

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 as 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,

(C) Densitometric analysis of the western blot results showing that 7 days after sciatic nerve injury, PMP22 and MPZ exhibit significantly higher expression in animals treated with hydrogel plus AS-RNA GapMers than in injured animals or injured animals treated with hydrogel plus scrambled GapMers. The results are normalized to the expression of actin and presented as mean ± SD from three independent experiments. Significance was calculated with a one-way ANOVA ($p < 0.001$, $F(3, 4) = 25.87$ for PMP22, $p = 0.0019$, $F(2,6) = 21.3$ for MPZ) followed by a post hoc Dunnett test (**$p < 0.005$).

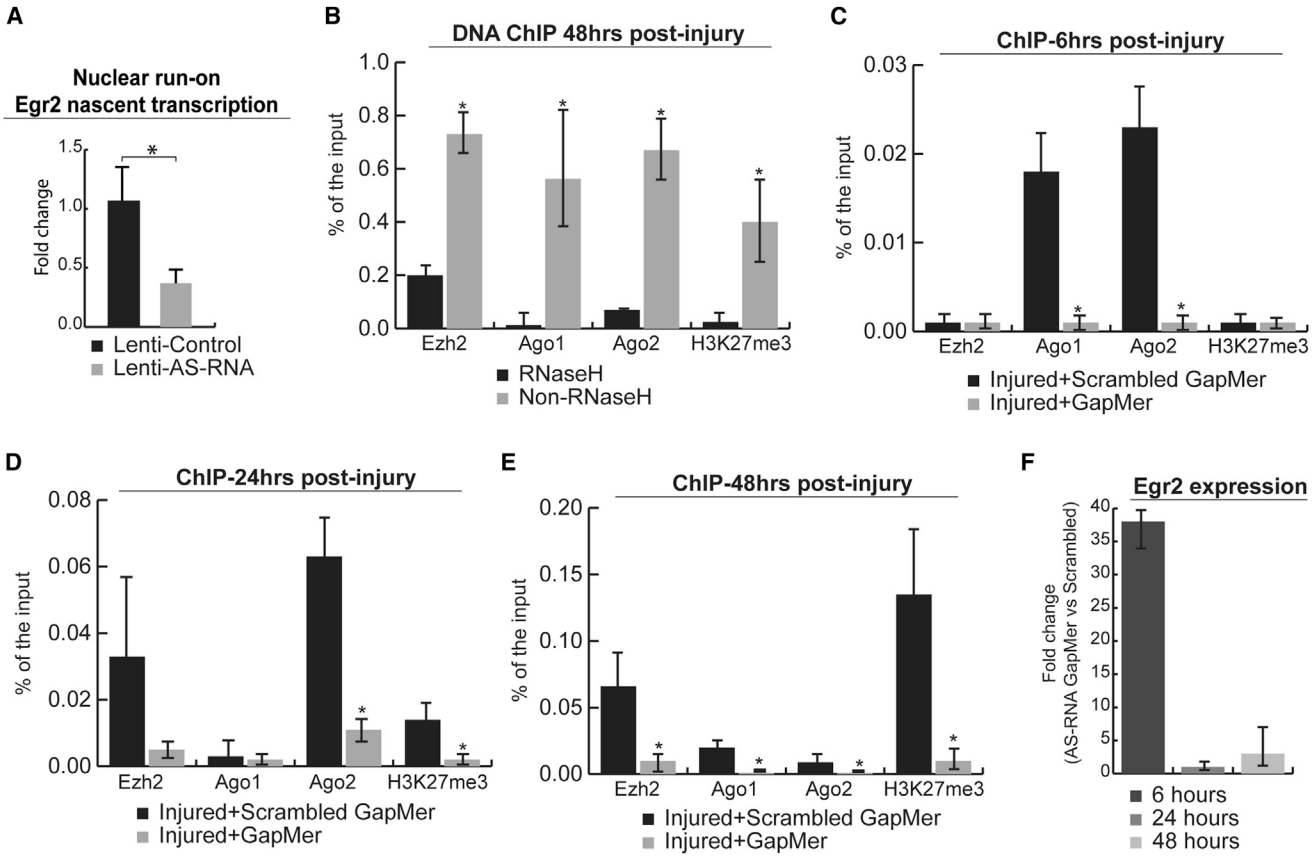


Figure 5. Egr2-AS-RNA Mediates Gradual Recruitment of a Remodeling Complex on the *Egr2* Promoter

(A) Expression of Egr2-AS-RNA induces a statistically significant decrease in nascent transcription of *Egr2* as quantified by qPCR (n = 8 in control cells, n = 9 in Egr2-AS-RNA cells, 3 independent experiments, p = 0.0012, t = 3.967, df = 15).

(B) In vivo ChIP using sciatic nerve chromatin with antibodies against EZH2, AGO1, AGO2, and H3K27Me3 with or without RNase H. Graph shows the mean ± SD from three independent experiments using sciatic nerves from three animals per experiment. Normalization was performed with values acquired from ChIP with isotype-matched IgG. Significance was calculated with an unpaired two-tailed Student's t test (p = 0.040, t = 4.836, df = 2 for EZH2; p = 0.044, t = 2.902, df = 4 for AGO1; p = 0.045, t = 4.514, df = 2 for AGO2; and p = 0.0012, t = 4.348, df = 4 for H3K27).

(C–E) In vivo DNA ChIP using chromatin from injured sciatic nerves 6, 24, and 48 hr after injury. At 6 hr post-injury, only AGO1 and AGO2 are present on Egr2 promoter, and inhibition of Egr2-AS-RNA expression inhibits their recruitment on the promoter (3 independent experiments; unpaired two-tailed Student's t test, p = 0.012, t = 5.710, df = 4 for AGO1; and p = 0.023, t = 2.105, df = 4 for AGO2) (C). At 24 hr post-injury, AGO2 and H3K27me3 are present, and inhibition of AS-RNA expression abolishes their recruitment on the *Egr2* promoter (3 independent experiments; unpaired two-tailed Student's t test, p = 0.029, t = 2.832, df = 6 for AGO2; and p = 0.049, t = 2.232, df = 10 for H3K27me3) (D). At 48 hr post-injury, EZH2, AGO1, AGO2, and H3K27me3 are all recruited on the *Egr2* promoter, and this depends on the presence of Egr2-AS-RNA, since inhibition of AS-RNA expression results in inhibition of EZH2, AGO1, AGO2, and H3K27me3 binding on the *Egr2* promoter (3 independent experiments; unpaired two-tailed Student's t test, p = 0.033, t = 3.188, df = 4 for EZH2; p = 0.017, t = 3.921, df = 4 for AGO1; p = 0.018, t = 3.826, df = 4 for AGO2; and p = 0.035, t = 2.714, df = 6 for H3K27me3) (E).

(F) Transcript expression of *Egr2* 6 hr, 24 hr, and 48 hr post-injury using RNA from injured sciatic nerves treated with AS-RNA GapMers or scrambled GapMers. The results are presented as fold change of *Egr2* transcript expression in AS-RNA-GapMer-treated nerves versus scrambled-GapMer-treated nerves. The experiment was repeated three times, and the data are presented as mean ± SD. Fold change above 2 was set arbitrarily and is used in conjunction with $p < 0.05$ to determine significant differences in gene expression.

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

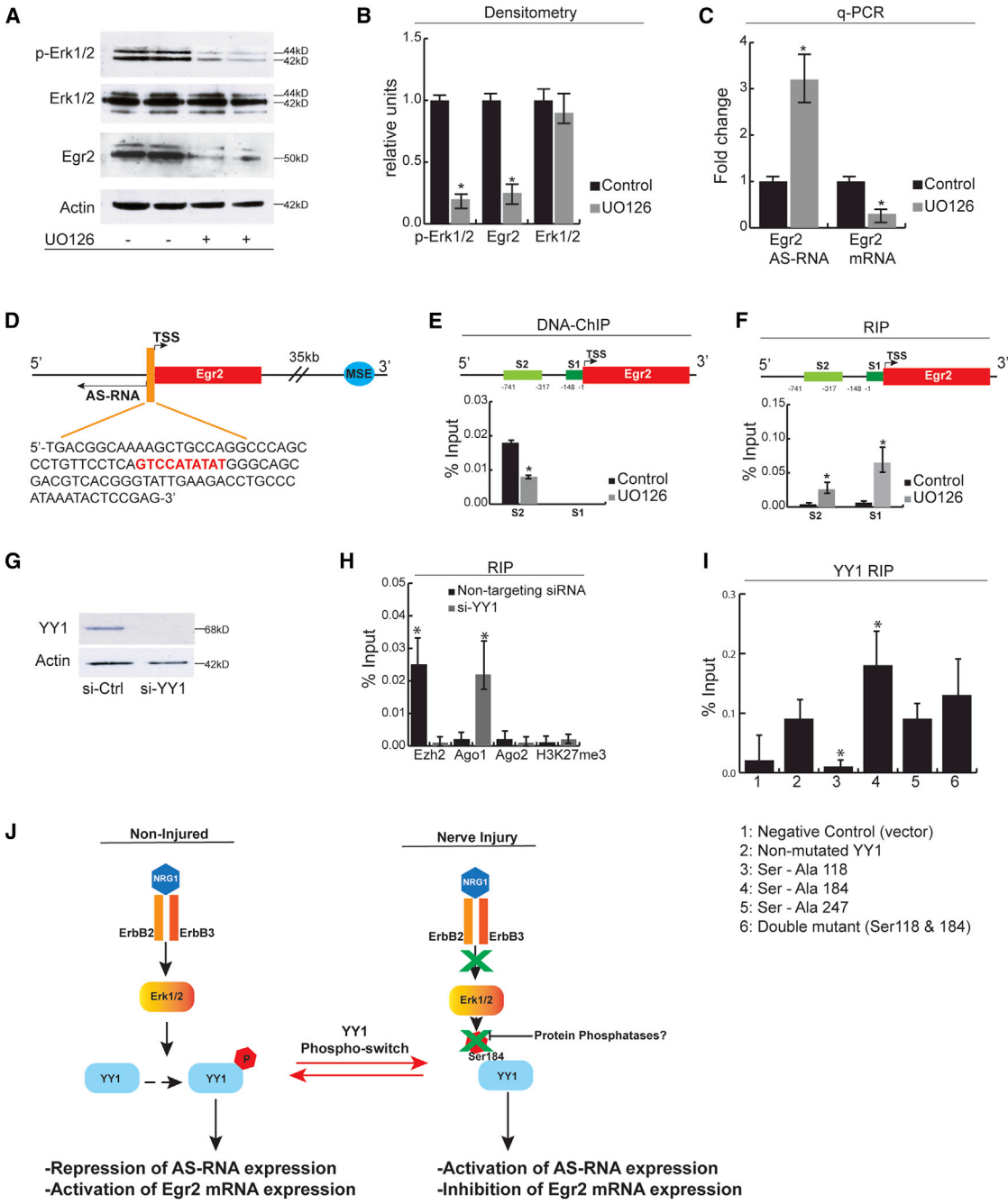


Figure 6. Role of NRG1-ERK1/2 Signaling and YY1 in the Regulation of Egr2-AS-RNA Expression

(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 lncRNA in the regulation of *Egr2* transcription.

We discovered a *cis*-acting lncRNA 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 lncRNA 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 biologic 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

(E) YY1 DNA-ChIP using chromatin isolated from SCs that were untreated or treated with UO126 in the presence of NRG1 overnight. The association of YY1 with the S1 and S2 regions of the *Egr2* promoter was examined with qPCR. YY1 associates only with the S2 region of the *Egr2* promoter, and the association is partially inhibited after treatment with UO126. The experiments were repeated three times, and results are presented as mean ± SD. Significance was calculated with a Student's t test ($p = 0.0012$, $t = 28.96$, $df = 12$).

(F) RNA immunoprecipitation (RIP) using RNA isolated from SCs that were untreated or treated with UO126 in the presence of NRG1 overnight. The association of YY1 with the S1 and S2 regions of AS-RNA was examined with qPCR. The experiments were repeated three times, and results are presented as mean ± SD. Significance was calculated with a Student's t test ($p = 0.0085$, $t = 3.842$, $df = 6$ for S2 and $p = 0.0362$, $t = 3.099$, $df = 4$ for S1).

(G) SCs transfected with YY1 siRNA for 48 hr show inhibition of YY1 protein expression as compared to SCs transfected with non-targeting siRNAs. Actin was used as a loading control.

(H) RIP with antibodies against EZH2, AGO1, AGO2, and H3K27me3 using RNA from YY1 siRNA or non-targeting siRNA transfected SCs. Results are presented as mean ± SD from three independent experiments (unpaired two-tailed Student's t test; $p = 0.04$, $t = 2.445$, $df = 8$ for EZH2 and $p = 0.014$, $t = 3.095$, $df = 8$ for AGO1).

(I) RIP using DDK antibody (OriGene) followed by qPCR detection of Egr2-AS-RNA. Loss of Ser118 inhibits the binding of YY1 to Egr2-AS-RNA as compared to non-mutated YY1-DDK. However, loss of Ser184 induced a significant increase in the binding of YY1 to Egr2-AS-RNA, while Ser247 has no effect compared to the non-mutated construct. The double mutation of Ser118 and Ser184 results in increased binding of YY1 to Egr2-AS-RNA compared to the non-mutated protein. The experiment was repeated three times, and results are presented as mean ± SD (one-way ANOVA [$(5,12) = 3.398$, $p = 0.0383$] followed by post hoc Dunnett test [*$p = 0.0281$]).

(J) Schematic representation of the AS-RNA-mediated regulation of *Egr2* expression. In non-injured nerves, NRG1 signaling to ERK1/2 phosphorylates YY1, which in turn activates the transcription of *Egr2* and represses the expression of the AS-RNA (left). Nerve injury inhibits the NRG1-ERK1/2 signaling axis, which in possible conjunction with protein phosphatases blocks Ser184 phosphorylation of YY1. This results in activation of the YY1 phospho-switch and association of non-phosphorylated YY1 with AS-RNA and activation of AS-RNA expression. Loss of YY1 phosphorylation and an increase in the expression of the AS-RNA results in inhibition of *Egr2* transcription (right).

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 exploration of an antisense lncRNA 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 datasets (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 mpileup 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 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 purified SC cultures, 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

Conceptualization, N.T.; Investigation, M.M.-M., T.M.O., J.P.Z., A.O., and J.K.N.; Writing – Original Draft, N.T. and M.M.-M.; Writing – Review and Editing, N.T.; Funding Acquisition, N.T.; Resources, N.T., and R.L.; Supervision, N.T. and R.L.

ACKNOWLEDGMENTS

We thank Dr. Steven Toms and members of the Tapinos lab for critically reviewing the manuscript. We also thank Dr. Fajardo for computational analysis of the RNA-seq data. This work was supported by internal funds from the Geisinger Clinic to N.T.

Received: January 16, 2017
Revised: April 25, 2017
Accepted: July 24, 2017
Published: August 22, 2017

REFERENCES

Adilakshmi, T., Sudol, I., and Tapinos, N. (2012). Combinatorial action of miRNAs regulates transcriptional and post-transcriptional gene silencing following in vivo PNS injury. PLoS ONE *7*, e39674.

Amaral, P.P., Clark, M.B., Gascoigne, D.K., Dinger, M.E., and Mattick, J.S. (2011). lncRNAdb: a reference database for long noncoding RNAs. Nucleic Acids Res. *39*, D146–D151.

Arthur-Farraj, P.J., Latouche, M., Wilton, D.K., Quintes, S., Chabrol, E., Banerjee, A., Woodhoo, A., Jenkins, B., Rahman, M., Turmaine, M., et al. (2012). c-Jun reprograms Schwann cells of injured nerves to generate a repair cell essential for regeneration. Neuron *75*, 633–647.

Bellone, E., Di Maria, E., Soriani, S., Varese, A., Doria, L.L., Ajmar, F., and Mandich, P. (1999). A novel mutation (D305V) in the early growth response 2 gene is associated with severe Charcot-Marie-Tooth type 1 disease. Hum. Mutat. *14*, 353–354.

ENCODE Project Consortium, Birney, E., Stamatoyannopoulos, J.A., Dutta, A., Guigó, R., Gingeras, T.R., Margulies, E.H., Weng, Z., Snyder, M., Dermitzakis, E.T., Thurman, R.E., et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature *447*, 799–816.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185–196.

D'Antonio, M., Michalovich, D., Paterson, M., Droggiti, A., Woodhoo, A., Mirsky, R., and Jessen, K.R. (2006). Gene profiling and bioinformatic analysis of Schwann cell embryonic development and myelination. Glia *53*, 501–515.

Decker, L., Desmarquet-Trin-Dinh, C., Taillebourg, E., Ghislain, J., Vallat, J.M., and Charnay, P. (2006). Peripheral myelin maintenance is a dynamic process requiring constant Krox20 expression. J. Neurosci. *26*, 9771–9779.

Ghislain, J., Desmarquet-Trin-Dinh, C., Jaegle, M., Meijer, D., Charnay, P., and Frain, M. (2002). Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development. Development *129*, 155–166.

Guertin, A.D., Zhang, D.P., Mak, K.S., Alberta, J.A., and Kim, H.A. (2005). Microanatomy of axon/glial signaling during Wallerian degeneration. J. Neurosci. *25*, 3478–3487.

Hawkins, P.G., and Morris, K.V. (2008). RNA and transcriptional modulation of gene expression. Cell Cycle *7*, 602–607.

He, Y., Kim, J.Y., Dupree, J., Tewari, A., Melendez-Vasquez, C., Svaren, J., and Casaccia, P. (2010). Yy1 as a molecular link between neuregulin and transcriptional modulation of peripheral myelination. Nat. Neurosci. *13*, 1472–1480.

Jang, S.W., Srinivasan, R., Jones, E.A., Sun, G., Keles, S., Krueger, C., Chang, L.W., Nagarajan, R., and Svaren, J. (2010). Locus-wide identification of Egr2/Krox20 regulatory targets in myelin genes. J. Neurochem. *115*, 1409–1420.

Janowski, B.A., Huffman, K.E., Schwartz, J.C., Ram, R., Nordsell, R., Shames, D.S., Minna, J.D., and Corey, D.R. (2006). Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. Nat. Struct. Mol. Biol. *13*, 787–792.

Jeon, Y., and Lee, J.T. (2011). YY1 tethers Xist RNA to the inactive X nucleation center. Cell *146*, 119–133.

Kassardjian, A., Rizkallah, R., Riman, S., Renfro, S.H., Alexander, K.E., and Hurt, M.M. (2012). The transcription factor YY1 is a novel substrate for Aurora B kinase at G2/M transition of the cell cycle. PLoS ONE *7*, e50645.

Kim, D.H., Saetrom, P., Snøve, O., Jr., and Rossi, J.J. (2008). MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc. Natl. Acad. Sci. USA *105*, 16230–16235.

Klase, Z., Kale, P., Winograd, R., Gupta, M.V., Heydarian, M., Berro, R., McCaffrey, T., and Kashanchi, F. (2007). HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. BMC Mol. Biol. *8*, 63.

Le, N., Nagarajan, R., Wang, J.Y., Araki, T., Schmidt, R.E., and Milbrandt, J. (2005). Analysis of congenital hypomyelinating Egr2Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell differentiation and myelination. Proc. Natl. Acad. Sci. USA *102*, 2596–2601.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R.; 1000 Genome Project Data Processing Subgroup (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078–2079.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics *30*, 923–930.

Lu, J., and Tsourkas, A. (2009). Imaging individual microRNAs in single mammalian cells in situ. Nucleic Acids Res. *37*, e100.

Makhlouf, M., Ouimette, J.F., Oldfield, A., Navarro, P., Neuillet, D., and Rougeulle, C. (2014). A prominent and conserved role for YY1 in Xist transcriptional activation. Nat. Commun. *5*, 4878.

Mercer, T.R., Dinger, M.E., Sunkin, S.M., Mehler, M.F., and Mattick, J.S. (2008). Specific expression of long noncoding RNAs in the mouse brain. Proc. Natl. Acad. Sci. USA *105*, 716–721.

Morris, K.V. (2009). RNA-directed transcriptional gene silencing and activation in human cells. Oligonucleotides *19*, 299–306.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods *5*, 621–628.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197–208.

Murphy, P., Topilko, P., Schneider-Maunoury, S., Seitanidou, T., Baron-Van Evercooren, A., and Charnay, P. (1996). The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. Development *122*, 2847–2857.

Nagarajan, R., Svaren, J., Le, N., Araki, T., Watson, M., and Milbrandt, J. (2001). EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. Neuron *30*, 355–368.

Newbern, J.M., Li, X., Shoemaker, S.E., Zhou, J., Zhong, J., Wu, Y., Bonder, D., Hollenback, S., Coppola, G., Geschwind, D.H., et al. (2011). Specific functions for ERK/MAPK signaling during PNS development. Neuron *69*, 91–105.

O'Shea, T.M., Aimetti, A.A., Kim, E., Yesilyurt, V., and Langer, R. (2015). Synthesis and characterization of a library of in-situ curing, nonswelling ethoxylated polyol thiol-ene hydrogels for tailorable macromolecule delivery. Adv. Mater. *27*, 65–72.

Omoto, S., and Fujii, Y.R. (2005). Regulation of human immunodeficiency virus 1 transcription by nef microRNA. J. Gen. Virol. *86*, 751–755.

Palazzo, A.F., and Lee, E.S. (2015). Non-coding RNA: what is functional and what is junk? Front. Genet. *6*, 2.

Parkinson, D.B., Bhaskaran, A., Arthur-Farraj, P., Noon, L.A., Woodhoo, A., Lloyd, A.C., Feltri, M.L., Wrabetz, L., Behrens, A., Mirsky, R., and Jessen, K.R. (2008). c-Jun is a negative regulator of myelination. J. Cell Biol. *181*, 625–637.

Poitelon, Y., Lopez-Anido, C., Catignas, K., Berti, C., Palmisano, M., Williamson, C., Ameroso, D., Abiko, K., Hwang, Y., Gregorieff, A., et al. (2016). YAP and TAZ control peripheral myelination and the expression of laminin receptors in Schwann cells. Nat. Neurosci. *19*, 879–887.

Quek, X.C., Thomson, D.W., Maag, J.L., Bartonicek, N., Signal, B., Clark, M.B., Gloss, B.S., and Dinger, M.E. (2015). lncRNAdb v2.0: expanding the reference database for functional long noncoding RNAs. Nucleic Acids Res. *43*, D168–D173.

Ramsköld, D., Wang, E.T., Burge, C.B., and Sandberg, R. (2009). An abundance of ubiquitously expressed genes revealed by tissue transcriptome sequence data. PLoS Comput. Biol. *5*, e1000598.

Rangnekar, V.M., Aplin, A.C., and Sukhatme, V.P. (1990). The serum and TPA responsive promoter and intron-exon structure of EGR2, a human early growth response gene encoding a zinc finger protein. Nucleic Acids Res. *18*, 2749–2757.

Rizkallah, R., and Hurt, M.M. (2009). Regulation of the transcription factor YY1 in mitosis through phosphorylation of its DNA-binding domain. Mol. Biol. Cell *20*, 4766–4776.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. *29*, 24–26.

Simon, M.D., Wang, C.I., Kharchenko, P.V., West, J.A., Chapman, B.A., Alekseyenko, A.A., Borowsky, M.L., Kuroda, M.I., and Kingston, R.E. (2011). The genomic binding sites of a noncoding RNA. Proc. Natl. Acad. Sci. USA *108*, 20497–20502.

Smale, S.T., and Kadonaga, J.T. (2003). The RNA polymerase II core promoter. Annu. Rev. Biochem. *72*, 449–479.

Stassart, R.M., Fledrich, R., Velanac, V., Brinkmann, B.G., Schwab, M.H., Meijer, D., Sereda, M.W., and Nave, K.A. (2013). A role for Schwann cell-derived neuregulin-1 in remyelination. Nat. Neurosci. *16*, 48–54.

Svaren, J., and Meijer, D. (2008). The molecular machinery of myelin gene transcription in Schwann cells. Glia *56*, 1541–1551.

Tan, Y., Zhang, B., Wu, T., Skogerbø, G., Zhu, X., Guo, X., He, S., and Chen, R. (2009). Transcriptional inhibiton of Hoxd4 expression by miRNA-10a in human breast cancer cells. BMC Mol. Biol. *10*, 12.

Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R.M., Loeb, J.A., Shrager, P., et al. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. Neuron *47*, 681–694.

Timmerman, V., De Jonghe, P., Ceuterick, C., De Vriendt, E., Löfgren, A., Nelis, E., Warner, L.E., Lupski, J.R., Martin, J.J., and Van Broeckhoven, C. (1999). Novel missense mutation in the early growth response 2 gene associated with Dejerine-Sottas syndrome phenotype. Neurology *52*, 1827–1832.

Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A.B., Seitanidou, T., Babinet, C., and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. Nature *371*, 796–799.

Uhler, J.P., Hertel, C., and Svejstrup, J.Q. (2007). A role for noncoding transcription in activation of the yeast PHO5 gene. Proc. Natl. Acad. Sci. USA *104*, 8011–8016.

Verheijen, M.H., Chrast, R., Burrola, P., and Lemke, G. (2003). Local regulation of fat metabolism in peripheral nerves. Genes Dev. *17*, 2450–2464.

Volders, P.J., Helsens, K., Wang, X., Menten, B., Martens, L., Gevaert, K., Vandesompele, J., and Mestdagh, P. (2013). LNCipedia: a database for annotated human lncRNA transcript sequences and structures. Nucleic Acids Res. *41*, D246–D251.

Volders, P.J., Verheggen, K., Menschaert, G., Vandepoele, K., Martens, L., Vandesompele, J., and Mestdagh, P. (2015). An update on LNCipedia: a database for annotated human lncRNA sequences. Nucleic Acids Res. *43*, D174–D180.

Warner, L.E., Mancias, P., Butler, I.J., McDonald, C.M., Keppen, L., Koob, K.G., and Lupski, J.R. (1998). Mutations in the early growth response 2 (EGR2) gene are associated with hereditary myelinopathies. Nat. Genet. *18*, 382–384.

Wu, T.D., and Nacu, S. (2010). Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics *26*, 873–881.

Yoon, J.H., Kim, J., and Gorospe, M. (2015). Long noncoding RNA turnover. Biochimie *117*, 15–21.

Zorick, T.S., Syroid, D.E., Arroyo, E., Scherer, S.S., and Lemke, G. (1996). The transcription factors SCIP and Krox-20 mark distinct stages and cell fates in Schwann cell differentiation. Mol. Cell. Neurosci. *8*, 129–145.