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Locus-wide identification of EGR2/Krox20 regulatory targets in myelin genes

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Abstract

Myelination of peripheral nerves by Schwann cells depends upon a gene regulatory network controlled by Egr2/Krox20, which is specifically required for Schwann cells to initiate and maintain myelination. To elucidate the mechanism by which Egr2 regulates gene expression during myelination, we have performed chromatin immunoprecipitation analysis on myelinating rat sciatic nerve in vivo. The resulting samples were applied to a tiled microarray consisting of a broad spectrum of genes that are activated or repressed in Egr2-deficient mice. The results show extensive binding within myelin-associated genes, as well as some genes that become repressed in myelinating Schwann cells. Many of the Egr2 peaks coincide with regions of open chromatin, which is a marker of enhancer regions. In addition, further analysis showed that there is substantial colocalization of Egr2 binding with Sox10, a transcription factor required for Schwann cell specification and other stages of Schwann cell development. Finally, we have found that Egr2 binds to promoters of several lipid biosynthetic genes, which is consistent with their dramatic upregulation during the formation of lipid-rich myelin. Overall, this analysis provides a locus-wide profile of Egr2 binding patterns in major myelin-associated genes using myelinating peripheral nerve.

Keywords

Chromatin; Krox20; Schwann; myelination; ChIP

Introduction

During the postnatal development of the PNS, Schwann cells establish a one-on-one relationship with neurons and initiate the formation of the multilayered membrane structure around large diameter axons known as the myelin sheath (reviewed in Jessen and Mirsky 2005). The integrity of myelin is required for rapid, saltatory nerve conduction of action

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potentials. Defective formation and maintenance of myelin by Schwann cells is a major cause of human peripheral neuropathies, such as Charcot-Marie-Tooth and Dejerine-Sottas disease (reviewed in Suter and Scherer 2003; Wrabetz et al. 2004). Profiling studies have provided extensive characterization of gene expression changes during the myelination process, including induction of major myelin genes such as *Myelin Protein Zero* and *Connexin 32*, as well as genes involved in lipid synthesis to support the synthesis of myelin membrane by Schwann cells (Nagarajan et al. 2002; Verheijen et al. 2003).

To coordinate these gene induction events, peripheral nerve myelination entails intricate networks of transcription factors, including Egr2/Krox20, Oct6/Scip/Pou3f1, Brn2/Pou3f2, Sox10, SREBP factors, NFATc3/c4 and NF-κB (Topilko et al. 1994; Bermingham et al. 1996; Jaegle et al. 1996; Britsch et al. 2001; Jaegle et al. 2003; Nickols et al. 2003; Kao et al. 2009). Although many of these transcription factors are expressed in several cell types, it is thought that combinatorial actions of these transcriptional regulators contribute to tissue-specificity of the transcriptome in myelinating Schwann cells (reviewed in Svaren and Meijer 2008). At least some of these combinatorial interactions involve Egr2/Krox20 (hereafter referred to as Egr2), which is significantly induced at the onset of myelination. Consistent with this, Egr2 null and hypomorphic mice display a severe hypomyelination phenotype in peripheral nerve (Topilko et al. 1994; Le et al. 2005a; Decker et al. 2006), and target gene analysis indicates that Egr2 induces many of the major myelin genes (Nagarajan et al. 2001; Parkinson et al. 2004; Le et al. 2005a). Egr2 is also required for high levels of expression of several lipid biosynthetic genes—including *HMG CoA reductase* and *Stearoyl CoA desaturase* (Nagarajan et al. 2001; Le et al. 2005a; LeBlanc et al. 2005). Mutations of the *EGR2* gene itself are associated with a spectrum of human peripheral neuropathies (Warner et al. 1998; Bellone et al. 1999; Timmerman et al. 1999; Pareyson et al. 2000; Mikesova et al. 2005). While a large number of genes are altered in Egr2-deficient mice, it is unclear if most of them are directly or indirectly regulated by Egr2.

Several lines of evidence suggest that Egr2 also represses other genes as myelination proceeds. Successful myelination depends upon interaction of Egr2 with the Nab1/2 transcriptional corepressors (Russo et al. 1995; Svaren et al. 1996). Targeted disruption of *Nab1/2* causes myelination defects in peripheral nerve (Le et al. 2005b), and similar results have recently been obtained using knockins of Nab-resistant alleles of Egr2 (Desmazieres et al. 2008; Baloh et al. 2009). Accordingly, Egr2 and Nab proteins are required to downregulate genes that become repressed as myelination proceeds, such as *Pou3f1/Oct6/Scip*, *Sox2*, and *Id2*/*Id4* (Zorick et al. 1999; Le et al. 2005a; Le et al. 2005b; Mager et al. 2008).

Myelination is coordinated by induction and modulation of specific transcription factors, which bind to cognate elements within dynamically regulated myelin genes. Therefore, such elements constitute the sites where positive and negative effects are integrated to establish the correct expression level of myelin genes. Another critical component of transcriptional regulation in Schwann cells is Sox10, a high mobility group (HMG) box-containing transcription factor required for Schwann cell specification and later stages of Schwann cell development (Kuhlbrodt et al. 1998; Britsch et al. 2001; Schreiner et al. 2007; Finzsch et al. 2010). Binding sites for Egr2 and Sox10 have been identified in a composite configuration in several myelin genes such as *Connexin32*, *Mbp*, *Mpz*, and *Mag* (Bondurand et al. 2001; Denarier et al. 2005; LeBlanc et al. 2007; Jang and Svaren 2009). However, it remains poorly understood how and to what extent Egr2 regulates expression of its target genes in conjunction with Sox10 during PNS myelination.

To identify Egr2 binding sites, we have developed a method for in vivo chromatin immunoprecipitation (ChIP) on rat sciatic nerve during the peak of PNS myelination

(postnatal day 10–15) (Jang et al. 2006). It should be noted that the success of these ChIP assays depends in part on the large proportion of myelinating Schwann cells in sciatic nerve, which is the only cell type in peripheral nerve that expresses Egr2 (Zorick et al. 1996; Topilko et al. 1997). Since standard ChIP assays targeted at selected sites is impractical for efficient discovery of novel sites, we performed a more unbiased screen for Egr2 binding sites by coupling in vivo ChIP on myelinating rat sciatic nerve with a tiled microarray (hereafter referred to as ChIP-chip). The array represents gene sets that are induced or repressed in Egr2-deficient peripheral nerve, many of which have yet to be analyzed for the mechanism of their regulation. The analysis allowed us to test if Egr2-regulated genes are generally associated with Egr2 binding in upstream, intragenic, and downstream regions. Finally, the locus-wide analysis of Egr2 binding sites provided the basis to test if Egr2 is generally colocalized with Sox10 binding in genes that are regulated during myelination.

Materials and Methods

In vivo chromatin immunoprecipitation

In vivo ChIP assays on rat myelinating sciatic nerve were performed on Sprague-Dawley rat pups at postnatal day 15 (P15) as previously described (Jang et al. 2006; Jang and Svaren 2009), except with the omission of herring sperm DNA in the blocking procedure. All experiments on rats were performed in strict accordance with experimental protocols approved by the Institutional Animal Care and Use Committee, University of Wisconsin, School of Veterinary Medicine. The antibodies used in this study include Egr2 antibodies (Abcam cat. no.43020 and Covance cat no. PRB-236P, designated antibodies 1 and 2, respectively), Sox10 (Santa Cruz Biotechnology cat# sc-17342X), and control IgG (normal rabbit IgG: Millipore 12–370 and normal goat IgG: Santa Cruz Biotechnology cat# sc-2028). Following ChIP, quantitative PCR was performed in duplicate to calculate the fold recovery of a given segment relative to the non-specific IgG, using the Comparative Ct method (Livak and Schmittgen 2001). Primer sequences used in this study are available upon request.

ChIP analysis using tiled microarrays

To combine ChIP with microarray analysis, amplicons were first generated from ChIP products by whole genome amplification (Sigma). Labeling of the samples with Cy5 (experimental-Egr2) or Cy3 (control-total input) followed by microarray hybridization was performed as described (Jang and Svaren 2009) by Nimblegen, using a custom microarray designed with isothermic probes staggered by 17 bp representing the selected gene loci listed in Table 1. Complete listing and coordinates of tiled regions in the rn4 genome build are provided in Supplemental Table S1. Gaps in the tiling represent repetitive DNA regions for which unique, optimal probes could not be designed. The enrichment ratio of Cy5 to Cy3 was plotted on a $\log_2$ scale and further processed to display a moving average using a window size of 5 probes. Peak finding was performed using the CMARRT (Correlation, Moving Average, Robust and Rapid method on Tiling array) algorithm, which identifies peaks in ChIP-chip data based on a moving average model that incorporates the correlation structure of tiled microarrays (Kuan et al. 2008), with a false discovery rate of 0.05. Displayed peaks are those that are common to the two independent replicates with each antibody. All raw data sets for the custom tiled array are available from the NCBI Gene Expression Omnibus website: accession number: GSE23648.

In vivo FAIRE

The *in vivo* FAIRE assay on rat myelinating sciatic nerve was performed at P14 using pooled nerves from 4 rat pups. Freshly dissected sciatic nerves were minced in phosphate-buffered saline (PBS) containing 1% formaldehyde for 5 minutes at room temperature

(22°C). The nerves were washed with cold PBS, pooled, and frozen at −8°C. The nerves were thawed and homogenized in buffer (150 mM NaCl, 10% glycerol, 50 mM Tris pH 8.0) containing protease inhibitor cocktail (Sigma, St Louis, MO, USA; 5μl of cocktail per ml of buffer). The cells were then lysed using incubation in three buffers, L1, L2 and L3 as previously described (Giresi and Lieb 2009). After cell lysis, cells were sonicated using the Bioruptor (Diagenode), and extracted with phenol:chloroform as previously described (Giresi and Lieb 2009). The protocol only differed in that the cells were sonicated in 2 ml of Lysis Buffer 3 on high with 30 seconds on followed by 30 seconds off, all on ice. 20% of the crosslinked chromatin was used as an input control, and the samples were reverse crosslinked at 65°C overnight.

Transfection Assays

The B16 (mouse melanoma) cell line was grown and transfected as described (Jones et al. 2007; LeBlanc et al. 2007). The reporter constructs contain the following coordinates from the rat genome (Rn4 build in UCSC Genome Browser), cloned upstream of the pGL4 luciferase reporter. The Ndrg1and Mag constructs also contain a minimal promoter.

Ndrg1 +9.3 kb: chr7:104335109-104335666; Ndrg1 +20.6 kb: chr7:104324019-104324460 c-jun: chr5:115361190-115362027; Id4: chr17:22528145-22528659; mouse Mag: mm9 chr7:31697999-31698377

Sequence analysis

Sequences obtained from rat genomic regions of Egr2 enrichment called as peaks in the ChIP-chip analysis were evaluated by comparison with the previously defined Egr2 binding sites (Nardelli et al. 1992; Swirnoff and Milbrandt 1995), using Transfac matrix M00246, or Egr1 position weight matrices defined recently (Badis et al. 2009). Analysis for composite elements containing Sox10 and Egr2 sites was performed as described using a constructed SOX dimer binding (Jones et al. 2007), allowing for 3–6 bp spacing between inverted repeats of the Sox10 monomeric site.

Results

Design of genomic tiling array with candidate Egr2 target genes

In an attempt to obtain an unbiased survey of Egr2 binding patterns in genes regulated during myelination, we combined in vivo Egr2-ChIP with a high-resolution custom microarray representing a broad representation of genes that are induced or repressed in peripheral nerve of Egr2-deficient mice (Le et al. 2005a; Le et al. 2005b). Overall, these genes are categorized into five functional groups in Table 1. In addition, the array included additional rat orthologues of genes that are mutated in human peripheral neuropathies (e.g. *Egr2*, *Litaf*). The custom microarray incorporated tiled probes representing ~50–100 kb windows surrounding the genes of interest in the rat genome. A complete listing of tiled genes and the chromosomal coordinates are included in Supplemental Table S1.

Immunoprecipitations were performed using two independent Egr2 antibodies with sciatic nerve samples obtained from pooled littermate samples (~10–12) at P15. The immunoprecipitations were performed on two independent litters for each antibody. The ChIP samples were labeled and co-hybridized to the tiled microarray along with corresponding input DNA as the control. These data were analyzed by the CMARRT algorithm, which identifies peaks in ChIP-chip data based on a moving average model that incorporates the correlation structure of tiled microarrays (Kuan et al. 2008). All previously identified Egr2 binding sites in *Mag*, *Mbp*, *Mpz*, *Nab1*, *Nab2*, *Dhh*, and *Prx* genes (Denarier et al. 2005; Jang et al. 2006; Jones et al. 2007; Srinivasan et al. 2007; Jang and Svaren 2009)

were identified as peaks in at least 3 out of 4 ChIP-chip analyses. Overall, the degree of overlap between the two Egr2 antibodies was fairly high as 65.5% of the Egr2 Ab.2 peaks overlapped with an Egr2 Ab.1 peak (Table 2). In general, the signal to noise ratio for Ab. 2 was greater than that of Ab. 1, resulting in a larger Ab.2 peak set identified by the CMARRT analysis. Western analysis shows that both antibodies detect Egr2, but Ab. 2 is somewhat more specific (Supplementary Figure S1).

Egr2 binding to the *Connexin 32* promoter

Mutations of the human *Connexin 32* gene (*GJB1*, gap junction protein, beta1) cause an X-linked form of Charcot-Marie-Tooth Disease (CMT1X), which is the 2nd most common form of inherited peripheral myelinopathy. *Connexin 32* expression is induced during myelination, and its expression in mouse peripheral nerve is dependent upon Egr2 (Le et al. 2005a; Le et al. 2005b). Previous studies of the *Connexin 32* gene had identified two alternate promoters, but the P2 promoter is used exclusively in peripheral nerve (Neuhaus et al. 1995). The P2 promoter has binding sites for both Egr2 and Sox10, and this promoter has been shown to respond to these factors in transient transfection assays (Bondurand et al. 2001; Musso et al. 2003). Analysis of the Egr2 ChIP-chip data showed one enriched region of Egr2 binding over the P2 promoter region (Figure 1). In contrast, there does not appear to be any significant binding in the P1 promoter region of the *Connexin 32* locus. An independent ChIP-chip assay was performed with a second antibody (Egr2 Ab.2) exhibited essentially the same major peak, although there were some minor differences perhaps reflecting some differences in non-specific binding.

A general characteristic of regulatory elements is that they lie within nucleosome-free regions. To map regions of open chromatin we used FAIRE (formaldehyde-assisted identification of regulatory elements), which identifies regions of DNA that are not efficiently crosslinked to histones. Data from the ENCODE project has shown that FAIRE sites largely colocalize with DNaseI hypersensitive sites (Giresi et al. 2007; Giresi and Lieb 2009). Interestingly, FAIRE analysis of the *Connexin 32* locus identified a single major peak that coincides with the previously identified binding sites for Egr2 and Sox10 (Bondurand et al. 2001; Musso et al. 2003), confirming previous reports that this site is a functional enhancer. The FAIRE peak is more narrowly focused over the reported Egr2 binding sites than the ChIP-chip analysis of Egr2 itself. Moreover, this analysis did not identify any additional regulatory elements outside of the P2 promoter region in the 50 kb tiled window surrounding the *Connexin 32* gene.

Egr2 regulation of *Ndrg1*

Figure 2 shows Egr2 binding patterns in the *Ndrg1* gene (N-myc downstream regulated gene), which is mutated in a recessive peripheral neuropathy classified as Charcot-Marie-Tooth disease type 4D (Kalaydjieva et al. 1996; Kalaydjieva et al. 1998; Kalaydjieva et al. 2000; Berger et al. 2002). Targeted disruption of this gene leads to progressive demyelination in peripheral nerves, and its expression is dynamically regulated during peripheral myelination (Nagarajan et al. 2002; Verheijen et al. 2003; Okuda et al. 2004). Although *Ndrg1* is downregulated in Egr2-deficient mice, the sites of regulation have not yet been identified. The ChIP-chip assay identified several regions of Egr2 enrichment called as peaks throughout the *Ndrg1* locus (Fig. 2). One major peak was found in the promoter region, which corresponds to a previously identified Egr1 site (Zhang et al. 2007), and other peaks were found in intron-associated sites. Several peaks correlate with open chromatin as defined by FAIRE analysis (i.e. promoter region, and intronic sites at ~104,324,000 and ~104,335,000). These data provide the first evidence for direct regulation of *Ndrg1* by Egr2 during peripheral nerve myelination and are consistent with the observed downregulation of *Ndrg1* in Egr2-deficient mice (Le et al. 2005a).

Egr2 binding to genes repressed during myelination

There is an extensive program of gene repression that occurs during the myelination process (reviewed in Jessen and Mirsky 2008). Many of these genes have been shown to antagonize the myelination process, such as *Sox2* and *c-jun* (Parkinson et al. 2004; Le et al. 2005a). Interestingly, many such genes are normally repressed during myelination and become induced in peripheral nerve of mice that are deficient in Egr2/Krox20 (marked as bold text in Table 1), leading to the proposal that Egr2 is directly involved in gene repression (Zorick et al. 1999; Le et al. 2005a; Le et al. 2005b; Decker et al. 2006; Desmazieres et al. 2008; Mager et al. 2008). Development of our ChIP-chip array allowed us to examine locus-wide Egr2 binding patterns in several such genes. The examples shown are from *Id4* and *c-jun* (Figures 3 and 4). Interestingly, binding of Egr2 is observed as a major peak in the promoter regions of both genes, with another notable peak peak within the c-jun coding region. The major binding of Egr2 in the promoters of both genes is observed within the context of open chromatin as determined by the FAIRE analysis. These data are consistent with direct repression of these genes by Egr2 in myelinating Schwann cells.

Distribution of Egr2 binding within target genes

Of all the Egr2-regulated genes listed in Table 1, all but two of the genes (*Nefl* and *Lpl*) had at least one Egr2 peak identified in the tiled region surrounding the gene in at least 3 out of the 4 datasets by CMARRT analysis. We analyzed the distribution of Egr2 binding sites in target gene loci with respect to the transcription start site (Figure 5A). The Egr2 peaks do not exhibit a significant preference for upstream regions with a high proportion of its binding sites found in intragenic regions. This is consistent with our previous analysis of selected genes (Jang et al. 2006; LeBlanc et al. 2006; Jones et al. 2007), in which functional Egr2 binding sites have been found within introns.

In the two repressed genes presented in Figures 3 and 4, there was a predominant peak in the promoter region. Overall, Egr2 binding was observed in virtually all of the repressed genes included on the custom microarray. A similar location analysis was therefore performed with the 15 genes on the array that are elevated in Egr2-deficient nerves (Le et al. 2005a; Le et al. 2005b; Mager et al. 2008), and are therefore potentially repressed by Egr2. In contrast to the total set, there seems to be more significant shift to binding to the promoter and upstream regions (Figure 5B), suggesting that repression of genes by Egr2 during myelination may involve more promoter-proximal mechanisms of interaction. However, it remains to be seen if this correlation observed in 15 genes will also be observed in a more genome-wide analysis.

Colocalization of Egr2 and Sox10 in regulated genes

Cooperative regulation by Egr2 and Sox10 has been suggested by protein interaction studies and analysis of myelin gene regulatory elements in which their respective binding sites are juxtaposed (Bondurand et al. 2001; Denarier et al. 2005; Wissmuller et al. 2006; Jones et al. 2007; LeBlanc et al. 2007). In an attempt to construct a more comprehensive integrated map of Egr2 and Sox10 interplay, we performed a similar ChIP-chip analysis of Sox10 binding in P15 rat sciatic nerve. Peak analysis for Sox10 was performed using the CMARRT algorithm as described above, and overlapping peaks of Egr2 and Sox10 binding (found in both replicates) were identified by comparison with the Egr2 data sets. The results of this overlap analysis (requiring overlap of ≥1 bp) are summarized in Table 2. Approximately 65% and 73% of the Sox10 peaks overlapped with the Egr2 Ab.1 and Egr2 Ab.2 peak sets, respectively. The increased percentage overlap with Egr2 Ab. 2 reflects the larger number of peaks found in both replicates using this antibody. Using the somewhat larger Egr2 peak sets as a starting point, approximately 32% (Ab. 1) and 26% (Ab. 2) of the Egr2 peaks overlapped with a Sox10 peak. To assess the statistical significance of the overlap, randomly

sampled regions (with matched chromosome and peak width) were analyzed for similar levels of overlap. After 3000 iterations, none of the randomly sampled peak sets attained a similar degree of overlap (i.e. >26%). Overall, these data suggest that the relatively high degree of binding overlap between Egr2 and Sox10 binding is significantly greater than would be expected by chance.

We have recently proposed that many myelin gene regulatory elements contain a conserved composite module of Egr2 and Sox10 binding sites, and that this module may have predictive value in identifying myelin gene regulatory elements (Jones et al. 2007). In particular, this module incorporated an inverted pair of Sox10 binding sites with a 3–6 bp spacing (Jones et al. 2007), based on previous work showing binding of Sox10 dimers to such inverted pairs of sites (Peirano and Wegner 2000). We performed binding site analysis on a high confidence peak set, consisting of 97 overlap regions that were positive in both replicates for Egr2 Ab.1, Egr2 Ab.2, and Sox10 (see Supplemental Table S2 for complete listing). Approximately 88% of these peaks had at least one binding site that conformed ($p<0.001$) to the defined Egr2 binding site (Swirnoff et al. 1998), and 80% had at least one match to a recently defined matrix for the closely related Egr1 factor (Badis et al. 2009). More than half of the peaks had at least one match to the inverted dimeric Sox10 binding site ($p<0.0001$). There did not seem to be any apparent preference for a particular spacing between the Sox10 monomeric sites in this peak set. At a lower stringency ($p<0.001$), all but one of the peaks in this set had at least one match to the Sox10 site, but many of these could be Sox10 monomeric sites, which also are functionally active in myelin gene regulatory elements (Peirano and Wegner 2000). Overall, this analysis identified a number of novel composite elements containing Egr2 and dimeric Sox10 binding sites, some of which are listed in Table 3.

Transfection analysis of Egr2 binding sites

Transient transfection assays have shown that Egr2 can activate the Connexin 32 promoter (Bondurand et al. 2001; Musso et al. 2003), and that the Ndrg1 proximal promoter region is responsive to the closely related Egr1 (Zhang et al. 2007). To explore the function of some of the novel binding sites identified in our analysis, we cloned selected sites upstream of a luciferase reporter gene, and tested their ability to respond to Egr2 expression in transfection assays using the B16 melanoma cell line, which expresses Sox10 (Slutsky et al. 2003). An intronic Egr2 binding site in the Mag gene—first identified in a previous study (Jang et al. 2006)—was strongly activated by Egr2 (Figure 6). Similarly, an intronic binding site in *Ndrg1* (+9.3 kb) was activated to a high level, whereas another binding region at +20.6 kb did not respond in this assay. This correlates with an apparently stronger ChIP signal at +9.3 kb as measured by qPCR validation (data not shown). We also tested the two repressed promoters (c-jun and Id4), but we observed only a ~2.8-fold activation of the Id4 promoter by Egr2 in a transfection assay. The c-jun promoter was not activated, but its basal activity was extremely high, indicating that endogenous activators may preclude activation by Egr2 in this assay.

Egr2 regulation of lipid biosynthesis genes

The peripheral nerve myelin membrane is extremely lipid-rich (70–80% of total dry mass), and the lipid content is unusually rich in cholesterol (composing 20–30% of total lipid, reviewed in Garbay et al. 2000). Interestingly, most of the cholesterol used for myelination is internally synthesized rather than being externally supplied from the circulation (Jurevics and Morell 1994). Egr2 is a convergent point of this coordination as several lipid synthesis genes are downregulated in Egr2-deficient mice (Nagarajan et al. 2001; Le et al. 2005a; LeBlanc et al. 2005). The ChIP-chip analysis revealed Egr2 binding sites in many of the lipid biosynthetic genes, implying that Egr2 directly regulates these genes. We confirmed

binding of Egr2 to several of these promoters—*HMG CoA Reductase, HMG CoA Synthase, Lanosterol Demethylase, Squalene Epoxidase, and Stearoyl CoA Desaturase*—by quantitative PCR analysis of independent ChIP assays (Figure S2). Of the genes involved in lipid synthesis included in our array, 16 out of 17 had at least one Egr2 peak within 1 kb of the transcription start site.

Discussion

Profound peripheral nerve defects in knockout mice (Topilko et al. 1994; Le et al. 2005a; Decker et al. 2006) and the genetic mutations associated with human peripheral neuropathies (Warner et al. 1998; Bellone et al. 1999; Timmerman et al. 1999) have implicated Egr2 as a master regulator of the myelination program in the PNS (reviewed in Svaren and Meijer 2008). Expression profiling studies have yielded an array of genes that are dynamically regulated during myelination by Schwann cells and also sensitive to Egr2 expression (Nagarajan et al. 2001; Nagarajan et al. 2002; Verheijen et al. 2003; Le et al. 2005a; D'Antonio et al. 2006). However, for most of these genes, it is not known if they are directly or indirectly regulated by Egr2. In this study, application of in vivo ChIP to microarray analysis revealed sites of Egr2 binding in a wide range of target genes identified by previous analysis of Egr2-deficient mice.

FAIRE analysis showed that many peaks of Egr2 binding lie within areas of open chromatin, which generally correlates with regulatory regions (Giresi et al. 2007; Giresi and Lieb 2009). Interestingly, many of the peaks of Egr2 binding revealed by ChIP-chip analysis extended over several hundred basepairs (e.g. Figure 1), which may reflect some bridging interactions (through various cofactors) to adjacent DNA segments. In contrast, the FAIRE analysis often provided a more finely focused peak that could be used to more accurately pinpoint Egr2 sites.

There seems to be considerable heterogeneity in the location of Egr2 binding sites. The simplest case is that of the *Connexin 32* gene, in which both Egr2 and Sox10 bind to the proximal promoter region of the regulated P2 promoter. Interestingly, *Connexin 32* is the only myelin gene in which noncoding promoter mutations have been identified associated with human peripheral neuropathy (Bondurand et al. 2001; Houlden et al. 2004). In contrast, activation of other myelin genes such as *Ndrg1* (Figure 2) and *Mpz* (Jang and Svaren 2009) is associated with multiple distributed Egr2 binding sites, including intragenic regions (often in conserved sites within introns). Based on similar analyses of other transcription factors, it is expected that not all sites are functionally required for regulation of specific genes in Schwann cells. It has been proposed that at least some binding sites may simply reflect the substantial affinity of transcription factors for DNA in general, but that functional sites are bound at quantitatively higher levels (Li et al. 2008). Accordingly, a strong site in the Ndrg1 gene was more highly activated in our tranfection assay compared to another intragenic site with a lower signal in our quantitative PCR validation. However, it is possible that weaker binding sites may still contribute to gene activation in the context of the whole locus.

The ChIP-chip analysis showed that Egr2 binds to the promoters of many of the lipid biosynthesis genes included on this array. Previous studies have indicated that the major regulators for lipid biosynthetic genes—sterol regulatory element binding proteins (SREBPs)—are required for efficient myelination (Verheijen et al. 2003; Verheijen et al. 2009). Since SREBPs generally bind to proximal promoter regions of their target genes (Seo et al. 2009), this may indicate that Egr2 interacts with SREBP factors in these promoters. Consistent with this, some lipid biosynthetic genes are synergistically activated by Egr2 and SREBPs in transfection experiments (LeBlanc et al. 2005). Egr2 has a binding specificity that overlaps to some extent with that of Sp1, which has been reported to bind adjacent to

SREBPs on the promoters of many genes involved in lipid metabolism (reviewed in Shimano 2001). Therefore, it is possible that Egr2 utilizes some previously defined Sp1 binding sites to augment the activation of lipid biosynthetic genes to the high levels required for myelination.

Tissue-specific expression of myelin genes in Schwann cells depends upon combinatorial interactions between transcription factors. For example, both Egr2 and Sox10 are present in tissues that do not express myelin genes (e.g. T cells and melanocytes, respectively). Therefore, expression of either of these factors alone is not sufficient for high level expression of myelin genes. We had proposed that a cluster of conserved Egr2 and Sox10 dimeric sites may be a common theme in regulatory elements of developmentally regulated myelin genes in Schwann cells (Jones et al. 2007). The ChIP-chip analysis revealed extensive colocalization of Egr2 with Sox10, and binding site analysis identified putative binding sites for Sox10 in most Egr2 binding peaks. The extensive colocalization of Egr2 with Sox10 at various sites may reflect direct interaction of the two factors (Wissmuller et al. 2006; Jones et al. 2007; LeBlanc et al. 2007).

Many Egr2 target genes are dynamically induced during myelination since they are required for the formation of myelin structure. On the other hand, some genes are shown to be downregulated as myelination proceeds, including *Sox2*, *Id2*, *Id4*, and *c-jun* (Stewart 1995; Shy et al. 1996; Verheijen et al. 2003; Le et al. 2005a). Based on the elevated levels of these genes in peripheral nerve of Egr2- and NAB-deficient mice, it has been proposed that Egr2 and associated NAB corepressors downregulate their expression (Zorick et al. 1999; Le et al. 2005a; Le et al. 2005b; Decker et al. 2006; Mager et al. 2008). Our previous work showed that Egr2 and its corepressors, Nab1 and Nab2, bind to the *Id2* gene in a developmentally-regulated manner as it is being repressed in peripheral nerve (Mager et al. 2008). The ChIP-chip analysis extends these findings by showing Egr2 binds to the promoters of virtually all of the genes (included on our array) that decline as myelination proceeds. It has not been determined how Egr2/Nab complexes repress some genes, while Egr2 activates myelin genes. Interestingly, Sox10 binding was also observed in repressed genes. Sox10 has been shown to be sumoylated on several conserved lysines, which is generally correlated with gene repression (Taylor and Labonne 2005; Girard and Goossens 2006), although further analysis will be required to determine if this plays a role in gene repression during myelination.

Overall, genome-wide mapping of Egr2 binding using ChIP-chip (or ChIP-Seq) in a physiologically relevant system will help elucidate the cognate elements that mediate gene regulation by Egr2 during PNS myelination. Moreover, it is expected that combining such studies with analyses of open chromatin (by DNaseI or FAIRE), cross-species sequence conservation, and colocalization with other transcription factors will accelerate discovery of the regulatory elements required for the developmental regulation of myelination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Egr2	early growth response 2
FAIRE	formaldehyde-assisted identification of regulatory elements
ChIP	chromatin immunoprecipitation
Ndrg1	N-myc downstream regulated gene 1
Nab	NGFI-A/Egr binding protein

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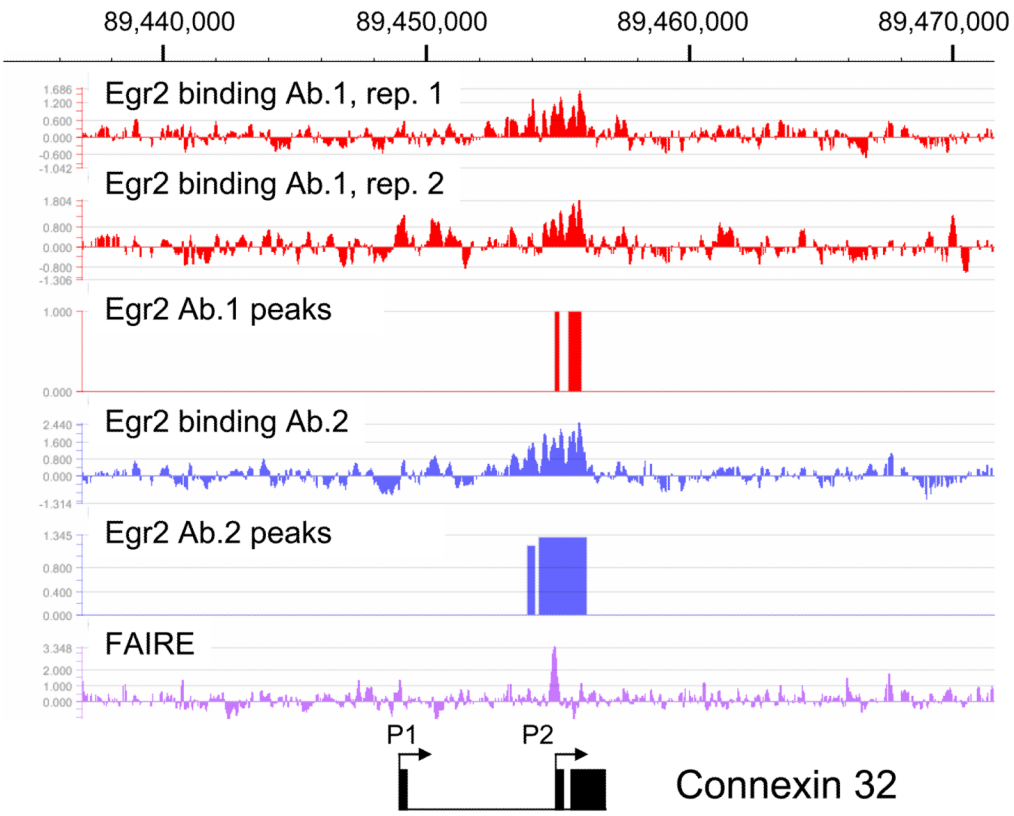

Figure 1. Egr2 binding in the *Connexin 32* (*Gjb1*) locus

Diagram shows peak data for binding of Egr2 within the rat *Gjb1* locus on chromosome X. In vivo ChIP assays were performed on rat sciatic nerve at postnatal day 15 (P15) using two independent antibodies to Egr2. The ChIP samples were labeled with Cy5 (Egr2) or Cy3 (total input DNA) for hybridization to the genomic tiling array. The enrichment ratio of Cy5 to Cy3 was plotted on a $\log_2$ scale and further processed to display a 5 point moving average. The top two tracks show replicates of Egr2 antibody 1, and the 4th track shows an independent assay with Egr2 antibody 2. Peak analysis was performed as described in the Methods section at a false discovery rate of <0.05. The bottom track shows analysis of sciatic nerve chromatin using the FAIRE technique to identify disruptions in nucleosomal structure. The two alternate promoters in the *Gjb1* locus are shown, along with the 3 exons (filled boxes). The P2 promoter is active in peripheral nerve.

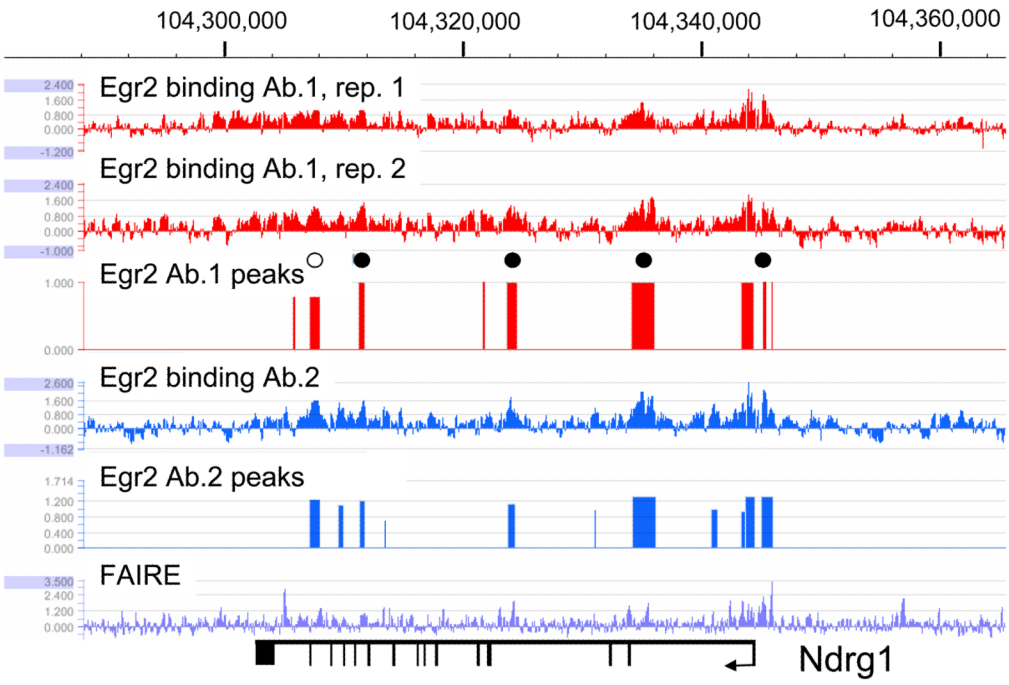

Figure 2. Egr2 is associated with the proximal promoter and intragenic region of the *Ndrg1* locus

In vivo ChIP-chip data for binding of Egr2 to the *Ndrg1* gene in rat sciatic nerve at postnatal day 15 (P15) was provided by the experiment described for Figure 1. Chromosome 7 numbering is from the Rn4 genome build, and exons are indicated by vertical lines in the diagram at the bottom. Circles in the diagram indicate selected peaks at which QPCR reactions on independent ChIP samples were used to validate binding (data not shown). Filled circles represent confirmed sites, and open circles indicate sites at which binding above background was not confirmed. Selected peaks were identified in both replicates for each Egr2 antibody.

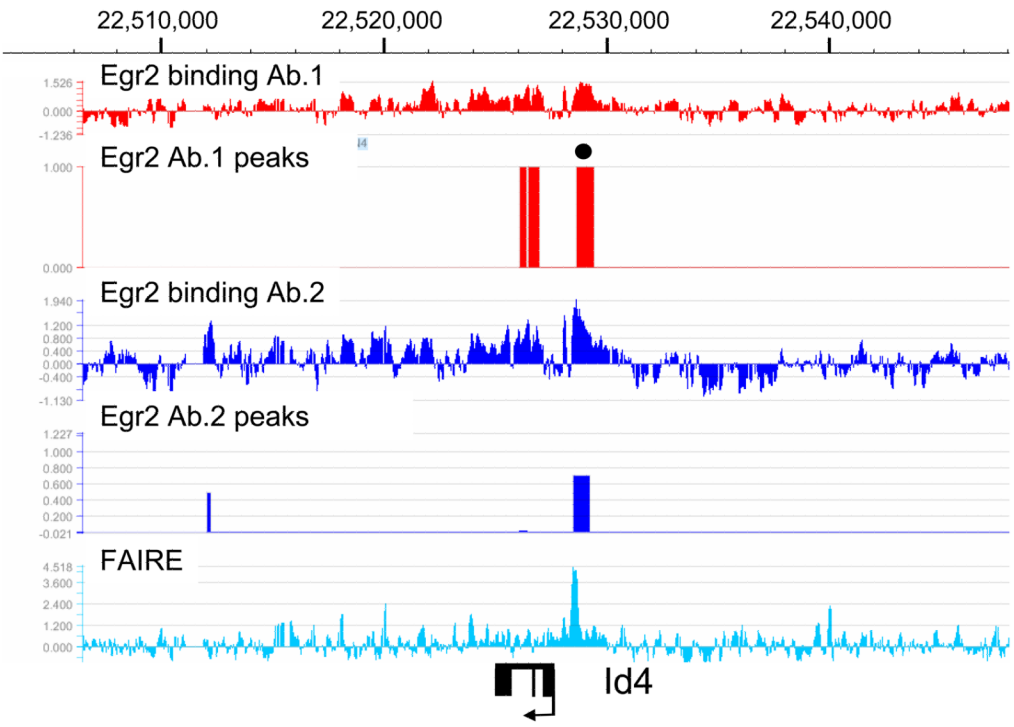

Figure 3. Egr2 binds to the repressed *Id4* gene

Binding of Egr2 is shown in the *Id4* gene on chromosome 17, which is activated in Egr2-deficient mice. Analysis and peak finding were performed as described in Figure 1. One peak (filled circle) was selected for validation by the ChIP-qPCR assay (data not shown). The FAIRE data show one major peak of open chromatin in the Id4 promoter. Exons for Id4 are indicated by filled rectangles.

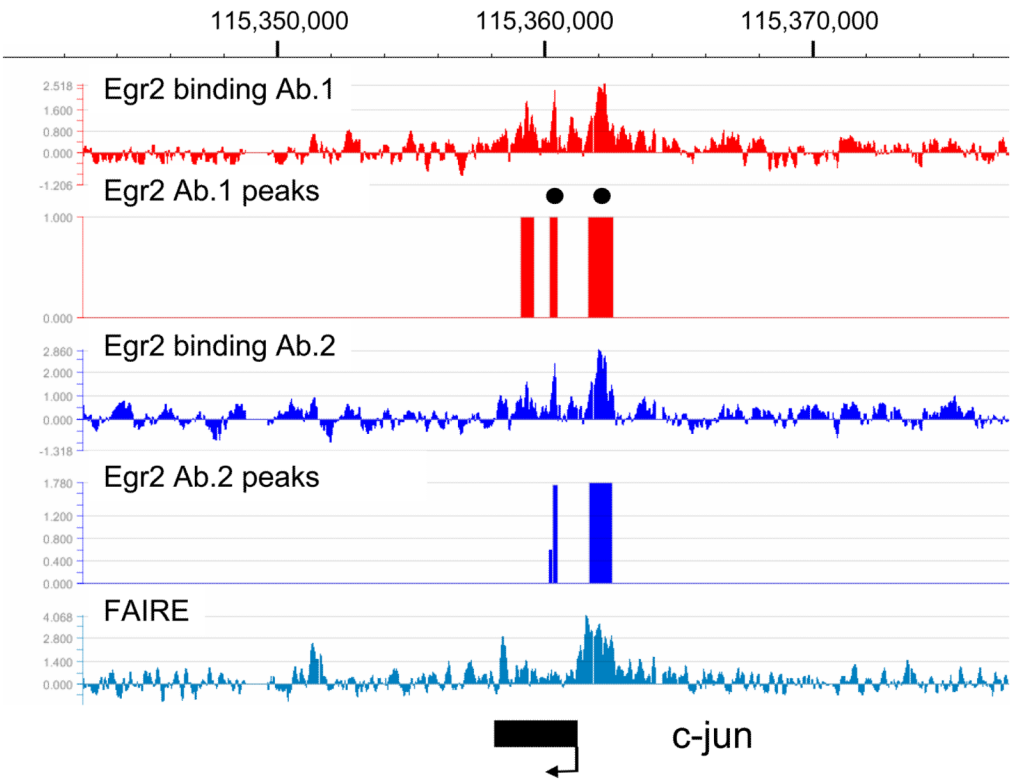

Figure 4. Egr2 binding in the *c-jun* locus

ChIP-chip analysis shows Egr2 binding patterns in the *c-jun* locus on chromosome 5, which is repressed by Egr2. Analysis and peak finding were performed as described in Figure 1. Two peaks (filled circles) were selected for validated by the ChIP-qPCR assay (data not shown). The diagram at the bottom indicates the position of the single exon constituting the *c-jun* gene.

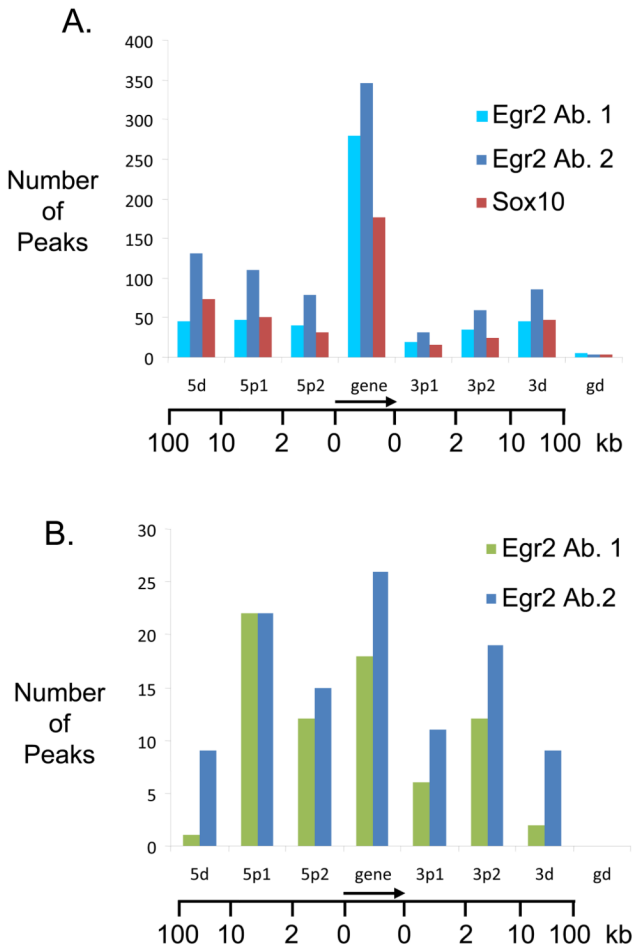

Figure 5. Location profile of Egr2 binding sites

A. The graph describes location of sites associated with Egr2 and Sox10 with respect to transcription start sites of genes present within the tiled regions of the custom array. Binding sites are classified as 5d (10kb–100kb upstream), 5p1 (2kb–10kb upstream), 5p2 (less than 2kb upstream), gene (any exon or intron), 3p1 (less than 2kb downstream), 3p2 (2kb–10kb downstream), 3d (10kb–100kb downstream), and gd (greater than 100kb).

B. The tiled array contains 15 genes that are elevated in the Egr2-deficient peripheral nerve (bold text in Table 1) and therefore apparently repressed by Egr2 activity. A similar location analysis was performed on this subset of genes as described in panel A.

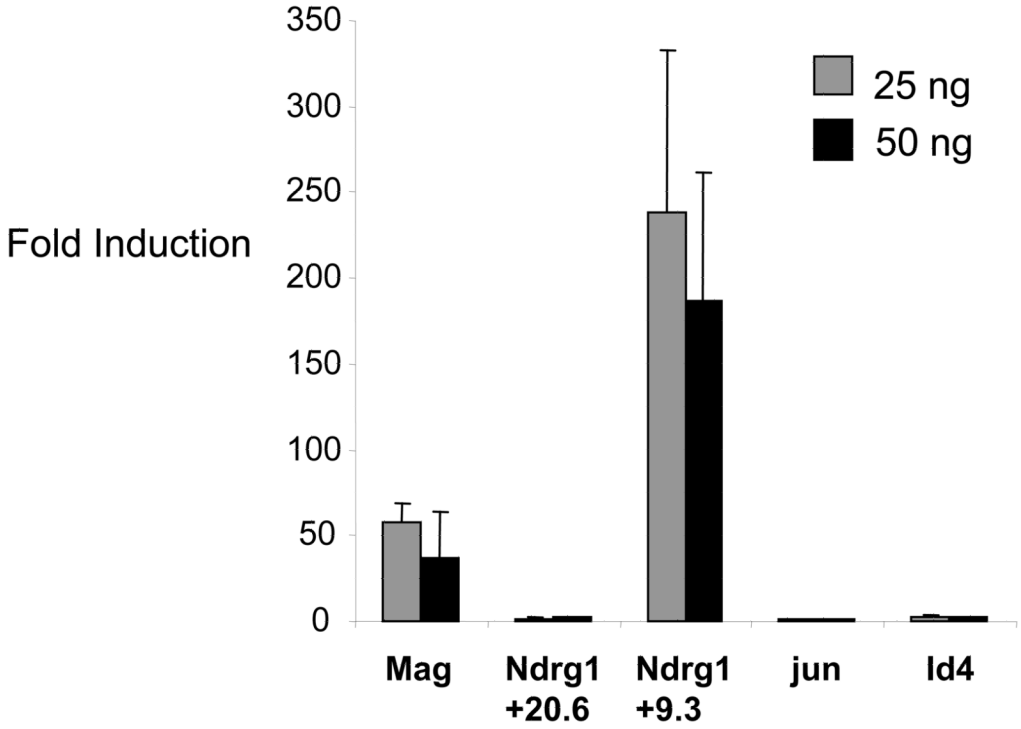

Figure 6. Transfection analysis of novel Egr2 binding sites

The indicated Egr2 binding sites were cloned upstream of a luciferase reporter gene and were transfected into B16/F10 cells along with increasing amounts (25 or 50 ng) of Egr2. Fold induction was calculated relative to the reporter activity in the absence of Egr2 for each construct. Id4 was activated 2.85- and 2.5-fold, respectively. Means and standard deviations for 6 independent assays are shown (for Mag, n=4).

Table 1

Egr2-regulated genes present on custom tiled array

Myelin Genes
*Connexin 32, Gjb1**
*N-myc downstream regulated gene, Ndrg1**
*Periaxin, Prx**
*Peripheral Myelin Protein 22, Pmp22**
Peripheral Myelin Protein 2, Pmp2
*Myelin Protein Zero, Mpz**
Myelin Basic Protein, Mbp
Myelin protein 11 kd, Mp11
Myelin-associated glycoprotein, Mag
Mal, myelin and lymphocyte protein
Plasmolipin, Pllp
Transcription Factors
NGFI-A binding protein 1, Nab1
NGFI-A binding protein 2, Nab2
Pou3f1/Scip
Pou3f2/Brn2
Inhibitor of differentiation 1, Id1
Inhibitor of differentiation 2, Id2
Inhibitor of differentiation 3, Id3
Inhibitor of differentiation 4, Id4
Sox2
T-box protein 2, Tbx2
c-myc
Early Growth Response, Egr1
c-jun
Genes mutated in peripheral neuropathies
*Neurofilament light, Nefl**
*Sox10**
*Myotubularin-related protein 2, Mtmr2**
*Early Growth Response 2, Egr2**
*Set-binding factor 2, Sbf2/Mtmr13**
lipopolysaccharide-ind. TNF factor, Litaf*
Signaling
Nerve growth factor receptor, Ngfr
Mapk8 interacting protein/jip1, Mapk8ip
Eph receptor B6, EphB6
Membrane metalloendopeptidase, Mme
Desert Hedgehog, Dhh
Ras homolog in diabetes, Rad

L1 cell adhesion molecule, L1cam

Lipid Synthesis/Transport

HMG CoA Synthase, Hmgcs1

HMG CoA Reductase, Hmgcr

Phosophomevalonate kinase, Pmvk

Mevalonate decarboxylase, Mvd

Isopentenyl diphosphate isomerase, Idi1

Farnesyl diphosphate synthase, Fdps

Farnesyl diphosphate farnesyl trans., Fdft1

Squalene epoxidase, Sqle

Lanosterol synthase, LSS

Lanosterol demethylase (Cyp51)

NADP-dependent steroid dehyd., NSDHL

Sterol C5 desaturase, Sc5d

Stearoyl CoA desaturase, Scd2

Lipoprotein lipase, Lpl

Spot14, Thrsp

Malic Enzyme, Me1

LDL receptor, Ldlr

UDP glycosyl transferase, Ugt8

Cell Cycle

Cdk inhibitor p21, Cdkn1a

Cdk inhibitor p57, Cdkn1c

Cyclin D1, Ccnd1

Genes in bold text are induced in Egr2-deficient peripheral nerve (Le et al. 2005a; Le et al. 2005b), and those in normal text are reduced (with the exception of *Nab2*). Genes that are mutated in human peripheral neuropathies are marked with an asterisk. Additional genes in this category are listed in the lower left column. A complete listing of tiled genes and the chromosomal coordinates are included in Supplemental Table S1.

Table 2

Peak overlap analysis in sciatic nerve ChIP-chip data

1st ↓ 2nd →	Egr2-Ab.1	Egr2-Ab.2	Sox10
Egr2-Ab.1	--	65.51%	32.35%
Egr2-Ab.2	47.98%	--	25.56%
Sox10	64.39%	72.68%	--

Each of the numbers describes the percentages of peaks within a given dataset listed in the first column that overlap with peaks in an independent data set listed in the first row. For example, 64.39% of the Sox10 peaks were found to overlap with peaks in the Egr2 Ab.1 data set. Each of the peak sets are defined as those peaks that were positive in two independent replicate experiments for that antibody. The minimum required overlap is 1 bp.

Table 3

Predicted Composite Binding Sites for Egr2 and Sox10

Gene	Predicted Sox10 sites	Predicted Egr2 sites
Ndrg1 (+9.5 kb)	AACAAAGtcagTTTTATA CACAAAGgccaCAGTGAG	**GCGTAGGCG**
Jun promoter	GAAAATGaagTGTTGTG	**GAGGGGGAG**
Id4 promoter	AACAAGA	**GCGCGGGCG** **GCGAGGGCG**
Mapk8ip (+5kb)	AACAAAAggctcTTATGAA	ATGGGGGCA

Predicted binding sites for Sox10 were identified in comparison to a matrix of inverted dimeric sites (Jones et al. 2007), based on previous characterization of Sox10 binding sites (Peirano and Wegner 2000). Egr2 sites in bold text have been shown previously to bind Egr2 in vitro (Nardelli et al. 1992; Swirnoff and Milbrandt 1995). Predicted binding sites for all overlapping peaks are presented in Supplementary Table 5.