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Active Gene Repression by the Egr2·NAB Complex during Peripheral Nerve Myelination*[S]

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Gennifer M. Mager[‡1], Rebecca M. Ward[§], Rajini Srinivasan[§], Sung-Wook Jang[¶], Lawrence Wrabetz[‖], and John Svaren[§2]**

*From the [‡]Molecular and Cellular Pharmacology Training Program, [§]Department of Comparative Biosciences, [¶]Graduate Program in Cellular and Molecular Biology, and **Waisman Center, University of Wisconsin, Madison, Wisconsin 53706 and the [‖]Dipartimento di Ricerca Biologica e Technologica, San Raffaele Scientific Institute, Milan 20132, Italy*

The Egr2/Krox20 transactivator is required for activation of many myelin-associated genes during peripheral nerve myelination by Schwann cells. However, recent work has indicated that Egr2 not only activates genes required for peripheral nerve myelination but may also be involved in gene repression. The NAB (NGFI-A/Egr-binding) corepressors interact with Egr2 and are required for proper coordination of myelin formation. Therefore, NAB proteins could mediate repression of some Egr2 target genes, although direct repression by Egr2 or NAB proteins during myelination has not been demonstrated. To define the physiological role of NAB corepression in gene repression by Egr2, we tested whether the Egr2·NAB complex directly repressed specific target genes. A screen for NAB-regulated genes identified several (including *Id2*, *Id4*, and *Rad*) that declined during the course of peripheral nerve myelination. *In vivo* chromatin immunoprecipitation analysis of the myelinating sciatic nerve was used to show developmental association of both Egr2 and NAB2 on the *Id2*, *Id4*, and *Rad* promoters as they were repressed during the myelination process. In addition, NAB2 represses transcription by interaction with the chromodomain helicase DNA-binding protein 4 (CHD4) subunit of the nucleosome remodeling and deacetylase chromatin remodeling complex, and we demonstrate that CHD4 occupies NAB-repressed promoters in a developmentally regulated manner *in vivo*. These results illustrate a novel aspect of genetic regulation of peripheral nerve myelination by showing that Egr2 directly represses genes during myelination in conjunction with NAB corepressors. Furthermore, repression of *Id2* was found to augment activation of *Mpz* (myelin protein zero) expression.

The early growth response-2 (Egr2/Krox20)[3] transcriptional activator is critically required for peripheral nerve myelination by Schwann cells. Peripheral nerve from *Egr2/Krox20*-deficient mice is characterized by arrested myelination and decreased myelin protein levels (1, 2), and recent analysis has indicated that *Egr2/Krox20* is required for maintenance of peripheral myelin (3). In addition, mutations in *EGR2* have been identified in several patients with myelin disorders such as Charcot-Marie-Tooth (CMT) disease, Dejerine-Sottas syndrome, and congenital hypomyelinating neuropathy (4–6) (reviewed in Ref. 7). Egr2 is induced in Schwann cells at the onset of myelination (1, 8) and is required for activation of a variety of genes involved in peripheral nerve myelination *in vivo* including *Mpz* (myelin protein zero) and *Pmp22* (peripheral myelin protein-22), as well as genes involved in lipid biosynthesis (1, 2, 9). Recent work has demonstrated sites of Egr2 occupancy within activated myelin genes *in vivo* (10, 11).

One of the *EGR2* mutations associated with a very severe congenital hypomyelinating neuropathy (I268N (4)) prevents binding of EGR2 to NGFI-A/Egr-binding (NAB1 and NAB2) corepressors (12), which repress Egr-mediated transcription (13–15). The importance of NAB corepressors to the regulation of peripheral nerve myelination by Egr2 was recently confirmed by the demonstration that a double knock-out of the *NAB1*/*NAB2* genes results in a phenotype very similar to that of the Egr2 knock-out, *i.e.* early lethality and peripheral neuropathy resulting from arrested myelination (16).

Expression analysis of Egr2-deficient mice identified several genes induced in the absence of Egr2 (2, 3), and some of these are also induced in NAB knock-out mice (16). Based on expression data, it has been proposed that Egr2 acts as a repressor of specific genes in peripheral myelin development. However, these analyses have compared expression patterns of wild type (myelinating) Schwann cells with Egr2-deficient Schwann cells that 1) fail to myelinate, 2) have increased rates of proliferation and apoptosis, and 3) exhibit up-regulation of some transactivators such as Sox2 and Scip/Oct6 (2, 3, 17). Therefore, given

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

This paper is dedicated to the memory of Brad Sevetson.

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[2] To whom correspondence should be addressed: 2015 Linden Dr., University of Wisconsin, Madison, WI 53706. Tel.: 608-263-4246; Fax: 608-263-3926; E-mail: jpsvaren@wisc.edu.

[3] The abbreviations used are: EGR, early growth response; CHD, chromodomain helicase DNA binding; NAB, NGFI-A/Egr1-binding protein; Rad, Ras homolog in diabetes; TSA, trichostatin A; ChIP, chromatin immunoprecipitation; NuRD, nucleosome remodeling and deacetylase; MTA2, metastasis-associated antigen 2; PMP, peripheral myelin protein; Hsp, heat shock protein; NF-L, neurofilament L; NF-M, neurofilament M; CMT, Charcot-Marie-Tooth; P, postnatal day (*e.g.* P7); RT, reverse transcription; siRNA, small interfering RNA.

the profound differences in Schwann cells used in these comparisons, it is not clear that Egr2 is directly involved in repressing any genes.

The goal of this study was to define the molecular role of Egr2 and NAB proteins in repression of gene expression during myelination. Although Egr2 has been considered principally as an activator of myelin gene expression, our study reveals that an Egr2·NAB complex directly represses specific genes during peripheral nerve myelination *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture and Microarray Analysis—Primary rat Schwann cells were cultured and infected as described (18) using recombinant adenoviruses prepared using the AdEasy system (19). For microarray analysis, rat Schwann cells were infected for 48 h with recombinant adenovirus (1.5×10^9 plaque-forming units/ml) expressing Egr2 in the presence or absence of a virus expressing dominant-negative NAB2 E51K (5×10^9 plaque-forming units/ml). Digoxigenin-labeled cRNA was prepared and analyzed by chemiluminescent detection on an Applied Biosystems 1700 rat array (GenUs Biosystems, Chicago). GeneSpring software was used to analyze for genes that differed more than 2-fold in samples expressing dominant-negative NAB2, and that had a signal/noise ratio of >4 in the induced sample (see supplemental Table 1). Treatments with trichostatin A (TSA) were performed as described (20). For expression of dominant-negative Egr2, recombinant adenovirus was prepared to express the DNA-binding domain of Egr2 (amino acids 330–470). The control siRNA (a medium GC content control) and *Id2* Stealth siRNAs were obtained from Invitrogen. Rat Schwann cells were either cultured in Dulbecco's modified Eagle's medium with 5% bovine growth serum (Hyclone) or transferred to a modified N2 medium (50% Dulbecco's modified Eagle's medium and 50% Ham's F12 medium with an insulin/transferrin/selenium supplement) prior to transfection with 80 pmol of either control or a pool of three *Id2* siRNAs with Lipofectamine 2000 and then treated 24 h later with forskolin (2 μM)/insulin-like growth factor-1 (150 ng/ml) or dimethyl sulfoxide as a control for an additional 24 h.

Quantitative PCR—cDNA was prepared from total RNA and analyzed by quantitative RT-PCR using SYBR Green dye as described (9) on a TaqMan 7000 sequence detection system (Applied Biosystems). Relative amounts of the indicated genes between samples were determined using a comparative Ct method (21) and normalized to relative levels of 18 S rRNA. Primer sequences are available upon request. The *Krox20* null (1) allele (*Krox20*-null mice were the gift of Dr. Giovanni Levi, Genova, Italy) was maintained on the DBA2/B6 genetic background to promote survival of *Egr2/Krox20*-null animals. For *Egr2/Krox20* wild type and homozygous-null P7 littermates, total RNA was prepared from pools of 20 sciatic nerves as described (9).

In Vivo Chromatin Immunoprecipitation—Sciatic nerves were pooled from P5 and P15 Sprague-Dawley rat pups (from ~10–13 pups at each time point). Freshly dissected nerves were immediately minced in phosphate-buffered saline containing 1% formaldehyde for 25 min at room temperature. Cross-linked chromatin was prepared, sonicated, and immunoprecipitated as described previously (10, 20) with 2 μg of anti-Egr2/Krox20 (Covance), anti-Nab2 (Santa Cruz Biotechnology, sc-22815), anti-CHD3/4 rabbit polyclonal (Santa Cruz sc-11378), anti-MTA2 goat polyclonal (Santa Cruz sc-9447), normal rabbit IgG (Upstate), or normal goat IgG (Santa Cruz sc-2028) control antibody. After reversal of cross-links and DNA purification, quantitative PCR was performed on samples in duplicate using primers to the *Rad* (Ras homolog in diabetes) and *IMG2a* promoters (20): *Id2*, −2607/−2551 bp, forward (GAAAGGCGAGAGCTCCCAAT) and reverse (GGGCGAGTCTCAAGGTCTTCT); +42/+92 bp, forward (CGGCCTTTCCTCCTACGAG) and reverse (CGGACCTCACCGGACTGA); *Id4*, −427/−363 bp, forward (GCGCGGCTCTACAAATACTGC) and reverse (AACTGTGCCTCCCAGCTCAAC); and −2098/−2041 bp, forward (TGCACACACAAAAGCAAAGGA) and reverse (GGCCACTGTCCCTAATTATTGG). Values are expressed as percent recovery compared with the input into the immunoprecipitation.

Promoter Analysis—Sequence analysis for Egr2 binding sites in aligned genomic sequence was performed using the rVISTA program (22), and putative Egr2 sites were confirmed by comparison with the previously defined consensus Egr2 binding site (23). The rat *Rad* promoter (−325 to +25) was cloned into the KpnI/HindIII sites of pGL2 (Promega). A deletion of Egr2 binding sites (−195 to −110) from the *Rad* promoter was created by site-directed mutagenesis. The pGLId2–2750 promoter construct (provided by Antonio Iavarone, Columbia University (24)) was modified to include nucleotides up to −3029.

Transfection Assays—S16 rat Schwann cells (25) were transfected as described (20) using LT-1 transfection reagent (Mirus) according to the manufacturer's protocol with 250 ng of the indicated luciferase reporter plasmid, 100 ng of a LacZ reporter driven by a cytomegalovirus promoter, the indicated expression plasmids, and pBluescript as required to make a total of 1 μg of DNA/well. After 48 h, luciferase activity was measured and normalized to β-galactosidase activity.

RESULTS

Identification of NAB-regulated Genes—As described above, profiling studies have identified a number of genes induced in peripheral nerve of *Egr2*-deficient mice (2, 3). To identify candidate genes that are directly repressed by Egr2 in a NAB-dependent manner, we employed a screen in which primary rat Schwann cells were infected with recombinant adenoviruses to express Egr2 in the absence and presence of dominant-negative NAB2 (15), which eliminates the function of both NAB1 and NAB2. This dominant negative contains a point mutation that selectively prevents interaction with EGR factors, yet it retains the ability to multimerize with other NAB proteins.

In initial experiments, dominant-negative NAB2 had little effect on the activation by Egr2 of a previously identified NAB-regulated gene, *Pthrp* (parathyroid hormone-related peptide) (16). However, the effect of dominant-negative NAB2 cannot be observed if ectopic expression increases Egr2 to superstoichiometric levels relative to the level of endogenous NAB proteins. Therefore, the experiment was repeated with reduced concentrations of Egr2-expressing virus, and the effect of dominant-negative NAB2 was much more evident at lower levels of

Egr2 expression (Fig. 1). RNA samples from cells with the lower levels of Egr2 expression were therefore used in a candidate screen for NAB-regulated Egr2 target genes. Rat Schwann cells were infected with Egr2-expressing adenovirus in the absence and presence of dominant-negative NAB2, and the resulting microarray analysis identified ~700 genes (supplemental Table 1) that are induced more than 2-fold by dominant-negative NAB2, although many of these may not be direct target genes. In addition, the *Pthrp* assays in Fig. 1 also revealed that the regulation of these genes in cultured Schwann cells is dependent on the relative ratios of NAB to Egr2 expression. Since ectopic expression of Egr2 does not necessarily recapitulate physiological levels of Egr2 and NAB expression, further analysis was directed toward identifying which of these genes may be directly down-regulated by the Egr2·NAB complex during myelination *in vivo*.

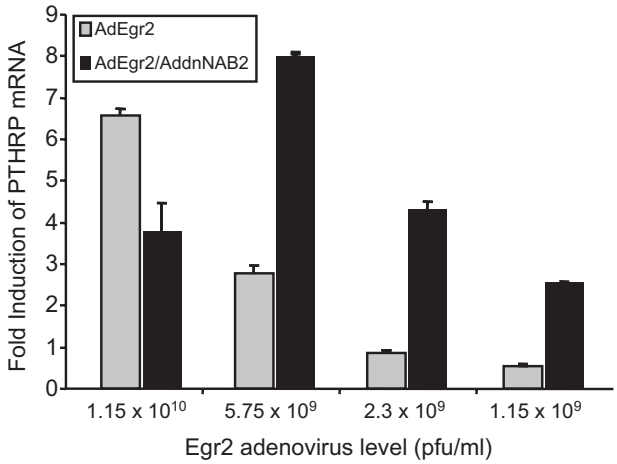

FIGURE 1. **Use of dominant-negative NAB2 to identify NAB-regulated target genes in Schwann cells.** Primary rat Schwann cells were infected with decreasing concentrations (as indicated) of adenovirus expressing Egr2 in the absence and presence of adenovirus expressing dominant-negative NAB2 (E51K; 4.6×10^9 plaque-forming units (*pfu*)/ml). After 48 h, total RNA was isolated for each sample, and quantitative RT-PCR was used to determine endogenous levels of parathyroid hormone-related peptide (*PTHRP*). After normalizing to 18 S rRNA, -fold induction was determined relative to untreated control.

Analysis of Candidate Genes Repressed by the Egr2·NAB Complex—The list of NAB-repressed genes was compared with previously published microarray analyses of peripheral myelination to identify NAB-regulated genes that decrease during the course of peripheral nerve myelination (26–28), consistent with their inhibition by an Egr2·NAB complex (Table 1). For example, the AP-2γ and Scip/Oct6 transcription factors decline during the course of peripheral nerve myelination (8, 29). We also examined whether any of these genes were up-regulated in a similar profiling analysis of peripheral nerve injury (30), as Egr2 declines during the demyelination period subsequent to nerve injury (8, 31) This analysis showed that expression of several NAB-regulated genes is inversely correlated with Egr2 levels *in vivo* (Table 1). In addition, several of these genes are up-regulated in the NAB knock-out mice (16).

Of the NAB-regulated genes selected by these criteria (Table 1), the Id proteins (inhibitors of DNA binding/differentiation) are particularly interesting, not only for their ability to promote proliferation (reviewed in Ref. 32) but also because Id2 and Id4 inhibit oligodendrocyte differentiation (33–36). Schwann cells express all members of the Id family, but Id2 and Id4 levels decline during the course of myelination and are expressed at relatively low levels in mature nerve (26, 37, 38). Another gene, *Rad* (Ras homolog in diabetes), modulates Rho signaling (39), which controls Schwann cell migration and the length of myelinated segments (40, 41).

Interestingly, several genes that are induced in the absence of Egr2 were not identified in this screen for NAB-regulated genes. For example, recent work has shown that *Sox2* is down-regulated during myelination and is reactivated in the absence of Egr2 (2, 3), but *Sox2* was not identified in this analysis. Similarly, other developmentally down-regulated genes identified by cluster analysis of myelinating peripheral nerve (26, 28) were not identified in the screen. In addition, only some of the Egr2-

TABLE 1

Microarray analysis of NAB-regulated Egr2 target genes

Microarray analysis was used to identify genes that are derepressed by dominant-negative (dom. neg.) NAB2 in primary rat Schwann cells. Unless otherwise cited, the listed genes include those that have been shown to decrease during myelination (development) in previously published microarray analyses of peripheral nerve myelination in development (26) or to increase during the demyelination subsequent to nerve injury (30). Asterisks indicate genes that are known to be mutated in cases of human peripheral neuropathies. Down-regulation of Id proteins and AP-2γ was shown previously (29, 37, 38) The last column indicates those genes that were reported to be induced in the sciatic nerve of the *NAB1/NAB2* double knock-out (KO) mouse (16). NR means that the gene was either not represented or not reported in previous microarray analyses, and ↔ denotes no significant change. PMP2 is listed as a control in some experiments.

NAB-regulated Egr2 target genes	Fold induction dom. neg. NAB2	Development	Injury	*NAB* KO
Signaling proteins				
Hck	71	Decrease	↔	Increase
Rad	4.3	Decrease	Increase	Increase
IGFBP3	9.1	Decrease	Increase	NR
Transcriptional regulation				
Id4	2.9	Decrease	Increase	Increase
Id2	2.1	Decrease	Increase	Increase
Sox11	12.2	Decrease	↔	NR
Pou3f1; *Scip*	5.8	Decrease	Increase	Increase
myc	5.2	Decrease	Increase	Increase
AP-2γ	10.1	Decrease (29)	NR	NR
Hey1	6.8	Decrease (27)	Decrease	NR
Other				
Myelin P2: *Pmp2*	1.3	Increase	Decrease (73)	NR
Neurofilament M: *Nef3*	11.35	Decrease (28)	Decrease (28)	NR
Neurofilament L: *Nefl**	10.9	NR	Decrease (28)	NR
Heat shock protein: *Hspb8**	40.9	Increase	NR	NR

regulated genes are derepressed by dominant-negative NAB2. For example, *Pmp2* (peripheral myelin protein P2) is a developmentally regulated myelin gene (42), that is induced by Egr2 expression in primary Schwann cells (Ref. 18 and Fig. 8*A*). However, dominant-negative NAB2 does not affect the expression level of *Pmp2* significantly.

Finally, this screen also identified potential regulation of neuropathy-associated genes by the Egr2·NAB complex (Table 1). For example, neurofilament L (*NF-L*) was not previously identified as an Egr2 target gene, although it is mutated in CMT2E and interacts with MTMR2 (Myotubularin-related 2), which is also mutated in CMT (43). Both *NF-L* and *NF-M* are expressed not only in neurons but also in Schwann cells (44, 45), and their expression is reduced after peripheral nerve injury (28). *NF-M* had also been identified in the initial screen of Egr2 target genes (18). The heat shock protein HspB8 has also been identified as mutated in inherited peripheral neuropathies (46, 47). However, the expression pattern of these genes suggests that they are not developmentally repressed, and therefore they were not analyzed further.

Expression of NAB-regulated Target Genes in Egr2-null Mice—If the Egr2·NAB complex actively represses a gene during peripheral nerve myelination, such a gene should be induced in both *Egr2* and *NAB* knock-out mice. Indeed, several of the NAB-regulated genes (*Id2*, *Id4*, *Hck, Pou3f1/Scip*, *myc*) are up-regulated in mice with a hypomorphic allele of Egr2 (2, 16), consistent with active repression by the Egr2·NAB complex during myelination. However, partial expression of Egr2 in the *Egr2* hypomorph could complicate interpretation of these data. Therefore, mRNA expression levels of several selected genes were evaluated in *Egr2/Krox20*-null mice (1) at P7 to determine whether these and other genes identified in our screen are up-regulated in the complete absence of Egr2 *in vivo*. The expression of *Rad*, *Hck*, *Id2*, *Id4*, and *Sox11* increased severalfold in Egr2-null mice compared with wild type littermates (Fig. 2*A*). Because the expression of many of these genes continues to decline beyond P7 (26), the increased expression in the absence of Egr2 could be higher at later time points, but the *Egr2* knockout mice do not remain viable much beyond P7 (1). In contrast, using the same samples, we have shown that a developmentally increased gene, *Mpz*, is dramatically decreased (11), and levels of *Pmp2* are also reduced in the Egr2 null samples (Fig. 2).

To independently test the role of Egr2 in the regulation of these genes, we infected rat Schwann cells with adenovirus expressing a dominant-negative Egr2, consisting of only the DNA-binding domain of Egr2 (48), which blocks binding of endogenous Egr2 and inhibits both transactivation and recruitment of NAB corepressors. Levels of the *Rad*, *Id2*, and *Id4* genes were induced in the presence of dominant-negative Egr2 (Fig. 2*B*). As a negative control, dominant-negative Egr2 expression did not affect the levels of *UbcE2L3* (ubiquitin-conjugating enzyme E2L3), which is expressed at moderately high levels with little variation during peripheral nerve myelination (9, 26). In summary, these data are consistent with the hypothesis that Egr2 can be an active repressor, in conjunction with NAB proteins, to repress expression of specific genes (*Rad*, *Hck*, *Id2*, *Id4*, *Sox11*) during myelination of peripheral nerve.

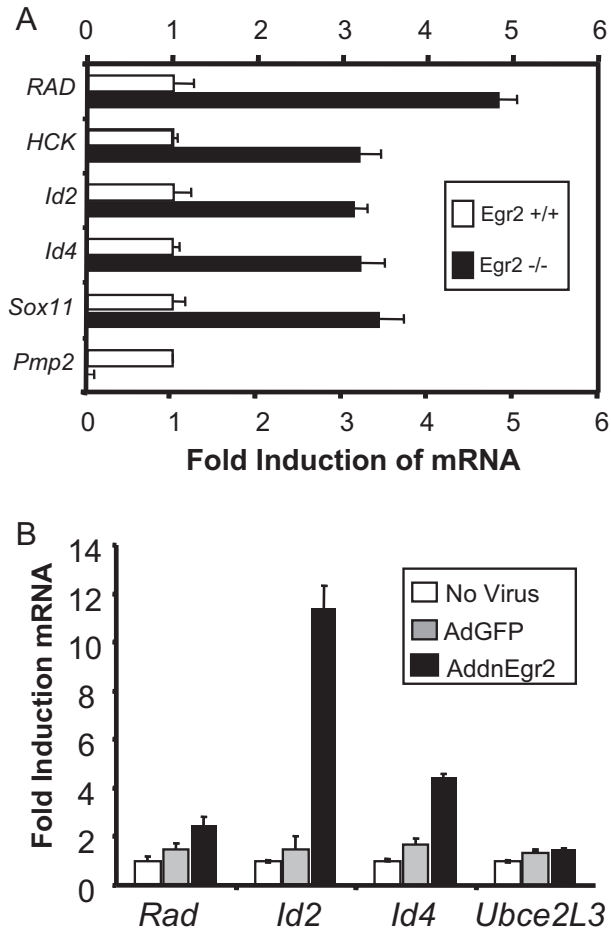

FIGURE 2. **Loss of Egr2 induces expression of NAB-regulated genes.** *A*, sciatic nerves were isolated from wild type (+/+), and *Egr2* knock-out (−/−) mice at postnatal day 7. The relative levels of each gene, for each genotype, are indicated relative to the wild type sample, which is set as 1. Levels are determined from a pool of at least 10 mice/genotype, normalized to 18 S rRNA. *B*, rat Schwann cells were infected with adenoviruses expressing GFP or dominant-negative Egr2 as indicated. Total RNA was isolated for each sample, and quantitative RT-PCR was used to determine endogenous levels of *Rad*, *Id2*, *Id4*, and *Ubce2L3*. After normalizing to 18 S rRNA, -fold induction was determined relative to uninfected control for each gene. Means ± S.D. was determined for duplicate measurements in two replicate experiments.

Egr2 and NAB2 Bind and Repress Promoters during Myelination of Peripheral Nerve—Although several of the NAB-regulated genes were induced in the absence of Egr2, these results did not demonstrate that an Egr2·NAB complex directly represses these genes. To test whether Egr2 or NAB2 regulates the promoters of these genes, we used *in vivo* chromatin immunoprecipitation (ChIP) assays of myelinating peripheral nerve, in which formaldehyde is used to covalently cross-link DNA with associated proteins. Because Egr2 expression is low at P0 in rat sciatic nerve and increases as myelination initiates during the first 7–10 days after birth (8), we tested whether Egr2 occupancy at a promoter increases as the promoter is repressed during myelination. Consistent with the developmental regulation of Egr2 levels, we observed little or no binding of Egr2 to other sites (*Mpz* (11)) at P5, compared with much higher levels at P15 (data not shown). Therefore, freshly dissected sciatic nerves were pooled from ~10–13 rat pups at ages P5 (early myelination) or P15 (late myelination) and minced in 1% form-

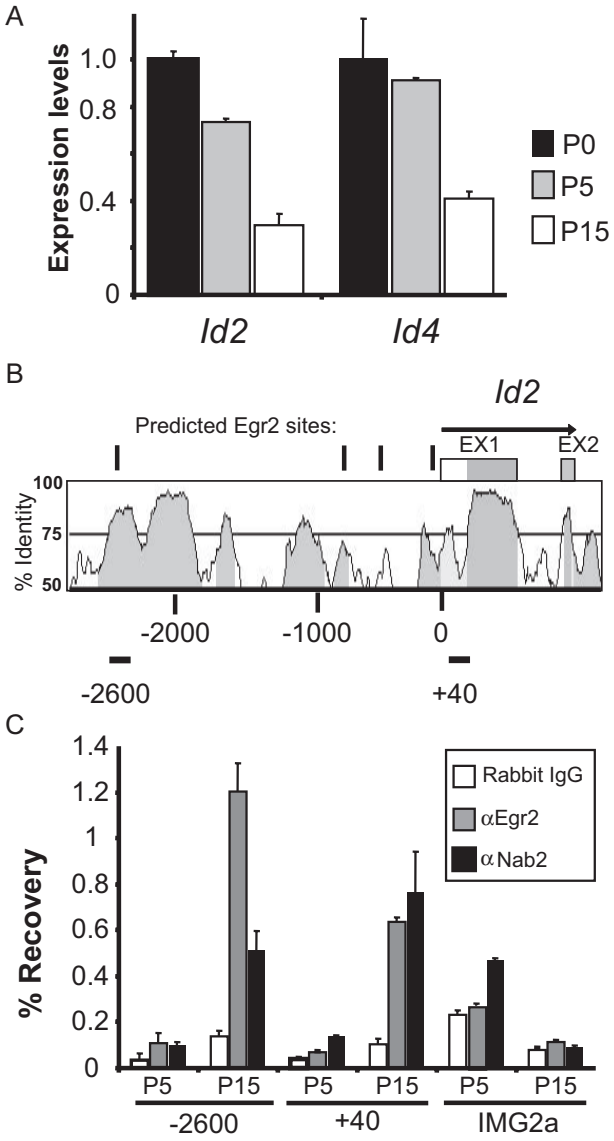

FIGURE 3. **Developmental regulation of Egr2 and NAB2 binding to the *Id2* promoter *in vivo*.** *A*, relative levels of gene expression for *Id2* and *Id4* in sciatic nerves from P0, P5, and P15 rats were determined by quantitative PCR. Levels are calculated relative to the P0 sample, which was set as 1 for each gene. Reactions were performed in triplicate, and the standard error is indicated. *B*, the plot shows percent identity of the rat and human *Id2* loci. The first two exons of the *Id2* gene, as well as about 3 kb of the upstream sequence, are shown. Predicted Egr2 binding sites are indicated by *vertical lines* above the homology plot. *Horizontal lines* indicate the amplicon locations used in the ChIP assays. *C*, cross-linked chromatin was prepared from pooled sciatic nerves obtained from rat litters at P5 and P15. Sonicated chromatin was immunoprecipitated with antibodies directed against Egr2, Nab2, or purified rabbit IgG as a negative control. Purified DNA was then analyzed by quantitative PCR using the indicated primers. The *y* axis indicates the percentage of DNA recovered relative to the input sample. The data are representative of three independent sets of pooled nerves at each time point. The silent *IMG2a* gene was used as a negative control.

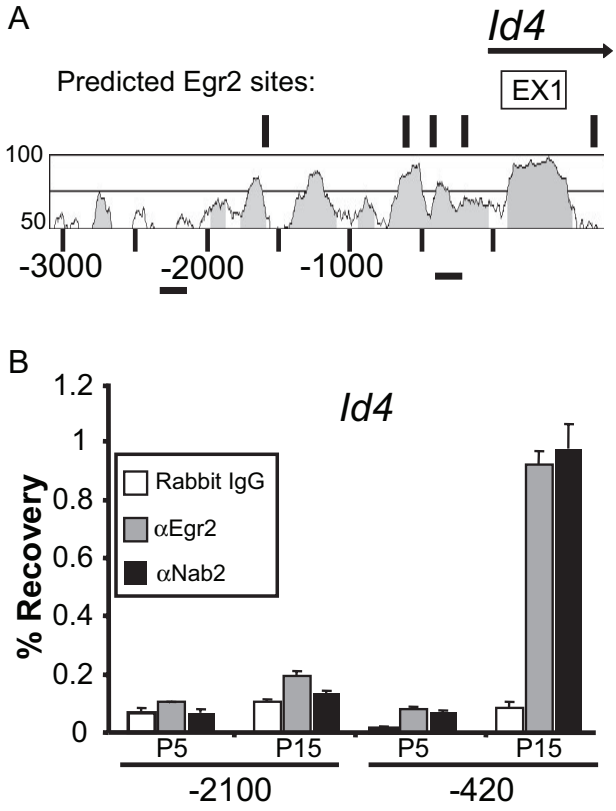

FIGURE 4. **Developmental regulation of Egr2 and NAB2 binding to the *Id4* promoter *in vivo*.** *A*, the plot shows the percent identity of the rat and human *Id4* loci from 3 kb upstream through the first exon of the *Id4* gene. Predicted Egr2 binding sites are indicated by the *vertical lines* above the homology plot. *Horizontal lines* indicate the amplicon locations used in the ChIP assays. *B*, with the same set of samples and protocol as described for Fig. 3, ChIP assays were performed using the indicated primer sets to evaluate the binding of Egr2 and NAB2. The data are representative of three independent sets of pooled nerves at each time point. The negative control ChIP assays using a silent gene (*IMG2a*) are shown in Fig. 3.

aldehyde. After cross-linking, sonicated chromatin from the pooled nerves was immunoprecipitated with antibodies to Egr2, NAB2, or IgG control. After washing and reversal of the cross-links, purified DNA was analyzed by quantitative PCR using primers designed to specific promoter sites.

We initially focused on *Id2* and *Id4* as potential direct targets of Egr2·NAB repression (Fig. 3*A* and Refs. 26, 37, and 38). Analysis of the *Id2* promoter identified several conserved Egr2 binding sites upstream of the transcription start site (Fig. 3*B*) in the mouse, rat, and human *Id2* genes. The ChIP assay revealed significant enrichment of Egr2 at −2600 and the proximal promoter compared with the IgG control immunoprecipitation (Fig. 3*C*), with intermediate levels at the −847/−797 site (data not shown). In addition, our analysis revealed that enrichment of Egr2 at −2600 and in the proximal promoter increases dramatically from P5 to P15. NAB2 enrichment on the *Id2* promoter parallels that of Egr2, increasing over development, with the highest level of binding over background at the proximal promoter. As negative controls, neither factor is significantly enriched in the silent immunoglobulin 2A gene (*IMG2a*).

Analysis of the *Id4* promoter identified three predicted Egr2 binding sites within 600 bp of the promoter (Fig. 4*A*). Significant binding of Egr2 and NAB2 compared with the IgG control was observed using a −427/−363 primer set, and the level of occupancy was much higher at P15 compared with that at P5 (Fig. 4*B*). Egr2 and NAB2 were not significantly enriched using a primer set positioned further upstream (−2100). Overall, these data indicate increasing association of Egr2·NAB2 with promoters of the *Id2* and *Id4* genes, as they are repressed during peripheral nerve myelination.

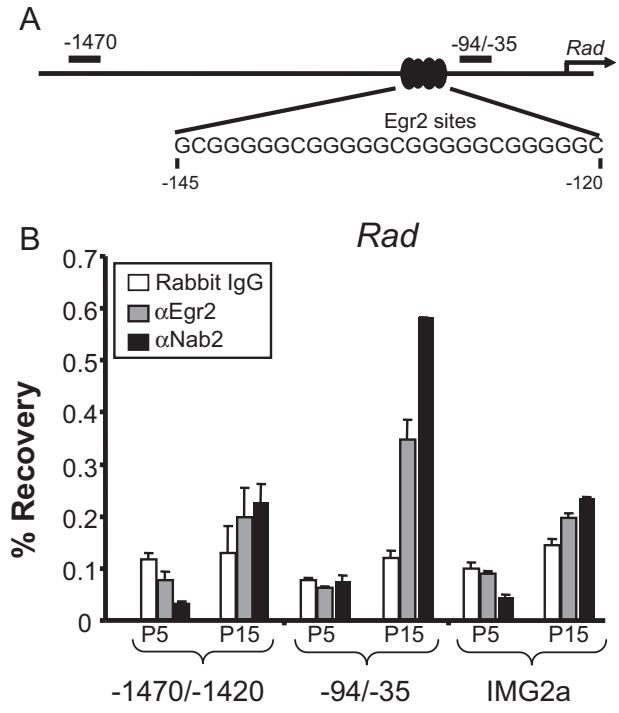

FIGURE 5. **Developmental regulation of Egr2 and NAB2 binding to the *Rad* promoter *in vivo*.** *A*, the diagram shows the location and sequence of conserved Egr2 binding sites in the *Rad* promoter relative to the transcription start site. The *short lines* indicate the amplicon locations used to detect binding in the ChIP assays. *B*, cross-linked chromatin was prepared from pooled sciatic nerves obtained from rat litters at P5 and P15. Sonicated chromatin was immunoprecipitated with antibodies directed against Egr2, Nab2, or purified rabbit IgG as a negative control. Purified DNA was then analyzed by quantitative PCR using the indicated primers. The *y* axis indicates the percentage of DNA recovered relative to the input sample. The data are representative of two independent sets of pooled nerves at each time point. Primer sets are situated at −1470 to −1420 and −94 to −35 relative to the *Rad* transcription start site.

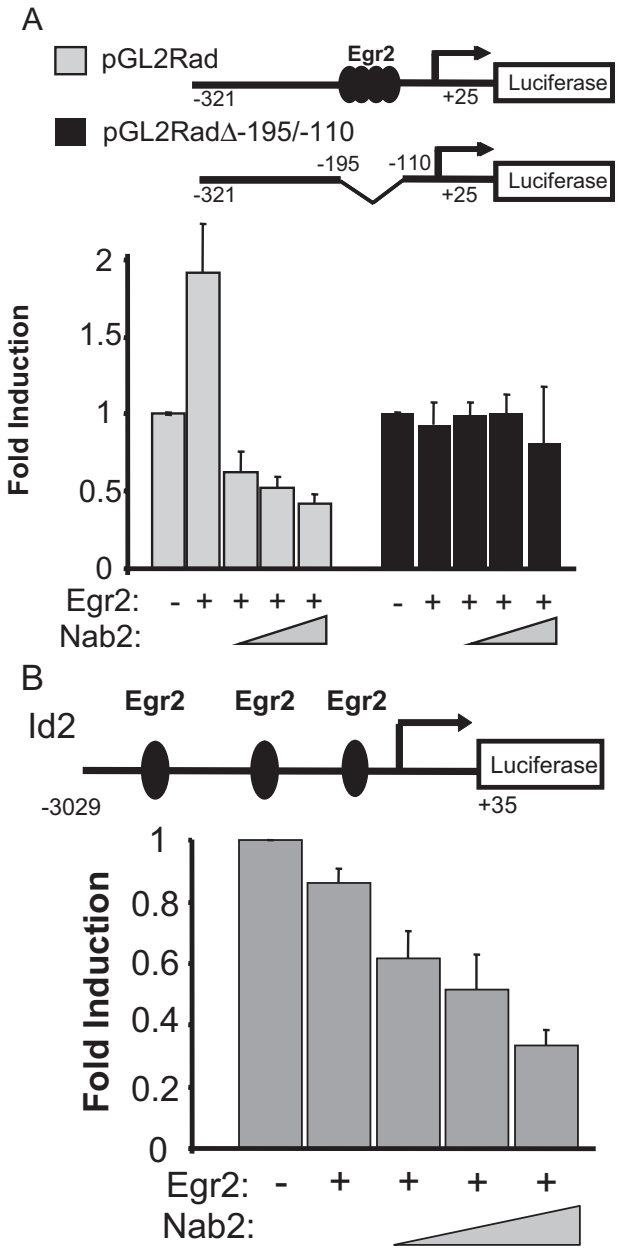

FIGURE 6. **The Egr2·NAB binding sites within *Rad* and *Id2* are functional.** *A*, the S16 Schwann cell line was transfected with the luciferase reporter plasmids containing the rat *Rad* promoter (pGL2Rad), a mutant promoter with the Egr2 binding site deleted (pGL2RadΔ-195/−110), and expression plasmids for Egr2 (50 ng) and NAB2 (10, 20, and 40 ng), as indicated. Means ± S.D. of duplicate measurements for three replicate experiments are shown. *B*, S16 cells were transfected with the luciferase reporter plasmid containing the human *Id2* promoter (pGL3-hId2) and expression plasmids for Egr2 (200 ng) and NAB2 (20, 50, and 100 ng) as indicated. Means ± S.D. of duplicate measurements of three replicate experiments are shown.

Rad is another NAB-regulated gene that is repressed during the course of myelination (26) and is up-regulated in *Nab1/Nab2* knock-out mice (16). Using primers positioned within the *Rad* proximal promoter (Fig. 5*A*, −94/−35), similar ChIP assays demonstrated increased enrichment of Egr2 in P15 rat pups as compared with P5 nerves (Fig. 5*B*). Accordingly, there is a 5.5-fold developmental increase in NAB2 occupancy in this same region (Fig. 5*B*). As negative controls, neither factor is significantly enriched in the −1470/−1420-bp region of the *Rad* locus or in *IMG2a*. The ChIP signals observed were likely derived from myelinating Schwann cells, as a significant proportion of sciatic nerve is composed of myelinating Schwann cells. Moreover, Egr2 and NAB2 are expressed only in myelinating Schwann cells (8, 16, 31). Overall, these data are consistent with direct repression of these genes during development by the Egr2·NAB complex, because both proteins become increasingly associated with the promoters of *Rad*, *Id2*, and *Id4* as they are repressed during peripheral nerve myelination.

Repression of the Rad and Id2 Promoters by the Egr2·NAB Complex—To determine whether the Egr2·NAB complex represses transcription through these identified control elements, we cloned ~400 bp of the rat *Rad* promoter into a luciferase reporter plasmid (pGL2Rad). In transfection assays in the S16 Schwann cell line, Egr2 alone induced *Rad* promoter activity ~2-fold over basal levels (Fig. 6*A*), but coexpression with NAB2 repressed the promoter in a dose-dependent manner. Deletion of the Egr2 binding sites from the promoter (pGL2RadΔ−195/−110) eliminated Egr2 activation and also attenuated the repression by NAB2. Ectopic expression of NAB2, in the absence of ectopic Egr2, does not repress reporter activity (data not shown). Similar experiments using the *Id2* promoter showed that it is not induced above background by Egr2 alone but, rather, is repressed by coexpression of Egr2 and

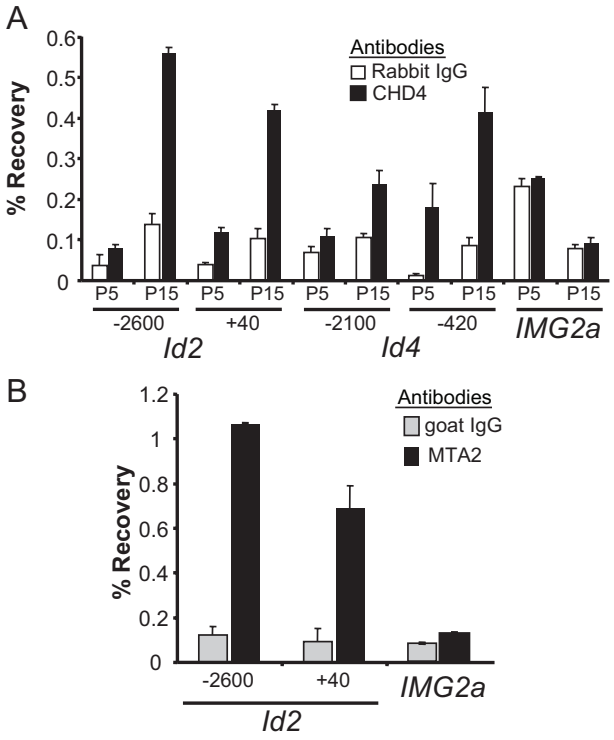

FIGURE 7. **Developmental regulation of NuRD recruitment on the *Id2* and *Id4* promoters.** *A*, ChIP assays were used to detect binding of CHD4 to potential binding sites within *Id2* and *Id4* in pooled sciatic nerves from P5 and P15 rat litters. The data are representative of three independent sets of pooled nerves at each time point. *B*, binding of MTA2 was detected by ChIP assay at the same *Id2* site in pooled sciatic nerves from P15 rat litters. The data are representative of two independent sets of pooled nerves at each time point.

NAB2 (Fig. 6*B*). Again, NAB2 alone does not repress reporter activity (data not shown). These data indicate that *Rad* and *Id2* are directly repressed during sciatic nerve myelination by the Egr2·NAB complex.

The NuRD Complex Assembles on NAB-regulated Promoters—We have recently shown that NAB2 represses transcription by interaction with the CHD4 (chromodomain helicase DNA-binding) subunit of the NuRD (nucleosome remodeling and deacetylase) complex (20). To determine whether CHD4 was also recruited to Egr2-repressed promoters in a developmentally regulated manner, a similar ChIP assay for CHD4 was used. CHD4 binding was observed using the primer sets at −2600 and proximal promoter of *Id2* and −427/−363 in *Id4* (Fig. 7*A*). Importantly, binding of CHD4 increased from P5 to P15, paralleling the developmental regulation of Egr2 and NAB2 binding at the same site. As a negative control, there was no enrichment of CHD4 on the silent *IMG2a* promoter. Prior studies revealed significant binding of CHD4 to the proximal promoter of Rad at P11 (20), and our current studies show this occupancy parallels that of Egr2 and NAB during myelination (P5 to P15, data not shown).

As CHD4 is part of the repressive NuRD remodeling complex, we sought to determine whether the NuRD complex as a whole is recruited to Egr2-repressed promoters. Therefore, similar ChIP assays were performed for another NuRD component, metastasis-associated antigen 2 (MTA2). MTA2 is expressed at relatively high levels and is moderately induced in myelinating sciatic nerve (26). This assay found significant occupancy of the *Id2* promoter by MTA2, indicating that the whole NuRD complex is recruited to Egr2-repressed promoters (Fig. 7*B*).

To test the functional requirement of CHD4 for repression of endogenous *Id2*, we adenovirally infected rat primary Schwann cells with Egr2 in the presence or absence of a dominant-negative form of CHD4 (Δ1–1280), which abrogates NuRD-dependent repression by NAB proteins (20). Expression of *Id2* increases by ~2-fold in the presence of dominant-negative CHD4, an effect that is not seen in the absence of ectopic Egr2 (Fig. 8*A*). By comparison, the *Pmp2* gene is activated by Egr2 but is not further induced by dominant-negative CHD4.

Because the NuRD complex also contains histone deacetylase subunits, we tested whether expression of *Id2* is regulated by histone deacetylase activity. Expression of *Id2* increases ~6-fold in primary rat Schwann cells exposed to the histone deacetylase inhibitor TSA, suggesting that NuRD is required for Egr2·NAB-dependent repression (Fig. 8*B*). As shown previously (20), treating rat Schwann cells with TSA causes a similar induction of *Rad* expression. In comparison, the Egr2-activated gene *Pmp*2 is only minimally affected by TSA treatment. These data further support the hypothesis that Egr2 and NAB proteins directly repress specific target genes during myelination by recruiting the NuRD complex.

Id2 Antagonizes Myelin Protein Zero Induction in Primary Schwann Cells—Direct repression of specific genes by the Egr2·NAB complex suggests that their continued expression would potentially interfere with induction of myelin genes during Schwann cell differentiation. Therefore, we tested whether induction of *Mpz* was limited by the expression of Id2. *Mpz* expression is highly induced by a combination of forskolin and insulin-like growth factor-1 in primary Schwann cells (49, 50), and such treatments were performed in the presence of siRNA directed against *Id2*. As shown in Fig. 9, basal and induced expression of *Mpz* is increased when Id2 is depleted by RNA interference, as compared with the control siRNA, suggesting that Id2 expression limits the induction of the most highly expressed myelin gene in Schwann cells. As reported previously (37, 38), treatment with forskolin increases expression of *Id2*, and the siRNA results indicate that induction of *Mpz* is significantly limited by the presence of Id2. Levels of *Id4* were unchanged in the presence of Id2 siRNA, indicating that the siRNA was specific for *Id2* knockdown (Fig. 9*B*).

DISCUSSION

Cellular differentiation in general is accompanied not only by gene activation but also by gene repression (*e.g.* of cell cycle genes). Recent work has suggested that factors traditionally characterized as activators can also play important roles as repressors in a promoter-dependent fashion. For example, recent analyses of the estrogen receptor have identified many genes that are repressed by estrogen action (51). To assess the possibility of active gene repression by the Egr2·NAB complex in peripheral nerve myelination, a critical observation was that some NAB-regulated genes are induced in the *Egr2* knock-out, which is consistent with active repression of these genes during myelination (2, 16). Expression analyses have identified several genes in which expression is inversely correlated with the pres-

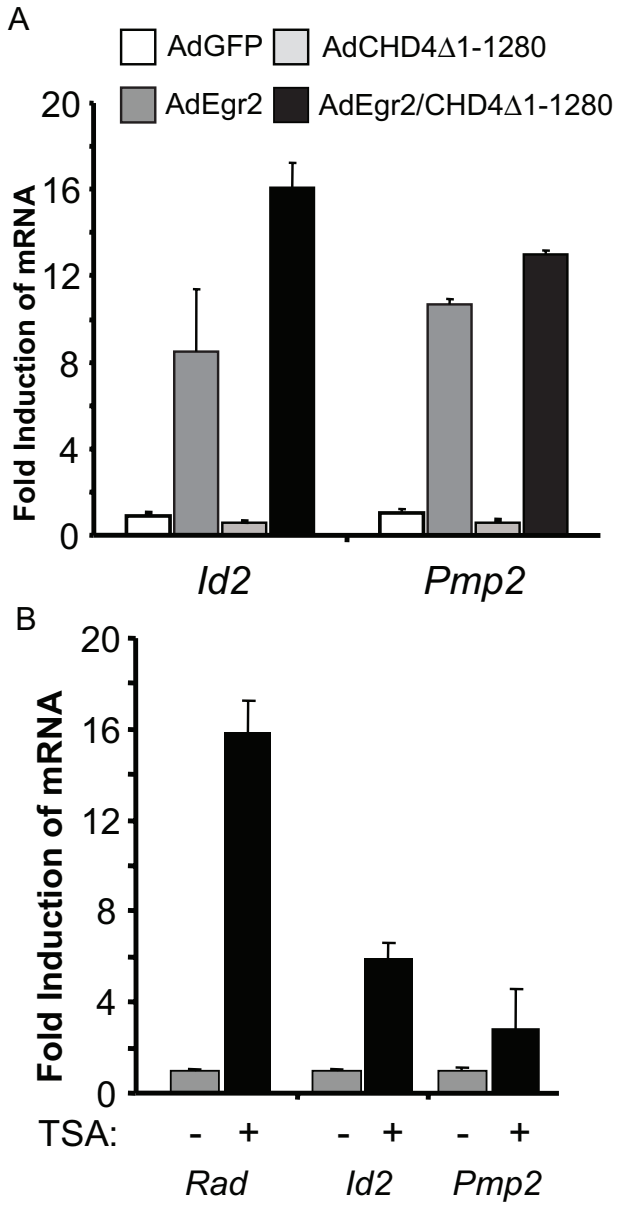

FIGURE 8. **Functional regulation of *Id2* expression by the NuRD complex.** *A*, primary rat Schwann cells were infected with recombinant adenovirus (1×10^9 plaque-forming units/ml) expressing CHD4Δ1–1280 in the presence or absence of AdEgr2 virus. Means ± S.D. were determined from duplicate measurements in a replicate experiment; these data are representative of several independent experiments. *B*, primary rat Schwann cells were treated with 1 mg/ml TSA for 24 h. Total RNA was isolated for each sample, and quantitative RT-PCR was used to determine endogenous levels of *Rad*, *Id2*, and *Pmp2*. After normalization to 18 S rRNA, -fold induction was determined relative to untreated control for each gene. Means ± S.D. were determined for duplicate measurements; these data are representative of several independent experiments.

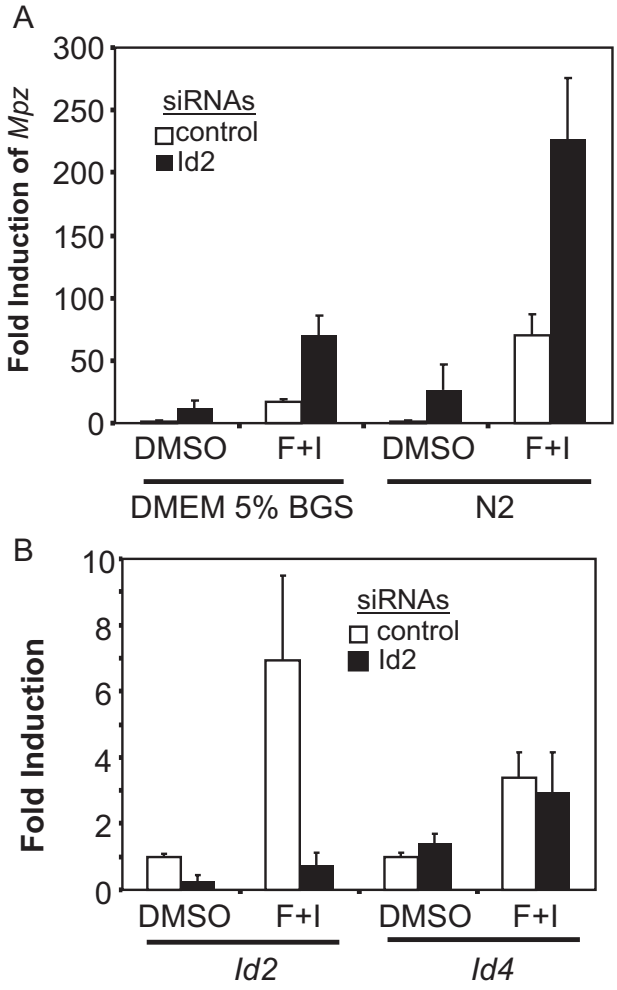

FIGURE 9. **Id2 antagonizes induction of the endogenous the *Mpz* gene.** *A*, primary rat Schwann cells cultured in serum-supplemented medium (Dulbecco's modified Eagle's medium (*DMEM*) and 5% bovine growth serum (*BGS*)) or N2 medium were transfected with control or Id2 siRNA for 48 h and then treated with either vehicle control (dimethyl sulfoxide (*DMSO*)) or forskolin and insulin-like growth factor-1 (*F+I*) for 24 h. Total RNA was isolated for each sample and analyzed by quantitative RT-PCR After normalization to 18 S RNA levels, the -fold induction of *Mpz* was calculated relative to dimethyl sulfoxide-treated control siRNA samples. Means ± S.D. were determined for duplicate measurements from two independent experiments. *B*, the relative levels of *Id2* and *Id4* were determined from three independent RNA interference experiments (including the two experiments in *panel A*) in serum-supplemented medium.

ence of Egr2 (*Sox2*, *jun*, *L1* (2, 3, 52, 53)), and several of these genes were induced in the *NAB1/NAB2* knock-out (16). However, expression analysis alone cannot determine whether the Egr2·NAB complex is directly involved in repressing these genes or whether they are merely up-regulated as a secondary consequence of arrested myelination.

To address this problem, ChIP assays were used to examine myelinating sciatic nerve *in vivo*, and the results show that *Id2*, *Id4*, and *Rad* promoters are occupied by EGR2 and NAB2 directly and that this regulation is dynamic, as it increases as myelination progresses. These results provide the first molecular mechanism for gene repression by Egr2 and associated NAB corepressors during peripheral nerve myelination. Moreover, we have recently shown that NAB2 interacts with the CHD4 subunit of the NuRD complex (20), which represses transcription by nucleosome mobilization and histone deacetylation (reviewed in Ref. 54). Our results demonstrate developmentally regulated CHD4 occupancy on actively NAB2-repressed promoters, with a developmental time course similar to that of Egr2 and NAB2 (Fig. 7). These results not only support the model of an active Egr2-NAB2 repressor complex but also elucidate a unique role of chromatin remodeling complexes in formation of peripheral nerve myelin.

The involvement of NAB proteins in regulation of peripheral nerve myelination was first highlighted by identification of the EGR2 I268N mutation associated with congenital hypomyelinating neuropathy (4). Several studies of NAB proteins suggest that they form a negative feedback loop that limits activation of Egr target genes (14, 55, 56). In contrast, our data support a model in which NAB proteins do not merely serve to limit Egr2 activation of myelin target genes but also co-induction of Egr2 and NAB proteins is directly involved in active gene repression during peripheral nerve myelination (57). A similar role of the Egr2·NAB complex has been proposed in hematopoietic development (48).

One of the surprising observations of the *NAB* knock-out mouse was that major Egr2 target genes (*e.g. Mpz*) were not overexpressed but rather were reduced (16). Although it remains possible that NAB proteins may act as coactivators in certain contexts, our work indicates that at least some genes, such as *Id2*, must be repressed by Egr2·NAB2 to allow efficient activation of major myelin genes like *Mpz*. This is consistent with observations that overexpression of *jun* and *Sox2* inhibits induction of myelin genes (2, 58). Moreover, one of the genes that is repressed by the Egr2·NAB complex is *Oct6/Scip*, and artificial maintenance of its expression in Schwann cells inhibits the myelination process (59), consistent with a requirement for down-regulation of specific factors. These examples provide an interesting parallel to recent work showing that Id2 and Id4 inhibit oligodendrocyte differentiation (33–36) as well as myelin basic protein promoter activity in oligodendrocytes (60). Overall, there is a growing list of regulators (Jun, Sox2, Scip/Oct6, and Id2) that appear to antagonize the myelination process and may thereby coordinate a demyelination program.

It is likely that repression of *Id2* has a broader significance in relation to demyelinating neuropathies, because Id2 is up-regulated in several models of demyelination. These include not only *Egr2/Krox20*-deficient mice (2, 16) but also both mouse and rat models of CMT1a involving *Pmp22* overexpression as well as homozygous deletion of the *Pmp22* gene (61, 62).

Repression of *Id2/Id4* expression may be required for Schwann cells to withdraw from the cell cycle as they initiate myelination, as levels of *Id2* correlate with proliferation in cultured Schwann cells (37). Interestingly, Schwann cells of *Egr2/Krox20*-null mice not only fail to myelinate but also exhibit higher levels of proliferation and apoptosis (1–3, 17). At this point, the target(s) of Id2/Id4 repression in Schwann cells remains unidentified, although one candidate is the Reb/Tcf12 bHLH (basic helix-loop-helix) factor, which is expressed in Schwann cells (37). Id family members can negatively regulate genes encoding cyclin-dependent protein kinase inhibitors (*e.g.* p16 and p21), by antagonizing both bHLH and Ets transcription factors (63–66). Id2 and Id4 can also regulate the cell cycle through interactions with unphosphorylated retinoblastoma (67). Therefore, down-regulation of *Id2/Id4*, as well as *myc* (Table 1), could be involved in exit from the cell cycle.

The regulation of *Rad* may reflect Egr2-regulated events on the cell surface, as Rad interacts with and inhibits the Rho effector, Rho kinase α (ROCK2) (39). Because the Rho pathway is involved in Schwann cell migration and is required during initiation of myelination to regulate the length of myelinated segments (40, 41), repression of *Rad* by EGR/NAB could play a role in regulating Rho pathway activation.

It appears that the NAB proteins may not be required for all gene repression events triggered by Egr2 in myelination, as many developmentally down-regulated genes identified by cluster analysis of myelinating peripheral nerve (26, 28) were not identified in our screen for NAB-regulated genes. For example, repression of *Sox2* and the JNK (c-*jun* NH_2-terminal kinase) pathway could involve additional cofactors (such as Ddx20 (68)) or intermediary steps.

Although our data indicated direct repression of specific target genes by an Egr2·NAB complex during peripheral nerve myelination, we did not observe derepression of major myelin genes such as *Mpz* and *Pmp2*. Some evidence suggests that NAB proteins can act as coactivators in certain contexts (16, 69). Nonetheless, NAB activation has not been demonstrated on any myelin target gene (11, 52). Overall, it appears that the NAB expression does not similarly affect all Egr2 target genes, and future work will be directed toward understanding how NAB proteins differentially affect Egr2 target gene expression.

In addition to the NAB-repressed genes, our study also identified several novel Egr2 target genes, namely *HspB8*, *NF-L*, and *NF-M*. Mutations in both *HspB8* and *NF-L* have been identified in inherited peripheral neuropathies (as reviewed in Ref. 7). Although the neurofilament proteins (NF-L, NF-M, and NF-H) are important components of the axonal cytoskeleton, there is also evidence that *NF-L* and *NF-M* mRNAs are expressed in Schwann cells (44, 45). Our results suggest a mechanism for the axonally dependent induction of NF-L and NF-M in Schwann cells (44). Interestingly, NF-L has been shown to interact with MTMR2 in both neurons and Schwann cells (43), and recessive mutations in *MTMR2* cause CMT4B1 neuropathy (reviewed in 7, 70). Recently, knock-out of the mouse *MTMR2* gene resulted in myelination defects (71), and further experiments indicate that specific loss of *MTMR2* in Schwann cells is sufficient to cause a peripheral neuropathy (72). The identification of *HspB8* as an Egr2-regulated gene is interesting not only for its mutation in peripheral neuropathies (46, 47) but also because of its functional role in cytoskeletal formation. Overall, these findings indicate that genes involved in cytoskeletal rearrangements could be an additional component of the Egr2-regulated target gene network in peripheral nerve myelination.

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