NAB2 Represses Transcription by Interacting with the CHD4 Subunit of the Nucleosome Remodeling and Deacetylase (NuRD) Complex

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Early growth response (EGR) transactivators act as critical regulators of several physiological processes, including peripheral nerve myelination and progression of prostate cancer. The NAB1 and NAB2 (NGFI-A/EGR1-binding protein) transcriptional corepressors directly interact with three EGR family members (Egr1/NGFI-A/zif268, Egr2/Krox20, and Egr3) and repress activation of their target promoters. To understand the molecular mechanisms underlying NAB repression, we found that EGR activity is modulated by at least two repression domains within NAB2, one of which uniquely requires interaction with the CHD4 (chromodomain helicase DNA-binding protein 4) subunit of the NuRD (nucleosome remodeling and deacetylase) chromatin remodeling complex. Both NAB proteins can bind either CHD3 or CHD4, indicating that the interaction is conserved among these two protein families. Furthermore, we show that repression of the endogenous Rad gene by NAB2 involves interaction with CHD4 and demonstrate colocalization of NAB2 and CHD4 on the Rad promoter in myelinating Schwann cells. Finally, we show that interaction with CHD4 is regulated by alternative splicing of the NAB2 mRNA.

By virtue of their ability to regulate the early growth response (EGR) family of transactivators, NAB (NGFI-A-binding protein) corepressors play an important role in regulating inflammation, nervous system function, and prostate cancer development. The NAB1 and NAB2 corepressors interact with a conserved domain found within Egr1 (also called NGFI-A/zif268), Egr2/Krox20, and Egr3 (1–3). The remaining family member, Egr4/NGFI-C, shares substantial homology with other EGR family members but lacks the NAB interaction domain and is therefore resistant to NAB repression.

Members of the EGR family play diverse physiological roles, including having both positive and negative effects on growth. For example, Egr1 null fibroblasts bypass senescence because of reduced expression of the p53 gene (4). On the other hand, EGR1 overexpression is also involved in the development of prostate cancer (5, 6), as it regulates several growth factor genes (7–10). The other EGR family member that has been studied intensively is Egr2/Krox20. Targeted disruption of the mouse Egr2 gene resulted in defects in hindbrain segmentation, bone development, and peripheral nerve myelination by Schwann cells (11–14). A number of Egr2 target genes in the hindbrain and Schwann cells have been identified, including several Hox family members, EphA4, and myelin-associated genes such as myelin protein zero and myelin basic protein (13, 15–21).

Several experiments in various systems have established that NAB corepressors are important regulators of EGR activity. NAB1 and NAB2 both repress EGR activation of several promoters (3, 22–26). Interestingly, although EGR1 is overexpressed in prostate cancer (5, 6), NAB2 expression is reduced in a majority of prostate cancer samples (27). This observation is consistent with the idea that derepression of EGR1 activity is a progression factor in prostate cancer. The importance of NAB regulation is underscored by the identification of a recessive mutation in the NAB-binding domain of EGR2 (I268N) in a family affected with an inherited form of congenital hypomyelinating neuropathy (28, 29). Congenital hypomyelinating neuropathy resembles the non-myelinating phenotype of the peripheral nervous system observed in Egr2/Krox20-deficient mice (13). Similarly, a double knock-out of the NABI and NAB2 genes causes early lethality and impaired myelination (30), indicating that NAB corepressors are required for peripheral nerve myelination.

Although diverse physiological data have demonstrated that NAB corepressors play a critical role in regulation of EGR activity, the molecular mechanism by which these corepressors act has remained elusive. NAB1 and NAB2 share a high degree of homology (2) and are able to homo- and heteromultimerize with each other (22). NAB1 and NAB2 are nuclear proteins, and they repress when tethered to active promoters by fusion to a Gal4 DNA-binding domain (DBD) (31). Therefore, we hypothesized that NAB proteins recruit other proteins in order to regulate EGR activity. We now show that the C-terminal domain of NAB2 interacts with the chromodomain helicase DNA-binding protein 4 (CHD4) subunit of the nucleosome remodeling and deacetylase (NuRD) complex and that this interaction is required for repression by this domain.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The yeast two-hybrid screen (32) was performed in the Molecular Interaction Facility, University of Wisconsin Biotechnology Center. Mouse embryonic and brain libraries in pGAD-T7Rec (BD Biosciences) were screened with a construct containing amino acids 130–525 of the NAB2 protein fused to the GAL4 DNA-binding domain in pBUTE (a kanamycin-resistant version of GAL4 bait vector pGBDUC1). Approximately 18 million clones were screened via
NAB Proteins Interact with CHD4

mating in yeast strain P′694. After isolation of prey plasmids from positive pools, 18 plasmids were positive after retransformation into the bait-containing strain. Of these, two contained clones of mouse CHD4.

**Plasmids**—Segments of the CHD4 (containing amino acids 1281–1915) and CHD3 (Δ1–1311) genes were cloned in-frame with an N-terminal 3× FLAG epitope in the pCDNA3.1 vector (Invitrogen). Human HA-NA2 (containing amino acids 34–525) was generated by introducing a C-terminal HA epitope. Deletion of amino acids 251 to 353 in HA-NA2 was used to create NAB2ΔCND2 (NCD1+CID), and NAB2ΔCID (NCD1+NCD2) lacks amino acids 386–525. The last 18 amino acids are excluded in the construct NAB2Δ507–525. The CID construct consists of amino acids 357–525, including the nuclear localization sequence to permit nuclear translocation. For NAB2 constructs lacking NCD1, translation initiation occurred at Met-141. A naturally occurring splice variant of NAB2 lacks amino acids 426–489 (exon 6 of NAB2). The indicated NAB2 segments were fused to the Gal4 DBD in PM1 (31).

The altered specificity version of Egr2 was generated by mutagenesis of the second zinc finger from SRSDDHHTTHIR to SQSVHLQHSR, as described for Egr1 (34). The corresponding reporter plasmid was created by inserting nine repeats of an altered EGR2 binding site (GGCGT-GAGCC) into the pGL2 vector (Promega) containing the adenovirus E1B TATA element. For mammalian two-hybrid experiments, amino acids 1281–1915 of CHD4 were fused to the Gal4 DNA-binding domain in the PM1 vector. The NAB2VP16 construct was created by fusing the VP16 activation domain to the C terminus of NAB2, as described for the NAB1-VP16 construct (31). Constructs for NAB1ΔNCD1–Δ2–210, the luciferase reporter containing the thymidine kinase promoter with a 1.5-kb minimal TATA element have been described previously (31).

**Coimmunoprecipitation Analysis**—QT6 (Quail fibroblast) or 293T cells were cultured as described previously (35), plated at a density of 5×10⁵ cells/ml, and transfected using LT-1 (Mirus) transfection reagent. After 48 h, cells were washed once in phosphate-buffered saline and extracted with lysis buffer containing 6% glycerol, 20 mM Tris, pH 7.5, 5 mM MgCl₂, 0.1% Nonidet P-40, and 200 mM NaCl. Cross-links were reversed by heating at 65 °C for 5 h, and DNA was purified using the QIAquick PCR purification kit (Qiagen). Quantitative PCR was performed on the same samples of input DNA. Sequence analysis of the Rad1470 gene identified potential Egr2 sites conforming to the previously defined consensus Egr2 binding site (38). Primers used for analysis are: Rad -1470, ACCCCACCACAGTG

**PCR**—Primers flanking the exon 6 region of NAB2 (GGTTGGAGAAGACAGTTCACAAATGA and GGCAGCGGTCCAGCAA) were used to amplify full-length and alternatively spliced forms of NAB2 from cDNA prepared from various mouse tissues.

**Chromatin Immunoprecipitation Assays**—All experiments were performed in strict accordance with experimental protocols approved by the University of Wisconsin School of Veterinary Medicine. After euthanasia of Sprague-Dawley rat pups at postnatal day 11 (P11), sciatic nerves were dissected (pooled from seven pups) and immediately minced in phosphate-buffered saline containing 1% formaldehyde for 25 min at room temperature. Nerves were washed in phosphate-buffered saline, resuspended in 150 mM NaCl, 10% glycerol, 50 mM Tris, pH 8.0 (with a 1:1000 dilution of Sigma protease inhibitor mixture), and homogenized using the Tissue Tearor (biospec). Triton X-100 was added to 0.3%, and the lysate was sonicated in the presence of 100 mg of glass beads, alternating 10-s pulses with 50 s of cooling for a total of 20 min. Sonicated chromatin (containing 300 μg of protein as determined by the Bio-Rad protein assay) was used for each immunoprecipitation, and 10% of this amount was saved as an input. Lysates were incubated with 2 μg of anti-Egr2/Krox20 (Covance), anti-Nab2 (Santa Cruz), anti-CHD3/4 rabbit polyclonal (Santa Cruz sc-11378), or normal rabbit IgG (Upstate) control antibody. Immune complexes were collected with 25 μl of a protein G-agarose slurry (Pierce) blocked with herring sperm DNA (Fisher) and 0.5 mg of bovine serum albumin. Beads were washed in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris 8.1, 150 mM NaCl) and high salt buffer (same buffer containing 500 mM NaCl) followed by 0.25 mM LiCl, 1% Igepal, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8.1. All buffers contained a 1:1000 dilution of Sigma Protease inhibitor mixture. Complexes were eluted with 1% SDS, 0.1 mM NaHCO₃, and 200 mM NaCl. Cross-links were reversed by heating at 65 °C for 5 h, and DNA was purified using the QIAquick PCR purification kit (Qagen). Quantitative PCR was performed on the same samples in duplicate. Values are expressed as percent recovery relative to the input DNA. Sequence analysis of the Rad gene identified potential Egr2 sites conforming to the previously defined consensus Egr2 binding site (38). Primers used for analysis are: Rad -1470, ACCCCACCACAGTG.
RESULTS

CHD4 Interacts with NAB2—NAB1 and NAB2 contain two highly conserved domains called NAB conserved domains 1 and 2 (NCD1 and NCD2). NCD1 is necessary for interaction with EGR proteins and is also required for multimerization of NAB proteins (2, 22). Repression by NAB1 was shown to require NCD2 as well as other regions near the C terminus (31). To identify interacting proteins that might mediate repression by NAB2, we employed a yeast two-hybrid screen of mouse brain and embryonic libraries for proteins that interact with amino acids 130–525 of NAB2. This screen identified two independent clones containing the C-terminal end of CHD4 (beginning at amino acids 1281 and 1290, respectively). The CHD4 diagram indicates the conserved chromodomains, PHD zinc finger, and ATPase domains. B, for the mammalian two-hybrid assay, JEG3 cells were transfected with a luciferase reporter (containing five Gal4 binding sites) along with 40 ng of CHD4 (amino acids 130–525) was used to screen mouse brain and embryo libraries, and the yeast two-hybrid screen identified two independent clones containing the C-terminal end of CHD4 (amino acids 1281 and 1290, respectively). The CHD4 diagram indicates the conserved chromodomains, PHD zinc finger, and ATPase domains. B, for the mammalian two-hybrid assay, JEG3 cells were transfected with a luciferase reporter (containing five Gal4 binding sites) along with 40 ng of CHD4 (amino acids 130–525) was used to screen mouse brain and embryo libraries, and the yeast two-hybrid screen identified two independent clones containing the C-terminal end of CHD4 (amino acids 1281 and 1290, respectively). The CHD4 diagram indicates the conserved chromodomains, PHD zinc finger, and ATPase domains.

Deletion analysis of NAB1 implicated the NCD2 domain and the extreme C terminus in transcriptional repression (31). Based on these studies, HA-tagged versions of NAB2 were generated that contained a deletion of the NCD2 domain, the C terminus (amino acids 386–525), or the C-terminal 18 amino acids (Fig. 2). To eliminate the possibility that any of these proteins might interact indirectly with CHD4 as a result of multimerizing with endogenous NAB proteins, the NCD1 domain was deleted from these constructs. Interestingly, analysis of these mutant proteins revealed that the NCD2 domain was not essential for interaction with CHD4 (Fig. 2B). Deletion of the 18 amino acids at the C terminus of the NAB2 protein also failed to disrupt interaction with CHD4. However, removal of amino acids 386–525 of the NAB2 protein prevented interaction with CHD4. Furthermore, expression of amino acids 386–525, together with the nuclear localization signal, was sufficient for interaction with CHD4. This C-terminal region of NAB2 was therefore designated as the CID (CHD4-interacting domain). We also tested whether NAB2 could interact with endogenous CHD4 by transfecting cells with the indicated NAB2 constructs in the absence of transfected CHD4. After immunoprecipitation of NAB2 with anti-HA, we were able to detect associated CHD3/CHD4, which was not observed using a NAB2 construct in which the CID was deleted (Fig. 2C).

NAB/CHD Interaction Is Conserved among Family Members—The antibody used in the endogenous assay in Fig. 2C detects both CHD3 and CHD4. CHD4 shares several regions of homology with CHD3, including not only the ATPase, PHD, and chromodomains but also...
NAB Proteins Interact with CHD4

A. NAB2\NCD1

B. NAB2\NCD1\NCD2

C. NAB2\NCD1\NCD2\NCD1\CID

B. NAB2\CID

NAB Proteins Interact with CHD4

FIGURE 2. Identification of CHD4-interacting domain of NAB2. A, each of the indicated NAB2 deletion constructs contained the HA epitope and the nuclear localization sequence (NLS) to allow translocation into the nucleus. B, QT6 cells were transfected with 1 μg of each of the deletion constructs of NAB2 along with 1 μg of 3× FLAG-tagged CHD4Δ1–1280 as indicated. Lysates were immunoprecipitated with anti-HA, and bound proteins were immunoblotted with anti-FLAG antibody to test for interaction. Immunoblotting analysis with anti-FLAG (4% input) and anti-HA (40% input) confirmed that the CHD4 and NAB2 constructs were expressed in the indicated lysates. All lanes within a given panel were taken from the same exposure. C, 293T cells were transfected with 10 μg of either HA-NAB2\NCD1 or HA-NAB2\NCD1\CID as indicated. Lysates were immunoprecipitated with anti-HA antibody, and CHD proteins were detected using an anti-CHD3/4 antibody (top panel); input lanes represent 35% of the protein in the immunoprecipitation (IP) lanes. Inputs and immunoprecipitates were also probed with an anti-HA antibody (bottom panel); input lanes represent 17.5% of the protein in the immunoprecipitation lanes. All lanes within a given panel were taken from the same exposure.

FIGURE 3. NAB and CHD family members share capacity for interaction. QT6 cells were transfected as indicated with 1 μg of 3× FLAG-tagged CHD3Δ1–1311 and/or HA-NAB2 (A) or 1 μg of 3× FLAG-tagged CHD4Δ1–1280 and/or 1 μg of HA-NAB1\NCD1 (B). Immunoprecipitations with the anti-HA antibody (IP) were blotted with an anti-FLAG antibody. Expression of the indicated proteins in the lysates was confirmed by immunoblotting with anti-FLAG and anti-HA. Input lanes represent 4% (Flag panel) and 40% (HA panel) of the amount of lysate used in the immunoprecipitation, and all lanes are from the same exposure.

a stably associated subunit (43, 44, 46–48). Accordingly, the observed coimmunoprecipitation of transfected NAB2 and CHD4 might reflect a more transient association involved in NuRD recruitment to EGR target genes rather than a stable complex. Therefore, we proceeded to test whether endogenous CHD4 is a functional requirement for repression by NAB2.

Dominant negative CHD4 mutants were used to test the involvement of CHD4 in repression by NAB2. We tested whether expression of the CHD4 C terminus (CHD4Δ1–1280) could interfere with NAB repression in a dominant negative manner, because it binds to NAB2 but lacks the ATPase and other domains required for CHD4 function. In addition, we created a full-length dominant negative CHD4 protein by mutating a conserved lysine (Lys-750) in the ATPase domain (referred to as CHD4K750C). Analogous mutations have been successfully used to create dominant negatives to study ATPase-dependent chromatin remodeling complexes in yeast, Drosophila, and mammalian systems (49–51). A similar CHD4 mutant has been used to test the involvement of CHD4 in repression of methylated DNA (52). As expected, the K750C mutation did not affect the ability of CHD4 to associate with NAB2, as assessed by coimmunoprecipitation (data not shown).

We evaluated the ability of the two CHD4 mutant constructs to affect NAB2 repression using an Egr2-dependent reporter assay. To provide an assay with sufficient dynamic range to study NAB repression of EGR activity, we generated an altered specificity mutant of Egr2 (Alt. Egr2) that is analogous to one described previously for Egr1 (34). This mutant, containing four point mutations in the second zinc finger, activates through a variant EGR binding site that is not bound efficiently by endogenous EGR proteins, allowing much greater activation of the reporter. In this altered specificity system, NAB2 represses Egr2 activity down to basal levels (Fig. 4). Cotransfection with either CHD4 mutant partially alleviated this repression, suggesting that CHD4 is required for repression by NAB2. Several controls tested the specificity of the mutant CHD4 constructs. First, expression of wild type CHD4 had little effect on repression. Second, the CHD4 mutants were also tested with an Alt. Egr2 construct containing the I268N mutation, which abrogates the ability of NAB proteins to bind and repress Egr2 activity (29). Importantly, expression of the mutant CHD4 constructs did not alter the activity of Alt. Egr2 containing the I268N mutation (data not shown), other regions within the C terminus (42). In addition, CHD3 has been found in NuRD-like complexes (43–45), suggesting that its molecular role is at least partially redundant to that of CHD4. To test a potential interaction of CHD3 with NAB2, a comparable portion of the C-terminal domain of CHD3 (CHD3Δ1–1311) was epitope-tagged and found to associate with NAB2 (Fig. 3A) in a coimmunoprecipitation assay. In addition, NAB1 and NAB2 share a considerable degree of homology, and in functional assays, we have never observed any substantial differences in their ability to repress EGR-mediated transcription (2, 3). As shown in Fig. 3B, NAB1 can also interact with CHD4, suggesting that NAB1 and NAB2 share the capacity to repress transcription by interacting with CHD3 and CHD4.

Disruption of NAB2 Function by Dominant Negative CHD4 Constructs—There have been several biochemical characterizations of mammalian NuRD complexes, but none of these studies has identified NAB2 as
indicating that the derepression of Egr2 activity caused by mutant CHD4 proteins is NAB-dependent. Finally, expression of the mutant constructs did not alter reporter activity in the absence of Egr2 (data not shown), and therefore we concluded that both CHD4 mutants specifically affect NAB repression in a dominant negative manner.

Blotting of lysates from cells transfected in parallel indicated that the CHD4 constructs did not affect expression levels of Egr2 or NAB2. Interestingly, we have consistently observed that expression of the CHD4 C terminus (Δ1–1280) lowers the expression level of endogenous CHD4. One potential cause could be displacement of endogenous CHD4 from the NuRD complex, rendering it more labile.

Identification of Two Independent Repression Domains in NAB2—The results of the deletion analysis of Fig. 2 were somewhat unexpected, because NCD2, a highly conserved domain in NAB1 and NAB2, was not required for interaction with CHD4. However, these data were consistent with the previous deletion analysis of NAB1, which suggests that repression by NAB1 cannot be attributed solely to NCD2, as other C-terminal regions of NAB1 are involved (31). We tested the possibility that there are at least two independent repression domains within NAB2 by fusing either the NCD2 or the CID with the Gal4 DBD. These constructs were tested for their ability to repress the thymidine kinase promoter containing upstream Gal4 binding sites. As shown in Fig. 5, the NCD2 of NAB2 does repress, consistent with previous analysis of NAB1 (38), and the isolated CID can also repress the thymidine kinase promoter. The Gal4 DBD alone or the CID without the Gal4 DBD failed to repress the promoter. These data indicate that there are at least two separable repression domains in NAB2, one of which encompasses the CHD4 interaction domain.

CHD4 Is Required for Repression Mediated by the CID of NAB2—To test the idea that repression by the C-terminal domain of NAB2 uniquely requires CHD4, different NAB2 domains were tested for their ability to repress in the presence of dominant negative CHD4 K750C (Fig. 6A). To independently test the requirement for the NCD2 and the CID, we utilized deletion constructs which contained NCD1 (for interaction with Egr2) along with either NCD2 (NCD1+NCD2) or CID (NCD1+CID). Consistent with the analysis of Gal4 fusion constructs (Fig. 5), NAB2 constructs containing either NCD2 or CID were still able to repress transcription. Repression by the CID was alleviated by expression of CHD4K750C; however repression by NCD2 was unaffected by expression of either CHD4K750C (Fig. 6A) or the CHD4 C terminus (data not shown), indicating that CHD4 is uniquely required for repression by the CID of NAB2.

We independently tested this model by depleting endogenous CHD4 using siRNA. We assayed the ability of the CID to repress Alt. Egr2 by transfecting the NAB2 CID-containing construct in the presence of CHD4 siRNA. With siRNA-mediated depletion of endogenous CHD4, NCD1+CID was unable to repress Alt. Egr2 activity (Fig. 6B). We tested for nonspecific effects of the siRNA using several controls. First, a pool of non-targeting siRNA had no effect on repression by the CID of NAB2. Second, the CHD4 siRNA had no effect on repression by NCD1+NCD2 (data not shown). Finally, expression of mouse CHD4 was able to restore repression by NCD1+CID in the presence of siRNA targeted against human CHD4. Interestingly, transfection of mouse CHD4 also enhanced the ability of NCD1+CID to repress Egr2 activity. Therefore, we conclude that repression by the C-terminal end of NAB2 is dependent on its interaction with CHD4. The CHD4 siRNA had no effect on repression by full-length NAB2 (data not shown), indicating that repression by the two domains was redundant in this assay. In addition, the lack of effect might be due to partial depletion of CHD4 levels by siRNA.

Repression by the NAB2 CID Requires Histone Deacetylase Activity—The NuRD complex contains not only CHD4 but also the histone deacetylases HDAC1 and HDAC2 (43, 44, 47). If the NAB2 CID represses transcription through recruitment of CHD4, this would predict that the associated histone deacetylases in the NuRD complex are required for the repression mechanism. To test for this requirement, we repeated the repression assay in the presence of trichostatin A (TSA), an inhibitor of histone deacetylase activity. As shown in Fig. 6C, repression through the NAB2 CID is sensitive to the inhibitor, indicating that the
NAB Proteins Interact with CHD4

CHD4 Is Required for Repression by the CID of NAB2—The diagram indicates the two NAB2 deletion constructs used for transfection assays. The extent of the deletions is the same as in Fig. 2, except that these constructs contain NCD1. JEG3 cells were transfected with the altered (Alt.) Egr2 reporter and 20 ng of altered Egr2. Transfections included 20 ng of the indicated NAB2 constructs: NCD1 + NCD2 (gray bars) or NCD1 + CID (black bars). The indicated samples were cotransfected with 200 ng of CHD4K750C, wild type (WT) CHD4, or vector (CMVSPORT). The results are normalized to the luciferase activity of the reporter plasmid alone. Means ± S.D. of two replicate experiments are shown. B. JEG3 cells were transfected with altered Egr2 reporter, 20 ng of altered Egr2, and 20 ng of NCD1 + CID. Indicated samples were co-transfected with either siRNA directed against human CHD4 (black bars) or non-targeting siRNA (white bars). Where indicated, cells were also transfected with 20 ng of wild type mouse CHD4. C. JEG3 cells were transfected with the altered Egr2 reporter, 20 ng of altered Egr2, and 20 ng of either full-length NAB2, NCD1 + CID, or NCD1 + CID. Indicated samples (white bars) were treated with 1 μM TSA for 24 h prior to harvest.

FIGURE 6. CHD4 is required for repression by the CID of NAB2. A, the diagram indicates the two NAB2 deletion constructs used for transfection assays. The extent of the deletions is the same as in Fig. 2, except that these constructs contain NCD1. JEG3 cells were cotransfected with the altered (Alt.) Egr2 reporter and 20 ng of altered Egr2. Transfections included 20 ng of the indicated NAB2 constructs: NCD1 + NCD2 (gray bars) or NCD1 + CID (black bars). The indicated samples were cotransfected with 200 ng of CHD4K750C, wild type (WT) CHD4, or vector (CMVSPORT). The results are normalized to the luciferase activity of the reporter plasmid alone. Means ± S.D. of two replicate experiments are shown. B. JEG3 cells were transfected with altered Egr2 reporter, 20 ng of altered Egr2, and 20 ng of NCD1 + CID. Indicated samples were co-transfected with either siRNA directed against human CHD4 (black bars) or non-targeting siRNA (white bars). Where indicated, cells were also transfected with 20 ng of wild type mouse CHD4. C. JEG3 cells were transfected with the altered Egr2 reporter, 20 ng of altered Egr2, and 20 ng of either full-length NAB2, NCD1 + CID, or NCD1 + CID. Indicated samples (white bars) were treated with 1 μM TSA for 24 h prior to harvest.

Analysis of NAB1/NAB2 knock-out Schwann cells indicated that the Rad gene is repressed by NAB proteins during peripheral nerve myelination (30). Therefore, we also assayed Rad expression in primary rat Schwann cells. Because primary rat Schwann cells transfect very inefficiently, recombinant adenoviruses were used to express Egr2 and dominant negative NAB2, which eliminates the function of both NAB1 and NAB2 (22). Activation of endogenous Rad expression by Egr2 is enhanced by dominant negative NAB2, confirming that it is a NAB-regulated gene (Fig. 7C). Importantly, a similar derepression of Rad activation by Egr2 is observed with expression of dominant negative CHD4—1280 (Fig. 7D), indicating that repression of Rad expression by endogenous NAB proteins is at least partially dependent upon CHD4. Moreover, activation of Rad expression by Egr2 is also enhanced by TSA (Fig. 7E), which is consistent with previous data showing that repression through the CID is HDAC-dependent.

CHD4 Binds to an Egr2 Binding Site in the Rad Promoter—To test whether the effect of CHD4 on NAB repression was mediated directly on the Rad promoter, chromatin immunoprecipitation assays were used to determine whether these proteins are found colocalized with Egr2 on the Rad promoter. We recently adapted this technique to assay binding of Egr2 in myelinating sciatic nerve (21), composed of >80% Schwann cells. The P11 time point was chosen because it coincides with the peak of active myelination, and Egr2 and NAB proteins are maximally induced by this time point (54). Freshly dissected sciatic nerves from P11 rat pups were treated with formaldehyde to achieve crosslinking. Chromatin was then sonicated and immunoprecipitated with antibodies directed against Egr2, NAB2, CHD4, or IgG control. After reversing the cross-links, purified DNA was analyzed by quantitative PCR using primers designed to particular regions within the Rad locus.

Sequence analysis identified potential Egr2 binding sites at −130 and −1470 of the Rad promoter. However, the chromatin immunoprecipitation assay revealed that Egr2 is enriched at −130 but not at −1470 (Fig. 8), suggesting that the −130 site is the true Egr2 binding site. The results also show occupancy of both NAB2 and CHD4 at this proximal site. There is no enrichment of either protein on the −1100 bp site or the control IMG2a promoter compared with the control immunoprecipitation. Taken together, these data indicate that NAB2 and CHD4 colocalize to an Egr2 binding site 90 bp upstream of the start site within the Rad promoter, where they interact to repress transcription.

Interaction of NAB2 with CHD4 Is Regulated by Alternative Splicing—Characterization of the NAB2 gene identified a splice variant that lacks exon 6 (55). Loss of exon 6 causes an in-frame deletion of a considerable portion of the CHD4-interacting domain (amino acids 426–489). We first determined that a similar alternative splicing occurs in several mouse tissues, using an RT-PCR assay (Fig. 9A) with primers...
flanking exon 6. In addition, several EST (expressed sequence tag) sequences corresponding to this alternatively spliced form have been identified in human sciatic nerve and mouse thymus and neurospheres (GenBank\textsuperscript{TM} BQ956141, AI117547, and CX201479, respectively). We tested the ability of the NAB2 splice variant to interact with CHD4 (Fig. 9B). Although the expression level of NAB2\textasciitilde\textasciitilde exon6 was somewhat higher than that of wild type NAB2, CHD4 did not communoprecipitate with NAB2\textasciitilde\textasciitilde exon6. We also tested whether the splice variant of NAB2 requires CHD4 activity and found that repression by this form of NAB2 was unaffected by the dominant negative CHD4\textasciitilde K750C (Fig. 9C).

Therefore, we have concluded that alternative splicing of NAB2 creates a CHD4-independent form of NAB2.

**DISCUSSION**

These results have uncovered a novel mechanism of NAB2 repression involving CHD4, which has been implicated in repression by other transcriptional repressors such as hunchback, Tramtrack69, KAP1, Ikaros, Aiolos, and FOG1 (45, 53, 56–59). Interestingly, several of these corepressors also interact with the C-terminal regions of CHD4, CHD3, and dMi2. CHD4 is an integral component of the NuRD complex, which represses transcription using both histone deacetylation and nucleosome mobilization. Both NAB1 and NAB2 interact with CHD4, and NAB2 also associates with the closely related protein CHD3, which has been identified as a component of NuRD-like complexes (43–45). The evolutionary conservation of the CHD/NAB interaction highlights the importance of this interaction for NAB repression. The mechanism of NAB repression is somewhat similar to that of the KAP1 corepressor, which interacts with the KRAB domain found in a large number of zinc finger proteins (but not in EGR proteins). A tandem PHD finger/bromodomain within KAP1 represses transcription by interacting with the CHD3 subunit of a NuRD-like complex (45). One significant difference in our study is that NAB2 interacts with both CHD3 and CHD4, whereas KAP1 interacts only with CHD3 (45).

**FIGURE 7. CHD4 is required for NAB repression of endogenous Egr2 target genes.** A, 293 cells were transfected with 100 ng of Egr2 and, where indicated, 100 ng of NCD1\textasciitilde CID, 500 ng of mouse CHD4, and either non-targeting (NT) or human CHD4 (hCHD4) siRNA (62.5 pm). At 48 h after transfection, total RNA was isolated for each sample, and quantitative RT-PCR was used to determine endogenous levels of RAD. After normalizing to 18 S rRNA, fold induction was determined by comparison with untreated control. Means ± S.D. represent duplicate assays in two replicate experiments. B, quantitative RT-PCR was used to determine endogenous levels of CHD4 in the same samples used in A. After normalizing to 18 S rRNA, levels of CHD4 expression were determined relative to a control culture (con) transfected with vector alone, which was set as 100%. Means ± S.D. were determined for duplicate measurements of two independent experiments. In D, rat Schwann cells were infected with two different amounts of a recombinant adenovirus (1 or 5 \times 10^8 plaque-forming units/ml) expressing CHD4\textasciitilde1\textasciitilde1280 in the presence or absence of the AdEgr2 virus. In E, Schwann cells were infected with adenovirus for 24 h, after which 1 \mu M TSA was added for another 24 h. GFP, green fluorescent protein.

**FIGURE 8. In vivo recruitment of NAB2 and CHD4 to Egr2 binding sites in the Rad promoter.** Cross-linked chromatin was prepared from sciatic nerves of P11 rat pups and immunoprecipitated with antibodies for Egr2, Nab2, CHD3/4, or rabbit IgG (as a negative control). Purified DNA was then analyzed by quantitative PCR using primer sets targeted at potential Egr2 binding sites at −130 and −1470 relative to the Rad transcription start site. Occupancy is expressed as the amount of DNA recovered relative to the input sample. IMG2a, control immunoglobulin G2a promoter. The results are representative of three independent experiments.
Although several independent experiments show that repression by NAB2 CID requires CHD4, it should be noted that the NAB/CHD4 interaction is sensitive to CHD4 expression levels, because repression by the CID can be further enhanced by exogenous expression of CHD4 (Figs. 6B and 7A). Therefore, the NAB/CHD4 interaction may be a relatively weak one that facilitates NuRD recruitment to NAB-regulated promoters, which subsequently would be stabilized by the intrinsic affinity of the NuRD complex for nucleosomes (60). Accordingly, NAB proteins have not been biochemically defined as integral subunits of mammalian NuRD complexes (43, 44, 46–48), which may also reflect the affinity of the NuRD complex for nucleosomes (60). Accordingly, NAB proteins interact with CHD4.

Our data demonstrate that NAB2 has at least two independent repression domains. The repression mechanisms of these two domains can be distinguished because repression by the CID is uniquely affected by: (a) dominant negative forms of CHD4, (b) siRNA directed against CHD4, and (c) inhibition of histone deacetylase activity. The mechanism by which NCD2 represses transcription remains uncharacterized, although NCD2 bears some homology with the Dr1/NC2β transcriptional repressor (31). It was somewhat unexpected to find that NAB2 has two potentially redundant repression mechanisms, although several other repressors (e.g. FOG1, KAP1) also exhibit multimodal repression (45, 53).

As a result of this redundancy, the presence of NCD2 compensated for the loss of CHD4 repression in our transfection assays. However, we anticipate that endogenous NAB-regulated promoters specifically require either one or both mechanisms. Indeed, we did observe loss of NAB repression of the endogenous Rad gene in Schwann cells expressing dominant negative CHD4, indicating that endogenous CHD4 levels play an important role in regulation of the genomic Rad locus. Furthermore, the in vivo chromatin immunoprecipitation assays showed that NAB2 and CHD4 are specifically localized on an Egr2 binding site in the Rad promoter.

The identification of two repression domains within NAB2 has important consequences regarding the mechanism by which NAB proteins regulate EGR target promoters. Although multimORIZATION is not required for interaction with CHD4, the multimORIZATION status of NAB proteins, mediated by NCD1 (22), increases the diversity of complexes that can be recruited through a single EGR binding site. Our proposed model in Fig. 10 indicates that one subunit of a NAB multimer could recruit the NuRD complex, whereas the other subunit might augment repression through interaction of proteins with NCD2. Second, it is expected that differential recruitment of chromatin remodeling complexes at different EGR target promoters could significantly alter NAB activity. For example, NAB proteins do not repress all EGR target promoters equally, and in at least two promoters, NAB proteins can even augment EGR-mediated transcription (3). In this regard, it is interesting that recent evidence has suggested that CHD4 may also have positive effects on gene regulation, depending on the promoter context (61, 62). Finally, alternative splicing of NAB2 mRNA could alter regulation of EGR target genes by preventing recruitment of CHD4.

There are several physiological contexts in which the NAB/CHD connection may play an important role, including development of prostate cancer (27) and regulation of cardiac hypertrophy (63). Furthermore, recent evidence has shown that interaction of NAB corepressors with Egr2 is required for peripheral nerve myelination (28–30). Interestingly, microarray profiling has shown that CHD4 is expressed at a very high level during active myelination of peripheral nerve (54), consistent with its interaction with NAB2 in regulation of EGR2 activity. Future work will be directed toward understanding the scope and mechanism of NAB function in these contexts.
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