The early growth response 2 (Egr2/Krox-20) transcription factor is essential for myelination of the peripheral nervous system and segmentation of the vertebrate hindbrain. To probe the mechanism by which Egr2 is regulated, we used a yeast two-hybrid assay and identified an RNA helicase, Ddx20 (DP103/Gemin3), as an Egr2-interacting protein. Mammalian two-hybrid assays indicated that Ddx20 can interact with Egr1, Egr3, and Egr4, in addition to Egr2, making it the only known cofactor that interacts with all four Egr family members. Using several Egr2 target promoters, we found that Ddx20 repressed Egr2-mediated transcriptional activation with significant promoter specificity. In addition, Ddx20 repressed Egr2-mediated activation of the endogenous insulin-like growth factor 2 (Igf2) gene. Interestingly, the C-terminal segment of Ddx20, which lacks the DEAD box helicase domain, was sufficient for its robust and specific repression. We also examined possible interactions between Ddx20 and NAB proteins, the only other known corepressors of the Egr family, and found that these two corepressors act independently. Finally, transcriptional repression assays performed in the presence of a histone deactetylase inhibitor (trichostatin A) indicate that although repression of certain promoters by Ddx20 requires histone deactetylase activity, another repression mechanism must also be involved. Because Egr2 is critical for hindbrain development and peripheral nerve myelination, modulation of Egr2 by Ddx20 may play an important role in maintaining the correct expression level of Egr2 target genes.

The members of the early growth response (Egr) family of transcription factors are rapidly induced by various extracellular signals such as growth factors and hormones, as well as developmental and environmental signals. Once induced, Egr proteins activate genes that cause cellular responses such as proliferation, differentiation, or apoptosis depending upon the stimulus. The Egr proteins share a highly conserved DNA-binding domain consisting of three C2H2 zinc fingers that recognize a GC-rich sequence, as well as shorter regions of homology outside of the DNA-binding domain (1, 2). Although all four Egr family members share a similar DNA-binding specificity (3), targeted disruption of these genes demonstrates distinct physiological roles for these proteins (4). One of the most dramatic phenotypes in this family arises from targeted disruption of Egr2/Krox-20 (hereafter referred to as Egr2); homozygous null mutants of Egr2 all die within 1 or 2 weeks after birth and exhibit defects in hindbrain patterning, peripheral nerve myelination, and bone formation (5–8).

A series of elegant experiments have described the role of Egr2 in establishing the segmentation pattern of the developing vertebrate hindbrain. Targeted disruption of Egr2 causes the disappearance of rhombomeres r3 and r5, hindbrain segments in which Egr2 is normally expressed. Egr2 regulates several homeobox genes (Hoxa2, Hoxb2, and Hoxb3) (9–11), which help determine the anterior-posterior positional identity of the rhombomeres. Another target gene of Egr2 in the developing hindbrain is Ephrin A4 (EphA4) (12), which encodes a transmembrane tyrosine kinase receptor. EphA4, along with other members of the Eph family and their ligands, is involved in restricting the intermingling of cells between odd- and even-numbered rhombomeres (13). Finally, ectopic expression of Egr2 in r2, r4, and r6 confers odd-numbered rhombomere characteristics upon even numbered rhombomeres (14).

Egr2-null mice also exhibit defects in peripheral nerve myelination, which is essential for rapid conduction of nerve impulses along an axon. In these mice, Schwann cells are blocked at an early stage of myelination with a reduction of major myelin proteins such as myelin protein zero and myelin basic protein (7). Microarray analysis of primary Schwann cells with ectopic expression of Egr2 demonstrates that Egr2 induces several genes necessary for myelination, such as myelin protein zero (MPZ), peripheral myelin protein (PMP22), and Connexin 32 (15). Additional genes induced by Egr2 expression include genes that encode enzymes necessary for synthesis of the lipids that comprise myelin.

Based upon the myelination defects observed in Egr2-null mice, human patients with the peripheral myelinopathies—Charcot-Marie Tooth disease, Dejerine-Sottas syndrome, and congenital hypomyelinating neuropathy—were examined for mutations in EGR2. These diseases, ranging in severity from the more mild condition, Charcot-Marie Tooth disease, to the more severe myelinopathies, Dejerine-Sottas syndrome and congenital hypomyelinating neuropathy, are most commonly caused by mutations in MPZ, PMP22, and Connexin 32 (16, 17). However, mutations in the human EGR2 gene have been reported in multiple cases of peripheral myelopathy (18–24). Most of these patients have mutations in the DNA-binding domain of EGR2 that significantly reduce its DNA-binding activity. Because heterozygous Egr2 knockout mice exhibit a
normal phenotype (7), it is surprising that the DNA-binding domain mutations are dominant (25). Recently, however, it has been demonstrated that one of the dominant EGR2 mutants can interfere with the ability of wild-type Egr2 to activate a subset of Egr2 target genes in primary Schwann cells (15). One possible explanation for the dominant-negative activity is that EGR2 requires a cofactor or cofactor complex that is sequenced by the mutant EGR2 proteins.

Although much progress has been made in understanding the physiological role of Egr2 and its regulation of target genes involved in hindbrain patterning and peripheral nerve myelination, the mechanism by which EGR2 protein regulates target genes remains relatively obscure. NAB1 and NAB2 are currently the only known corepressors of Egr2 activity (26, 27). NAB proteins bind directly to a conserved domain found in Egr1, Egr2, and Egr3 and repress Egr- mediated transcriptional activation of many Egr target genes. However, NAB proteins were recently found to activate rather than repress Egr activity on promoters of the luteinizing hormone β (LHβ) and fas ligand (fasL) genes (28). One of the congenital hypomyelinating neuropathy-associated EGR2 mutations abrogates the EGR2/NAB interaction, suggesting that this interaction is essential for properly regulating EGR2 target genes in Schwann cells (25).

The activation of Egr2 target genes depends not only upon the levels of Egr2 that are induced but also upon the relative levels of Egr2 cofactors. Identification of other cofactors that regulate Egr2 will be required to develop a mechanistic understanding of how Egr2 regulates its transcriptional network. To this end, we have identified and characterized an Egr2 corepressor, Ddx20 (DP103/Gemin3).

MATERIALS AND METHODS

Yeast Two-hybrid Screen—A high throughput yeast two-hybrid screen (29) was performed in the Molecular Interaction Facility, UW-Madison. The mouse embryonic and pre-B and T cell libraries are in pACT and pACT2 (30) or pGAD-T7Rec (BD Biosciences) prey vectors. Amino acids 180–470 of the Egr2 protein containing the S362R, D383Y mutations were fused to the GAL4 DNA-binding domain in pBUTE (a kanamycin-resistant version of GAL4 bait vector pGBDUC1), and the construct was transformed into yeast strain PJ694 (31). Approximately 18 million clones were screened via mating. After isolation of prey plasmids from positive pools, 30 prey plasmids were positive after retransformation into the bait-containing strain as well as a strain containing an additional Egr2 bait (Egr2NL180.R409W).

Plasmids—The HA-Egr1, HA-Egr2, HA-Egr3, and HA-Egr4 constructs were described previously (28). VP16-Ddx20.612–825 was generated by ligating the Ddx20.612–825 fragment isolated in the yeast two-hybrid screen into pVP16. Flag-Ddx20.612–825 contains an N-terminal 3xFLAG epitope fused to amino acids 612–825 of Ddx20 in the pCDNA3.1 vector. Full-length VP16-Ddx20 and Flag-Ddx20 were described previously (32). The full-length NAB2 construct contains the entire mouse Nab2 open reading frame in the pcDNA3.1 expression vector. The EphA4 promoter construct was created by cloning a 475-base pair enhancer fragment from the mouse EphA4 gene (containing multiple Egr2 binding sites) upstream of a minimal E1B TATA element in the pGL2 vector. The NAB2 reporter construct contains −750 to +250 of the mouse NAB2 promoter, cloned into KpnI-XhoI pGL2. The fasL, LHβ, β, FG2, and 4xEgr.syn luciferase reporters have been described previously (28, 33, 34). The adenovirus major late (5XGal4AdML) and thymidine kinase (5XGal4TK) luciferase reporter plasmids, each containing five Gal4 binding sites (35), and the Gal4-Rb plasmid (36) were also described previously. For Gal4 tethering experiments, amino acids 612–825 of Ddx20 were fused to the Gal4 DNA-binding domain in the pM2 vector.

Cell Culture and Transfections—For reporter assays, JEG-3 cells (a human trophoblast cell line) were cultured in minimal essential medium supplemented with 10% fetal bovine serum. The JEG-3 cells were plated at 3 × 10⁴ cells/well in a 12-well plate. Cells were transfected with the indicated expression plasmids, 250 ng of the indicated luciferase reporter plasmids, 100 ng of a cytomegalovirus-driven lacZ reporter and pBluescript as required to make a total of 1 μg of DNA per transfection using either LT1 (Mirus, Madison, WI) or calcium phosphate.

For Fig. 6, trichostatin A was added 24 h after transfection. Forty-eight hours after transfection, the cells were harvested and the levels of luciferase and β-galactosidase activity were measured and analyzed as described previously (34, 37).

Quantitative Real-time PCR—HeLa cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and were plated at 5 × 10⁵ cells/ml in a 6-well plate. After 24 h, the cells were transfected as described above. Forty-eight hours after transfection, RNA was isolated from the cells using the TRIzol reagent (Invitrogen) and analyzed by quantitative PCR as described previously (38), using primers to IFG2 (forward, AGGAAATGCTGCAAGCT; reverse, CGGAGATTTTGCGGATGGA). Primers to 18 S rRNA were used as a control for the amount of cDNA in each reaction (forward, CCAGCGTGAGTGGAAAATCC; reverse, CGAAACCTGACTTGTTCTGTT) and primers to lacZ were used as a control for transfection efficiency (forward, CGCGCTGATTCCCTCGGAT; reverse, ACGAACGCGCCTGTAAGAC).

RESULTS

Egr2 Interacts with Ddx20—To probe the mechanism by which Egr2 regulates its target genes, we screened for proteins that interact with Egr2 using the yeast two-hybrid screen. Amino acids 180–470 of Egr2 (removing the N-terminal activation domain; Fig. 1A) were fused to the Gal4 DNA-binding domain, and this construct was used to screen mouse embryonic and mouse B and T cell libraries. Previous work demonstrated that expression of the Egr1 DNA-binding domain in yeast dramatically retarded their growth (39), and that mutations of the DNA-binding domain relieve the growth impairment. Therefore, an additional modification of the Egr2 bait construct was the insertion of a neuropathy-associated mutation (S382R, D383Y), which significantly impairs the ability of Egr2 to bind DNA (18).

Of the 30 positive clones identified, Ddx20 (also known as DP103 and Gemin3) was isolated once with the original bait construct (Egr2NL180.S382R.D383Y). Ddx20 belongs to the DEAD box family of proteins named for the highly conserved Asp-Glu-Ala-Asp motif that is embedded within a domain that often exhibits ATPase and RNA helicase activities (Fig. 1A). Consistent with the presence of these motifs, Ddx20 has both ATPase and helicase activities (40, 41). To eliminate the possibility that Ddx20 interacted specifically with the Egr2 mutant used in the screen, a confirmation assay was performed with a second Egr2 bait (Egr2NL180.R409W), which contained a mutation (R409W) in the third zinc finger corresponding to a Charcot-Marie Tooth disease-associated mutation (18). Recently, other groups have shown an interaction between Ddx20 and several transcriptional activators: Epstein-Barr virus protein EBNA2 and EBNA3C (40), survival motor neuron (SMN) protein (42, 43), steroidogenic factor-1 (SF-1) (32), and mitogenic Ets transcriptional suppressor (MET5) (44). EBNA2, EBNA3C, SMN, SF-1, and METS all interact with the C-ter-
minal half of Ddx20. Consistent with these findings, the portion of Ddx20 isolated in the yeast two-hybrid screen consisted of amino acids 612 to the end of Ddx20 (Ddx20.612–825).

To further test the interaction between Egr2 and Ddx20 in a co-immunoprecipitation experiment, QT6 cells were cotransfected with HA-tagged full-length Egr2 and Flag-tagged Ddx20.612–825. Egr2 was precipitated with anti-FLAG affinity beads only in the presence of Ddx20.612–825 (Fig. 1B). To determine whether full-length Ddx20 and Egr2 could interact, we repeated the assay with full-length Ddx20 fused to an N-terminal FLAG epitope and demonstrated that Egr2 precipitated with the anti-FLAG affinity beads only when Ddx20 was present. In addition, we used anti-HA affinity beads to precipitate Flag-Ddx20.612–825 (Fig. 1C) or Flag-Ddx20 (data not shown) indicating that the co-immunoprecipitation results are specific.

The Egr2/Ddx20 interaction was independently tested using a modified mammalian two-hybrid assay. A luciferase reporter plasmid containing four Egr binding sites upstream of a minimal prolactin promoter (4xEgr.syn), 25 ng of a plasmid expressing the indicated Egr construct, and the indicated amounts of a plasmid expressing full-length Ddx20 fused at its N terminus to the VP16 activation domain (Ddx20-VP16). Results are normalized to the luciferase activity of the reporter plasmid alone. Means and standard deviations of two replicate experiments are shown. VP16-Ddx20 itself activated 4xEgr.syn ~6-fold over reporter alone (data not shown). This slight activation is probably caused by interaction with endogenous Egr proteins.

To further test the interaction between Egr2 and Ddx20 in a co-immunoprecipitation experiment, QT6 cells were cotransfected with HA-tagged full-length Egr2 and Flag-tagged Ddx20.612–825. Egr2 was precipitated with anti-FLAG affinity beads only in the presence of Ddx20.612–825 (Fig. 1B). To determine whether full-length Ddx20 and Egr2 could interact, we repeated the assay with full-length Ddx20 fused to an N-terminal FLAG epitope and demonstrated that Egr2 precipitated with the anti-FLAG affinity beads only when Ddx20 was present. In addition, we used anti-HA affinity beads to precipitate Ddx20.612–825 in the presence of HA-Egr2 (Fig. 1C), and the results confirmed that Egr2 interacts with the C terminus of Ddx20. Anti-Flag affinity beads were unable to precipitate HA-Egr2 (Fig. 1B), whereas anti-HA affinity beads were unable to precipitate either Flag-Ddx20.612–825 (Fig. 1C) or Flag-Ddx20 (data not shown) indicating that the co-immunoprecipitation results are specific.

The Egr2/Ddx20 interaction was independently tested using a modified mammalian two-hybrid assay. A luciferase reporter plasmid containing four Egr binding sites upstream of a minimal promoter (4xEgr.syn) was cotransfected with an Egr2 expression plasmid, and the promoter activation was assayed in the presence of a Ddx20/VP16 fusion protein. If the Ddx20/VP16 fusion can interact with Egr2, the luciferase activity should be increased above the activity observed with Egr2 alone. As shown in Fig. 2B, coexpression of Ddx20-VP16 with
**Egr2** activated the 4xEgr.syn luciferase activity >4-fold over Egr2 alone, suggesting that Ddx20-VP16 interacts with Egr2 in mammalian cells.

**Ddx20 Interacts with Egr1, Egr3, and Egr4**—Egr2 shares a highly conserved DNA-binding domain with the other members of the Egr family (Egr1, Egr3, and Egr4) as well as other shorter conserved motifs (1, 2). The modified two-hybrid assay was used to test whether Ddx20 interacts with other Egr family members. Cotransfection of Ddx20-VP16 and Egr1 activated the 4xEgr.syn luciferase activity 2-fold over Egr1 alone (Fig. 2A). Addition of Ddx20-VP16 and Egr3 stimulated the 4xEgr.syn luciferase activity 5-fold over Egr3 alone (Fig. 2C). Similarly, Ddx20-VP16 and Egr4 stimulated the reporter >3-fold over Egr4 alone (Fig. 2D). Thus, our data suggest that Ddx20 interacts with all four members of the Egr family making Ddx20 the only known cofactor to interact with Egr4.

**Ddx20 Represses the Activity of Egr2 in a Promoter-dependent Manner**—Ddx20 represses the activity of steroidogenic factor-1 (SF-1) (32) and forms a corepressor complex with the mitogenic Ets transcriptional suppressor (METS) (44). Therefore, we tested whether Ddx20 would also repress Egr2-mediated transcriptional activation. When the 4xEgr.syn luciferase reporter was cotransfected with Egr2, the reporter was stimulated 13-fold above background. In the presence of either full-length Ddx20 or Ddx20.612–825, we observed only a slight repression of Egr2 activity (Fig. 3A).

Because 4xEgr.syn is a synthetic promoter, we also examined the effect of Ddx20 on Egr2-mediated transcriptional activation of Egr2 target genes—EphA4 (12) and fibroblast growth factor 2 (FGF2/bFGF) (45, 46). Reporter constructs for these two genes were created by cloning their Egr2 responsive regions upstream of the luciferase gene. Egr2 stimulated the activity of the EphA4 reporter construct 30-fold above background. Ddx20 repressed this activity in a dose-dependent fashion, with the highest amount of Ddx20 repressing the Egr2 activity by 6-fold (Fig. 3B). Interestingly, the C-terminal fragment of Ddx20 (612–825) isolated from the yeast two-hybrid assay also repressed Egr2 to approximately the same degree (Fig. 3B). Similar results were observed with the FGF2 promoter construct; a 32-fold stimulation in the presence of Egr2 was repressed by 5- or 6-fold upon addition of increasing amounts of Ddx20 or Ddx20.612–825, respectively (Fig. 3C). In addition, we also examined the ability of DP103 to repress the NAB2 promoter, because previous work has shown that Egr2 regulates expression of NAB2 in both primary Schwann cells and the developing hindbrain (15, 47). In contrast to the EphA4 and FGF2 promoter constructs, the NAB2 promoter construct, although stimulated over 30-fold in the presence of Egr2, did not exhibit a reduction in stimulation with increasing concentrations of either Ddx20 or Ddx20.612–825 (Fig. 3D). These data suggest that Ddx20 is a repressor of Egr2 activity and that the C-terminal 213 amino acids are sufficient for repression. Furthermore, the ability of Ddx20 to repress Egr2 activity seems to depend upon the promoter context.

**Fig. 3.** Ddx20 represses Egr2 activity in a promoter selective manner. JEG-3 cells were transfected with a luciferase reporter plasmid driven by four synthetic Egr binding sites (4xEgr.syn) (A), the EphA4 enhancer (B), the FGF2 promoter (C), or the NAB2 promoter (D), along with 25 ng of a plasmid expressing Egr2, and the indicated amounts of a plasmid expressing either full-length Ddx20 or Ddx20.612–825. Results are normalized to the luciferase activity of the reporter plasmid alone. Means and standard deviations of two replicate experiments are shown.
Ddx20 Repression Is Independent of NAB Proteins—The only other described repressors of Egr2 are the NAB1 and NAB2 proteins (26, 27). One possible model is that Ddx20 might synergize with NAB proteins to repress Egr2 activity and/or modify the effect of NAB proteins on Egr2. Therefore, we examined whether Ddx20 could interact with NAB proteins to repress Egr2-mediated transcriptional activation of the EphA4 reporter construct. Combining suboptimal amounts of Ddx20 and Nab2 did not significantly increase the level of repression of either protein alone (Fig. 4A). Similar results were seen when higher amounts of Ddx20 and Nab2 were used in the assay (Fig. 4B), suggesting that Nab2 and Ddx20 do not synergistically repress Egr2-mediated induction of the EphA4 promoter.

Our assays suggest that repression of Egr2 activity by Ddx20 is promoter dependent. Similarly, previous work identified two promoters, LH-β and fasL, on which Nab proteins stimulate rather than repress Egr2-mediated transcriptional activation (28). We used these two promoters linked to a luciferase reporter to explore the following questions: 1) Do Nab2 and Ddx20 interact to stimulate Egr2 activity on certain promoters or 2) Does Ddx20 repress Nab2-enhanced Egr2 activity? As shown in Fig. 4C, Egr2 alone activated the LH-β reporter 4-fold, whereas addition of Ddx20 repressed the activation to background levels. In contrast, Nab2 stimulated the activation 16-fold, a 4-fold increase over Egr2 alone. Addition of Ddx20 reduced the NAB2-enhanced activity from 16- to 5-fold above background (Fig. 4C). Similar results were obtained with a reporter construct containing the luciferase reporter gene under control of the fasL promoter (Fig. 4D). Thus, unlike Nab2, Ddx20 repressed Egr2 activation of the LH-β or fasL promoters; in fact, Ddx20 was able to repress the activity of the Egr2/Nab2 activation complex on both promoters. In summary, Ddx20 and Nab2 do not seem to act synergistically to either repress or stimulate Egr2 activity, indicating that the effects of Ddx20 are largely independent of NAB proteins.

Ddx20 Represses Egr2-mediated Activation of the Endogenous IGF2 Gene—To explore whether Ddx20 was also able to repress endogenous genes, we used quantitative real-time PCR to examine the relative levels of endogenous Egr2 target genes. We first examined the EphA4, FGF2, and FasL genes because they were the promoters repressed in our reporter assays (Figs. 3 and 4). In the multiple cell lines we tested, endogenous levels of these genes were already high and were not further activated by ectopic Egr2 expression. However, when we analyzed the levels of another Egr target gene, IGF2 (38, 48), we found that Egr2.I268N induced IGF2 gene expression 17-fold over background levels. An Egr2 construct containing the I268N mutation was chosen for this assay to isolate the effects of Ddx20 from those of endogenous Nab proteins, because I268N abrogates the interaction between Egr2 and Nab proteins (25). The addition of Ddx20 or Ddx20.612–825 reduced the IGF2 gene activation to 3- or 4-fold over background respectively (Fig. 5). Thus, full-length Ddx20, as well as its C-
terminal 213 amino acids, are able to repress Egr2-mediated activation of endogenous IGF2 gene expression.

Repression by Ddx20 Involves Histone Deacetylase Proteins—Because previous work demonstrated that Ddx20 binds histone deacetylases (HDAC) 2 and 5 (44), we tested the hypothesis that Ddx20 represses Egr2 activity by recruiting histone deacetylases. Ddx20 and Ddx20.612–825 repressed Egr2-mediated activation of the EphA4 reporter construct (normalized to 100%; Fig. 6A) by ~80%, but in the presence of the histone deacetylase inhibitor, trichostatin A (TSA), the repression was only 60%. To eliminate any possible interference by histone deacetylases that might potentially be recruited by NAB proteins, the experiment was repeated with Egr2.1268N and similar results were obtained (Fig. 6A). We next examined the effect of TSA on the ability of Ddx20.612–825 to repress Egr2-mediated activation of the FGF2 reporter construct, and we observed a significant alleviation of repression by TSA (Fig. 6B).

When Ddx20 is tethered to a Gal4 DNA-binding domain (Gal4-Ddx20), it acts as an active repressor of a constitutively active thymidine kinase reporter construct containing five Gal4 binding sites (32, 44). We used both a thymidine kinase (5XGal4TK) and an adeno-virus major late (5XGal4AdML) luciferase reporter construct to examine whether active repression by Gal4-Ddx20.612–825 was sensitive to TSA. Although Gal4-Ddx20.612–825 repressed both reporter constructs, the addition of TSA did not significantly affect Ddx20-mediated repression of either reporter construct (Fig. 6C), suggesting that another mechanism is involved in Ddx20-mediated repression of both constitutively active promoters. As a control to ensure that the TSA was functional, we used the retinoblas-toma protein fused to Gal4 (Gal4-Rb) to repress both 5XGal4TK and 5XGal4AdML. The addition of TSA alleviated Gal4-Rb repression on 5XGal4TK but not 5XGal4AdML (Fig. 6C), as has been reported previously (36). Therefore, whereas histone deacetylase recruitment plays a role in the mechanism by which Ddx20 represses transcriptional activation, at least part of the mechanism of Ddx20 repression seems to be independent of histone deacetylases.

DISCUSSION

In this study, we have identified and characterized a novel corepressor of Egr2 known as Ddx20. Initial characterization of the role of Ddx20 in transcriptional regulation focused on its interaction with two Epstein-Barr viral proteins (EBNA2 and EBNA3C) (40), as well as with the SMN protein (42, 43). More recently, Ddx20 has been found to interact with two other cellular DNA-binding proteins, SF-1 (32) and METS (44). In addition to its interaction with Egr2, Ddx20 seems able to interact with other members of the Egr family, Egr1, Egr3, and Egr4 (Fig. 2). This interaction would be the first to be shared in common with all four members of the Egr family.

The functional tests of an Egr2/Ddx20 interaction revealed that Ddx20 could efficiently repress several Egr target promoters, including promoters derived from the FGF2, LH-β, fasL, and EphA4 genes, as well as endogenous IGF2. In contrast, we observed only minimal repression of a synthetic promoter construct containing four consensus Egr binding sites (4xEgr.syn). We tested a NAB2 promoter construct as well, because previous work demonstrated that Egr2 regulates NAB2 expression (15, 47), and subsequent characterization of the NAB2 pro-
motomer revealed several Egr binding sites. 2 Although we observed a large activation of the NAB2 promoter by Egr2, Ddx20 was unable to repress this activity; in fact, there seemed to be a modest potentiation effect. Therefore, it seems that the effects of the Ddx20 interaction with Egr2 are promoter-specific. One possible explanation for promoter-specific repression is that the promoter itself might determine the ability of Egr2 to interact with Ddx20. However, although Ddx20 did not significantly repress the 4xEgr syn reporter construct (Fig. 3A), we found that Egr2 and Ddx20 were able to interact on the same reporter in our modified mammalian two-hybrid assay (Fig. 2B). These data suggest that the effects of promoter context on Ddx20 activity occur subsequent to the interaction of Ddx20 with Egr2. Similarly, Ddx20 repression of SF-1-mediated transcriptional activation is also dependent upon promoter context; the SF-1 target gene steroidogenic acute regulatory protein is not repressed by Ddx20 in contrast to the other tested SF-1 targets (41).

The promoter specificity of the effects of Ddx20 on Egr2 activity is similar to observations made with the NAB1 and NAB2 proteins. Both of these proteins have generally been found to repress Egr2 (as well as Egr1 and Egr3) transactivation. However, there are two notable exceptions, the fasL and LH-β promoters, where NAB proteins augment activation of these promoters by Egr proteins (28). To test whether Ddx20 is involved in a mechanism by which promoter context switches NAB proteins from corepressors to coactivators, we examined the ability of Ddx20 to repress the NAB-potentiated Egr2 activity on the LH-β and fasL promoters. Ddx20 did repress the activation of these two promoters by the Egr2/NAB complex (Fig. 4, C and D). Therefore, our results indicate that Ddx20 and Nab2 do not act synergistically and are independent regulators of Egr2 activity. Another aspect of this independence is the demonstration that the NAB2 promoter was not repressed by Ddx20 (Fig. 3D), because repression of this promoter by Ddx20 would indirectly lead to loss of NAB activity.

Many questions remain regarding the mechanism by which Ddx20 represses transcription. The DEAD box domain resides in the N-terminal half of Ddx20, but consistent with results obtained with SF-1 (32), the C-terminal 213 amino acids of Ddx20 (Ddx20ΔC, 121–235) are sufficient to repress Egr2 activity, indicating that helicase activity is not required for repression. Thus, although Ddx20 may function as a helicase, it also has an independent transcriptional repression domain. Ddx20 (Gen3) is found in large complexes with several proteins, including the SMN protein, which is required for pre-mRNA splicing and possibly for the recycling or regeneration of snRNPs (42, 43). Furthermore, Ddx20 has also been found in micro-RNA ribonucleoprotein complexes (49). The helicase activity of Ddx20 may function to restructure the RNA in these ribonucleoprotein complexes (43, 49).

Recent work by Rajendran et al. (50) demonstrated that DP97 (DDX54), a DEAD box RNA helicase, represses the transcriptional activity of various nuclear receptors including the estrogen receptor, the progesterone receptor and the retinoic acid receptor α. As is the case with Ddx20, the C terminus of DP97, which lacks the helicase motifs, is sufficient for repression. Despite these functional similarities between Ddx20 and DP97, a BLAST search revealed no significant sequence homology between the C termini of the two proteins. It will be interesting to determine whether other RNA-helicase corepressors, such as Ddx20 and DX54, exist and what role, if any, the RNA helicase activity plays in transcription. Despite its lack of homology to other known proteins, the C terminus of Ddx20 is an active repressor (Fig. 6C) (32, 44). One recent study showed that Ddx20 interacts with the nuclear receptor corepressor Sin3A and HDAC2 and HDAC5 (44). Because these proteins have been found in chromatin remodeling complexes, these data suggest that Ddx20 may form a chromatin-remodeling complex that is recruited to specific promoters through an interaction between Ddx20 and a transcription factor. We tested the hypothesis that the repression by Ddx20 of Egr2 activity occurred through recruitment of histone deacetylases to the target genes. Again, we observed promoter-specific effects in that the repression of the EphA4 construct was only minimally affected by the histone deacetylase inhibitor, TSA, whereas the repression of the FGFR2 construct seemed to be almost entirely dependent on histone deacetylase activity. Therefore, recruitment of histone deacetylase proteins seems not to be the sole mechanism by which Ddx20 represses transcriptional activation.

Our data have identified Ddx20 as an Egr2 corepressor that exhibits activities distinct from those of the previously described Nab1 and Nab2 proteins. The existence of several Egr2 cofactors may be necessary to achieve the proper balance of Egr2 activity during peripheral nerve myelination, because aberrant expression levels of several Egr2 target genes can cause peripheral myelinopathies (16). Therefore, future work will be directed toward understanding how the Ddx20 and NAB cofactors maintain proper levels of Egr2 target gene expression.

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