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Targets of the Nuclear Factor I Regulon Involved in Early and Late Development of Postmitotic Cerebellar Granule Neurons

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Recent studies have shown that the nuclear factor I (NFI) family controls multiple stages of the postmitotic differentiation of cerebellar granule neurons (CGNs). Regulation of cell–cell signaling is an integral part of this NFI program, which involves expression of the cell adhesion molecules N cadherin and ephrin B1 throughout postmitotic CGN development. Here, we identify two additional downstream targets of NFI that are involved in extracellular CGN interactions. The cell adhesion molecule Tag-1 is highly enriched in CGNs undergoing parallel fiber formation and is down-regulated prior to onset of radial migration. We found that Tag-1 expression was strongly reduced by NFI dominant repression in immature primary CGNs and in the cerebella of E18 *Nfib*-null mice. Transient transfection and chromatin immunoprecipitation suggested that the *Tag-1* gene is directly regulated by NFI. Furthermore, functional, *Nfi* knockout and chromatin immunoprecipitation studies implicated Wnt7a as a direct target of NFI in maturing CGNs. Wnt7a is secreted by developing CGNs and is required for maturation of mossy fiber–CGN synaptic rosettes. Consistent with this, synapsin I was greatly reduced within the internal granule cell layer of P17 *Nfia*-null mice. These findings indicated that NFI controls CGN postmitotic maturation through a combination of extracellular signaling molecules that operate either continuously to regulate multiple stages of development (N cadherin and ephrin B1) or primarily at early (Tag-1) or late (Wnt7a) maturation steps. They also illustrate the importance of NFI as a critical link between cell-intrinsic mechanisms and cell–cell interactions in the development of the mouse cerebellum.

Key words: cell adhesion molecule; gene transcription; neuronal development; axon formation; synaptogenesis

Nervous system development involves a complex interplay between cell–cell interactions and cell-autonomous events. Much remains to be learned regarding the intrinsic factors and their interactions with external signals that control different aspects of neuronal differentiation. Granule neurons of the cerebellum (CGNs) undergo a series of coordinated differentiation events during postnatal development. Within the premigratory zone (PMZ), immature CGNs extend bipolar processes, and their cell bodies then migrate radially from the PMZ through the forming molecular layer (ML) until they reach the internal granule cell layer (IGL). Upon onset of radial migration, CGN axons form fascicles of parallel fibers (Altman, 1972). Within the IGL, CGNs complete their differentiation by extending claw-like dendrites that form synapses with incoming extracerebellar mossy fibers as well as with GABAergic interneurons. The synaptic fields between mossy fibers and CGN dendrites take the form of rosettes, which are interdigitated, multisynaptic structures that increase the mossy fiber synaptic surface area (Hamori and Somogyi, 1983).

CGN differentiation is driven by a combination of cell-intrinsic mechanisms (Lin and Bulleit, 1996; Powell et al., 1997; Yacubova and Komuro, 2002; Kawaji et al., 2004) and local cellular interactions (Gao et al., 1991; Gao and Hatten, 1993; Lu et al., 2004). For example, newly forming parallel fibers undergoing fasciculation exhibit direct axonal contacts between neighboring CGNs (Altman, 1972; Berglund et al., 1999). Direct interactions between CGNs and radial glia also have

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been implicated in radial migration (Rakic and Sidman, 1973). Cell adhesion molecules have a central role in mediating these various cell contact-dependent events, including Tag-1/contactin-2, contactin, N cadherin, and ephrin B1. Locally secreted factors are also critical for CGN development, including sonic hedgehog, which is released by Purkinje neurons and promotes CGN progenitor proliferation (Wallace, 1999; Wechsler-Reya and Scott, 1999). Similarly, Wnt7a is secreted by CGNs and is important for mossy fiber remodeling and maturation of mossy fiber–CGN synaptic rosettes (Hall et al., 2000). CGNs thus provide an excellent model for investigating the interplay between intrinsic and extrinsic regulation of various phases of neuronal development.

The nuclear factor I (NFI) family of transcriptional regulators is composed of four separate genes (*Nfia*, *Nfib*, *Nfic*, and *Nfix*), and several members have been directly implicated in nervous system development (das Neves et al., 1999; Driller et al., 2007; Campbell et al., 2008; Kumbasar et al., 2009). We recently found that these factors are an important part of the intrinsic program controlling CGN differentiation in vivo. All NFI members are markedly elevated in CGNs and are expressed throughout their postmitotic development (Wang et al., 2004, 2007), suggesting a broad role in CGN differentiation. Functional studies confirmed that NFI proteins regulate multiple aspects of postmitotic CGN maturation, including parallel fiber formation and alignment, migration, and dendrite formation (Wang et al., 2004, 2007). These diverse actions of NFI are mediated in part by N cadherin and ephrin B1, indicating an important link between NFI and cell–cell interactions in differentiating CGNs. Both of these cell adhesion molecules are expressed throughout CGN postmitotic differentiation, and they regulate both early and late aspects of maturation, including axon extension and parallel fiber fasciculation, radial migration, and dendritogenesis (Wang et al., 2007). Here, we expand the mediators of NFI action on CGN cell–cell interactions to include the cell adhesion molecule Tag-1 and the secreted protein Wnt7a. In contrast to N cadherin and ephrin B1, these proteins are involved primarily in specific phases of CGN maturation, parallel fiber formation (Tag-1) and synaptogenesis (Wnt7a).

MATERIALS AND METHODS

Plasmids

DNAs for lentiviruses expressing hemagglutinin (HA)-tagged versions of the NFI dominant repressor (NFI/EnR) or the *Drosophila* engrailed repressor domain alone (EnR) for NFIA and NFIB expression plasmids have been previously described (Wang et al., 2004, 2007). A β-galactosidase reporter construct containing a 13.8-kb human *TAX-1* promoter fragment (Bizzoca et al., 2003) was kindly provided by Dr. G. Gennarini (University of Torino).

Immunostaining

Immunofluorescence assays for the axonal markers phosphoneurofilament and Tag-1 were performed by using 4% paraformaldehyde-fixed CGN cultures or frozen sections from E18 *Nfib*-null and wild-type mouse cerebella fixed in 4% paraformaldehyde and embedded with 30% sucrose as previously described (Wang et al., 2004, 2007). Synapsin I and Wnt7a immunohistochemistry was performed on paraffin sections from paraformaldehyde-fixed P17 *Nfia* knockout and wild-type cerebella. For antigen retrieval, sections were microwaved twice for 5 min in 10 mM sodium citrate buffer (pH 6.0). Samples were blocked with 10% normal goat serum and incubated with primary and Cy3-conjugated/horseradish peroxidase (HRP)-conjugated secondary antibodies. HRP activity was revealed with diaminobenzidine (DAB) tetrachloride chromogen (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The following primary antibodies were used: anti-Tag-1 (clone 4D7; 1:100), pan-axonal neurofilament monoclonal antibody (pNFL; SMI-312; 1:1,000), antisynapsin I polyclonal antibody (1:100; Sigma, St. Louis, MO), and anti-Wnt7a polyclonal antibody (sc-23260; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence, nuclei were stained with bisbenzimide (Sigma; 1 μg/ml) following treatment with secondary antibodies. For Wnt7a immunohistochemistry, nuclei were counterstained with hematoxylin. Measurements of axon length were performed on captured images of phosphoneurofilament staining from 100–150 cells as described previously (Ronn et al., 2000).

Virus Production

The production of lentiviruses enveloped with VSV-G was achieved by cotransfection of the lentiviral plasmid (15 μg), the packaging construct pCMV Δ8.91 (10 μg), and the pMD.G VSV-G viral envelope expression vector (5 μg) into 293T cells using the Calcium Phosphate ProFection Mammalian Transfection System (Promega, Madison, WI; Wang et al., 2004). All viral supernatants were harvested, passed through 0.45-μm-pore-size filters, and concentrated by ultracentrifugation. Transduction efficiencies of CGN cultures were ~90%, as in previous studies (Wang et al., 2007).

Animals

CGN cultures were prepared from CD1 mice. $Nfia^{-/-}$ mice and control $Nfia^{+/+}$ littermates were on a C57Bl/6NTac background (Shu et al., 2003), whereas $Nfib^{-/-}$ mice and wild-type littermates were on a C57BL6/129S background (Steele Perkins et al., 2005). All protocols employed for mouse studies were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School and were in full compliance with the National Institutes of Health *Guide for the care and use of laboratory animals*.

Cell Culture

Purified mouse CGNs were isolated and cultured in Neurobasal medium containing B-27 serum-free supplement, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (pen-strep; Invitrogen, Grand Island, NY) and 0.45% D-glucose as described previously (Wang et al., 2004). Briefly, dissected mouse cerebella were trypsinized and tritu-

rated in calcium-magnesium-free phosphate-buffered saline (PBS; pH 7.4). CGNs were enriched by Percoll (Sigma) gradient centrifugation and preplating on poly-D-lysine-coated Petri dishes. For reaggregate cultures, cell aggregates were formed by incubating purified CGN progenitors on uncoated tissue culture dishes in MEM medium (Sigma) containing 10% fetal bovine serum, 0.45% D-glucose, pen-strep, and 2 mM L-glutamine at a cell density of 4×10^6/ml overnight (Wang et al., 2007). Reaggregates were then washed and cultured in Neurobasal/B27 medium on poly-D-lysine-coated plates at 37°C/5% CO_2.

Isolation of RNA and RT-PCR

Total RNA was extracted from tissues and cells using Tri reagent (Sigma). First-strand cDNAs were synthesized with random hexamers by using the SuperScript RT-PCR system (Invitrogen, Carlsbad, CA). Tag-1 transcripts were assayed by semiquantitative RT-PCR with the following primers: CCTGTGAGGCGGCTGAT (forward), TGTCCCTCCGTG GCGTA (reverse). Real-time RT-PCR for Wnt7a was performed in triplicate on a Bio-Rad iCycler system using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) with the following primers: GCTAGGCTACGTGCTCAAGG (forward), CCTGTCACTGGGTCCTCTTC (reverse). 18S ribosomal RNA was assayed for normalization purposes. Quantification of Wnt7a transcripts was determined by using the $2^{-\Delta Ct}$ method as previously described (Wang et al., 2004).

Transient Transfections

JEG3 cells were obtained from ATCC and cultured in DMEM and 10% FBS in 24-well plates. The human *TAX-1* promoter plasmid was cotransfected with NFIA, NFIB, or control expression plasmids at a ratio of 3:1 using FuGENE 6 Transfection Reagent as previously described (Wang et al., 2004). A luciferase-expressing plasmid (pHR′-CMV-LUC-W-Sin18) was cotransfected as a normalization control. Protein was extracted 48 hr posttransfection. Luciferase and β-galactosidase activities were determined in 10 μl of cell extract using the luciferase assay system (Promega, Madison, WI) and Galacto-Light and Galacto-Light Plus Systems (Applied Biosystem, MA), respectively.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed as previously described (Wang et al., 2004). Briefly, cells or minced cerebellar tissue were cross-linked with 1% formaldehyde for 10 min at 37°C and lysed in SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture mix). Chromatin was sonicated to an average length of 600 bp. After overnight incubation with NFI antibody (Wang et al., 2004) at 4°C, immunocomplexes were collected with protein A agarose (Millipore, Temecula, CA). DNA was extracted and assayed by PCR for mouse *Tag-1* genomic sequences with the following primers: distal region, GAGCCGCGTTCCTATT (forward), CACGACAGGTGCA GGG (reverse); proximal region, TCCAGAATTTCAGAAG CAAG (forward), TGAGCGGAGCCTGCGA (reverse). The *Wnt7a* promoter region was examined with the following primers: AAAGGCCAGAATGCACCAC (forward), GATTGC ACCTAAGGCGAGAC (reverse). Mouse *Bf2* genomic sequences (forward: GGGGTTGGTTTCGTTC; reverse: AAGTC AGGGTTGCAGCATAG) were assayed as a negative control.

Statistical Analysis

In all studies, a minimum of three independent experiments was performed. Data are presented as the mean ± SE and were compared for statistical significance by unpaired, two-tailed *t*-test analysis (Gould, 2002). $P < 0.05$ was considered significant.

RESULTS

NFI Functions Upstream of Tag-1 During Axon Outgrowth

We recently demonstrated that NFI regulates multiple aspects of parallel fiber formation in CGNs, acting at one or more steps in this process to regulate axon extension as well as their parallel alignment or fasciculation (Wang et al., 2007). To probe further the mechanism of NFI action in axonogenesis in CGNs, we examined the effect of repressing NFI trans-activation on the expression of two proteins, phosphoneurofilament (pNFL), a general axonal marker, and Tag-1, which is expressed early during CGN axon formation (Wolfer et al., 1994) and has been implicated in parallel fiber alignment (Baeriswyl and Stoeckli, 2008). Cells were transduced with lentiviruses expressing the NFI/EnR dominant repressor or a control EnR repressor domain alone. When dissociated cultures were transduced, staining for pNFL showed no obvious alterations in axon formation (Fig. 1a). These findings mirror previous studies showing that NFI is not required for axon extension by dissociated CGNs on a laminin surface (Wang et al., 2007).

In contrast to pNFL, Tag-1 immunostaining was dramatically reduced by the dominant repressor in dissociated cultures (Fig. 1b). This preferential inhibition of Tag-1 relative to the general axonal marker pNFL suggested that Tag-1 expression may be specifically regulated by NFI trans-activation during early axon extension in immature CGNs. To address this, we examined the effect of NFI/EnR on Tag-1 mRNA in CGN cultures. Reaggregate suspension cultures were used for these studies to foster CGN progenitor proliferation (Gao et al., 1991) and early onset of NFI/EnR expression prior to plating, which induces growth arrest and differentiation. In control cultures, Tag-1 gene expression increased during axon extension over the first 2 days of in vitro culture (2 DIV; Fig. 2a) and subsequently declined, consistently with previous reports (Tarnok et al., 2005). The NFI dominant repressor blocked this early increase in Tag-1 gene expression observed on 2 DIV (Fig. 2b), suggesting that Tag-1 lies downstream of NFI in early-differentiating CGNs.

Tag-1 Expression Is Diminished in Nfib-Deficient Mice

To explore further the regulatory relationship between NFI proteins and Tag-1, we examined Tag-1

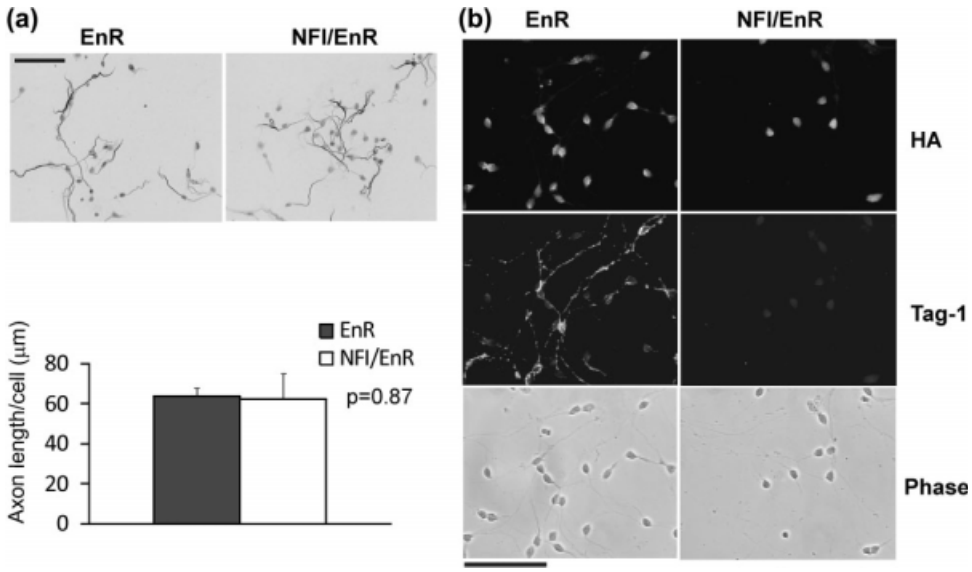

Fig. 1. NFI regulates Tag-1 protein in immature CGNs. **a:** NFI dominant repressor does not significantly affect axon extension in dissociated CGNs on a PLY surface. Top: Dissociated CGNs were quantitatively infected with HA-tagged NFI/EnR or EnR virus on 0 DIV and stained for pNFL on 2 DIV. Bottom: Quantification of axonal length in CGN cultures transduced with lentivirus expressing either Nfi/EnR or EnR. **b:** NFI dominant inhibition impairs Tag-1 expression. Dissociated CGNs transduced with NFI/EnR or EnR viruses were stained with antibody against HA or Tag-1. Phase-contrast images of the same fields are shown below. Scale bars = 100 μm.

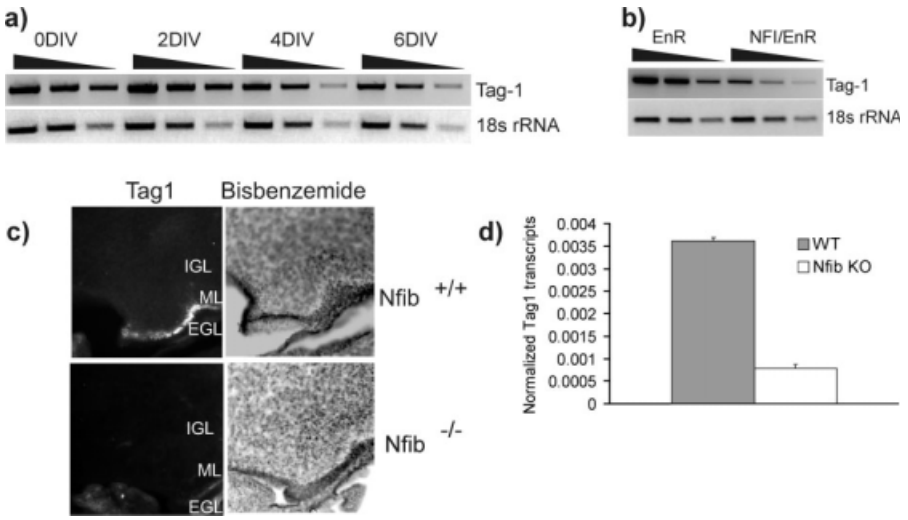

Fig. 2. Regulation of Tag-1 mRNA by NFI. **a:** Tag-1 transcripts were analyzed in developing CGN cultures by semiquantitative RT-PCR (threefold serial dilution). 18S rRNA was assayed as an internal control. **b:** Tag-1 transcripts were diminished by the NFI dominant repressor. CGN reaggregates were transduced with NFI/EnR or EnR lentivirus on 0 DIV. Tag-1 mRNA was measured by semiquantitative RT-PCR on 2 DIV. **c:** Tag-1 immunostaining is reduced in the cerebellum of *Nfib*-deficient mice. Sagittal sections of E18 $Nfib^{+/+}$ and $Nfib^{-/-}$ mice were stained with anti-Tag-1 antibody. Sections were costained with bisbenzimide to identify cell nuclei (right). Different cell layers are indicated. **d:** Cerebellar Tag-1 mRNA is markedly down-regulated in *Nfib*-null mice. Total RNA from E18 cerebella from $Nfib^{-/-}$ (KO) and $Nfib^{+/+}$ (WT) mice was assayed by qRT-PCR for Tag-1 transcripts.

expression in *Nfi* knockout mice. We focused on *Nfia*- and *Nfib*-null mice, because these exhibit cerebellar defects (Wang et al., 2007), whereas *Nfic*- and *Nfix*-null mice show no obvious alterations of this structure (Wang et al., 2007; R. Gronostajski, unpublished observations). Cerebella of *Nfia*-null mice were examined at P8, when parallel fiber formation is highly active. No significant differences were apparent in Tag-1 immunostaining in different folia of the vermis for wild-type and $Nfia^{-/-}$ mice (data not shown). *Nfib* knockout mice die at birth, precluding the analysis of Tag-1 expression during postnatal development. We therefore examined these

mice at E18, when axon formation in *Nfib*-null mice is known to be affected (Wang et al., 2007). Tag-1 immunostaining within the deep EGL/PMZ region was markedly reduced in the posterior cerebellum of E18 *Nfib*-deficient mice (Fig. 2c). The thickness of the EGL did not show substantial differences between wild-type and *Nfib*-null mice (data not shown), indicating that CGN progenitor formation was not grossly altered, which is consistent with previous findings (Wang et al., 2007). This decrease in Tag-1 protein was accompanied by an approximately fivefold reduction in Tag-1 mRNA in *Nfib* $^{-/-}$ relative to *Nfib* $^{+/+}$ E18 cerebella (Fig. 2d). Together these findings indicated that NFIB is an important upstream regulator of Tag-1 in differentiating granule neurons, although NFIA is not absolutely required for Tag-1 expression during postnatal parallel fiber emergence within the PMZ.

NFI Proteins Regulate the Tag-1 Promoter In Cellulo and Are Bound to It In Vivo

To examine whether NFI directly regulates Tag-1 transcription, transient transfection studies were performed using an expression plasmid for either NFIA or NFIB and a human *TAX-1* promoter construct containing 11 kb of the 5′-flanking region (Bizzoca et al., 2003). Cotransfection into JEG3 cells demonstrated that both NFIA and NFIB activate the *TAX-1* promoter by approximately six- to eightfold (Fig. 3a). Sequencing of the 5′-flanking region for the mouse, rat, and human genes revealed four potential NFI binding sites that were highly similar in both nucleotide sequence and location for all three genes (Fig. 3b). These sites were distributed within two subregions, one more distal (~1 kb upstream) to the reported transcription start site for the mouse gene and a proximal promoter region flanking the start site.

We next performed ChIP assays to determine whether NFI proteins were bound in vivo to these two genomic subregions in mouse CGNs. Analysis of 2-DIV CGN cultures detected robust binding of endogenous NFI proteins to both the distal and the proximal segments of the mouse *Tag-1* gene, but not to a genomic region lacking NFI consensus sites (*Bf2* gene; Fig. 3c). Furthermore, in vivo binding of NFI proteins to the *Tag-1* gene was confirmed in the intact cerebellum of P6 mice (Fig. 3c), when CGNs are extending parallel fibers and *Tag1* gene expression is robust (Bizzoca et al., 2003). These results, in conjunction with promoter, NFI dominant repressor and *Nfi* knockout studies, indicated a direct regulatory interaction between NFI trans-activation and the up-regulation of the *Tag-1* gene during early CGN differentiation in vivo.

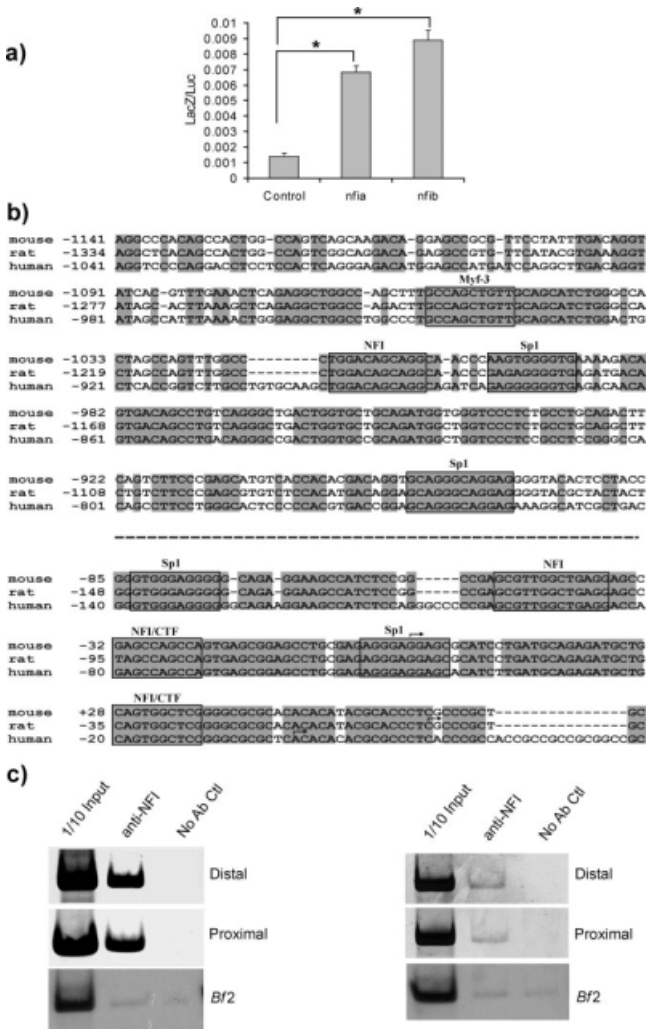

Fig. 3. NFI proteins trans-activate and bind to the *Tag-1* gene. **a:** JEG3 cells were cotransfected with a human *TAX-1*/β-galactosidase reporter construct along with an expression plasmid for NFIA, NFIB, or a control vector. A luciferase-expressing vector was cotransfected as an internal control. β-Galactosidase activity was measured after 48 hr and normalized relative to luciferase (luc) activity. The asterisk indicates significant difference ($P < 0.01$). **b:** Multispecies sequence alignment of the distal (top) and proximal (bottom) *Tag-1* promoter region revealed conserved transcription factor binding sites (boxes). Shading indicates sequence identities among rat, mouse, and human, and arrows indicate predicted transcriptional start sites. **c:** In vivo binding of NFI to the distal and proximal regions containing NFI sites was analyzed by ChIP assay in 2-DIV CGN cultures (left) and cerebella of P6 mice (right). Lower NFI ChIP signal in P6 cerebellar chromatin relative to CGN cultures likely reflects the substantial cellular heterogeneity at this age. Unrelated *Bf2* genomic sequences were not significantly precipitated by NFI antibodies.

Nfia Deficiency Affects a Presynaptic Marker Within the IGL

In addition to defects in parallel fiber formation and migration, *Nfia*-null CGNs also exhibit diminished dendrite formation during the third postnatal week, specifically within more anterior lobules of the cerebellar vermis (Wang et al., 2007). Here, we examined the degree to which mossy fiber-CGN synapse formation was affected within the *Nfia*-null cerebellum using synapsin I, a presynaptic marker for mossy fiber–CGN rosettes (Hall et al., 2000). Staining for synapsin I was greatly diminished within the IGL of *Nfia*-deficient mice at P17 (Fig. 4a). In particular, the number of synapsin I-

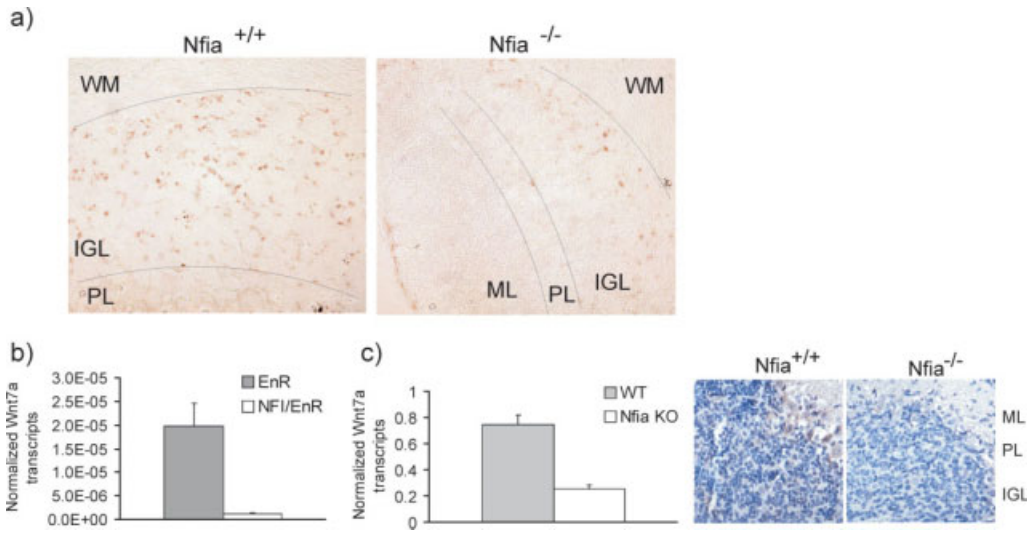

Fig. 4. Synapsin I and Wnt7a expression are regulated by NFI proteins in CGNs. **a:** Saggital sections from the central vermis of wild-type (*Nfia*$^{+/+}$) and knockout (*Nfia*$^{-/-}$) P17 mouse cerebella were stained for synapsin I protein. The different regions of the postnatal cerebellum are indicated for clarity: WM, white matter; IGL, internal granule cell layer; PL, Purkinje cell layer; ML, molecular layer. **b:** CGNs were transduced on 0 DIV with lentiviral expression vectors for NFI dominant repressor or control (EnR only) proteins. Cells were harvested on 6 DIV and assayed by qRT-PCR for Wnt7a mRNA. **c:** Wnt7a mRNA and protein are reduced in *Nfia* knockout mouse cerebellum. Left: Total RNAs from P17 wild-type (WT) or *Nfia*-null cerebella were assayed by qRT-PCR for Wnt7a transcripts. Right: Wnt7a was examined by immunohistochemistry in *Nfia*-deficient and wild-type cerebella at P17. Nuclei were counterstained with hematoxylin.

positive synaptic rossettes was greatly reduced. Also, labeled rosettes were localized mainly to the deeper segment of the *Nfia*-null IGL, whereas staining in wild-type mice occurred in both deeper and more superficial aspects of this structure (Fig. 4a). This might have reflected a more selective defect or a delay in the appearance of synapsin I protein within rosettes associated with later-arriving CGNs, insofar as this upper region of the IGL tends to be populated by CGNs that are born and differentiate later than those generally found more deeply within this structure (Altman, 1972; Jones et al., 2000). Furthermore, synapsin I signal was decreased in both posterior and anterior lobules to a similar extent (data not shown), suggesting that this defect was not solely an indirect effect of reduced dendrite extension from CGNs in the IGL.

Wnt7a Is an Apparent Direct Target of NFI Proteins in Maturing CGNs

Synapsin I within IGL rosettes is expressed by presynaptic neurons located in multiple extracerebellar regions. Altered gene expression in postsynaptic CGNs of *Nfia* knockout mice might contribute to reduced synapsin I staining within in the IGL. In particular, Wnt7a is a direct regulator of mossy fiber rosette formation in the postnatal IGL (Hall et al., 2000). CGN cultures transduced with the Nfi dominant repressor showed a dramatic inhibition of Wnt7a mRNA (Fig. 4b). In addition, Wnt7a mRNA and protein were markedly reduced in P17 cerebella from *Nfia*-null mice (Fig. 4c). Wnt7a protein was decreased in the IGL as well as in the Purkinje and molecular layers. Thus, expression of Wnt7a requires NFI transactivation in primary CGNs and specifically NFIA in the developing postnatal mouse cerebellum, where granule neurons are the major site of Wnt7a expression (Lucas and Salinas, 1997). Reduced Wnt7a expression likely contributes to decreased synapsin I expression within IGL rosettes in *Nfia*-null cerebellum, as observed in *Wnt7a*-null mice (Hall et al., 2000), although an additional effect of *Nfia* deficiency on synapsin I gene expression within presynpatic neurons cannot be ruled out.

Bioinformatic analysis of the mouse *Wnt7a* gene identified two NFI consensus sites within its 5′-flanking region (Fig. 5a). ChIP assays showed that NFI proteins bound robustly to the proximal site within chromatin of mature mouse CGN cultures (Fig. 5b). Furthermore, direct in vivo binding of NFI was detected in P21 mouse cerebellum (Fig. 5b), when Wnt7a expression is highly up-regulated in CGNs within the IGL (Lucas and Salinas, 1997). Only very weak NFI binding was detected within the region spanning the upstream consensus site in CGN cultures and P21 mouse cerebellum (data not shown). These findings are consistent with Wnt7a being a direct target of the NFI family in later-maturing CGNs in the IGL.

DISCUSSION

Recent studies have shown that NFI proteins have a central role in directing the intrinsic differentiation of CGNs in vivo (Wang et al., 2004, 2007). Based on the analysis of gene knockout mice here and in earlier stud-

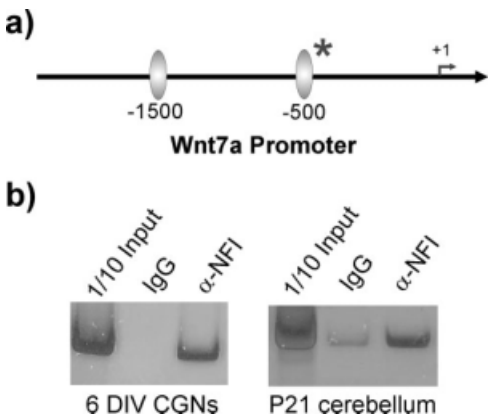

Fig. 5. NFI proteins bind to the mouse *Wnt7a* proximal promoter in vivo. **a:** Diagram showing the locations of NFI consensus sites within the mouse *Wnt7a* 5′-flanking region. The asterisk indicates the consensus site to which NFI binding was detected in vivo. **b:** ChIP assays of the proximal Wnt7a NFI site. Left: NFI binding to chromatin prepared from 6-DIV CGN cultures. Right: In vivo NFI binding is also detected in the intact P21 mouse cerebellum. IgG antibodies were used as a nonspecific control. No significant binding was detected to the upstream NFI consensus site in a (data not shown).

ies, NFIA and NFIB appear to be particularly important for CGN maturation. The cell adhesion molecules N cadherin and ephrin B1 were previously identified as key mediators of NFI regulation (Wang et al., 2007). These proteins control multiple stages of CGN development, including axon extension, migration, and dendritogenesis, reflecting their expression throughout the postmitotic differentiation of these cells. The present findings expand the repertoire of downstream NFI genes in maturing postmitotic CGNs to include Tag-1 and Wnt7a. As for N cadherin and ephrin B1, the *Tag-1* and *Wnt7a* genes are bound by NFI in vivo indicating that they are direct targets of this transcription factor family. Moreover, regulation of Wnt7a and synapsin I by NFI proteins directly implicates this transcription factor family in glomerular synaptogenesis and synaptic function.

In contrast to N cadherin and ephrin B1, Tag-1 and Wnt7a have more selective actions during specific phases of CGN development. Tag-1 is preferentially expressed during the early postmitotic period when immature CGNs are present in the PMZ (Pickford et al., 1989). This cell adhesion molecule was recently implicated in regulating the alignment of newly forming parallel fibers in the developing chick cerebellum (Baeriswyl and Stoeckli, 2008). Tag-1 may therefore be an important downstream effector of the Nfi family in regulating parallel fiber fasciculation and orientation in the developing cerebellum. Although a similar phenotype was not reported for the cerebellum of *Tag-1*-null mice, this may reflect compensatory mechanisms arising during earlier development (Fukamauchi et al., 2001). *Nfia*-deficient mice also exhibit disrupted orientation of newly extending parallel fibers (Wang et al., 2007). This reflects at least in part altered N cadherin and ephrin B1 function (Wang et al., 2007) as opposed to Tag-1, which is unaltered in these mice. Axon extension is disrupted in *Nfib*-null mice (Wang et al., 2007), and the latter do not survive postnatally (Steele Perkins et al., 2005). Both features make the assessment of CGN axon alignment in *Nfib*-null mice technically unfeasible.

Wnt7a is transiently up-regulated in mouse CGNs within the IGL as they undergo synaptogenesis during the third postnatal week (Lucas and Salinas, 1997) and is required for synapsin I expression and formation of synaptic rossettes within the IGL (Hall et al., 2000). Reduced expression of synapsin I in CGNs from P17 *Nfia*-deficient mice is consistent with a mediator role for Wnt7a in Nfi regulation of synapsin I appearance in glomeruli within the IGL. Thus, formation of both CGN dendrites (Wang et al., 2007) and mossy fiber-CGN synaptic proteins appear to be regulated by NFI factors.

Our findings indicate that NFI intrinsically controls cell–cell interactions by means of both membrane-bound and secreted proteins during CGN postmitotic development. These mediators are expressed and act either throughout CGN maturation (e.g., N cadherin and ephrin B1) or in a more selective manner during early (parallel fiber formation; Tag-1) or late (synaptogenesis; Wnt7a) developmental stages. These findings illustrate the complex regulatory actions of the Nfi family and their downstream effectors during postmitotic CGN differentiation. Together with earlier results, the present work also suggests a broad role for NFI proteins in the normal functioning of the cerebellum. By controlling the extension and fasciculation of parallel fibers, they are directly implicated in establishing CGN-Purkinje cell synaptic interactions and communication. Furthermore, NFI regulation of CGN dendritogenesis and glomerular synapse formation via Wnt7a directly affects mossy fiber excitatory inputs to CGNs. NFI proteins also bind to and regulate the expression of the $GABA_A$ receptor $\alpha 6$ subunit (*Gabra6*) gene (Wang et al., 2004), which controls CGN excitability via extrasynaptic tonic inhibition from Golgi interneurons (Brickley et al., 2001). Thus, the NFI family regulates events important for both excitatory and inhibitory synaptic interactions controlling CGN excitability and outputs, which in turn are critical for cerebellar function and communication with extracerebellar structures.

Finally, NFI proteins are enriched in postmitotic CGNs relative to other cerebellar cell types (Wang et al., 2004). The *Gabra6* gene is expressed primarily in CGNs as well as cochlear neurons (Kato, 1990; Varecka et al., 1994), both of which derive from a common lineage (Pierce, 1967; Altman and Bayer, 1985). Similarly, both Tag-1 and Wnt7a are highly expressed in CGNs (Pickford et al., 1989; Lucas and Salinas, 1997). These findings, together with our observations that NFI proteins bind directly to consensus sites within the mouse *Gabra6*, *Tag-1*, and *Wnt7a* genes, suggest that this transcription factor family directly contributes to elevated or cell-specific expression of a subset of critical genes in granule neurons within the developing mouse cerebellum.

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