

# Reciprocal autoregulation by NFI occupancy and ETV1 promotes the developmental expression of dendrite-synapse genes in cerebellar granule neurons

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**ABSTRACT** Nuclear Factor One (NFI) transcription factors regulate temporal gene expression required for dendritogenesis and synaptogenesis via delayed occupancy of target promoters in developing cerebellar granule neurons (CGNs). Mechanisms that promote NFI temporal occupancy have not been previously defined. We show here that the transcription factor ETV1 directly binds to and is required for expression and NFI occupancy of a cohort of NFI-dependent genes in CGNs maturing *in vivo*. Expression of ETV1 is low in early postnatal cerebellum and increases with maturation, mirroring NFI temporal occupancy of coregulated target genes. Precocious expression of ETV1 in mouse CGNs accelerated onset of expression and NFI temporal occupancy of late target genes and enhanced Map2(+) neurite outgrowth. ETV1 also activated expression and NFI occupancy of the *Etv1* gene itself, and this autoregulatory loop preceded ETV1 binding and activation of other coregulated target genes *in vivo*. These findings suggest a potential model in which ETV1 activates NFI temporal binding to a subset of late-expressed genes in a stepwise manner by initial positive feedback regulation of the *Etv1* gene itself followed by activation of downstream coregulated targets as ETV1 expression increases. Sequential transcription factor autoregulation and subsequent binding to downstream promoters may provide an intrinsic developmental timer for dendrite/synapse gene expression.

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## INTRODUCTION

Timing mechanisms are now recognized as fundamentally important requirements for neuronal development (Martynoga *et al.*, 2012; Kumamoto *et al.*, 2013). In particular, neurons elaborate den-

drites and form numerous synaptic connections, and the timing of these events is critical for establishing appropriate neural circuitry (Deguchi *et al.*, 2011; Tripodi and Arber, 2012). This requires precise temporal regulation of numerous genes (Balamotis *et al.*, 2012), and disruption of these timing mechanisms can dramatically alter neural circuitry (Hippenmeyer *et al.*, 2005). Further, dysregulated gene expression during critical temporal windows of synaptic maturation has been implicated in neurodevelopmental disorders (NDs; Meredith *et al.*, 2012). The mechanisms that temporally coordinate dendrite and synapse formation and related gene expression remain poorly understood.

Cerebellar granule neurons (CGNs) undergo a well-defined postnatal program of development in which subsets of genes are expressed in distinct temporal patterns linked to specific stages of maturation—for example, progenitor proliferation within the

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Abbreviations used: CGN, cerebellar granule neuron; DIV, days *in vitro*; IGL, internal granule cell layer; ND, neurodevelopmental disorder; NFI, Nuclear Factor One.

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external germinal layer (EGL), axon formation in the premigratory zone, radial migration, and finally dendrite/synapse formation by postmigratory CGNs within the internal granule cell layer (IGL) (Goldowitz and Hamre, 1998; Furuichi *et al.*, 2011). Further, much of this developmentally regulated gene expression is reproduced in primary CGN cultures (Ding *et al.*, 2013), providing an excellent system for elucidating mechanisms underlying temporal programming of dendrite and synapse formation.

Transcription factor and chromatin interactions have a central role in dendrite and synapse formation during neuronal development (Santiago and Bashaw, 2014; Vogel-Ciernia and Wood, 2014), and evidence for their involvement in the timing of these events has recently come to light. Nuclear Factor One (NFI) proteins, and in particular NFIA, regulate CGN dendritogenesis both in vivo and in purified cultures (Wang *et al.*, 2007, 2010). Recent studies revealed that this is mediated by an NFI-dependent developmental switch that controls the timing of dendritogenesis in postmigratory CGNs maturing within the internal granule cell layer during the second and third postnatal weeks (Ding *et al.*, 2013). Although NFI proteins are constitutively expressed in the nucleus throughout CGN differentiation and in vitro NFI DNA binding activity is similar in early- and later-maturing cerebellum (Wang *et al.*, 2011), late gene activation is specifically linked to temporally up-regulated occupancy of NFI sites within late gene promoters. Onset of NFI temporal occupancy is orchestrated in part by voltage-sensitive and maturation-dependent dismissal of NFATc4 repressor bound to late gene promoters, permitting subsequent NFI occupancy of late-expressed genes (Ding *et al.*, 2013).

The aforementioned studies left open the question of whether additional factors control NFI temporal binding and switch programming in CGNs. For example, up-regulation of specific subsets of late-expressed genes likely occurs with distinct time frames, requiring the operation of additional mechanisms to fine-tune late temporal gene expression. This may include *trans*-activators that promote these events subsequent to NFATc4 dismissal from NFI-late genes. ETV1 is a member of the ETS family of *trans*-factors that is temporally up-regulated in postmigratory CGNs maturing within the IGL (Sato *et al.*, 2005; Schuller *et al.*, 2006). Knockdown recently implicated ETV1 in the activity-dependent up-regulation of gene expression during CGN maturation (Abe *et al.*, 2011). Here we used gene-knockout mice, chromatin immunoprecipitation (ChIP), and lentiviral transduction of neuronal cultures to investigate the role of ETV1 in promoting the NFI switch program and NFI temporal binding in maturing CGNs.

## RESULTS

### **Etv1 regulates the NFI switch in CGNs maturing in vivo**

*Etv1*-knockout mice survive into the second postnatal week (Cave *et al.*, 2010), during which the NFI switch program and dendrite formation are underway in CGNs within the IGL (Altman and Bayer, 1997). Because *Etv1* gene expression is detected only in CGNs and not other cerebellar cell types in the mouse (Sato *et al.*, 2005; Schuller *et al.*, 2006), cerebella from these mice should reflect largely CGN-intrinsic deficits in ETV1 function. Transcripts for the NFI-switch late genes *Gabra6*, *Rps6ka1*, *Nptx1*, *Wnt7a*, and *Ets2* were markedly down-regulated in *Etv1*-null mouse cerebellum at postnatal day 10 (P10) and P11 (Figure 1A). Note that expression of each of these genes is either unique or highly predominant in CGNs within the IGL of the developing mouse cerebellum (Sato *et al.*, 2005; Cerebellar Development Transcriptome Database [www.cdtb.neuroinf.jp/CDT/Top.jsp]; MGI:3511933 [www.informatics.jax.org/assay/MGI:3511933]). In contrast, the non-NFI switch gene

*Rbfox3/NeuN*, which is a temporally up-regulated specific marker for postmitotic mouse CGNs (Weyer and Schilling, 2003), was unaffected by *Etv1* deficiency (Figure 1A). Thus these results did not reflect generalized changes in cerebellar or CGN gene expression. Consistent with this, no significant differences were observed in the thickness and cell densities of the EGL, molecular layer, and IGL of P10 *Etv1*-deficient and wild-type mice (Supplemental Figure S1). Further, the early-expressed NFI-switch genes *Dcx*, *Neurod6*, and *Tgfb2* were up-regulated in *Etv1*-knockout cerebellum (Figure 1A), indicating that ETV1 also regulates a switch program that overlaps with the NFI-switch regulon (Ding *et al.*, 2013).

The effects of in vivo *Etv1* deficiency on the NFI-late genes *Gabra6*, *Nptx1*, *Ets2*, and *Wnt7a* mirror those found in CGN cultures using *Etv1* siRNAs and are consistent with ETV1 activation of several NFI-late gene promoters in transfection studies (Abe *et al.*, 2011). Of interest, the NFI-late gene *Nab2* was not significantly affected in *Etv1*-knockout mouse cerebellum (Figure 1A), revealing selectivity in ETV1 regulation of NFI-late genes in vivo.

### **Grin2c and Tiam1 are Etv1-dependent NFI-switch late genes**

Promoter cotransfection experiments and small interfering RNA (siRNA) studies previously identified *Grin2c* and *Tiam1* as ETV1-regulated late target genes in maturing CGNs (Abe *et al.*, 2011). We extend these results here by showing that both genes are strongly down-regulated in *Etv1*-null mouse cerebella (Figure 1A). We also find that *Grin2c* and *Tiam1* are regulated as part of the NFI switch program, using an NFI dominant repressor lentivirus, which represses genes activated by all NFI family members, in CGN cultures and by analysis of P15 *Nfia*-knockout mouse cerebellum (Figure 1B). Further, both genes undergo NFI temporal occupancy (Figure 1C). Thus *Grin2c* and *Tiam1* are part of the ETV1/NFI temporal coregulon.

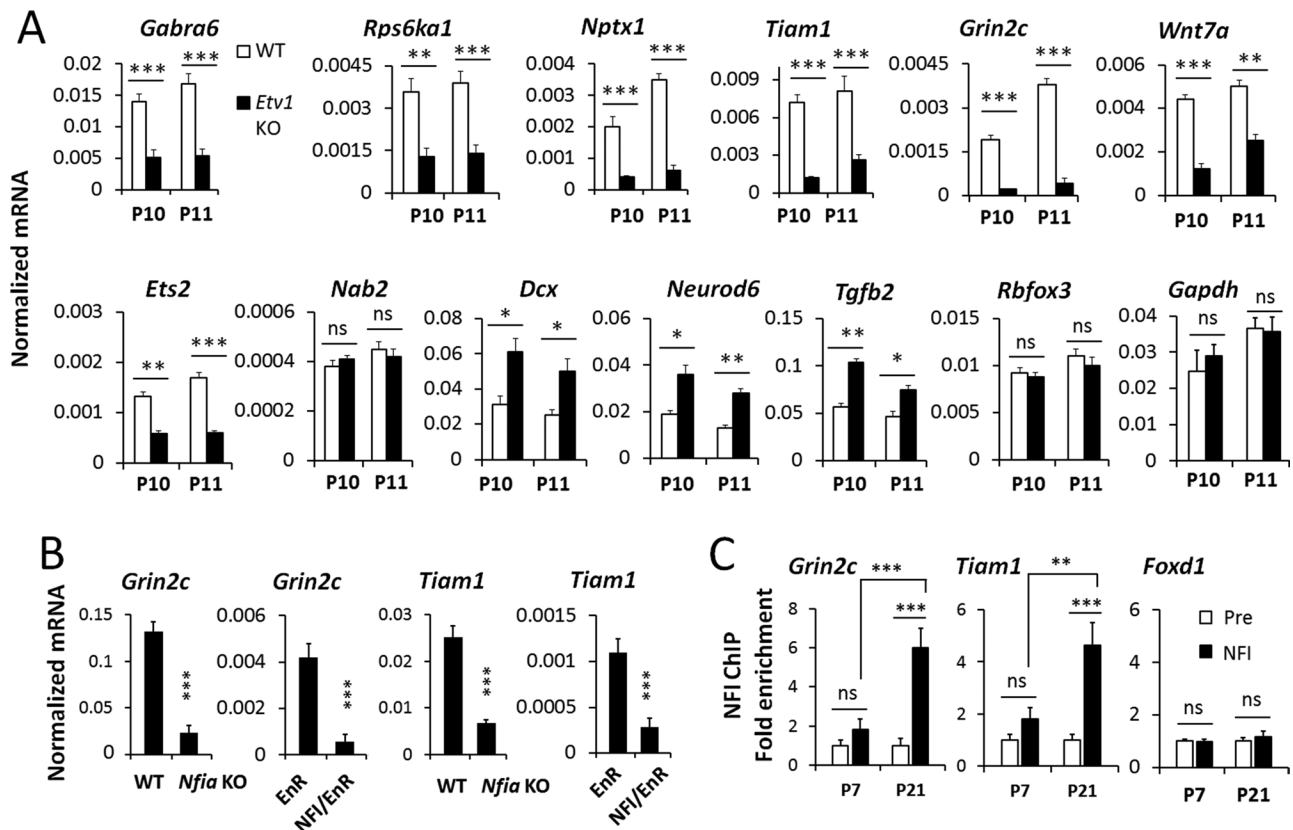
### **Effects of precocious ETV1 expression in immature CGNs**

ETV1 protein and *Etv1* mRNA are low in immature CGNs and increase with maturation (Sato *et al.*, 2005; Abe *et al.*, 2012). We therefore examined the effect of elevated early expression of ETV1 on endogenous late genes in immature CGNs. Premature expression of ETV1 increased late gene expression at 2 and 3 d in vitro (DIV), when endogenous *Etv1* mRNA is normally low (Abe *et al.*, 2012; Figure 2A). This included both *Grin2c* and *Tiam1* but not *Rbfox3* (Figure 2A). Collectively these gain- and loss-of-function results indicate both a requirement and an activating role for ETV1 in NFI-late gene regulation in immature CGNs.

The NFI switch program enhances dendritogenesis in developing CGNs (Wang *et al.*, 2007; Ding *et al.*, 2013). We therefore examined whether ETV1 itself regulates the maturation of neurites expressing Map2, a dendritic marker. Transduction of 0-DIV CGN cultures with ETV1 produced a modest but significant increase in Map2(+) neurite length at 2 DIV (Figure 2B). Thus elevation of ETV1 expression in immature CGNs accelerates both neurite formation and NFI-late gene expression linked to this process. These in cellulo gain-of-function results are consistent with reduced maturation of CGN primary dendrites observed in vivo using *Etv1* siRNAs (Abe *et al.*, 2011).

### **ETV1 promotes NFI temporal occupancy**

Because NFI temporal binding is a hallmark of the NFI late gene program, we next asked whether ETV1 acts by enhancing NFI binding to target promoters. ChIP assays revealed that NFI occupancy was dramatically reduced in P10 *Etv1*-knockout cerebellum for *Gabra6*, *Rps6ka1*, *Nptx1*, *Grin2c*, and *Tiam1*, whereas no specific NFI binding was observed for the *Nab2* gene at this early age in



**FIGURE 1:** ETV1 and NFI coregulate a subset of late temporal genes. (A) RT-qPCR results for selected NFI switch-program and control genes in P10 and P11 *Etv1*-knockout cerebellum. (B) RT-qPCR assays of *Grin2c* and *Tiam1* transcripts in wild-type (WT) and *Nfia*-knockout (*Nfia* KO) cerebellum at P15 and of 6-DIV CGN cultures transduced on 0 DIV with lentiviruses expressing NFI dominant repressor (NFI/EnR) or its negative control (EnR). (C) ChIP-qPCR analysis of NFI binding to the *Tiam1* and *Grin2c* promoters in the developing cerebellum at P7 and P21, expressed as the enrichment relative to control values (set as 1). *Foxd1* genomic sequences were assayed as a negative control. Pre, preimmune serum. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; ns, no significant difference.

either wild-type or *Etv1*-null mice (Figure 3A). Reduced NFI occupancy of late genes was not related to decreased amounts of total NFI or NFIA proteins in *Etv1*-knockout cerebella (Figure 3B). Conversely, early expression of ETV1 in immature CGNs stimulated NFI binding to these late genes at 2 DIV (Figure 3C).

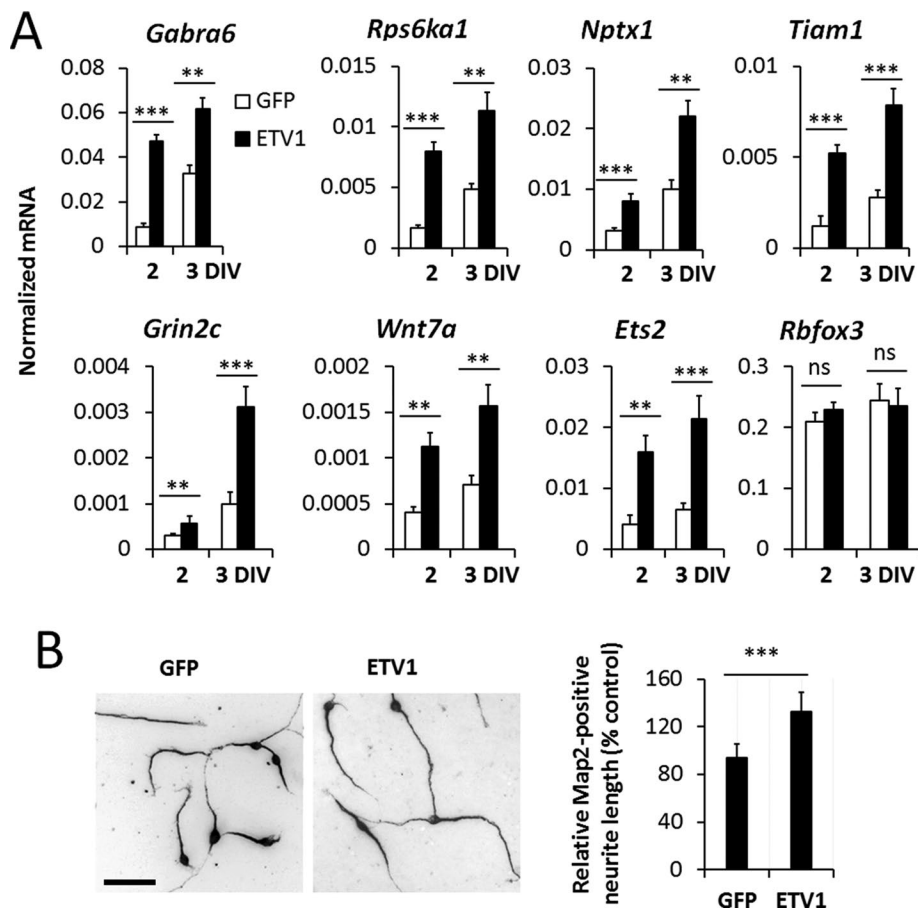
ETV1 was shown to activate promoter constructs for numerous late genes (*Grin2c*, *Gabra6*, *Tiam1*, *Nptx1*) in a heterologous cell line, and it binds to the *Gabra6* and *Grin2c* genes in P21 mouse cerebellum (Abe et al., 2011). We confirmed that ETV1 occupies sites within these two gene promoters in mouse cerebellum, as well as those for the late genes *Rps6ka1*, *Nptx1*, and *Tiam1* (Figure 4, A and B). For each gene, ETV1 binding was temporally up-regulated in parallel with its increased expression (P7–P21), whereas no specific binding was observed for nonexpressed *Foxd1* genomic sequences. Further, specific ETV1 occupancy was substantially reduced in cerebella of *Etv1*-knockout mice (Figure 4C). Together with promoter transfection studies (Abe et al., 2011), these findings support a direct activating role for ETV1 in regulating a substantial segment of the NFI late gene program and, critically, in promoting NFI temporal occupancy of numerous late genes as CGNs mature.

#### ETV1 is an NFI-switch late gene

We previously identified *Etv1* as a potential NFI-late gene in microarray studies ([www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42018](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42018)). We first confirmed that *Etv1* gene expression is up-

regulated as CGNs mature in vivo (P7 vs. P21) and in culture (0 DIV, consisting of CGN progenitors and immature premigratory CGNs; 6 DIV, more-mature CGNs; Figure 5A). Western analyses further confirmed that nuclear levels of ETV1 protein (~50–55 kDa) increased with cerebellar maturation between P7 and P21 (Figure 5B). Two isoforms of ETV1 were detected, one with a faster migration predominating at P7, and the other a slower and more abundant form present at P15 and P21. This more abundant species may reflect in vivo-phosphorylated ETV1, which exhibits increased transcriptional activity and has been detected in CGN cultures after brain-derived neurotrophic factor (BDNF) stimulation (Abe et al., 2012). This is not further addressed here. In contrast, nuclear levels of NFIA were relatively unchanged (or slightly declined), and nuclear NFATc4 strongly decreased between P7 and P21 (Figure 5B). The reduction in nuclear NFATc4 is consistent with decreasing NFATc4 occupancy of late genes and declining calcineurin phosphatase activity with maturation of the mouse cerebellum (Ding et al., 2013).

*Etv1* gene expression was strongly reduced in 6-DIV CGNs transduced on 0 DIV with an NFI-dominant repressor lentivirus and also in the cerebella of P15 *Nfia*-knockout mice, when expression of late genes is normally elevated (Figure 5C). We also identified an NFI consensus site within the proximal *Etv1* promoter. Because the NFI ChIP antibody used here recognizes both NFIA and NFIB (Ding et al., 2013), we performed ChIP analysis of this site using an NFIA-specific



**FIGURE 2:** Overexpression of ETV1 up-regulates NFI-late genes and neurite formation in maturing CGNs. (A) Transcript levels for the indicated genes for wild-type 6-DIV CGN cultures transduced on 0 DIV with lentiviruses expressing GFP or ETV1 protein and assayed on 2 and 3 DIV. (B) Left, photomicrographs of Map2 immunofluorescence in wild-type 2-DIV CGNs transduced on 0 DIV with GFP- or ETV1-expressing lentiviruses. Bar, 100  $\mu$ m. Right, quantification of the effects of ETV1 overexpression on Map2(+) neurite length in wild-type CGN cultures expressed relative to control (GFP). Data are from three biological replicates (averages of 32 [GFP] and 26 [ETV1] transduced cells were analyzed in each experiment) and expressed relative to GFP(+) cells. \*\*\* $p$  < 0.001, \*\* $p$  < 0.01; ns, no significant difference.

antibody. As observed for other NFI-late genes (Ding *et al.*, 2013), NFIA exhibited delayed occupancy of the *Etv1* promoter region in vivo (Figure 5D).

Consistent with this regulatory interaction, both ETV1 and NFIA proteins are widely expressed in CGN nuclei within the IGL of the P15 mouse cerebellum (Figure 6). This agrees with previous in situ hybridization results showing broad but selective detection of *Etv1* mRNA within the mouse IGL (Sato *et al.*, 2005; Schuller *et al.*, 2006), as well as widespread immunostaining of NFIA within the IGL of P6 mouse cerebellum (Wang *et al.*, 2007). Because only rabbit antibodies were found suitable for immunohistochemistry for either protein, it was not possible to show NFIA and ETV1 coexpression directly in the same cells. However, immunostaining for the two proteins was detected in nearly all CGN nuclei throughout the IGL (Figure 6), consistent with the presence of NFIA and ETV1 together in nuclei of most postmigratory CGNs in the IGL.

To study the functional effect of NFIA and ETV1 coexpression on late-gene transcription, we performed transient transfections using a 6-kb mouse *Gabra6* promoter-luciferase reporter plasmid (Wang *et al.*, 2004). Transfections were performed in the JEG3 cell line, since these cells have low endogenous NFI protein levels (Chaudhry

*et al.*, 1998). Both ETV1 and NFIA stimulated *Gabra6* promoter activity when transfected individually (Figure 7A). To study the effects of coexpression, we expressed NFIA with and without increasing amounts of ETV1 vector to mirror developmental changes occurring in CGNs. Under these conditions, *Gabra6* promoter activity increased relative to its activation with either factor alone, and this effect was more than additive at lower levels of ETV1 expression vector (Figure 7B). Thus ETV1/NFIA exhibited a transcriptional interaction under these conditions.

We also attempted to determine whether NFIA and ETV1 proteins directly interacted in the developing mouse cerebellum. However, we were unable to detect NFIA and ETV1 interactions on chromatin using sequential ChIP and native coimmunoprecipitations in P21 mouse cerebella. These results may reflect in part the limited sensitivity in detecting ETV1 protein using available antibodies. Transient or indirect functional interactions between NFIA and ETV1 in vivo cannot be ruled out.

#### ETV1 regulates NFI occupancy of its own promoter in maturing CGNs

Similar to other NFI-regulated late genes, lentiviral expression of human ETV1 in early-developing CGNs (2–3 DIV) increased endogenous mouse *Etv1* transcripts (Figure 8A). ChIP assays also revealed ETV1 binding to a region of the proximal *Etv1* promoter containing multiple ETS binding sites during mouse cerebellar development that increased in parallel with temporal expression of ETV1 (Figure 8B). These findings were consistent with the notion that temporal increases in *Etv1* expression in maturing

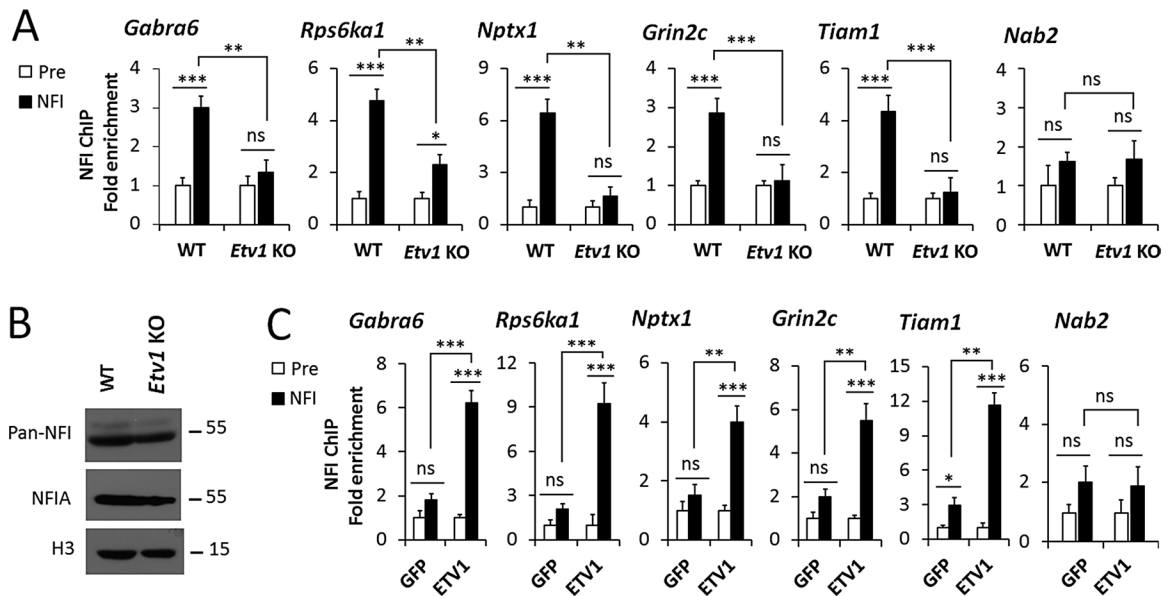
CGNs are driven at least partly by direct ETV1 binding and auto-regulation. This is further supported by ETV1 activation of the transfected *Etv1* promoter (Abe *et al.*, 2011).

Next we examined the effect of ETV1 on NFI binding to the *Etv1* promoter. Early ETV1 expression increased NFI occupancy of the *Etv1* promoter in immature CGNs (Figure 8C), and NFI binding to this site decreased in P10 *Etv1*-knockout mouse cerebellum (Figure 8D). Collectively these findings argued that ETV1 temporally up-regulates its own gene expression in maturing CGNs via positive feedback involving in part ETV1 enhancement of NFI binding to the *Etv1* promoter.

#### Variable timing patterns for ETV1 occupancy in vivo

*Etv1* mRNA is already detectable throughout the IGL of P7 mouse cerebellum (Sato *et al.*, 2005; Schuller *et al.*, 2006), suggesting that *Etv1* expression precedes activation of downstream NFI-late genes. Temporal onset of *Etv1* transcripts was in fact accelerated during early IGL maturation (P8–P13) compared with several NFI-late genes (Figure 9A) and strongly so relative to *Gabra6* and *Grin2c*. Further, ETV1 occupancy of its own promoter preceded and was temporally distinguishable from that of other NFI-late genes based on ChIP



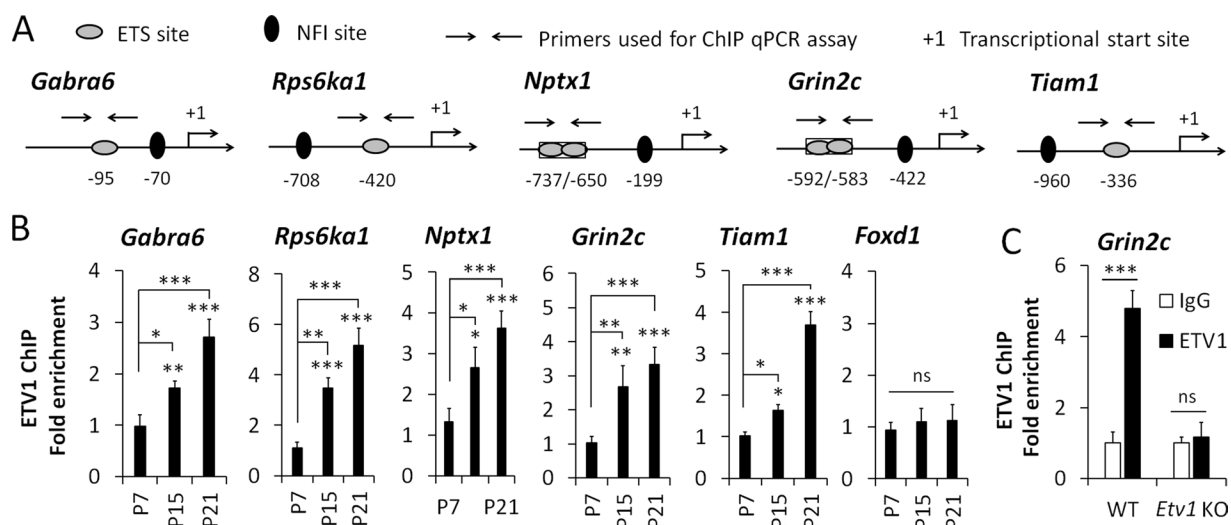


**FIGURE 3:** ETV1 facilitates NFI occupancy of NFI-late gene promoters. (A) ChIP assays of NFI binding to several target genes in P10 *Etv1*<sup>-/-</sup> (*Etv1* KO) cerebellum. The *Nab2* gene showed no specific occupancy at P10 or at P11 (unpublished data). Pre, preimmune serum. (B) Western blot analysis of NFIA and total NFI proteins (pan-NFI) in P10 cerebella of *Etv1*-knockout mice (*Etv1* KO) relative to wild type (WT). Histone H3 (H3) served as a loading control. Identical results were obtained in two biological replicate experiments. (C) ChIP analysis of NFI binding to late genes in 2-DIV wild-type CGN cultures transduced on 0 DIV with lentiviral vectors expressing ETV1 protein relative to GFP. \*\*\**p* < 0.001, \*\**p* < 0.01, \**p* < 0.05; ns, no significant difference.

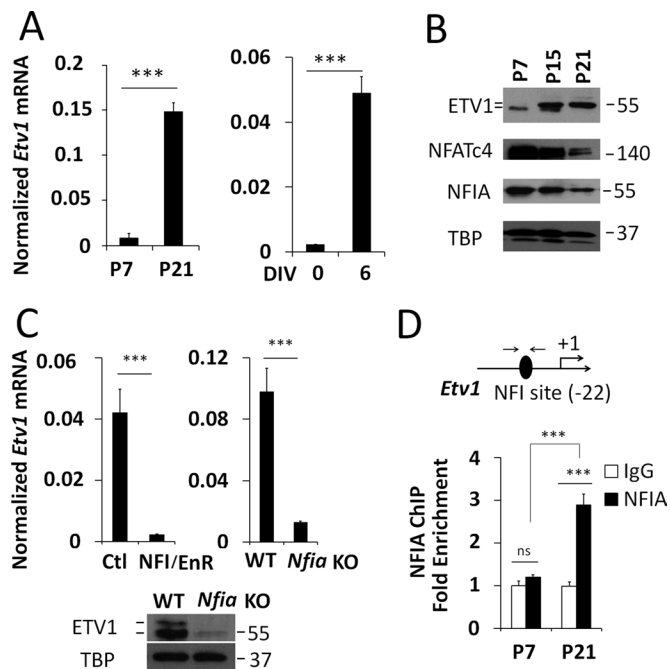
(Figure 9B). Of note, there was ~1-d latency between increases in *Etv1* mRNA and ETV1 occupancy of its own promoter that preceded ETV1 binding of other late-gene promoters (compare fractional binding levels between P9 and P10; Figure 9B). Thus ETV1 preferentially binds to its own promoter relative to sites in the other NFI-late genes during earlier stages of cerebellar development, likely contributing to more-accelerated *Etv1* gene expression.

These differential rates of ETV1 binding are consistent with an autoregulatory mechanism that contributes to accelerated

*Etv1* expression relative to other NFI-late genes targeted by ETV1. Further, they suggest that ETV1 autoregulation is an important determinant of the timing of NFI occupancy and expression of ETV1-dependent NFI-late genes. Of interest, gene expression was considerably more delayed for *Nab2* relative to other NFI-late genes (Figure 9A). The more protracted temporal pattern for *Nab2* may account for its weak NFI occupancy at P11 (Figure 1A) and be related to its insensitivity to *Etv1* deficiency (Figure 3A).



**FIGURE 4:** ETV1 binding to coregulated NFI-late genes increases as CGNs mature. (A) Schematic of ETV1- and NFI-binding sites in the proximal promoter regions of several NFI-switch late genes. (B) ChIP analysis of ETV1 binding to the indicated genes during postnatal development of the mouse cerebellum. (C) ChIP assays showing depletion of ETV1 binding to the *Grin2c* gene in P11 *Etv1*-null mouse cerebella. \*\*\**p* < 0.001, \*\**p* < 0.01, \**p* < 0.05; ns, no significant difference.



**FIGURE 5:** *Etv1* is an NFI-regulated late gene. (A) *Etv1* transcripts increase as CGNs mature in developing mouse cerebellum (P7/P21) and culture (0/6 DIV). (B) Comparison of developmental changes in nuclear levels of ETV1, NFIA, and NFATc4 proteins in the developing cerebellum (P7, P15, and P21). A transition from a faster- to a slower-migrating form of ETV1 is apparent with maturation (shown by bars). TBP served as a soluble nuclear protein loading control. Data are reflective of two biological replicate experiments. (C) *Etv1* gene expression in wild-type 6-DIV CGN cultures transduced on 0 DIV with lentiviruses expressing NFI dominant repressor (NFI/EnR) relative to EnR control (EnR), as well as in the cerebellum of P15 *Nfia*-knockout (*Nfia* KO) mice relative to wild type (WT). (D) Top, schematic showing an NFI-binding site within the proximal *Etv1* 5'-flanking region relative to the transcription start site (+1) and the position of primers (arrows) used for ChIP qPCR assays. Bottom, ChIP analysis of NFIA binding to the *Etv1* promoter in the developing mouse cerebellum at P7 and P21. \*\*\* $p < 0.001$ ; ns, no significant difference.

### NFATc4 controls *Etv1* expression in maturing CGNs

NFATc4 represses NFI-switch late genes in immature CGNs by binding to and preventing NFI occupancy of their promoters (Ding et al., 2013). It is subsequently dismissed from these promoters as CGNs differentiate. We therefore examined whether *Etv1* gene expression was similarly regulated by NFATc4. In CGN cultures, *Etv1* expression was strongly suppressed by constitutively active NFATc4 (Figure 10A), as previously found for other NFI-late genes (Ding et al., 2013). *Etv1* mRNA was also inhibited by culturing CGNs in membrane-depolarizing concentrations of KCl (25 mM; Figure 10A), which enhances NFATc4 nuclear localization via activation of calcineurin (Ding et al., 2013). Further, expression of a dominant-interfering form of NFATc4 substantially reversed this depolarization-induced inhibition (Figure 10A). Conversely, *Etv1* mRNA was strongly up-regulated in *Nfatc4*-knockout cerebellum during the postnatal week 2 (Figure 10A), when NFATc4 normally binds to and represses late genes in CGNs (Ding et al., 2013).

NFAT consensus sites were identified upstream of the *Etv1* promoter, and NFATc4 occupied this region in the P7 but not the P15 mouse cerebellum (Figure 10B). Of significance, NFIA occupancy of the *Etv1* promoter was enhanced in *Nfatc4*-null cerebella

(Figure 10B). Thus NFATc4 binds to the *Etv1* gene and represses its expression and NFI occupancy in vivo, and NFATc4 departure coincides with up-regulation of these events during mouse cerebellar development. We also found that the NFI/ETV1 late genes *Grin2c* and *Tiam1* were up-regulated in P10 mouse cerebella of *Nfatc4*-null mice (Figure 10C) and that NFATc4 temporally occupies their promoters in P7 but not P15 mouse cerebellum (Figure 10D). Further, loss of NFATc4 enhanced NFIA occupancy of the *Grin2c* and *Tiam1* genes in the developing cerebellum (Figure 10E). NFIA occupancy was also increased for the *Rps6ka1* gene in *Nfatc4*-null mouse cerebellum (Figure 10E), which was not previously documented.

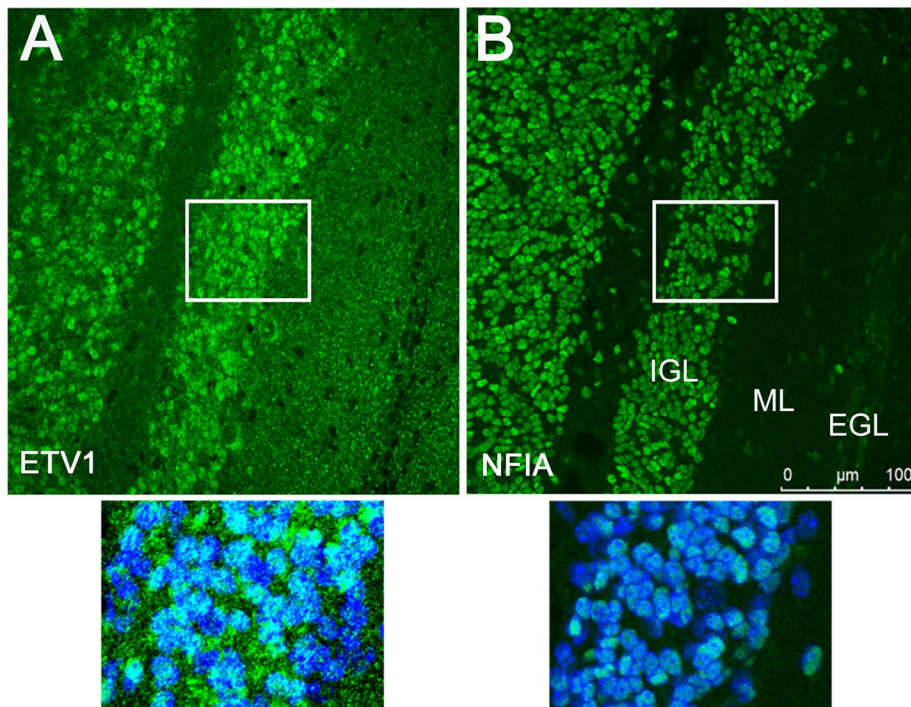
These findings raised the possibility that ETV1 promoted NFI occupancy of the *Etv1* and other late genes indirectly by displacing bound NFATc4. However, ChIP analysis revealed that NFATc4 binding to late-gene promoters decreased in *Etv1*-knockout cerebellum (Figure 10F), indicating that the effects of *Etv1* deficiency on NFI occupancy were not mediated via enhanced NFATc4 occupancy. This result may reflect compensatory mechanisms activated in response to the loss of late gene expression in maturing *Etv1*-deficient CGNs. NFATc4 mRNA levels were not significantly altered in P10 and P11 *Etv1*-null mouse cerebella (Supplemental Figure S2), indicating that the effects of *Etv1* deficiency on NFATc4 binding lie downstream of *Nfatc4* gene regulation.

### DISCUSSION

Postmigratory CGNs elaborate dendrites and form synapses with excitatory mossy fibers and inhibitory Golgi type II cells postnatally (Zheng et al., 1993; Mellor et al., 1998). These events are important for cerebellar circuitry and are temporally linked to the onset of eye-blink conditioning and increased motor activity (Tia et al., 1996). NFI proteins, and in particular NFIA, control the temporal expression of genes required for dendritogenesis and synapse formation in post-migratory CGNs, and the calcineurin/NFATc4 pathway regulates the timing of this expression by blocking NFI occupancy and late-gene expression in immature CGNs (Ding et al., 2013). Other NFI family members, including NFIB, also may contribute to NFI temporal occupancy.

The present findings provide deeper insight into regulation of the NFI temporal occupancy program during postmigratory CGN development. We identify ETV1 as a positive regulator of NFI temporal binding to several dendrite/synapse-related genes, showing that ETV1 regulates, directly binds to, and facilitates NFI occupancy of a subset of NFI-late gene targets. Together with promoter transfection studies done here and by others (Abe et al., 2011), these findings support a role for ETV1 in temporal activation of NFI binding and expression of coregulated genes in CGNs. Of importance, *Etv1* is itself an NFI-late gene, and ETV1 autoregulates its expression in maturing CGNs and promotes NFI binding to its own promoter. Also of importance, *Etv1* gene autoregulation and its occupancy by NFI precede the activation of downstream ETV1-dependent NFI-late genes. Further, our data also revealed that loss of NFATc4 occupancy, which normally leads to up-regulated NFI-late gene expression and NFI binding (Ding et al., 2013), is not sufficient by itself to stimulate these events in the absence of ETV1. This is consistent with an activating function of ETV1 in promoting NFI occupancy of late genes.

ETV1 and NFIA are present in most nuclei of postmigratory CGNs, and both proteins bind to and regulate the *Etv1* and several other coregulated late genes and promoters in the postnatal cerebellum (see also Abe et al., 2011). These findings argue that ETV1 enhances NFI binding to coregulated late genes within individual CGN nuclei. Our data do not allow us to distinguish whether these



**FIGURE 6:** ETV1 and NFIA are both widely expressed within the IGL of the developing mouse cerebellum. Immunostaining for ETV1 (A) and NFIA (B) proteins was performed on adjacent coronal sections of P15 mouse cerebella using rabbit antibodies in both cases (shown in green). Top, widespread staining of both proteins within the IGL, which consists mainly of maturing postmigratory CGNs. Bottom, higher-magnification images of IGL immunostaining (regions indicated by white rectangles at top) and costaining with the nuclear marker DAPI (blue). Background signal was higher for ETV1 immunostaining due to reduced signal-to-noise relative to the NFIA antibody. No–primary antibody controls yielded no detectable signal.

events involve direct physical interactions between NFIA and ETV1 or occur via intermediary factors such as chromatin-remodeling complexes or other *trans*-factors.

Our findings suggest a possible stepwise model for temporal activation of NFI-late genes involving reciprocal, mutually dependent interactions between NFI occupancy and ETV1 (Figure 11). In premigratory and immature postmigratory CGNs (P7), NFATc4 occupies the *Etv1* and other NFI-late genes, repressing NFI binding and gene expression. ETV1 protein and its occupancy of target genes are low at this stage. As maturation proceeds in the IGL (P8–P11), NFATc4 is dismissed from the *Etv1* and other late-gene promoters due to declining calcineurin activity, which is permissive for NFI binding (Ding *et al.*, 2013). Initial up-regulation of ETV1 and its binding enhances NFI occupancy of the *Etv1* promoter, which in turn further increases *Etv1* gene expression via a positive feedback loop (Figure 11, step 1). In an overlapping second phase (P11–P21), elevated ETV1 expression and binding activates other NFI-late genes in part by promoting NFI occupancy (Figure 11, step 2).

A significant implication of this model is that initiation of *Etv1* autoregulation is a prerequisite for expression of dendrite/synapse-related genes coregulated by ETV1 and NFI. Diminished calcineurin signaling and NFATc4 departure is necessary but not sufficient to activate this subset of NFI-late genes (Figure 10F). We show here that both expression and NFI occupancy of this gene subset are modulated by ETV1. Furthermore, this model implies that NFI occupancy of this late-gene subset is itself autoregulatory. That is, the NFI occupancy program simultaneously is dependent on and promotes *Etv1* autoregulation and vice versa, reflecting an intrinsic interdependence of these processes.

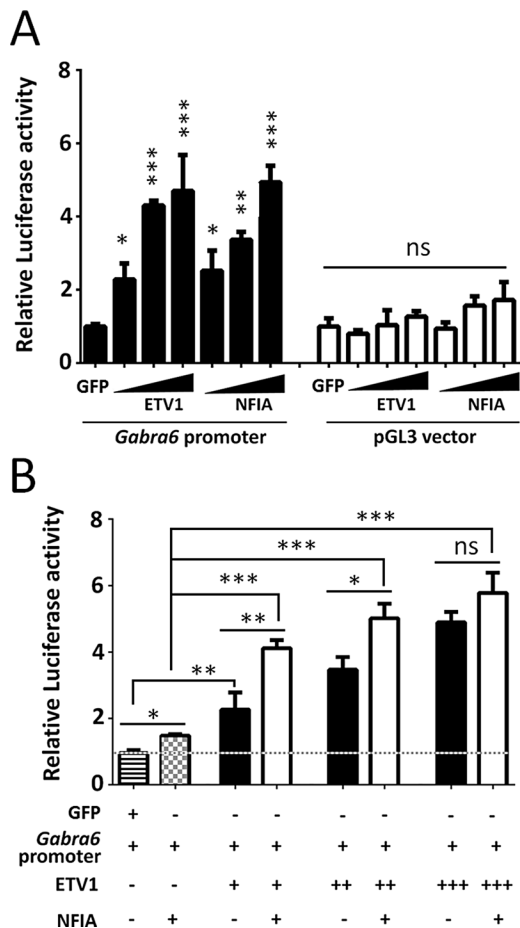
Based on our model, ETV1 and its auto-regulation may function as an integral timing mechanism for NFI occupancy and temporal expression of dendrite/synapse-related genes. Preferential binding of ETV1 to its own promoter would increase its occupancy by NFI and drive accelerated up-regulation of *Etv1* expression in early-maturing CGNs, thereby regulating the timing of its own expression. Increasing ETV1 levels in turn would promote temporally coordinated NFI occupancy and expression of a subset of dendrite/synapse-related genes that function in postmigratory CGNs maturing within the IGL. Some NFI-regulated late genes are not dependent on ETV1 *in vivo* and likely have different regulatory timing requirements and functions. ETV1 may thus provide both a selective and delayed timing mechanism for *activating* a subset of NFI-late genes in addition to the earlier and more widespread temporal effects on late-gene expression mediated by calcineurin/NFATc4 derepression. Of interest, precocious expression of ETS transcription factors perturbs synaptic connectivity of mouse sensory neurons (Hippenmeyer *et al.*, 2005). Thus ETS-factor temporal expression affects synaptogenesis in both developing central and peripheral neurons. Whether ETV1 and NFIA also regulate the magnitude of late-gene expression in the mature cerebellum and not just its timing remains an open

question, since neither *Etv1*- nor *Nfia*-null mice survive beyond the second to third postnatal week.

Multiple mechanisms may contribute to preferential early binding of ETV1 to its own promoter, including unique synergism between ETV1-binding sites (Figure 8B) and their interactions with co-regulator sites as well as with chromatin-modifying complexes. ETV1 autoregulation also may be modulated by posttranslational modifications in maturing CGNs. BDNF, a regulator of CGN differentiation, transiently phosphorylates ETV1 in CGNs and increases ETV1 transcriptional activity in heterologous cells (Abe *et al.*, 2012). BDNF/TRKB signaling may therefore augment ETV1 autoregulation in immature CGNs by stimulating ETV1 activity when its expression levels are low.

CGN differentiation and its timing, including the NFI switch, are reproduced in purified CGN cultures (Trenkner *et al.*, 1984; Gao and Fritschy, 1995; Mellor *et al.*, 1998; Yacubova and Komuro, 2002; Ding *et al.*, 2013), implicating cell-intrinsic processes in these events. Thus CGNs provide a valuable system for elucidating how distinct temporal stages are controlled in developing neurons (Kilpatrick *et al.*, 2012). Here we used this system to provide evidence that a transcription factor autoregulatory loop coupled with differential occupancy rates may provide a timing mechanism for developmental stage-specific gene expression in late-maturing neurons. Transcriptional positive feedback loops play important roles in diverse cellular events, including circadian oscillators (Shearman *et al.*, 2000), commitment to cell division (Eser *et al.*, 2011), and specification of peripheral nervous system cell types (Grocott *et al.*, 2012). Of interest, the ETV1 autoactivation loop preceding activation of downstream genes observed here in CGNs mirrors the “positive feedback first”





**FIGURE 7:** ETV1 and NFIA coactivate late-gene transcription. (A) Expression plasmids for ETV1, NFIA, or GFP were transfected individually into JEG-3 cells along with a 6-kb mouse *Gabra6* promoter construct (left) or promoterless pGL3 plasmid (right). (B) The effect of ETV1 and NFIA coexpression on *Gabra6* promoter activity was determined using a constant amount of NFIA plasmid and increasing amounts of ETV1 expression plasmid. The dotted line highlights promoter activity above that for the GFP control plasmid for each condition. Luciferase activity is expressed as the fold increase of GFP control activity throughout. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; ns, no significant difference.

events seen in autoregulatory control of commitment to cell cycle progression (Eser et al., 2011).

Altered timing of dendrite and synapse maturation and circuitry formation occurs in several NDs (Geschwind and Levitt, 2007; Leonardo and Hen, 2008; Harlow et al., 2010; LeBlanc and Fagioli, 2011; Penzes et al., 2011; Addington and Rapoport, 2012; Till et al., 2012; Uhlhaas and Singer, 2012), and disrupted synaptogenic gene expression has been implicated in these disorders (Brown, 2011; Addington and Rapoport, 2012; Meredith et al., 2012; Tsigelny et al., 2013). In addition to movement control, the cerebellum participates in language, cognition, mood, and attention, likely via cerebellar-thalamo-cortical connections (O'Halloran et al., 2012). Alterations in cerebellar structure and its connectivity also occur in several NDs (Fatemi et al., 2012; O'Halloran et al., 2012; Rogers et al., 2013a,b). Further, NFIA gene variants and deletions have been linked to delayed acquisition of language/motor skills and NDs (Mikhail et al., 2011; Iossifov et al., 2012; Prasad et al., 2012; Lee et al., 2013). An intriguing question is whether disruption of NFIA

dependent synaptogenic timing mechanisms in CGNs contributes to altered cerebellar circuitry and associated ND phenotypes.

## MATERIALS AND METHODS

### Animals and primary cultures

Cerebellar tissues were obtained from postnatal knockout mice and wild-type littermates of varying genetic backgrounds: *Etv1*( $-/-$ ) were 129/Sv and C57B/6J hybrids (Arber et al., 2000), *Nfatc4*( $-/-$ ) (Ding et al., 2013) were C57Bl/6, and *Nfia*( $-/-$ ) were C57Bl/6NTac (Shu et al., 2003). Mouse CGNs were prepared from P6 CD1 mouse pups of either sex and cultured as previously described (Ding et al., 2013; Selvakumar and Kilpatrick, 2013). Individual culture experiments were performed using cells prepared from the same litter. Cells were plated at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> onto chamber slides or cell culture dishes coated with poly-D-lysine/laminin (Invitrogen, Carlsbad, CA) in Neurobasal medium (Invitrogen) containing B-27 serum-free supplement (50x; Invitrogen).

### Plasmids and cell lines

Plasmid FU-ETV1-CRW containing full-length human ETV1 coding sequences was generously provided by Owen N. Witte (University of California, Los Angeles, Los Angeles, CA). Self-inactivating lentiviruses expressing green fluorescent protein (GFP), hemagglutinin (HA)-tagged NF1 dominant repressor (NF1/EnR), or *Drosophila* engrailed repressor domain alone (EnR), FLAG-tagged NFATc4 proteins (constitutively active [NFATc4-Ala] and dominant-negative [dnNFAT]) were described previously (Wang et al., 2004; Ding et al., 2013). Human embryonic kidney 293T cells were grown in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen).

### Lentivirus preparation and transduction of primary CGNs

Lentiviruses were generated by cotransfection of 293T cells with lentiviral expression plasmid, psPAX2, and pCMV-VSVG and then concentrated and titered as previously described (Wang et al., 2005; Ding and Kilpatrick, 2013b). CGN cultures were transduced on day 0 in vitro using multiplicity of infection of ~3, yielding 80–90% cell transduction.

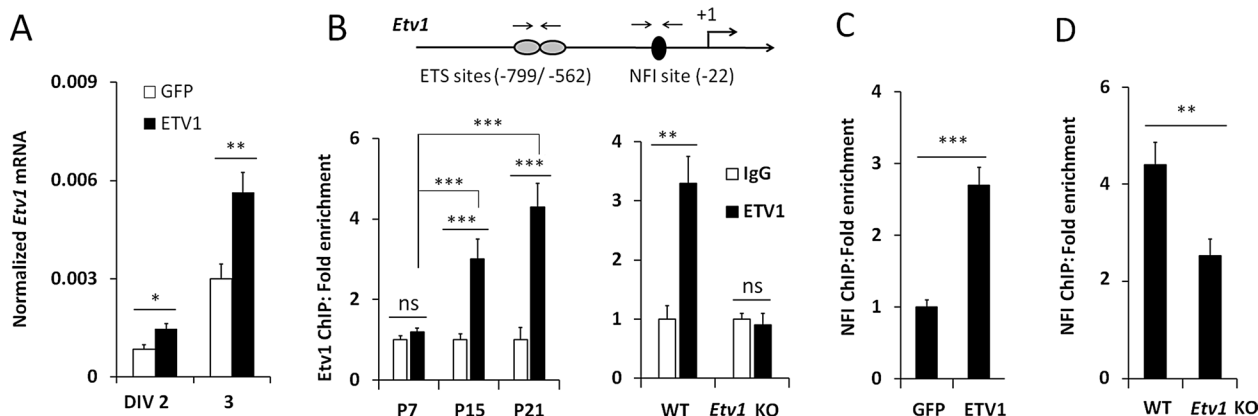
### RNA isolation and real-time quantitative PCR

RNA was extracted from tissues or cultured cells, and cDNAs were prepared as described previously (Ding et al., 2013). Real-time quantitative PCR (RT-qPCR) was performed in triplicate using primers, SYBR Green PCR master mix (Qiagen, Valencia, CA), and a StepOne-Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). Transcript data were analyzed using the  $2^{-\Delta\Delta C_t}$  method and then normalized to 18S rRNA as previously described (Ding et al., 2013). PCR primer sequences are available upon request.

### ChIP assays

ChIP was performed as previously described (Ding et al., 2013). For tissues, nuclei were first purified by Percoll gradient centrifugation (Wang et al., 2011; Ding and Kilpatrick, 2013a) or differential centrifugation ( $1400 \times g$  for 3 min, 4°C) in 0.25 M sucrose. Samples were assayed by real-time PCR, and data from triplicate biological replicates were expressed as the fold enrichment relative to antibody controls. ChIP antibodies were to ETV1 (sc-28681; Santa Cruz Biotechnology, Dallas, TX), *Xenopus* NFIB1, which recognizes mammalian NFIA and NFIB (Ding et al., 2013), NFIA (39036; Active Motif, Carlsbad, CA), and NFATc4 (sc-1153; Santa Cruz Biotechnology). The term NF1 is used to distinguish ChIP assays employing the *Xenopus* NFIB1 antibody from those specifically assaying NFIA



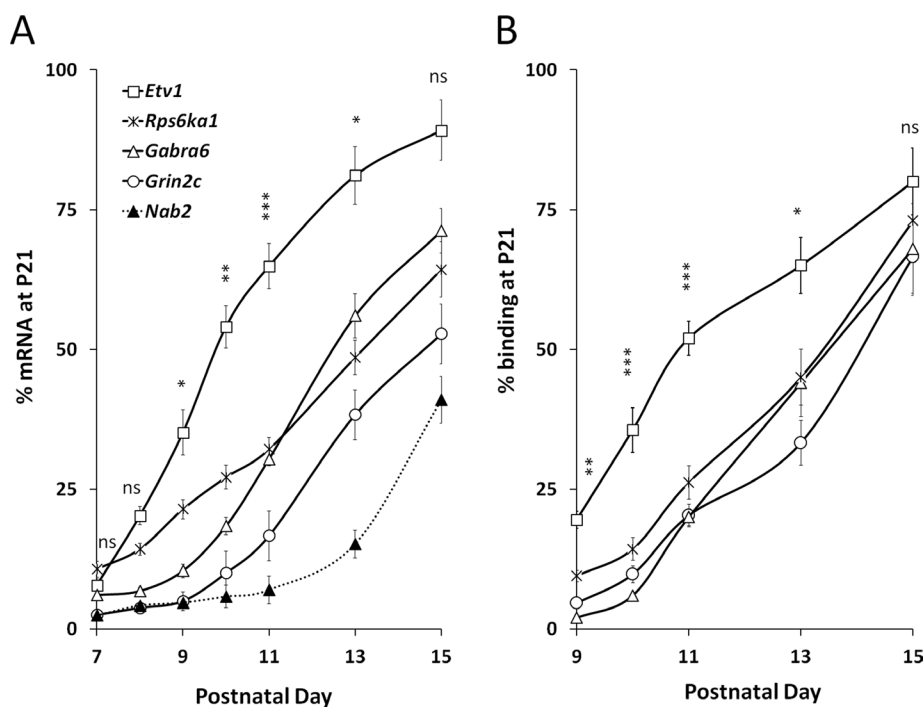


**FIGURE 8:** The *Etv1* gene undergoes autoregulated NFI temporal occupancy in maturing CGNs. (A) Wild-type CGN cultures were transduced with lentiviruses expressing GFP or human ETV1 protein on 0 DIV and then assayed by RT-qPCR using primers to noncoding mouse *Etv1* sequences not present in the lentiviral expression vector. (B) Top, locations of multiple ETS-binding sites and the NFI-binding site within the *Etv1* proximal promoter. Arrows show the positions of primers used for ChIP qPCR. Bottom, ChIP assays of ETV1 occupancy of the *Etv1* promoter in the maturing mouse cerebellum. (C) ChIP qPCR assay of NFI binding to its site within the *Etv1* promoter in wild-type 3-DIV CGN cultures transduced on 0 DIV with GFP- or ETV1-expressing lentivirus. (D) NFI binding to the *Etv1* promoter in P10 *Etv1*-knockout (*Etv1* KO) mouse cerebellum. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; ns, no significant difference.

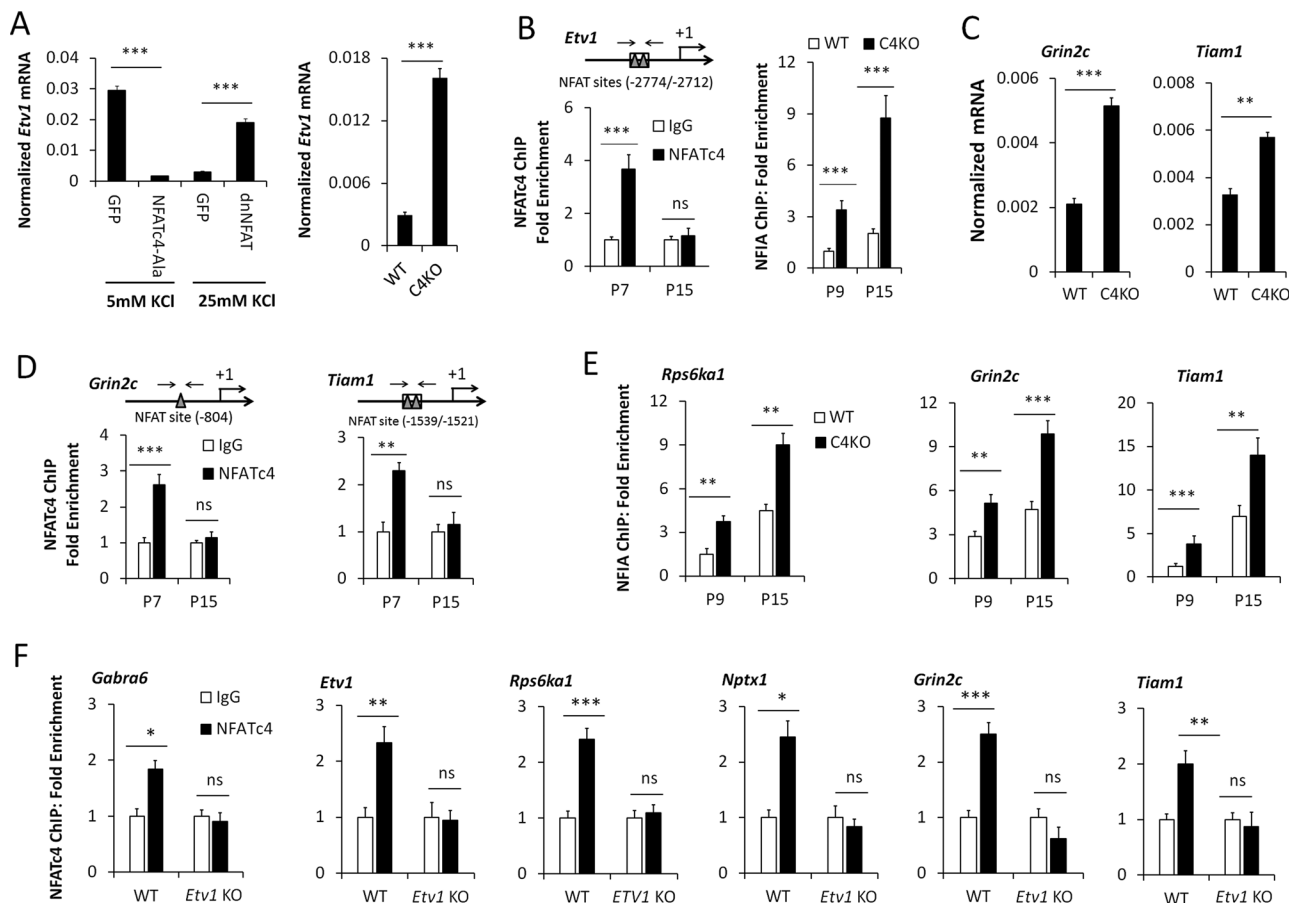
occupancy. Note that the NFI and NFIA antibodies both detect binding to the same genomic regions in ChIP assays (Ding et al., 2013). Preimmune serum or normal rabbit immunoglobulin G (IgG; PP64; Millipore, Billerica, MA) was used as negative control. PCR primer sequences for ChIP assays are available upon request.

### Immunostaining

Immunofluorescence of neuronal cultures was performed as in previous studies (Ding et al., 2013). Briefly, primary CGNs were cultured on chamber slides coated with poly-D-lysine/laminin (Invitrogen) and then fixed on 2 DIV with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized with 1% Triton X-100 solution and incubated with 5% normal goat serum followed by primary antibodies at 4°C overnight and then with Cy3-conjugated goat anti-rabbit IgG (NG1807775; Millipore). Anti-Map2 antibody (AB5622; Millipore) was used to stain neurites, and bisbenzimidazole (1 µg/ml; Sigma-Aldrich, St. Louis, MO) was used to stain nuclei. Pictures were captured with a fluorescence and phase contrast microscope (Leica DM IRE2; Leica Microsystems, Buffalo Grove, IL). Neurite length was measured with Image Pro Plus 6.0 software. Lentiviral titrations used antibodies for ETV1 (sc-28681; Santa Cruz Biotechnology), HA (NFI/EnR and EnR; C29F4, Cell Signaling Technology, Boston, MA), or FLAG (NFATc4 proteins; M2 antibody; Sigma-Aldrich).



**FIGURE 9:** Temporal expression and ETV1 occupancy of the *Etv1* gene are accelerated relative to other NFI-late genes. (A) Transcripts for *Etv1* and several other NFI-late genes were assayed at different postnatal ages by RT-qPCR. (B) ChIP assays of ETV1 temporal binding to the *Etv1* and other NFI-late genes. Data are expressed relative to values at P21, when IGL formation and CGN late gene expression are largely complete (Altman and Bayer, 1997; Furuichi et al., 2011; Ding et al., 2013). Data were derived from three biological replicates, and significance is shown for the difference between *Etv1* values and that for the gene showing the least significant difference at each time point. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; ns, no significant difference.



**FIGURE 10:** NFATc4 regulates expression and NFI occupancy of the *Etv1*, *Grin2c*, and *Tiam1* genes in maturing CGNs. (A) RT-qPCR assays of the regulation of *Etv1* mRNA by NFAT proteins. Left, CGNs were transduced on 0 DIV with lentiviruses expressing GFP, constitutively active NFATc4 (NFATc4-Ala), or dominant-negative NFAT (dnNFAT) and then cultured to 6 DIV in medium containing either normal (5 mM) or depolarizing (25 mM) KCl concentrations. Right, assay of *Nfatc4*-knockout (C4KO) mouse cerebellum at P10 relative to wild type (WT). (B) Left, NFATc4 binding to the *Etv1* gene in developing mouse cerebellum between P7 and P15. Shown above is the location of ChIP PCR primers relative to NFAT-binding sites examined in the *Etv1* promoter. Right, NFIA binding to the *Etv1* promoter in *Nfatc4*-knockout (C4KO) mouse cerebellum relative to WT at P9 and P15. (C) RT-qPCR assays of the *Tiam1* and *Grin2c* genes in cerebella of C4KO compared with WT at P10. (D) ChIP assays of NFIA binding to the indicated genes in C4KO cerebellum relative to WT at P9 and P15. The locations of PCR primers relative to NFAT-binding sites are shown above. (E) NFATc4 temporal ChIP in the developing mouse cerebellum for the *Rps6ka1*, *Grin2c*, and *Tiam1* genes. (F) ChIP assays of NFATc4 binding to the *Etv1* and several other NFI-late genes in *Etv1*-knockout mouse cerebellum at P10. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ; ns, no significant difference.

Covance, Dedham, MA) or NFIA (1:500; 39036; Active Motif) or with no primary antibody overnight at 4°C in 2% normal goat serum, 0.1% Triton X-100, 0.02% sodium azide, and PBS. Only rabbit antibodies were found to be suitable for immunostaining of ETV1 and NFIA proteins. After being washed in PBS, slides were incubated with goat Alexa Fluor 488-conjugated anti-rabbit IgG F(ab')<sub>2</sub> (1:500; A11070; Invitrogen), and 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml). Confocal fluorescence microscopy was performed using a Nikon A1 confocal microscope.

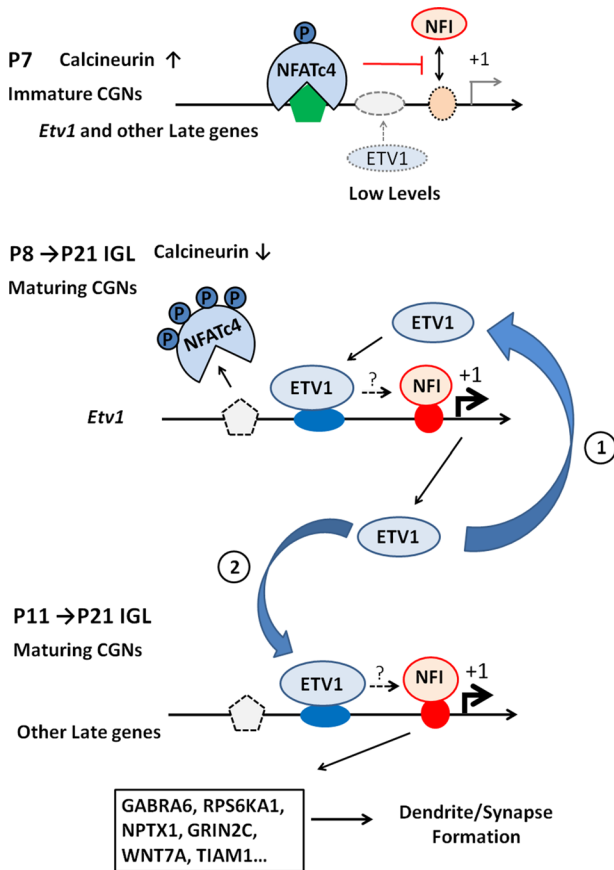
### Western blotting

For analyses of knockout mice, proteins were prepared from frozen cerebellar tissues of postnatal *Etv1*(<sup>-/-</sup>) mice and wild-type littermates by extraction with RIPA buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors (11 873 580 001; 454 Life Sciences, Branford, CT). Extracts were centrifuged at 1000 × *g* for 10 min at 4°C, and the supernatant was collected. Protein samples were

separated on 8% SDS-polyacrylamide gels and transferred onto Pure Nitrocellulose membranes (GE Water & Process Technologies, Boulder, CO). After blocking for 1 h, blots were incubated with primary antibodies at 4°C overnight and then with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Bound antibodies were detected with a chemiluminescent substrate (Thermo Scientific, Waltham, MA). For temporal analyses, nuclear extracts were prepared from P7, P15, and P21 mouse cerebella as previously described (Wang et al., 2011). Primary antibodies used were as follows: pan-NFI antibody (1:1000; sc-5567; Santa Cruz Biotechnology), anti-histone H3 (1:1000; PAB0653; Abnova, Taipei City, Taiwan), NFIA (39036; Active Motif), NFATc4 (ab99431; Abcam, Cambridge, MA), ETV1 (sc-28681; Santa Cruz Biotechnology), and TBP (8515; Cell Signaling).

### Promoter cotransfections

JEG-3 cells were transfected with a luciferase reporter plasmid containing 6 kb of the mouse *Gabra6* gene inserted into pGL3



**FIGURE 11:** Possible sequential model for developmental regulation of NFI switch programming by ETV1. (A) In the early postnatal cerebellum (P7), elevated calcineurin (CaN) activity promotes NFATc4 translocation to the nucleus and its occupancy of late genes, preventing NFI binding and gene activation. ETV1 protein is low at this stage. (B) As CGNs mature (P8–P21), decreasing calcineurin activity reduces NFATc4 occupancy. This is permissive for NFI binding, resulting in increased NFI occupancy of the *Etv1* promoter and enhanced gene expression. Initially, increasing ETV1 protein preferentially targets its own gene promoter and further facilitates NFI occupancy and *Etv1* gene expression in CGNs in a positive feedback manner (step 1). In step 2 (P11–P21), elevated ETV1 protein subsequently binds to and promotes NFI occupancy of a subset of other NFI-late genes, resulting in enhanced mature gene expression and dendrite/synapse formation. ETV1 may promote NFI occupancy (dotted arrows) in CGNs via direct interactions with NFI and/or indirectly via other transcriptional mechanisms (see Discussion). This uncertainty is indicated by question marks above the arrows.

(Wang et al., 2004) along with various combinations of expression plasmids for NFIA (Wang et al., 2004) and ETV1 or GFP expression vectors using TransIT-LT1 reagent (Mirus Bio, Madison, WI). pBlue-script plasmid was used to maintain a constant amount of transfected DNA in all samples. This DNA did not affect promoter activity or its activation by expression vectors. Promoterless pGL3 plasmid was used as a negative control. Luciferase activity was measured ~72 h after initiating transfection using a firefly luciferase assay kit (Mirus Bio) according to the manufacturer's instructions.

### Statistics

Unpaired two-tailed Student's *t* tests were run for comparisons of one experimental sample with its control. One-way analysis of variance was performed when comparing multiple samples, with either

a Tukey (when comparing samples to each other) or Dunnett (when comparing samples to a control) post hoc tests. Results were expressed as mean  $\pm$  SD, and  $p < 0.05$  was considered significant.

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