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Transcriptional Inhibition of REST by NeuroD2 during Neuronal Differentiation

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Abstract

For a progenitor cell to become a neuron, three activities must occur: neuronal differentiation program must be activated, elements repressing neuronal differentiation must be deactivated and competing differentiation programs must be silenced. It is known that NeuroD2 and related bHLH transcription factors induce neuronal differentiation, REST represses neuronal differentiation, and Zfhx1a prevents myogenic gene expression. We demonstrate that NeuroD2 suppresses REST during differentiation in culture. In the hippocampus of NeuroD2 knockout mice, higher level of REST is detected. Functional significance of NeuroD2-REST interplay is uncovered by showing that forced expression of REST interferes with neuronal differentiation in culture. NeuroD2 inhibits REST indirectly by involving the inhibitor of myogenic genes, Zfhx1a, which binds response elements in REST 5′-UTR. Our study supports a model wherein NeuroD2 induces transcription of neuronal genes and Zfhx1a, which in turn de-represses neuronal differentiation by down-regulating REST, and suppresses competing myogenic fate.

Keywords

NeuroD2; REST; Zfhx1a; Basic Helix Loop Helix Transcription Factor; Neurogenesis

INTRODUCTION

Basic Helix-Loop-Helix (bHLH) transcription factors control a broad array of cellular processes including proliferation, lineage commitment and terminal differentiation (Atchley and Fitch, 1997; Weintraub et al., 1973). Based on sequence homology, spatio-temporal patterns of gene expression and roles during development, several families of bHLH factors have been defined (Massari and Murre, 2000). Over the past decade, the neuroD family has emerged as one of the key players in the development of the nervous system (Ross et al., 2003). Members of this family are expressed in the developing, as well as the adult nervous system, and have been shown to control genesis and maintenance of neuronal identity (Lee,

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1997). Loss-of-function studies have unraveled distinct roles for these transcription factors at specific points during development (Lin et al., 2005; Miyata et al., 1999; Naya et al., 1997; Olson et al., 2001).

NeuroD2 is a member of the neuroD family, whose exogenous expression in Xenopus embryos and P19 embryonal carcinoma cell line is sufficient to induce neurogenesis (Farah et al., 2000; McCormick et al., 1996). Studies in mice have shown NeuroD2 to be necessary for the proper development and survival of the cerebellar and hippocampal granular neurons (Olson et al., 2001), the neurons of the basolateral amygdaloid nucleus (Lin et al., 2005), the proper development of thalamocortical connections (Ince-Dunn et al., 2006), and the hypothalamic-pituitary-thyroid axis (Lin et al., 2006). While loss-of-function studies have helped to identify developmental deficits caused by loss of NeuroD2 expression, the fine molecular events orchestrated by NeuroD2 remain obscure. It is known that, similar to other bHLH factors, NeuroD2 directly binds E boxes within the regulatory regions of certain target genes and stimulates their expression (Lin et al., 2005; Lin et al., 2004). As is the case with other fate-determining bHLH factors (Bergstrom et al., 2002), not all neuronal differentiation genes are direct targets of NeuroD2.

In recent years, RE1-Silencing Transcription Factor (REST; also known as NRSF) has emerged as a transcriptional master switch, which inhibits the expression of neuronal genes in nonneuronal cells (Chong et al., 1995; Schoenherr and Anderson, 1995). REST is a zinc-finger protein, which binds the 23 bp RE1 element (NRSE) present in the regulatory sequences of many neuron specific genes (Chong et al., 1995; Schoenherr and Anderson, 1995; Schoenherr et al., 1996). Co-factors with histone deacetylase activity have been shown to coalesce around REST and form a transcriptional repression complex (Ballas et al., 2001). In a series of exquisite experiments, Ballas et al. have shown REST protein to decrease and eventually clear from the promoter of many neuronal genes as neuronal progenitors mature (Ballas et al., 2005). Many targets of REST have been identified and studied; however, regulation of REST expression itself remains vague.

Here, we show that NeuroD2 inhibits the expression of REST as part of its neurogenic program. Our studies point to Zfhx1a as an intermediary transcriptional repressor linking NeuroD2 activity to the inhibition of REST expression. Zfhx1a is a zinc-finger homeodomain transcription factor, which represses the expression of myogenic and other genes (Postigo and Dean, 1997,1999). Based on our studies, Zfhx1a is upregulated during NeuroD2 induced neurogenesis. We show that Zfhx1a represses REST regulatory sequences, REST transcription and protein levels. In our NeuroD2 knock out mouse model, loss of NeuroD2 expression results in elevated levels of REST expression demonstrating the physiological significance of this interplay.

MATERIALS and METHODS

Expression and Reporter Constructs ND2, E12, GFP, REST, 5′ REST regulatory sequences

pCS2+MTNeuroD2, pCS2+E12 and pCS2+GFP were described previously (Farah et al., 2000; Lee et al., 1995). Murine REST construct was a gift of Dr. D.J. Anderson (Paquette et al., 2000). The 4.2kb REST promoter sequence as reported by Koenigsberger et al. (Koenigsberger et al., 2000) was cloned in three fragments into the pGL3-Basic vector (Promega). Each fragment was PCR amplified (Platinum *Pfx*, Invitrogen) from mouse genomic DNA (primers for −4.2kb to −2.4kb fragment: TCCAGAGGGCTCAGGTTTAATTCC and CCAGGAGATCCAGGTCATTGACT; primers for −2.4kb to −856 bp fragment: AAGTCAATGACCTGGATCTCCTGG and CCTCGCTACCCGCGTCCGATCG; primers for −1.06 kb to 0 fragment: CGAGTTACGGAGCGAGTCACG and

CGTACGGATCCCCTTCTTCCCACA). These fragments were sequentially introduced into the pGL3-Basic vector via the TOPO cloning kit (Invitrogen).

Cell culture, Transfection, and Neurogenesis Assay

P19 embryonal carcinoma cells were maintained at subconfluent density, according to ATCC culturing condition, in DMEM supplemented with 7.5% calf serum and 2.5% fetal bovine serum (FBS and BCS; Hyclone, Logan, UT). For transfections, P19 cells were plated on poly-L-lysine (Sigma) coated cell culture plates at a density of 40 cell/cm^2. Equal amounts (total 2.1 μg DNA in 35mm dish and 6 μg in 10cm dish) of NeuroD2 and E proteins were incubated with serum free DMEM and FuGENE 6 transfection reagent according to manufacturer's directions (Roche). The neurogenesis assay was performed as previously described (Farah et al., 2000). Each experiment contained 3 replicates per condition and each experiment was repeated 3 times. Each plate was analyzed for neurogenesis by scoring 300 cells from random visual fields for TUJ1 expression and neuronal arborization. To enrich for transfected cells, the MACSelect4 system (Miltenyi Biotech) was used according to the manufacturer's protocol.

Primary granular precursor cells were isolated from wild type mice at postnatal day 5 (P5) and cultured overnight in media containing purified Sonic Hedgehog protein (Shh) (need manufacturer) (1.5 μg/mL) and (put in serum condition) serum. After 16hrs, serum supplemented media was removed and replaced with serum-free media conaining Shh. These culture conditions were maintained for 3 to 5 days as described previously (Kenney et al. 2000). For infection, Shh-conditioned medium was removed (and stored for future use) and cells were incubated with retroviral supernatants for 8 hours. Viral media was removed and Shh-conditioned medium was replaced. GNPs were infected on three sequential days leading to an infection rate of >60% verified by parallel cultures infected with green fluorescent protein (GFP). Real time PCR studies were completed on the second and third day post-infection.

Microarray

P19 cells were plated on 10 cm laminin coated plates and co-transfected with 1.6 μg pCS2 control or pCS2+NeuroD2, 1.6 μg E12, 1.6 μg pCS2 and 1.2 μg pMACS 4.1 plasmid for cell selection. 48 hours after transfection, transfected cells were selected using the MACSelect 4 cell selection kit and an autoMACS magnetic cell sorter according to manufacturer's instructions (Miltenyi Biotech, Auburn, California). Total RNA was extracted from selected cells using RNeasy Mini Kits (Qiagen, Valencia, California) according to manufacturer's instructions. Double stranded cDNA was synthesized from 15 μg of total RNA using the Superscript Choice System (Gibco). Biotinylated cRNA was synthesized using the T7 Megascript System (Ambion, Austin, Texas) and purified using the RNeasy RNA clean-up protocol (Qiagen). 10 μg biotinylated cRNA was fragmented and hybridized to Affymetrix mouse U74A oligonucleotide arrays (Santa Clara, California) as previously described (Luthi-Carter et al, 2000).

Northern Analysis and Quantitative Real Time PCR

Total RNA was isolated using the RNAeasy kit (Qiagen) according to the manufacturer's instructions. For each condition, 18 μg of total RNA was resolved on 1.2% formaldehyde agarose gel and blotted onto nitrocellulose membranes (Nytran). Membranes were pre-hybridized and hybridized in ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion). To detect REST, a 500 bp probe fragment was PCR amplified using primers GGCAGTCTTCTGGAGGAGGCA and GCTCTTCAGATTCGGCTTCGTAC. ^{32}P-labeled probe was prepared using the Amersham random priming kit according to manufacturer's guide.

Real Time PCR was done as described before (Hallahan et al., 2004). Briefly, total RNA from sorted P19 NeuroD2 transfectants was converted to cDNA using the ABI Taqman Reverse Transcription kit (Applied Biosystems). ABI SYBR green Master Mix was used to set up reactions and run on an ABI 7000 Sequence Detection System. The sequences of the forward and the reverse primers used for real time PCR analysis of Zfhx1a were GCCAAACGGAAACCAGGATGA and GTGAGGCCTCTTACCTGTGTG respectively. For each condition, triplicate reactions were set up and normalized to S16 control. The data were analyzed using the ABI GeneAmp SDS software.

Western Analysis

Total protein was collected from cells using RIPA buffer supplemented with Complete mini protease inhibitor (Roche). 20μg of each condition was resolved on 10% SDS-PAGE acrylamide gel. The contents of the gel were transferred to a Protran nitrocellulose membrane (Whatman, NJ) and immunoblotted with REST antibody (Upstate, Catalog number: 07-579) according to manufacturer's recommendations.

Luciferase Reporter Assay

The Dual Luciferase Kit (Promega) was used to assay the activity of the various reporter constructs. Briefly, for each condition, triplicate or quintuplicate 35 mm plates were set up and transfected with the appropriate luciferase reporter constructs, modifying constructs (NeuroD2, E12, Zfhx1a) and the renilla internal control construct (Promega). The total amount of DNA was equalized in each condition by adding empty pCS2 vector. The cells from each plate were lysed and analyzed according to manufacturer's guide. For each condition, the average ratio of the luciferase to renilla activity is reported.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was done as described previously (Lin et al., 2005) by using the anti-NeuroD2 antibody (Sigma). Briefly, P19 cells were transfected with NeuroD2/E12 and enriched by sorting, as described above, 48 hours after transfection. The positive fraction was fixed in 2% paraformaldehyde before sonication yielding an average of 400-500bp fragments. The lysate was pre-cleared using the anti-rabbit IgG crosslinked to agarose beads (Sigma), followed by incubation with anti-NeuroD2. The antibody was pulled down using the anti-rabbit IgG crosslinked to agarose beads. DNA fragments were de-crosslinked from proteins and purified over PCR purification column (Qiagen). Primers flanking the E-box (CANNTG) region were used to PCR amplify the substrate DNA.

Laser Capture Microdissection

Total postnatal day 5 mouse brains were embedded in polyethylene glycol freezing media (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA) and flash frozen in isopentane that was prechilled in liquid nitrogen. Specimens were stored at −80°C until laser-capture microdissection (LCM). At the time of LCM, 8-μm frozen sections were prepared and immediately fixed in cold 95% ethanol. Sections were briefly (5 to 10 seconds) stained with hematoxylin using the Arcturus HistoGene Staining Solution (Arcturus Bioscience, Mountain View, CA) and dehydrated in 100% ethanol followed by xylenes (described in the Arcturus HistoGene LCM Frozen Section Staining Kit protocol). IGL and EGL cell layers were distinguished based on the hematoxylin staining pattern and these cell layers from 9 consecutive sections were isolated by LCM using the Arcturus PixCell II instrument. To monitor proper isolation of cell layers, digital photos were taken from sections before and after LCM. Each capture did not last longer than 20 minutes to minimize RNA damage. Capture settings were 55 mW beam, 1.5 ms pulse, and 15 μm spot size.

Isolated cells were lysed using Arcturus RNA Extraction Buffer and, RNA was isolated using the Arcturus PicoPure RNA Isolation Kit. The samples were treated with DNAse using the Qiagen RNase-Free DNAse Set (Qiagen Inc, Valencia, CA). RNA was amplified with one cycle using Ambion MessageAmp aRNA Kit (Ambion Inc, Austin, TX). Sample quality and quantity were assessed by agarose gel electrophoresis and absorbance at A260. Amplified RNA was used for cDNA synthesis and real-time PCR.

Immunohistochemistry

Four micron sections of formalin-fixed paraffin-embedded tissue were cut and baked for at least 1 hr at 60 degrees C (can be done up to overnight for better section adherence, with no loss of staining). The sections were then de-paraffinized with xylene and rehydrated in a series of graded ethanol solutions (100%, 95%, and 80%, respectively) followed by distilled water.

Antigen retrieval in Dako Target Retrieval Solution (Citrate pH 6.0) was then performed for 20 minutes, in a Black and Decker vegetable steamer, followed by a 20 minute cooling on the bench. The slides were then placed in 1X Dako Wash Buffer for 5 minutes prior to loading on the Dako Autostainer (this is a Tris-Buffered-Saline-Tween solution – catalog # S3006).

All of the following incubations were performed at room temperature on the Dako Autostainer: Endogenous peroxidase activity was blocked for 8 minutes with 3% hydrogen peroxide followed by a 5 minute wash using 1X Dako Wash Buffer. This same buffer was used for 5 minutes between all subsequent steps (except between the protein block and the primary antibody, where the reagents are just blown off the slides and not replaced by buffer). A protein block step consisting of 15% normal swine serum and 5% human serum in 1X Tris Buffer (Dako catalog # S3001) with 1% BSA was then performed for 10 minutes. Tissues were then stained with the REST antibody at a dilution of 1:150 (6.67 micrograms/ml) diluted in 1X Tris Buffer with 1% BSA for 60 minutes; or rabbit IgG (negative control) matched to the protein concentration of the primary antibody for 60 minutes. Rabbit Envision + labeled polymer (Dako catalog #K4003) was applied for 30 minutes. Finally, Dako Liquid DAB+ Substrate Chromagen System (Dako catalog # K3468) was applied for 2 applications of 4 minutes each, with no rinse in between. The slides were rinsed in distilled water and 1X Dako Wash Buffer and then counterstained with Dako Automation Hematoxylin (Dako catalog # S3301) for 2 minutes. The slides were then washed in 1X Dako Wash Buffer, distilled water, dehydrated in graded ethanol, cleared in xylene, and then coverslipped.

RESULTS

Genes Regulated by NeuroD2

To study broad changes in gene expression induced by NeuroD2, P19 cells were co-transfected with NeuroD2 and E12. P19 cells offer a suitable culture model for studying transcriptional changes initiated by NeuroD2/E12 because they have undetectable levels of endogenous NeuroD2 and E12 (Figure 1A and 1B) and have been shown to form neurons in response to exogenous NeuroD2/E12 expression (Farah et al. 2000). Total RNA was isolated from these cultures and used as substrate in microarray analyses. Parallel cultures were evaluated for neurogenesis based on axonal arborization and β-tubulin expression.

To understand global changes in gene expression induced by NeuroD2 during neurogenesis, high density oligonucleotide arrays representing 10,000 genes were used (Affymetrix, U74A array, mouse). We established the relative expression of genes in an enriched population of NeuroD2/E12 transfectants versus P19 cells transfected with an empty expression vector at forty eight hours post-transfection, when approximately 65% of NeuroD2/E12 transfectants become neurons. For each condition, triplicate plates were set up. Differentially expressed

genes were ranked by the consistency of the Affymetrix MAS 5.0 difference call metric, based upon all nine possible pair wise comparisons of experimental and control conditions. As NeuroD2 guides cells through neuronal differentiation, it influences the expression of many genes belonging to various functional families. Table 1 summarizes NeuroD2/E12 transcriptional changes of some known key participants of cellular differentiation. A comprehensive list of all transcriptional changes is available in supplementary data Table 1.

NeuroD2 Suppresses REST Expression

As a bHLH transcription factor, NeuroD2 is believed to induce neurogenesis by modulating the expression of a network of necessary genes for the acquisition of neuronal identity. Some of these genes are direct targets of NeuroD2 transcriptional activation, while many others are subject to indirect mechanisms of transcriptional control. Identifying master transcriptional switches regulated by NeuroD2 will help us to understand the *en masse* regulation of some of the indirect targets of NeuroD2 activity.

In our cDNA microarray study of NeuroD2-induced neurogenesis, REST is down regulated in seven out of nine pair wise comparisons between NeuroD2/E12 transfectants and control condition, indicating that REST is likely regulated by NeuroD2/E12. Northern analysis confirms our microarray finding of REST expression, showing a 1.78 fold (+/− 0.38, p 0.0056) reduction in REST transcript level in NeuroD2/E12-induced neurons (Figure 1C). Western analysis of protein samples shows a 7.18 fold (+/− 2.5, p 0.0042) decline in REST protein levels during NeuroD2/E12 induced-neurogenesis as well (Figures 1D).

To test whether inhibition of REST activity is necessary for NeuroD2/E12 induced neurogenesis, P19 cells were co-transfected with NeuroD2/E12 and murine REST constructs. Neurogenesis was assayed by evaluating axonal arborization and β-tubulin expression, a neuronal marker, and reported as a percentage of neurons per total number of transfectants, as measured by co-transfection of GFP. Co-expression of REST and NeuroD2/E12 significantly reduces the number of neurons formed in culture by 30% ($p<0.005$), confirming that suppression of REST is a necessary component of the NeuroD2-initiated neurogenic program (Figure 1E & F).

Loss of NeuroD2 and Effect on REST Expression

We explored the effect of NeuroD2 on REST expression level *in vivo* by studying the brain of NeuroD2 null mice. In our studies, we focused on the amygdala, hippocampus and the cerebellum which are areas of the brain with known abundance of NeuroD2 expression and areas where loss of NeuroD2 expression results in a phenotype (Lin et al., 2005; Lin et al., 2004; Lin et al., 2006; Olson et al., 2001). No significant change in REST expression was detected in the cerebellum and the amygdala of 21 day old NeuroD2 null mice, possibly due to compensation by NeuroD1 or other family members. In the dentate gyrus of the hippocampus, however, an elevated level of REST expression was detected in the brains (three animals from three independent litters) of 17-21 day old NeuroD2 null animals (Figure 2).

NeuroD2 suppresses REST Transcription

Different mechanisms may account for NeuroD2-induced reduction in REST transcript and protein levels. For instance, REST protein is down regulated independent of its transcript level by an ubiquitin-independent proteolytic mechanism (Ballas et al., 2005). REST activity may also be controlled by small modulatory dsRNA species (Kuwabara et al., 2004). Other possible mechanisms include transcriptional inhibition and a resultant reduction in protein level, or post-transcriptional mechanisms involving translation or mRNA stability. We reasoned that by assaying the activity of *cis*-regulatory regions controlling REST expression in response to

NeuroD2, we will be able to determine whether NeuroD2 inhibits transcription of REST among other mechanisms.

To test this possibility, we generated a reporter construct containing the 4.2kb REST 5′ regulatory sequence (Koenigsberger et al., 2000) inserted upstream of the luciferase gene and labeled pGL3-REST regseq (Figure 3A). This construct was introduced into P19 cells alone or together with NeuroD2/E12. Co-expression of NeuroD2/E12 and the luciferase construct resulted in 3.81 fold reduction in the activity of the 4.2kb REST regulatory region (p 0.0057), showing that NeuroD2/E12 inhibits REST transcription (Figure 3B).

A survey of the 4.2kb REST regulatory sequence reveals the presence of eight E boxes, including a NeuroD2 preferred E box (CAGATG), in the space between −4.2kb and −2kb region. To address whether these E boxes mediate a direct inhibitory effect of NeuroD2 on REST expression, we tested a variant of the pGL3-REST regseq containing only the −4.2kb to −2kb region (pGL3-F1). We reasoned that if the E boxes are involved in NeuroD2 induced REST suppression, this fragment should recapitulate the effect of NeuroD2 on the full length reporter construct. Interestingly, pGL3-F1 does not afford adequate baseline reporter expression to allow subsequent study of the inhibitory effect of NeuroD2/E12 on its activity. To test the effect of E-boxes within the 5′ REST regulatory sequences, the pGL3-REST regseq construct was divided into two segments, pGL3-F1 (which contains eight putative E boxes) and pGL3-F2F3. The effect of NeuroD2 on the activity of either construct was assayed. The pGL3-F1 fragment supports minimal baseline activity insufficient for further analysis. The pGL3-F2F3 fragment supports strong activity and recapitulates the effect of NeuroD2 on the full-length reporter construct indicating that the E-boxes are not necessary for inhibition of reporter activity. More importantly, the construct containing the region between −2kb and 0 (pGL3-F2F3) is suppressed 5.6 fold (p 0.0045) by NeuroD2/E12, showing that this region is sufficient for the transcriptional inhibition of REST by NeuroD2 (Figure 3C).

To evaluate the possibility of direct NeuroD2 involvement in inhibition of REST expression, we performed chromatin immunoprecipitation studies against NeuroD2 in differentiating P19 cells. NeuroD2 does not bind the identified E boxes in the REST 5′ regulatory sequence demonstrating that the transcriptional effect of NeuroD2 on REST expression is indirect and requires intermediary factors (Figure 3D).

Putative Zfhx1a Binding Sites Identified in the REST Regulatory Sequence

To identify potential factors mediating the inhibitory effect of NeuroD2 on REST expression, the 4.2kb REST 5′ regulatory region was analyzed using TFSEARCH database (Heinemeyer et al., 1998). This search identifies a number of potential transcription binding sites (Table 2), a majority of which have been shown to act as positive regulators of transcription and are expressed in the nervous system. Comparison of the human and the mouse 4.2kb REST 5′ regulatory sequences shows that there are two regions of high evolutionary conservation (supplementary data, Figure 1). Many of the potential transcription binding sites fall within these two conserved regions. Two criteria were applied to narrow our search for factors mediating the inhibitory effect of NeuroD2 on REST expression. First, candidates ought to possess known transcriptional repression activity as documented by other studies, and second, the factor must be a target of NeuroD2. Zfhx1a is a transcriptional repressor that fits these criteria.

Three consensus Zfhx1a binding sites, CACCT (Sekido et al., 1994), were identified in the region within −2kb to 0 of the murine REST 5′ regulatory sequence by TFSEARCH. The three putative Zfhx1a binding sites are scattered an average of 167 nucleotides apart. Two of these sites are relatively preserved between human and mouse (Supplemental Data, Figure 1).

NeuroD2-Induced Neurogenesis Upregulates Zfhx1a Expression

Zfhx1a is among the genes that were identified as increased by NeuroD2 expression in P19 cells in the microarray studies. Zfhx1a is upregulated in eight out of nine pair wise comparisons between NeuroD2/E12 transfectants and control cultures in the array study. Other studies have reported changes in Zfhx1a expression during neuronal differentiation of P19 cells (Yen et al., 2001). Real-time PCR results on cDNA samples from NeuroD2-induced P19 neuronal cells demonstrate a 3.37 fold increase in Zfhx1a transcript 48 hours after transfection (Figure 4A).

To rule out the possibility that induction of Zfhx1a expression is itself independent of REST downregulation, we analyzed the 2kb region upstream of the Zfhx1a open reading frame for the presence of RE1 consensus element and did not find one (not shown). This finding is consistent with a study by Bruce et al (Bruce et al., 2004) who conducted a genome-wide search for RE1 elements and open reading frames near these RE1 sites. Zfhx1a is not on the list of genes with close proximity to a REST binding site.

Zfhx1a Suppresses REST Expression

To study the effect of Zfhx1a on REST expression, murine Zfhx1a was transiently transfected into P19 cells along with the Rest regulator fragment construct pGL3-F2F3 and activity of this reporter was assayed for luciferase activity. Zfhx1a inhibits the activity of the REST regulatory sequence construct 6 fold (p 0.009) similar to NeuroD2/E12 (Figure 4B). Next, we tested the effect of Zfhx1a on endogenous REST transcript level. Northern analysis confirms that REST expression is attenuated 2 fold (+/− 0.31, p 0.009)) in response to exogenous expression of Zfhx1a in P19 cells (Figure 4C). Western analysis of REST protein shows a concomitant 2.5 fold (+/− 0.59, p 0.01) reduction in REST protein level (Figure 4D). These findings suggest a transcriptional regulatory effect of Zfhx1a on REST. These findings, together with the findings showing upregulation in Zfhx1a expression during NeuroD2 induced neurogenesis, are consistent with a model where NeuroD2 employs Zfhx1a as a mediator to inhibit REST activity during neurogenesis.

To ask whether Zfhx1a is the only pathway bridging NeuroD2 activity and REST expression, we used a dominant negative form of Zfhx1a containing only the C-terminal zinc finger domain. In the presence of exogenous NeuroD2 in P19 cells, the dominant negative variant of Zfhx1a did not antagonize the inhibitory effect of NeuroD2 on REST expression suggesting the availability of an alternate mechanism mediating the effect of NeuroD2 on REST expression (supplementary data, Figure 2). In these experiments, the activity of dominant negative Zfhx1a was confirmed by showing that its co-expression with full length Zfhx1a interferes with downregulation of REST.

Zfhx1a Recognizes Its Cognate Binding Sites in the REST Regulatory Sequence

Sekido et al. showed that Zfhx1a binding to its cognate DNA element requires the consensus CACCT sequence (Sekido et al., 1994). We identified three such elements in the −2kb to 0 region of the REST 5′ regulatory sequence. To test whether the effect of Zfhx1a on REST expression and reporter construct activity is through the interaction of Zfhx1a with these *cis*-elements, we mutated the three CACCT boxes to CCCTT either individually (Mut 1, Mut 2, Mut 3) or in adjacent dual combinations (Mut 1&2 and Mut 2&3) and assayed the effect of Zfhx1a on these mutant reporter constructs.

Zfhx1a inhibits the wild type REST reporter construct activity, but it fails to inhibit the Zfhx1a binding site mutants with a similar magnitude. The failure to inhibit reporter activity appears to be most robust when the first and the second mutants (Mut 1&2) are combined suggesting that the full inhibitory activity of Zfhx1a requires both binding sites (Figure 5).

NeuroD2, Zfhx1a and REST expression in vivo

The developing mouse cerebellum is a unique post-natal model of neuronal maturation. Within the first three weeks of life, the external germinal layer (EGL) undergoes massive proliferation to progressively spawn a population of cells, which migrate inward through the molecular layer, and form the internal granular layer (IGL). As these cells transit from the outer to the inner layer, they stop dividing and acquire neuronal identity, providing a window into some of the cellular and molecular changes a cell experiences as it matures into a neuron. We used this system to study the expression of message and protein for NeuroD2, Zfhx1a and REST during various stages of neuronal maturation in the developing cerebellum. Furthermore, we forced primary granular precursor cells cultured from 5 day old cerebellum to undergo neuronal differentiation by manipulating culture conditions. We accomplished this with the addition or removal of Sonic Hedgehog (Shh) or by overexpressing NeuroD2. We then measured the resulting changes in the expression of NeuroD2, Zfhx1a and REST.

By using Nestin and NeuN as cellular markers of undifferentiated and differentiated neuronal cells respectively, we identified the fifth postnatal day as a time, when both EGL and IGL are notably present and distinguishable in the developing cerebellum (Supplementary Figure 3A). Then, using Laser Capture Microdissection, we isolated cells from the EGL and the IGL layers of the cerebellum of five day old mice (Supplementary Figure 3B) and used RNA from these samples as template in quantitative real time PCR to assay the expression of Math 1(a bHLH factor highly expressed in neuronal progenitor cells of EGL), NeuroD2 (whose transcript has been shown to be present in both EGL and IGL), Zfhx1a, and REST (Figure 6A). We found that Math1 is 25 times more abundant in the EGL compared to the IGL. This expected finding validates our approach. NeuroD2 levels are equal in EGL and IGL. This finding is consistent with earlier studies of NeuroD2 message expression in the developing central nervous system. There is a 4 fold enrichment of the Zfhx1a transcript level in the IGL region compared to EGL. An elevated level of Zfhx1a in the IGL, where fully differentiated granular neurons reside compared to the EGL, where neuronal precursors are dividing, is entirely consistent with the proposed model of NeuroD2/Zfhx1a/REST molecular interplay. Interestingly, a higher REST transcript level is detected in IGL compartment compared to EGL, which is contrary to our earlier findings and the function of REST in general.

To address this inconsistency we sought to understand the expression of NeuroD2/Zfhx1a and REST proteins in the developing cerebellum using immunohistochemistry (Figure 6B). Interestingly, NeuroD2 is expressed both in EGL and IGL, but the cellular localization of NeuroD2 differs in these two layers. The immunohistochemical expression pattern shows that NeuroD2 staining is localized to the nucleus in the inner EGL plate and IGL, where post-mitotic migrating neurons reside. NeuroD2 staining is excluded from the nucleus in proliferating, undifferentiated GNPs of the outer EGL. This finding reconciles the paradoxical earlier observation that the NeuroD2 gene is expressed in EGL. Cellular trafficking of NeuroD2 protein away from the nucleus explains how a transcription factor that induces cell cycle arrest and neuronal differentiation can be expressed in EGL, where generally granular precursor cells undergo massive proliferation and remain undifferentiated until they migrate to the inner layers of the EGL.

Similar to the laser capture microdissection results, Zfhx1a expression is detected in the inner EGL and IGL, where mature neurons reside. Additionally a small number of individual nuclei, scattered in regular intervals within the EGL were detected whose identity is unknown.

REST expression in the EGL was limited to the outer layer, where cells are actively proliferating and have not begun the differentiation process. Furthermore, the Purkinje cell layer, residing closely adjacent to the IGL, abundantly expresses REST. Since separation of intimately positioned cell populations such as the IGL and the Purkinje cell layer is almost

impossible, this explains the higher level of REST message detected in the IGL sample from laser capture microdissection.

Changes in NeuroD2, Zfhx1a and REST Expression during Neuronal Differentiation of Primary Granule Neuron Precursor Cell Culture (PGNP)

Granular Precursor Cells can be isolated from a developing cerebellum and maintained as primary cultures. These cultures represent a physiologically relevant model for studies of neurons *ex vivo*. In the presence of sonic hedgehog (Shh), these cultures can be maintained in the proliferative phase, whereas withdrawal of Shh guides cells in the culture towards terminal neuronal differentiation. Additionally, forced gene expression can be used to induce changes in target gene expression and manipulate cell fate.

We maintained control cultures in the presence of Shh for 72 hours and induced neuronal differentiation in experimental cultures by withdrawing Shh for 48 and 72 hours and measured the resulting changes in the expression of NeuroD2, Zfhx1a and REST (Figure 7A). Math 1 was used a positive control. There was a gradual 6.77 fold increase in NeuroD2 levels with an accompanying 3.29 fold increase in Zfhx1a levels and 4.21 fold reduction in REST levels.

We examined the effect of NeuroD2 on REST expression directly in this culture model system by overexpressing NeuroD2 in PGNP cultures (Figure 7B). Forced expression of NeuroD2 in the primary granular cultures results in findings similar to induced differentiation by withdrawing Shh. There is a 2.77 fold increase in NeuroD2 expression accompanied by a 2.8 fold increase in Zfhx1a expression and a 3.36 fold decrease in REST levels.

DISCUSSION

During the development of the nervous system, transformation of a progenitor cell into a mature neuron is made possible by complex transcriptional changes that eventually result in the expression of neuron specific markers. Discoveries of the past decade reveal that the availability of various neurogenic bHLH transcription factors in exquisitely timed waves orchestrates changes in the expression of key contributors to neuronal differentiation. On the surface, the role of these bHLH factors appears simple. Generally thought of as positive regulators of transcription, one can imagine that by recognizing binding sites in the promoter of various markers of neuronal activity and inducing their expression, they encourage immature cells along the path of neuronal differentiation. A deeper consideration and study of neurogenesis however, reveals that differentiation is as much dependent on upregulation of proneural genes as on inhibition of repressors (Roopra et al., 2001). The findings we present here begin to reconcile these two faces of neuronal differentiation.

NeuroD2 is necessary for the development and survival of different populations of neurons. In choosing to detail the transcriptional changes induced by NeuroD2, we were mindful of accumulating evidence that during differentiation the expression of clusters of genes is subject to common mechanisms of transcriptional control by master switches (Tapscott, 2005). We found that NeuroD2 inhibits the expression of REST, a transcriptional repressor of many neuronal genes in nonneuronal cells. Identifying a link between NeuroD2 and REST, helps us map two master switches within the transcriptional landscape of a maturing neuron. This brings us a step closer to distilling differentiation into a circuit wherein changes in the expression of hundreds of targets are achieved by turning a series of stereotyped switches on and off.

We show that NeuroD2 downregulates REST transcription indirectly, and at least partially, through the activity of Zfhx1a. Zfhx1a itself is a transcriptional repressor of many genes, some of which are involved in muscle differentiation. This finding suggests an integrated model of neuronal fate determination or maintenance where the switch for neuronal gene derepression

(REST) and an inhibitor of a competing cellular fate (Zfhx1a) converge. This finding offers a clue in understanding the exclusive nature of neuron and muscle cell generation and maintenance during development. Interestingly, Watanabe et al. have shown that overexpression of REST alone is sufficient to convert myoblasts into functionally active neurons, highlighting the fine line that separates these two cellular fates.

The sum of expression data of NeuroD2, Zfhx1a and REST during neuronal differentiation, from either the developing cerebellum *in vivo*, or the differentiating PGNP cultures is consistent with the model of molecular interplay between NeuroD2, Zfhx1a and REST proposed here. In the developing cerebellum, nuclear NeuroD2 signal is detected only in the inner EGL and the IGL, where post-mitotic and mature neurons reside. Zfhx1a expression profile is similar to NeuroD2. Conversely, there is very little overlap between REST and NeuroD2/Zfhx1a domain of expression. REST is exclusively detected in the outer EGL plate, where cells are actively proliferating, and in the Purkinje cell layer. In PGNP cultures, neuronal differentiation either by changing culture conditions or by overexpression of NeuroD2 results in a consistent increase in Zfhx1a expression along with a corresponding reduction in REST expression.

The model presented here would predict that loss of NeuroD2 results in changes in the expression of REST. To evaluate this possibility *in vivo*, we studied the brains of NeuroD2 null animals with particular attention paid to the regions where loss of NeuroD2 has been shown to result in developmental deficits. The hippocampus was the only region where a notable increase in REST expression was detected. This may be due to possible overlapping functions among other members of the NeuroD2 family in other regions of the brain. Consistent with this observation, neurogenin 1 and neuroD1, but not math1, regulate the expression of REST in culture (Supplementary Figure 4).

While it is important to understand how positive markers of a particular cellular fate are induced during differentiation, it is also important to learn how other cellular fates are actively repressed such that a cell does not get lost along the differentiation axis. Our findings establish a link between two nodal points in neuronal differentiation. More importantly, we show that this molecular interplay is at least partly achieved through the activity of Zfhx1a, which has been shown to inhibit muscle and other genes. Based on this model, as NeuroD2 inhibits REST, it also actively blocks the expression of muscle genes through Zfhx1a, and ensures commitment to the neuronal differentiation path.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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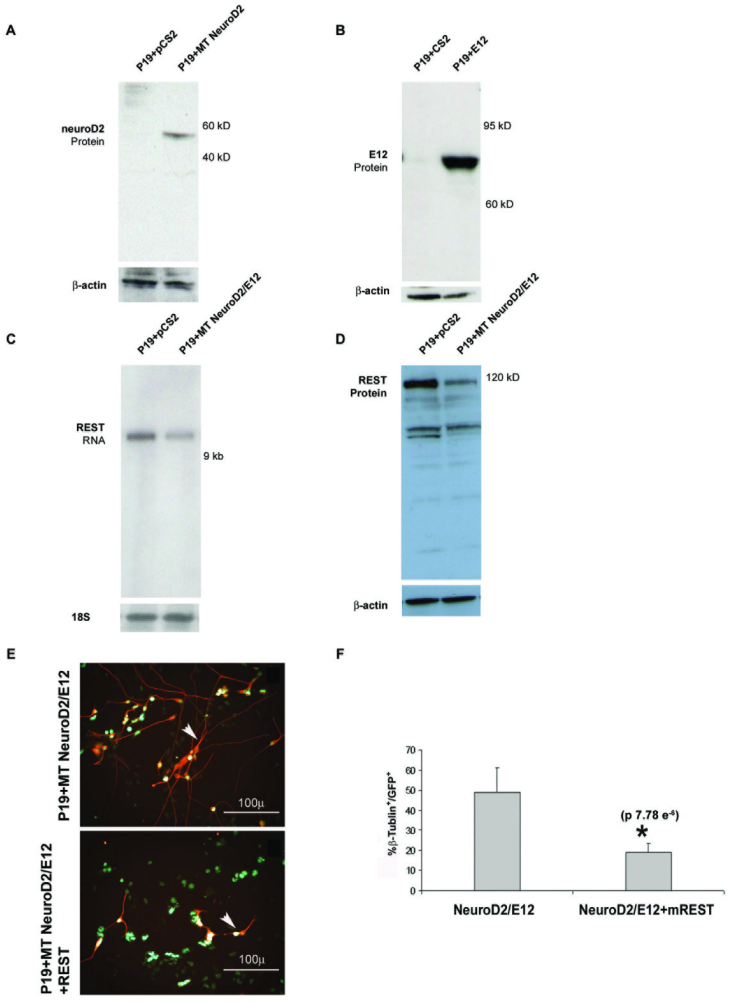


Figure 1. NeuroD2 downregulates REST during neurogenesis

P19 cells lack detectable levels of NeuroD2 (A) and E12 (B) protein in western analysis. As P19 cells are directed to form neurons by ectopic expression of NeuroD2, the level of REST transcript detected by northern analysis decreases (C). REST protein level is also downregulated as detected by western analysis (D). Concomitant forced expression of NeuroD2 and murine REST interferes with neuronal differentiation of P19 cells (E). Neuronal formation is measured by expression of β-tubulin III, a neuronal marker (red), and axonal arborization (arrow). Green represents co-transfection of GFP to mark transfected cell. The graph shows a significant reduction in the number of neurons formed when exogenous REST is co-expressed with NeuroD2/E12 (F). * denotes $p \leq 0.005$

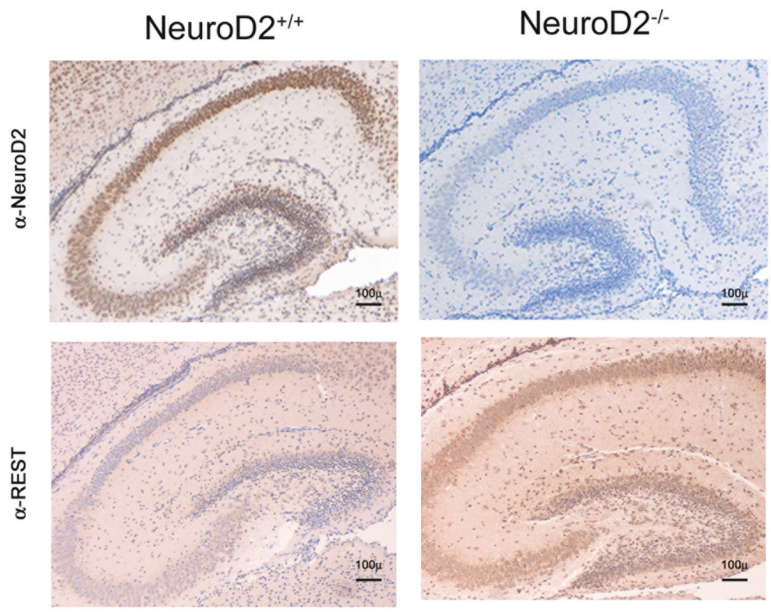


Figure 2. Higher level of REST is detected in the hippocampus of NeuroD2 P5 null animals

NeuroD2 is expressed in the granular cell layer of the hippocampus. Loss of NeuroD2 expression results in higher levels of REST in the hippocampal granule cell layer. Images are representitive of 3 animals per condition. Comparisons were made between wild type and NeuroD2 null slices placed on the same slide.

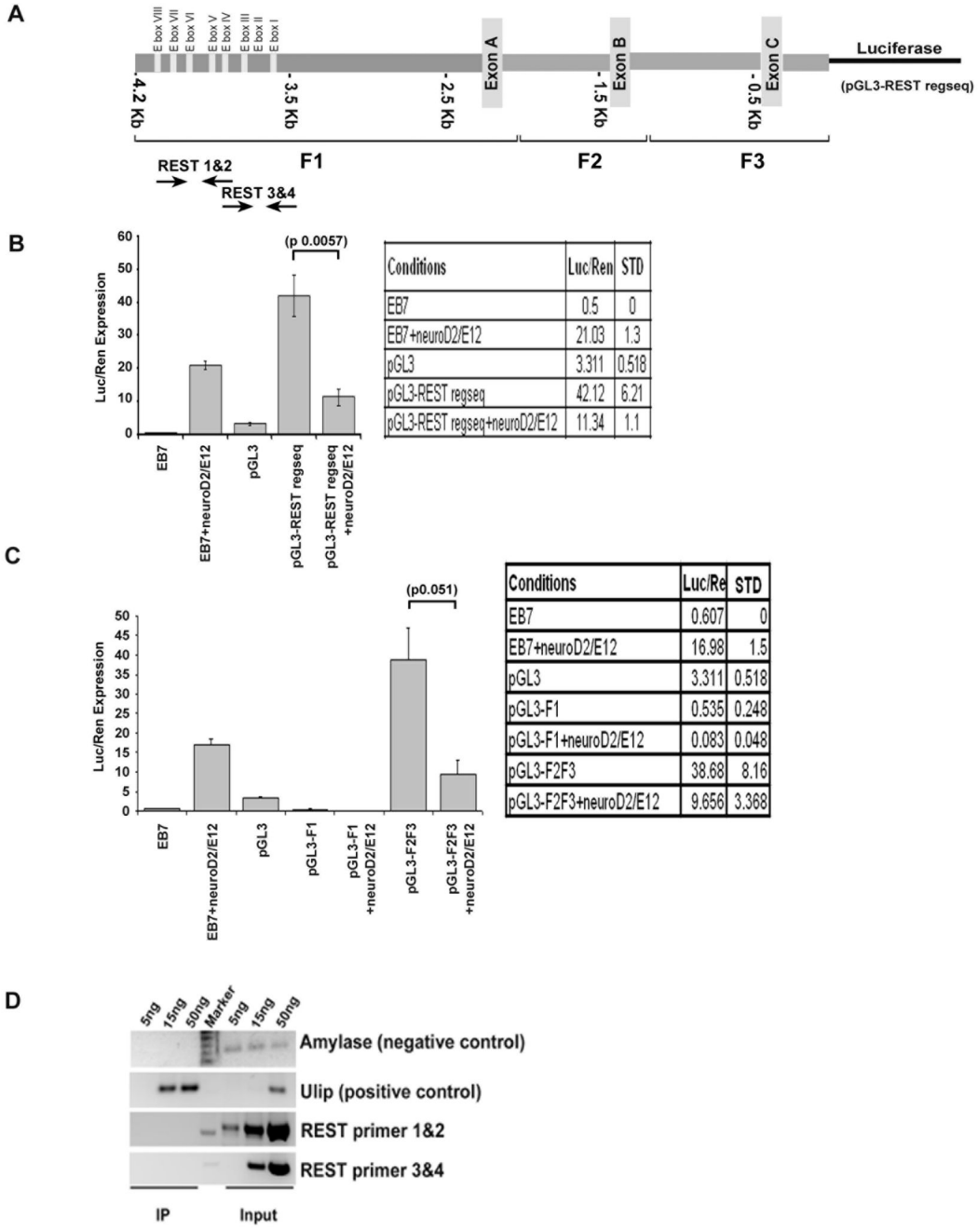


Figure 3. NeuroD2 inhibits REST regulatory sequence indirectly

4.2 Kb 5′ REST regulatory sequence containing three untranslated exons (Exon A, B, C) and eight E boxes was cloned into the pGL3-Basic luciferase vector and labeled pGL3-REST regseq (A). The PGL3-REST regseq construct is active in P19 cells. The activity of this construct is reduced when P19 cells are induced to differentiate into neurons by NeuroD2/E12 (B). EB7, containing tandem E boxes, was used as a positive control for NeuroD2 activity. The effect of NeuroD2 on the activity of Rest regulatory sequence fragments were assayed (C). ChIP studies on the E-box region of the endogenous REST regulatory sequences in NeuroD2 transfected cells shows that NeuroD2 does not bind the E-boxes (D). The location of primers used for ChIP PCR reactions is indicated in panel A. (ChIP = chromatin immunoprecipitation).

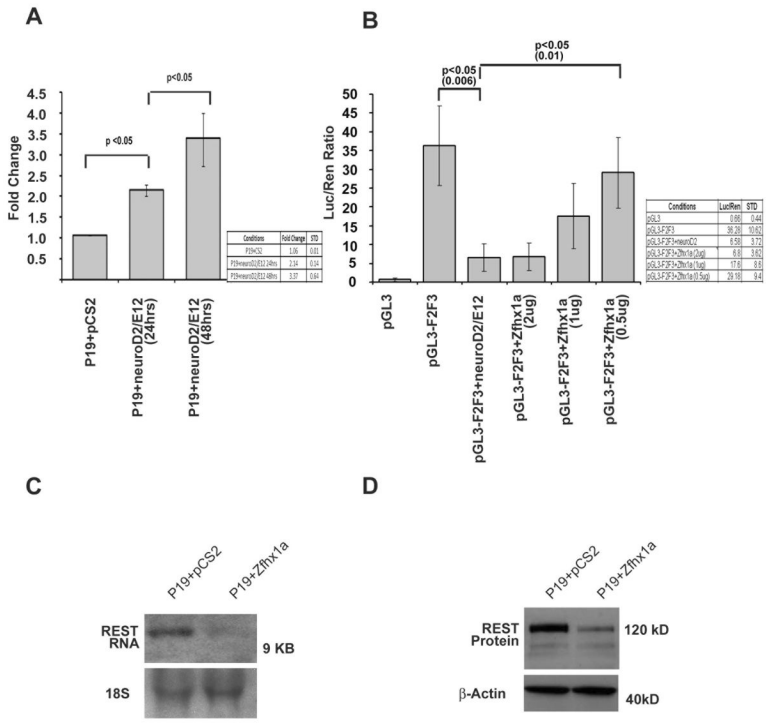
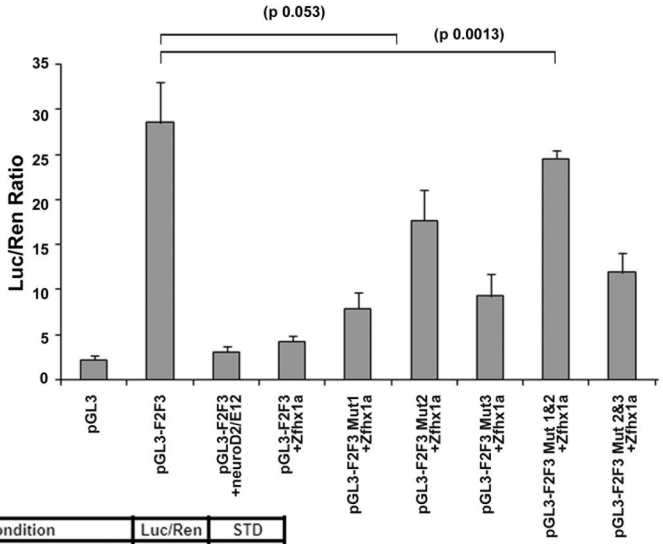


Figure 4. Zfhx1a is upregulated by NeuroD2 and inhibits REST expression

Real Time PCR confirms a steady increase in Zfhx1a transcript level in P19 cells transfected with NeuroD2 (A). Zfhx1a inhibits the activity of the Rest regseq pGL3-F2F3 reporter construct in a dose dependent manner (B). Overexpression of Zfhx1a in P19 cells inhibits the expression of REST transcript (C) and protein (D).



Condition	Luc/Ren	STD
pGL3	2.21	0.4065
pGL3-F2F3	28.56	4.53
pGL3-F2F3+neuroD2/E12	3.05	0.61
pGL3-F2F3+Zfhx1a	4.25	0.5894
pGL3-F2F3Mut 1 + Zfhx1a	7.83	1.77
pGL3-F2F3Mut 2 + Zfhx1a	17.69	3.33
pGL3-F2F3Mut 3 + Zfhx1a	9.282	2.366
pGL3-F2F3Mut 1&2 + Zfhx1a	24.52	0.9165
pGL3-F2F3Mut 2&3 + Zfhx1a	11.91	2.18

Figure 5. Zfhx1a requires two intact binding sites to inhibit REST 5′ regulatory sequences

The three putative Zfhx1a binding sites in the REST 5′ regulatory sequences were mutated to test their necessity for Zfhx1a inhibition (the mutated E box is denoted by an X). Mut 1&2 are least responsive to Zfhx1a inhibitory activity. The horizontal bars indicate direct comparison between the two series.

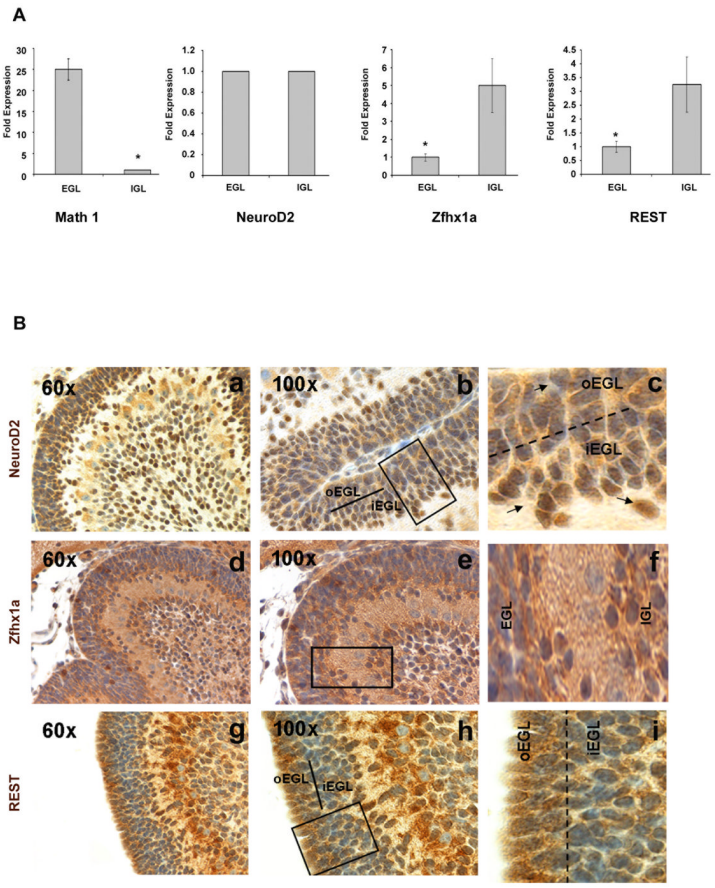


Figure 6. NeuroD2, Zfhx1a and REST expression during neuronal differentiation in the developing cerebellum

NeuroD2, Zfhx1a and REST qRT-PCR measures mRNA expression in EGL and IGL of the developing cerebellum. Math1 was used as a positive control in these experiments since Math1 is enriched in the EGL. * denotes $p<0.05$ (A). Immunohistochemistry for NeuroD2, Zfhx1a and REST on wild type P5 S129/C57b hybrid background (B). NeuroD2 protein is expressed in both the EGL and the IGL; however, nuclear NeuroD2 staining is detected only in the inner EGL (arrows), where post-mitotic, migrating cells reside (a-c). Zfhx1a is detected in the nucleus of most cells of the IGL and also in the nuclei of rare cells in the EGL (d-f). REST expression is limited to the outer EGL, where cells are farthest from differentiation (g-i). REST is also detected in the Purkinje cell layer. (oEGL = outer external granular layer, iEGL = inner external granular layer)

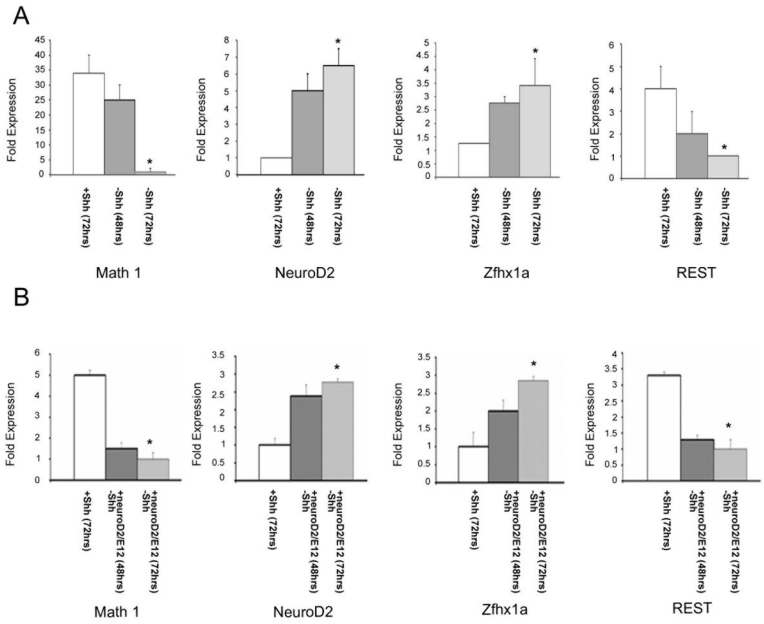


Figure 7. Expression of NeuroD2, Zfhx1a and REST in primary granular precursor cultures during induced neuronal differentiation by Shh withdrawal, and in response to forced expression of NeuroD2

Both NeuroD2 and Zfhx1a are upregulated in response to Shh withdrawal and neuronal differentiation while REST levels decline, * denotes $p<0.05$ (A). PGNP cultures were infected with pBABE-NeuroD2 and the resulting changes in the expression of NeuroD2, Zhfx1a and REST were measured 48 hours and 72 hours post-infection, * denotes $p<0.05$ (B).

Table 1

Representative Genes Regulated by NeuroD2/E12

Gene ID	Symbol	Name and Function	Direction*
Transcription Factor			
55502	Hes6	Hairy and enhancer of split 6	I
58158	NeuroD4/Math3	Neurogenic differentiation 4	I
6935	Zfhx1a/TCF8	Zinc finger homeobox 1a	I
84504	Nkx6-2	Nk6 Transcription Factor	I
666	Sox11	SRY-box containing gene 11	I
5978	REST	RE1-Silencing Transcription Factor	D
1051	C/EBP	CCAAT/enhancer binding protein	D
467	Atf3	Activating Transcription Factor 3	D
Signaling/Intracellular Cascade			
3481	Igf2	Insulin like Growth Factor 2	I
8153	RhoN	Ras Homolog N	I
5333	Plcd	Phospholipase C, delta	I
6004	Rgs16	Regulator of G protein Signaling 16	I
306	Anxa3	Annexin A3	D
311	Anxa11	Annexin A11	D
1382	Crabp2	Cellular Retinoic Acid Binding Protein II	D
Cytoskeletal Element			
1641	Dcx	Doublcortin	I
1627	Dbn1	Drebrin	I
5414	4-Sep	Septin 4	I
7414	Vcl	Vinculin	D
Membrane Protein/Ion Channel			
4826	Nnat	Neuronatin	I
4242	Mfng	Manic Fringe	I
28514	Dll1	Delta-Like 1	I
9543	Punc	Putative neuronal Cell Adhesion Molecule	I
781	Cacna2d1	Voltage-dependent Calcium Channel	I
22795	Nid2	Nidogen 2	D
4072	Tacstd1	Tumor-associated Calcium Signal Transducer 1	D
6515	Slc2a3	Solute Carrier Family 2-member 3	D
64116	Slc39a8	Solute Carrier Family 39-member 8	D
Secretory Granule			
1114	Chgb	Chromogranin B	I
6447	Sgne1	Secretory granule neuroendocrine protein 1	I
6812	stxbp1	Syntaxin binding protein 1	I
6616	Snap25	Synaptosomal-associated protein 25	I

* I denotes an increase and D denotes a decrease on the array.

Table 2

REST Regulatory Sequence Transcription Factor Binding Sites

Transcription Factor	Binding Site	Transcriptional Activator or Repressor
Oct-1	TGGGTAATGAGGG	Activator/Repressor
Sox-5	GTATTGTTTT	Activator
CREB	TGACGTGT	Activator/Repressor
Zfhx1a	CACCT/AGGTG	Repressor
C/EBP	TGTTTGGTATTTT	Activator
AP-1	TTGACTCAG	Activator
Sp1	GGGGCGGGAT	Activator
YY1	TGAGACCATTTAGAAAC	Activator/Repressor
Elk-1	AAACTTCCGGGCCA	Activator