Published in final edited form as:
Dev Neurobiol. 2012 June ; 72(6): . doi:10.1002/dneu.20979.

Reciprocal actions of ATF5 and Shh in proliferation of cerebellar granule neuron progenitor cells

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Abstract

Precise regulation of neuroprogenitor cell proliferation and differentiation is required for successful brain development, but the factors that contribute to this are only incompletely understood. The transcription factor ATF5 promotes proliferation of cerebral cortical neuroprogenitor cells and its down-regulation permits their differentiation. Here, we examine the expression and regulation of ATF5 in cerebellar granule neuron progenitor cells (CGNPs) as well as the role of ATF5 in the transition of CGNPs to post-mitotic cerebellar granule neurons (GCNs). We find that ATF5 is expressed by proliferating CGNPs in both the embryonic and post-natal cerebellar external granule layer (EGL) and in the rhombic lip, the embryonic structure from which the EGL arises. In contrast, ATF5 is undetectable in post-mitotic GCNs. In highly enriched dissociated cultures of CGNPs and CGNs, ATF5 is expressed only in CGNPs. Constitutive ATF5 expression in CGNPs does not affect their proliferation or exit from the cell cycle. In contrast, in presence of sonic hedgehog (Shh), a mitogen for CGNPs, constitutively expressed ATF5 promotes CGNP proliferation and delays their cell cycle exit and differentiation. Conversely, ATF5 loss-of-function conferred by a dominant-negative form of ATF5, significantly diminishes Shh-stimulated CGNP proliferation and promotes differentiation. In parallel with its stimulation of CGNP proliferation, Shh enhances ATF5 expression by what appeared to be a post-transcriptional mechanism involving protein stabilization. These findings indicate a reciprocal interaction between ATF5 and Shh in which Shh stimulates ATF5 expression and in which ATF5 contributes to Shh-stimulated CGNP expansion.

Keywords

ATF5; sonic hedgehog (Shh); cerebellum; cerebellar granule neuron progenitor cells; cerebellar granule neurons; external granule layer (EGL)

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This article is dedicated to the memory of Ira Black, whose studies in neurodevelopment were both pioneering and inspirational.

INTRODUCTION

Generation of the proper numbers of cells to form the nervous system requires a regulated balance between neuroprogenitor cell expansion and differentiation. Our understanding of the factors that contribute to this balance is incomplete. Although initially considered as a cell survival gene (Persengiev et al., 2002), the CREB/ATF transcription factor family member ATF5 has been more recently implicated as a protein that plays an important role in influencing the transition between neuroprogenitor cells and differentiated neurons and glia in the cerebral cortex (Angelastro et al., 2003, 2005; Mason et al., 2005; reviewed by Greene et al., 2009). ATF5 protein is highly expressed by proliferating neuroprogenitors in the embryonic and perinatal rodent cerebral cortical ventricular and subventricular zones and is undetectable when such cells differentiate into neurons, astrocytes and oligodendroglia (Angelastro et al., 2003, 2005; Mason et al., 2005). Constitutive expression of exogenous ATF5 in cerebral cortical neuroprogenitor cells *in vitro* and *in vivo* blocks their differentiation and exit from the cell cycle. Conversely, interference with ATF5 expression or function accelerates cerebral cortical neuroprogenitor cell cycle exit and their differentiation into neurons and glia (Angelastro et al., 2003, 2005; Mason et al., 2005). These findings support the ideas that ATF5 functions in cerebral cortical neuroprogenitor cells to maintain their proliferation and to block their differentiation and that ATF5 must be down-regulated in order for such cells to exit the cycle and differentiate. In this way, ATF5 appears to be an important regulator of proper genesis of those cells that form the cerebral cortex.

Several growth factors have been identified that cause ATF5 down-regulation and that promote neuroprogenitor cell differentiation. These include NGF and NT3 for neuronal progenitors and CNTF for astrocyte progenitors (Angelastro et al., 2003, 2005). Little is known, in contrast, about the signals that positively regulate ATF5 expression.

The present study addresses several questions about ATF5 in the developing nervous system. For one, is ATF5 expressed in areas of the developing brain in addition to the cerebral cortex and, for another, if so, what role does it play there? In addition, what extrinsic factors are responsible for maintaining ATF5 expression in proliferating neuroprogenitor cells? One area of the brain whose development has been intensively studied and that appears appropriate to address these questions is the cerebellum. The most abundant neuron type found in the cerebellum (as well as in the entire mammalian brain) is the cerebellar granule neuron (CGN) and there appears to be tight and well regulated control of CGN numbers (reviewed by Hatten and Heintz, 1995; Altman and Bayer, 1997; Chizhikov and Millen, 2003; Sotelo, 2004; Sillitoe and Joyner, 2007). During embryogenesis, proliferating precursor cells destined to form CGNs are produced in the rhombic lip (Alder et al., 1996; Altman and Bayer, 1997; Wingate and Hatten, 1999). These migrate from the rhombic lip to form a secondary germinal zone, the external granule layer (EGL). The EGL persists after birth (for about 2 weeks in the mouse) and continues to act as a germinal area (Gao and Hatten, 1994; Wingate and Hatten, 1999). The EGL contains a single proliferating cell type, the cerebellar granule neuroprogenitor cell (CGNP) that gives rise only to CGNs, (Zhang and Goldman, 1996a, b) which in turn migrate to and form the inner granule layer (IGL). Abundant evidence indicates that CGNP proliferation is driven at least in part, by sonic hedgehog (Shh) released from nearby processes of cerebellar Purkinje neurons (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999).

The well-defined path by which CGNPs are generated and by which they develop into CGNs, the known role of Shh in promoting CGNP proliferation and the ability to

recapitulate these events in highly enriched cultures of CGNPs have led us to examine ATF5 in the context of the CGNP to CGN transition. Here, we describe ATF5 expression in the developing cerebellum, demonstrate its regulation by Shh and examine its role in CGNPs and in Shh-promoted CGNP proliferation.

MATERIALS AND METHODS

Immunohistochemistry on mouse cerebellar sections

For embryonic study, mouse embryos were removed and dropped-fixed up to 48 hours in 4% paraformaldehyde/PBS (PFA/PBS). For postnatal study, mice were perfused with 4% PFA/PBS. The brains were removed and further fixed overnight in 4% PFA/PBS. After fixation, embryos/brains were rinsed with 1xPBS and transferred to 10% sucrose/1xPBS for cryoprotection until they sunk and then transferred to 30% sucrose/1xPBS until they sunk. Following dehydration in the sucrose, the embryos/brains were embedded in OCT cryosectioning matrix and frozen in 2-methyl butane containing dry ice. Specimens were sectioned on a cryostat at 12 to 14 μm thickness for developmental expression studies.

For tissue staining, sections underwent an antigen retrieval step in citrate buffer (10mM Citric Acid, pH 6.0) using a rice cooker, which brought the temperature of the buffer to 100°C. After the antigen retrieval step, sections were permeabilized with 1xTBS + 0.4% Triton X-100 for 20 minutes at room temperature (RT). Sections were then blocked with SuperBlock® in 1xTBS (Pierce) + 0.1% Triton X-100 for one hour at RT. Sections were incubated with primary antibody overnight at 4°C in a blocking solution. For co-localization experiments performed with ATF5 antibody, sections were blocked for endogenous Avidin/Biotin binding (Vector Labs cat#SP-2001) after the normal blocking step since biotinylated secondary antibody was used to enhance ATF5 detection. The generation of the ATF5 antisera is described in Angelastro et al. (2003) and was produced in rabbit. The rabbit anti-ATF5 837 was used for all experiments. For ATF5 co-localization experiments (used at 1:1000), the following antibodies were used: mouse anti-Ki67 (Vector Labs) at 1:100; mouse anti-NeuN (Santa Cruz) at 1:500; mouse anti-TuJ1 (class III β-tubulin) (Sigma) at 1:1000. Ki67 staining at E14.5 and E16.5 were performed with the rabbit anti-Ki67 (Vector Labs) antibody at 1:1000 dilution. For controls and to confirm specificity of primary antibodies, adjacent sections were incubated without primary antibodies.

Before the application of secondary antibodies, sections were washed 3X with 1xTBS + 0.1% Triton X-100 and incubated with secondary antibodies in a blocking solution for one hour at RT. For ATF5 co-localization experiments, goat anti-rabbit biotinylated antibody was used at 1:200 dilution (Vector Labs) in conjunction with the secondary antibody, Alexa Fluor® 568 (Molecular Probes/Invitrogen) at 1:1000 dilution. Sections were washed and Fluorescein Avidin D (Vector Labs) was used at 1:1000 dilution and incubated for 1 hour at RT. Sections were washed and mounted with a hard mounting medium containing DAPI. (Vectorshield Hardset, Vector Labs). Sections were stored at 4°C and images were taken within one week of staining.

Cerebellar cell cultures

Dissociated cultures of cerebellar cells were prepared from P4–6 C57BL/6J mice as described in detail by Lee et al. (2009). These were enriched for CGNPs (Lee et al., 2009). All cultures were prepared without the use of Percoll gradients as has been the case for past studies on regulation of proliferation and differentiation of CGNPs (Kenney and Rowitch, 2000; Pons et al., 2001; Kenney et al., 2003).

For retroviral infection experiments on GNPs, cells were infected at the time of plating with the addition of concentrated retroviruses to the medium. When present, Shh protein was applied directly to the medium at the time of plating at a concentration of 150 nM (3 μg/ml).

For cell counting and infection experiments using cultured cerebellar cells, cells were grown on poly-D-lysine coated glass coverslips placed in 24-well plates (Lee et al., 2009). Cells on coverslips were fixed with ice-cold 4% PFA/PBS for 10 minutes at RT. Cells were washed 3X quickly with 1xPBS, permeabilized with 1xTBS + 0.4% Triton X-100 for 20 minutes at RT, and quickly washed with 1xTBS. Cells were blocked with SuperBlock® in 1xTBS + 0.1% Triton X-100 for an hour at RT. Incubations with primary antibodies were performed overnight at 4°C in a blocking solution. For assessment of ATF5 and Ki67 positive cells in cerebellar cultures, cells were stained with rabbit anti-ATF5 (1:1000) and mouse anti-Ki67 (1:100 from Vector Labs) antibodies. For infection experiments where cell proliferation was assessed, cells were stained with rabbit anti-GFP (1:1000 from Invitrogen) and mouse anti-Ki67 (1:100 from Vector Labs) antibodies. After primary antibody incubation, cells were washed with 1xTBS + 0.1% Triton X-100 and incubated with secondary antibodies in a blocking solution for an hour at RT. Secondary antibodies used were Alexa Flour 488 and Alexa Flour 568 (Molecular Probes, Invitrogen). Cells were washed with 1xTBS + 0.1% Triton X-100 and the coverslips were mounted on a slide using a mounting medium containing DAPI. Slides were stored at 4°C and images were taken within one week after staining.

Imaging mouse cerebellar sections and cultured GNPs on coverslips

Imaging cell cultures and brain sections was carried out with Axiovision or OpenLab software through Axiophot Camera and a Zeiss Axioplan 2 microscope.

Retroviral constructs

Design of the long-length form of ATF5 and the d/n-ATF5 (AZIP) constructs are described in Angelastro et al. (2003). The short-length form of ATF5 lacks the first 100 amino acids found in the long-length form of ATF5. The long-length form of ATF5 has a FLAG-tag sequence incorporated into the N-terminus right after the first Kozak sequence and also contains a second Kozak sequence. The short-length form of ATF5 has the FLAG-tag sequence at the N-terminus incorporated right after the second Kozak sequence. The constructs encoding the two forms of LAG-ATF5 do not include the 5′ regulatory portions of the ATF5 gene.

Both forms of ATF5 were cloned into retroviral vector pWZL IRES-GFP, which has been shown to infect CGNPs in cell culture with high efficiency (Kenney et al., 2003). This is a bicistronic vector with IRES-dependent eGFP expression. ATF5 cassettes (Angelastro et al., 2003) were excised from retroviral vector pQCX-eGFP using the restriction enzyme Xho I and cloned into a multi-cloning site in the pWZL IRES-GFP vector linearized with Xho I. Correct orientation and sequence were confirmed through sequence analysis. d/n-ATF5 was delivered via the pLe-eGFP vector as previously described (Angelastro et al., 2003). Viral production was as previously described (Kenney et al., 2003) and concentrated by centrifugation at 50,000 × g at 4°C. The final titer was 1×10^7 virus particles/ml. In the present studies, the infection efficiency in cerebellar cultures was approximately 30%. For retroviral infection experiments on CGNPs, cells were infected at the time of plating with the addition of 2 μl of concentrated retroviruses to the medium per well in 24-well plates. For western blotting experiments, 10 μl of concentrated retroviruses was added to the medium per well in 6-well plates at the time of plating. When present, Shh protein was applied directly to the medium at the time of plating at a concentration of 150 nM (3 μg/ml)

Western Immunoblotting

Cerebellar cells were grown in 6-well tissue culture plates coated with poly-D-lysine. Cells were rinsed 3X with 1xPBS before being lysed with 100 μl of lysis buffer (Cell Signaling) containing 1 mM PMSF. Samples were sonicated for 30 seconds, centrifuged for 15 minutes at 4° C, and the supernatant collected. The Bradford Assay system (Pierce) was used to measure protein concentration. The desired volume of sample was added to NuPAGE® LDS Sample Buffer (NuPage, Invitrogen) with 5% beta-mercaptoethanol and boiled for 15 minutes. Samples were resolved on 4–12% or 12% bis-tris SDS-PAGE gradient gels (NuPage, Invitrogen). Separated proteins were transferred to nitrocellulose membrane, and blocked for 1 hour in 5% bovine serum albumin in 1xTBS + 0.2% Tween-20 (5% BSA/TBST). The membrane was incubated with primary antibody overnight at 4°C in a blocking buffer and washed with 1xTBST. Secondary antibody was bound in blocking buffer for 1 hour. Following washes in 1xTBST, membranes were developed with ECL or Pierce reagent. Primary antibodies are as follows: mouse anti-FLAG (Sigma) at 1:1000; mouse anti-beta-tubulin (Sigma) at 1:1000; rabbit anti-GFP (Invitrogen) at 1:1000.

Quantitative Real Time PCR

TRI Reagent (Molecular Research) was used to collect RNA samples. cDNA was transcribed from total RNA with First-Strand cDNA Synthesis for Quantitative RT-PCR Kit (Marlingen Biosciences). Primers for Gli1 and GAPDH and qRT-PCR parameters are described in Galvin et al. (2007). Analyses of amplification curves of real-time fluorescence and of melting curves were performed as described previously (Troy et al., 2001). Primers targeting mouse ATF5 were designed using the Primer 3 design program. Primers for ATF5 were as follows:

Forward: 5′ GAC CGC AAG CAA AAG AAG AG 3′

Reverse: 5′ CAG CCT GGA CCT GTA CCC TA 3′

Conditions for qRT-PCR were as follows: preheat at 95°C for 30 seconds; cycling 40 cycles of 93°C for 10 seconds, 63°C for 30 seconds, 72°C for 20 seconds and 89° for 20 seconds. qRT-PCR runs were performed three independent times and GAPDH was used to normalize input cDNA.

Statistics

For cell culture experiments involving quantification of stained cells, two to three coverslips per condition were used. For experiments with two coverslips per condition, 6 to 8 random images were taken per coverslip. For experiments with three coverslips per condition, 3 random images were taken per coverslip. Each image contained 75–150 cells. The results are reported as means ± SEM. Student's t test was performed as unpaired, two-tailed sets of arrays and presented as probability (p) values.

RESULTS

ATF5 Protein Expression in the Embryonic Mouse

Precursor cells that are destined to become cerebellar granule neurons (CGNs) arise from the rhombic lip, a germinal domain located in the caudo-medial edges of the cerebellar primordium lining the fourth ventricle (Alder et al., 1996; Altman and Bayer, 1997; Wingate and Hatten, 1999). In the mouse, this occurs between E12.5 and E17 (Machold and Fishell, 2005). Starting at about E13–14 in the mouse, granule neuron precursors migrate tangentially from the rhombic lip over the cerebellar anlage to form a secondary germinal zone, the external granule layer (EGL). EGL cerebellar granule neuron progenitors (CGNPs)

continue to proliferate until the second post-natal week in mouse and give rise only to CGNs (Gao and Hatten, 1994; Zhang and Goldman, 1996a, b; Wingate and Hatten, 1999).

We first examined ATF5 protein expression in the developing mouse cerebellum at E14.5 because this is soon after precursor cells leave the rhombic lip RL) to form the EGL and because at this age, the vast majority of rhombic lip cells are destined for the EGL (Altman and Bayer, 1997; reviewed by Hatten and Heintz, 1995; Machold and Fishell, 2005). Immunostaining revealed nuclear ATF5 expression in the rhombic lip as well as in a subset of cells in the nascent EGL (Fig. 1a, aa, B, C). At this stage, ATF5 staining in the EGL was relatively faint compared with later ages (Fig. 1B). No staining was seen when the primary ATF5 antiserum was omitted (data not shown). ATF5 immunostaining was also seen in the cerebellar ventricular zone which gives rise to GABAergic cerebellar neurons including Purkinje cells (Fig. 1a, C) (Hoshino et al., 2005; Hoshino, 2006; reviewed by Millen and Gleeson, 2008). Immunostaining of adjacent sections for the proliferation marker Ki67 confirmed that ATF5 is expressed within germinal regions in the VZ, RL and nascent EGL at E14.5 (Fig. 1d, dd).

At E16.5 nuclear expression of ATF5 in the EGL was markedly more intense and extensive compared to E14.5, with the majority of cells at this stage being ATF5+ (Fig. 1E, e, F). ATF5 expression in the EGL appeared to be highest in cells nearest to the rhombic lip (Fig. 1E). Staining was absent when the primary ATF5 antiserum was omitted (Fig. 1H). At this stage, the rhombic lip was also positive for ATF5. As at E14.5, Ki67 staining of adjacent sections revealed that ATF5 expression was present in regions of high cell proliferation (Fig. 1G, g). Similar observations were made at E17.5.

ATF5 expression in the postnatal mouse cerebellum

Proliferation of CGNPs continues in the postnatal cerebellum, peaking by P5–7 in the mouse and largely ending by P15. During this time, CGNPs proliferate in the outer EGL and leave the cell cycle as premigratory CGNs in the inner EGL. They then migrate radially down Bergmann radial fibers to reach their final terminal territory, the inner granule layer (reviewed by Hatten and Heintz, 1995; reviewed by Chizhikov and Millen, 2003; reviewed by Sotelo, 2004; reviewed by Sillitoe and Joyner, 2007).

At P1, ATF5 was widely present in all regions of the mouse cerebellar EGL (Fig. 2A). In contrast, there was relatively little ATF5 expression in the cerebellum outside of the EGL. Examination of adjacent sections further showed that the area of ATF5 expression in the P1 cerebellum corresponded to that of Ki67 (Fig. 2B), indicating ATF5 is likely to be expressed by proliferating CGNPs in the EGL.

Peak genesis and expansion of CGNPs in the murine EGL occurs between P5 to P7. Therefore it is not surprising that at P6, strong nuclear expression of ATF5 was detected in the EGL (Fig. 2C, F). ATF5 expression was seen in the majority of the cells at this time (Fig. 2C) and double staining indicated that it was present in cells that were also Ki67 positive (Fig. 2D, G, E, H). Although the majority of ATF5+ cells were also Ki67+, there were also a few ATF5+ cells that were not Ki67+ (Fig. 2H).

In addition to its expression in the EGL at P6, ATF5 was also detected at this stage in widely dispersed cells within the cerebellum that are outside of the EGL (Fig. 2C; white arrow). To determine whether these might be CGNs, co-immunostaining was carried out in P6/7 cerebellum for ATF5 and Tuj1 or NeuN, markers for post-mitotic/differentiated CGNs, respectively (Weyer et al., 2003). However, there was no evident co-staining of ATF5 with Tuj1 at P6 (Supplementary Fig. 1A, B, C) or with NeuN at P7 (Supplementary Fig. 1D, E, F). Such observations support the idea that ATF5 is expressed by proliferating CGNPs and

is down-regulated in post-mitotic and differentiated granule neurons. The identities of the ATF5-positive cerebellar cells outside the EGL at P6/7 are presently unclear, but they may correspond to oligodendroglial precursor cells that prior work showed to be ATF5 positive in the cortex (Angelastro et al., 22005, Mason et al., 2005) or to interneuron progenitors that proliferate post-natally in the developing cerebellum (Zhang and Goldman, 1996a, b).

At P9, as at P6, ATF5 was widely expressed in the EGL (Fig 3A, D) and double staining indicates that it was found in cells that are Ki67 positive (Fig. 3B, C, E, F). At this stage, the inner and outer portions of the EGL can be clearly distinguished, with Ki67 positive CGNPs limited to the outer EGL before they move to the inner EGL (where they begin to differentiate into post-mitotic CGNs; reviewed by Hatten and Heintz, 1995; Goldowitz and Hamre, 1998; Sotelo, 2004). Significantly, staining for Ki67 and nuclei (DAPI) indicated that ATF5 expression was relegated to the outer EGL and was not detected in the inner EGL (Fig. 3C, F). This is again consistent with the idea that ATF5 is expressed by CGNPs, and down regulated when they leave the cycle and become CGNs. By P9, there were also relatively few cerebellar cells outside the EGL that detectably expressed ATF5 and this corresponded to significantly diminished Ki67 staining (Fig. 3B, E).

By P15, the murine EGL is only a few cells thick and is at its widest in the central lobes of the cerebellum. There were relatively few Ki67 positive cells in the EGL at this time (Fig. 3G). Correspondingly, there were also relatively few ATF5-positive cells in the EGL at this time and when present, expression appeared to be lower compared with P1–9 (Fig. 3H). Little or no expression was found in the P15 cerebellum outside the EGL. Similarly, staining of adult cerebellum revealed no detectable ATF5 in the IGL or molecular layer (data not shown).

ATF5 expression in cultured CGNPs in absence of Shh

Our *in vivo* studies indicate that ATF5 is expressed in proliferating CGNPs and is down regulated when they exit the cell cycle and differentiate into CGNs. We next determined whether this also occurs *in vitro* and whether ATF5 expression is affected by external factors that regulate CGNP proliferation and differentiation.

A modified protocol (Lee et al., 2009), based on past procedures (Messer, 1977; Hatten, 1985; Hatten et al., 1998; Manzini et al., 2006) was used to establish highly enriched dissociated cultures of CGNPs and CGNs from P4–6 mouse cerebellum. Staining with calbindin and GFAP indicated that these cultures contain < 0.8% Purkinje cells and < 4% astrocytes, respectively, and that astrocyte number did not significantly change over time under the culture conditions employed here (Supplementary Figure 2C, D). The cultures were established at high density to promote proliferation (Gao et al., 1991) and in some cases as described below, proliferation was significantly enhanced by addition of Shh (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Nevertheless, even at high density and with Shh, the CGNPs ultimately underwent spontaneous differentiation into CGNs (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Manzini et al., 2006).

We initially compared ATF5 and Ki67 expression in cerebellar cultures in the absence of exogenous Shh. The dissociated cerebellar cells adhere within 2 hrs of plating. At this time about 60% of the cells were Ki67 positive and about 26% expressed ATF5 (Fig. 4A). Of the ATF5-positive cells, counts revealed that 98–100% were also Ki67+ (Fig. 4B), indicating that ATF5 is almost exclusively expressed by proliferating cells. Expression of both Ki67 and ATF5 substantially dropped over the next 2 days *in vitro* and was nearly absent by 3 days (Fig. 4A). As at 2 hours, ATF5 expression up to 3 days of culture was limited to proliferating cells (Fig. 4B). These findings are consistent with past reports that cultured

CGNPs differentiate into post-mitotic CGNs (Wechsler-Reya and Scott, 1999) and with our *in vivo* observations that ATF5 is expressed by CGNPs, but not by CGNs.

While nearly all ATF5+ cells were Ki67+, the converse was not seen from 2 hrs to 3 days after plating when only 20–40% of Ki67 labeled cells were positive for ATF5 (Fig. 4B). This observation raised the possibilities either that not all proliferating cells in the cultures express ATF5 or (given that Ki67 marks cells during many phases of the cell cycle), that ATF5 is highly expressed only during particular phases of the cycle. In consonance with the latter possibility, CGNPs undergoing mitosis (characterized by distinctive DAPI nuclear staining *in vivo* (Fig. 4D) and *in vitro* (Fig. 4C) consistently showed the most intense immunostaining for ATF5 (Fig. 4c, d). To better understand the latter observation, cultured cerebellar cells were co-stained for ATF5 and the G2/M marker PH3 (phospho-histone H3 (Ser10) (Cheung et al., 2000) (Fig. 4E–J). As DAPI staining, this revealed that essentially all cells in G2/M were ATF5+ at 2 and 24 hrs after plating (Fig. 4L). In contrast, at these times only about 10–15% of ATF5+ cells were also PH3+ (figure 4L). This indicates that ATF5 is expressed at stages of the cycle in addition to G2/M. However, because it appears that only a subpopulation of Ki67 positive cells in the cultures also detectably expressed ATF5, we cannot presently distinguish whether ATF5 is expressed at specific stages of the cycle in addition to G2/M in all CGNPs, or whether it is expressed in non-G2/M phases in some CGNPs, but not others.

Effect of ATF5 over-expression on CGNP proliferation in absence of Shh

Constitutive ATF5 expression in neuroprogenitor cells in primary cortical cell cultures and in the cortical SVZ *in vivo* was sufficient to maintain their proliferation and to block their differentiation (Angelastro et al., 2003, 2005). We therefore sought to determine whether constitutive ATF5 expression is sufficient to maintain proliferation of cultured CGNPs. To achieve this, we generated bicistronic retroviruses expressing FLAG-tagged ATF5 and eGFP. Such viruses should lead to expression only in proliferating cells such as CGNPs and not in post-mitotic CGNs. The ATF5 transcript has two potential Kozak start sites giving rise to two different proteins: 30 kDa (long form) and 22 kDa (short form) (Hansen et al., 2002). Recent findings indicate that the short form of the protein is substantially more stable than the long form (Uekusa et al., 2009). Because the functions of the two ATF5 protein forms have not been evaluated, we prepared retroviral expression constructs corresponding to both the long or short forms of the protein. Consistent with a difference in stability, initial expression studies in 293 EBNA cells indicated substantially higher expression of the short form of the protein despite similar levels of infection for both the long- and short-form constructs (Supplementary Fig. 3). We next infected cerebellar cultures with both constructs. Although the short form of ATF5 protein was readily detected, we could not detect the long form, even though eGFP expression indicated a substantial level of infection (Supplementary Fig. 4). Thus, it appears that the short form of ATF5 is differentially expressed in CGNPs as in 293 cells. This is consistent with a past study in which only the endogenous short form was detected in PC12 cells (Angelastro et al., 2003). It is important to note, however, that since our constructs encoded N-terminally FLAG-tagged proteins, our approach would not detect the short form of the protein produced from the second start site of the long form transcript.

We next infected newly dissociated cerebellar cultures (maintained without Shh) with retroviruses expressing either the long or short form transcripts of ATF5 and determined two days later the proportion of infected cells (marked by eGFP) that were Ki67+. At this time point, only about 2–3% of cells infected with control eGFP virus were positive for Ki67 (Supplementary Fig. 5). Although there was a trend to higher Ki67 expression with either form of ATF5 transcript, this was not statistically significant.

Regulation of ATF5 expression by Shh

Shh is a major mitogen for CGNPs *in vivo* and prolongs their proliferation *in vitro* (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Given the presence of ATF5 in CGNPs, we next sought to determine whether Shh regulates ATF5 expression in such cells. Cerebellar cells were therefore cultured with or without Shh and monitored over 72 hrs for expression of Ki67 and ATF5 (Fig. 5A). While Shh did not affect the degree of Ki67 expression at 2 hrs, it significantly maintained it at higher levels at 24 hr and more so at 48 and 72 hrs after plating. Although Shh slowed cell cycle exit, as previously reported, it did not prevent it (Wechsler-Reya and Scott, 1999). Similarly, Shh significantly enhanced ATF5 expression between 24–72 hours after plating (Fig. 5A). For instance, at 72 hrs, 4-fold more cells expressed ATF5 in Shh treated cultures, compared with untreated controls (Fig. 5A). To more fully examine the relationship between the effects of Shh on cell cycle and ATF5 expression, we carried out additional experiments at 48 hr after plating (Fig. 5B–G, H, I). At this time, the increases in proportions of cells expressing Ki67 or ATF5 in presence of Shh were similar (4.5- and 5.6-fold, respectively). As without Shh, in presence of Shh nearly all ATF5+ cells were also Ki67+, while only 25–30% of Ki67+ cells expressed ATF5 (Fig. 5I). Taken together, these findings indicate that Shh acts to maintain both CGNP proliferation and ATF5 expression and does so to similar degrees. Moreover, just as CGNPs ultimately leave the cell cycle and differentiate even in continued presence of Shh, they also lose ATF5 expression in parallel.

To better understand the mechanism by which Shh enhances ATF5 expression, we asked whether this occurs at the level of transcription. Although Shh increased levels of Gli1 transcripts in our cultures as previously reported (reviewed by Ruiz i Altaba et al., 2002), it had no significant effect on ATF5 transcript levels at 3, 24 or 48 hours of treatment (Supplementary Fig. 6). We next examined the influence of Shh on expression of exogenous ATF5 protein in cerebellar cultures infected with retrovirus encoding the short form of this protein. Because the construct does not include promoter or other regulatory elements of the ATF5 gene, any effects of Shh on exogenous ATF5 expression are likely to occur post-transcriptionally. When cell lysates were collected and assessed by western immunoblotting at 48 hours after infection and treatment, Shh produced on average (N=3 independent experiments) a statistically significant 4-fold increase in expression of exogenous ATF5 (Supplementary Fig. 6; Fig. 5J). Taken together, these findings suggest that Shh enhances ATF5 expression in CGNPs by a post-transcriptional mechanism.

ATF5 over-expression enhances Shh-induced CGNP proliferation

The correlation between ATF5 expression and Shh-stimulated CGNP proliferation raised the possibility that ATF5 may play a role in this effect. To begin to assess this, we infected newly dissociated cerebellar cells with retroviruses encoding either the long or short forms of ATF5 and assessed Ki67 expression after two or three days in culture with or without Shh. As in our previous experiments (Supplementary Fig. 5), ATF5 failed to promote CGNP proliferation in absence of Shh (Fig. 6G–J). In contrast, expression of either transcript form of ATF5 in the presence of Shh resulted in an approximate doubling (compared with controls) of the proportion of infected cells that were positive for Ki67 at both 48 and 96 hours of culture (Fig. 6G–J). Although we failed to observe expression of the transfected long-form of the protein in cerebellar cultures (Supplementary Fig. 4), as noted above, we cannot rule out that the second start site of the long-form transcript was employed in the cells to produce the short form of the protein.

ATF5 loss-of-function interferes with Shh-stimulated CGNP proliferation

The observations that Shh regulates endogenous ATF5 expression and that ATF5 over-expression potentiates Shh-promoted CGNP proliferation raises the possibility that ATF5

may participate in the proliferative actions of Shh on CGNPs. To evaluate this, we used a previously described and characterized dominant-negative (d/n) form of ATF5 (Angelastro et al., 2003, 2005). In the case of cortical neuroprogenitor cells, d/n- ATF5 promotes cell cycle exit and differentiation both *in vitro* and *in vivo* (Angelastro et al., 2003, 2005). A retroviral construct of d/n-ATF5 shows strong expression in 293 cells (Supplementary figure 3). This likely reflects enhanced stability of the protein due to its N-terminal truncation.

Prior studies have shown that ATF5 loss-of-function can promote apoptotic death of tumor-derived cells, but not of non-transformed cells. For instance, interference with ATF5 expression or function kills cultured glioblastoma and breast cancer cells, but spares cortical neuroprogenitor cells, normal mammary gland epithelial cells and activated astrocytes (all of which express ATF5) (Angelastro et al., 2003; 2006; Monaco et al., 2007). To assess the effect of d/n-ATF5 on CGNP viability, newly-dissociated cerebellar cells were infected with retroviruses expressing either d/n-ATF5 or eGFP, and infected cells were scored after 48 hours of culture for apoptotic nuclei. This revealed that d/n-ATF5 had no significant effect on cell death either in presence or absence of Shh (Fig. 7A). Thus, as with cortical progenitor cells, ATF5 loss-of-function does not affect viability of cultured CGNPs.

We next assessed the effect of d/n-ATF5 on proliferation of cultured CGNPs in either the presence of absence of Shh. While there was no detectable effect of the d/n-ATF5 on CGNP Ki67 expression without Shh, ATF5-loss of function significantly decreased Shh-stimulated Ki67 expression by about 35% over four independent experiments (Fig. 7B). Thus, endogenous ATF5 appears to participate in the pathways by which Shh promotes CGNP proliferation.

DISCUSSION

The particular aims of the current study were to determine whether and where ATF5 is expressed in the development of mouse cerebellar granule neurons, to examine how ATF5 expression is regulated in developing CGNPs and to ascertain whether and the extent to which ATF5 contributes to CGNP proliferation.

ATF5 appeared to be present from the earliest stages of EGL formation as well as in the rhombic lip that gives rise to this structure during embryogenesis. Cellular expression appeared to increase as EGL development progressed and to reach maximal expression at P6–P7 when CGNP genesis peaks (reviewed by Hatten and Heintz, 1995). In contrast, ATF5 was not detected in post-mitotic CGNs within either the inner EGL (prior to migration and maturation) or in the IGL (after migration and maturation).

ATF5 appeared to be present only in proliferating CGNPs. However, not all proliferating CGNPs expressed detectable ATF5. This suggested either that ATF5 is present only during particular stages of the cell cycle or that it is expressed by only a subpopulation of CGNPs. We determined that nearly all CGNPs in the G2/M phase of the cell cycle are strongly ATF5+, thus indicating that essentially all CGNPs express ATF5 during at least one stage of their division.

We employed cultures of highly enriched cerebellar CGNPs and CGNs to explore the regulation of ATF5 expression as well as its role in CGNP proliferation. Such cultures have proved to closely reproduce many of the aspects of CGNP proliferation and differentiation that occur *in vivo*. For instance, they respond to the presence of Shh and, even in the continued presence of Shh, eventually exit the cell cycle and differentiate into CGNs. We found, as *in vivo*, that ATF5 was expressed by proliferating CGNPs and then was down-regulated when such cells differentiated into post-mitotic CGNs.

In parallel with its capacity to stimulate/maintain CGNP proliferation, Shh significantly slowed the loss of ATF5 expression that takes place in the cerebellar cultures over time. This did not appear to be at the level of transcription. Our findings that Shh increased expression of exogenous ATF5 protein encoded by a construct lacking noncoding elements of the ATF5 gene or message suggests that Shh may instead act to stabilize ATF5 protein. Consistent with this possibility, ATF5 is subject to proteasomal degradation (Wei et al., 2008; Uekusa et al., 2009). Moreover, although Shh signaling is associated with transcriptional regulation, it also has reported effects on protein stability. For instance, Shh suppresses degradation of the transcription factor Gli2 (Pan et al., 2006) and of the signaling protein IRS1 (Parathath et al. 2008). It was recently shown that ATF5 binds and is stabilized by HSP70 (Li et al., 2011). While it has not been reported that Shh induces HSP70, this or other Shh-regulated genes may mediate the Shh-promoted elevation of ATF5 expression.

The presence of ATF5 in proliferating CGNPs and its regulation by Shh, which is a mitogen for such cells, suggested that ATF5 may contribute to CGNP expansion. We found here that constitutive ATF5 expression alone was not sufficient to affect CGNP proliferation and that these cells exited the cell cycle despite its presence. However, when Shh was present, constitutively expressed ATF5 enhanced CGNP proliferation while d/n-ATF5 significantly diminished CGNP proliferation. These findings therefore implicate ATF5 as a positive regulator of CGNP replication and indicate that it contributes to Shh-mediated CGNP expansion.

The role of ATF5 in CGNP proliferation is consistent with prior observations that over-expression of ATF5 is sufficient to drive proliferation and block differentiation of neuroprogenitor cells from the cerebral cortical ventricular and subventricular zones and that ATF5 down regulation or loss-of-function accelerates VZ and SVZ cell differentiation (Angelastro et al., 2003, 2005; Mason et al., 2005). Such observations however also point to differences in the roles of ATF5 in expansion of cerebral neuroprogenitors and in CGNPs. ATF5 appears to be necessary as well as sufficient for cerebral neuroprogenitor proliferation whereas in CGNPs it does not appear to be sufficient to promote cell division and contributes to this only in presence of Shh.

In summary, our findings reveal reciprocal actions of ATF5 and Shh in cerebellar development. Shh stabilizes the levels of ATF5 protein and ATF5 increases Shh-stimulated CGNP proliferation. We can postulate that when CGNPs become refractory to Shh, they also lose ATF5 expression, exit the cell cycle and undergo terminal differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. M. Chiara Manzini for her superb guidance and assistance with cerebellar cell culture.

Sources of financial support: R01-NS33689 (LAG), R21CA126924-02 (JMA), R01-NS061070 (AMK), and RO1-NS16951 (CAM). HYL was supported in part by endocrinology training grant DK07328.

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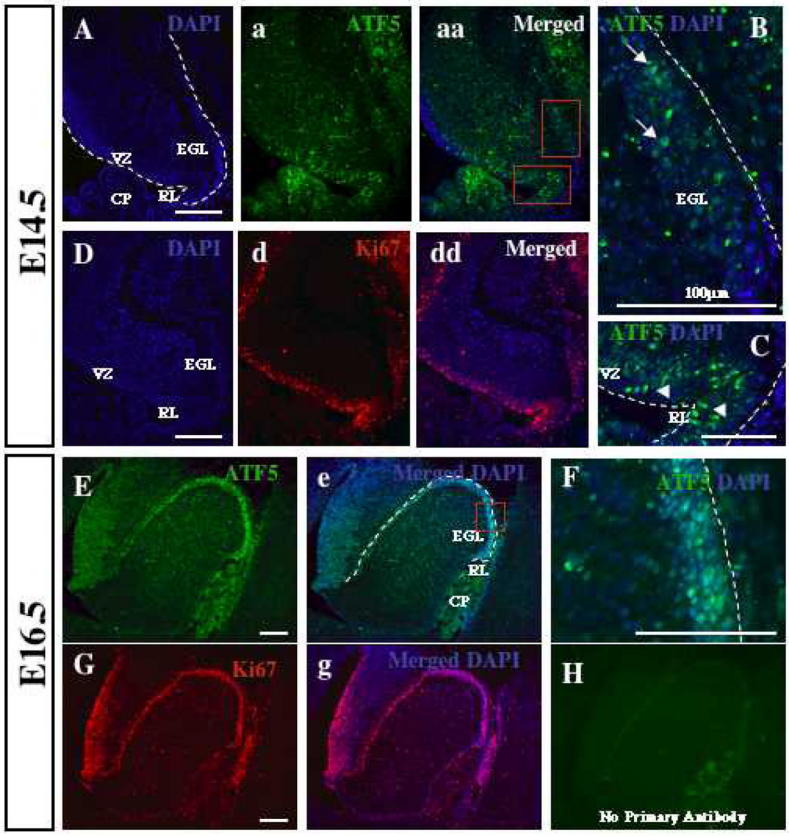

Figure 1. ATF5 protein is expressed in proliferating cerebellar precursor cells during murine embryonic cerebellum development at E14.5 and E16.5

Sagittal sections from the E14.5 (A–D) and E16.5 (E–H) mouse cerebellum were immunostained for ATF5 and the cell cycle marker, Ki67. (A, D) Nuclear staining with DAPI for visualizing the morphology of the E14.5 cerebellum. (a, aa, B, C) Immunostaining of ATF5 in the RL, EGL, and the VZ at E14.5. (B) Magnified image of the upper rectangle in panel aa. ATF5 positive cells in the nascent EGL, as indicated by white arrows, are few and ATF5 expression is less intense than compared to its expression at later embryonic stages. (C) Magnified image of the lower rectangle in panel aa. ATF5 positive cells are found in the VZ and the RL as indicated by white arrowheads. Nuclear expression of ATF5 in the VZ and the RL is higher than its expression in the EGL at this time. (d, dd) Ki67 staining in the RL, VZ and the EGL indicate that these regions contain cells undergoing proliferation. The regions of ATF5 expression correlates with cell proliferation. (E, e) ATF5 expression at E16.5 is present in the EGL and the intensity of its expression is higher in cells nearest to the RL. (F) Higher magnification image from panel *e* shows that ATF5 expression is nuclear (co-staining with DAPI) and is present in a majority of the cells in the EGL. (G, g) Ki67 expression in the adjacent section indicates high levels of proliferative activity in the E16.5 EGL. The zone of ATF5 expression correlates with this region of high cell proliferation. (H) ATF5 immunostaining is absent when primary ATF5 antibody is omitted. The white dotted lines mark the developing cerebellum. Scale bars in panels A, D, E, and G represent 200 μm. Scale bars in panels B, C, and F represent 100 μm. EGL=external granule layer, CP= choroid plexus, RL= rhombic lip, VZ= ventricular zone.

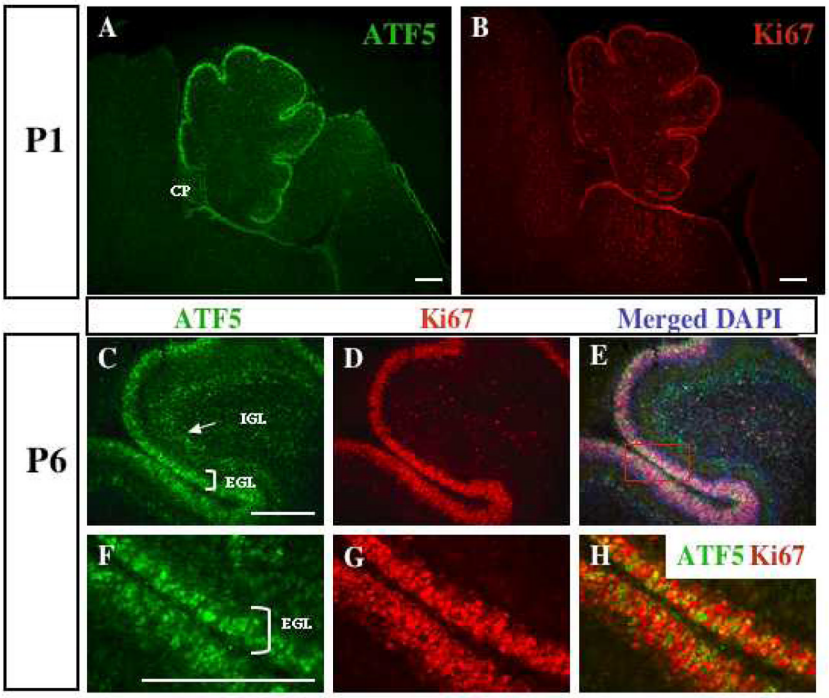

Figure 2. ATF5 protein is highly expressed by proliferating CGNPs in the murine EGL at P1 and P6

Sagittal sections from the P1 (A, B) and P6 (C–H) mouse cerebellum were immunostained stained for ATF5 and the cell cycle marker, Ki67. (A) At P1, ATF5 expression is found in all lobes of the P1 mouse EGL. (B) ATF5 staining is correlated with areas of cell proliferation as indicated by Ki67 expression present in an adjacent section. (C–H) Co-staining for ATF5 and Ki67 in the P6 mouse cerebellum. ATF5 expression is most intense at P6 in proliferating CGNPs in the EGL. (C) ATF5 expression is seen within the EGL as well as in cells outside this region (white arrow). (D, E) Co-staining with DAPI indicates that Ki67 and ATF5 are co-localized in proliferating CGNPs within the EGL. (F, G, H) Magnified images from panels C and D without DAPI staining show staining of (F) ATF5, (G) Ki67 and (H) merged ATF5 with Ki67. This indicates that ATF5 is present in a subfraction of proliferating CGNPs. Scale bars represent 200 μm. EGL=external granule layer, CP= choroid plexus, IGL=inner granule layer.

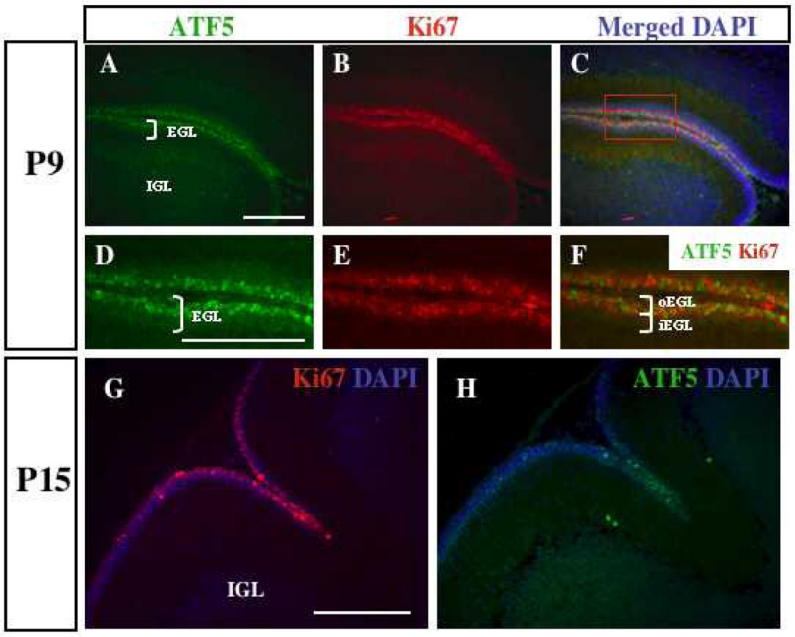

Figure 3. ATF5 protein is expressed by proliferating CGNPs in the murine EGL at P9 and P15

Sagittal sections from the P9 (A–F) and P15 (G, H) mouse cerebellum were immunostained for ATF5 and the cell cycle marker, Ki67. (A–C) ATF5 is expressed by proliferating CGNPs in the P9 EGL, specifically in the oEGL where the proliferating CGNPs are found. ATF5 expression in the EGL is lower than at P6 and very few proliferating cells are found outside of the EGL as indicated by lack of Ki67 expression outside this region. (D–F) These images are magnified from those in panels A and B and indicate the presence of Ki67 and ATF5 expression in the oEGL and not in iEGL where post-mitotic premigratory CGNs reside. ATF5 co-staining with Ki67 indicates that ATF5 is associated with proliferating CGNPs. (G) By P15, a majority of the cells have migrated out of the EGL into the IGL. The EGL is only a few cells thick but still contain CGNPs, which are undergoing proliferation as indicated by Ki67 staining. (H) There is lower ATF5 expression at P15 and this is found in a few cells within the EGL. Scale bars represent 200 μm. EGL= external granule layer, oEGL=outer external granule layer, iEGL=inner external granule layer, IGL=inner granule layer.

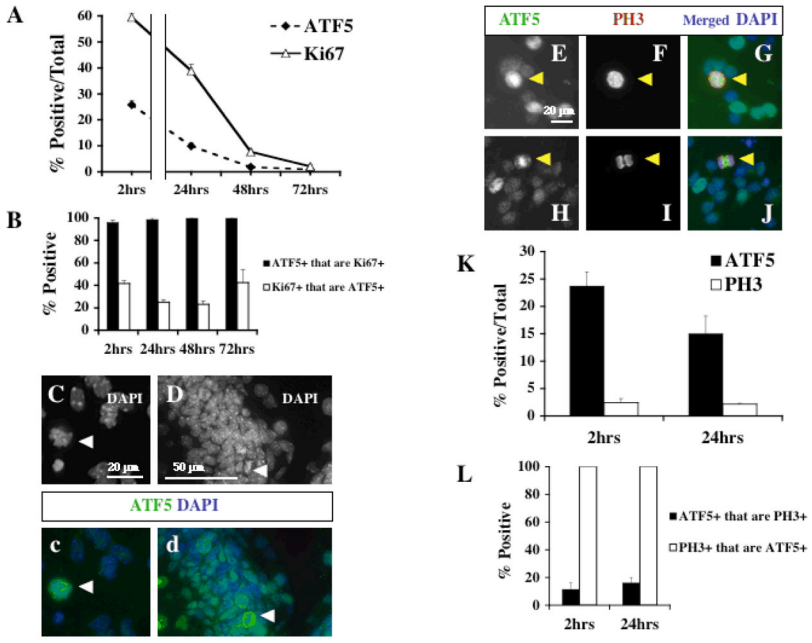

Figure 4. ATF5 and cell cycle marker expression in cerebellar cell cultures

(A) Proportion of total cells in cerebellar cultures (without Shh) positive for ATF5 and Ki67 expression at time points 2, 24, 48, and 72 hours after plating. Dissociated cerebellar cells were plated on glass coverslips coated with Poly-D-Lysine. After plating, cells were fixed and immunostained for ATF5 and Ki67 and with DAPI at indicated time points. Quantification (vs. total DAPI positive cells) indicates that at two hours after plating, about 26% of the cells are positive for ATF5 and 60% of the cells are undergoing proliferation as indicated by Ki67 staining. Over time, the numbers of cells expressing ATF5 falls as well as numbers of cells undergoing proliferation. By 72 hours in culture, very few cells are proliferating or expressing ATF5. (B) Nearly all ATF5 positive cells in cerebellar cultures are Ki67 positive while only a fraction (30–40%) of the Ki67-positive cells are ATF5-positive. Graphs in A and B represent data from one experiment ± SEM. The experiment was done in triplicate with three coverslips per time point with three images were taken per coverslip. About 300 cells were counted for each time point. Comparable results were found in two independent replicate experiments. (C, c) CGNPs undergoing mitosis in cell culture (white arrowhead) consistently stain strongly for ATF5 protein expression (white arrowhead). (D, d) CGNPs undergoing mitosis in the P6 mouse cerebellum (white arrowhead) stain strongly for ATF5 expression (white arrowhead). (E–J) In cerebellar cell cultures (24 hours in culture), cells positive for the G2/M phase marker PH3, express high levels of ATF5 protein (yellow arrowhead). (K) Proportion of total cells in cerebellar cultures (without Shh treatment) positive for ATF5 and PH3 expression at time points 2 and 24 hours after plating. At 2 and 24 hours, only 2% of the cells are in G2/M. (L) Quantification of co-staining indicates that all PH3 positive cells in cerebellar cultures are positive for ATF5 expression, but that only a fraction of ATF5 positive cells are positive for PH3. Graphs in K and L represent one experiment with SEM. The experiment was done in triplicate with three coverslips per time point and three images were taken per coverslip. About 200–250 cells were counted for each time point. Comparable results were found in an independent replicate experiment.

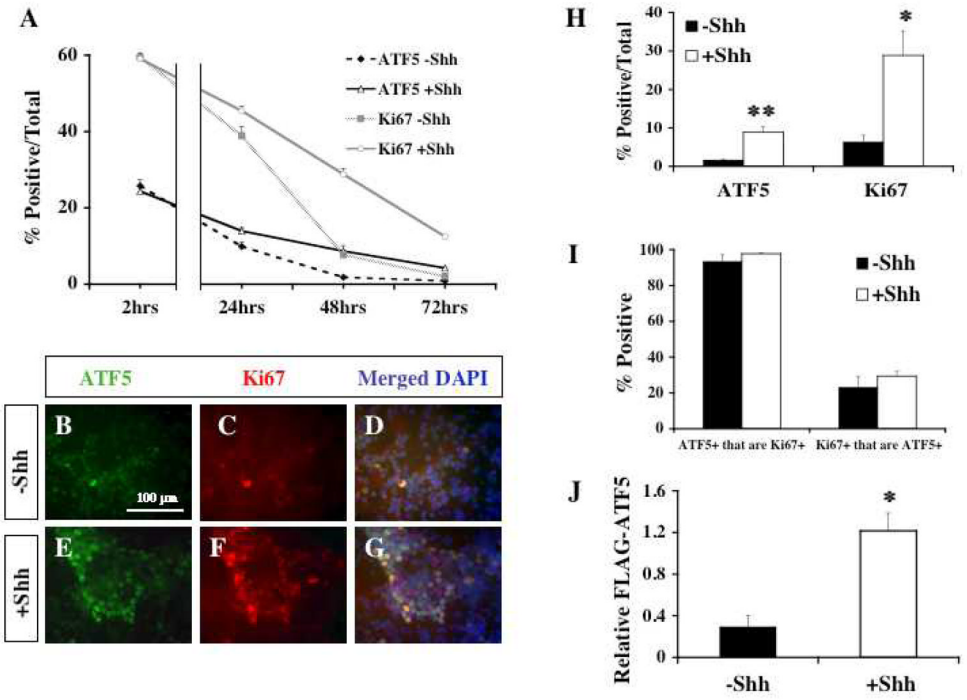

Figure 5. Effect of Shh on proportion of cultured cerebellar cells that are positive for expression of Ki67 and ATF5

(A) Proportion of total cells in cerebellar cultures (with and without Shh) positive for ATF5 and Ki67 expression at time points 2, 24, 48, and 72 hours after plating. Graph represents data from one experiment ± SEM. The experiment was done in triplicate with three coverslips per time point with three images taken per coverslip. Approximately 300 cells were counted for each time point. Comparable results were found in two independent replicate experiments. (B, C, D) Fluorescence photomicrographs of cultured cerebellar cells stained for expression of Ki67, ATF5 and with DAPI after 48 hours in the absence of Shh. Note that few cells are undergoing proliferation and are positive for ATF5. (E, F, G) Fluorescence photomicrograph of cultured cerebellar cells stained for expression of Ki67, ATF5 and with DAPI after 48 hours in the presence of Shh (3 μg/ml; 150 nM). Note increase in proportions of cells positive for Ki67 and ATF5 staining. (H) Shh treatment increases the proportion of cultured cerebellar cells that express ATF5 and Ki67 at 48 hours *in vitro*. Values are means of counts from three independent experiments and error bars represent SEM. Significance compared to Shh untreated cultures; *p=0.026, **p=0.006 (Student's t-test). (I) Shh does not affect the proportion of ATF5 positive cultured cerebellar cells that co-express Ki67 or the proportion of Ki67 cells that co-express ATF5. Values are means of counts from three independent experiments and error bars represent SEM. (J) Effect of Shh on expression of exogenous short-form ATF5-FLAG in cultured CGNPs. Cultures were infected with retrovirus for 48 hrs in the presence or absence of Shh. Cell lysates were collected and analyzed by western immunoblotting as described in Methods and in the text. The graph represents means of relative FLAG-ATF5 expression (normalized to eGFP expression) from three independent experiments and error bars represent SEM. Significance compared to Shh untreated cultures; *p=0.01(Student's t-test).

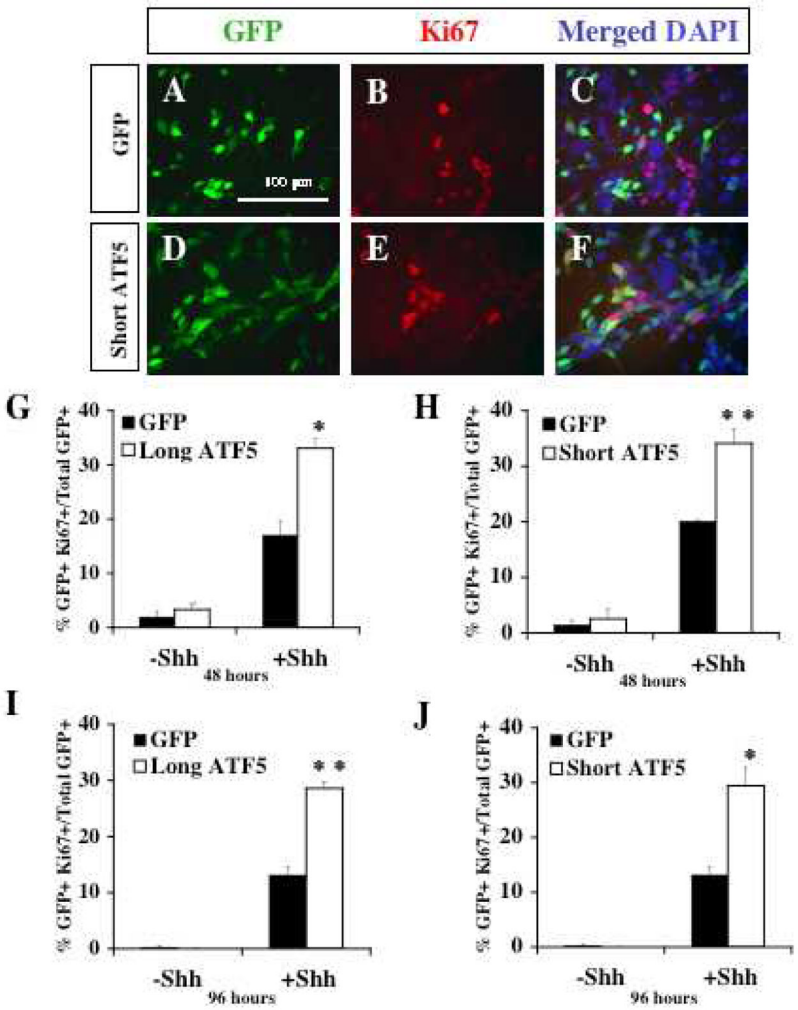

Figure 6. Shh mediated CGNP proliferation is potentiated by exogenous ATF5 at 48 hours and 96 hours

Cultured CGNPs were infected with retrovirus encoding the long and short forms of ATF5 or eGFP alone in the presence and absence of Shh. Forty-eight hours after infection, eGFP-positive (infected) cells were evaluated for expression of the proliferation marker Ki67. (A–F) Fluorescence photomicrographs of cells infected with control empty eGFP vector or vector expressing the short form of ATF5 and stained for eGFP and Ki67 and with DAPI. (G, H) Quantification of proportions of infected (eGFP positive) cells that were Ki67 positive after infection with retroviruses expressing the indicated constructs and cultured for 48 hours with or without Shh. Values represent means ± SEM for 4 (G) or 3 (H) independent experiments. *p = 0.02, **p = 0.005 for difference from control cells infected with eGFP alone (Student's t-test). (I, J) Quantification of proportions of infected (eGFP positive) cells that were Ki67 positive after infection with retroviruses expressing the indicated constructs and cultured for 96 hours with or without Shh. Values represent means from 3 (I) or 4 (J) independent experiments ± SEM. *p=0.003, ** p=0.001 for difference from control cells infected with eGFP alone (Student's t-test).

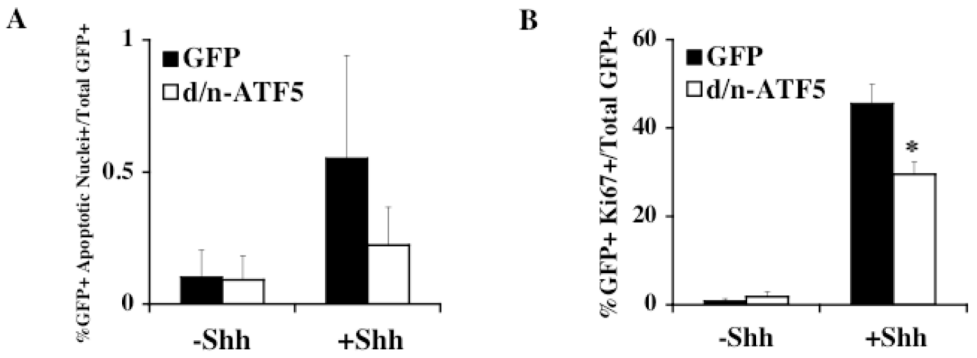

Figure 7. Interference with ATF5 function does not cause death of cultured GNPs, but diminishes their proliferative response to Shh

(A) Quantification of proportions of infected (eGFP positive) cells that have apoptotic nuclei after infection with retroviruses expressing d/n-ATF5 or eGFP and cultured for 48 hours with or without Shh. Values represent means from 4 independent experiments ± SEM. (B) Quantification of proportions of infected (eGFP positive) cells that are Ki67 positive after infection with the retroviruses expressing d/n-ATF5 or eGFP and cultured for 48 hours with or without Shh. Values represent means from 4 independent experiments ± SEM. Difference between control and d/n-ATF5 infected cultures *p=0.02 (Student's t-test).