

AF4 Is a Critical Regulator of the IGF-1 Signaling Pathway during Purkinje Cell Development

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Deregulation of the insulin-like growth factor 1 (IGF-1) signaling pathway is a recurrent finding in mouse models and human patients with cerebellar ataxia and thus represents a common pathological cascade in neuronal cell death that may be targeted for therapy. We have previously identified a point mutation in AF4, a transcription cofactor of RNA polymerase II elongation and chromatin remodeling, that causes progressive and highly specific Purkinje cell (PC) death in the ataxic mouse mutant *robotic*, leading to the accumulation of AF4 in PCs. Here we confirm that the spatiotemporal pattern of PC degeneration in the robotic cerebellum correlates with the specific profile of AF4 upregulation. To identify the underlying molecular pathways, we performed microarray gene expression analysis of PCs obtained by laser capture microdissection (LCM) at the onset of degeneration. *Igf-1* was significantly downregulated in robotic PCs compared with wild-type controls before and throughout the degenerative process. Consistently, we observed a decrease in the activation of downstream signaling molecules including type 1 IGF receptor (IGF-1R) and the extracellular signal-regulated kinase (ERK) 1 and ERK2. Chromatin immunoprecipitation confirmed that *Igf-1* is a direct and the first validated target of the AF4 transcriptional regulatory complex, and treatment of presymptomatic robotic mice with IGF-1 indeed markedly delayed the progression of PC death. This study demonstrates that small changes in the levels of a single transcriptional cofactor can deleteriously affect normal cerebellum function and opens new avenues of research for the manipulation of the IGF-1 pathway in the treatment of cerebellar ataxia in humans.

Introduction

AF4, LAF4, and AF5q31 are commonly involved in chromosome translocations with the mixed-lineage leukemia gene responsible for acute lymphoblastic leukemia (ALL) (Taki et al., 1999; von Bergh et al., 2002). Together with FMR2, transcriptionally silenced in FRAXE mental retardation (Géczy et al., 1997), these proteins form the AF4/LAF4/FMR2 (ALF) family of conserved transcription cofactors. We have recently established that the ALF proteins function in a large transcriptional regulatory complex that mediates chromatin remodeling and stimulates RNA polymerase II elongation (Bitoun et al., 2007). These studies have been greatly facilitated by the use of the AF4 mutant mouse, *robotic*, previously isolated as a novel model of cerebellar ataxia. Purkinje cell (PC) degeneration starts around weaning age in robotic mice, leading to impaired motor coordination at 5 weeks, followed by progressive cell death from 8 weeks that most severely affects the anterior lobes of the cerebellum while sparing lobe X (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). The robotic mutant also displays general growth retardation, defects in T-cell maturation, and cataracts (Isaacs et al., 2003).

The robotic mutation disrupts the binding of AF4 to SIAH ubiquitin ligases, resulting in a marked decrease in proteasomal turnover of the mutant protein (Oliver et al., 2004). In the cerebellum *Af4* is specifically expressed in PCs (Isaacs et al., 2003), and we have demonstrated that accumulation of robotic AF4 and colocalization of known transcriptional complex components occur in these cells (Bitoun et al., 2007). We also observed increased levels of the AF4 transcription regulatory complex in the robotic thymus. Importantly, overexpression of wild-type AF4 in neuronal N2a cells results in a seven-fold increase in reporter gene transcription, which is further enhanced three-fold with a robotic AF4 construct (Bitoun et al., 2007). Based on these observations, we anticipated that the accumulation of AF4 in the robotic mouse leads to the chronic activation of downstream gene targets in all sites of normal expression. The transcriptional targets of AF4 and the mode of cell death observed in *robotic* remain unclear, however.

Here we provide the first detailed characterization of AF4 expression during postnatal PC development and show that the spatiotemporal pattern of PC degeneration coincides with the abnormal levels of AF4 in the robotic cerebellum. Due to the highly specific expression of AF4, we used a combination of laser capture microdissection (LCM) and microarray profiling of robotic PCs. This strategy revealed that *Igf-1* is a direct target of the AF4 transcriptional regulatory complex and that reduced activation of the IGF-1 signaling pathway takes place in robotic PCs before and throughout the degeneration process. Our studies have uncovered an essential role for AF4 in the regulation of the IGF-1 signaling pathway during postnatal PC development, upon which depend the correct functioning

Received Oct. 19, 2009; accepted Oct. 28, 2009.

This work was supported by the Medical Research Council. We thank the Wellcome Trust Integrative Physiology Initiative in Ion Channels and Diseases of Electrically Excitable Cells for use of the microarray facility.

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DOI:10.1523/JNEUROSCI.5188-09.2009

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and survival of mature PCs. This novel approach to investigate the degenerative pathways in an ataxic mutant demonstrates that subtle changes in a single transcriptional cofactor can have a dramatic impact on brain function.

Materials and Methods

Animals. All studies were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. The *robotic* mutant mouse, a progeny of an *N*-ethyl-*N*-nitrosourea-injected BALB/c male and a C3H/HeH female, has been described previously (Isaacs et al., 2003). The genotypes of individual animals were confirmed by PCR and sequencing as previously described (Oliver et al., 2007).

Antibodies. The polyclonal rabbit AF4 antiserum has been described previously (Bitoun et al., 2007). Primary antibodies to IGF-1 (Abcam), phospho-IGF-1R (Abcam), phospho-p44/42 MAPK (Cell Signaling Technology), and calbindin D28K (Swant) were used in conjunction with horseradish peroxidase-conjugated (GE Healthcare) and Alexa Fluor 594- and 488-conjugated (Invitrogen) secondary antibodies.

LCM and RNA isolation. Brains were excised, embedded in OCT compound (TissueTek) and stored at -80°C until sectioning. Sagittal cerebellum sections ($10\ \mu\text{m}$) were cut, mounted onto PEN membrane-coated slides (Carl Zeiss), fixed in a graded series of ice-cold ethanol solutions (95, 75, 50%) for 30 s each, and stained for 1 min in 1% cresyl violet acetate (Sigma). Sections were rinsed in 50% ethanol solution and dehydrated in 75, 95, and 100% ethanol solutions for 30 s each. Sections were further dehydrated twice in xylene for 30 s and air dried for 5 min before microdissection. PCs were microdissected using the P.A.L.M Micro Beam system in combination with Robo software (Carl Zeiss) and directly catapulted into the RLT lysis buffer of the RNeasy Micro kit (Qiagen). Collected cells were frozen on dry ice and stored at -80°C until RNA extraction was performed from pooled samples. For microarray analysis, ~ 2500 PCs were collected within the same day from freshly cut, fixed, and stained sections. For *Af4* gene expression analysis, 350–500 PCs were collected from all 10 cerebellum lobes or from groups of lobes. Total RNA was extracted using the RNeasy Micro kit (Qiagen) with additional treatment with proteinase K, and the quality was assessed on a 2100 BioAnalyzer using the RNA 6000 Pico Assay (Agilent Technologies).

qRT-PCR. Total RNA was reverse transcribed using Sensiscript reverse transcriptase (Qiagen). Total cDNA and genomic DNA were subjected to real-time PCR on an ABI PRISM 7000 sequence detection system using SYBR green PCR master mix (Applied Biosystems). β -Actin mRNA levels were used as internal normalizing control. Primers used were *Af4*-forward 5'-GGCCTCAGAGTCAGAAAG-3', *Af4*-reverse 5'-CAACGCAAGCA-TAAACAGC-3', *Igf-1*-forward 5'-TGGATGCTCTTCAGTTCGTG-3', *Igf-1*-reverse 5'-GTCTTGGGCATGTCAGTGTG-3', *Igfbp-3*-forward 5'-GAAAGCGGGGCTTCTGCT-3', *Igfbp-3*-reverse 5'-CTCTGCACGCTG-AGGCAAT-3', β -actin-forward 5'-CTGGCCTCACTGTCCACCTT-3', and β -actin-reverse 5'-CGGACTCATCGTACTCCTGCTT-3'. To determine significant differences in expression for the tested genes between samples, the Welch *t* test was applied (Miller, 1997).

cRNA amplification, microarray hybridization, and data analysis. Samples were prepared from four pairs of wild-type and robotic litter-matched mice. Labeled cRNA was generated from 19 ng total RNA using the two-cycle target labeling and control kit (Affymetrix) and the MEGAscript T7 kit (Ambion) according to the manufacturers' instructions. cRNA was fragmented and hybridized to GeneChip Mouse 430 2.0 arrays following the manufacturer's protocol (Affymetrix). Arrays were washed, stained, and scanned using the GeneChip Fluidics Station 450 and Scanner 3000 (Affymetrix). Data were normalized in GeneSpring GX 7.3 (Agilent Technologies) using guanine cytosine robust multiarray analysis (GCRMA), and differentially expressed genes were identified using the Welch *t* test with a *p* value cutoff of ≤ 0.05 and a fold change difference between robotic and wild-type PCs cutoff of ≥ 1.5 . The genes were sorted according to their gene ontology using GenMAPP's MAPPFinder (Salomonis et al., 2007). Only ontologies with ≥ 3 genes changing and a permuted *p* value ≤ 0.05 were selected.

Immunohistochemistry. Mice were transcardially perfused with 4% PFA and brains were dissected and postfixed overnight before embed-

ding in paraplast wax. Ten-micrometer sections were cut, mounted on lysine-coated slides, deparaffinized in xylene, and rehydrated through graded alcohol series (100, 95, 80, 70%) before antigen retrieval was performed in boiling 10 mM sodium citrate pH 6.0 for 20 min. Sections were blocked for 1 h at room temperature in PBS supplemented with 0.3% Triton X-100 and 2% normal goat serum and then incubated with primary antibodies between 1 and 3 overnights at 4°C depending on the antibody used. Antibodies were diluted in blocking solution according to the manufacturer's instructions. The AF4 antiserum was used at a dilution of 1:750. Following extensive washes in PBS, sections were incubated overnight at 4°C with the appropriate Alexa Fluor-conjugated secondary antibodies, washed again, and mounted in Histomount (RA Lamb). Sections were observed under an Axioplan II imaging microscope equipped with an AxioCam and images were captured using the AxioVision software (Carl Zeiss Ltd).

ChIP. Assays were performed using the ChIP-IT enzymatic kit according to the manufacturer's instructions (Active Motif). Briefly, fresh tissues (60–100 mg) were finely minced and incubated for 10 min at room temperature with gentle shaking in 1% formaldehyde solution to cross-link protein–DNA interactions. The cross-linking reaction was stopped by addition of glycine to a final concentration of 0.25 M. Minced tissues were homogenized, and cross-linked chromatin was sheared by enzymatic digestion following the manufacturer's recommendations to yield an average fragment size of 150 bp. Samples were incubated with 3 μg of antibody (AF4 antiserum or preimmune serum) or PBS diluent and provided magnetic beads and placed on a rotator at 4°C for 4 h. Cross-linking reversal was achieved as recommended and proteins were removed by proteinase K treatment. DNA was purified using the DNA extraction mini kit (Qiagen). Primer sets used for qPCR were designed to amplify DNA fragments of 50–150 bp from the 5' untranslated (5'UTR), coding, or 3'UTR regions of the *Igf-1* and *Trpc3* loci (sequences available upon request). The specific binding of the AF4 transcriptional complex to the *Igf-1* locus was calculated by subtracting the noise determined with the different negative controls (preimmune serum and PBS diluent) from the value of the signal obtained with the AF4 antiserum, and the enrichment was expressed as a percentage of input.

Analysis of serum IGF-1 protein levels. Blood was collected from postnatal day 21 (P21) mice and the serum fraction was prepared using standard procedures. Levels of IGF-1 in serum were measured by ELISA using a human IGF-1 kit (Ray Bio).

IGF-1 treatment of robotic mice. P7 robotic mice and littermate controls were injected daily subcutaneously for 4 weeks with recombinant human IGF-1 (GroPep) at 1 mg/kg or with the vehicle (0.1% DMSO aqueous solution) ($n \geq 7$ in each group).

Results

Af4 gene expression is tightly regulated during postnatal PC development

Using *in situ* hybridization, we have previously shown that expression of *Af4* in the mouse cerebellum is strictly restricted to PCs with reduced levels in lobe X (Isaacs et al., 2003). To establish an accurate expression profile of this gene during postnatal cerebellum development, quantitative RT-PCR (qRT-PCR) was performed from PCs obtained by LCM. Data showed that *Af4* expression peaks at P14 and quickly declines thereafter, reaching virtually undetectable levels by P56 (Fig. 1A). A more detailed analysis of distinct regions of the cerebellum revealed that expression is mainly confined to the anterior lobes I–V (Fig. 1B). Collectively, these data demonstrate a highly specific spatiotemporal expression of the *Af4* gene during postnatal development of PCs.

Accumulation of AF4 protein causes irreversible PC dysfunction in the robotic cerebellum

Strikingly, the distinctive pattern of PC loss observed in the robotic cerebellum, in which anterior lobes are most prominently affected (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), coincides with the normal expression

pattern of *Af4* described earlier (Fig. 1), implying that protein levels become toxic for PCs above a certain threshold. The fact that the first signs of PC degeneration are observed at P21 when *Af4* expression undergoes a dramatic downregulation further supports a model of proteotoxicity.

To determine whether the robotic mutation affects *Af4* gene expression levels in PCs during the degenerative process, transcript levels were measured by qRT-PCR from wild-type and robotic laser-microdissected PCs between P7 and P56. No significant difference, however, was observed in the pattern or levels of expression at any of the time points analyzed (Fig. 2).

We have previously demonstrated that the AF4 protein, however, accumulates in PCs of the robotic cerebellum as evidenced by immunohistochemical staining at 5 weeks of age (Bitoun et al., 2007; Bitoun and Davies, 2009). Unfortunately, the amount of protein available from up to 60,000 pooled laser-microdissected PCs was not sufficient to quantify the relative levels of AF4 by Western blotting. Therefore, to determine whether the onset of PC loss at 8 weeks of age is the result of sustained high toxic levels of AF4 protein, or simply the inevitable outcome of prolonged cell dysfunction initiated 5 weeks earlier, we performed a time course analysis of AF4 expression in the cerebellum by immunohistochemistry between P7 and P56 (Fig. 3). Presumably because of the post-translational regulation of AF4 by the proteasome (Oliver et al., 2004), peak expression of the wild-type protein was observed at P21, 1 week later than that of transcripts (Fig. 1). As expected, AF4 protein levels were markedly higher in robotic PCs compared with wild-type at P21 but these remained abnormally elevated up to P35 before declining back down to normal low values at P56. The fact that the accumulation of AF4 protein in robotic PCs coincides precisely with the onset of degeneration at P21 substantiates the hypothesis that toxic AF4 levels are the primary disease-causing mechanism. In addition, the fact that PC death proceeds at P56 despite the return of AF4 protein levels to normal low values indicates that, once initiated, cell dysfunction is irreversible. Collectively, these observations demonstrate the requirement of finely tuned levels of AF4 for the correct functioning and survival of mature PCs. However, in the robotic mouse, motor dysfunction precedes visible PC death by as long as 3 weeks, indicating that the deregulation of AF4 transcriptional activity primarily affects PC function, rather than cell survival per se.

Microarray analysis of robotic PCs at onset of degeneration reveals reduced *Igf-1* gene expression

To identify the deregulated genes and associated signaling pathways involved in PC dysfunction in the robotic cerebellum, we performed microarray analysis of robotic PCs obtained by LCM at P21 when signs of degeneration first appear (supplemental Fig. S2A, available at www.jneurosci.org as supplemental material). RNA samples obtained from ~2500 PCs collected for each of 4 wild-type and robotic littermates were compared for gene expression changes using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. Data were normalized using GCRMA with a cutoff p value of 0.05 ($p \leq 0.05$) and cutoff fold expression score of 1.5 (fold change in robotic PC ≥ 1.5). A total of 498 differentially expressed genes were identified between wild-type and robotic PCs, of which one-third (153) were downregulated (supple-

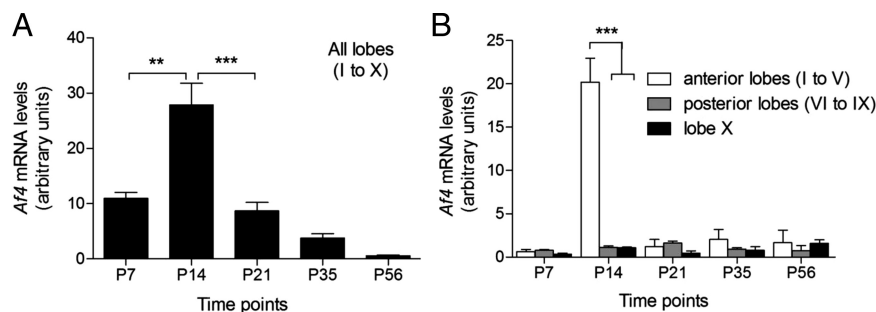


Figure 1. Spatiotemporal expression of the *Af4* gene in PCs during postnatal cerebellum development. **A**, **B**, *Af4* transcript levels were determined by qRT-PCR from PCs of wild-type mice obtained by LCM ($n = 3$). Analysis was performed from all 10 lobes (**A**), or from individual cerebellar regions (**B**). Results are shown \pm SD. The asterisks indicate significant differences between time point expression values (** $p < 0.01$, *** $p < 0.001$).

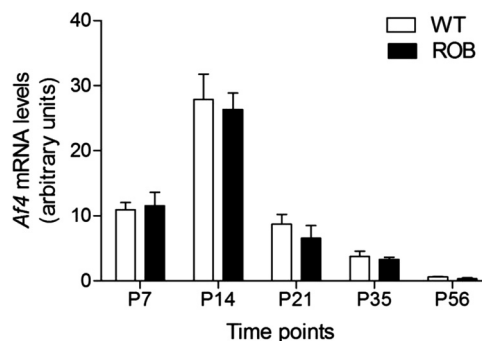


Figure 2. *Af4* gene expression is not altered in robotic PCs. *Af4* transcript levels were determined by qRT-PCR from PCs of wild-type (WT) and robotic (ROB) mice obtained by LCM ($n = 3$). Results are shown \pm SD.

mental Fig. S2B, available at www.jneurosci.org as supplemental material).

The gene list obtained was further examined based on a combination of expression change (≥ 2.0 -fold), PC-specific cerebellum expression pattern, known link to ataxia or neurodegeneration and/or relevance to PC function. This analysis generated a shortlist of 22 genes for which expression changes in robotic PCs were validated by qRT-PCR at P21 from independent laser-microdissected cell samples (data not shown), confirming that the microarray data were reproducible. One gene in particular not only fulfilled all the selection criteria mentioned above, but also bore functional relevance to the other non-neurological features of the robotic phenotype (growth retardation, T-cell development defects and cataracts). *Igf-1*, a well known and well documented promoting agent for PC development and survival (Torres-Aleman et al., 1992; Fukudome et al., 2003), was found to be downregulated ~2.7-fold in robotic PCs both by microarray and qRT-PCR at P21 (Fig. 4). In the cerebellum, *Igf-1* is specifically expressed in the soma of PCs with reduced levels in lobe X (Lee et al., 1992; Bondy et al., 1992; Cerebellar Development Transcriptome Database, <http://www.cdtb.brain.riken.jp>), bearing a striking resemblance to *Af4* expression pattern. Although no overt degenerative phenotype has been described in the cerebellum of the various engineered *Igf-1* knock-out (KO) mice (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993), deregulation of the IGF-1 signaling pathway was recently reported in PCs of mouse models of spinal cerebellar ataxia (SCA) types 1 and 7 (Gatchel et al., 2008).

To investigate whether misregulation of *Igf-1* gene expression occurred throughout the course of the degeneration process, we

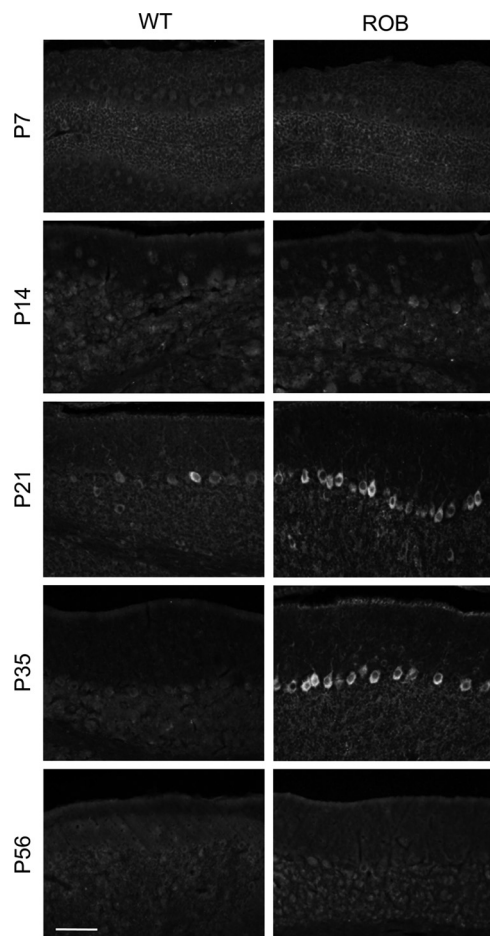


Figure 3. AF4 protein accumulates in robotic PCs. Immunostaining of AF4 in parasagittal cerebellum sections from wild-type (WT) and robotic (ROB) mice. To enable a direct comparison, pictures were taken using the same exposure time. The localization of AF4 to PCs was confirmed by double staining for calbindin (data not shown). Images show lobe III only and are representative of the results obtained for WT and ROB littermates from three independent litters. Scale bar, 100 μ m.

measured transcript levels by qRT-PCR from robotic PCs obtained by LCM during postnatal cerebellum development (Fig. 4). Levels of *Igf-1* expression were consistently reduced by 1.7- to 3.7-fold between P7 and P35 in robotic PCs compared with wild-type controls, with the most dramatic difference occurring at P14 when *Af4* gene expression peaks (Fig. 1). Interestingly, *Igf-1* gene expression was not found to be statistically different in robotic PCs at P56, a time point by which we observed a return of AF4 protein levels to normal values (Fig. 3). Importantly, the fact that a significant difference in *Igf-1* gene expression levels can already be detected in robotic PCs as early as P7, up to 2 weeks before the onset of degeneration, strongly implicates the deregulation of this trophic factor in the pathogenesis of PC dysfunction.

The data above suggest that AF4 mediates the repression of *Igf-1* gene transcription in PCs. To determine whether *Igf-1* is indeed a direct target of the transcription regulatory complex in which AF4 functions, ChIP analysis was performed from wild-type and robotic cerebellum extracts using an AF4 antiserum (Fig. 5). Binding was identified across the *Igf-1* locus but predominantly localized within the proximal transcribed region of the gene. Consistently, a similar distribution was previously reported across actively transcribed loci for the elongation factor P-TEFb and the histone methyltransferase DOT1 (Ni et al., 2004; Steger et

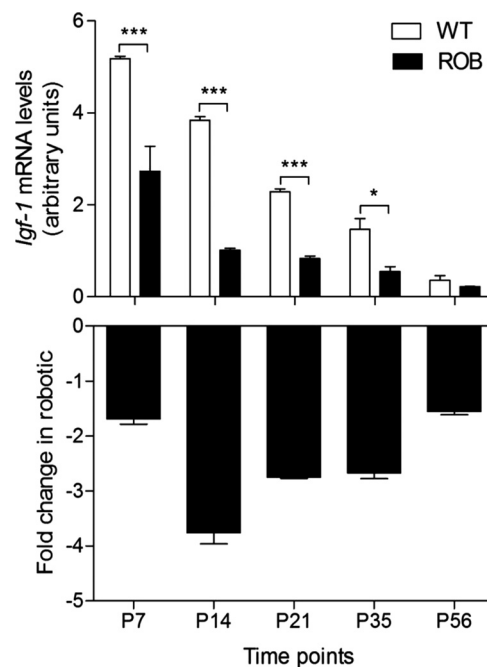


Figure 4. *Igf-1* gene expression is decreased in robotic PCs. *Igf-1* transcript levels were determined by qRT-PCR from PCs of wild-type (WT) and robotic (ROB) mice obtained by LCM ($n = 3$). Results are shown \pm SD. The asterisks indicate significant differences between WT and ROB expression values (* $p < 0.05$, *** $p < 0.001$).

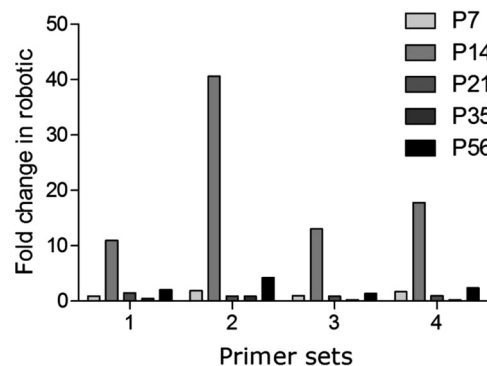


Figure 5. The AF4 transcriptional regulatory complex binds to the *Igf-1* locus in the cerebellum. Cerebellum from wild-type and robotic mice at the indicated age ($n = 3$ in each group) were subjected to ChIP analysis using the AF4 antiserum. The isolated chromatin was used as a template for qPCR with 4 primer sets designed within the 5' untranslated (1), coding (2 and 3), and 3' untranslated (4) regions of the *Igf-1* genomic sequence. Nonspecific binding was determined through the use of negative ChIP controls and was subtracted, as described in Materials and Methods. Results are expressed as a percentage of input, in fold change of wild-type in robotic.

al., 2008) to which AF4 is directly or indirectly associated within the transcription regulatory complex (Bitoun et al., 2007). Regulation of *Igf-1* expression was observed throughout postnatal development of the cerebellum, with a particularly high activity at P14 when *Af4* transcript levels peak (Fig. 1). Importantly, binding events were consistently more frequent in the robotic cerebellum, up to 40 times at P14, confirming a direct impact of AF4 levels on *Igf-1* gene transcription. In addition to the internal ChIP negative controls used (see Materials and Methods), the specificity of the AF4 antiserum was further verified through the lack of PCR amplification from the same chromatin samples of the PC-specific gene *Trpc3* (transient receptor potential channel 3), for which the

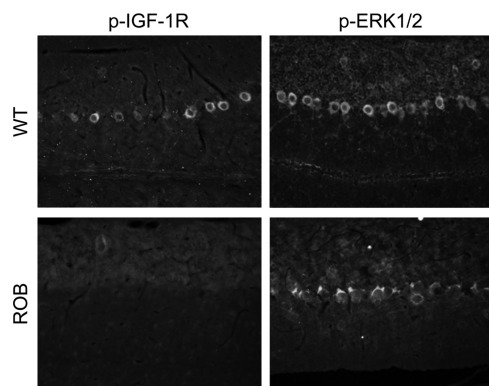


Figure 6. Activation of the IGF-1 signaling pathway is reduced in robotic PCs. Immunostaining of parasagittal cerebellum sections of P21 wild-type (WT) and robotic (ROB) mice for p-IGF-1R and p-ERK1/2. To enable a direct comparison between WT and ROB, pictures were taken using the same exposure time. Pictures show lobe III only and are representative of the results obtained for WT and ROB littermates from three independent litters. Scale bar, 100 μ m.

expression was found unchanged in our microarray data (data not shown).

The IGF-1 signaling pathway is deregulated in the robotic mouse

To provide further evidence of the functional implication of reduced levels of IGF-1 in the pathogenesis of the robotic cerebellum, we investigated whether the activation of known signaling molecules downstream of IGF-1 including type 1 IGF receptor (IGF-1R), and the extracellular signal-regulated kinases ERK1 and ERK2 (Torres-Aleman et al., 1998) was also affected in robotic PCs. Immunohistochemical staining of cerebellum sections indeed showed a marked decrease in the levels of phosphorylated IGF-1R (p-IGF-1R) and -ERK1/2 (p-ERK1/2) in robotic PCs at P21 (Fig. 6), confirming the deregulation of the IGF-1 signaling pathway in robotic PCs at the onset of cell degeneration.

IGF-1 activity is modulated by a family of high affinity IGF-binding proteins (IGFBP 1–6) (Shimasaki and Ling, 1991). Association with IGFBPs, in particular IGFBP-3 the most abundant, negatively regulates its bioavailability to IGF-1R (Ferry et al., 1999). In turn, IGF-1 can regulate *Igfbp-3* transcript levels *in vitro* (Bale and Conover, 1992). Interestingly, the spatiotemporal PC-specific expression of IGFBP-3 is very reminiscent of that of both AF4 and IGF-1 (Ye et al., 2003). Together with the concomitant decrease in the levels of IGF-1 and IGFBP-3 in the cerebellum of diabetic rats (Busiguina et al., 1996), these findings strongly suggest that IGF-1 also regulates *Igfbp-3* gene expression in PCs. Consistent with this postulate, we found that *Igfbp-3* transcript levels are downregulated ~ 2.3 -fold in robotic PCs at P21 both by microarray and qRT-PCR (Fig. 7). To provide further evidence of a direct correlation between *Igf-1* and *Igfbp-3* levels in robotic PCs, *Igfbp-3* mRNA levels were determined by qRT-PCR from cells obtained by LCM at earlier and later time points (Fig. 7). Robotic expression levels were indeed significantly reduced by over two- to three-fold between P7 and P21. Based on the observations above, we speculate that the reduction in IGF-1 levels in robotic PCs in turn leads to reduced *Igfbp-3* gene expression.

The IGF-1 signaling pathway is also a key regulator of general body growth, lymphoid development and lens differentiation, and its dysfunction in the relevant tissues could thus account for the non-neurological features of growth retardation, T-cell maturation defects and cataracts observed in the robotic mouse. IGF-1 is an anabolic factor essential for embryonic and postnatal

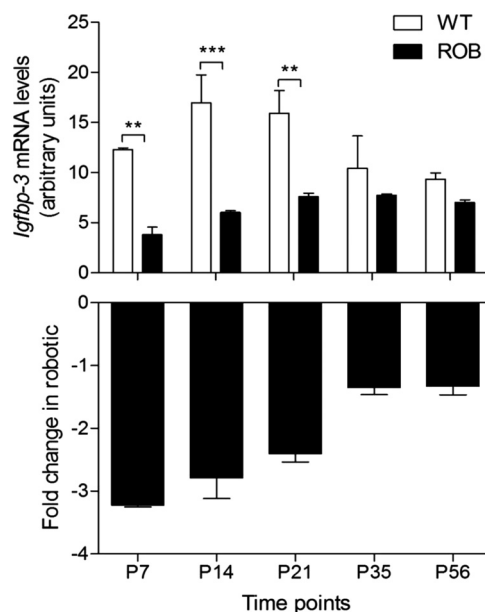


Figure 7. *Igfbp-3* gene expression is decreased in robotic PCs. *Igfbp-3* transcript levels were determined by qRT-PCR from PCs of wild-type (WT) and robotic (ROB) mice obtained by LCM ($n = 3$). Results are shown \pm SD. The asterisks indicate significant differences between WT and ROB expression values (** $p < 0.01$, *** $p < 0.001$).

growth and development, as illustrated by a severe growth deficiency in the various *Igf-1* null mice (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993). The robotic mouse shows a similar reduced size (60% of control) and, in agreement with the postulate above, we detected a marked 60% reduction in serum IGF-1 levels at P21 compared with wild-type littermates (control 118.31 ± 5.9 ng/ml; robotic 47.37 ± 17.2 ng/ml; $n = 3$ in each group). IGF-1 plays important regulatory roles in the early stages of both B- and T-cell development (Landreth et al., 1992; Welniak et al., 2002) as well as lens development (Walker et al., 2002; Ebong et al., 2004). In addition, a number of studies have reported deregulation of the IGF-1 signaling pathway in both cases of ALL (Blatt, 2000; Khatib et al., 2004) and cataract formation (Ruberte et al., 2004; Kubo et al., 2005). *Af4* is highly expressed in developing thymocytes (Chen et al., 1993; Baskaran et al., 1997), and at lower levels in the spleen and bone marrow (Isnard et al., 1998). Consistently, the *Af4* KO mouse shows altered lymphoid development including a severe reduction in the number of T-cell progenitor cells in the thymus, and pre-B and mature B-cells in the bone marrow (Isnard et al., 2000). While we also identified subtle defects in early T-cell maturation in the robotic mouse (Isaacs et al., 2003), we have not investigated whether B-cell development is also impaired in this mutant. We have previously demonstrated *Af4* gene expression in the lens (Isaacs et al., 2003) but, unlike the robotic mouse, the *Af4* KO does not develop cataracts or show any overt impairment in lens development (unpublished observation). As in PCs, *Af4* transcript levels were not affected in the thymus, bone marrow, or lens of robotic mutants (data not shown). To determine whether AF4 also regulates *Igf-1* gene expression in these tissues, we first examined the transcript levels in robotic and *Af4* KO mice by qRT-PCR; in the cerebellum, thymus, and bone marrow at P21, and in the lens at P70 when the relevant pathologies have previously been characterized (Isaacs et al., 2003). Data showed statistically significant differences in the levels of *Igf-1* expression in both robotic and *Af4* heterozygous (*Af4*^{+/+}) and homozygous

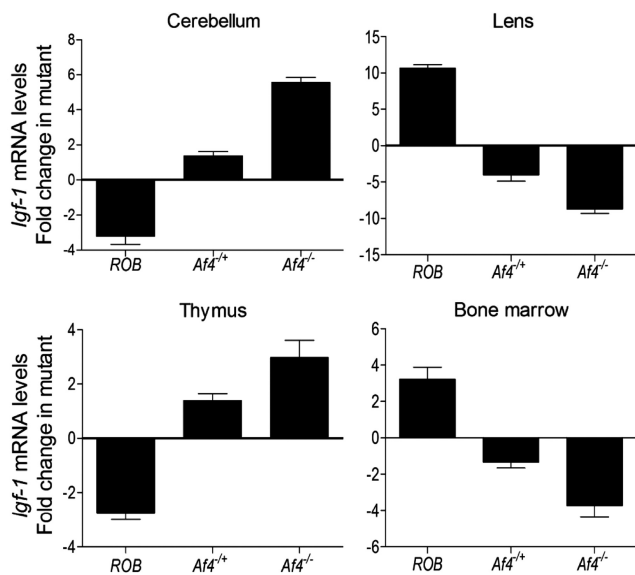


Figure 8. *Igf-1* gene expression is altered in neuronal and non-neuronal affected tissues of the robotic and *Af4* KO mice. *Igf-1* transcript levels were determined by qRT-PCR from tissues of robotic (ROB), and *Af4* heterozygous (*Af4*^{+/+}) and homozygous (*Af4*^{-/-}) KO mice ($n = 3$). Analysis was performed in the cerebellum, thymus, and bone marrow at P21, and in the lens at P70. Results are shown in fold change of wild-type in robotic or KO mutants \pm SD.

(*Af4*^{-/-}) KO mice tissues featured in the robotic phenotype compared with wild-type samples (Fig. 8). In contrast, no variation in the levels of *Igf-1* expression was observed in the small intestine of robotic mutants in which expression of *Af4* is not detected (data not shown). Importantly, *Igf-1* gene expression changes were in opposite directions in robotic and *Af4* KO mice thus strongly correlating with the levels of AF4 present in the various genotypes. To confirm that *Igf-1* is also a direct transcriptional target of the AF4 regulatory complex in the thymus, bone marrow and lens, ChIP assays were performed from these tissues as described earlier using P42 wild-type and robotic mice ($n = 3$ in each group). Binding to the proximal coding region of *Igf-1* (detected with primer set 2) was observed both in the thymus and bone marrow, with a six- and four-fold greater complex occupancy in robotic samples, respectively. However, we failed to demonstrate a similar regulation of *Igf-1* gene expression by the AF4 transcriptional complex in the lens due to the limiting amounts of starting material and the low levels of AF4 expression in this tissue (data not shown). These data confirm that altered *Igf-1* gene expression also occurs in the non-neuronal tissues affected as part of the robotic phenotype, strongly suggesting that deregulation of the IGF-1 signaling pathway is also responsible for the lymphoid developmental defects and cataract formation in the robotic mouse.

Treatment of the robotic mouse with IGF-1 delays the progression of PC death

To obtain *in vivo* evidence that reduced levels of IGF-1 and deficits in downstream signaling are indeed responsible for the onset of PC death in *robotic*, presymptomatic P7 mutant and control mice were injected daily with recombinant IGF-1 or vehicle for 4 weeks. The start and length of treatment were chosen based on the normal expression profile of *Igf-1* to encompass the time points when deregulation of transcript levels is observed in robotic mice (Fig. 4). In addition, previous reports have shown efficient delivery of IGF-1 to the mouse cerebellum using the subcutaneous route of administration used (Fernandez et al.,

1998; Nahm et al., 2003). Following treatment, motor coordination of IGF-1- and vehicle-injected groups was assessed using an accelerating rotarod and a static rod. At P42, no significant difference was observed in the performance of the two groups of robotic mutants (data not shown). Although ataxia develops from P28 to P35 in the robotic mouse and is clearly noticeable by visual observation, we have been unable in the past to obtain a statistical difference between wild-type and robotic mice before P70 using these tests (Oliver et al., 2007). Animals were therefore aged to P70 and locomotor activity analysis was repeated. Again, no significant difference was observed between IGF-1 and vehicle-treated robotic animals (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). It should be noted that at both P42 and P70, the ataxic gait of IGF-1-treated mice was visually indistinguishable from that of vehicle-treated robotic littermates, indicating that the delivery of exogenous IGF-1 to PCs at P7 is not sufficient to prevent PC dysfunction and its progression into ataxia from occurring. Following assessment of motor coordination, P70 mice were killed and the cerebellum was examined for signs of PC degeneration and death by immunohistochemical staining for calbindin (Fig. 9A). As expected, vehicle-treated robotic mice had suffered a small but significant loss of PCs in the anterior lobes, while surviving cells showed the typical signs of degeneration with swelling of bodies and dendrites, and thickening of axonal torpedoes. Remarkably, the cerebellum of IGF-1-treated mice showed little or no cell loss, with a marked reduction in the early signs of degeneration, indicating that administration of IGF-1 had effectively slowed down the degenerative process, in particular the progression of cell death. To ensure that the observed effect was mediated by the recombinant IGF-1 administered, we verified uptake by PCs and confirmed upregulation of the IGF-1 signaling pathway through increased levels of p-IGF-1R and p-ERK1/2 (Fig. 9B).

Discussion

Deregulation of the IGF-1 signaling pathway secondary to disease-specific mutations is a recurrent finding in mouse models and human patients with cerebellar ataxia, and thus represents a common pathological cascade in neuronal cell death that may be targeted for treatment (Fernandez et al., 2005; Gatchel et al., 2008). In this study, we report the identification of AF4 as the first negative transcriptional regulator of the IGF-1 pathway in the cerebellum. Using a targeted *in vivo* approach coupling LCM of PCs with gene arrays, we show that deficits in the activation of this pathway are involved in the early pathogenesis of PC degeneration that takes place in the cerebellum of the robotic mouse, an ataxic model in which we had previously identified a novel mutation in *Af4* that stabilizes protein levels in PCs (Oliver et al., 2004; Bitoun et al., 2007).

To dissect the role of AF4 in the pathogenesis of the robotic cerebellum, we first established its spatiotemporal expression during normal postnatal PC development. The pattern of *Af4* gene expression remarkably coincides with the regional susceptibility of the anterior lobes to degeneration, indicating that the accumulation of AF4 protein becomes toxic for PCs above a certain threshold. The lack of neurological impairment in the *Af4* KO mouse (Isnard et al., 2000) is also consistent with a toxic gain-of-function mechanism for the robotic mutation. In addition, the onset of degeneration at P21 correlates with the first detection of increased levels of robotic AF4 in PCs at this time point. Importantly, this finding argues against an active role of AF4 in PC development and rather suggests that this transcription cofactor is required for the maintenance of mature PC phys-

iology. Compartmentalized patterns of cerebellar expression and regional PC degeneration are often described in ataxic mutants (Herrup and Kuemerle, 1997; Sarna and Hawkes, 2003), but never have these two phenomenon been reported together. Therefore, the robotic mouse represents the first reported model of cerebellar ataxia in which the regional expression of the causative gene in the cerebellum determines the susceptibility of expressing cells to degeneration. *Af4* thus belongs to a critical pool of genes whose protein patterning defines subsets of PCs in discrete areas of the cerebellum that may have functionally distinct properties, potentially underlying synaptic circuit formation and refinement.

Attempts to delineate the degenerative pathways in other mouse models of cerebellar ataxia have typically used expression arrays from whole cerebellum for the PC degeneration (Ford et al., 2008), SCA1 (Goold et al., 2007) and SCA7 (Gatchel et al., 2008) mutants, or from SCA3 transgenic neuronal cell lines (Evert et al., 2003). However, due to the highly specific expression pattern of AF4, the only accurate way to identify genes potentially regulated by this protein, without the confounding effect of the remaining 98% of the cerebellum cell volume, was to use LCM in combination with expression arrays. Although surprising at first given the stimulatory role of the AF4 complex on transcription (Bitoun et al., 2007), this analysis revealed that AF4 functions both as a positive and negative regulator of gene transcription. Indeed, methylation of H3K79 by DOT1, although typically associated with transcriptionally active chromatin, can also be found in repressed genes (Zhang et al., 2006). In addition, AF5q31 which is the closest ALF family member to AF4 was previously shown to repress transcription of HIV-I (Niedzielski et al., 2007). Among the numerous differentially expressed genes in robotic PCs, *Igf-1* was a very strong candidate as the known downstream signaling cascades could account for both the neurological (PC degeneration) and non-neurological features of the robotic (growth retardation, impaired T-cell development and cataracts) and *Af4* KO (impaired B-cell development) mutants. We confirmed that *Igf-1* is a direct target of the AF4 transcriptional complex in PCs, thymus, and bone marrow, and presumably in the lens in which we also detected altered *Igf-1* expression levels. However, while the AF4 complex mediates repression of *Igf-1* transcription in the cerebellum and thymus, it seems to have an opposing effect in the bone marrow and lens. Further studies will be required to investigate the transcriptional regulatory roles of the AF4 complex on *Igf-1* gene expression specifically in non-neurological tissues.

IGF-I stimulates neurogenesis and synaptogenesis in many parts of the CNS (O'Kusky et al., 2000), and specifically promotes differentiation and survival of PCs in the cerebellum (Torres-Aleman et al., 1994; Fukudome et al., 2003). One prime finding of this study is that decreased *Igf-1* expression occurs not only throughout the PC degenerative process but also before its onset.

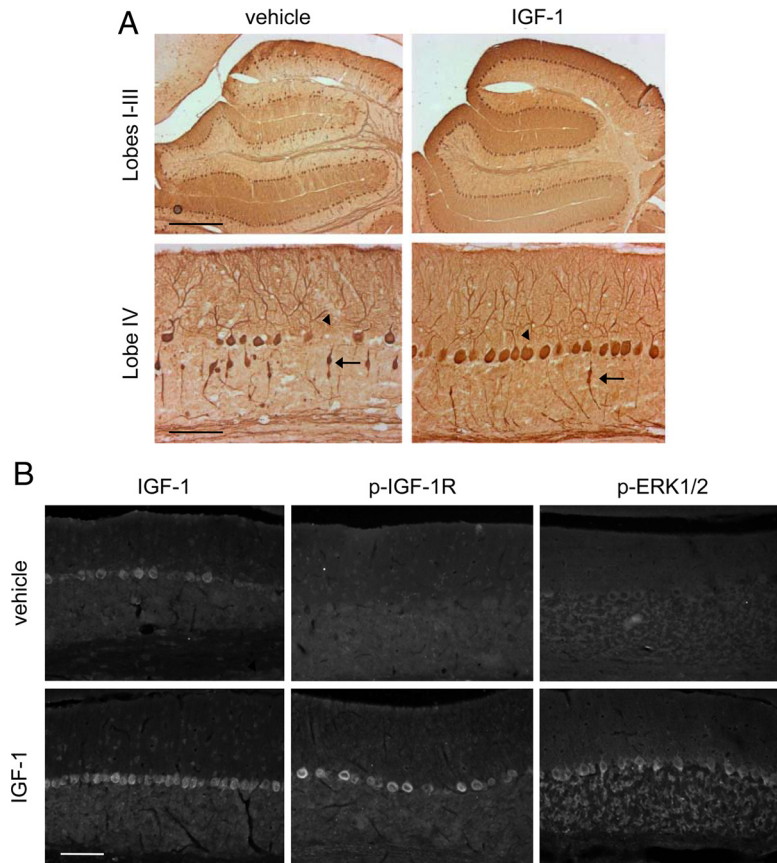


Figure 9. Treatment of robotic mice with IGF-1 slows down PC death. P7 robotic mice were injected daily for 4 weeks with IGF-1 at 1 mg/kg or the vehicle ($n \geq 7$ in each group). **A**, Analysis of PC degeneration in the cerebellum following IGF-1 treatment at P70. Parasagittal sections were immunostained for calbindin. Typical signs of PC degeneration including swelling of PC dendrites (arrow) and axonal torpedoes (arrowhead) can be observed. Scale bars, 1 mm (lobes I–III); 100 μ m (lobe IV). **B**, Analysis of IGF-1 pathway activation in the cerebellum following IGF-1 treatment at P70. Parasagittal sections were immunostained for IGF-1, p-IGF-1R, and p-ERK1/2. To enable a direct comparison, pictures were taken using the same exposure time. Scale bar, 100 μ m. In **A** and **B**, images are representative of the results obtained for three animals from each group.

Together with the reduced activation of signaling components downstream of IGF-1 in PCs at P21, these data strongly suggest that the deregulation of the IGF-1 pathway is an important primary causative event in the pathogenesis of the robotic cerebellum. Although treatment of P7 presymptomatic robotic mice with IGF-1 did not prevent PC degeneration and gait ataxia from developing, it markedly delayed the progression into PC death. Despite a partial protection, these data still provide strong *in vivo* evidence for the implication of the IGF-1 pathway in the pathological cascade that leads to PC death. Until this study, the mode of cell loss in the robotic cerebellum had remained unclear. We have previously excluded an apoptotic mechanism based on the lack of DNA fragmentation or activation of the proapoptotic key markers caspase-3 and Bcl-2 (Isaacs et al., 2003). The swelling of PC bodies and dendrites observed in the early stages of the degeneration process, however, tends to suggest autophagic or necrotic pathways. In agreement with these observations, decreased levels of IGF-1 have previously been associated with neuronal cell death by necrosis (Villalba et al., 1997). Recent studies have also shown that IGF-1 regulates autophagic vesicle turnover and that their accumulation leads to PC death (Bains et al., 2009). Ultimately, ultrastructural studies might help determine whether these two types of death take place in robotic PCs.

Although mutations in *AF4* have not been identified yet in ataxic patients, the IGF-1 deficiency degenerative pathway that

takes place in the robotic mouse model may prove directly relevant to one of the many forms of human cerebellar ataxia (Fernandez et al., 2005). Given the degree of sequence conservation within the ALF family (Bitoun and Davies, 2005), this study also potentially bears important implications for other disorders of the CNS such as mental retardation to which both LAF4 and FMR2 have been linked (Géczy et al., 1997; Steichen-Gersdorf et al., 2008). Increasing evidence indeed suggests that the IGF-1 pathway plays an important role in cognitive function, especially in learning and memory. Consistent with high expression of IGF-1R in the hippocampus, IGF-1 deficiency disorder is characterized by growth retardation, sensorineural deafness and mental retardation (van Nieuwpoort and Drent, 2008). Interestingly, cases of familial satellited chromosome 4q to which *AF4* localizes have been reported in the literature: two with mild mental retardation, one with growth retardation, and one with both cerebellar ataxia and mental retardation (Babu et al., 1987; Estabrooks et al., 1992; Faivre et al., 2000). Like AF4, MeCP2 which is mutated in Rett mental retardation syndrome is a dual transcriptional regulator that couples DNA methylation-mediated chromatin remodelling to transcriptional silencing and activation (Chahrouh et al., 2008). Strikingly, gene profiling recently identified *Af4* as one of seven genes upregulated by >2-fold in the cerebellum of the *MeCP2* KO Rett mouse model (Urduingio et al., 2008). In addition, ChIP assays established that *Af4* is a direct target of MeCP2 transcriptional repression in this tissue. This finding raises the possibility that the *MeCP2* KO mouse shares some features with the robotic mouse, in particular those relevant to the function of AF4 in the cerebellum such as ataxia. Consistent with this postulate, PC loss occurs in the cerebellum of Rett patients (Oldfors et al., 1990) and IGF-1 treatment of *MeCP2* mutant mice also partially rescues Rett syndrome symptoms including a marked improvement in locomotor activity (Tropea et al., 2009). Recently, a loss-of-function mutation in *AF9* was described in a patient with neuromotor development delay, cerebellar ataxia, mental retardation, severe delay in language acquisition and communication skills, and epileptic seizures (Pramparo et al., 2005). In the light of the functional interaction between the ALF proteins and AF9 (Bitoun et al., 2007), and their overlapping expression in the cerebellum and hippocampus (Vogel and Gruss, 2009), we predict that such mutation would lead to the continuous turnover of AF4 and FMR2 by the proteasome in these tissues, presumably resulting in a combination of features from both the robotic mouse and FRAXE patients in which *FMR2* is silenced (Mulley et al., 1995; Musumeci et al., 2001).

With the identification of AF4 as a negative regulator of *Igf-1* gene expression in the cerebellum, and possibly in other regions of the brain, this study opens new avenues of research into the manipulation of the IGF-1 signaling pathway for the treatment of a whole range of human degenerative diseases (Ebert et al., 2008; Palazzolo et al., 2009), in particular for cerebellar ataxia (Fernandez et al., 2005; Zhong et al., 2005; Vig et al., 2006).

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