A novel function of Ataxin-1 in the modulation of PP2A activity is dysregulated in the spinocerebellar ataxia type 1

Ivelisse Sánchez1,2,3,4, Patricia Piñol1,2, Marc Corral-Juan2,3, Massimo Pandolfo5, & Antoni Matilla-Dueñas2,3*

1Translational Neurosignaling group
2Basic, Translational, and Molecular Neurogenetics Unit in Neurosciences, Health Sciences Research Institute Germans Trias i Pujol (IGTP), Badalona, Barcelona, Spain
3Neuroscience Department, Universitat Autònoma de Barcelona, Spain.
4Laboratory of Systems Molecular Signalling and Chemical Biology Department of Anatomy and Neurobiology Boston University School of Medicine (BUMC) Boston, MA, USA 5Department of Neurology and Institute of Neuroscience Université Libre de Bruxelles, Belgium.

CORRESPONDING AUTHOR* Address correspondence to Dr. Antoni Matilla-Dueñas, Health Sciences Research Institute Germans Trias i Pujol (IGTP), Ctra. de Can Ruti, Camí de les Escoles s/n, Badalona, Barcelona, Spain, Telephone: +34 934 978 687, Fax: +34 934 978 654, E-mail: amatilla@igtp.cat; amatilla@btnunit.org.
ABSTRACT

An expansion of glutamines within the human ataxin-1 protein underlies spinocerebellar ataxia type 1 (SCA1), a dominantly inherited neurodegenerative disorder characterized by ataxia and loss of cerebellar Purkinje neurons. Although the mechanisms linking the mutation to the disease remain unclear, evidence indicates it involves a combination of both gain and loss of functions of ataxin-1. We previously showed that the mutant ataxin-1 interacts with Anp32a, a potent and selective PP2A inhibitor, suggesting a role of PP2A in SCA1. Herein we found a new function of ataxin-1 (Atxn1): the modulation of Pp2a activity and the regulation of its holoenzyme composition, with the polyglutamine-mutation within Atxn1 altering this function in the SCA1 mouse cerebellum before disease onset. We show that ataxin-1 enhances Pp2a-bβ expression and down-regulates Anp32a levels without affecting post-translational modifications of Pp2a catalytic subunit (Pp2a-c) known to regulate Pp2a activity. In contrast, mutant Atxn1 induces a decrease in Y307-phosphorylation in Pp2a-c, known to enhance its activity, while reducing Pp2a-b expression and inhibiting Anp32a levels. qRT-PCR and ChIP analyses show that ataxin-1-mediated regulations of the Pp2a-bβ subunit, specifically bβ2, and of Anp32a occur at the transcriptional level. The Pp2a pathway alterations were confirmed by identified phosphorylation changes of the known Pp2a-substrates, Erk2 and Gsk3β. Similarly, mutant ataxin-1-expressing SH-SY5Y cells exhibit abnormal neuritic morphology, decreased levels of both PP2A-Bβ and ANP32A, and PP2A pathway alterations, all of which are ameliorated by overexpressing ANP32A. Our results point to dysregulation of this newly-assigned function of ataxin-1 in SCA1 uncovering new potential targets for therapy.
INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited rare disorder caused by an expansion of glutamines within the ataxin-1 (ATXN1) protein (1). SCA1 is a fatal progressive neurodegenerative disease of late onset characterised by cerebellar ataxia accompanied by varying degrees of oculomotor deficits, pyramidal and extrapyramidal signs, and peripheral neuropathy (2). The primary neurodegeneration in SCA1 patients and animal models is observed in Purkinje cells (PC) and dentate nucleus neurons in the cerebellum, with pathological loss of cerebellar Purkinje cell dendrites, reduced dendritic trees, decreased formation of proximal spines, abnormal variable accumulation of neurofilaments, and early formation of axonal spheroids (3, 4). Although several molecular pathways affected in SCA1 are shared with other polyglutaminopathies, including other ataxias (5-7), the events responsible for the early pathogenic changes in SCA1 remain unclear. Transcriptional dysregulation has been proposed as a primary and early mechanism in SCA1 cerebellar pathogenesis. In fact, several important pathways implicating glutamate, drd2, notch, and wnt signalling have been shown transcriptionally dysregulated in the SCA1 mouse model (8-10). Recent data has shown that although SCA1 is mainly caused by a gain of function of mutant ataxin-1, dysregulation of normal ataxin-1 functions, particularly its role in transcription, modulate SCA1 pathogenesis (11). This could be explained by the fact that the presence of the expanded polyglutamine induces opposite effects in Atxn1 protein interactions. Whereas its interactions with Sp1 and Capicua are shown to diminish (9, 12), binding to RBM17 and ANP32A appear enhanced (13, 14). The mutation may then confer Atxn1 the ability to both activate and repress gene expression by altering the biological functions of the interacting proteins.

In addition to forming part of the INHAT complex by which it exerts repression of gene transcription, Anp32a, also known as Lanp/PHAPI/I1PP2A/pp32/mapmodulin (15), is a selective endogenous inhibitor of the protein phosphatase 2 (Pp2a) by binding its catalytic subunit (16). Anp32a is highly expressed in the brain regions predominantly affected in SCA1 including the motor cortex, brainstem, and cerebellum (15). Anp32a appears to mediate neurite formation by its INHAT function and actively translocates to the
cytoplasm during neuronal differentiation to mediate neurite growth through cytoskeleton regulation (15, 17, 18). We and others previously showed that ANP32A interacts with ATXN1, with stronger affinity towards the mutant form (14, 19). This led us to propose that alterations of ANP32A biological functions, including PP2A activity inhibition, could be dysregulated by the polyglutamine-expanded mutation within ATXN1 during the pathogenesis process in SCA1 (14). However, this hypothesis or the functional consequences of the interaction and the possible underlying mechanisms have not been explored. The heterotrimeric protein phosphatase 2 (PP2A) is an ubiquitous and conserved serine/threonine phosphatase with broad substrate specificity as it regulates a myriad of cellular functions including transcription, translation, cell cycle, signal transduction, metabolism, cell growth, and apoptosis (20). Among the direct and indirect targets of PP2A are proteins in oncogenic signalling cascades, such as Raf and MEK, and in other signalling pathways such as AKT, beta-catenin, ERK, and GSK3β. PP2A is highly regulated at the developmental, transcriptional, and post-translational levels through the availability of the different subunits (21, 22). The core enzyme consist of a catalytic (C) 36 kDa and scaffold structural (A) 65 kDa subunits which form a heterotrimeric holoenzyme upon binding to a regulatory B subunit. The scaffold (A) subunit (α or β), the catalytic (C) subunit isoforms (α or β), and the four regulatory (B) subunits families described to date, B (B55/PR55), B’ (B56/PR61), B” (PR72, PR130, PR59/PR48), B’” (PR93/SG2NA/PR110/striatin), with B and B’ being the most abundant regulatory subunits in the brain, each consist of 2-7 isoforms not including splice variants, alternate translation products, post-translational modifications, and other protein and chemical modifiers (20). All of these combine to yield a large number, at least 70, of highly diverse PP2A holoenzyme subtypes. The combination of the distinct catalytic-structural subunit isoforms dictate the regulatory subunit which will bind to form the holoenzyme and in turn the bound regulatory subunit appears to define the cellular and subcellular localizations of PP2A thereby narrowing its target specificity (23). Post-translational modifications such as the methylation of residue L309 and phosphorylation at Y307 in the catalytic subunit, regulate its binding to the regulatory subunits, with the binding to B subunits being selectively inhibited by increasing levels of phospho-Y307 and decreased L309 methylation (22, 24). The regulatory subunits Bβ, Bγ, Bα, B’β and
B’δ are highly expressed in the cerebellum, with Bβ being restricted to Purkinje cells (25). Fine regulation of the Bβ subunit appears critical for normal functioning of the cerebellar cortex, since a mutation consisting of an expanded CAG repeat in the Bβ promoter region underlies spinocerebellar ataxia type 12 (SCA12) characterized by cerebellar atrophy and Purkinje cell dysfunction (26). Although the effect of the SCA12 mutation on Bβ subunit expression levels in patients is not yet clear, in vitro data shows up-regulation of expression driven by the PPP2R2B promoter with expanded CAG repeats (27). Recent evidence shows that Bβ consists of 2 isoforms with both being found in the cytoplasm, and Bβ2 becoming recruited to the mitochondria during different stimuli including stress, to regulate mitochondria fusion/fission balance through dephosphorylation of Drp1 (28). Furthermore, regulatory subunits B and B’ have been shown to regulate Erk2 and GSK3β activity and to modulate neurite extension (29-33). Interestingly, the activity of the PP2A-B’d holoenzyme was found modulated by dopamine signalling through the PKA phosphorylation of B’δ (34). Although our work and that from other laboratories previously indicated that dopamine signalling, specifically through Drd2, is most likely altered in SCA1 (9, 35), it remains undetermined whether the PP2A-B’d is altered in SCA1. Our previous studies describing the interaction between ATXN1 and ANP32A suggested a possible involvement of PP2A in SCA1 (14). However, the effect of this interaction on the activation state of Pp2a and its mediated signalling pathways in the SCA1 mice have not been investigated. In addition, recent studies show that ATXN1, specifically the S776 phosphosite associated with ATXN1 toxicity, is a substrate of PP2A in the nucleus and that this phosphosite appears protected in the cytoplasm from dephosphorylation by the ATXN1 interaction with 14-3-3 protein (36-38). Taken together these studies suggested a role for PP2A in SCA1 pathogenesis.

Thus, here we sought to determine the effects of Atxn1 and its mutation on Pp2a activity in the SCA1 mouse cerebellum. Unexpectedly, we found that ataxin-1 modulates Pp2a activity by transcriptionally regulating the Pp2a holoenzyme components bβ2 and its endogenous inhibitor, Anp32a. In addition, we show that Atxn1, previously shown to activate the expression of Drd2 (9), regulates the activation
inducing-phosphorylation of b\(\delta\) at S566. Furthermore, we found that the polyglutamine-expanded mutation within Atxn1 modulates these regulatory functions. Pp2a pathway dysregulation was confirmed by identified phosphorylation changes of the known Pp2a-substrates Erk2 and Gsk3\(\beta\) in the SCA1 mouse cerebellum before disease onset. Similarly, mutant ATXN1-expressing SH-SY5Y cells exhibit abnormal neuritic morphology, decreased levels of both PP2A-B\(\beta\) and ANP32A, and PP2A pathway alterations which are shown ameliorated by overexpressing ANP32A. Our results point to dysregulation of this newly-assigned function of ataxin-1 in SCA1 and suggest new potential targets for therapy.
RESULTS

Ataxin-1 regulates Pp2a activity and the polyglutamine-expansion mutation enhances this function.

To determine whether Pp2a activity was regulated by Atxn1 expression, we immunoprecipitated Pp2a from cytosolic extracts of the cerebellum of 5 weeks-old Atxn1 KO and WT mice (Supplementary material, Fig. S1A,B). We found that the Pp2a specific activity was significantly decreased in the Atxn1 KO mice (30%) compared to WT mice (F (1,6) = 26.607, p = 0.002) (Fig. 1A). We then asked whether ATXN1 levels beyond those in wild-type mice could modulate Pp2a activity and measured the Pp2a activity in the cerebellum of 5 weeks-old ATXN1 overexpressing transgenic mice, A02. One-way ANOVA and the Tukey HSD post-hoc test revealed that the Pp2a specific activity was increased by 59% in the cerebellum of the wild-type ATXN1 overexpressing mice A02 compared to WT mice (F (2,11) = 17.794; Tukey HSD: WT vs A02, p = 0.027) (Fig. 1A). We then sought to determine the effect of the mutant ATXN1 by measuring the Pp2a activity from cytoplasmic lysates of the same age, 5 weeks-old SCA1 transgenic mouse model, B05 well before it presents the ataxic phenotype. Interestingly, we found that the Pp2a activity was substantially higher (238%) in the mutant ATXN1 overexpressing SCA1 mice B05 compared to wild-type mice, and significantly higher than in the wild-type ATXN1 overexpressing mice A02 (Tukey HSD: B05 vs WT, p <0.001; B05 vs A02, p = 0.032) (Fig. 1A). This dysregulation in Pp2a activity was also detected in the cerebellum of the B05 mice at 12 weeks of age when the mice are exhibiting the ataxic phenotype (F(2,8) = 13.757, p = 0.003; B05 vs WT, p = 0.003 and A02 vs B05, p = 0.006) (data not shown). To investigate the mechanism underlying the Atxn1-mediated regulation of Pp2a activity, we first examined the total catalytic subunit (Pp2a-c) levels and post-translational modifications of Pp2a-c known to regulate its activity. Pp2a-a and Pp2a-c or its relative levels of demethylation appeared unaffected in the Atxn1 KO mice (Pp2a-a: F(2,14) = 1.289, p = 0.306; Pp2a-c: F(1,6) = 1.396 , p = 0.282; demethylated Pp2a-c: F(1,6) = 2.523; p = 0.163) (Supplementary material, Fig. S1C and Fig. 1B). However, we found a significant decrease in the levels of the phosphotyrosine Y307 in Pp2a-c, known to restrict the regulatory subunit binding specificity of Pp2a-c, only in the B05 mice expressing mutant...
ATXN1 (WT vs KO: $F_{(1,6)} = 0.021, p = 0.890$; WT vs A02 vs B05: $F_{(2,10)} = 7.465, p = 0.010$, Tukey HSD: WT vs B05, $p = 0.011$, WT vs A02, $p = 0.629$, A02 vs B05, $p = 0.036$) ([Fig. 1B](#)). These data suggest that the increased Pp2a activity in the SCA1 B05 mice may, at least in part, be due to decreased levels of phospho-Y307 Pp2a-c since it allows a wider range of regulatory binding to the core Pp2a enzyme (23).

**Ataxin-1 enhances Pp2a-b and represses Anp32a expression in the mouse cerebellum**

We further assessed the levels of the regulatory subunits and found that in the cerebellum of mice lacking Atxn1 there was a significant decrease (30%) in Pp2a-b subunit protein compared to WT mice ($F_{(1,6)} = 9.629, p = 0.021$). On the other hand, Pp2a-b subunit protein levels in mice overexpressing ATXN1 (A02) increased (23%) while they remained unchanged in the mutant ATXN1 overexpressing mice (B05) relative to WT mice ($F_{(2,12)} = 6.699, p = 0.013$, Tukey HSD: WT vs A02, $p = 0.019$; WT vs B05, $p = 1.00$; A02 vs B05, $p = 0.023$) ([Fig. 1C,D](#)). Furthermore, of all three isoforms of Pp2a-b identified in the cerebellum ($\alpha$, $\beta$, and $\gamma$) only Pp2a-b$\beta$, known to be highly expressed in cerebellar purkinje cells (25), appeared regulated by Atxn1 as it was significantly altered in the Atxn1 KO mice ([Fig. 1E](#)) (Pp2a-b$\beta$: $F_{(1,6)} = 7.568, p = 0.033$; Pp2a-b$\gamma$: $F_{(1,6)} = 0.108, p = 0.754$; Pp2a-b$\alpha$: $F_{(1,6)} = 1.007, p = 0.354$). These data indicate that ataxin-1 regulates the Pp2a-b levels in the mouse cerebellum and that the polyglutamine-expanded mutation appears to interfere with this function of ataxin-1.

We then examined the possible role of the endogenous inhibitor of Pp2a, Anp32a, in the Atxn1-mediated modulation of Pp2a activity. We assessed the levels of Anp32a in the cerebellum of the Atxn1 KO and Atxn1 overexpressing transgenic mice. We detected approximately a three-fold increase in Anp32a levels in the Atxn1 KO compared to WT mice ($F_{(1,6)} = 8.262, p = 0.043$) ([Fig. 1F](#)). These data point to a role of Atxn1 in the regulation of Anp32a, specifically, it suggests that Atxn1 suppresses Anp32a expression. This was further supported by the finding that when wild-type (A02) or mutant (B05) ATXN1 was overexpressed in the cerebellum there was a 4- and 2-fold decrease in Anp32a levels, respectively ($F_{(2,12)} = 6.560, p = 0.011$; Tukey HSD: WT vs A02, $p = 0.012$; WT vs B05, $p = 0.042$) ([Fig. 1F](#)). Taken together these data suggest that while the expression of WT, but not the mutant ATXN1, induces Pp2a-b$\beta$, both
WT and mutant ATXN1 down-regulate Anp32a levels. Interestingly, analysis of the cerebellum of Atxn1 heterozygous (+/-) mice, which have one copy of the endogenous Atxn1 gene, shows WT levels of Anp32a (WT vs Atxn1 heterozygous: F(1,6) = 0.330, p = 0.587) (Supplementary material, Fig. S2). However, one copy of the Atxn1 gene does not appear sufficient to achieve the same levels as those in WT mice for Pp2a-bβ (WT vs Atxn1 heterozygous: F(1,6) = 7.367, p = 0.035) (Supplementary material, Fig. S2). We then asked whether transcriptional regulation underlies the Pp2a-bβ and Anp32a protein level changes. For these experiments we examined the Pp2a-bβ and Anp32a mRNA levels from the cerebellum of the WT and Atxn1 KO mice. qRT-PCR analysis indicates that Atxn1 regulates Pp2a-bβ, specifically isoform 2, and Anp32a protein levels in the 5-weeks-old mouse cerebellum at the transcriptional level (Fig. 2A) (Ppp2r2b2: F(1,6) = 44.739, p = 0.001; Anp32a: F(1,6) = 6.103, p = 0.040). These changes were not observed in either the striatum (Ppp2r2b2: F(1,10) = 3.297, p = 0.099; Anp32a: F(1,10) = 0.559, p = 0.472) or hippocampus (Ppp2r2b2: F(1,10) = 0.00, p = 0.996; Anp32a: F(1,8) = 0.495, p = 0.502) of the Atxn1 KO mice indicating this effect to be brain area specific (Supplementary material, Fig. S3). Moreover, ChIP analysis of preparations from the WT and Atxn1 KO mice revealed that Atxn1 occupies the Pp2a-bβ2 and Anp32a promoters in the mouse cerebellum at 5 weeks of age, further supporting a role for Atxn1 in the transcriptional regulation of Anp32a and Pp2a-bβ (Fig. 2B). We then went on to test whether overexpression of the wild-type and mutant ATXN1 in a human neuronal cell model was able to induce changes in PP2A-Bβ and ANP32a proteins levels mirroring the changes in A02 or B05 mice cerebella. Analysis of cellular lysates from retinoic acid differentiated SH-SY5Y cells transiently expressing ATXN1[30Q] or ATXN1[80Q] revealed changes in PP2A-Bβ and ANP32a protein levels similar to those observed in vivo (F(2,6) = 33.980, p = 0.001; F(2,6) = 4.716, p = 0.040) (Fig. 2C,D). The Tukey HSD test shows that overexpression of wild-type ATXN1 induces a significant increase in Pp2a-bβ (ATXN1[30Q] vs empty vector, p = 0.001; ATXN1[30Q] vs ATXN1[80Q], p = 0.003) and a slight decrease in ANP32A levels compared to the control cells transfected with empty vector (ATXN1[30Q] vs empty vector, p = 0.023). On the other hand, expression of ATXN1[80Q] induced a significant decrease in ANP32A levels both compared to the control cells and to the wild-type ATXN1
overexpressing cells (p = 0.015; p = 0.024) but was unable to induce Pp2a-bβ expression (ATXN1[80Q] vs WT, p = 0.173). These data also support a role of the mutation in the interference of the wild-type ATXN1 function in inducing Pp2a-bβ expression (Fig. 2C). Furthermore, as revealed by the ChIP analysis of the Atxn1 WT and KO mice, ATXN1 was also found to occupy the PPP2R2B and ANP32A promoters in the differentiated SH-SY5Y cell culture system (Fig. 2D). Interestingly, in both mice and the human cells, there appears to be proportionately less mutant ATXN1 bound to the promoter regions analysed, although there are equal levels of the WT and mutant ATXN1 in the nuclear fractions (F(1,4) = 0.668, p = 0.460) (Supplementary material, Fig. S4). Ongoing studies aim to understand the mechanisms underlying the differential transcriptional regulation from the Pp2a-bβ and Anp32a promoters by ataxin-1.

The Pp2a holoenzyme composition and signalling are altered in the cerebellum of SCA1 mice

To determine whether the identified Atxn1-mediated changes in Pp2a-b and Anp32a levels in the cerebellum were also reflected in the assembled Pp2a holoenzyme, we analysed Pp2a-a antibody immunoprecipitates from the WT, Atxn1 KO and the ATXN1 transgenic mice (Fig. 3A,B). We found that the levels of the Pp2a-b regulatory subunit (WT vs KO: F(1,6) = 11.081, p = 0.016; WT vs A02 vs B05: F(2,10) = 21.436, p < 0.001) and Anp32a (WT vs KO: F(1,6) = 9.613, p = 0.021; WT vs A02 vs B05: F(2,10) = 4.485, p = 0.041) in the Atxn1 KO and transgenic mice, respectively, mostly parallels levels detected in the cytoplasmic extracts (Fig. 1C and Fig. 3A,B). Specifically, the Pp2a holoenzyme in the cerebellum of the Atxn1 KO contained significantly lower levels of Pp2a-b (KO vs WT: p = 0.016) and higher levels of Anp32a compared to WT (KO vs WT: p = 0.021). In the ATXN1-overexpressing A02 mice, the Pp2a holoenzyme contained more bound Pp2a-b subunit than WT or the B05 mice (A02 vs WT: p = 0.008; A02 vs B05: p < 0.001) and less Anp32a than WT mice (A02 vs WT: p = 0.040). The Pp2a holoenzymes in mice overexpressing the mutant ATXN1 contained less Pp2a-b compared to WT or A02 mice (B05 vs WT: p = 0.033; B05 vs A02: p < 0.001) and less Anp32a compared to WT mice (B05 vs WT: p = 0.039). We then asked whether there were any changes in bδ subunit levels in the Pp2a holoenzymes, although
we detected no differences in any of the cytoplasmic extracts (WT vs KO: $F_{(1,6)} = 2.523, p = 0.163$; WT vs A02 vs B05: $F_{(2,13)} = 2.383, p = 0.131$) (Fig. 1C,D). Likewise, no differences were noted in Pp2a-b'δ levels in Pp2a-a immunoprecipitates (WT vs KO: $F_{(1,6)} = 0.056, p = 0.821$; WT vs. A02 vs B05: $F_{(1,6)} = 0.313, p = 0.596$) from any of the cerebellar samples we analysed (Fig. 3A,B). We then examined the proportion of the activating phosphosite Pp2a-b'δ (S566), known to be regulated by Drd2 signalling through PKA (34). Drd2 levels were previously reported decreased due to ataxin-1 loss of function in the Atxn1 KO and in B05 SCA1 mice, and increased in the A02 mice (9). In fact, we found that levels of phosphorylated Pp2a-b'δ are highest in the cytoplasmic extracts of the cerebellum of the KO and B05 (WT vs KO: $F_{(1,4)} = 12.578, p = 0.024$; WT vs A02 vs B05: $F_{(2,10)} = 8.046, p = 0.008$; Tukey HSD: WT vs B05 $p = 0.040$) and in Pp2a-a immunoprecipitates (WT vs KO: $F_{(1,6)} = 46.400, p = 0.001$; WT vs A02 vs B05: $F_{(2,10)} = 21.436, p < 0.001$; Tukey HSD: WT vs B05, $p = 0.033$; WT vs A02, $p = 0.008$) compared to their respective WT mice control (Fig. 3A,B and 3C,D). This evidence supports our previous hypothesis that PKA signalling is dysregulated, as a consequence of the altered Drd2 levels, in both the Atxn1 KO and SCA1 B05 mice (9). Furthermore, that the levels of Anp32a, the endogenous inhibitor of Pp2a which is bound to the Pp2a holoenzyme, are lower in mice overexpressing WT and mutant ATXN1 (A02 and B05, respectively), indicates that these have different subtypes of active Pp2a as defined by their bound regulatory subunits (Fig. 3A,B). Specifically, the data from these studies suggest that the Pp2a holoenzymes in the Atxn1 KO contain high levels of bound Anp32a, phospho-Pp2a-b’δ, and phospho-Y307 Pp2a-c, and low Pp2a-bβ, in mice overexpressing wild-type ATXN1 it is composed of low Anp32a and phospho-Pp2a-b’δ, and high Pp2a-bβ and phospho-Y307 Pp2a-c. However, in the mutant ATXN1 overexpressing SCA1 mice (B05), the Pp2a holoenzymes appear to have low levels of Anp32a, Pp2a-bβ phospho-Y307 Pp2a-c, and high levels of phospho-Pp2a-b’δ. Very interestingly, the Pp2a holoenzymes in the cerebellum of the Atxn1 KO and the ATXN1 mutant overexpressing mice show similar proportions of both Pp2a-bβ and phospho-Pp2a-b’δ but differ in levels of the endogenous inhibitor, Anp32a and the B-subunit inhibitory phosphosite Y307 on Pp2a-c. We have now confirmed these Pp2a regulatory subunit levels changes in SCA1 patient fibroblast samples (data not shown). Overall, these data indicate that
Atxn1 modulates the levels and the composition of the Pp2a holoenzyme and, importantly, point to an Atxn1 mutation-mediated dysregulation of the targets and downstream signalling pathways regulated by Pp2a.

Pp2a is a known negative regulator of Erk2, which is also regulated by Anp32a, and is also a positive regulator of GSK3\(\beta\) activity (29, 30, 39). Therefore, we confirmed the functional effects of the ataxin-1 mediated alterations in Pp2a activity and the modulation of its holoenzyme composition by identified changes in activating Erk phosphosites (Thr202/Tyr204) and in the activity inhibiting GSK3\(\beta\) serine-9 phosphorylation in the mouse cerebellum of the Atxn1 KO, A02, and B05 mice (Fig. 3C,D). Levels of phosphorylated Erk2 (pErk2) but not Erk1(pErk1) were higher in the cerebellum of the Atxn1 KO compared to WT mice which express Atxn1 (pErk2: \(F(1,6) = 10.849, p = 0.017\); pErk1: \(F(1,6) = 0.014, p = 0.911\)). On the other hand, mice overexpressing wild-type ATNX1 (A02) did not exhibit alterations on pErk2 levels compared to WT mice while the mutant ATXN1 expressing mice (B05) showed a slight but significant decrease (\(F(2,9) = 6.830, p = 0.016\); Tukey HSD: B05 vs WT \(p = 0.045\), A02 vs WT \(p = 0.826\), and B05 vs A02 = 0.018). We then analysed the levels of the phospho-ser9 in GSK3\(\beta\) (pGSK3\(\beta\)) and found it significantly increased (50%) in the Atxn1 KO mice cerebella which shows the lowests levels of Pp2a activity compared to wild-type (\(F(1,10) = 9.570, p = 0.011\)). However, no significant differences were noted in mice overexpressing wild-type ATXN1 (A02) or in ATXN1[30Q] expressing SH-SY5Y cells (WT vs A02 vs B05: \(F(2,12) = 4.088, p = 0.044\), Tukey HSD for A02 vs WT, \(p = 0.439\); ATXN1[30Q] vs ATXN1[80Q] vs empty vector: \(F(2,6) = 6.957, p = 0.010\), Tukey HSD for ATXN1[30Q] vs empty vector: \(p = 0.709\)). Interestingly as also seen for pErk2 levels, phospho-ser9 GSK3\(\beta\) levels were significantly decreased in the cerebellum of the B05 mice (Tukey HSD: B05 vs WT, \(p = 0.04\) and B05 vs A02, \(p = 0.010\)) and in the ATXN1[80Q] expressing SH-SY5Y cells (Tukey HSD: ATXN1[80Q] vs empty vector \(p = 0.007\)). These data show that both pErk2 and pGSK3\(\beta\) levels are increased in mice with lower than normal Pp2a activity as has been previously shown for pErk2 in the Pp2a activity deficient L199P transgenic mice (29). However, decreases in the phosphorylation levels of Erk2 and GSK3\(\beta\) resulting in Erk2 inactivation and GSK3\(\beta\) activation respectively, may be under a more stringent regulatory control. In
fact, we detect decreases in Erk2 and GSK3β activity-modulating phosphosites only in the B05 cerebellum, which shows a 238% increase in Pp2a activity compared to a 59% activity increase in the A02 over that in the WT mice. That levels of the GSK3β phosphorylated serine-9 are decreased in the B05 but not in the A02 mice cerebella indicates that this alteration is not caused by increments in the ataxin-1 transgene levels, but rather that it is a result of acquired properties of the mutant ataxin-1. Our results suggest that these acquired properties of the mutant ATXN1 may include its enhanced ability to suppress Anp32a expression, together with its inability to induce Pp2a-b expression, its induction of increased levels of active Pp2a-b’δ, and its ability to bind b-type regulatory subunits (lower phospho-Y307 Pp2a-c).

**ANP32A overexpression restores GSK3β ser9 phosphorylation and neurite morphology alterations induced by mutant ataxin-1**

We then tested whether expression of the endogenous PP2A inhibitor ANP32A in the SH-SY5Y expressing ATXN1[30Q] or ATXN1[80Q] neuronal cell lines would induce increased phospho-ser9 GSK3β. We found that overexpression of ANP32A in ATXN1[80Q] expressing SH-SY5Y cells restores phospho-ser9 GSK3β levels (empty vector vs ATXN1[30Q] vs ATXN1[80Q], each without and with ANP32A: F(2,12) = 12.547, p < 0.001; Games-Howell: ATXN1[80Q] vs ATXN1[80Q] with ANP32A p = 0.008; empty vector vs ATXN1[80Q] with ANP32A, p = 0.338) (Fig. 3C,E; Supplementary material, Fig. S5). This suggests that Anp32a is able to inhibit the Pp2a holoenzyme(s) involved in dephosphorylation of the ser9 phosphosite in GSK3β.

Because GSK3β has been previously shown to play a role in neurite extension and stability, and we found that expression of the mutant ATXN1 altered the levels of the activity-modulating phosphosite ser-9 in GSK3β, we investigated whether the expression of ATXN1[80Q] in differentiated SH-SY5Y cells had any effect on neurite morphology, specifically neurite length and presence of spines (Fig. 4, Supplementary material Fig. S6). Indeed, expression of ATXN1[80Q] resulted in a decreased number of neurites with spines (F(5,25) = 8.307, p < 0.001; Tukey HSD: ATXN1[80Q] vs empty vector, p < 0.001) and shorter overall neurite length (F(5,1338) = 15.810, p < 0.001; Tukey HSD: ATXN1[80Q] vs empty vector, p <
0.001; ATXN1[80Q] vs ATXN1[30Q], p < 0.001) (Fig. 4A-C). These morphological changes did not appear to be caused by cell death as no changes in cell soma size or pyknotic nuclei count were detected in any of the samples 48 hours after transfection (data not shown). An inverse relationship between Pp2a activity and Anp32a levels was noted in the cerebellum and in a retinoic acid differentiated human SH-SY5Y neuronal cell model of SCA1, and Anp32a co-expression restored GSK3β ser-9 phosphorylation levels. Therefore, we investigated whether increasing ANP32A levels in the ATXN1[80Q] expressing SH-SY5Y cells by co-transfection had any effect on the neurite morphology alterations (Fig. 4, Supplementary material Fig. S6). Remarkably, overexpression of ANP32A significantly ameliorated the neurite length changes (Tukey HSD: ATXN1[80Q] with ANP32A vs empty vector p = 0.205; ATXN1[80Q] vs ATXN1[80Q] with ANP32A, p = 0.033). The neuritic spine alterations detected in this SCA1 neuronal cell model were also ameliorated by co-expression with ANP32A (Tukey HSD: empty vector vs ATXN1[80Q] with ANP32A, p = 0.558; ATXN1[80Q] vs ATXN1[80Q] with ANP32A, p = 0.043 (Fig. 4A-C). That increasing the expression of the PP2A inhibitor ANP32A, induces an increase in levels of the activity inhibitory phosphosite GSK3β ser9, suggests that ANP32A regulates its dephosphorylation. Interestingly, both GSK3β and ANP32A have been shown to exert differential effects on neurite formation, extension and stability, as well as spine density (17, 18, 33). These data suggest that GSK3β activity is increased in the SCA1 mouse model (B05) but not in the WT ATXN1 overexpressing (A02) mice due to the mutant ATXN1-mediated modulation of Pp2a holoenzyme composition in addition to its effect on the expression of the inhibitor Anp32a. Furthermore, expression of Anp32a was sufficient to increase GSK3β phosphorylation levels at serine-9 which would then lower its activity. In agreement with our results, lithium, an inhibitor of GSK3β activity, was found to ameliorate SCA1 motor deficits (4).
DISCUSSION

The aim of this study was to determine whether ataxin-1, in addition of being a substrate for Pp2a in the nucleus as has been recently reported (38), also has a regulatory role on Pp2a activity and whether the expanded polyglutamine within ataxin-1 modulates these effects. Based on the strong inhibition exerted by Anp32a on Pp2a activity and our previous evidence showing that mutant ataxin-1 containing the polyglutamine-expansion interacted strongly with Anp32a/Lanp in cells (14, 16, 40), we hypothesized that mutant ataxin-1 may exert a regulatory effect on Pp2a activity in the mouse cerebellum. Indeed this was the case since we found that ataxin-1 has a significant effect on Pp2a activity in the mouse cerebellum, however it had a more significant and broader effect on Pp2a than we anticipated. Here we also found that Atxn1 regulates the expression of at least two important modulators of Pp2a substrate specificity and activity: the β regulatory subunit and the endogenous Pp2a inhibitor, Anp32a also known as Lanp/L1PP2A/PHAPI/pp32/mapmodulin. We show that the Pp2a in the cerebellum from mice lacking Atxn1 has significantly less activity than in wild-type mice, while the Pp2a from mice overexpressing wild-type ATXN1 (A02) is more active. In the SCA1 B05 mice expressing the polyglutamine-expanded mutant form of human ataxin-1, Pp2a activity is induced by almost three fold. Therefore, the polyglutamine mutation appears to enhance the ataxin-1-mediated positive regulation of Pp2a activity. Analysis of the components of the Pp2a holoenzyme revealed that the Pp2a catalytic subunit shows lower levels of phosphorylated Y307 only in the SCA1 mice expressing mutant ATXN1. It has been previously shown that Y307 phosphorylation inhibits the Pp2a-b regulatory subunits binding to the catalytic-scaffold dimer (24) so that the decreased phosphorylation of the Pp2a-c in the SCA1 mice may allow for a wider range of regulatory subunit binding to the core enzyme dimer thereby contributing to the enhanced Pp2a activation detected. Src-family kinases are known to phosphorylate the Y307 site in the Pp2a-c, however the nature of modulators of these kinases in the cerebellum is not clear (22). Interestingly, VEGF an angiogenic factor with neurotrophic effects, which has been implicated and found to ameliorate pathogenesis in SCA1, activates src-family kinases and thus could also have inhibitory effects on Pp2a
through Y307 phosphorylation thereby potentially normalizing the Pp2a activity levels in the SCA1 mice (41, 42). Furthermore, the analysis of the Atxn1-mediated changes in the Pp2a holoenzyme components also revealed that Atxn1 regulates the expression of the Pp2a-bβ2 regulatory subunit and its endogenous inhibitor, Anp32a. While Atxn1 induces the expression of bβ2 it down-regulates the expression of Anp32a in the cerebellum. However, the mutation within Atxn1 appears to enhance the Anp32a down-regulating effect since the SCA1 mouse (B05) has 30 copies of the transgene while the A02 mouse is known to have at least 3 times more (43) and the effects on Anp32a levels are not significantly different in both strains. In contrast, the mutant Atxn1 appears to loose its ability to induce the expression of Pp2a-bβ2. These effects were also seen in the cell culture model where the same amount of plasmid was transfected for both wild-type and mutant ATXN1. Likewise, this resembles the effects we previously observed with the ataxin-1 mutation on the Drd2 gene promoter (9).

Anp32a, albeit widely distributed in the brain, it is highly expressed in brain regions affected in SCA1 such as the cerebellum, motor cortex, and the brainstem (15). It is known to selectively inhibit Pp2a activity by binding the catalytic subunit (16). Therefore increased levels of Anp32a and its binding to Pp2a-c in the Atxn1 KO mice are expected to induce decreased Pp2a activity while the opposite is true in the cerebellum of the A02 and the SCA1 B05 mice where Anp32a levels are lower than in wild-type mice. The Pp2a regulatory subunit bβ2 has been shown to be highly and selectively expressed in purkinje cells in the cerebellum (25). It is expressed as two isoforms bβ1 and bβ2, both being present in the cytoplasm and the bβ2 being able to translocate to the mitochondria where it plays a role in its fusion/fission cycles. It is noteworthy to mention that another spinocerebellar ataxia, SCA12, which like SCA1 is characterized by loss of cerebellar purkinje cells, is caused by a CAG expansion within the promoter of the Bβ1 gene and in vitro studies suggest that this mutation could induce up-regulation of Bβ1 expression (27). However, the specific effect of the mutation in SCA12 and how it may affect the expression of the other isoform, bβ2, is unknown. Here we found that the Atxn1-induced changes in bβ2 and Anp32a are at the transcriptional level as demonstrated by changes in their respective mRNAs mirroring the protein levels.
Interestingly, our data shows that Atxn1 occupies both the Anp32a and bβ2 promoters leading us to propose that Atxn1 regulates *Anp32a* as part of an inhibitory transcription complex and in contrast it is also able to bind to an activating transcriptional complex regulating the *Ppp2r2b2* and other genes including *Drd2* as we have previously shown (9).

In addition to transcriptional regulation, analysis of the Pp2a holoenzyme also revealed that another Pp2a regulatory subunit, b'δ is post-translationally regulated in both the Atxn1 KO and SCA1 mice suggesting it may be due to the loss of function of mutant Atxn1 on Drd2 expression (9, 35). We previously showed that Atxn1 activates the expression of Drd2 and that the mutation within Atxn1 compromises this function (9). The b'δ subunit is phosphorylated by PKA and this phosphorylation has been shown to increase the PP2a-b'δ holoenzyme activity (34). PKA activity is known to be suppressed during Drd2 mediated signalling, thus the decrease in Drd2 in the cerebellum in both the Atxn1 KO and SCA1 mice may dysregulate PKA activation (35). We propose that this increase in PKA activity may thus result in the increased levels of the phosphorylated b'δ protein in both, the Atxn1 KO and SCA1 B05 mice. Overall, our results point to an alteration not only in the levels of Pp2a activity in the cytoplasmic fractions from the SCA1 B05 mice but also in the composition of the holoenzyme, with less inhibitory regulation by Anp32a, and less bβ2 but more active b'δ regulatory subunit bound holoenzyme. In addition, there are proportionately more b-type regulatory subunit containing Pp2a holoenzymes since there are lower levels of the b-type subunit binding inhibitory phosphosite Y307 on the catalytic subunit in the SCA1 mice (B05). All of these account for the higher overall Pp2a activity in the SCA1 with distinct Pp2a holoenzyme specificity than the A02 or WT mice. The proposed model and summary of the findings is shown in Fig. 5.

Alterations in the Pp2a regulated pathways, specifically Erk2 and Gsk3β were identified by quantification of phosphosite levels known to modulate their respective activation state. Gsk3β phosphorylation in ser9, indicative of inactive enzymatic activity, is found increased in the cerebellum of the Atxn1 KO mice while the mutant ATXN1 over-expressing mice and cells show lower phospho-ser9 in GSK3β suggesting its
enhanced activation. Likewise at the Thr202/Tyr204, indicative of activation, is increased in fractions from the cerebellum of Atxn1 KO mice and slightly decreased in the SCA1 mice (B05). That the Erk2 signaling pathway is regulated by Pp2a has been demonstrated in the Pp2a-α dominant negative (L199P) Pp2a transgenic mice, a Pp2a-b’β and Pp2a-b knockdown cell culture and drosophila genetic model (29, 39, 44, 45). In contrast, the Pp2a-b’δ null mouse shows alterations on the Gsk3β but not in Erk2 activation (30). Interestingly, in the Pp2a-b’δ null mice the Gsk3β activation was found altered in the cortex and other brain regions but not in the cerebellum (30), suggesting some functional redundancy in the cerebellum. Therefore, the activation of the Pp2a-b’δ holoenzyme appears to be tightly regulated, thus pointing to an important role in the mouse cerebellum.

The PP2A B’δ and B’β have been shown to regulate the TrkA-mediated neurite growth (32, 46). Furthermore, GSK3β has been shown to mediate the trkA-induced neurite growth through MAP1B phosphorylation (33). Interestingly, while Anp32a appears to inhibit neurite formation in NGF treated PC12 cells (17), it was shown to promote MAP1B-induced neurite extension in differentiated mouse neuronal cell line neuro2a (18). These seemingly contradictory results have been previously described as GSK3β appears to have distinct roles in neurite formation, extension, and stabilization, with high GSK3β activity favouring neurite formation whereas low activity favouring neurite extension or stability in differentiated neuronal cells (33, 47). Interestingly, both the wild-type (A02) and mutant ATXN1 mice overexpressing mice (B05) had low Anp32a levels, but only the B05 mice showed reduced phospho-GSK3β or phospho-Erk2 levels. This suggests that Anp32a levels are not sufficient to account for the alteration and consequent activation of GSK3β and decreased Erk2 activity. The same was observed in the SH-SY5Y SCA1 cell model. These data then highlight the role for the alterations in the regulatory subunit specific Pp2a holoenzymes by the mutant ataxin-1 and suggests that the specific active Pp2a holoenzyme(s) involved in dephosphorylation of ser9 in GSK3β is enriched in the mutant ataxin-1 expressing mice or cell model. Therefore, we reasoned that because Anp32a inhibits Pp2a by binding to the catalytic subunit and Anp32a is down-regulated in the cerebellum of the SCA1 mice and in mutant
ATXN1 expressing differentiated SH-SY5Y cells, restoring Anp32a levels may increase GSK3β phosphorylation levels thus decreasing its activity and preventing the abnormal neuritic morphology induced by the expression of the mutant ATXN1. Indeed, this was the case, we found that the neurite length shortening and spine loss were ameliorated in ATXN1[80Q] expressing SH-SY5Y cells co-transfected with Anp32a. That co-expression of Anp32a was able to increase GSK3β phosphorylation levels in both the WT and mutant ATXN1 expressing SH-SY5Y cells shows that there appears to be no alterations in the GSK3β phosphorylating kinase in either, but rather that the mutant ATXN1 has an effect on the GSK3β dephosphorylation. Therefore, taken together these studies point to different potential targets of therapeutic intervention related to the altered Pp2a activity in SCA1, namely the Y307 Pp2a-c phosphorylation, phospho-Pp2a-bδ and the Pp2a-bβ2 holoenzyme levels, and the increased GSK3β activity. The latter, has been previously explored since lithium was found to ameliorate to some degree the motor deficits in the SCA1 B05 mouse model (4). We propose that using a more specific compound inhibitor of GSK3β or modulators of the other targets mentioned above may serve to restore some of the signaling pathways and functionality altered in purkinje neurons of SCA1 patients. Interestingly, dysregulation of PP2A and GSK3β have also been found in other neurodegenerative disorders such as Alzheimer's disease and some genetic linkage data has suggested ATXN1 as a risk factor in some patients (52).

In conclusion, this study assigns a new biological function onto ataxin-1: the regulation of Pp2a activity and its components, specifically the regulatory subunit Pp2r2b isoform 2 (bβ2) and the Pp2a endogenous inhibitor Anp32a, both of which are highly enriched in cerebellar purkinje cells. In addition, ataxin-1 was found to regulate the inhibiting Y307 phosphosite in Pp2a-c and levels of the activating S566 phosphosite in the Pp2a regulatory subunit Pp2a-bδ. We demonstrate that these functions of Atxn1 are dysregulated in the polyglutamine-expanded mutant ataxin-1 in SCA1 neuronal cell lines and SCA1 transgenic mice. Our results point to the dysregulation of this newly assigned function of ataxin-1 in the cerebellar neurodegeneration of SCA1 and uncovers new possible targets for therapy.
MATERIALS AND METHODS

Mice. Five weeks-old inbred Atxn1-null (Scal KO°) and transgenic mice carrying the mutant SCA1 allele with 82 CAG repeats (B05) or an interrupted wild-type SCA1 allele with 30 repeats (A02) driving the expression in cerebellar purkinje cells of mutant or wild-type Ataxin-1 containing 82 or 30 glutamines respectively were previously described (43, 48). The SCA1 mice and respective controls (B05, A02, and FVB/NJ WT) were provided by Dr. Massimo Pandolfo (Institute of Neuroscience, Université Libre de Bruxelles, Belgium). All animal procedures were carried out in accordance with EU and local regulations and approved by the appropriate Ethical Committees.

Protein sample preparations and immunoblotting. Proteins from mouse cerebella or cultured cells were extracted by homogenization in hypotonic lysis buffer: 10 mM Heps, 10 mM KCl, 2 mM EGTA, 320 mM sucrose, 5 mM MgCl₂, 1 mM DTT, 25 mM NaF, 2.5 mM NaVO₃ and protein inhibitor cocktail (Roche). Hypotonic lysis fractions were generated by differential centrifugation at 4 ºC. Briefly, cerebellar tissue or cell pellets were dounced homogenized with hypotonic buffer (35 mg of tissue/ml of buffer) and incubated on ice for 15 min. After centrifugation at 500 g for 10 min to yield S1 and P1 fractions the supernatant was centrifuged again to remove residual nuclei. The remaining S1 fraction was centrifuged at 900 g 10 min to yield the S2 fraction used as cytoplasmic fractions in these studies. Residual membranes were extracted from the P1 fraction with 0.1% triton in hypotonic buffer after centrifugation at 500 g for 10 min to yield the nuclear pellet. We extracted the nuclei with 1% triton in hypotonic buffer, 10 min to obtain the nuclear triton soluble (NS) and insoluble fractions (N). Protein samples were mixed with 2X SDS-sample buffer (625 mm Tris pH 6.8, 10% SDS, 50% glycerol, 1 mM DTT and bromophenol blue) and separated by electrophoresis before transfer to PVDF membranes (Life technologies). Primary antibodies used were antibodies to β-actin (AC15, Sigma), Flag M2 (A-1978, Sigma-Aldrich), VDAC1 (28-243, ProSci), ERKs and phosphorylated ERKs (4695 and 9106, Cell Signaling technology), total GSK3β and phospho-GSK3β ser9 (9832 and 5558, Cell Signaling technology), PP2A-C (2038, Cell Signaling technology), PP2A-C mAb ID6, demethylated PP2A-C, PP2A-A, PP2A-B, PP2A-B’ (05-421,
21

05-577, 05-657, 05-592, and 07-334, Merck-Millipore), PP2A-C phospho-Y307 (1155-1, Epitomics), Atxn1 (N78/8, N78/3, NeuroMab), anti-ANP32A mAb DC63 (49) (generous gift from Dr. Michal Novak, Institute of Neuroimmunology, Slovak Republic) and anti-Phapi (3145P, ProSci), PP2A-B alpha, beta, gamma (generous gift from Dr. Brian Wadzinski, Vanderbilt University Medical Center, TN, USA), PP2A-B56 delta and p-S566-PP2A-B56 delta (generous gift from Dr. Angus Nairns, Yale School of Medicine and Dr. Paul Greengard, Rockefeller University, USA), and phospho-ATXN1 S776 (generously provided by Dr. Harry Orr, Institute of Human Genetics and University of Minnesota, USA). Infrared-dye conjugated secondary antibodies (Li-Cor Biosciences) were detected using the Odyssey analyzer (Li-Cor Biosciences). Immunoblot images were quantified using the software v2-1 (Li-Cor Biosciences). Statistical analyses were performed using the SSPS statistical software with significance set at p < 0.05.

**Immunoprecipitations.** Hypotonic lysates were brought to 0.1% triton and fresh protease inhibitors were added (Roche) prior to clearing with 50 μls of magnetic protein A/G beads (LSKMAGA02 and LSKMAGG08, Merck-Millipore). The protein was incubated overnight with 4 μl of antibody, PP2A-C mouse mAb ID6 (05-421, Merck-Millipore, rabbit polyclonal PP2A-C or PP2A-A antibodies (2038, Cell Signaling technology and 05-577, Merck-Millipore) prior to incubation with protein A/G magnetic beads and the immunoprecipitates analysed by immunoblotting. Sixty micrograms of protein were used for Pp2a activity assays and 250 μg for analytical immunoprecipitations.

**PP2A activity assays.** We used a phosphatase activity assay for measuring the PP2A-activity in tissue extracts. The procedures of sample preparation, immunoprecipitation, and substrate incubation were performed according to the manufacturer’s protocol (17-313, Merck-Millipore). Briefly, Pp2a-c was immunoprecipitated from 60 μgs of pre-cleared protein extract with ID6 Pp2a-c antibody and the precipitate was incubated with a synthetic phosphorylated peptide for 10 min. The colour development of liberated phosphate was performed in 96-well plastic-Falcon plates and the absorbance (630 nm) was measured using the Varioskan plate reader (Thermo Scientific). Linearity of the test was confirmed in a range of 40-100 μg protein used for the initial immunoprecipitation. Liberated phosphate was read off from a standard curve (0–1,000 pmoles phosphate in 125 μl) and specific activity was expressed as:
liberated phosphate in pmoles/ relative levels of Pp2a-c.

**Cell culture, transfections, immunofluorescence, and microscopy.** Four micrograms of flag tagged ATXN1[30Q] or ATXN1[80Q] plasmid DNAs (generous gift from Dr. Huda Zoghbi, Department of Molecular and Human Genetics, Baylor College of Medicine, TX, USA) were co-transfected with 4 μgs of Anp32a (17) or empty vector plasmid in addition to EGFP into SH-SY5Y cells grown in DMEM medium containing 10% FBS (Sigma), 2 mM glutamine, 50 μg/ml Penicillin/Streptomycin (Life technologies) at 70% confluence in 10 cm culture dishes using Lipofectamine 2000 (Life technologies) and OptiMEM medium (Life technologies) according to the manufacturer’s instructions. Four hours after transfection, the culture medium was replaced with SH-SY5Y culture medium B: Neurobasal, B27 supplement, 10 μM retinoic acid, 2 mM glutamine, 50 μg/ml Penicillin/Streptomycin. Forty-eight hours after medium change cells were harvested for protein preparations or fixed with 2% paraformaldehyde for morphology assessment or immunofluorescence. Transfection efficiency was around 60%. For immunofluorescence fixed transfected cells were permeabilized with 0.1% triton in PBS for 1 hour and following washing and blocking with 5% goat serum in PBS for 30 min cells were incubated with antibody to phospho-GSK3β (ser9) in PBS with 1% goat serum. Immunoreactivity was detected with texas red antibody to mouse IgG and visualized using a Zeiss AxioObserver Z1 inverted Microscope (Zeiss).

**Neuritic morphology assesment.** Neurite length (2 or more soma sizes) and neurites with spines in at least half their length were analysed using the Axiovision software (Zeiss). EGFP positive cells were analysed in 4-6 random areas within 4 wells (200-550 cells) for each transfected sample. Experiments were done in duplicate with similar results.

**Quantitative RT–PCR.** qRT-PCR was performed to test levels of gene expression in 4-7 WT and 4-7 ataxin-1 KO mice cerebellum, hippocampus, and striatum samples. We performed a single step reverse transcriptase PCR reaction (Qiagen) with 7900 HT fast real-time PCR system (Applied Biosystems), continuous fluorescence detection and SYBR green fluorescence reagents as specified by the
manufacturer in a final reaction volume of 20 µl. Primer sequences were designed for each target gene containing minimal internal structures (i.e. hairpins and primer dimers) and optimal melting temperatures with each Tm within 1 °C of the other using PrimerEXpress (Applied Biosystems) to target splice sites within the RNA sequence. The primer sequences are described in Supplementary Table 1. Samples were compared using the relative comparative CT method (50). Each primer was used at a final concentration of 0.1 mM and the qRT-PCR conditions are as follows: 95 °C for 30 sec, and 40 cycles of 95 °C for 5 sec, 62 °C for 20 sec, 72 °C for 15 seconds.

**Chromatin immunoprecipitation (ChIP).** SH-SY5Y cells were cultured in 100 cm plates as mentioned above and transfected with 4 µg of Flag tagged ATXN1[30Q], ATXN1[80Q] or empty vector as described earlier (9). Forty-eight hours after transfection, cells were fixed with 1% paraformaldehyde and further processed for ChIP analysis. Immunoprecipitations were carried out using 4 µg of antibody to flag (M2, Sigma) or control serum. Input and immunoprecipitated DNAs were subjected to PCR amplification with the primers listed in Supplementary Table 2. For *in vivo* ChIP assays, whole cerebella from 5-weeks-old WT and KO mice were dissected out and cut into 50 mg pieces. Cerebellar tissues were washed with ice-cold PBS containing protease inhibitors (PBS/Pi), resuspended in PBS/Pi containing 1% formaldehyde, incubated at room temperature for 15 min, centrifuged at 3,000 g and washed with PBS/Pi. Further processing of the cells and cerebellar samples was done using the Magna ChIP Kit components (16-663, Merck-Millipore) as previously described (9) using the primers listed in the Supplementary Table 2. PCR amplifications were performed with two DNA input volumes and conditions chosen that did not saturate the PCR reaction. Briefly, chromatin extracts from three cerebellar WT and KO samples were amplified independently in parallel using two DNA amounts as input in the PCR reaction (1 and 2 µl). The Transcriptional Regulatory Element Database (TRED, http://rulai.cshl.edu/TRED) was searched to obtain the promoter sequences of the genes in this study. The retrieved predicted promoter regions were further analysed by aligning the putative open reading frames (ORFs) with *ClustalW2* (51). Retrieved sequences were subsequently analysed with ConSite to obtain DNA-binding profiles for Sp1, HBP1 and RA/thyroid
hormone nuclear receptors as in (9). Primers used for ChIP experiments are listed in Supplementary Table 2. Manual hotstart was done at 95 ºC for 2 min prior to Taq addition, followed by 95 ºC for 5 min, and 40 cycles: 95 ºC for 30 sec, 58 ºC for 60 sec, 72 ºC for 40 sec. 0.1 mM of each primer, 5% Dimethyl sulfoxide (DMSO), 2% formamide, and 0.2 μl Ecotaq (Bioline, UK) were used per each reaction.

Statistics. Statistical data were analysed with SPSS 21.0 (IBM corp). Data were tested for normal distribution and equality of variances using the Shapiro–Wilks' $W$-test and the Levene's test of homogeneity of variances, respectively. Normally distributed data were analysed by one-way ANOVA followed by post-hoc comparisons with either the Tukey HSD (honestly significant difference test) for groups of data with equal variances or, alternatively, with the Games–Howell test in one-way ANOVA analyses with unequal variances. Statistical significance was defined as a p-value < 0.05. s.e.m. denotes for standard error of the mean.
ACKNOWLEDGEMENTS

We thank all the members of theBTN Unit for their helpful comments and special thanks to Q. Caus-Capdevila for her assistance with the SH-SY5Y ChIP experiments. We are grateful to Drs. Huda Y. Zoghbi (Department of Molecular and Human Genetics and Jan and Dan Duncan Neurological Research Institute, Baylor College of Medicine, TX, USA), Harry T. Orr (Institute of Human Genetics, University of Minnesota, USA), Michal Novak (Institute of Neuroimmunology, Slovak Republic), Brian Wadzinski (Vanderbilt University Medical Center, TN, USA), Angus Nairns (Yale School of Medicine, CT, USA), Paul Greengard (Rockefeller University, NY, USA), and for reagents used in this study. This work was funded by the Spanish Ministry of Science and Innovation (BFU2008-00527/BMC to A.M-D and I.S), the Carlos III Health Institute (CP08/00027 to A.M-D), the Iberoamerican Programme for Science, Technology and Development (CYTED) (RIBERMOV, 210RT0390 to A.M-D and I.S), the European Commission (EUROSCA project, LHSN-CT-2004-503304 to A.M-D and M.P), the Fundació de la Marató de TV3 (Televisió de Catalunya, 100730, to A.M-D and I.S), and BUMC start-up funds (I.S). We are indebted to the ataxia patients for their support and motivation. Antoni Matilla-Dueñas is a Miguel Servet Investigator in Neurosciences of the Spanish National Health System.

AUTHOR CONTRIBUTIONS

I.S. and A.M-D. conceived and designed experiments. I.S., P.P., and M.C-J. performed experiments. M.P. provided critical reagents. I.S. and A.M-D. analysed all the data and wrote the manuscript. A.M-D. and I.S. obtained funding and A.M-D. supervised the work.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.
REFERENCES


49 Kovacech, B., Kontsekov, E., Zilka, N., Novak, P., Skrabana, R., Filipcik, P., Iqbal, K. and Novak,


Legends to Figures

Figure 1. Ataxin-1 regulates Pp2a activity, its regulatory subunit bβ and the endogenous inhibitor Anp32a. (A) Pp2a was immunoprecipitated from the cerebella cytosolic fractions of wild-type, Atxn1 KO mice, and ATXN1[30Q] (A02), and mutant ATXN1[82Q] (B05) transgenic mice and its activity determined using an exogenous phosphopeptide. Relative specific activity data is expressed as pmoles of phosphate released relative to levels of immunoprecipitated Pp2a-c. Top panels show levels of ataxin-1 in samples used. (B) Levels of demethylated and phospho-Y307 Pp2a-c in cytoplasmic fractions from cerebella of Atxn1, KO, A02, and B05 mice. (C) Pp2a subunits a, b, and bδ relative levels in cytoplasmic fractions from Atxn1 KO, WT, ATXN1[30Q] (A02), and ATXN1[82Q] (B05) mouse cerebellum. Data is expressed relative to Pp2a-c levels. (D) Representative sample of Pp2a components analysed in c. (E) Levels of Pp2a-b alpha, beta, and gamma in the cerebellum of Atxn1 KO mice. (F) Anp32a levels in the cytoplasmic fraction of cerebella from WT, KO, A02, and B05 mice. Mice were studied at 5 weeks of age. All data are normalized to values obtained for their respective wild-type mice controls; C57B6/J mice (n = 4) for Atxn1 KO (n = 4) and FVB for A02 and B05 (n = 5 each) which is set to 1 and denoted by dotted lines. One-way ANOVA and post-hocs Tukey HSD, *p < 0.05, **p < 0.005 compared to wild-type controls. Error bars denote s.e.m.

Figure 2. Ataxin-1 regulates Pp2a regulatory subunit bβ and the endogenous inhibitor Anp32a at the transcriptional level and occupies their promoters. (A) qRT-PCR of mRNA encoding Pp2a catalytic subunit alpha (Ppp2ca) and regulatory subunits b beta isoforms-1 (Ppp2r2b-1), isoform-2 (Ppp2r2b-2) or both (Ppp2r2b), alpha (Ppp2r2a), and gamma (Ppp2r2c) are shown. Data expressed relative to B-microglobulin (B2m) (B) ChIP analysis of the Anp32a, Pp2a-bβ and Pp2a-c promoters in Atxn1 KO mice. (C) SH-SY5Y cells transfected with WT ATXN1[30Q] or mutant ATXN1[80Q] show decreased levels of ANP32A while only ATXN1[30Q] expressing cells showed increased levels of PP2A-B. (D) ChIP analysis of ANP32A and PP2R2B-2 promoters in ATXN1[30Q] and ATXN1[80Q]
expressing SH-SY5Y cells. All data are normalized to values obtained for their respective control which are set to 1 and denoted by dotted lines. Mice were studied at 5 weeks of age. Wild-type mice controls C57B6/J mice (n = 4) for Atxn1 KO (n = 4) and FVB for A02 and B05 (n = 5 each) or control SH-SY5Y cells transfected with empty vector (n = 3) for SH-SY5Y cells expressing ATXN1[30Q] (n = 3) or ATXN1[80Q] (n = 3). One-way ANOVA and post-hocs Tukey HSD, *p < 0.05, **p < 0.005 compared to wild-type controls. Error bars denote s.e.m.

**Figure 3.** Pp2a holoenzyme composition and signalling are altered in SCA1. (A, B) Pp2a-a immunoprecipitated from cerebellar cytoplasmic fractions of WT, Atxn1 KO, ATXN1[30Q] (A02), and ATXN1[82Q] (B05) expressing mice were probed with antibody to Pp2a-c, Pp2a-b, phospho- and non-phospho-Pp2a-b'δ or Anp32a. (C, D) Levels of phospho-Erk1, -Erk2, GSK3β (ser9), Pp2a-b'δ. (E, F) Levels of p-GSK3β in SH-SY5Y expressing ATXN1[30Q] or ATXN1[80Q] alone or in addition to exogenous ANP32A. All data are normalized to values obtained for their respective control which are set to 1 and denoted by dotted lines. Mice were studied at 5 weeks of age. Wild-type mice controls C57B6/J mice (n = 4) for Atxn1 KO (n = 4) and FVB for A02 and B05 (n = 5 each) or control SH-SY5Y cells transfected with empty vector (n = 3) for SH-SY5Y cells expressing ATXN1[30Q] (n = 3) or ATXN1[80Q] (n = 3), and Anp32a co-transfected SH-SY5Y cells expressing ATXN1[30Q] (n = 3) or ATXN1[80Q] (n = 3). One-way ANOVA and post-hocs Tukey HSD or Games-Howell, *p < 0.05. Error bars denote s.e.m.

**Figure 4.** Neuronal morphological alterations in ATXN1[30Q] and ATXN1[80Q] expressing differentiated SH-SY5Y cells and the effects of ANP32A co-expression on neurite morphology. The soma diameter, neurite length, and neurites with spines were measured in over 200-500 cells from 4-6 random fields per well. (A) The percent of cells with spines in at least one third of the length of their neurites and (B) the percent of cells with neurites larger than two soma sizes were determined. (C)
Representative images of the transfected cells. Area enlarged is denoted in the box and shown to the right. Scale bars (top right) = 20 μm and 5 μm, respectively. For neurite length measures: SH-SY5Y cells with empty vector (n = 230), ATXN1[30Q] (n = 240), ATXN1[80Q] (n = 127) and Anp32a co-transfected SH-SY5Y cells with empty vector (n = 107) or ATXN1[30Q], (n = 366) or ATXN1[80Q] (n = 184). For percent of neurites with spines determination: SH-SY5Y cells with empty vector (n = 197), ATXN1[30Q] (n = 188), ATXN1[80Q] n= 184 and Anp32a co-transfected SH-SY5Y cells with empty vector (n = 332) or ATXN1[30Q] (n = 108) or ATXN1[80Q] (n = 185). One-way ANOVA and post-hoc Tukey HSD: *p < 0.05, **p < 0.005. Error bars denote s.e.m.

**Figure 5. Proposed model of the ataxin-1 induced alterations of Pp2a activity and holoenzyme composition in the cerebellum of 5 weeks-old mice.** Wild-type ataxin-1 induces the expression of the Pp2a-bβ2 protein from the Ppp2r2bβ2 gene. In addition, it suppresses the expression of Anp32a resulting in active Pp2a catalytic subunits, enriched in the Y307 phosphosite which inhibits its binding to b-type Pp2a regulatory subunits. We previously showed that Atxn1 regulates the expression of Drd2 (9), a negative regulator of PKA which is known to phosphorylate the Pp2a regulatory subunit b'δ at the activation inducing phosphosite S566. On the other hand, the mutant Atxn1 is unable to induce either the expression of the Ppp2r2β2 or Drd2 genes (loss of function), exerts an enhanced suppression of Anp32a (gain of function) and induces a decrease in Y307 phophosite (gain of function) on the Pp2a catalytic subunit. Therefore, while mutant Atxn1 expressing neurons would have more Pp2a holoenzymes with b-type regulatory subunits of which a low proportion would be of the bβ2 type and more active b'δ containing Pp2a holoenzymes than wild-type Atxn1 expressing neurons.

**ABBREVIATIONS**
Anp32a, mouse acidic nuclear protein of 32 kDa; ANP32A, human acidic nuclear protein of 32 kDa; A02, mice overexpressing wild-type human ataxin-1 containing 30 glutamines in cerebellar purkinke cells; Atxn1, mouse ataxin-1; ATXN1, human ataxin-1; B05, mice overexpressing mutant human ataxin-1
containing 82 glutamines in cerebellar Purkinje cells; Erk, extracellular signal-regulated kinases; Gsk3β, mouse glycogen synthase kinase-3; GSK-3β, human glycogen synthase kinase-3β; KO, knock-out; Pp2a, mouse protein phosphatase 2 holoenzyme; PP2A, human protein phosphatase 2A holoenzyme; Pp2a-a, mouse Pp2a scaffold subunit; PP2A-A, human PP2A scaffold subunit; Pp2a-b, mouse Pp2a regulatory subunit b; PP2A-B, human PP2A regulatory subunit B; Pp2a-c, mouse Pp2a catalytic subunit; PP2A-C, human PP2A catalytic subunit; SCA1, spinocerebellar ataxia type 1; WT, wild-type.
Sanchez et al., Fig. 1-HMG-2013-D-00243
Sanchez et al., Fig. 2-HMG-2013-D-00243
A

B

C

empty vector  ATXN1[30Q]  ATXN1[80Q]

Sanchez et. al., Fig. 4-HMG-2013-D-00243
Sanchez et al., Fig. 5-HMG-2013-D-00243