Regulation and Subcellular Compartmentalization of Ataxin-1 Phosphorylation at Serine776

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Shaojuan Lai

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Abstract

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant cerebellar ataxia caused by the expansion of a CAG repeat encoding an abnormally long polyglutamine tract in Ataxin-1 protein. Although many studies demonstrate that subcellular distribution of Ataxin-1 and protein folding/degradation pathways modulate neurodegeneration, the mechanism of pathogenesis is not completely understood. Phosphorylation of Ataxin-1 at Serine776 (S776) was previously shown to regulate Ataxin-1's functions and SCA1 pathogenicity. In addition, mice expressing human wild type Ataxin-1-[30Q] with a mutation replacing S776 with a phosphomimicking aspartic acid show similar SCA1 pathology as Ataxin-1-[82Q] mice. Here I investigated the mechanism by which phosphorylation of Ataxin-1 at S776 is regulated. I found in the cerebellum a large proportion of Ataxin-1 is phosphorylated at S776 with phosphorylated S776 enriched in the nucleus. While the kinase activity for Ataxin-1 at S776 is localized to the cerebellar cytoplasm, the phosphatase activity is restricted to the nucleus. PP2A was shown to be the phosphatase for phosphorylated S776 Ataxin-1 (Ataxin-1-pS776). 14-3-3, a protein enriched in the cytoplasm, blocks dephosphorylation of Ataxin-1-pS776 by PP2A in the cytoplasm and may affect the shuttling of Ataxin-1 to the nucleus. This work suggests that Ataxin-1 after it is phosphorylated in the cytoplasm, shuttles to the nucleus where it is dephosphorylated by PP2A. The separation of phosphorylation and dephosphorylation of S776-Ataxin-1 into two subcellular compartments may suggest that they regulate different Ataxin-1 functions in different subcellular compartments.

Table of Contents

List of Tables

List of Figures

Abbreviations

[30Q]	30 CAG (encoding glutamine) repeat units
[82Q]	82 CAG (encoding glutamine) repeat units
AAV	Recombinant adeno-associated virus
Akt	AKR mouse thymoma
A776	Alanine 776
A or PR65 subunit	Scaffolding subunit
Ataxin-1-pS776	Phospho-S776-Ataxin-1
AXH	Ataxin-1 and HMG-box protein 1
B subunit	Regulatory subunit
BSA	Bovine serum albumin
C subunit	Catalytic subunit
CAG	Cytosine-adenine-guanine
CAT	Cytosine-adenine-thymine
CER	Cytoplasmic Extraction Reagent
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescence protein
GST	Glutathione-S-transferase
HD	Huntington's disease
HeLa	Henrietta Lacks (human) derived cell line
JNK	c-Jun N-terminal kinase

KDa	Kilodalton
Mn2+/Mg2+	Manganese/magnesium ion
NLS	Nuclear localization signal
OA	Okadaic acid
PBS	Phosphate buffered saline
P.I &II	Phosphatase inhibitors I&II
PKA	Cyclic AMP-dependent protein kinase
PKB	Protein kinase B
PP2A	Protein phosphatase 2A
PP5	Protein phosphatase 5
PPMs	Mn2+/Mg2+-dependent protein phosphatases
PPPs	Phosphoprotein phosphatases
PSKs	Protein serine/threonine kinases
PSP	Protein serine/threonine phosphatase
PTKs	Protein tyrosine kinases
PTP	Protein tyrosine phosphatase
RMB17	RNA binding motif protein 17
S776	Serine776
SAP	Shrimp alkaline phosphatase
SAR	Self association region
SCA1	Spinocerebellar ataxia type 1
shRNA	short hairpin RNA

SPF45	Splicing factor 45
SUMO	Small ubiquitin like modifier
UHM	U2AF homology motif
ULM	UHM ligand motif
U2AF65	U2 small nuclear ribonucleoprotein auxiliary factor 65KDa subunit

CHAPTER 1

GENERAL INTRODUCTION

1. I: Neurodegenerative Disorders

Neurodegenerative disorders are characterized by neuronal dystrophy and neuronal loss. Currently, several million Americans are affected by neurodegenerative diseases. This incidence is expected to increase as the population ages. Studying these disorders is not only beneficial for a better understanding of the disease pathogenesis from a neurobiology and cell biology standpoint, it also reveals potential pathways for therapeutic development.

Many of these diseases are genetic, with a subgroup caused by an expansion of cytosine-adenine-guanine (CAG) trinucleotide repeats. This repeat encodes an expanded polyglutamine tract in the disease protein, thus, these diseases are also known as polyglutamine diseases. To date, there are nine distinct polyglutamine disorders of which the involved genes have been identified. These include Huntington's disease (HD) (Huntington's disease collaborative research group, 1993), spinal and bulbar muscular atrophy (La Spada et al., 1991), dentatorubralpallidoluysian atrophy (Koide et al., 1994; Nagafuchi et al., 1994), and six spinocerebellar ataxias, spinocerebellar ataxia type 1 **(SCA1)** (Orr et al., 1993), type 2 (Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996), type 3 (Kawaguchi et al., 1994), type 6 (Zhuchenko et al., 1997), type 7 (David et al., 1997; Koob et al., 1998) and type 17 (Nakamura et al., 2001).

Although genetic evidence consistently demonstrates that these are gain of function diseases, the complete mechanism and the molecular basis of each remain to be elucidated. In 1994, a pathogenic mechanism that mutant proteins harboring the polyglutmaine expansion are more prone to self-associate and form aggregates or

inclusions was proposed (Perutz et al., 1994). This quickly became the most prominent mechanism after aggregates were discovered in several neurodegenerative diseases. However, over the years, the causative relationship between aggregates and pathogenesis has not been established. Investigators began to question whether the large aggregates or inclusions are the direct etiology for pathogenesis or just an outcome of disease development (Cummings et al., 1999; Klement et al., 1998; Saudou et al., 1998; Slow et al., 2005). Research also focused on identifying cellular components that interact with the disease-causing protein and, thereby, identifying pathways critical for pathogenesis (Colomer et al., 1997; Goehler et al., 2004; Kaytor et al., 2005; Matilla et al., 1997; Ravikumar et al., 2004; Steffan et al., 2001). One pathway critical for pathogenesis involves post-translational modifications of disease-causing protein. In particular, studies indicate that phosphorylation of disease causing-protein is critical in the neurotoxicity of two polyglutamine diseases, HD and SCA1 (Emamian et al., 2003; Luo et al., 2005; Schilling et al., 2006). Thus, this thesis focuses on the regulation of Ataxin-1 phosphorylation at S776, an important regulator for both Ataxin-1 protein function and SCA1 pathogenesis, in the mammalian cerebellum.

1. II: SCA1

SCA1 is an autosomal dominant inherited neurodegenerative disease caused by expansion of CAG repeats in the Ataxin-1 gene. The CAG repeats encode a polyglutamine expansion in the Ataxin-1 protein. In normal alleles, the length of CAG varies from 6-44. In mutant alleles, the repeat length expands to 40-83 repeats (Chung et al., 1993; Orr et al., 1993). The repeat size in unaffected and affected alleles overlaps in

the 39 to 44 range. However, the repeat in unaffected alleles is interrupted by at least one CAT encoding histidine (Chung et al., 1993; Mittal et al., 2005; Orr et al., 1993).

Clinically, SCA1 is characterized by ataxia, dysphagia, dysarthria, and progressive motor dysfunction. Symptoms usually present in the 3rd to 4th decade of life and progress until death. Initially, SCA1 patients have uncoordinated movements, slurred speech, abnormal gait, and mild cognitive impairment (Zoghbi and Orr, 2000). Later, as the disease progresses, patients lose their capability to work and take care of themselves. Generally, patients with longer repeats experience an earlier age of onset, more rapid disease progression and more severe symptoms. Pathologically, SCA1 neurodegeneration affects the cerebellar Purkinje cells, inferior olivary complex, and pontine nuclei (Robitaille et al., 1995). The red nucleus, dentate nucleus, and dorsal spinal cord are affected as well but to a lesser extent. Currently, patients with SCA1 can not be treated effectively with treatments limited to ameliorating symptoms, relieving pain and increasing mobility. This is partly due to the lack of full understanding of Ataxin-1 functions.

1. III: Function of Ataxin-1

The functions of Ataxin-1 are thought to be related to SCA1 disease. Ataxin-1 knock out mice (Sca1-/-) have memory deficits demonstrating that the function of Ataxin-1 is important for memory (Matilla et al., 1998). Ataxin-1 contains a functional classical arginine-lysine nuclear localization signal (NLS). The NLS is important for mutant expanded Ataxin-1 [82Q] to cause disease since presence of mutant Ataxin-1 in the nucleus is required to induce neurodegeneration (Klement et al., 1998). In the

nucleus, Ataxin-1 plays a role in regulating RNA metabolism and gene expression. Expression of the expanded polyglutamine tract in mutant Ataxin-1 interferes with these normal functions of Ataxin-1 and its protein-protein complexes, which might contribute to SCA1 pathogenesis.

RNA metabolism. In vitro, Ataxin-1 has RNA binding activity that is inversely correlated with the length of polyglutamine tract (Yue et al., 2001). In addition, Ataxin-1 may play a role in RNA metabolism, including RNA stability and transport, by associating with promyelocytic leukaemia protein (a nuclear matrix-associated protein) and the nuclear matrix (Skinner et al., 1997).

RNA splicing. Ataxin-1 may also have a function in RNA splicing. Recently, researchers identified an Ataxin-1 interacting protein named RNA binding motif protein 17 (RBM17), also known as splicing factor 45 (SPF45) (Lim et al., 2008). RBM17 is a splicing factor that binds to single-stranded 3'AG at the exon/intron border and has a role in the regulation of alternative splicing (Corsini et al., 2007; Lallena et al., 2002; Sampath et al., 2003). More recently, Ataxin-1 was shown to interact with another splicing factor, U2 small nuclear ribonucleoprotein auxiliary factor 65-kilodalton (KDa) subunit (U2AF65) (de Chiara et al., 2009). Thus, Ataxin-1 may affect RNA splicing via interaction with these two splicing factors.

Regulation of transcription in the nucleus. Ataxin-1 is thought to regulate gene expression by interacting with several transcription factors including both transcriptional activators and repressors (Goold et al., 2007; Lam et al., 2006; Okazawa et al., 2002; Serra et al., 2006; Tsai et al., 2004; Tsuda et al., 2005). For example, Ataxin-1,

independent of glutamine repeat length, is able to repress transcription by interacting with polyQ binding protein, the corepressors silencing mediator of retinoid and thyroid hormone receptors and histone deacetylase 3 (Okazawa et al., 2002; Tsai et al., 2004). Most of the interactions above are mediated through Ataxin-1's AXH domain (Ataxin-1 and HMG-box protein 1) (Mizutani et al., 2005; Tsuda et al., 2005).

The role of Ataxin-1 in RNA binding and transcriptional regulation is further supported by the observation that Ataxin-1 is covalently modified by the protein small ubiquitin like modifier (SUMO) (Riley et al., 2005). SUMO targets Ataxin-1 to specific nuclear proteins, then Ataxin-1 can exert its function of transcriptional regulation since many of these Ataxin-1 targeted proteins play a role in transcription (Seeler and Dejean, 2003).

1. IV: SCA1 and Phosphorylation of Ataxin-1 at S776

Protein phosphorylation, a reversible post-translational mechanism, is an important means of regulating protein/protein interactions, protein degradation and function (Hunter, 2000). Ataxin-1 was shown to be phosphorylated at two serine sites, S239 and S776 (Chen et al., 2003; Emamian et al., 2003; Vierra-Green et al., 2005). Although the role of phosphorylation at S239 is not understood, phosphorylation of Ataxin-1 at S776 has a critical role in regulating its function as well as SCA1 pathogenesis (Chen et al., 2003; Duvick et al., 2010; Emamian et al., 2003; Lam et al., 2006; Lim et al., 2008) (Table 1).

Phosphorylation at S776 affects the capability of Ataxin-1 to associate with other cellular proteins to form large soluble complexes (Lam et al., 2006). Notably, the

interaction of Ataxin-1 with the splicing factor RBM17 is increased with a phospho-mimicking aspartic acid at position 776 (Lim et al., 2008). In addition, a phosphorylation resistant Ser to Ala (S776A) substitution enhances SUMOylation of Ataxin-1 in tissue culture cells (Riley et al., 2005).

In transgenic mice a S776A mutation largely mitigates the ability of Ataxin-1 [82Q] to induce neurodegeneration even though the mice express mutant Ataxin-1 [82Q] in Purkinje cell nuclei (Emamian et al., 2003). The A776-Ataxin-1 [82Q] mice displayed a very mild disease phenotype even late in life. Behaviorally, these mice are not distinguishable from wild type controls. Pathologically, these mice develop very mild SCA1 phenotype. These findings suggest the importance of S776 for neurotoxicity of mutant Ataxin-1 in vivo. Support for this conclusion also came from the D776-Ataxin-1 [30Q] model, which expresses wild type Ataxin-1 [30Q] containing a phospho-mimetic aspartic acid at position 776 (Duvick et al., 2010). S776D enhances pathogenesis induced by Ataxin-1 [82Q] and converts wild type Ataxin-1 [30Q] to a pathogenic protein.

From what was described above, phosphorylation of Ataxin-1 at S776 is not only a critical regulator for Ataxin-1's functions but also important for SCA1 pathogenesis. Thus insight into the cellular pathways that regulate the phosphorylation of Ataxin-1 at S776 is crucial for better understanding the protein itself and disease process of SCA1.

1. V: Regulation of Protein Phosphorylation

The phosphorylation state of a protein is a dynamic process dictated by both protein kinases and phosphatases (Cohen, 1989). Thus, regulation of protein

phosphorylation involves a protein kinase and a protein phosphatase. A substrate protein is converted from the dephosphorylated form to the phosphorylated form by a protein kinase, and the phosphorylated form returns to the dephosphorylated form by dephosphorylation due to a protein phosphatase.

The fully sequenced human genome contains 518 putative protein kinases (Johnson and Hunter, 2005; Lander et al., 2001; Venter et al., 2001), which can be divided into protein serine/threonine kinases (PSKs), protein tyrosine kinases (PTKs) and dual-function kinases. Among these kinases the majority of them (428) are PSKs.

Protein kinases contain a conserved catalytic domain, which catalyzes the transfer of the γ-phosphate of ATP to the hydroxyl moiety on serine, threonine or tyrosine residue in protein substrates. In the cells, protein kinases exist in either active or inactive state. Switch between the two states is regulated by a wide range of mechanisms including regulation by phosphorylation, regulation by additional domains that may target other molecules, binding and regulation by additional subunits, and control by protein–protein association (Johnson, 2009). Interestingly, most PSKs undergo autophosphorylation, which is often associated with enhanced kinase activity (Hanson and Schulman, 1992; Seger et al., 1991). One example is CaMKII, where autophosphorylation results in an extended kinase activity independent of the presence of Ca^{2+} and calmodulin.

The mechanism by which phosphorylation at S776 confers toxicity to mutant expanded Ataxin-1 in neurodegeneration was previously depicted in a model by Chen et al., 2003 (Figure 1). In this model, oncogene from AKR mouse thymoma (Akt), or

Protein Kinase B (PKB) was the kinase phosphorylating Ataxin-1 at S776. Phosphorylation of Ataxin-1 induced the binding of 14-3-3 and redistributed 14-3-3 to the nucleus. 14-3-3 stabilized Ataxin-1 and triggered the accumulation of Ataxin-1 in the nucleus, thus causing disease. However, data supporting this model came from transfected tissue culture cells and a *Drosophila* model of SCA1. Recent data with mouse cerebellar lysate suggests that while phosphorylation of S776 is associated with a stabilization of Ataxin-1 in Purkinje cells, Akt is not the in vivo kinase (Jorgensen et al., 2009). In contrast, current data indicates cyclic AMP-dependent protein kinase (PKA) is the relevant kinase in vivo. Immunodepletion and inhibition of PKA decreased phosphorylation of Ataxin-1-pS776. In contrast, inhibition of Akt either in vivo or in a cerebellar extract-based phosphorylation assay did not decrease the phosphorylation of Ataxin-1-pS776.

The phosphate group added by protein kinases on serine, threonine or tyrosine can be cleaved by protein phosphatases. Protein phosphatases can be classified into three major families on the basis of sequence, structure and catalytic mechanism. The three families are: the protein serine/threonine phosphatase (PSP) superfamily, the protein tyrosine phosphatase (PTP) superfamily, and the aspartate-based protein phosphatases with a DXDXT/V catalytic signature (Moorhead et al., 2007). Although the number of PTKs is roughly comparable to the number of PTPs (about 107) (Alonso et al., 2004), there are far fewer PSPs (around 30) than PSKs. This dichotomy is complemented by a variety of phosphatase holoenzymes formed by the association of a common catalytic

subunit with multiple forms of regulatory subunits. Thus, collectively, these holoenzymes have a wide range of substrates.

The regulatory subunits of phosphatases or domains in some phosphatases regulate the enzyme activity, localization to a specific cellular compartment and substrate specificity. The mechanism for how these regulatory subunits or domains regulate the holoenzyme is not totally elucidated. In some phosphatases, the presence of them can inhibit the holoenzyme's activity such as protein phosphatase 5 (PP5) (Kissinger et al., 1995; Yang et al., 2005). Other regulatory mechanisms such as post-translational modification of subunits and association of subunits with some other proteins may also be involved.

Dephosphorylation by phosphatases is essential for proper regulation of protein phosphorylation. Recent studies implicate PSPs in a multitude of cellular events, many of which take place in the nucleus (Moorhead et al. 2007). The brain contains multiple forms of protein serine/threonine phosphatases. These phosphatases differ in their regional distribution, substrate specificity and regulation by cellular messengers. Many physiological responses in the brain are exerted by regulating protein phosphatases.

In the diseased state, regulation of protein phosphorylation is frequently disrupted. Phosphorylation of Ataxin-1 at S776 is an important regulator of both Ataxin-1's neurotoxicity and SCA1 pathogenesis. Thus, my thesis is aimed to understand the regulation of Ataxin-1 phosphorylation at S776 in the cerebellum. I examined the cerebellar subcellular distribution of Ataxin-1 and phospho-S776-Ataxin-1 (Ataxin-1-pS776), and the subcellular localization of kinase and phosphatase activity for Ataxin-1

at S776. I also further confirmed PKA's role as kinase for S776-Ataxin-1 and identified protein phosphatase 2A (PP2A) as the phosphatase for Ataxin-1-pS776. The reason why PP2A's activity for dephosphorylation of Ataxin-1-pS776 is limited to only one subcellular compartment was also examined.

Ataxin-1 stability	↑
interaction with 14-3-3	↑
interaction with Capicua	↑
interaction with RBM17	↑
interaction with U2AF65	↓
SUMO	↓
SCA1 pathogenesis	↑

Table 1. The biological effects of Ataxin-1 phosphorylation at S776. Phosphorylation of Ataxin-1 at S776 increases the stability of Ataxin-1, enhances Ataxin-1 interaction with 14-3-3, Capicua, and RBM17 while decreases SUMO level of it. Most importantly, phosphorylation is necessary for mutant Ataxin-1 [82Q] to cause disease.

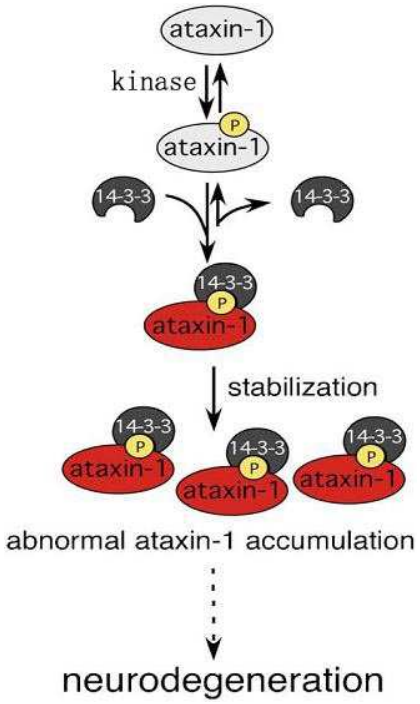

Figure 1. A model depicting a mechanism contributing to Ataxin-1-induced neurodegeneration. A kinase phosphorylation of Ataxin-1 at S776 allows 14-3-3 binding, which stabilizes Ataxin-1 perhaps by competing with factors mediating its degradation. The 14-3-3-bound mutant Ataxin-1 gradually accumulates and causes neurodegeneration. (modified from Chen et al., 2003)

CHAPTER 2

CONSERVATION AND SUBCELLULAR LOCALIZATION OF THE ATAXIN-1-pS776 PATHWAY

2. I: Introduction

S776 site is a key biochemical feature of Ataxin-1 and crucial for SCA1 pathogenesis in transgenic mice. Moreover this residue is present in Ataxin-1 homologs in different species, including mouse, xenopus and zebrafish, suggesting that this site is important for Ataxin-1 function (Figure 2A). Zebrafish is a well established model system for studies in biology. They were previously established as a useful model system for studying modifiers of polyglutamine toxicity (Miller et al., 2005). The cerebellum of Zebrafish, though less complicated than the cerebellum of mammals, contains a similar cellular architecture as mammalian cerebellum. Purkinje cells, molecular layer and granule cell layer are all well present (Miyamura and Nakayasu, 2001). Moreover zebrafish cerebellum expresses Ataxin-1. The two key biochemical features of human Ataxin-1 protein homolog, a NLS sequence and S776, are conserved in zebrafish Ataxin-1 family. However, whether zebrafish Ataxin-1 is phosphorylated at S776 remains to be clarified.

Normally, Ataxin-1 shuttles between the nucleus and cytoplasm (Irwin et al., 2005). In Purkinje cells wild type and mutant expanded Ataxin-1 are localized to both cytoplasm and nucleus (Servadio et al., 1995). However, mutant Ataxin-1 must enter the nucleus of Purkinje cells for it to cause disease (Klement et al., 1998). In addition, mutant expanded Ataxin-1 needs to be phosphorylated in order to cause disease (Chen et al., 2003; Emamian et al., 2003). The importance of the subcellular localization of Ataxin-1 and its phosphorylation at S776 led me to investigate the cerebellar subcellular

localization of Ataxin-1-pS776 and the enzyme activities responsible for its phosphorylation and dephosphorylation.

2. II: Materials and Methods

2. IIa: Cerebellum collection and cell culture

Zebrafish. Wild type zebrafish were purchased from Segrest Farms and raised in the University of Minnesota Zebrafish Core Facility.

Preparation of zebrafish brain lysates. Adult zebrafish were anesthetized using 4mg/mL Tricaine (Sigma) and decapitated with a razor blade. Two adult zebrafish brains were pooled together and homogenized with a cordless motor pestle (VWR #A6-LP-A0001) in brain lysis buffer (0.25 M Tris-Cl, pH 7.5) containing 1× protease inhibitors (Roche Biochemicals #11836170001) and phosphatase inhibitor cocktails 1 (Sigma #P2850) and 2 (Sigma # P5726). Homogenates were then prepared by three rounds of freezing in liquid nitrogen followed by thawing in a 37 °C water bath. Lysates were centrifuged at 2500 rpm for 10 min at 4°C, and the supernatant was transferred to a new gel-slick microfuge tube.

Cell culture and tranfection. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO_2. Cells were plated onto 60 mm dish and transfected 24 hr later using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. The following plasmids were used for transfection: A776-Ataxin-1 [82Q], S776-Ataxin-1 [82Q] and D776-Ataxin-1 [82Q].

Preparation of cell culture lysates. 48 hr after tranfection, cells were rinsed with 1X cold phosphate buffered saline (PBS) stored at 4 °C. 500 µL of cold lysis buffer (50 mM Tris-Cl, pH 7.5, 2.5 mM $MgCl_2$, 100 mM NaCl, 0.5% Triton) containing protease inhibitor and phosphatase inhibitor cocktails was added to 60 mm plate and rocked at 4 °C for 20 min. Plates were scraped and lysates were transferred to cold gel-slick microfuge. Lysates were triturated 10× through a 21 gauge needle, followed by 5× through a 25 gauge needle on ice. Lysates were spun 10 min at 4 °C 15,000 rpm and transferred to fresh gel-slick tube.

Preparation of cerebellar lysates. Mice were sacrificed with CO_2 according to University of Minnesota Institutional Animal Care and Use Committee guidelines. Fresh cerebella were removed, placed in 1.5 mL microfuge tubes and flash frozen in liquid nitrogen. 500 µL brain lysis buffer described above was added. Cerebella were homogenized and freeze-thawed three times in liquid nitrogen followed by thawing in a 37 °C water bath. Lysates were centrifuged at 2500 rpm for 10 min at 4 °C. The supernatant was transferred to a new 1.5 mL microfuge tube and kept on ice. For immunodepletion, cerebella were homogenized in NP-40 lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl and 1% NP-40) containing protease inhibitor and phosphatase inhibitor cocktails. Cerebellar lysates were kept in ice for 40 min and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new 1.5 mL microfuge tube and kept on ice.

Subcellular fractionation. Cerebellar nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotech #78833)

according to the manufacturer's protocol with slight modifications. In all dephosphorylation assays, Cytoplasmic Extraction Reagent I (CER I) and Nuclear Extraction Reagent (NER) were only added with protease inhibitor. Except for dephosphorylation assays, CER I and NER were added with protease inhibitor and phosphatase inhibitor cocktails. Throughout extraction, lysates and reagents were on ice and spun at 4 °C. Cerebella mass was weighed and CER I was added according to the instruction. Cerebella were homogenized with a motor pestle in CER I, vortexed for 15 s, and incubated on ice for 10 min. Ice cold CER II was added. Extracts were vortexed for 5 s, incubated on ice for 1 min and votexed for 5 s again. Extracts were centrifuged for 5 min at 15, 000 rpm. Supernatant was transferred to a clear pre-chilled tube and saved as cytoplasmic extract. Pellet was washed once with 200 μL ice-cold 1× PBS, flicked and centrifuged for 5 min at 13,000 rpm. Supernatant was discarded. Pellet was resuspended in ice-cold NER, vortexed every 10 min for 22 s for 40 min and centrifuged for 10 min at 15 000 rpm. Supernatant was transferred to a clear pre-chilled tube and saved as nuclear extract.

2. IIb: Protein and expression analysis

Western blotting. Protein concentration of lysates was determined using a Bradford protein assay (Bio-Rad). Prepared lysates were added with 10× NuPAGE® Sample Reducing Agent (Invitrogen #NP0004) and 4× LDS sample buffer (Invitrogen #NP0007), heated for 10 min at 90 °C, and loaded on 4-12% gradient NuPAGE® Novex Bis-Tris gels (Invitrogen #NP0321BOX). Gels were run in NuPAGE® MES SDS buffer (Invitrogen #NP0002) containing 0.1% NuPAGE® Antioxidant (Invitrogen #NP0005) at

120 V until the dye comes out of the gel bottom. Transfer cassette was set up with Invitrogen mini-gel transfer cassette system soaked in NuPAGE® Transfer Buffer (Invitrogen #NP00061) containing 0.1% antioxidant. Gels were transferred in the transfer buffer to nitrocellulose membranes (Whatman Protran BA 85) overnight at a constant 100 mAmp/cassette.

For PN1248 (antibody specific for Ataxin-1-pS776), 11750 (Ataxin-1 antibody), and anti-α-Tubulin (Sigma), blots were blocked 2 hr at room temperature in 5% weight/volume (w/v) low-fat, powdered milk in PBS with 0.1% volume/volume (v/v) Tween-20 (PBST) made with deionized water. First antibody in blocking milk was added to the membranes and incubated at room temperature for 2 hr. Membranes were washed with PBST 3× 15 min and incubated with secondary antibody (anti-mouse HRP (GE Healthcare #NA931V) or anti-rabbit HRP (GE Healthcare (#NA934V)) for 1 hr at room tempreture. Membranes were washed with PBST 3× 15 min, incubated with SuperSignal® West Pico Chemiluminescent substrate (Pierce #34080) for 5 min at room temperature, and then exposed to film (Kodak #165-1454). The blots probed for Atxain-1-pS776 and Ataxin-1 as loading control were probed for Atxain-1-pS776 first and then stripped and reprobed for total Ataxin-1.

For anti-IκB-α and Histione 3 (Cell Signaling), blots were blocked overnight at 4 °C in 3% w/v bovine serum albumin (BSA) in PBST. First antibody in blocking BSA was added to the membranes and incubated at 4 °C overnight. Membranes were washed with

PBST 4× 15 min and incubated with secondary antibody in 3% BSA for 1 hr at room tempreture. Membranes were washed with PBST 4× 15 min, incubated with SuperSignal® West Pico Chemiluminescent substrate for 5 min at room tempreture, and then exposed to film.

Immunodepletion. Protein G Sepharose beads (for IgG mouse and rabbit antibodies) were washed 3× with NP-40 lysis buffer. 50 µg cerebellar lysates made in NP-40 lysis buffer was filled up with lysis buffer to 120 µL. Lysates were precleared with washed protein G beads for 1 hr on a rugged rotator and spun. Supernatant was transferred to a new gel-slick tube, added with PN1248 antibody and incubated overnight on a rugged rotator at 4 °C. The next day, washed protein G sepharose beads were blocked with 1 mL 5% w/v BSA in 1× PBS for 2 hrs at 4 °C, washed 3× with 1 mL 1×PBS and made as 50% slurry. The lysates were added with BSA-blocked G beads and incubated for 2 hrs on a rugged rotator at 4 °C. Lysates were spun for 30 s at 15,000 rpm at 4 °C and supernatants were saved and loaded on a gel.

In vitro kinase assay—Cytoplasmic or Nuclear fractions as a source of kinase. 5 µg of purified Glutathione-S-transferase (GST)-Ataxin-1 [30Q] and 20 µg of mouse cerebellar cytoplasmic or nuclear fractions were combined with 0.1 mM ATP in lysis buffer (0.25 M Tris-Cl, pH 7.5 containing 1× protease inhibitors and 1× phosphatase inhibitor cocktails 1 and 2. The reaction was incubated for up to 1 hr at 30 °C and loaded on a 4-12% Bis-Tris polyacrylamide gel.

Endogenous dephosphorylation assay. Aliquots of cerebellar lysates containing both protease and phosphatase inhibitors, lysates lacking phosphatase inhibitors and lysates

lacking phosphatase inhibitors but containing shrimp alkaline phosphatase (SAP) (Promega) were incubated at 37 ºC for up to 1hr (as indicated in Figure 6). Dephosphorylation was terminated by adding sample buffer and heating in boiling water for 5 min. Western blot was performed as described above.

Aliquots of 60 μg cytoplasmic extracts and 15 μg nuclear extracts made without phosphatase inhibitors were incubated at 37 ºC for up to 2 hrs (as indicated in Figure 7). Dephosphorylation was terminated by adding sample buffer and heating in boiling water for 5 min. Western blot was performed as described above. For cytoplasmic extracts with R18, R18 (AnaSpec) was added to cytoplasmic extracts to a final concentration of 25 μM.

Statistic analysis and quantification. Densitometry was done with films scanned on a BioRad Gel Dock GS700 600 dpi flatbed densitometer. Western blot band density was analyzed with ImageQuant software. The level of Ataxin-1-pS776 was normalized to the level of Ataxin-1. The level of Ataxin-1 was normalized to the level of α-Tubulin. All experiments were carried out using three individual FVB mouse cerebella or independent cell transfections in triplicate. Statistical analysis was performed using Students t-test. Western blotting images are representative of repeated experiments.

2. III: Results

Conservation of S776 phosphorylation. To examine if Ataxin-1 is phosphorylated at S776 in the zebrafish brain, adult Zebrafish brains were removed and protein extract was analyzed by western blotting with an Ataxin-1 phospho-S776 specific antibody, PN1248 antibody (Emamian et al, 2003). Specificity of the antibody was tested with cellular lysate from cells either transfected with S776-Ataxin-1 or A776-Ataxin-1 or D776-Ataxin-1. As shown in Figure 2B, the antibody was specific in that it recognized only the S776 form of Ataxin-1. Moreover, treatment of cellular lysate expressing S776-Ataxin-1 with shrimp alkaline phosphatase dramatically decreased recognition of S776-Ataxin-1 by PN1248. Using this antibody to probe a Zebrafish brain protein extract, as shown in Figure 2C, a band was detected at around 85 KDa, the molecular weight of Ataxin-1, suggesting that Zebrafish endogenous Ataxin-1 is phosphorylated at S776 in vivo. Cerebellar lysates from wild type (FVB) and Ataxin-1 knockout (SCA1-/-) mice were included as controls. The conserved phosphorylation of zebrafish and human Ataxin-1 suggests that the pathway regulating Ataxin-1 phosphorylation is physiologically important and relevant.

The proportion of S776-Ataxin-1 phosphorylated in the murine cerebellum. To further characterize the phosphorylation of Ataxin-1 in vivo, the proportion of Ataxin-1 phosphorylated at S776 in the murine cerebellum was determined. The approach was to compare the amount of Ataxin-1 in a cerebellar extract to that in an extract immunodepleted for Atxain-1-pS776. As shown in Figure 3, immunodepletion of Ataxin-1-pS776 decreased the amount of Ataxin-1 detectable by 60%, suggesting that in the

cerebellum, a considerable amount of Ataxin-1 (60%) was phosphorylated at S776.

Subcellular location of the S776-Ataxin-1 kinase activity. Next the subcellular compartment where S776-Ataxin-1 is phosphorylated was determined. The approach applied was to separate wild type mouse cerebella into nuclear and cytoplasmic extracts and used the extracts as a source of the S776 kinase. The substrate for the kinase assay was exogenous recombinant Glutathione-S-Transferase (GST) tagged Ataxin-1 [30Q]. After the kinase reaction, samples were run on a polyacrylamide gel, transferred and probed with PN1248 antibody. As shown in Figure 4, only the cytoplasmic extract was capable of phosphorylating GST-Ataxin-1. The level of Ataxin-1 phosphorylated at S776 dramatically increased with the addition of ATP as compared to no cytoplasmic control (Jorgensen et al. 2009). Nuclear extracts had no effect on the phosphorylation of S776, indicating that the kinase activity for Ataxin-1 at S776 is restricted to the cerebellar cytoplasm. This conclusion was further supported by the result of the same assay run at different time points (Figure 4B). IκB-α and Histone 3 were used as cytoplasmic protein marker and nuclear protein marker, respectively, to assess fraction purity.

The subcellular distribution of Ataxin-1-pS776 in the cerebellum. In order to gain insight into whether phosphorylation of S776 might impact the cytoplasmic to nuclear transport of Ataxin-1 in the cerebellum, the cerebellar subcellular distribution of total Ataxin-1 and Ataxin-1-pS776 was examined. The amount of total Ataxin-1 and Ataxin-1-pS776 in the cerebellar cytoplasmic and nuclear fractions (Figure 5A) was calculated using the formula depicted in Figure 5B. In this formula, the total volume of cytoplasmic or nuclear fractions was divided by the corresponding cytoplasmic or nuclear

volume loaded in the gel for western blot. The resulting quotient was multiplied by the densitometry value of the corresponding western band. Thus, the total Ataxin-1 and Ataxin-1-pS776 in the cytoplasm and nucleus were expressed as densitometry readings. Using this formula to process western blot data, the amount of total Ataxin-1 localized to the cerebellar cytoplasm was 53% compared to 47% in the nucleus (Figure 5C-E). In contrast, a greater proportion of total Ataxin-1-pS776 (60%) was localized to the nucleus. Therefore, the relative amount of Ataxin-1-pS776 in the nucleus (1.66) was higher than that in the cytoplasm (1), suggesting that Ataxin-1-pS776 is enriched in the cerebellar nucleus.

Dephosphorylation of Ataxin-1-pS776 in the cerebellum. Next, the dephosphorylation of Ataxin-1-pS776 was examined. First, whether Ataxin-1-pS776 could be dephosphorylated was determined by comparing the Ataxin-1-pS776 level in a mouse cerebellar lysate containing phosphatase inhibitor cocktails with that in a lysate lacking the phosphatase inhibitor cocktails. As shown in Figure 6, one hour incubation at 37 °C reduced the amount of Ataxin-1-pS776 detected in the lysate lacking the phosphatase inhibitor cocktail. This result indicated the presence of an endogenous Ataxin-1-pS776 phosphatase in cerebellar lysates.

Subcellular location of the S776 phosphatase activity. To determine whether the dephosphorylation of Ataxin-1-pS776 is localized to a specific subcellular compartment in the cerebellum, mouse cerebellar cytoplasmic and nuclear extracts were incubated in the absence of phosphatase inhibitors at 37 °C for up to two hours. As shown in Figure 7, Ataxin-1-pS776 levels in the cytoplasmic fraction remained constant

over the two hour incubation period, indicating that dephosphorylation did not occur in the cytoplasmic fraction. In contrast, the amount of Ataxin-1-pS776 decreased in the nuclear fraction over the incubation period. From these results it was concluded that phosphatase activity that dephosphorylates Ataxin-1-pS776 is restricted to the nuclei of cerebellar cells.

2. IV: Discussion

Phosphorylation of S776 in Ataxin-1, the protein affected in the neurodegenerative disease SCA1, regulates its interaction with other cellular proteins and is crucial for pathogenicity induced by the mutant protein (Chen et al., 2003; Emamian, et al., 2003; Lim et al., 2008; Duvick et al., 2010). Results presented here indicate that phosphorylation at S776 is conserved in zebrafish brain and the majority of endogenous cerebellar Ataxin-1 is phosphorylated at S776. While the subcellular site of phosphorylation activity is the cytoplasm, Ataxin-1-pS776 is enriched in the nuclei of cerebellar cells. Moreover, it is nuclear and not cytoplasmic extracts that are able to dephosphorylate Ataxin-1-pS776.

Both mutant and wild type Ataxin-1 are phosphorylated at S776 site. In wild type FVB mice, about 60% of Ataxin-1-pS776 was found localized to the nucleus. It is yet to be determined whether the subcellular distribution of mutant Ataxin-1[82Q]-pS776 is similar to that reported here. However, it is possible that in SCA1 mice more mutant Ataxin-1-pS776 accumulates in the nucleus of Purkinje cells since nuclear export of mutant Ataxin-1 is greatly reduced by the expansion of its polyglutamine tract (Irwin et al. 2005).

Considering in the whole cerebellum, 60% of Ataxin-1 is phosphorylated at S776 and 60% of phosphorylated S776-Ataxin-1 is localized to the nuclei of cerebellar cells, thus around 36% (60% × 60%) of Ataxin-1 phosphorylated at S776 is localized to the nucleus while 24% (60%−36%) of it is in the cytoplasm. Since 47% of total Ataxin-1 is localized to the nuclei of cerebellar cells and 53% of it is in the cytoplasm, 77% (36%/47%) of Ataxin-1 in the nucleus is phosphorylated while 45% (24%/53%) of it in the cytoplasm is phosphorylated. Thus, around 23% (100%−77%) of Ataxin-1 in the nucleus is unphosphorylated and 55% (100%−45%) of it in the cytoplasm is unphophorylated.

A previous study demonstrated that Ataxin-1, like many other proteins, shuttles back and forth between the cytoplasm and nucleus (Irwin et al. 2005). The fact that the kinase activity for S776-Ataxin-1 is in the cytoplasm (Jorgensen et al 2009) and that Ataxin-1-pS776 is enriched in the nucleus suggests that Ataxin-1 after phosphorylation in the cytoplasm facilitates transport and accumulating in the nucleus. The nuclear localization of the phosphatase activity for Ataxin-1-pS776 suggests that it is dephosphorylated in the nucleus. Based on the data presented here, a model can be proposed (Figure 8).

The enrichment of Ataxin-1-pS776 in the nucleus suggests that Ataxin-1-pS776 shuttles to the nucleus quicker than it moves back to the cytoplasm, although the subcellular site of Ataxin-1 degradation needs to be considered. The nucleus is the subcellular location where Ataxin-1 forms aggregates and interacts with other proteins to form large protein complexes. Thus, the subcellular site of S776-Ataxin-1

phosphorylation is separated from that of its action. It is interesting that although the phosphatase activity for Ataxin-1-pS776 is localized to the nucleus; Ataxin-1-pS776 is enriched in the nucleus. Explanation for this may be related to the kinetics of Ataxin-1 shuttling in and out of the nucleus, and the relative rate of phosphorylation and dephosphorylation.

It is clear that the process of macromolecular trafficking across the nuclear membrane must be meticulously regulated in order to maintain the normal state of cells and respond to intracellular signals. Many levels of regulation for this exist. Even the context of NLS can influence the rate of transport to the nucleus (Nelson and Silver, 1989). In particular, post-translational modifications of amino acids flanking NLS are able to regulate nuclear traffic. The subcellular localization of many proteins, including the transcriptional regulators of NF-AT, Mig1, Swi5, and Swi6, appears to be regulated by phosphorylation in response to either cell cycle position or extracellular signals (Beals et al., 1997a; Beals et al., 1997b; De Vit et al., 1997; Moll et al., 1991; Sidorova et al., 1995).

Phosphorylation regulates nucleocytoplasmic transport by three basic mechanisms as shown in Figure 9 (Hood and Silver, 1999). First, regulation is unidirectional. In this case, proteins shuttle constitutively in one direction through the nuclear envelope but its movement in the other direction is regulated by phosphorylation. Phosphorylation renders the protein unrecognizable either by the import machinery or the export machinery. One example of this is Cyclin B (Pines and Hunter, 1994). The second type of regulation involves a binary switch. In this case, modified and unmodified forms

of the protein are differentially recognized by an importer and an exporter. In this mode the kinase and phosphatase activities for the protein are separated, one being in the cytoplasm, the other in the nucleus, like Ataxin-1. Examples of this regulation include transcription factors such as Hog1 and Pho4 (Ferrigno et al., 1998; Kaffman et al., 1998; O'Neill et al., 1996). For the third mode, substrate anchors to some stable structure in either the cytoplasm or the nucleus. Phosphorylation of either substrate or the anchor triggers their dissociation and allows the transport of substrate to the other compartment. An example for this is Spc1 (Sty1) (Gaits et al., 1998).

In summary, thus far, my work demonstrated that phosphorylation and dephosphorylation of S776-Ataxin-1 are separated into two distinct subcellular sites. Ataxin-1 after phosphorylation in the cytoplasm by PKA shuttles to and is enriched in the nucleus where it is dephosphorylated by a serine/threonine phosphatase.

However, the role and mode of phosphorylation in regulation of Ataxin-1 subcellular localization is not totally understood. The second type of regulation, a binary switch, could be involved since Ataxin-1's kinase and phosphatase activity are separated in two locations. Further study on whether dephosphorylation of Ataxin-1-pS776 in the nucleus can influence its export could help answer this question.

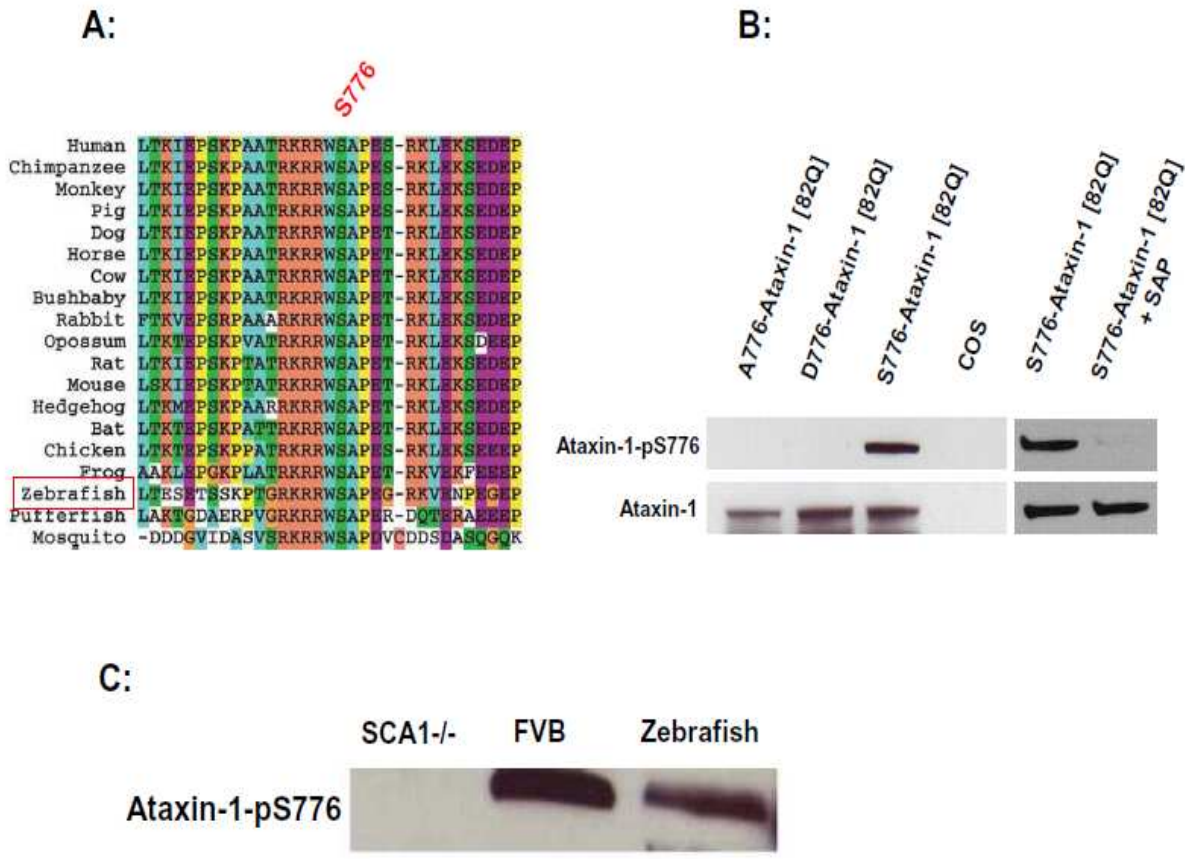

Figure 2. Conservation of S776 phosphorylation. A, S776 is evolutionarily conserved (de Chiara et al., 2009). B, The specificity of an Ataxin-1 phospho-S776 specific antibody, PN1248, was tested with COS cells expressing S776-Ataxin-1, A776-Ataxin-1 or D776-Ataxin-1. SAP: shrimp alkaline phosphatase. C, Endogenous Ataxin-1 in the brain of Zebrafish is phosphorylated at S776.

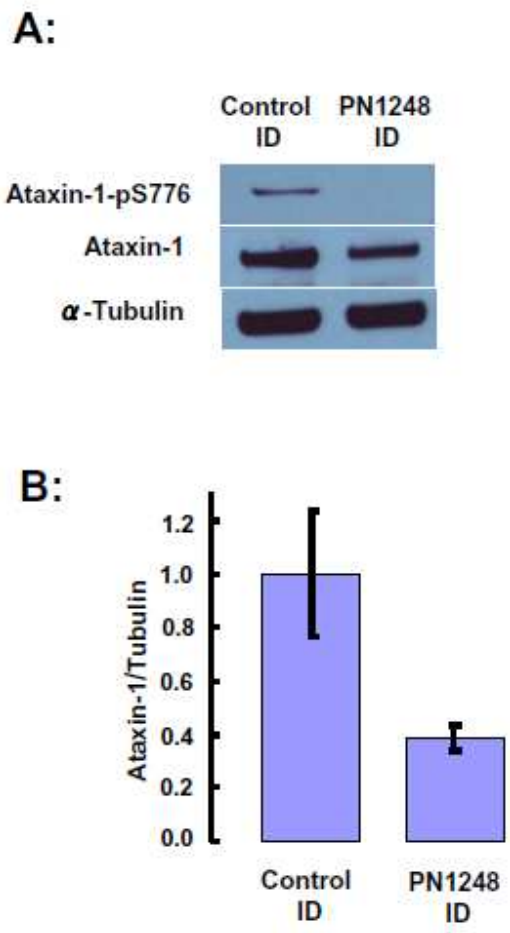

Figure 3. The proportion of S776-Ataxin-1 phosphorylated in the cerebellum. A, immunodepletion of Ataxin-1-pS776 decreased the amount of Ataxin-1 detected. B, quantitative analysis of the data in A, showing that immunodepletion of Ataxin-1-pS776 decreased the amount of Ataxin-1 detectable by 60%, suggesting that in the cerebellum, around 60% of Ataxin-1 is phosphorylated at S776.

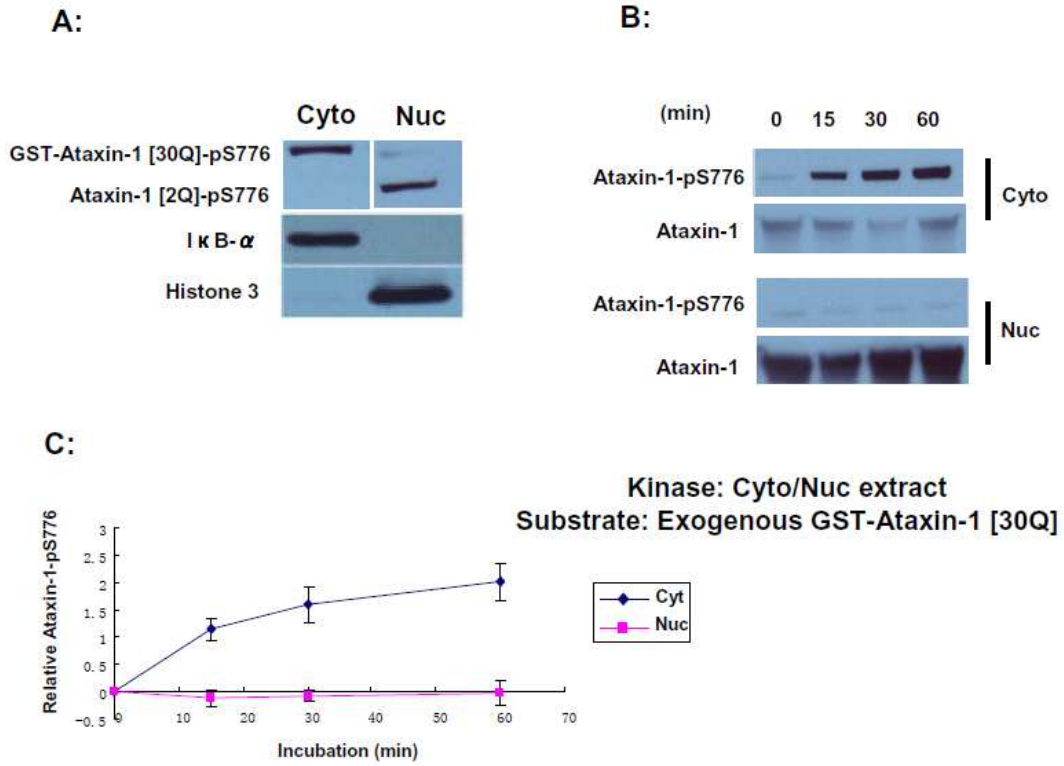

Figure 4. The kinase activity for S776-Ataxin-1 is in the cerebellar cytoplasm. A, in vitro kinase assay was run with cytoplasmic or nuclear extracts as a source of potential S776 kinase and GST-Ataxin-1 [30Q] as the substrate. The kinase activity was examined by western with PN1248 antibody. The cytoplasmic extract is capable of phosphorylating GST-Ataxin-1, while the nuclear extract can not, indicating that the kinase activity for Ataxin-1 at S776 is restricted to the cerebellar cytoplasm. Ataxin-1 [2Q] indicates mouse endogenous cerebelllar Ataxin-1. B, the same assay was run with different time points. C, quantitative analysis of the data in B. IκB-α and Histone 3 were used as cytoplasmic protein marker and nuclear protein marker, respectively, to assess fraction purity. n=3.

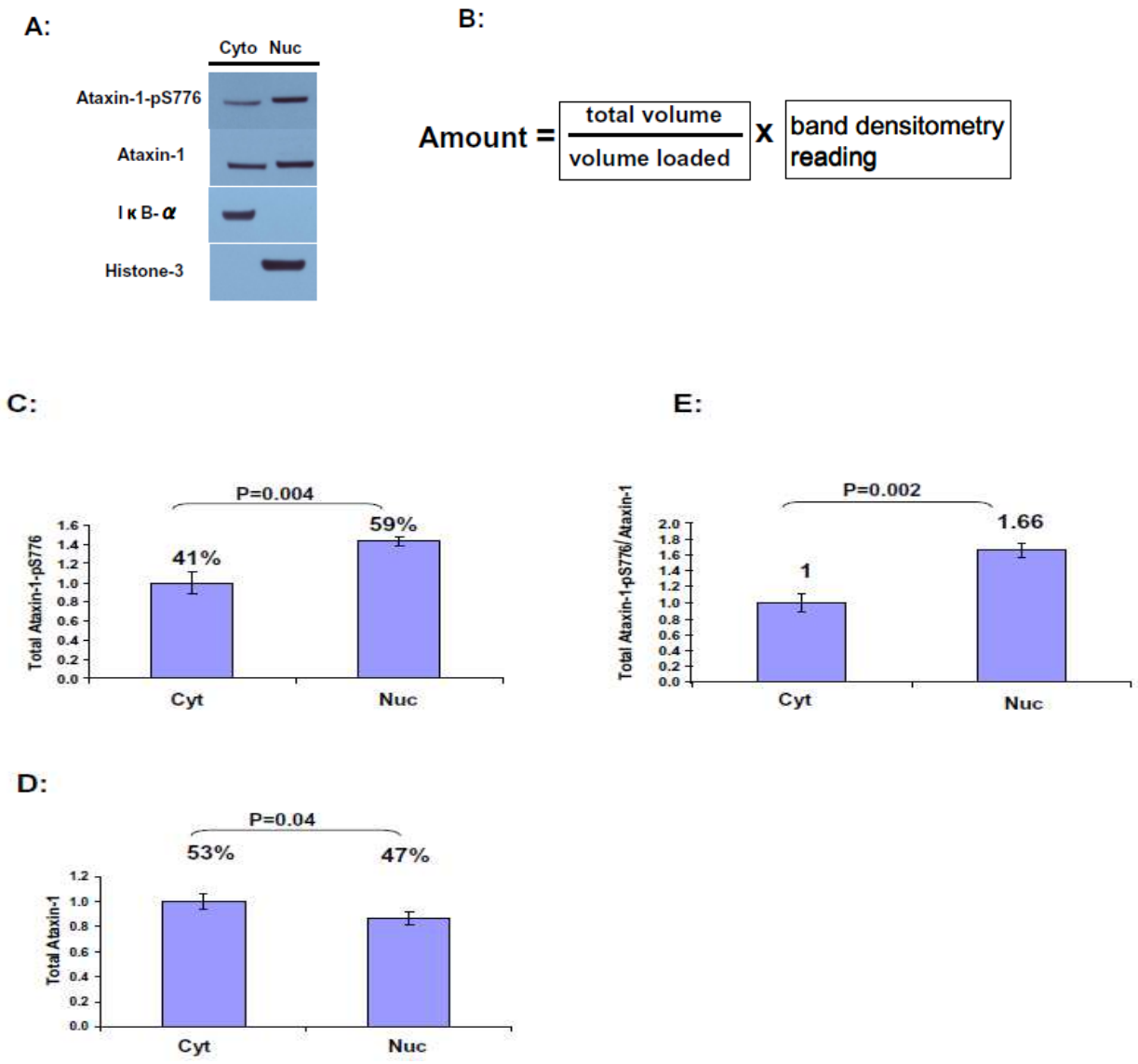

Figure 5. The subcellular distribution of total Ataxin-1 and Atxain-1-pS776 in the cerebellum. A, representative western blot of Ataxin-1-pS776 and Ataxin-1 in the mouse cerebellar cytoplasmic and nuclear extracts. B, the formula for calculating the total amount of Ataxin-1-pS776 and Ataxin-1 in the two sites. C, quantitative analysis of the total amount of Ataxin-1-pS776 (C), Ataxin-1 (D) and ratio (E) using the formula in B. Error bars indicate SEM, n =3.

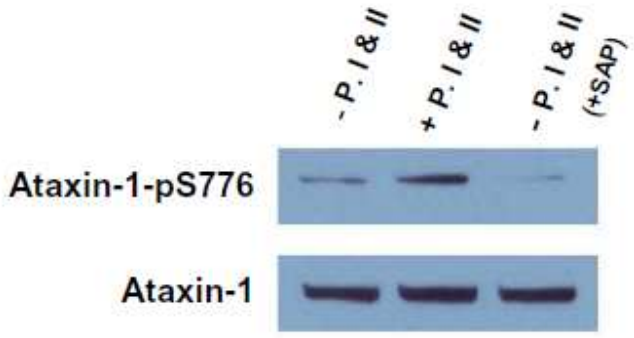

Figure 6. Dephosphorylation of Ataxin-1-pS776 in the cerebellum. The amount of Ataxin-1-pS776 detected in the lysate lacking phosphatase inhibitors was less than the lysate containing phosphatase inhibitors by one hour incubation at 37 °C. Addition of SAP further decreased Ataxin-1-pS776. This data suggests that an endogenous Ataxin-1-pS776 phosphatase is present in the cerebellar lysates. P.I &II: phosphatase inhibitors.

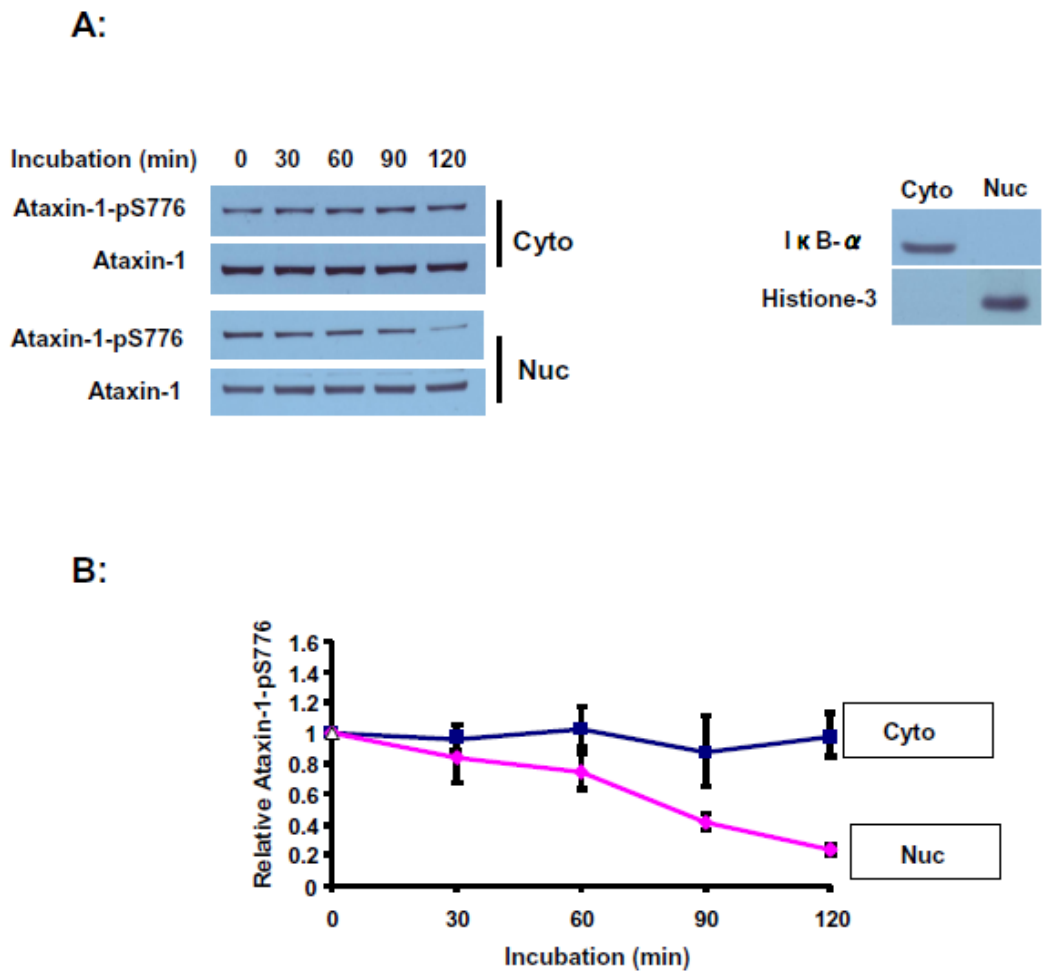

Figure 7. Ataxin-1-pS776 phosphatase activity is present in the cerebellar nucleus. A, cerebellar extract was separated into cytoplasmic and nuclear fractions and assessed for their ability to endogenously dephosphorylate Ataxin-1-pS776. Dephosphorylation of Ataxin-1-pS776 occurred only in the nuclear extract. B, the quantitative analysis of relative Ataxin-1 phosphorylation data at each time point from A as a time course curve, n=3.

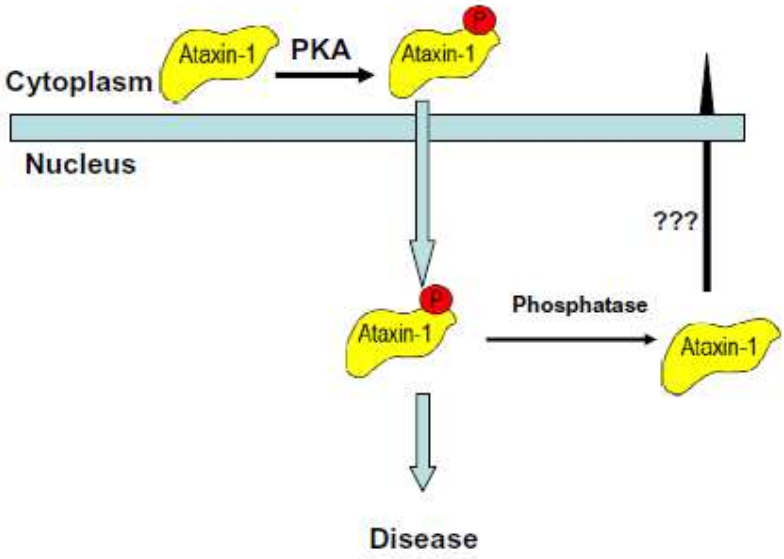

Figure 8. A proposed model. Ataxin-1 is phosphorylated at S776 in the cerebellar cytoplasm by PKA. Then phosphorylated Ataxin-1 shuttles to the nucleus and is enriched in the nucleus where it is dephosphorylated by a serine/threonine phosphatase.

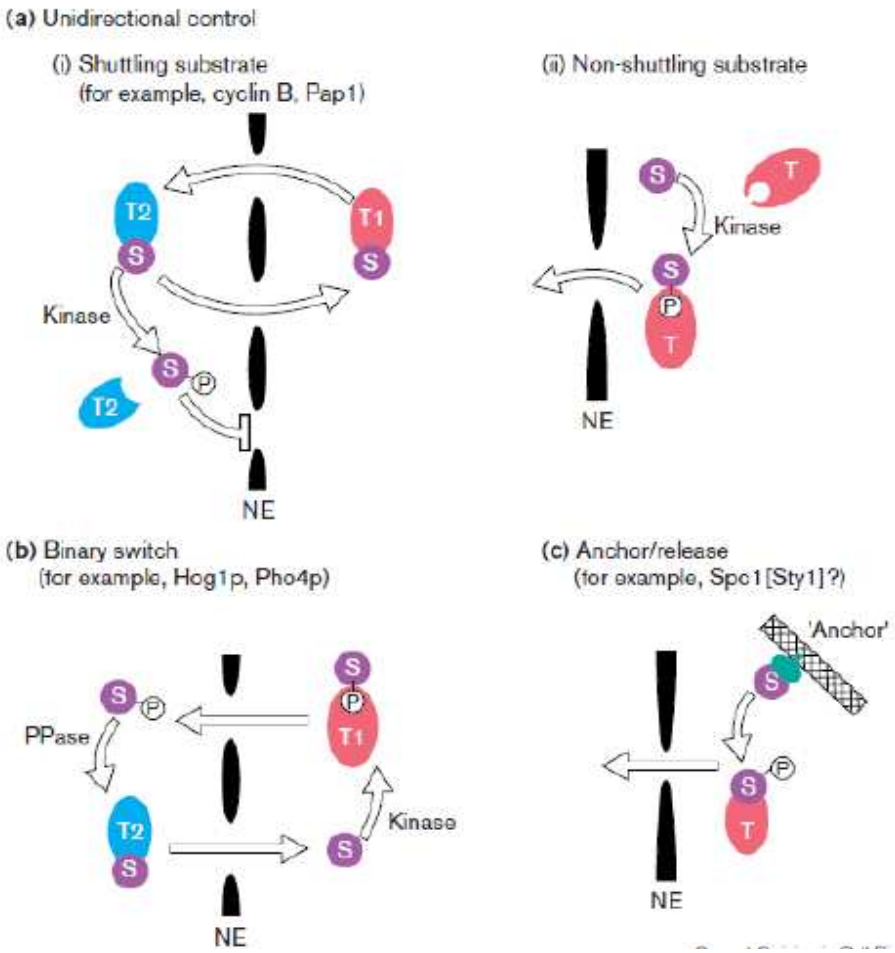

Figure 9. Phosphorylation regulates nucleocytoplasmic transport in three basic mechanisms. (a) Unidirectional control. Transport is regulated in one direction (i.e. import or export): the substrate (i) shuttles between the cytoplasm and nucleus, then is trapped in one compartment or (ii) undergoes a single regulated transport event. **(b)** Binary switch. Modified and unmodified forms of the substrate are differentially recognized by an importer and an exporter; signaling pathways determine the modification state of the substrate, and thus its intracellular localization. **(c)** Anchor/release. A substrate is anchored in either the cytoplasm or the nucleus by association with some cellular structure. Phosphorylation of the substrate releases it from the anchor, allowing it to be transported to the other compartment. T, T1 and T2 represent transport factors, S is the transport substrate, which is phosphorylated (P). NE, nuclear envelope; PPase, phosphatase. Note that nucleus and cytoplasm are interchangeable in these models. (Hood and Silver, 1999)

CHAPTER 3
THE ATAXIN-1-pS776 PHOSPHATASE AND ITS REGULATION

3. I: Introduction-the protein serine/threonine phosphatases (PSPs)

PSPs comprise the classic phosphoprotein phosphatases (PPPs) with the members of protein phosphatase 1 (PP1), PP2A, PP2B (commonly known as calcineurin), PP4, PP5, PP6, and PP7, and the manganese/magnesium ion (Mn^{2+}/Mg^{2+})-dependent protein phosphatases (PPMs), such as PP2C and pyruvate dehydrogenase (Shi, 2009) (Table 2).

PPP members form multiple holoenzymes through different combinations of a shared catalytic subunit with a wide variety of regulatory subunits. For example, the holoenzyme of PP2A contains a highly active core dimer composed of a catalytic subunit (C subunit) and a scaffolding subunit (A or PR65 subunit), and one of the many regulatory subunits (B subunit). PP2A has four families of B subunits; moreover, each family contains multiple isoforms and splice variants (Janssens and Goris, 2001; Lechward et al., 2001; Mumby, 2007). The regulatory subunits of PPP members are related with the holoenzyme activity, localization to a specific subcellular compartment, determination of substrate specificity, or being substrates themselves (Schonthal, 2001; Westermarck and Hahn, 2008); Janssens et al. 2005). For the members of the PPM family, while they do not have regulatory subunits, they contain various extension domains and conserved sequence motifs at their C and N termini that may have a similar role as PPP regulatory subunits (Schweighofer et al., 2004). Therefore, each holoenzyme is thought to have stringent substrate specificity and biological functions. Not only do the regulatory subunits or extension domains affect the holoenzyme activity and subcellular localization, other factors may also play a role. For example, Ca^{2+}-calmodulin by formation of a

complex with PP7 appears to have an inhibitory effect on PP7 phosphatase activity (Kutuzov et al., 2001). For PP1, there are two major nuclear targeting factors, NIPP1 and p99 (also known as PNUTS) (Jagiello et al., 1995). Moreover, currently, the list of PP1 subcellular targeting subunits is growing due to several subsequent studies (Jagiello et al., 1995; Kwiek et al., 2006; Llorian et al., 2004; Sagara et al., 2003; Tran et al., 2004; Trinkle-Mulcahy et al., 2006).

PPP members are involved in many cellular processes except PP7 which is unique to plants and thought to regulate blue-light signaling (Moller et al., 2003). For example, PP2A plays an important role in numerous cellular processes such as development, cell proliferation and death, cell mobility, cytoskeleton dynamics, control of the cell cycle, and regulation of numerous signaling pathways (Janssens and Goris, 2001). Although most of the PPP members are widely expressed in eukaryotic cells, individual phosphatases may have variable expression and subcelluar localization patterns depending on the type of cell and tissue. For example, while PP2A and PP5 are found in all mammalian tissues examined, they have a high expression in the brain. In addition, the expression pattern in different cellular compartments varies. For PP5, in humans and *S. cerevisiae,* it is both cytoplasmic and nuclear (Borthwick et al., 2001; Jeong et al., 2003). For PP2A, while the A and C subunits are found to be more concentrated in the nucleus than in the cytoplasm with no localization in nucleoli (Turowski et al., 1997), the subcellular residence of B subunits differs depending on isoform and splice variant. For example, B55δ, B56α, B56β and B56ε reside exclusively in the cytoplasm, whereas other B55 subunits, such as B56δ and B56γ are highly

enriched in the nucleus, and PR48 and PR90 are exclusively nuclear (McCright et al., 1996; Strack et al., 1999; Yan et al., 2000).

Phosphatases, except the PPM family and PP7, can be inhibited by several natural toxin inhibitors including microcystins, calyculins, tautomycin and okadaic acid (OA) (Dawson and Holmes, 1999). OA is a tumor-promoting C38 polyether fatty acid produced by marine dinoflagellates (Cohen et al., 1990; Dawson and Holmes, 1999; Suganuma et al., 1988). It is one of the most frequently used inhibitor for phosphatase research and exhibits variable inhibitory potential on different PPP family members. It inhibits PP2A most strongly with an inhibitory constant of approximately 0.1 nM (IC50≈0.1 nM), and PP4 and PP6 similarly as PP2A. It's less potent for PP1 and PP2B. It inhibits PP1 with an inhibitory constant of 10 nM (IC50 ≈10 nM), which is about 100-fold less potent than its inhibition for PP2A, and inhibits PP2B even more weakly (IC50 ≈1–2 μM) (Bialojan and Takai, 1988; Holmes and Boland, 1993; MacKintosh et al., 1990) (Table 2).

Having demonstrated that the phosphatase activity for Ataxin-1-pS776 is localized to the nuclei of cerebellar cells, the next step was to determine the phosphatase involved. In order to achieve this aim, a mouse cerebellar extract-based dephosphorylation assay and several other approaches were utilized.

3. II: Materials and Methods

Western blotting. Western blotting was done as described previously. First antibodies used were anti-PP2A-C and anti-PP6-C (Millipore); anti-PP1-C (Santa Cruz); anti-PP5-C (Bethyl lab); anti-PP4-C (R&D Systems), anti-14-3-3 ε, anti-14-3-3 β and anti-14-3-3 ζ (Santa Cruz). Antibodies were diluted with 5% (w/v) milk in PBST.

Endogenous dephosphorylation assay with OA. OA (Calbiochem) was added to each aliquot of nuclear extract to a final concentration of 100 nM; extracts were incubated at 37 ºC for different time periods up to 2 hrs (refer to Figure 10). Dephosphorylation was terminated by adding sample buffer and heating in boiling water for 5 min. Western blot was performed as described above. To narrow down the phosphatase candidates, aliquots of nuclear extracts were incubated at 37 ºC with increasing concentrations of OA (0-100 nM) for 2 hrs (Refer to Figure 11). Nuclear extract without the addition of OA and without incubation at 37 ºC was included as control.

In vitro phosphatase assay. The 1 hr cold kinase assay (40 µL) was set up as described previously. Glutathione-agarose resin (GST beads) (Invitrogen) was washed 3× with Tris-DTT buffer (20 mM Tris-CL, pH 7.5, 1 mM DTT) containing protease inhibitor only. Phosphorylated GST-Ataxin-1 from the kinase reaction was collected by GST-pull down for 35 min with 40 µL GST bead slurry in 400 µL Tris-DTT buffer. The beads were spun, washed 3× with Tris-DTT buffer and incubated with 0.25 U purified PP2A A/C core enzyme (Millipore 14-111) for 7.5 min at 30 °C in a reaction mixture containing 50 mM Tris, pH 7.0, 20 mM 2-mercaptoethanol, 2 mM $MnCl_2$ and 0.1% BSA (Deng et al., 2009). The beads lacking PP2A A/C enzyme and kept on ice were included as control. The

reaction was stopped by adding sample buffer and heating in boiling water for 5 min. Samples were subject to western blot analysis.

Transfection of siRNA. Hela cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Cells were transfected with scrambled siRNA or PP2A-C siRNA (Dharmacon) using QIAGEN HiPerFect transfection Reagent. Twenty-four hours post transfection, cells were transfected with Ataxin-1 plasmid using Invitrogen Lipofectamine PLUS reagent. 48 hrs after Ataxin-1 plasmid transfection, cells were lysed with the lysis buffer described above and lysate was used for western blot.

Immunodepletion. 0.05 mg of nuclear extract was diluted to 150 µL with brain lysis buffer. The extract was precleared with protein G Sepharose beads and spun. Supernatant was transferred to gel-slick tubes, PP2A-C antibody was added and the mixture was incubated for 3 hrs with rocking at 4 ºC. BSA-blocked protein G Sepharose beads were added for 2 hrs with rocking at 4 ºC and spun. The post-immunodepletion supernatant (post-ID supernatant) was saved and kept on ice. Aliquots of post-ID supernatants were transferred to new tubes and incubated at 37 ºC for up to 2 hrs (refer to Figure 15). Aliquots of post-ID supernatants were also analyzed by western blot for PP2A-C to test the efficiency of immunodepletion. Control ID was done the same way except that the same amount of lysis buffer instead of PP2A-C antibody was used.

Co-immunoprecipitation. 0.5 mg of mouse cerebellar lysate made with NP-40 lysis buffer was diluted with lysis buffer to 500 µL. The lysate was precleared for 1 hr with protein G Sepharose bead slurry and spun. The supernatant was saved. PP2A-C or PP5-C

antibody was added to the supernatant and rocked overnight at 4 ºC on a rugged rotator. The following day, the samples were added with BSA-blocked protein G Sepharose beads and incubated for 2 hrs. Lysates were spun, supernatant discarded and pellet washed 4× with lysis buffer, resuspended in sample buffer, boiled and supernatant was loaded on a gel. The negative control for co-immunoprecipitation was prepared with the same condition as described above, except that it was carried out without the addition of antibodies.

GST pull down. BSA-blocked GST beads were incubated with GST-Ataxin-1 in 500 µL NP-40 lysis buffer and rocked for 2 hrs at 4 ºC. GST beads were washed 3× and incubated with 500 µg cerebellar lysate in 500 µL buffer for 3 hrs at 4 °C on a rugged rotator. The sample was spun and supernatant was discarded. GST beads were washed 3× with lysis buffer, resuspended in sample buffer, boiled and supernatant was loaded on a gel.

Indirect cerebellar immunofluorescence. Mice were anesthetized with 5 µL of 100 mg/mL ketamine and 2 µL of 20 mg/µL xylazine. Sedated mice were perfused transcardially with 10% phosphate buffered formalin (Fisher #SF100-4). Brains were removed, stored overnight in 10% phosphate buffered formalin, and then transferred to PBS at 4 ºC for long-term storage. Brains were sectioned (50 µM sagittal sections) near vermis on a vibratome (speed set to 1.8 and amplitude to 6.5 on Vibratome Series 1000). Sections were collected in PBS. Epitope was unmasked by replacing PBS with 0.01 M Urea and heating in microwave for 15 s. The Urea solution was discarded. A blocking solution (2% goat sera, 0.3% Triton-X in PBS) was added to sections and incubated

overnight on a rotating rocker at 4 °C. The following day, first antibody was added to blocking solution at the following dilutions: mouse anti-14-3-3 β (Santa Cruz #SC-1657) at 1:100, rabbit anti-14-3-3 ζ (Santa Cruz #SC-1019) at 1:50 and rabbit anti-14-3-3 ε (Santa Cruz #SC-1020) at 1:100. Sections were incubated with the first antibodies on a rotator for 48-72 hrs at 4 ºC, washed 4× for 20 min each in 1 mL PBS on a rocker at room temperature, and incubated with secondary antibodies in blocking solution for 48 hrs at 4ºC. Secondary antibodies used were: Dylight 549-conjugated affinity pure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch #715-505-151) at 1:250 and Dylight 549-conjugated affinity pure donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch #711-505-152) at 1:250. After secondary antibody incubation, sections were washed 4× for 20 min each in 1 mL PBS and mounted on slides (Thermo Scientific 24x40) with 60 µL 50°C glycerol gelatin (Sigma #GG1-15ML) containing 4 mg/mL n-Propyl Gallate (n-PG). Images for the slides were taken with a FluoView inverted confocal, laserscanning microscope (Olympus #FV1000 IX2).

Statistic analysis and quantification. Densitometry was done with films scanned on a BioRad Gel Dock GS700 600 dpi flatbed densitometer. Western blot band density was analyzed with ImageQuant software. All densitometry analysis of western data was normalized to total Ataxin-1 levels. For Figure 22, total Ataxin-1 meant the sum of Ataxin-1 level from both cytoplasmic and nuclear fractions.

To calculate the IC50 of OA with cerebellar nuclear extract, the level of dephosphorylation at each OA concentration with incubation at 37°C for 2 hrs was

determined by subtracting the Ataxin-1-pS776 level at that point from that without incubation. The OA concentration that resulted in half inhibition of dephosphorylation was its IC50. All experiments were carried out using three individual FVB mouse cerebella or independent cell transfections in triplicate. Western blot images are representative of repeated experiments.

3. III: Results

Okadaic acid (OA) inhibition of S776 dephosphorylation. As indicated above for the PPP family of serine/threonine phosphatase, some phosphatases are not sensitive to inhibition by OA. Thus, as a first step towards identifying the candidate phosphatases for Ataxin-1-pS776, the nuclear dephosphorylation assay was treated with a high concentration of OA. As shown in Figure 10, the reduction in nuclear Ataxin-1-pS776 normally seen in this condition was blocked by 100 nM OA. Thus, the phosphatase for Ataxin-1-pS776 is OA sensitive. While PP2B is sensitive to OA, it is inhibited only at a very high concentration of OA (IC50 ≈1–2 μM) (Table 2). Thus, the data suggests PP1, PP2A, PP4, PP5 and PP6 as the possible phosphatase candidates.

To examine further the phosphatase involved in Ataxin-1-pS776 dephosphorylation, a cerebellar nuclear extract was treated for two hours with increasing concentrations of OA. As shown in Figure 11, treatment of nuclear extracts with OA resulted in increased Ataxin-1-pS776 in a dose-dependent manner. Starting at 1 nM, OA treatment increased the level of Ataxin-1-pS776 without affecting total Ataxin-1 level. Analysis of the ability of OA to block dephosphorylation of Ataxin-1-pS776 indicated an IC50 between 1 and 10 nM for the cerebellar nuclear Ataxin-1-pS776 phosphatase. OA inhibits several protein phosphatases with reported IC50s in this range as shown in Table 2. These IC50s were determined with pure phosphatases. It is suggested that IC50s with cellular extracts (nuclear extract here) are likely to be much higher (around a 10-100 fold increase) than IC50s determined with pure phosphatases (Boudreau and Hoskin, 2005; Fernandez et al., 2002; Jaramillo-Babb et al., 1996; Schonthal, 1998).

Interaction of PP2A with Ataxin-1. Protein phosphatases often form relatively stable complexes with their substrates (Bakan et al., 2008; Sontag et al., 1996; Zhu et al., 1999). To test which phosphatase candidate could form a complex with Ataxin-1, GST-Ataxin-1 [30Q] was used to 'pull down' endogenous phosphatases from a mouse cerebellar lysate (Figure 12A). While PP2A-C bound to GST-Ataxin-1 [30Q], other phosphatases expressed highly in the cerebellum, PP1-C, PP4-C, and PP5C, did not form detectable stable complexes with Ataxin-1. Moreover, endogenous Ataxin-1 could be co-immunoprecipitated along with endogenous PP2A from a wild type mouse cerebellar lysate (Figure 12B). In contrast, Ataxin-1 could not be co-immunoprecipitated with PP5-C. Based on this, PP2A was selected for study as a lead candidate cerebellar phosphatase for Ataxin-1-pS776.

PP2A effectively dephosphorylates Ataxin-1-pS776 in vitro. If PP2A is the phosphatase candidate for Ataxin-1-pS776, it should be able to dephosphorylate Ataxin-1-pS776. To test this hypothesis, the ability of PP2A to dephosphorylate Ataxin-1-pS776 was examined with an in vitro phosphatase assay. Ataxin-1-pS776 obtained from an in vitro kinase assay as described above was incubated with the PP2A core enzyme at 30 °C. After just a 7.5 min incubation, the PP2A core enzyme dramatically decreased the level of Ataxin-1-pS776 (Figure 13), demonstrating that PP2A can effectively dephosphorylate Ataxin-1-pS776. These results support PP2A as the phosphatase for Ataxin-1-pS776.

Depletion of PP2A decreases dephosphorylation of Ataxin-1-pS776. As an additional means of further investigating whether PP2A is the Ataxin-1-pS776 phosphatase, two approaches were used to examine the effect of depleting PP2A had on

Ataxin-1-pS776 dephosphorylation. First, a siRNA specific to PP2A-C was co-transfected into Hela cells along with a plasmid encoding Ataxin-1. Figure 14 shows that knockdown of PP2A-C significantly increased the amount of Ataxin-1-pS776 obtained from transfected HeLa cells. The PP2A-C siRNA treatment had no effect on total Ataxin-1 level and did not affect levels of the other phosphatases, PP4, PP5 and PP1.

Second, an immunodepletion strategy was utilized. Endogenous PP2A-C was immunodepleted from a mouse cerebellar nuclear extract and the post-immunodepleted extract was incubated at 37°C for up to two hours as described above. The ability of the PP2A-C depleted extract to dephosphorylate endogenous Ataxin-1-pS776 was compared to an extract that was mock depleted for PP2A. As shown in Figure 15, immunodepletion of PP2A substantially reduced the Ataxin-1-pS776 dephosphorylation ability of a cerebellar nuclear extract. Thus, by two approaches depletion of PP2A led to a decrease in Ataxin-1-pS776 dephosphorylation activity. These results are consistent with PP2A as the cerebellar Ataxin-1-pS776 phosphatase.

Mechanistic basis for lack of S776 dephosphorylation in the cytoplasm. In the previous chapter, it was shown that the phosphatase activity for Ataxin-1-pS776 was restricted to the cerebellar nucleus. Here the data demonstrated that the phosphatase is PP2A. Thus, Ataxin-1-pS776 is dephosphorylated by PP2A in the cerebellar nucleus. Why is the cytoplasmic Ataxin-1-pS776 not dephosphorylated by PP2A? There are several possible reasons.

To test whether the lack of dephosphorylation with a cytoplasmic extract was due to the absence of PP2A in the cytoplasm, the subcellular distribution of PP2A was

examined. Figure 16 showed that PP2A was detected in both the nucleus and cytoplasm. Several other OA sensitive phosphatases were examined as well. As shown in Figure 16, in addition to PP2A, PP1 also was enriched in the nuclei of cerebellar cells while PP5-C and PP4-C were more localized to the cytoplasm.

Perhaps, the holoenzymes of PP2A in the cytoplasm and nucleus differ in their regulatory subunits and, thus, differ in their substrate specificity (Kamibayashi et al., 1994; McCright et al., 1996; Strack et al., 1998; Tehrani et al., 1996). PP2A has many different regulatory subunits (Figure 17). One possibility is that only PP2A in the nucleus is able to interact with Ataxin-1 due to a difference in regulatory subunits. To test this hypothesis, GST-Ataxin-1 [30Q] was used to 'pull down' endogenous PP2A phosphatase from cerebllar cytoplasmic and nuclear extracts. As shown in Figure 18, both cytoplasmic and nuclear PP2A-C bound to GST-Ataxin-1 [30Q], suggesting that PP2A was able to interact with Ataxin-1 in both cerebellar cytoplasm and nucleus.

Finally, the hypothesis that a protein by associating with Ataxin-1-pS776 in the cytoplasm protects Ataxin-1-pS776 from dephosphorylation by PP2A was examined. It is known that 14-3-3 binds to phosphoserine/phosphothreonine motifs in a variety of cellular proteins, thus inhibiting their dephosphorylation (Fu et al., 2000; Muslin et al., 1996; Tzivion and Avruch, 2002; Yaffe et al., 1997). Moreover, it was previously shown that 14-3-3 binds to phosphorylated Ataxin-1 at S776 (Chen et al., 2003). To test whether association of 14-3-3 with Ataxin-1 interfered with the dephosphorylation of S776, a 14-3-3 specific inhibitor, R18 peptide, was added to cytoplasmic extracts run for the endogenous dephosphorylation assay described above. R18 is a 20-mer peptide that

specifically binds to 14-3-3 proteins without isoform selectivity (Masters and Fu, 2001). Moreover, it was shown that R18 was able to competitively interfere with 14-3-3/ligand interactions (Masters et al., 1999; Wang et al., 1999; Zhang et al., 1999). With the addition of R18, cytoplasmic Ataxin-1-pS776 was dephosphorylated upon incubation at 37ºC (Figure 19). This finding suggested that 14-3-3 bound to Ataxin-1-pS776 in the cytoplasm blocked accessibility of Ataxin-1-pS776 to PP2A, thus inhibiting Ataxin-1-pS776 dephosphorylation by PP2A.

The subcellular distribution of 14-3-3. Since nuclear Ataxin-1-pS776 can be dephosphorylated by PP2A, it raises the possibility that 14-3-3 is not present in the nucleus such that nuclear 14-3-3 can not interrupt Ataxin-1-pS776 dephosphorylation. To test whether 14-3-3 is deficient in the nucleus, the cerebellar cytoplasmic and nuclear distribution of 14-3-3 protein was examined. 14-3-3 has several isoforms in mammals (β, γ, ε, η, σ, τ and ζ). The isoforms with antibodies available were examined. As shown in Figure 20A&B, by western blotting of nuclear and cytoplasmic extracts, all 14-3-3 isoforms examined were almost exclusively localized to the cytoplasm. This result was supported by immunofluorescence data from mouse cerebella sections showing that almost all of 14-3-3 resides in the cytoplasm of cerebellar cells (Figure 20C). These results are consistent with an insufficient level of 14-3-3 in the nucleus to block dephosphorylation.

Effect of 14-3-3 binding on Ataxin-1 cytoplasmic and nuclear distribution: S776-Ataxin-1 [30Q] *vs.* D776-Ataxin-1 [30Q] *vs.* A776-Ataxin-1 [30Q]. As shown in Figure 21, the binding motif of Ataxin-1-pS776 for 14-3-3 is adjacent to Ataxin-1's NLS.

To investigate whether 14-3-3 association with Ataxin-1-pS776 could also affect Ataxin-1's localization to the nucleus, the cerebellar cytoplasmic and nuclear distribution of Ataxin-1 in mice expressing forms of Ataxin-1-[30Q] with varying abilities to bind 14-3-3. Mice expressing these forms of Ataxin-1 were bred to SCA1-/- background to get rid of mouse endogenous Ataxin-1 [2Q]. Previously, it was shown that D776-Ataxin-1 and A776-Ataxin-1 do not bind 14-3-3 while S776-Ataxin-1 does (Chen et al., 2003; de Chiara et al., 2009). As shown in Figure 22, D776-Ataxin-1 [30Q] and A776-Ataxin-1 [30Q], forms of Ataxin-1 that do not bind 14-3-3, were more predominantly localized to the nucleus than S776-Ataxin-1 [30Q] which binds 14-3-3. Thus, these results suggest that binding of 14-3-3 near NLS of Ataxin-1 affected Ataxin-1's NLS function. Further, these data suggest that for Ataxin-1 to be transported into the nucleus, 14-3-3 must be dissociated.

3. IV: Discussion

To provide further insight into regulation of S776 phosphorylation, this chapter examined the dephosphorylation process of Ataxin-1-pS776. Several lines of evidence were provided supporting PP2A as the phosphatase that dephosphorylates Ataxin-1-pS776. In vitro PP2A was able to readily dephosphorylate Atxain-1-pS776. Treatment with OA and selective siRNA knockdown of PP2A increased the amount of Ataxin-1-pS776 in cerebellar lysates and transfected HeLa cells, respectively. Notably, immunodepletion of PP2A decreased the ability of a cerebellar nuclear extract to dephosphorylate endogenous Ataxin-1-pS776. In addition, PP2A forms a complex with Ataxin-1 in the cerebellum. The reason why cytoplasmic Ataxin-1-pS776 can not be

dephosphorylated and the effect of 14-3-3 binding on Ataxin-1 cytoplasmic and nucleuar distribution were also examined.

PP2A is a major brain serine/threonine phosphatase, which participates in a variety of signaling pathways that modulate neuronal functions and activity (Price and Mumby, 1999). It is particularly abundant in the brain, accounting for about 1% of total cellular protein mass (Xu et al., 2008). Although many phosphatases including PP2A were present in the cerebellar cytoplasmic extract, we found that these phosphatases were unable to dephosphorylate Ataxin-1-pS776. The reason why cytoplasmic Ataxin-1-pS776 can not be dephosphorylated by PP2A was shown to be due to 14-3-3 binding, which protected Ataxin-1-pS776 from dephosphorylation by PP2A in the cytoplasm. This is supported by the fact that all 14-3-3 isoforms examined are almost exclusively localized to the cytoplasm.

The association of 14-3-3 to Ataxin-1-pS776 not only blocked the accessibility of it for PP2A but also affected Ataxin-1's NLS and its shuttling to the nucleus. Complex formation is one means known to regulate protein nucleocytoplasmic transport through intermolecular masking of either the NLS or NES of a protein (Sorokin et al., 2007). The NLS of Ataxin-1 contains S776 and is in the middle of or immediately adjacent to two other functional motifs, 14-3-3 binding motif and U2AF homology motif (UHM) ligand motif (ULM) as shown in Figure 21. Phosphorylation at S776 induced 14-3-3 to bind to Ataxin-1 at 14-3-3 binding motif (Chen et al., 2003). Binding of 14-3-3 to its binding motif adjacent to protein's NLS was shown to shield the recognition of NLS by nuclear import machinery, resulting in enhanced localization of protein in the cytoplasm

(Sekimoto et al., 2004; Yaffe, 2002). This could explain why S776-Ataxin-1 [30Q] was more localized to the cytoplasm than D776-Ataxin-1 [30Q] and A776-Ataxin-1 [30Q] due to their different binding abilities to 14-3-3. A similar example for this is c-Abl (Pendergast, 2005; Yoshida et al., 2005). Taken together, binding of 14-3-3 to Ataxin-1 has two effects: inhibition of dephosphorylation by PP2A and inhibition of Ataxin-1's shuttling to the nucleus.

Phosphorylation can regulate protein nucleocytoplasmic shuttling through several modes (Hood and Silver, 1999). In the case of Ataxin-1, phosphorylation at S776 induces 14-3-3 binding and binding of 14-3-3 affects Ataxin-1's nucleocytoplasmic shuttling. Thus, phosphorylation at S776 and Ataxin-1 complex formation with 14-3-3 regulate Ataxin-1's cytoplasmic transport to the nucleus. Ataxin-1 also interacts with U2AF65 and RBM17 via its ULM motif (de Chiara et al., 2009). The ULM motif overlaps with Ataxin-1's NLS and 14-3-3 binding motif. It is not clear whether Ataxin-1's NLS can mediate its nuclear export as seen in some proteins, for example heterogeneous nuclear ribonucleoprotein A1 (Michael et al., 1995). If it does, it will be interesting to know whether complex formation with RBM17 and U2AF65 in the nucleus affects Ataxin-1's nuclear export, and whether their effects on shuttling are mediated through phosphorylation at S776. Also it remains to be clarified whether dephosphorylation of Ataxin-1-pS776 in the nucleus affects its binding ability for U2AF65 and RBM17 in the nucleus.

The physical masking of NLS must be removed in order to allow recognition and binding by the import or export receptor. The data here suggest that in order for

Ataxin-1-pS776 to move into the nucleus efficiently, 14-3-3 needs to be dissociated. Considering Ataxin-1-pS776 is enriched in the nucleus, the dissociation process must be rapid. Association/dissociation of 14-3-3 with its client proteins is regulated. One regulation is dephosphorylation of client proteins by phosphatases, thus 14-3-3 can not bind. Another regulation is reversible phosphorylation of 14-3-3 itself. Protein kinase C, c-Jun N-terminal kinase (JNK), AKT, casein kinase 1 and Bcr are kinases able to phosphorylate specific 14-3-3 isoforms at certain sites (Aitken, 2006). Phosphorylation of 14-3-3 was shown to result in decreased binding to its client proteins. For example, phosphorylation of 14-3-3 by JNK released 14-3-3 binding to c-Abl, resulting in the translocation of c-Abl from cytoplasm to nucleus (Yoshida et al., 2005). In addition, phosphorylation may also influence the dimerization of 14-3-3 protein monomers. One example is S58D mutation in 14-3-3 zeta, which resulted in a compromised ability of it to dimerize (Powell et al., 2003). Since different heterodimers play an important role in controlling 14-3-3 binding properties, the change in its dimerization by phosphorylation may change its target protein or bind to the same target with different affinities (Wilker and Yaffe, 2004).

In summary, in this chapter, the first direct evidence was provided showing that PP2A is the phosphatase for Ataxin-1-pS776 in the cerebellum. A mechanistic basis for the lack of Ataxin-1-pS776 dephosphorylation in the cerebellar cytoplasm was also examined. 14-3-3 by associating with Ataxin-1-pS776 protects it from dephosphorylation by PP2A in the cytoplasm where 14-3-3 is much enriched. Association of 14-3-3 to Ataxin-1 also interferes its shuttling to the nucleus. These findings are important for

understanding the regulation of Ataxin-1 phosphorylation and kinetics of Ataxin-1 subcellular shuttling in the mammalian cerebella.

Phosphatase	Expression in Purkinje	OA IC50
PP1	High	~ 10 nM
PP2A	High	0.1-0.3 nM
PP4	High	0.1-0.3 nM
PP5	High	1-5 nM
PP6	Low	0.1-0.3 nM
PP7	No	Not sensitive
PP2B	High	1–2 μM
PP2C	High	Not sensitive

Table 2. Expression of protein serine/threonine phosphatases in Purkinje cells and their sensitivity to okadaic acid (OA). OA IC50s were determined with pure phosphatases. (Bialojan and Takai, 1988; Holmes and Boland, 1993; MacKintosh et al., 1990)

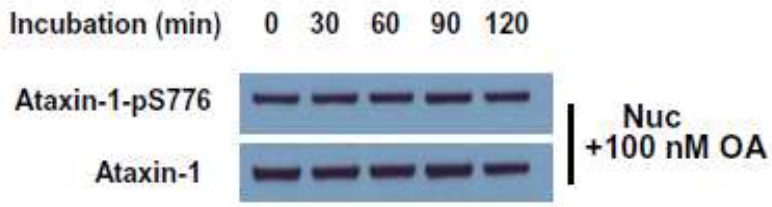

Figure 10. Dephosphorylation of Ataxin-1-pS776 in the nuclear extract is completely inhibited by the addition of 100 nM OA.

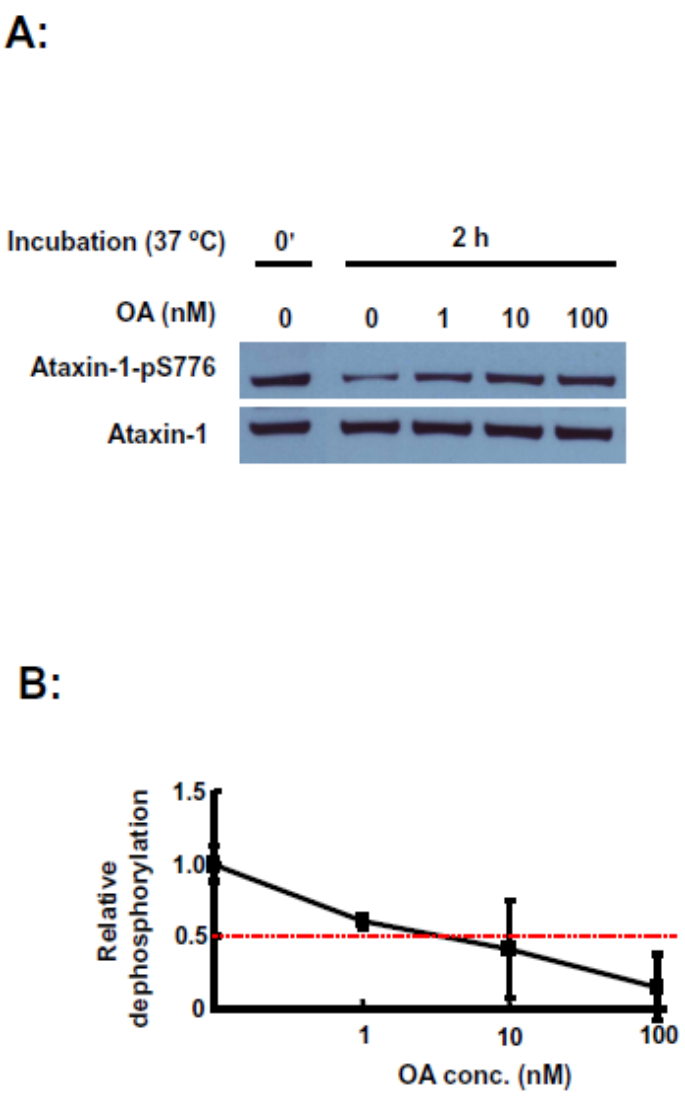

Figure 11. OA inhibits the Ataxin-1-pS776 phosphatase with an IC50 between 1 and 10 nM. A, Ataxin-1-pS776 dephosphorylation is inhibited by varying concentrations of OA (0-100 nM). B, the quantitative analysis of the data for A, n=3. The curve was made with Y as relative dephosphorylation.

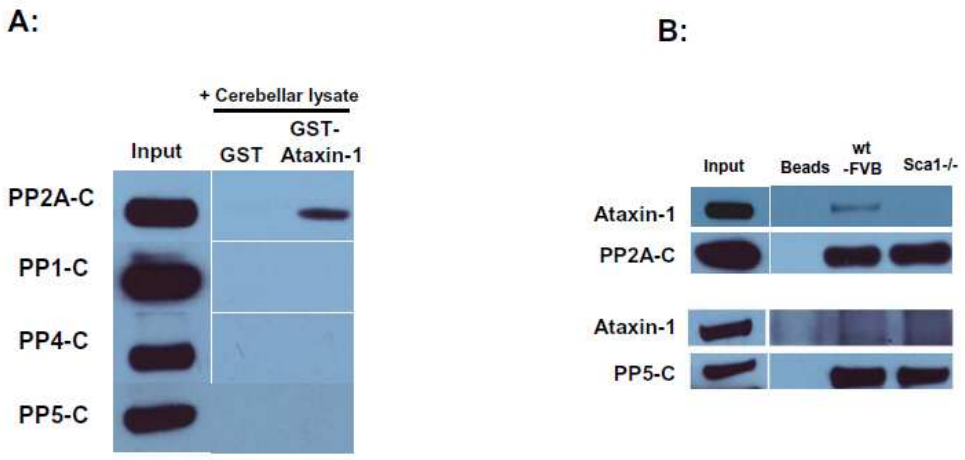

Figure 12. PP2A interacts with Ataxin-1. A, PP2A-C but not PP1-C or PP4-C or PP5-C in the cerebellar lysate bound to exogenous GST-Ataxin-1. GST alone protein was included as control. B, cerebellar lysates were immunoprecipitated with anti-PP2A-C antibody to capture PP2A-C and co-immunoprecipitated Ataxin-1 was detected by immunoblotting with anti-Ataxin-1 antibody. Co-immunoprecipitation of PP5-C and Ataxin-1 was included as control. Sca1-/-: Ataxin-1 knock out mice.

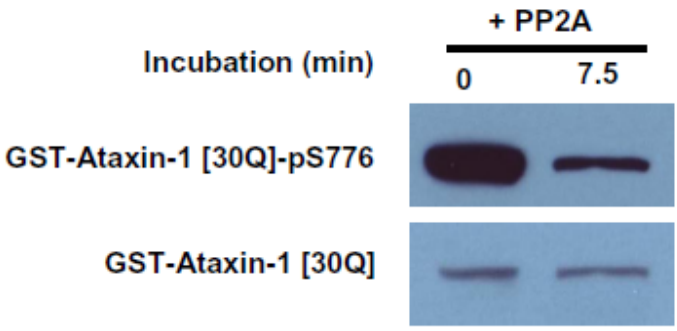

Figure 13. PP2A effectively dephosphorylates Ataxin-1-pS776. In vitro phosphatase assay showing that PP2A can directly and effectively dephosphorylate Ataxin-1-pS776.

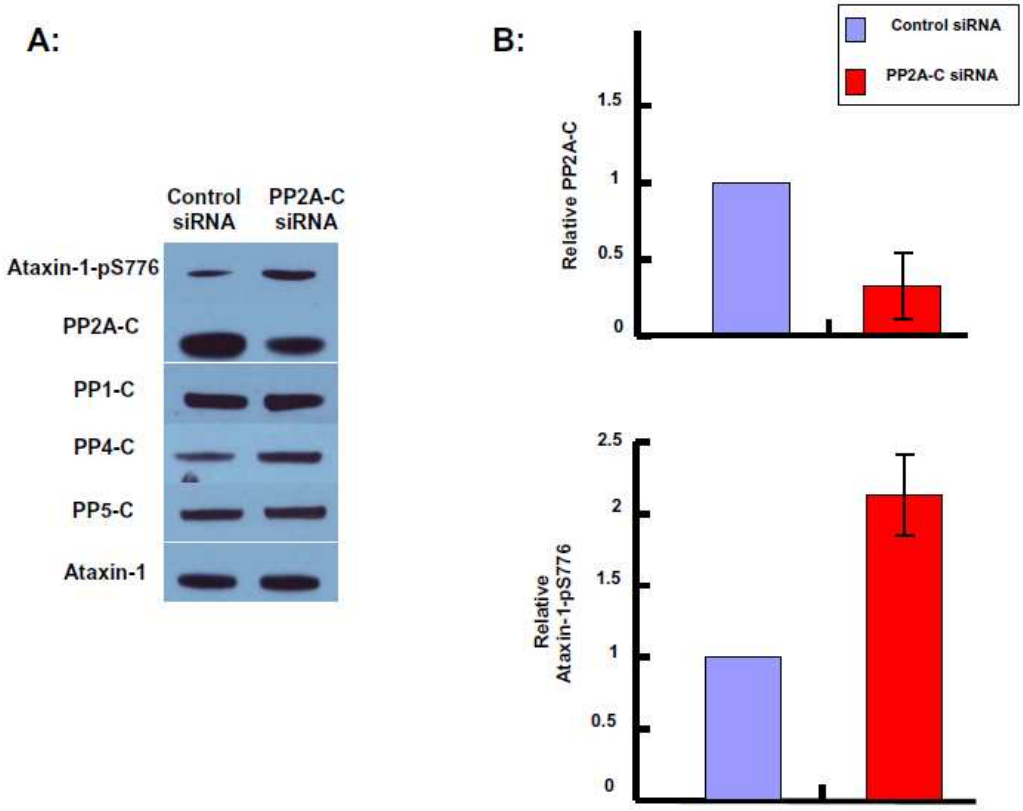

Figure 14. Knock down of PP2A significantly increases the level of Ataxin-1-pS776 in Hela cells. A, western blot analyses of Ataxin-1-pS776, total Ataxin-1, PP2A-C, PP1-C, PP4-C, and PP5-C of HeLa cells transfected with specific siRNA against PP2A-C, showing that down-regulation of PP2A-C increases Ataxin-1-pS776 but does not affect total Ataxin-1 level. B, the quantitative analysis of the relative amount of PP2A-C and relative phosphorylation data for A.

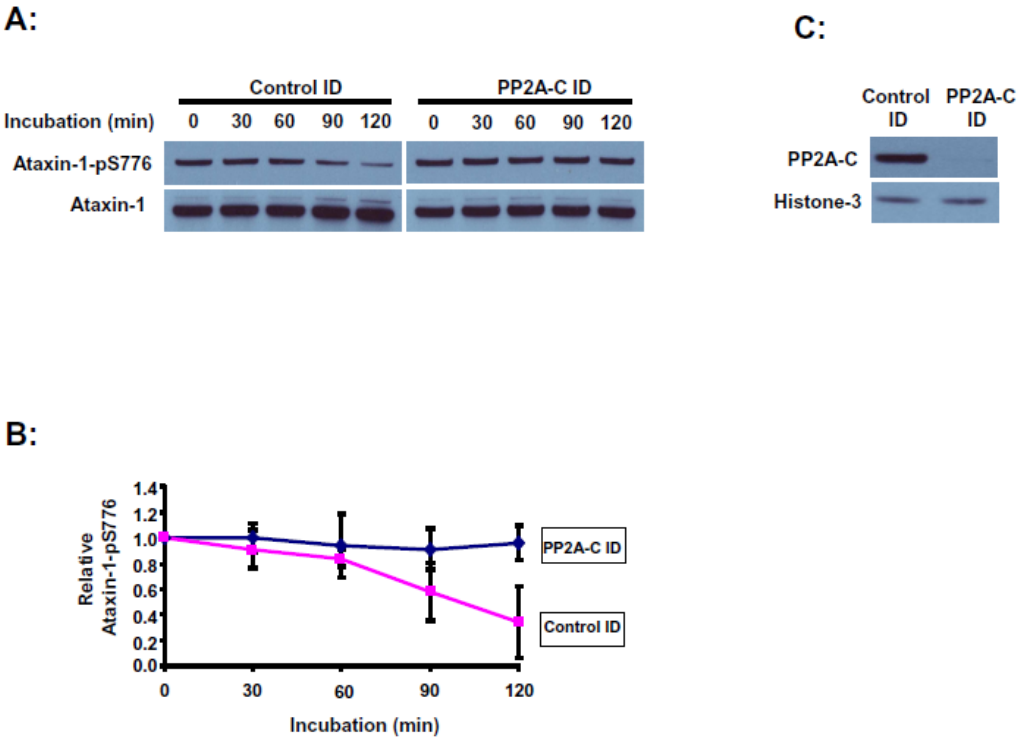

Figure 15. Depletion of PP2A-C decreases dephosphorylation of Ataxin-1-pS776. A, immunodepletion of PP2A-C from cerebellar nuclear extracts substantially decreases dephosphorylation of Ataxin-1 at S776. B, the quantitative analysis of relative Ataxin-1 phosphorylation data for A, n=3. C, western blot analysis of PP2A-C and Histone-3 in the post-ID supernatant showing that upon immunodepletion, PP2A-C was almost depleted while Histone-3 was not affected.

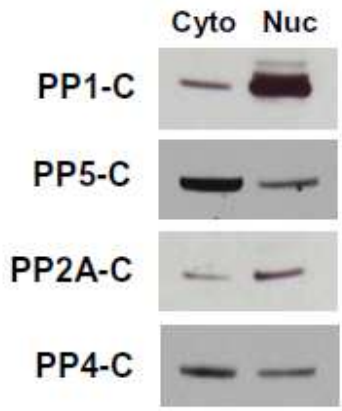

Figure 16. PP2A is expressed in both cerebellar cytoplasm and nucleus. Cytoplasmic (cyto) and nuclear (nuc) distribution of individual OA sensitive phosphatases in the cerebellum was shown. Here equal amount of protein was loaded for cytoplasmic and nuclear fractions.

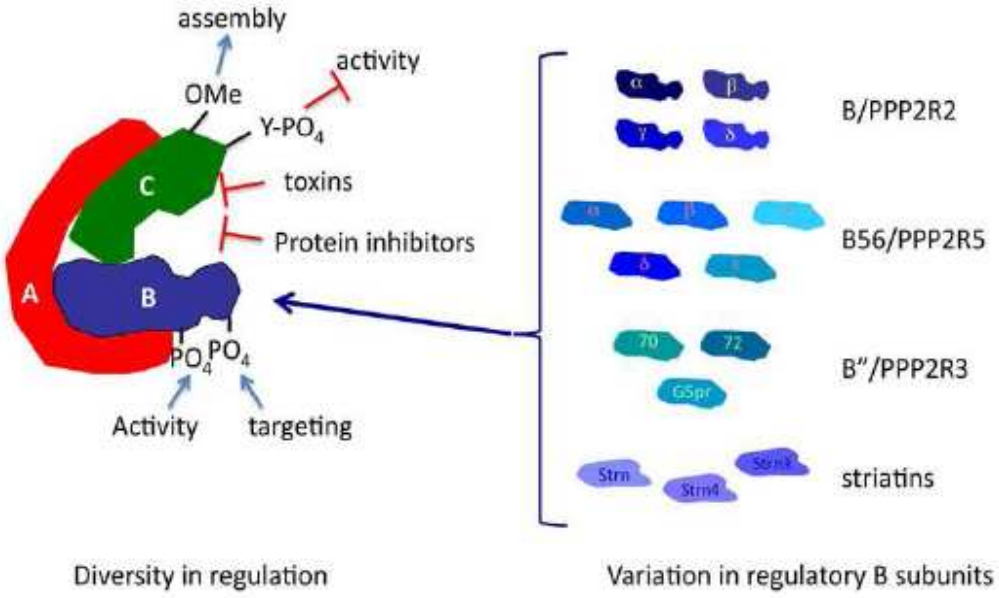

Figure 17. The diversity and complexity of PP2A. Numerous mechanisms control PP2A. Left: PP2A exists predominantly as a heterotrimer, with conserved A and C subunits and variable B subunits. This heterotrimer is regulated at multiple levels, including regulation of heterotrimer assembly, microbial toxins (such as okadaic acid and microcystin), protein inhibitors such as SET and CIP2A, and phosphorylation of the B and C subunits to regulate activity, assembly, and targeting. Right: the B subunits are encoded by at least 15 different genes, each with multiple splice variants. (Virshup and Shenolikar, 2009)

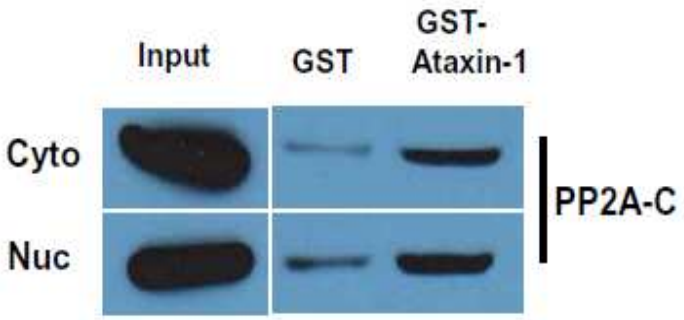

Figure 18. PP2A interacts with Ataxin-1 in both cerebellar cytoplasm and nucleus. GST-Ataxin-1 [30Q] was used to 'pull down' endogenous PP2A phosphatase from cytoplasmic (cyto) and nuclear (nuc) extracts. PP2A is able to interact with Ataxin-1 in both cerebellar cytoplasm and nucleus.

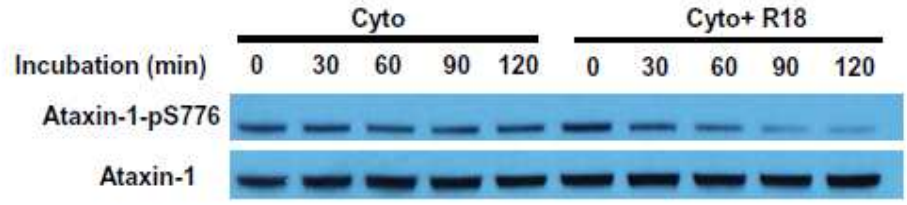

Figure 19. Dissociation of 14-3-3 and Ataxin-1 complex induces Ataxin-1-pS776 dephosphorylation in the cytoplasm. The addition of R18, a 14-3-3 specific inhibitor, resulted in cytoplasmic Ataxin-1-pS776 dephosphorylation.

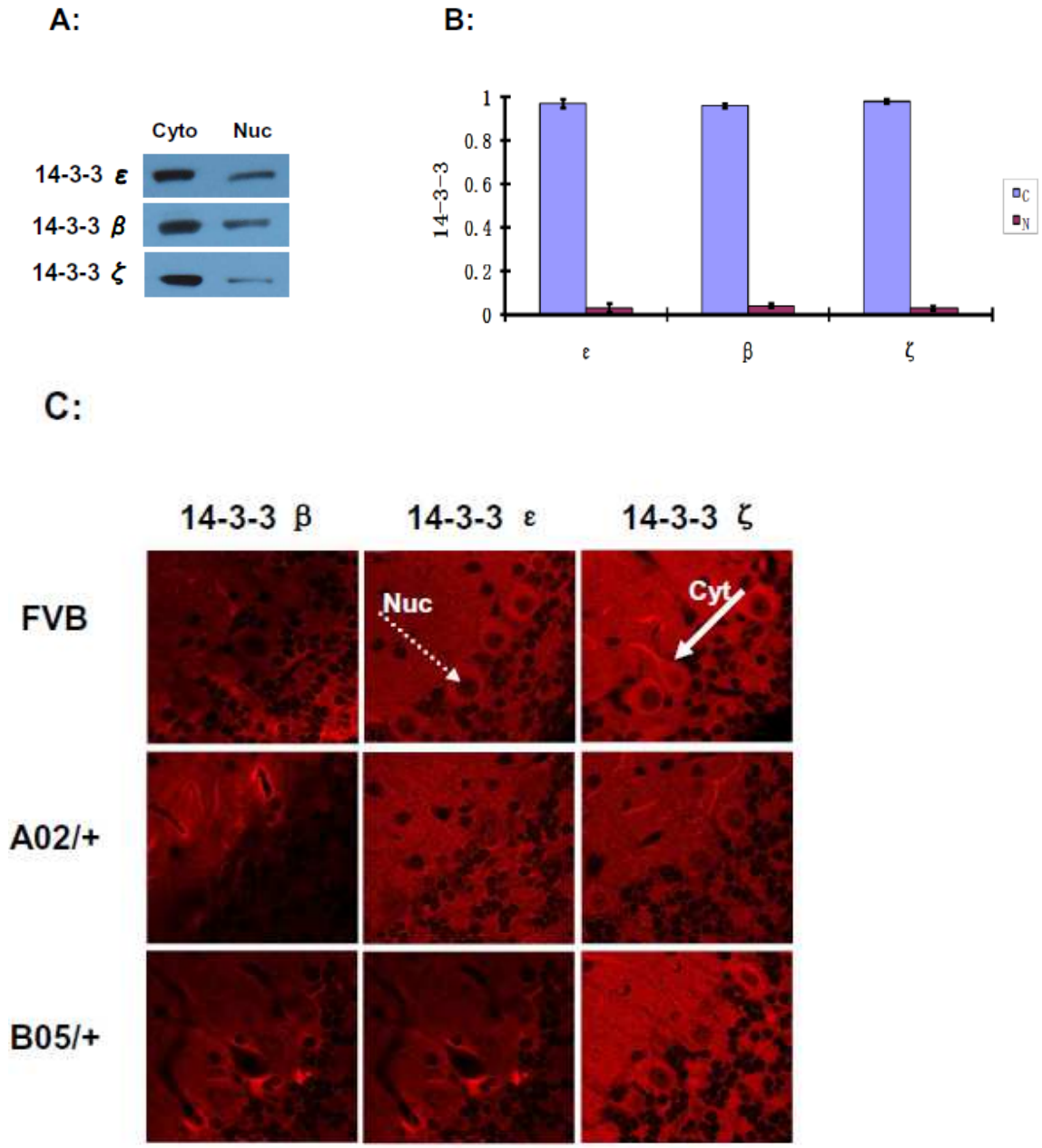

Figure 20. 14-3-3 is almost exclusively localized to the cerebellar cytoplasm. A, Western blot shows of the 14-3-3 isoforms examined, all of them are almost exclusively localized to the cytoplasm. Equal amount of protein was loaded for cytoplasmic and nuclear fractions. B, the quantitative analysis for A using formula in Figure 5B shows relative 14-3-3 distribution in the cytoplasm (C) and nucleus (N), n≥3. C, Immunofluorescence of mouse cerebellar sections shows the localization of 14-3-3 in the cytoplasm.

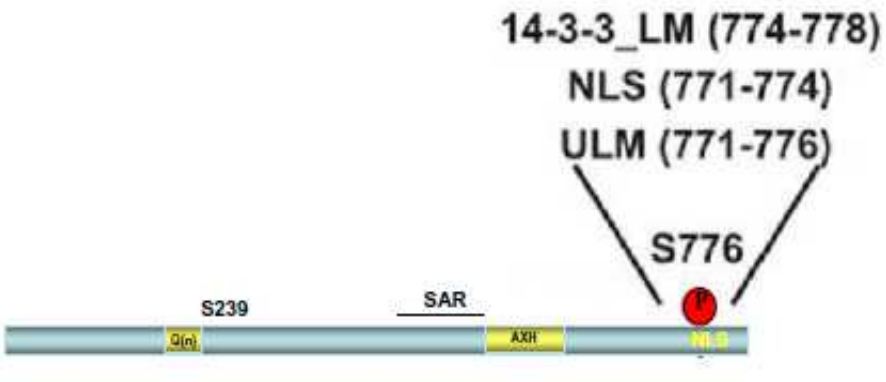

Figure 21. Ataxin-1 protein schematic. Indicated are Ataxin-1's polyglutamine tract (Q(n)), self-association region (SAR), Ataxin-1 and HMG-box protein 1 (AXH) domain, Nuclear localization signal (NLS) and two serine sites (S239 and S776) that can be phosphorylated. S776 is in the middle of or immediately adjacent to three motifs, 14-3-3 binding motif, NLS and UHM ligand motif. (Modified from de Chiara et al., 2009)

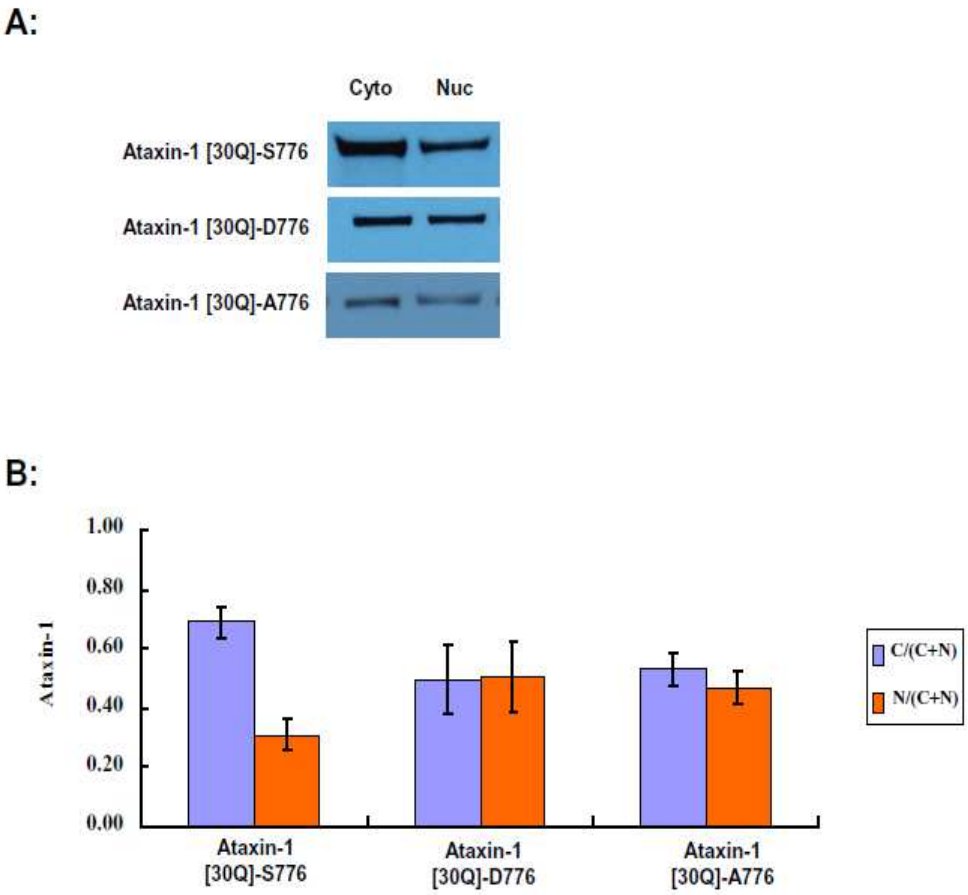

Figure 22. Effect of 14-3-3 binding on Ataxin-1 cytoplasmic and nuclear distribution: S776-Ataxin-1 [30Q] *vs.* D776- Ataxin-1 [30Q] *vs.* A776-Ataxin-1 [30Q]. A, A representative western blot shows that D776-Ataxin-1 [30Q] and A776-Ataxin-1 [30Q] are more localized to the nucleus than S776-Ataxin-1 [30Q]. B, the quantitative analysis for A shows relative Ataxin-1 distribution in the cytoplasm (C) and nucleus (N), n≥3.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4. I: Conclusions

The regulation of Ataxin-1 phosphorylation at S776 in the cerebellum was investigated in this thesis. While the kinase activity for Ataxin-1 at S776 is localized to the cerebellar cytoplasm, the phosphatase activity is in the nucleus. Ataxin-1 after phosphorylated at S776 in the cytoplasm shuttles to the cerebelllar nucleus and is enriched in the nucleus where it is dephosphorylated by PP2A. 14-3-3, a protein almost exclusively localized to the cytoplasm, associates with Ataxin-1-pS776, resulting in blocked accessibility of Ataxin-1-pS776 by PP2A in the cytoplasm and inhibited Ataxin-1-pS776 nuclear import. A proposed model incorporating these findings is shown in Figure 23.

4. II: Future Directions

The effect of reversible phosphorylation on Ataxin-1 nucleocytoplasmic trafficking. While some evidence was provided implicating that phosphorylation has an effect on Ataxin-1 nucleocytoplasmic shuttling, direct evidence for it was absent. A further understanding of whether reversible phosphorylation has an effect on Ataxin-1 nucleocytoplasmic trafficking, thereby subcellular localization, will require an experimental dissection of the effect of phosphorylation/dephosphorylation on the rate of Ataxin-1 import and export.

Experiments addressing this will involve using tissue culture cells, e.g. Hela cells, transfected with green fluorescence protein (GFP) tagged Ataxin-1 [30Q]-S776, Ataxin-1 [30Q]-A776, and Ataxin-1 [30Q]-D776 (Figure 24). Transfected cells would be examined by fluorescence recovery after photobleaching (FRAP) analysis using a

qualitative live cell nuclear shuttling assay (Howell and Truant, 2002). This technique has been successfully applied to show that Ataxin-1 shuttles back and forth in the cellular cytoplasm and nucleus (Irwin et al., 2005). It will be applied to measure the individual Ataxin-1 nuclear import and export rate. As stated above, A776-Ataxin-1 is phosphorylation resistant and can not be dephosphorylated, D776-Ataxin-1 mimics phosphorylation at S776 and can not be dephosphorylated either while S776-Ataxin-1 is phosphorylation reversible. The difference of import and/or export rate among these forms of Ataxin-1, if there is any, may implicate that phosphorylation and/or dephosphorylation has an effect on Ataxin-1's transport in and out of nucleus. A limitation of this assay will be that the substitution at S776 (S776D) may not properly mimic S776 phosphorylation.

Another approach will be regulating the kinase and phosphatase activity of S776-Ataxin-1 in the cells. Ataxin-1 is phosphorylated by PKA in the cytoplasm and dephosphorylated by PP2A in the nucleus. To regulate the kinase activity, PKA will be overexpressed, such as transfection of a plasmid encoding the catalytic subunit of PKA together with GFP-Ataxin-1-S776 plasmid into Hela cells, or knocked down by using PKA specific siRNA or some cell permeable kinase inhibitor, for example, staurosporine. Then FRAP will be applied to measure the change of Ataxin-1 import and export rate. To regulate the phosphatase activity, overexpressing PP2A catalytic subunit using plasmid of PP2A catalytic subunit, or knock down PP2A with siRNA or PP2A inhibitor such as OA, can be applied to Hela cells transfected with GFP-Ataxin-1-S776 plasmids. An alternative experiment, cytoplasmic and nuclear distribution of Ataxin-1-pS776 after the

above treatments, may help define the effects of reversible phosphorylation on Ataxin-1 shuttling. Ideally, combinational regulation of both PKA and PP2A activity, for example, increasing the expression of PKA and decreasing that of PP2A, would be used. These experiments will help provide evidence about whether reversible phosphorylation affects Ataxin-1 nucleocytoplasmic shuttling and help predict the mode of phosphorylation on Ataxin-1 trafficking.

The effect of complex formation on Ataxin-1 nucleocytoplasmic trafficking. It would be interesting to see whether complex formation affects Ataxin-1 nucleocytoplasmic trafficking. Ataxin-1 NLS contains phosphorylated S776 and is adjacent by 14-3-3 binding motif, and the U2AF65 and RBM17 binding motif. 14-3-3 by binding to protein sequences adjacent to NLS was shown to inhibit protein nuclear import (Sekimoto et al., 2004; Yaffe, 2002). Some evidence was provided in this thesis indicating that association with 14-3-3 may affect Ataxin-1's subcellular distribution. Experiments that help support this conclusion would involve regulating the expression levels of 14-3-3, such as overexpressing or knocking down 14-3-3 levels in Hela cells, and determining its effect on the rate of GFP-Ataxin-1-S776 import and export. A 14-3-3 specific inhibitor, R18, will also be applied.

Similar assays could be applied to regulate U2AF65 and RBM17 levels and examine their effects on Ataxin-1 nucleocytoplasmic trafficking. Ideally, combinational regulation of these proteins may provide a better resolution. For example, overexpression of 14-3-3 and downregulation of U2AF65 together to enhance 14-3-3 effect since studies show that 14-3-3 and U2AF65 compete for binding to Ataxin-1-S776 (de Chiara et al.,

2009). Again, cytoplasmic and nuclear distribution of Ataxin-1-pS776 after the above regulations could also be determined to help address the question. One limitation of this assay would be that the subcellular site of Ataxin-1 synthesis and degradation needs to be considered.

The same experiment stated above involving transfection of Hela cells with GFP-Ataxin-1 [30Q]-S776, Ataxin-1 [30Q]-A776, and Ataxin-1 [30Q]-D776 could also be performed. Both Ataxin-1 [30Q]-A776 and Ataxin-1 [30Q]-D776 do not bind 14-3-3 (Chen et al., 2003; de Chiara et al., 2009). In addition, Ataxin-1 [30Q]-D776 binds to RBM17 most strongly while Ataxin-1 [30Q]-A776 almost does not bind (Lim et al., 2008). Thus, this experiment would further define the effect of these protein complexes on Ataxin-1 trafficking. The aforementioned two future directions may converge since the effect of Ataxin-1 phosphorylation on its nucleocytoplasmic trafficking may be exerted through its phosphorylation effect on complex formation.

Examine the reasons why nuclear 14-3-3 does not affect PP2A dephosphorylation of Ataxin-1-pS776. While 14-3-3 is almost exclusively localized to the cytoplasm, a small proportion of it resides in the nucleus. It will be interesting to know the reasons why nuclear 14-3-3 does not affect PP2A dephosphorylation of Ataxin-1-pS776. GST pull down assay with phosphorylated GST-Ataxin-1 can be used to enrich endogenous 14-3-3 from either cerebellar cytoplasmic or nuclear extract. However, for this experiment much more nuclear extracts will be needed in order to get comparable level of nuclear 14-3-3 with cytoplasmic 14-3-3. Alternatively, GST-14-3-3 can be used to do the pull down. These experiments may provide evidence about whether no effect of

14-3-3 on pS776 dephosphorylation is due to no interaction of it with Ataxin-1-pS776 in the nucleus. Future work about the reasons may also involve experiments aimed to examine where the kinase/phosphatase activity for 14-3-3 is localized since phosphorylation of 14-3-3 by kinases was shown to dissociate 14-3-3 from its various target proteins (Sunayama et al., 2005; Tsuruta et al., 2004). Dephosphorylation by phosphatase confers 14-3-3 the ability again to bind target proteins.

Examine the regulation of 14-3-3 associating with Ataxin-1-pS776. It would be of great interest to understand how the association/dissociation of 14-3-3 with Ataxin-1-pS776 is regulated. Many questions still remain regarding this process. First, the 14-3-3 isoform(s) involved will need to be identified. Isoform specific antibodies can be used for experiments such as co-immunoprecipitation of 14-3-3 with Ataxin-1 from mouse cerebellar lysate. The results may turn out to be complex since some of 14-3-3 isoforms may have redundant functions (Aitken et al., 1992). However, it is still possible that one/some isoform(s) will preferentially bind. Some ambitious experiments involving identification of the kinase and phosphatase for 14-3-3 isoform(s), modulation of these enzyme activities and examining its effect on association with Ataxin-1-pS776 will provide evidence about whether 14-3-3 association with Ataxin-1-pS776 is regulated by its phosphorylation status. Conclusions from these experiments would be supported by the above proposed experiment aimed at examining the subcellular localization of 14-3-3 kinase/phosphatase activity.

Examine the role of PP2A in regulating Ataxin-1 phosphorylation and Ataxin-1 complex formation in the nucleus and SCA1. PP2A was demonstrated to be

the phosphatase for Ataxin-1-pS776 in the cerebellum. However, in vivo data showing how PP2A regulates Ataxin-1 phosphorylation, Ataxin-1 complex formation in the nucleus and SCA1 disease is lacking. Modulating PP2A levels in the cerebellum is a potential approach for this purpose. One method would be to generate recombinant adeno-associated virus (AAV) vectors expressing short hairpin RNAs against PP2A catalytic subunit, inject AAV viruses into mouse cerebellum and examine their effects on Ataxin-1 phosphorylation, Ataxin-1 complex formation and SCA1. This method has been successfully used in a SCA1 mouse model (Xia et al., 2004). Alternatively, a PP2A transgenic mouse model with a L199P mutation in its Cα subunit (PP2A L199P) would be used. These mice highly express a dominant negative mutant form of PP2A Cα in cortical, hippocampal, and cerebellar neurons and the activity of PP2A in the mouse brain was shown to be reduced to 66% (Kins et al., 2001). In order to examine PP2A's role in SCA1, PP2A L199P mice will need to be crossed with SCA1 mice to generate SCA1 mice with decreased PP2A activity. In addition, PP2A inhibitors such as OA can be used to treat the mice. Results from these experiments will provide evidence for the role of PP2A in the regulation of Ataxin-1 phosphorylation, Ataxin-1 complex formation and SCA1. Also, examination of whether PP2A differs in its ability in dephosphorylating unexpanded Ataxin-1 [30Q] and expanded Ataxin-1 [82Q] could provide more information.

Future efforts on how PP2A regulates Ataxin-1 phosphorylation should involve identifying the regulatory subunit or adaptor protein of PP2A for Ataxin-1-pS776. Experiments such as two-dimensional electrophoresis and mass spectrometry will help

this identification.

The proportion of Ataxin-1 phosphorylated at S776 in the cytoplasm and nucleus. Although by calculation 77% of Ataxin-1 in the nucleus was estimated to be phosphorylated while 45% of it in the cytoplasm is phosphorylated, no experimental data supporting this predication is currently available. Immunodepletion of Ataxin-1-pS776 in the cytoplasm and nucleus respectively with the use of PN1248 antibody will test whether this prediction is correct.

Clearly, many questions are unanswered regarding the regulatory mechanism of Ataxin-1 phosphorylation in the cerebellum. More future information is needed to better elucidate the regulatory process and its effects on Ataxin-1 shuttling and SCA1 pathogenesis. Studies like this will not only provide additional knowledge to understand the physiological function of Ataxin-1 and its role in SCA1 pathogenesis but also help develop therapeutic tools for this disease.

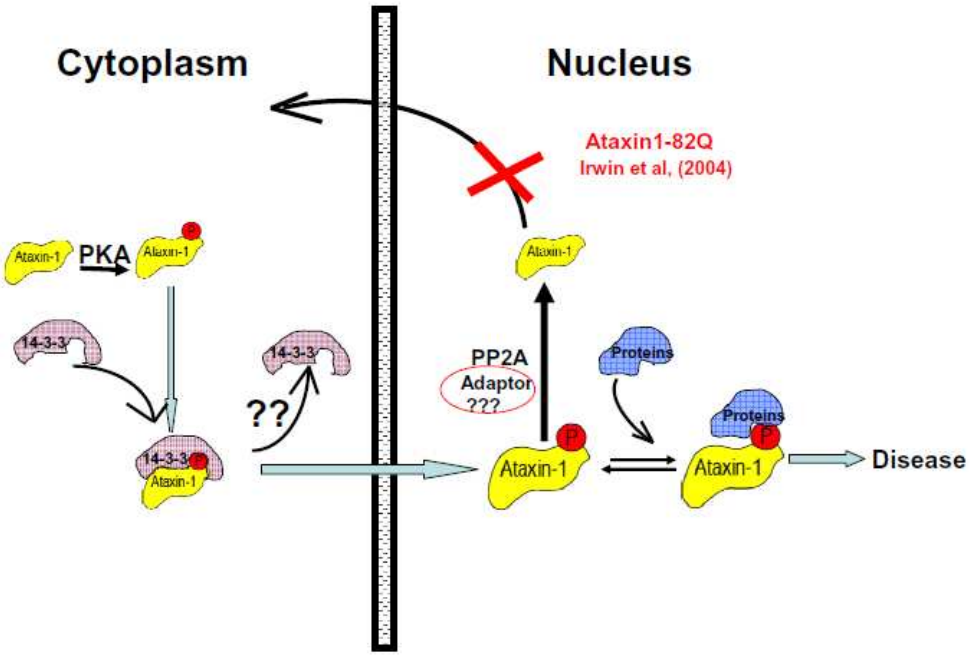

Figure 23. A proposed model. Ataxin-1 is phosphorylated by PKA in the cytoplasm. 14-3-3 associates with Ataxin-1-pS776 and protects it from dephosphorylation by PP2A in the cytoplasm. Association of 14-3-3 with Ataxin-1 also interferes its shuttling to the nucleus. In order for Ataxin-1-pS776 to shuttle into the nucleus, 14-3-3 needs to be dissociated. Ataxin-1-pS776 is enriched in the nucleus where it is dephosphorylated by PP2A.

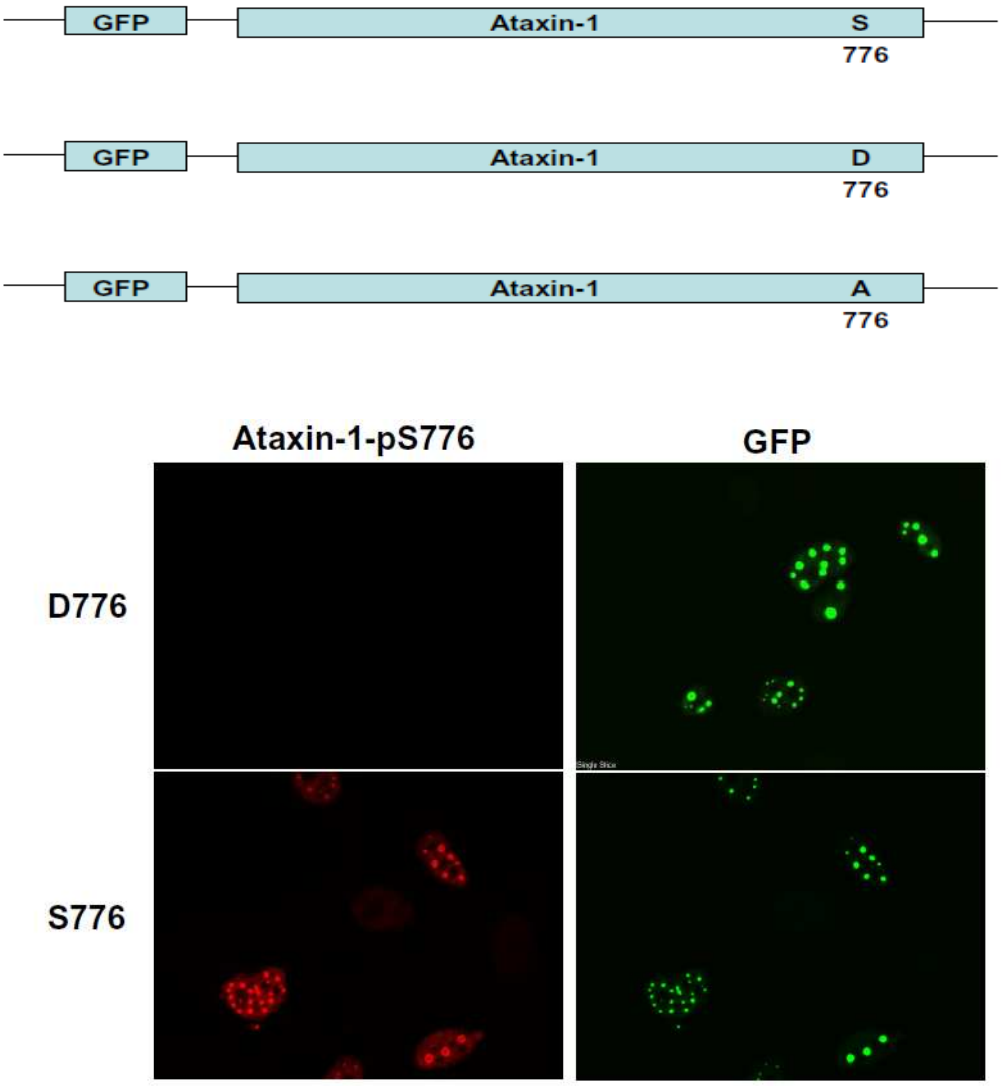

Figure 24. Schematics of GFP-S776-Ataxin-1 [30Q], GFP-D776-Ataxin-1 [30Q] and GFP-A776-Ataxin-1 [30Q] plasmids transfected into Hela cells. Immunofluorecence of GFP-S776-Ataxin-1 [30Q] and GFP-D776-Ataxin-1 [30Q] in Hela cells immunostained with PN1248 and GFP antibody.

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