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SCA1 – Phosphorylation, a Regulator of Atxain-1 Function and Pathogenesis

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Abstract

Spinocerebellar ataxia type 1 (SCA1) is one an intriguing set of nine neurodegenerative diseases caused by the expansion of a unstable trinucleotide CAG repeat where the repeat is located within the coding of the affected gene, i.e. the polyglutamine (polyQ) diseases. A gain-of-function mechanism for toxicity in SCA1, like the other polyQ diseases, is thought to have a major role in pathogenesis. Yet, the specific nature of this gain-of-function is a matter of considerable discussion. An issue concerns whether toxicity stems from the native or normal function of the affected protein versus a novel function induced by polyQ expansion. For SCA1 considerable evidence is accumulating that pathology is mediated by a polyQ-induced exaggeration of a native function of the host protein Ataxin-1 (ATXN1) and that phosphorylation of S776 regulates its interaction with other cellular protein and thereby function. In addition, this post translational modification modulates toxicity of ATXN1 with an expanded polyglutamine.

SCA1 – Overview of the Disease and Genetics

Spinocerebellar ataxia type 1 (SCA1) joined the ranks of the unstable nucleotide repeat disorders and specifically the CAG/polyglutamine (polyQ) diseases in 1993 (Orr et al., 1993). SCA1 patients have loss of coordination of the limbs and trunk, unstable gait, dysarthric speech, and nystagmus but may have other symptoms, including extrapyramidal dysfunction, dysautonomia, cognitive impairment, and motor and sensory impairments. SCA1 is characterized pathologically by loss of Purkinje cells in the cerebellar cortex and neuronal loss in brain stem nuclei and cerebellar dentate nuclei (Koeppen, 2005). Individuals carrying a mutant SCA1 allele can have symptoms starting as early as the first decade. By the sixth decade disease penetrance is essentially complete.

SCA1 genetics dates back to the mid-late 1970s when use of HLA serological typing as genetic markers revealed an autosomal dominant form of ataxia linked to the HLA complex on chromosome 6 (Yakura et al., 1974; Jackson et al., 1977). With the application of molecular genetic approaches, location of the *SCA1* gene was refined on the short arm of chromosome 6 to a region about 15 CM distal to HLA-A (Rich et al., 1987; Keats et al., 1991). With its cloning, the *SCA1* gene was found to span 450 kb of DNA at 6p22.3 and consists of nine exons (Banfi et al., 1994). The *SCA1* transcript is 10,660 bases in length

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with the first seven exons encoding the 5' untranslated region and exons eight and nine containing the Ataxin-1 (ATXN1) coding region (816 amino acids in a protein having a polyQ tract of 30 residues), and the 7,277 base 3' untranslated region. The polyQ stretch begins at amino acid 197 and is encoded within exon eight. Interestingly, there are binding sites for several miRNAs within the 3' untranslated region of the *SCA1* transcript that function to down-regulate ATXN1 levels (Lee et al., 2008).

Normal *SCA1* alleles contain from 6 to 42 CAG repeats with those greater than 21 being interrupted with 1 – 3 CAT trinucleotides. Disease alleles, on the other hand, are pure CAG tracts ranging from 39 to 82 units. Such interruptions are found in all of the longer unaffected alleles. In contrast, all affected alleles are pure CAG tracts (Chung et al., 1993). The presence of repeat interruptions particularly in the longer wild type allleles lead to the suggestion that the CAT interruptions have a critical role in maintaining the relative stability of normal alleles compared to mutant *SCA1* alleles.

Disease-related expansions have a direct relationship between length and severity/age-of-onset of disease, i.e. longer the longer the glutamine tract the more severe and earlier is the age of onset of disease. Expansion of the CAG repeat into the affected range besides encoding a pathogenic protein also enhances the instability of the DNA repeat such that changes in repeat length are found when transmitted from parent to offspring. Overall this instability of mutant alleles is the molecular basis of the genetic observation anticipation, an increase in disease severity/earlier age of onset as ones follows the disease for generation to generation in a family. In the case of SCA1, anticipation was first noted in 1950 in a large family with an inherited ataxia that subsequently proved to have SCA1 initially by virtue of a genetic linkage to the HLA complex on chromosome 6p (Schut, 1950; Haines et al., 1984). One point worth noting is that large repeat expansions are restricted to paternal transmissions so that in juvenile forms of SCA1 identified to date all stem from a father to offspring transmission.

Of Mice and Flies

Over the years several mouse models were generated in an effort to understand mechanisms underlying SCA1 disease and ATXN1 function. In modeling SCA1, as well as other neurodegenerative disorders, an issue is whether to express the mutant transgene in all neurons or cells of the CNS, or to target critical populations such as the Purkinje cell in the case of SCA1. The advantage of the former approach is that it more closely models the human disease, but in turn it makes interpretation of mechanisms more complex. The advantage of the latter approach is that Purkinje cells are critical cells for cerebellar function, they are affected in nearly all forms of SCA, and damage to them usually creates a behavioral phenotype.

The first SCA1 mouse model was generated in 1995 to test the CAG expansion, gain-of-function hypothesis. Burright et al. (1995) directed expression of ATXN1 in mouse Purkinje cells using a Purkinje cell-specific Pcp2/L7 promoter. Two types of lines were created; control lines with over-expression of the normal interrupted allele (ATXN1[30Q]) containing 30 repeats, $(CAG)_{12}$ CATCAGCAT$(CAG)_{15}$, and experimental lines expressing the expansion construct (ATXN1[82Q]) containing 82 uninterrupted CAG repeats. Progeny of the control ATXN1[30Q] lines, and the expansion ATXN1[82Q] lines inherited the transgene either paternally or maternally and in all animals the repeat was stable and showed no change in repeat size.

At one year of age, ATXN1[30Q] mice had a normal neurological phenotype, suggesting intact Purkinje cell function. In contrast, the mutant ATXN1[82Q] mice beginning around five weeks of age developed neurological abnormalities that progressed with age (Figure 1).

These included reduced cage activity and general uncoordinated movements. Footprint patterns indicated gait abnormalities and motor performance measured using an accelerating rotating-rod apparatus was abnormal as early as five weeks of age. (Clark et al.,1997). The motoric deficits progressed with age until mice were clearly ataxic by cage behavior at 12 weeks of age.

Pathologic changes were observed in the ATXN1[82Q] cerebellum as early as P25 when clear cytoplasmic vacuoles were found in some of the cell bodies of Purkinje cells (Clark et al., 1997). The degeneration progressed with age and by 27 weeks the majority of Purkinje cells had stunted, atrophic dendritic morphology. Notably, Purkinje cell loss was preceded by the ataxic phenotype (Figure 1). At 1 year, the cerebellar cortex was dramatically decreased in size secondary to both atrophy and loss of Purkinje cells, frequent heterotopic localization of Purkinje cells in the molecular layer, and diminished calbindin immunoreactivity in surviving Purkinje cells. In addition, ATXN1[82Q] expressing Purkinje cells accumulate ATXN1 and ubiquitin-positive aggregates, or neuronal intranuclear inclusions, that co-localize with the proteasome and the molecular chaperone, HDJ-2/HSDJ, demonstrating that there is ATXN1 misfolding in SCA1 disease (Cummings et al., 1998).

To determine if the neurological symptoms in SCA1 might be caused by an ATXN1 loss of function, *Sca1* null mice were generated (Matilla et al., 1998). *Sca1* exon 8 was targeted and deleted as the majority of the coding sequence resides in this exon. *Sca1*$^{(-/-)}$ mice were viable, and displayed both normal development and a normal life span. In addition, they failed to display ataxic symptoms or neurodegeneration even out to 30 months of age. Further behavioral studies, including the open field test, elevated plus maze, and Morris water maze suggest *Sca1*$^{(-/-)}$ mice have spatial learning deficits.

In order to generate a more accurate model of the effects of mutant ataxin-1 expression under control of its endogenous promoter, a knock-in model was generated by homologous recombination to inset an expanded CAG trinucleotide repeat into the mouse *Sca1* locus. The initial line generated, *Sca1*$^{78Q/2Q}$, while expressing endogenous levels of expanded ataxin-1 in the expected temporal and spatial patterns, lacked any ataxic phenotype or neuropathological abnormalities (Lorenzetti et al., 2000). A second model was generated with 154 CAGs (Watase et al., 2002). *Sca1*$^{154Q/2Q}$ mice had an altered phenotype that included muscle wasting, ataxia, abnormal gait, severe kyphosis and premature death between 35 and 45 weeks. *Sca1*$^{154Q/2Q}$ animals displayed significant motor deficits by rotating-rod analysis at 5 and 7 weeks as well as impaired spatial learning deficits. In addition, the *Sca1*$^{154Q/2Q}$ mice exhibited a decrease in long-term potentiation at 24 weeks. The *Sca1*$^{154Q/2Q}$ animals were distinct from the ATXN1[82Q] model in that they exhibited repeat instability similar to human patients (Watase et al., 2002). Brains from *Sca1*$^{154Q/2Q}$ animals were smaller than controls, though brain sections showed uniform atrophy rather than specific atrophy of the cerebellum more characteristic of SCA1 disease in humans. While slight Purkinje cell loss was observed, the molecular layer thickness was relatively intact even in 24-week-old animals.

Overall, pathology and disease in Pcp2-ATXN1[82Q] mice is similar to the typical mid to late-onset clinical SCA1 phenotype that arises from moderate CAG expansions. Neuronal damage is confined to specific neuronal groups (e.g. Purkinje cells in the disease. In contrast mice for the *Sca1*$^{154Q/2Q}$ knockin line have a more extreme CAG repeat expansion and exhibit a disease more similar to SCA1 juvenile or even infantile-onset disease where neuronal damage is much more widespread than in adult-onset disease, reaching into brain regions completely unaffected in older patients. This loss of specificity might result from the mutation being so severe that protein function and/or clearance is impaired even in neurons that normally express low levels of ATXN1.

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First Steps of Relevance to a Treatment

As one envisions moving towards a treatment for a neurodegenerative disease, the extent to which the disease is reversible after onset is of importance. To determine whether SCA1 disease is reversible, a conditional mouse model of SCA1 was developed using the tetracycline-regulated system (Zu et al., 2004). The approach used the *Pcp2/L7* promoter to drive a tetracycline-transactivator (Tta) transgene specifically in Purkinje cells. Tta functions to bind to a tetracycline response element (*TRE*) to induce expression of a second *TRE-ATXN11[82Q]* transgene solely in Purkinje cells. Administration of doxycycine (dox), a tetracycline derivative that crosses the blood brain barrier, abolishes the Tta/TRE interaction and turns off expression of ATXN1. Effects of reversal of transgene expression were assessed at early, mid, and late stages of disease(Zu et al., 2004). At the early stage, 6-week-old mice demonstrated the rotating-rod deficit could be reversed after 6 weeks of dox treatment. In addition, Purkinje cell pathology improved by assessment of molecular layer thickness and dendritic arborization, although heterotopic Purkinje cells were still present. At mid stage disease, 12 week on – 4 week off animals continued to have rotating-rod deficits similar to 12 and 16 week-on animals. After 8 weeks and 12 weeks of dox treatment, however, rotating-rod performance improved, but only partially. Interestingly, this 8-week timepoint coincided with the return of mGluR1α glutamate receptors at the Purkinje cell-parallel fiber synapses, albeit at a lower expression level. There was recovery of molecular layer thickness and improved arborization of Purkinje cell dendrites demonstrating that halting ATXN1[82Q] expression at the time of typical onset of ataxia prevents the progression of and partially reverses degenerative changes in Purkinje cells. When dox was given to animals at 32 weeks of age, there was no significant improvement of ataxia, but there was some improvement of Purkinje cell pathology. At all disease stages, Purkinje cells rapidly eliminated ATXN1[82Q]-containing nuclear inclusions upon administration of dox and cessation of ATXN1 expression. Thus, even at a late stage of disease Purkinje cells are able to clear ATXN1 with an expanded polyQ. Overall, these studies revealed that in fact Purkinje cell pathology induced by ATXN1 with an expanded polyQ could be reversed with cessation of ATXN1 protein synthesis. Interestingly, the capability of Purkinje cells to recover decreases with increasing age. It remains unclear whether this age dependence of recovery is due to affects of prolonged exposure to mutant ATXN1 and/or that older neurons in general are less able to repair damage.

The Tta/TRE conditional *SCA1* mice were also used to demonstrate a link between proper Purkinje cell development and susceptibility of adult neurons to toxic insults. The basis for this study was a finding that one of the initial transgenic mouse lines of the ATXN1[82Q] transgenic series (the so-called B04 line) while expressing less ATXN1[82Q] than other lines, had a more severe ataxia (Burright et al., 1995). Intriguingly, in the B04 mice ATXN1[82Q] expression begins 10 days earlier during postnatal development than in the other lines of the ATXN1[82Q] series, on postnatal day 2, raising the possibility that onset of transgene expression impacts disease severity in an adult. This idea was tested directly by delaying ATXN1[82Q] transgene expression during early postnatal development in the conditional mice.

In mice, the first three postnatal weeks are a critical period for cerebellar development. It is during this period that one of the two major inputs to Purkinje cells matures, climbing fibers from the inferior olives, and the cerebellum undergoes a dramatic increase in size being transformed from an immature smooth surfaced anloga at birth to the adult highly foliated structure (Goldowitz and Hammre, 1998). ATXN1[82Q] mice that do not express ATXN1 during this developmental period failed to develop the neurological and pathological features seen in animals that express ATXN1[82Q] during postnatal development (Serra et al., 2006). Additional studies implicated an alteration in function of the orphan nuclear

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receptor Rora in ATXN1[82Q], perhaps via an interaction of ATXN1 with the Rora cofactor Tip60 (Gehrking et al., 2011), as being key for this developmental affect on SCA1 pathogenesis in Purkinje cells. Mouse models expressing mutant forms of ATXN1 expressed decreased levels of Rora and therefore decreased levels of Rora-mediated genes. To evaluate the role of Rora in SCA1 pathogenesis, ATXN1[82Q] animals were crossed to staggerer heterozygous mice, which lack one functional copy of *Rora* (sg/+). ATXN1[82Q]:sg/+ mice had more severe Purkinje cell pathology suggesting that depletion of *Rora* enhances SCA1 disease. Crossing ATXN1[82Q] animals to *Tip60*$^{+/-}$ animals transiently delayed the ATXN1[82Q] cerebellar degeneration by increasing Rora and Rora - mediated gene expression (Gehrking et al., 2011).

One of the first potential pathogenic mechanism examined using *SCA1* transgenic mice was the role of the ATXN1 nuclear inclusions/aggregates. Neuronal aggregates containing the mutant protein occur in many of the inherited polyglutamine disorders and were raised as a possible common mechanism for neurodegeneration (Ross and Poirier, 2004). To examine the role the macroscopic aggregates play in SCA1, ATXN11[77Q]Δ mice were developed. ATXN11[77Q]Δ was generated by deleting the self-association region of ataxin-1 (Burright et al., 1997). In tissue culture cells ATXN1[77Q]Δ failed to form macroscopic inclusions as seen with ATXN1[82Q]. ATXN1[77Q]Δ transgenic mice expressed ATXN1 in the Purkinje cell cytoplasm and nucleus, similar to ATXN1[82Q] animals. Like as seen in tissue culture cells, ATXN1[77Q]Δ mice did not develop inclusions in their Purkinje cells. The absence of inclusions, however, did not protect ATXN1[77Q]Δ mice from developing Purkinje cell pathology or ataxia. Indeed, ATXN1[77]Δ animals had the same Purkinje cell atrophy seen in ATXN1[82Q] mice and performed as poorly as ATXN1[82Q] animals in motor performance tasks (Klement et al., 1998). Moreover, Purkinje cells that express ATXN1[82Q] but not the E6-AP ubiquitin-protein ligase Ube3A also have far fewer nuclear inclusions but markedly worse pathology (Cummings et al., 1999). Thus, several lines of investigation show that Purkinje cell pathology and motor deficits associated with SCA1 are not dependent on the formation of mutant ATXN1 inclusions.

Expressing full-length human *SCA1* genes in *Drosophila* is another strategy used to generate SCA1 animal models. This approach is particularly useful in the elucidation and dissection of genetic pathways that impact SCA1 pathogenesis. For example, genetic screens in the fly were the first to reveal that genes encoding proteins involved in RNA metabolism and transcription regulation modify development of SCA1 (Fernandez-Funez et al., 2000). As will become evident below, transcription and RNA processing are now thought to be two of the major pathways in which ATXN1 functions and in which alterations lead to disease induced by ATXN1 with an expanded polyQ tract.

Pathogenic Pathways in SCA1

An important step forward in dissecting SCA1 pathogenesis was taken when the role of the cellular localization of mutant ATXN1 on disease was assessed. Using the ATXN1[82Q] transgene, a lysine- to-threonine substitution was introduced at amino acid residue 772 to disrupt the nuclear localization signal (NLS) function (Klement et al., 1998). In mice expressing ATXN1[82Q]-K772T, ATXN1 localized primarily to the cytoplasm of Purkinje cells in contrast to ATXN1[82Q] mice with a functional NLS where significant levels of ATXN1 localized to the nucleus as well as to the cytoplasm. Importantly, ATXN1[82Q]-K772T animals failed to develop ataxia, as measured by rotating-rod deficits and ataxic cage behavior. In addition ATXN1[82Q]-K772T mice had no cerebellar pathology out to one year of age. This result lead to what has subsequently proven to be important hypothesis regarding the molecular basis SCA1 pathogenesis – localization of mutant ATXN1 to the nucleus is critical for disease indicating that it is a function(s) of ATXN1 in the nucleus that

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is altered in disease. With regards to ATXN1 in the nucleus, further studies showed that its dynamics is altered by expansion of the polyQ tract (Krol et al., 2008). Moreover, while mutant ATXN1 with an expanded polyQ tract is able to enter the nucleus, it's ability to be transported back into the cytoplasm is dramatically reduced (Irwin et al., 2005). Also, the length of the polyQ tract negatively affects the SUMOylation, a common posttranslational modification of nuclear proteins having a role in transcription (Muller at al., 2004), of ATXN1, which occurs when ATXN1 can be transported to the nucleus (Riley et al., 2005).

With regards to possible functions in the nucleus, ATXN1 interacts with RNA (Yue et al., 2001), and several regulators of transcription, SMRT (Tsai et al., 2004), Capicua (Lam et al., 2006), Gfi-1 (Tsuda et al., 2005), and the Ror□/Tip60 complex described above (Serra et al., 2006). In the case of Capicua and the Ror□/Tip60 complex, mutant ATXN1 seems to enhance their degradation. For Capicua, loss of ATXN1 decreases its steady-state level (Lam et al., 2006). ATXN1 also interacts with two RNA-splicing factors, RBM17 (Lim et al., 2008) and U2AF65 (de Chiara et al., 2009). Another ATXN1 interacting protein is the ATXN1 paralog Brother of ATXN1, BOAT or ATXN1L (Mitzutani et al., 2005).

An evolutionary conserved region within ATXN1 that drives some of these interactions is a 120-amino acid AXH domain, Ataxin-1/HBP1 (HMG box-containing protein-1 transcription factor), crystalizes as a dimer that contains an OB-fold (de Chiara et al., 2003). Presence in ATXN1 of an OB-fold, a structural motif found in many oligonucleotide-binding proteins, is consistent with it ability to bind RNA. In addition to the OB-fold, the AXH domain of ATXN1 was postulated to contain a protein interacting surface (de Chiara et al., 2003). Subsequently, the AXH domain was shown to be important for the interaction of ATXN1 with the *Drosophia*/mammalian transcription factor Senseless/Gfi-1 (Tsuda et al., 2005), the transcription repressor Capicua (Lam et al., 2006), and the transcription factors Ror□/Tip60 (Gehrking et al., 2011), as well as the ATXN1 parolog BOAT/ATXN1L (Mitzutani et al., 2005).

An indication that the AXH domain of ATXN1[82Q] has a role in SCA1 pathogenesis came for studies in *Drosophila* where expression of ATXN1[82Q] lacking the AXH domain no longer induced the pathogenic effects on the Senseless (Tsuda et al., 2005). As part of an effort to assess whether ATXN1 with an expanded polyQ induces neurotoxicity via formation of novel, aberrant versus native protein complexes, it was found that the majority of wild type Atxn1 as well as expanded ATXN1 assembled into large stable complexes (Lam et al., 2006). ATXN1 was shown to also interact with the transcription repressor Capicua through the AXH domain and modulate its activity in *Drosophila* and mammalian cells. Furthermore, Capicua co-eluted with the ATXN1 containing complexes by sizing chromatography. Lastly, this study found that ATXN1[82Q] with an alanine residue instead of the normal serine at position 776 no longer formed high molecular weight complexes, suggesting that it is mutant ATXN1 in a native multiprotein complex that is pathogenic.

Recently, it was demonstrated that reducing the amount of Capicua decreases severity of disease in *Sca1*$^{154Q/2Q}$ mice (Fryer et al., 2011). Fryer et al., showed that a 50% reduction in Capicua generated by crossing *Sca1*$^{154Q/2Q}$ animals with *Capicua*$^{+/-}$ mice was sufficient to improve motor performance as well as learning and memory and extend life span of mice carrying on mutant allele of *Sca1*. Remarkably, mild exercise also reduced Capicua levels and extended life span of *Sca1*$^{154Q/2Q}$ mice. The effect of exercise on life span last for an extended period of time, lasting well after exercise was ceased. This latter affect is due to the ability of exercise to increase concentrations of epidermal growth factor in brains that functions to reduce Capicua. Molecularly, Fryer et al. found that the effect of mutant ATXN1 on Capicua regulation transcription varied depending on the target gene. They suggested that polyglutamine-expanded Atxn1 causes Capicua to bind more tightly to

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certain transcriptional targets (hyper-repressing them) and at other transcriptional targets causing Cic to bind less to—and thus upregulate (derepress) them. It is the relief of the hyper-repression that seems to underlie the protective effect of a reduction in Capicua either genetically or by exercise.

Two other functions shown to impact SCA1 pathogenesis in mice are the neurotrophic and angiogenic vascular endothelial growth factor (VEGF) and calbindin-D28k pathways (Vig et al., 2011). In the case of VEGF, ATXN1 with an expanded polyQ repressed its transcription and genetic overexpression or infusion of recombinant of VEGF dampened pathology in *Sca1*$^{154Q/2Q}$ mice (Cvetanovic et al., 2011). Maintaining calcium homeostasis is critical for proper neuronal function and downregulaiton of genes encoding proteins involved in calcium homeostasis and signaling, including calbindin-D28k, is a feature of ATXN1[82Q] expressing Purkinje cells (Lin et al., 2000; Serra et al., 2004). The importance of Calbindin-D28k function in SCA1 was directly demonstrated by the finding that a partial genetic reduction of calbindin-D28k in SCA1 mice exacerbated disease severity (Vig et al., 2011).

Ser776 - ATXN1 Function and SCA1 Pathogenesis

Ser776 (S776) is one of seven endogenous sites of phosphorylation in ATXN1 (Emamian et al., 2003; Huttlin et al., 2010). Importantly, S776 is located within another highly conserved portion of ATXN1, a 12 amino acid segment (residues 768–780) towards the C-terminus of ATXN1 (Carlson et al., 2009; de Chiara et al., 2009). This segment of ATXN1 contains three overlapping functional motifs among which are the previously described nuclear localization sequence (NLS), amino acids 771–774 (Klement et al., 1998), a 14-3-3 binding motif, amino acids 774–778 (Chen et al., 2003), and a UHM ligand motif (ULM), amino acids 771–776 (de Chiara et al., 2009). ULMs are a protein-protein interaction motif found exclusively in proteins involved with RNA splicing (Corsini et al., 2007).

Interest in S776 and the role its phosphorylation may have in ATXN1 function was first stirred with the finding that in mice with Purkinje cell expression of ATXN1[82Q]-A776, a phosphorylation blocking amino acid substitution, there is substantially fewer nuclear inclusions compared to ATXN1[82Q] transgenic mice at 32 weeks. In addition, ATXN1[82Q]-A776 mice do not develop ataxic cage behavior, do not show deficits by rotating-rod testing, and have only mild Purkinje cell pathology (Emamian et al., 2003). Recently, it was shown that animals with a phospho-mimicking Asp776 (ATXN1[82Q]-D776) had a more severe disease than animals expressing ATXN1[82Q]-S776 in their Purkinje cells. More intriguingly, ATXN1[30Q]-D776 also had impaired motor coordination on the Rotarod compared to wild type and ATXN1[30Q] control animals (Duvick et al., 2010). The level of impairment was similar to that of ATXN1[82Q] animals and progressed as animals aged until they could no longer perform the task. Similarly, ATXN1[30Q]-D776 animals had a significantly widened gait typical of ATXN1[82Q] animals. Both ATXN1[30Q]-D776 and ATXN1[82Q]-S776 mice at 12 weeks had Purkinje cell atrophy. Thus by a single, possibly mimicking phosphorylation amino acid substitution at residue 776, an ATXN1 with a wild type polyQ tract was converted into a pathogenic protein.

So how does phosphorylation of S776 affect ATXN1 and to what extent does having an Asp at position 776 have a similar effect? Phosphorylation of S776 was first suggested to stabilize ATXN1 in tissue culture cells (Chen et al., 2003). In support of this idea are data from mice expressing various forms of ATXN1 (Jorgensen et al., 2009; Lai et al., 2011). In these studies the relative ratios of *SCA1* mRNA to ATXN1 in mice expressing Pcp2-ATXN1 transgene on a *Sca1*$^{-/-}$ background were used as a surrogate for protein stability. Based on this approach it was concluded that the phospho-resistant ATXN1-A776 (Ala) is far less stable than either phosphorylatible ATXN1-S776 or phospho-mimicking ATXN1-

D776. These results are consistent with phosphorylation of S776 stabilizing ATXN1 *in vivo* in Purkinje cells. More recent *in vitro* data showing that in the absence of protease inhibitors, inhibition of S776 phosphorylation results in a decrease in ATXN1 level over time. In contrast when protease activity is blocked, inhibition of S776 phosphorylation had no affect on ATXN1 levels (Lagalwar et al., submitted for publication). In sum, there is considerable and consistent evidence that phosphorylation of S776 stabilizes ATXN1 by blocking its proteolysis.

S776 and perhaps its phosphorylation also has a crucial role in regulating interactions of wild type and mutant ATXN1 with at least three cellular proteins. These include the phospho-serine/phospho-threonine binding protein 14-3-3 (Chen et al., 2003) - a regulator of many signal transduction pathways (Morrison, 2008), and splicing factors RBM17 (Lim et al., 2008) and U2AF65 (de Chiara et al., 2009). As of now characterization of the interaction of ATXN1 with U2AF65, constitutive component of the spliceosome (Hartmuth et al., 2002), is limited to one study (de Chiara et al., 2009). In a peptide-peptide interaction assay, phosphorylation of S776 in a nine-residue ATXN1 peptide reduced but did not eliminate binding to a U2AF65 UHM peptide. This study also showed that overexpression of wild type enhances U2AF65 splicing function in a transfected cells co-expressing splicing reporter minigene. Interestingly, ATXN1 with an expanded polyQ was not able to promote U2AF65 splicing function, suggesting that polyQ expansion in some way disrupts the ATXN1/U2AF65 interaction.

The interaction of ATXN1-pS776 with 14-3-3 is important from several perspectives. First, the ATXN1-pS776/14-3-3 complex is stable such that the two proteins co-immunoprecipitate (Chen et al., 2003). Moreover, the interaction of 14-3-3 with ATXN1 seems to be restricted to the cytoplasm of cells in the cerebellum (Lai et al., 2011). Binding of 14-3-3 to ATXN1 in the cytoplasm has two identified outcomes; it blocks the dephosphorylation of ATXN1-S776 in the cytoplasm and it covers the NLS thereby blocking transport of ATXN1 to the nucleus (Lai et al., 2011). In the latter case, it means that for ATXN1 to be transported to the nucleus 14-3-3 must first disassociate presumably by some yet to be identified regulated process. Notably, 14-3-3 does not bind to ATXN1-D776 (de Chiara et al., 2009). Yet, ATXN1-D776 is resistant to proteolysis similar to ATXN1-pS776 (Lai et al., 2011). Thus, 14-3-3 binding is not required for stabilization of ATXN1 but does act indirectly to stabilize ATXN1-pS776 by preventing its dephosphorylation. The finding that the relative amount of ATXN1-D776 found in the nucleus is increased is consistent with the inability of 14-3-3 to form a complex with ATXN1-D776, i.e. the NLS in ATXN1-D776 is not masked by 14-3-3 (Lai et al., 2011).

Another protein whose interaction with ATXN1 involves S776 is RBM17, RNA-binding motif protein 17/spf45 in *Drosophila*, (Lim et al., 2006; de Chiara et al., 2009). To date, RBM17 is the only protein whose interaction with ATXN1 is modified by the length of ATXN1's polyQ tract and the amino acid at position 776 (Lim et al., 2008). The ATXN1/RBM17 interaction increases with increasing length of the polyQ tract. In addition, the ATXN1/RBM17 is decreased dramatically with ATXN1-A776 regardless of polyQ tract length. Interestingly, replacing S776 with an aspartic acid reside results in a form of ATXN1-30Q, ATXN1[30Q]-D776, that has an enhanced interaction with RBM17 similar to that seen with ATXN1[82Q]-S776. Thus, the two forms of ATXN1 that are able to cause disease in Purkinje cells of transgenic mice, ATXN1[82Q]-S776 and ATXN1[30Q]-D776 (Burright et al., 1995;Duvick et al., 2010), are the both forms of ATXN1 that interact strongly with RBM17 (Lim et al., 2008). Moreover, a form of ATXN1 – ATXN1-A776 - that interacts weakly with RBM17 is unable to cause disease (Emamian et al., 2003; Lim et al., 2008). These results indicate that a gain of function pathogenic mechanism of mutant

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ATXN1 involves its interaction with RBM17, and suggests that the phosphorylation state of serine 776 is critical for the strength of this interaction.

The phosphorylation state of a protein is a dynamic process dictated by both protein kinases and phosphatases. S776 of ATXN1 lies within strong consensus phosphorylation sites for the kinases AKR mouse thymoma (Akt) and cyclic AMP-dependent protein kinase (PKA). While initial data using tissue culture cells and a *Drosophila* model of SCA1 indicated that Akt could phosphorylate S776 (Chen et al., 2003), more recent data favors PKA as the ATXN1-S776 kinase in the mammalian cerebellum (Jorgensen et al., 2009). Inhibition of Akt either *in vivo* or in a cerebellar extract-based phosphorylation assay did not reduce the amount of phospho-S776 ATXN1. Moreover, reduction of Akt in SCA1 transgenic mice was not protective against disease. These results argue against Akt as a kinase that phosphorylates S776 of ATXN1. In contrast, drugs that selectively inhibit PKA or immunodepletion of PKA blocked the ability of cerebellar extracts to phosphorylate ATXN1-S776. Thus, PKA is likely to be one kinase that phosphorylates ATXN1 at S776 in the cerebellum. Together the data suggest that the phosphorylation of S776 in ATXN1 by PKA and the subsequent interaction of ATXN1[82Q]-pS776 with RBM17 in the nucleus is a major contributor to at least Purkinje cell pathogenesis in SCA1.

Pathophysiology of SCA1

One fundamental question facing SCA1 and many of the neurodegenerative disorders concerns the pathophysiology of neuronal dysfunction. Is there dysfunction in a specific neuronal population or circuit initially that contributes the onset of behavioral abnormalities? One study examined Ca^{2+} dynamics and electrophysiological properties of Purkinje cells from adult (3–7 months) *ATXN1[82Q]* mice in slice preparations (Inoue et al., 2001). After adjustment for neuronal volume, the basic electrophysiological properties of mutant Purkinje cells were found to be essentially intact, including the responses to both major afferent projections, the climbing fibers (CFs) and parallel fibers (PFs). This study, in the end, suggested that the functioning of the intact cerebellar neuronal circuitry is affected in SCA1 mice.

Barnes et al. (2011) utilized flavoprotein autofluorescence optical imaging and field potential recordings *in vivo* to examine whether there are alterations in the climbing fiber-Purkinje cell and parallel fiber-Purkinje cell circuitry in early-stage (6-week-old) and mid-stage (12-week-old) disease mice prior to gross Purkinje cell pathology and death. The results show that ATXN1[82Q] preferentially affects the climbing fiber-Purkinje cell synapse early in the disease process while parallel fiber-Purkinje cell synaptic transmission is unaltered. As disease progresses out to 40 weeks of age (late-stage disease) there are signs of altered parallel fiber-Purkinje cell synaptic activity. The functional and morphological assessments revealed that the abnormalities in the climbing fiber-Purkinje cell circuit that are dependent on ATXN1[82Q] expression and entrance into the nucleus of PCs. Mice that express ATXN1[82Q] that fails to enter the nucleus of Purkinje cells show no signs of an altered climbing fiber-Purkinje cell circuit Moreover, the deficits in climbing fiber-Purkinje cell synaptic transmission required mutant ATXN1 expression during early postnatal development. These findings support a model of early SCA1 disease where abnormalities at the synapse contribute to disease initiation and motoric deficits. They implicate a compromised function at a specific type of synapse in the development of SCA1 and highlight the importance of identifying factors unique to the climbing fiber-Purkinje cell synapse that contribute to its dysfunction.

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Closing Comments – Future Directions

The data generated to date strongly support a model of SCA1 pathogenesis where four molecular features of mutant ATXN1 all must be present for disease; an expanded polyQ tract, a functional NLS, the AXH domain, and phosphorylatible Ser at residue 776. Together these contribute to critical events that occur in the nucleus of an affected neuron. Expansion of the polyQ tract differentially affects the function of ATXN1 depending on the context of the different native complexes it forms with other nuclear proteins. PolyQ tract expansion shifts the *status quo* by enhancing certain functional complexes/pathways perhaps at the expense of others. At the center of the postulated imbalance in native complex formation induced by mutant ATXN1 are the complexes it forms with the transcriptional repressor Capicua and the splicing protein RBM17 (Figure 2). While changes in both Capicua and RBM17 complexes contribute to disease, it is striking that a single amino change, S776 to D776, converts ATXN1 with a wild type polyQ tract to a protein that shares two features of ATXN1 with an expanded polyQ – a protein that interacts strongly with RBM17 and causes disease in murine Purkinje cells. Thus, it seems that an enhanced interaction of mutant ATXN1 with RBM17 is a major driver of disease at least in Purkinje cells. Since RBM17 is know to regulate alternative RNA splicing it seems very likely that alterations in splicing underlie the Purkinje aspect of SCA1.

Participation of S776 of ATXN1 and its phosphorylation in RBM17 interaction and SCA1 pathogenesis provides one potential therapeutic target for SCA1. Much effort has gone into and is going into understanding signaling pathways regulated by protein kinases. Protein kinases are one of the most active groups under investigation as drug target owing to their involvement in many pathological conditions. Yet there are substantial challenges that face targeting protein kinases in disorders of the brain like SCA1 (Chico et al., 2009). Not the least of which are penetration of the blood-brain barrier and selectively targeting a multi-functional kinase like PKA. Thus, it is important not to depend on any one single therapeutic target. Rather, multiple therapeutic avenues need to be explored. In this regard identification of the kinase(s) responsible for ATXN1-S776 phosphorylation in affected neurons is of importance as it affords an opportunity to elucidate processes/signals upstream to the kinase that regulate its action on ATXN1 that could result in viable drug targets. Likewise, elucidation of downstream events triggered by ATXN1-S776 phosphorylation is of value.

It is worth noting that all of the evidence on SCA1 using experimental models show a direct relationship between disease severity and level of mutant ATXN1 expression. Thus, identifying other pathways or strategies by which the levels of the disease-causing protein could be decreased would serve as additional novel drug targets and or therapeutic approaches. For example, the ability of RNA interference to inhibit polyQ-induced neurodegeneration caused by mutant ATXN1 in a mouse model of SCA1 (Xia et al., 2004) or the use of allele specific oligonucleotides as has been sued in a mouse model of ALS (Smith et al., 2006) offer two potential genetic therapeutic approaches for decreasing the function of mutant ATXN1.

Abbreviations

Akt	AKR mouse thymoma kinase
ATXN1	vascular endothelial growth factor human Ataxin-1
dox	doxycycine
NLS	nuclear localization sequence
PKA	cyclic AMP-dependent protein kinase

polyQ	polyglutamine
RBM17	RNA binding motif protein 17
SCA1	Spinocerebellar ataxia type 1
Tta	tetracycline-transactivator
TRE	tetracycline response element
VEGF	vascular endothelial growth factor

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Highlights

- ▶ An overview of the pathogenesis of the polyglutamine neurodegenerative disease spinocerebellar ataxia type 1 (SCA1).
- ▶ A study of all the molecular pathways that impact SCA1 pathogenesis.
- ▶ A discussion of the two major pathways that contribute to SCA1, transcriptional regulation mediated by ATaxin-1/Capicua and what is likely RNA processing involving Atxain-1/RBM17.
- ▶ A review of the importance of Ser 776 phosphorylation in regulation of Atxain-1 biology and SCA1 pathogenesis.
- ▶ An opinion is promoted, which encourages the identification and characterization of multiple potential therapeutic targets. .

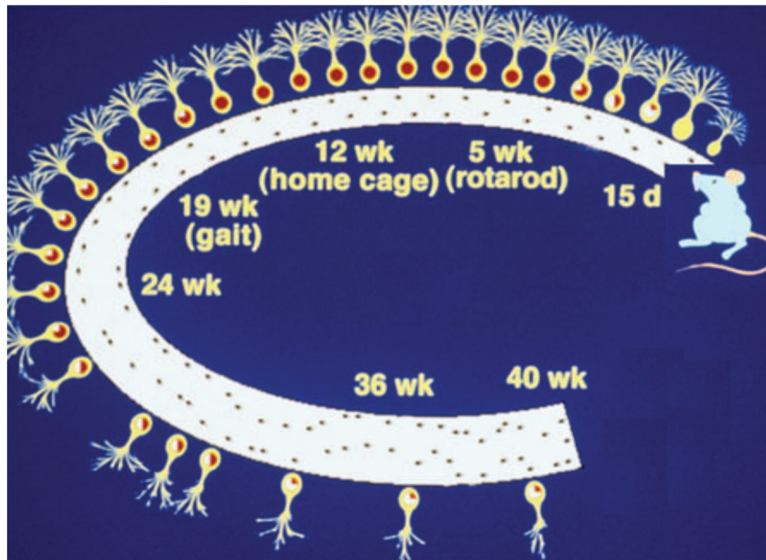

Figure 1. A Schematic Depiction of the Progressive Motoric Deficits and Purkinje Cell Pathology in ATXN1[82Q] Mice

Importantly all neurological alterations including overt ataxia appear prior to the onset of Purkinje cell death.

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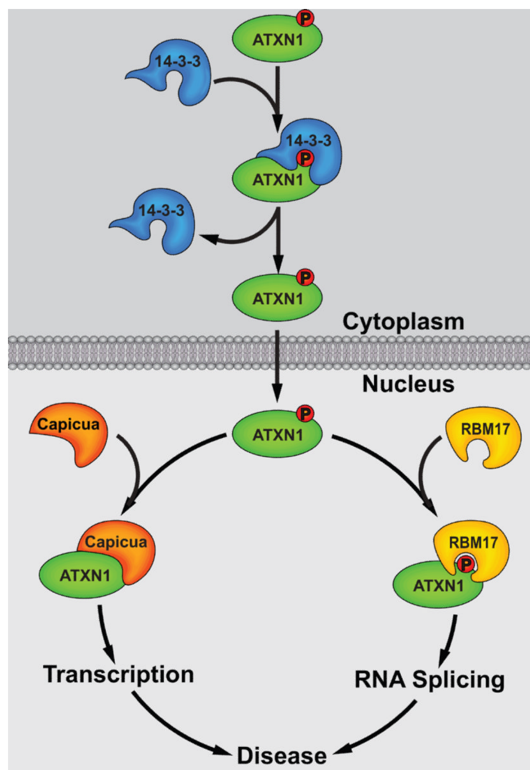

Figure 2. The Cellular Pathway by which S776 Phosphorylation of ATXN1 Impacts its Function and Pathogenesis in the Nucleus

In the nucleus two pathways involving the interaction of ATXN1 with either the transcriptional repressor Capicua or the RNA splicing factor RBM17 contribute to pathogenesis. Its is the RBM17-mediated pathway that appears to be the pathway that is impacted by S776 phosphorylation.

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