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Ataxin-2 Binds Alpha-Actinin-1

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Abstract: Ataxin-2 (human gene symbol *ATXN2*, protein ATXN2) is the disease protein of Spinocerebellar Ataxia type 2 (SCA2). The large expansions of a polyglutamine (polyQ) stretch above a threshold of ~33 glutamines cause the multi-system nervous atrophy SCA2, while intermediate expansions of 29-32 glutamines contribute to the risk of the motor neuron diseases Amyotrophic Lateral Sclerosis (ALS) and Fronto-Temporal Lobar Dementia (FTLD). To elucidate the cellular function of ATXN2, we further characterized its direct interaction with alpha-Actinin-1 (symbol ACTN1), which had been observed in high-throughput yeast-two-hybrid surveys. An endogenous complex of ATXN2 and ACTN1 proteins was detected by co-immunoprecipitation. *In vitro* GST-tag pull-down experiments showed that the Calponin-Homology-domain at the N-terminus of ACTN1 binds to the N-terminus of ATXN2. Although an impact of the polyQ expansion on the interaction was not evident in pull-down experiments, a recent characterization of aged *Atxn2*-CAG100-KnockIn mice provides evidence. Both proteins associated in the cytosol and at the plasma membrane, as determined by sedimentation experiments in mouse brain, and by immunofluorescence microscopy of a transfected monkey cell line and of rat primary hippocampal neurons. In view of the roles of ACTN1 for spine plasticity and postsynaptic receptor control via reassembly of cortical actin, our data help to explain the impaired dendrite maintenance in SCA2.

Keywords: olivo-ponto-cerebellar atrophy (OPCA); Purkinje cells; Calcium/calmodulin dependent protein kinase II (CAMK2); Plastin (Fimbrin); Hirano bodies; Huntington's disease.

1. Introduction

Spinocerebellar ataxia type 2 (SCA2) is an autosomal-dominant neurodegenerative disorder which is caused by the expansion of a (CAG)-triplet-repeat encoding a polyglutamine (polyQ) domain [1-3]. Upon large expansions beyond the critical threshold (~33Q) within the Ataxin-2 protein (human gene symbol *ATXN2*, protein symbol ATXN2), a multi-system atrophy in the nervous system ensues, which was previously described by clinical neurologists as cerebellar ataxia with slowed saccadic eye movements [4-9], by neuropathologists as olivo-ponto-cerebellar atrophy [10-26], and was defined by genetic analyses as SCA2 [27-34]. Upon intermediate expansions between 29Q and 32Q, the risk to be affected by tauopathies such as the motor neuron diseases Amyotrophic Lateral Sclerosis (ALS) and Fronto-Temporal Lobar Dementia (FTLD), or the Parkinson-plus variant PSP (Progressive Supranuclear Palsy) is increased [35-47]. Similar polyQ expansion mutations were identified in an increasing number of different genes to underlie neurodegenerative processes, and the resulting diseases were named 'polyglutamine diseases'. So far five other ataxias (SCA1, SCA3, SCA6, SCA7, SCA17), Huntington's disease (HD), spinobulbar muscular atrophy (SMA), and dentatorubro-pallidoluysian atrophy (DRPLA) [48, 49] have been reported. There are intense investigations to elucidate the mechanisms how expanded polyQ domains lead to neural dysfunction and finally cell loss in the nervous system [50-56].

When polyQ domains are expanded to pathogenic length, they show an increased propensity to change the protein conformation. The turnover of such proteins is altered and insoluble aggregates

are formed, which show an amyloid morphology [57, 58]. In post-mitotic neurons, this cumulative aggregation process may eventually fill the affected subcellular compartment and trigger the expulsion of aggregates from the cell via exosomes [59], as well as their internalization by adjacent microglia [60, 61]. The role of such aggregates (inclusion bodies) is controversially discussed. On the one hand, they mediate a toxic gain-of-function of the disease protein, sequester essential factors among its physiological interactome into the aggregation process [62], and represent a burden for the protein degradation systems via the ubiquitin-proteasome pathway as well as the autophagy pathway [63]; on the other hand, they were suggested to play also a protective role [64, 65].

Each of the nine polyQ diseases triggers a specific neurodegeneration pattern in the nervous system [66], so the affected neuron populations vary in dependence on the disease protein structure, expression profile, subcellular localization and interacting molecules. In the case of ATXN2, it was demonstrated that its cytosolic interactor protein PABPC1 [poly(A)-binding protein cytoplasmic 1] as well as nuclear RNA-processing factors are recruited into the inclusion bodies, but this sequestration process also extends to the calcium-dynamics endoplasmic reticulum factor ITPR1 and the mitochondrial autophagy effector Parkin [50, 51, 53, 54, 61, 67]. The spinocerebellar degeneration pattern of SCA2 is easily explained by the preferential expression of ATXN2 in the cerebellum together with specific brainstem and spinal cord neurons, e.g. cerebellar Purkinje cells and motor neurons [68]. However, ATXN2 is ubiquitously expressed and plays a major role also in female sexual organs, blood vessels and lung (<https://gtexportal.org/home/gene/ATXN2>). The subcellular distribution of ATXN2 was shown to be mainly associated with the rough endoplasmic reticulum [69-71], but some ATXN2 is also present at the plasma membrane [72, 73]. During periods of cellular damage, ATXN2 re-localizes to cytosolic stress granules where RNA quality control and triage take place [74, 75]. Indeed, human ATXN2 was demonstrated to bind mRNAs directly, with preference at their 3'-untranslated region [76, 77]. ATXN2 is phylogenetically conserved in all eukaryotic organisms [78]. For its orthologue in *Drosophila melanogaster* it was shown that Ataxin-2 physically assembles with ribosomes [76], but another effect of *D. melanogaster* Ataxin-2 on actin filament formation was never understood in modular detail, or reproduced in other organisms so far [79]. The known direct protein interactors for ATXN2 either confirm the role at the ribosome, like poly(A)-binding cytoplasmic protein 1 (gene symbol *PABPC1*) [80], ATXN2 binding protein 1 (A2BP1, aka Fox-1-Like RNA-Binding Protein 1, gene symbol *RBFOX1*) [81], and the RNA helicase DDX6 [74]. The protein interactors of ATXN2 also support an additional function related to endocytosis, in view of its direct association with Endophilin-A (gene symbols *SH3GL2*, *SH3GL3*) and a perhaps indirect association with Fimbrins (aka T-/L-Plastin, gene symbols *PLS3*, *LCP1*) [72, 82]. Interestingly, the interaction with Endophilin-A is in common between two polyQ disease proteins, namely ATXN2 and Huntingtin, and both non-expanded polyQ proteins become toxic in cells with deletion of the filopodia-enriched Fimbrin [82].

Specific protein sequence motifs within ATXN2 have been identified to be responsible for these interactions. The direct binding to RNAs is mediated by Lsm and LsmAD domains encoded by exons 3-8. Its indirect binding to mRNAs via direct binding to the poly(A)-tail associated PABPC1 is mediated by a Pam2 motif encoded by exon 16 [83]. The aggregation-prone polyQ domain is encoded in exon 1, but is not conserved even in mice. The exon 1 and the alternatively spliced exon 10 encode two proline-rich domain (PRD) of type II, which mediate the association with SH3 motifs within endocytosis factors like SH3GL2, SRC and GRB2 [72, 73]. With exception of the polyQ domain, all these motifs are conserved throughout all eukaryotic organisms, while varying in order [78].

In a large protein-interaction screen of proteins involved in the degeneration of cerebellar Purkinje neurons (Suppl. Table S3 in [84]), alpha-Actinin-1 (protein symbol ACTN1) was reported to directly associate with ATXN2, similar to several other protein fragments from A2BP1, ACTN2, ATXN1, BAT3, CHGB, FLJ35171, GFI1B, IDH3B, KIAA1279, NCOA4, SH3GL2, SH3GL3, TDRD7, ZNRD1 (Suppl. Tables S3/S4 in [84]). In this published survey, a stringent yeast-two-hybrid assay was employed to assess 23 human cDNAs encoding proteins directly involved in ataxias, including

ATXN2 and its truncation fragments, regarding their interactions with the human ORFeome1.1 [85] and an adult brain library. We focused additional validation work on ACTN1.

This protein occurs ubiquitously in many cell types, while its three homologs ACTN2-4 vary in distribution, two of them representing muscle-selective isoforms. All alpha-actinins form an antiparallel homo-dimer where each end makes contact with actin, while the rod-shaped central region regulates the geometry between actin filaments, an interaction that is diminished and abolished upon calcium elevation [86]. They promote actin turnover, prevent the formation of actin stress-fibers and of rigid adhesions [87, 88]. Alpha-actinins thus establish the symmetric architecture and mobility of the actin-network, integrating the mechanical forces to carry out membrane movements, in adaptation to extracellular cues [89-92]. Beyond binding to actin, they associate with cytoplasmic domains of transmembrane proteins, connecting receptors and ion channels with submembrane platform proteins, such as the postsynaptic integrator PSD-95 [93]. In a signalling function, they were reported to associate with nuclear hormone receptors and potentiate their basal transcription activity [94]. They thus serve as scaffold in the connection of perception signals to the cytoskeleton [95], mediate filopodia movement [96-98], and are important for the rearrangement of cortical actin to open space for vesicle fusion/fission, and to create a hollow lumen within membranes [99]. In view of the known interactions of ATXN2 with endocytosis factors, it is interesting to note that ACTN1 mediates the contracting force of cortical actin-myosin complexes, which acts on clathrin lattices to internalize the vesicle [100-103]. The endocytic internalization of the ADORA2A (adenosine A2A) receptor and the L-type Ca-channel was shown to be dependent on calcium and excitation, in a stimulus-response mediated via the interaction of calmodulin with ACTN1 [104, 105].

The protein sequence and structure of alpha-Actinin makes it a versatile docking station for a large number of other factors. Alpha-Actinin consists of an N-terminal Calponin-Homology (CH1/2) domain that serves as an protein-protein interaction module [106], four spectrin repeats ensuring a rod-like linear shape [107], and a C-terminal Calmodulin-like domain (CaM) capable of calcium-binding and sensing [95]. Alpha-Actinin is abundantly expressed with preference in cells under the influence of mechanical force. It is subcellularly located in the cytoplasm and at the migrating edge of plasma membranes, together with other actin-binding proteins such as VCN (Vinculin), GSN (Gelsolin), CFL1 (Cofilin), PDLIM1 (PDZ And LIM Domain 1, aka Elfin) under control of kinases such as LIMK1 (LIM motif containing kinase 1) [108-110]. Alpha-Actinin is concentrated in filopodia or in extending neurites, when the growth cone extends upon stimulation by trophic factors such as the Purkinje-neuron specific c-Kit [96, 111-115].

Our findings indicate that ATXN2 directly binds to ACTN1, so it may modulate its downstream functions e.g. during dendritic spine growth.

2. Results

2.1. Recombinant full-length alpha-Actinin-1 and Ataxin-2 interact in co-immunoprecipitates from HEK-293 cells.

To evaluate the coexistence of ATXN2 and ACTN1 in a protein complex for mammalian cells, we used overexpressed and tagged versions of full-length ATXN2 and ACTN1 in HEK-293 cells, investigating their association via co-immunoprecipitation. We precipitated ATXN2 with an antibody against its specific tag from the cell homogenates and detected ACTN1 in the precipitate by immunoblots (WB) (Figure 1). This observation confirms the published yeast-two-hybrid results and demonstrates that both proteins can interact in mammalian cells.

Figure 1. Recombinant Ataxin-2 and alpha-Actinin-1 form a protein complex in HEK-293 cells.

Plasmids coding for Myc-tagged wildtype (22Q) ATXN2 and for GFP-tagged ACTN1 were used to co-transfect HEK-293 cells. Input was controlled in the three lanes to the left, while immunoprecipitates from cell homogenates with anti-Myc antibody are shown in the right three lanes. Precipitates were analysed by SDS-PAGE and Western blotting. The band corresponding to GFP-ACTN1 can be detected selectively in the immunoprecipitate (IP) of Myc-tagged ATXN2(22Q), indicating the presence of a protein complex that contains both components.

2.2. Endogenous ACTN1 and ATXN2 interact in co-immunoprecipitates from mouse brain cytosolic fractions.

It was important to clarify if endogenous ATXN2 and endogenous ACTN1 exist in a physiological complex within the brain, the tissue most affected by SCA2. In view of the low abundance of ATXN2, a differential velocity centrifugation protocol (Figure 2A) was used for the subcellular fractionation of mouse brain. To differentiate between cytosolic and light membrane fractions, the cytosolic marker EEA1 (Early Endosome-Associated Protein P162) and the light membrane marker CANX (endoplasmic reticulum-associated Calnexin) were used. ATXN2 and ACTN1 co-fractionated in the cytosolic fraction (Figure 2B).

Immunoprecipitation of ACTN1 from the cytosolic fraction confirmed the co-precipitation of ATXN2. Conversely, upon precipitation of ATXN2 the ACTN1 protein was detected in association with it (Figure 2C). These findings substantiate the notion that an endogenous complex containing both proteins exists in brain.

Figure 2. ATXN2 and ACTN1 form an endogenous complex in mouse brain. (A) Differential velocity centrifugation protocol for brain fractionation. (B) Among various brain fractions, ATXN2 was detected by Western blot (WB) in the post-nuclear and the post-mitochondrial supernatants (SN), but not in the mitochondrial pellet. The cytosolic fraction, indicated by the marker protein EEA1, contained both ATXN2 and ACTN1, while the light membrane fraction contained only ATXN2. (C) Co-immunoprecipitates from mouse cytosolic fraction contained both ATXN2 and ACTN1. Empty beads were used for negative controls. Analysis of the precipitates was performed by SDS-PAGE and immunodetection with the indicated antibody. ATXN2 can be demonstrated in the ACTN1 precipitate and *vice versa*. These data prove the existence of an endogenous complex containing both proteins in the disease-relevant tissue.

2.3. *In GST-pull-down assays, the N-terminus of ATXN2 interacts with full-length ACTN1.*

Recombinant constructs tagged by GST (Glutathione-S-Transferase polypeptide) were used in pull-down assays to define the domain within ATXN2, which is responsible for the interaction. Full-length ACTN1 with GST-tag was combined with Myc-tagged ATXN2, either full-length or in fragments (Figure 3A). The fragments contained (i) the N-terminus of ATXN2 as the least conserved part of the protein, (ii) the polyQ domain either only with the like-Smith-antigen (Lsm) domain or (iii) also with its Lsm-associated domain (LsmAD), or (iv) the rest of the protein containing the Pam2-motif. Binding could be observed for three of the fragments which all contained the very N-terminal part of ATXN2 (Figure 3B). The addition of the Lsm and LsmAD domains did not have a strong effect on the interaction. The region essential for the interaction lies upstream from the polyQ region of ATXN2 in the N-terminal part. The function of this N-terminal sequence has not been defined so far.

Figure 3. ATXN2 interacts with ACTN1 through its N-terminal part. (A) Schematic representation of ATXN2 fragments tested for interaction with ACTN1 in GST-pull-down assays. (B) Input was controlled in the left panel, where the translation product of each Myc-tagged ATXN2 construct was detected via the Myc-tag antibody. In the middle panels, GST alone was loaded onto glutathione (GSH)-sepharose beads, as a negative control. In the right panels, GST-ACTN1 was loaded on GSH-sepharose beads. Loaded beads were then incubated with extracts from HEK-293 cells that overexpressed Myc-tagged constructs of ATXN2 either full-length or as the fragments shown in (A). Equal loading of the beads with GST and GST-ACTN1 was controlled by Western blots using anti-GST antibody. Detection with anti-Myc antibody showed binding between ACTN1 and all ATXN2 constructs, which encoded the full-length protein or contained the N-terminal part with amino acids (aa) 1-456.

2.4. The Actin-Binding-Domain of ACTN1 mediates the interaction with full-length ATXN2 in GST-pull-down assays.

ACTN1 contains six distinct functional domains, with repetition of two sequence motifs that are known to mediate protein-protein interactions. They include (i) the actin-binding-domain (ABD) with two Calponin-Homology motifs (CH1/2) at the N-terminus, which mediates the connection to the cytoskeleton via actin, and (ii) the four spectrin repeats forming the rod domain that serves as

scaffold for multiple protein interactions. In addition, calcium sensing occurs via a pair of EF-hand (EFh) motifs separated by a flexible linker region at the C-terminus, which provide binding sites for two Ca^{2+} ions and are homologous to the four EF-hand motifs in Calmodulin [116].

To determine the region on ACTN1 that mediates the interaction with ATXN2, we used GST-fusion constructs of full-length ACTN1 and of (i) the ABD, (ii) each of the four spectrin repeats, or (iii) the last two spectrin repeats together with the EF-hand region (Figure 4A). Equal binding of ATXN2 was observed for the full-length protein and for the ABD region. All other fragments of ACTN1 failed to pull down ATXN2 (Figure 4B). These data raise the possibility that ATXN2 competes with actin for the binding site on ACTN1, which would explain the actin-filament phenotype of *D. melanogaster* mutants of ATXN2 [79].

Figure 4. The N-terminal region of ACTN1 with two Calponin-Homology (CH1/2) motifs mediates the interaction with ATXN2. (A) Schematic representation of ACTN1 fragments that were tested for interaction with ATXN2 in GST pull-down assays. (B) GST-ACTN1 full-length and its GST-tagged fragments were bound to GSH-sepharose beads, which were then incubated with extracts from HEK-293 cells that overexpressed Myc-tagged full-length ATXN2. Equal loading of GST and the GST fusion proteins was controlled by an anti-GST antibody. Associated ATXN2 fragments were detected using anti-Myc antibody. ATXN2 was observed to bind to full-length ACTN1 or to the CH1/2 domain alone, in similar intensity. All other parts of ACTN1 failed to pull down ATXN2.

2.5. The pathological expansion of ATXN2 does not detectably alter the interaction in GST-pull-down assays.

It is crucial to investigate if a polyQ expansion in ATXN2 affects its binding to ACTN1. We used the largest expansion observed so far in the Cuban SCA2 population, where 79Q instead of 22Q triggered disease manifestation already at 2 years and caused death by 8 years of age [1, 9]. The two N-terminal ATXN2 fragments that had bound to ACTN1 were modified to contain 79Q, instead of the preexisting 22Q wildtype version (Figure 5A). This expansion size had no obvious effect on the binding to ACTN1 under the current GST pull-down conditions (Figure 5B), a disappointing finding in view of the proximity between the polyQ domain and the ACTN1-binding area within ATXN2, and given the high probability that an expansion would cause N-terminal misfolding. However, in a separate manuscript we showed that a 100Q expansion knocked into mouse Ataxin-2 triggers authentic SCA2 [53]. The investigation of spinal cord tissue from such *Atxn2*-CAG100-KnockIn mice at the age of 12 months via global transcriptome profiling and automated bioinformatics demonstrated significant dysregulations of several pathways under control of actin and alpha-actinin [61].

Figure 5. PolyQ expansion from 22Q to 79Q has no apparent effect on the interaction between ACTN1 and ATXN2 in GST pull-down assays. (A) Schematic representation of ATXN2 fragments tested for interaction with ACTN1. **(B)** Correct expression of all fragments was controlled as input (left panel). GST alone (middle panel) or GST-ACTN1 (right panels) were bound to GSH-sepharose beads. The beads coupled to GST or GST-ACTN1 were then incubated with extracts from HEK-293 cells that overexpressed Myc-tagged constructs of ATXN2 fragments with normal or expanded polyQ stretches as shown in (A). The N-terminal fragment upstream from the polyQ stretch was included as a positive control. Binding of the ATXN2 fragments was detected using anti-Myc antibody, binding and equal amounts of GST and GST-ACTN1 were confirmed with the anti-GST antibody. All fragments bound to GST-ACTN1. The expansion of the polyQ stretch did not visibly alter the binding of the ATXN2 fragments to GST-ACTN1.

2.6. Co-localization of ATXN2 and ACTN1 as recombinant proteins in COS-7 cells, and as endogenous proteins in rat hippocampal neurons.

In order to substantiate the biochemical findings, the subcellular localization of both proteins was examined further. Again with the recombinant constructs already used during co-immunoprecipitation experiments (Figure 1), ATXN2 and ACTN1 were overexpressed in COS-7 cells. Both proteins showed a largely cytoplasmic and membranous distribution, with overlap in

both regions. There was no apparent difference between the co-localisation of normal ATXN2 (22Q) and ACTN1 compared to the mutated form of ATXN2 (79Q) (Figure 6A). To also visualize this co-localisation for both endogenous proteins, as further confirmation of the co-immunoprecipitation results, rat hippocampal primary neurons were stained. Indeed, a co-localisation in the cytosol could be observed (Figure 6B). These data support the biochemical findings and corroborate the physiological existence of a ATXN2 / ACTN1 complex.

Figure 6. ACTN1 co-localizes with ATXN2 upon confocal microscopy. (A) COS-7 cells were transfected with HA-ACTN1 (green) and either Myc-ATXN2(22Q) or Myc-ATXN2(79Q) (red); they were counterstained with DAPI (blue) to illustrate the nucleus. Each of the recombinant proteins was detectable throughout the cytosol with perinuclear concentration and at the plasma membrane. In the merged pictures, the co-localization of the recombinant proteins appears in yellow. A distinct yellow co-localization signal was demonstrated at the leading cell growth edge, where plasma membrane cortical actin is concentrated. (B) In rat hippocampal neurons, endogenous ACTN1 (green) and ATXN2 (red) were stained, showing a diffuse cytosolic pattern for both proteins, with ATXN2 being less abundant in neurites than ACTN1. Specific signals at the plasma membrane were not obvious under non-overexpressed conditions, in the absence of growth factor deprivation. In the merged picture, the co-localization (yellow) was prominent in the perinuclear cytosol.

3. Discussion

This study examines the direct protein-protein interaction between Ataxin-2 and alpha-Actinin-1. We demonstrated that ATXN2 and ACTN1 associate in mammalian cells,

showed in GST-pulldown assays what specific domains in each factor are responsible for the interaction, and corroborated the coexistence of the endogenous proteins by differential fractionation and with microscopy, studying brain tissue and neurons as the principal targets of the relevant disease process.

So far, interactions and associations of ATXN2 to various proteins suggested two major roles for ATXN2 in cell physiology. On the one hand they included a quality control function for RNAs [74, 76, 80-82], on the other hand an inhibition of the RTK endocytosis process [82], in particular during cell stress. We now present data that support previous findings in *D. melanogaster* regarding the impact of ATXN2 on the actin-cytoskeleton [79]. The loss-of-function of Datx2, the single fly homolog of mammalian ATXN2 and ATXN2L, resulted in severe phenotypic consequences. Datx2 was found to be a dosage-sensitive regulator of actin filaments, affecting fly bristle formation and actin in eye-discs, in absence of a direct actin-interaction. Actin synthesis and abundance was not altered. Thus, the interaction of ATXN2 with ACTN1 may partially explain how cortical actin in growth processes is affected by mutations in Ataxin-2.

Our data also expand on a previous report that ATXN2 as well as Huntingtin interact directly with endophilin A1/A3 (SH3GL2/SH3GL3) and co-immunoprecipitates also with L-/T-Plastin (homologs of I-Plastin aka Fimbrin) in mouse brain [82]. This association was discovered because an unbiased survey of polyQ toxicity in yeast had identified the Fimbrin depletion to trigger vulnerability towards ATXN2, Huntingtin, Endophilin A1 and A3. The resulting growth deficit could be rescued by human L- and T-Plastin. This study also showed that a direct ATXN2-Plastin interaction was not detectable in yeast-two-hybrid assays, but ATXN2 overexpression in the mammalian cell lines HEK-293, SH-SY5Y and COS-1 led to higher T-Plastin (protein symbol PLS3) abundance upon immunofluorescence imaging [82]. As shown in Figure 7, both T- and L-Plastin have CH1/2 domains for actin-association in common with ACTN1, as well as sharing two EFh domains. L-/T-Plastin are actin-bundling proteins like alpha-actinins [95], control the distance between actin filaments and influence cell morphology [117, 118]. Indeed, alpha-actinins can prevent Fimbrin from actin-association [119, 120]. Although the identification of a specific domain that mediates the association between L-/T-Plastin and ATXN2 has not been possible, our data suggest that the actin-binding-domain could be involved again.

Figure 7. Alpha-Actinin-1 and T-/L-Plastin share the actin-binding-domain with two CH motifs, as well as the Ca²⁺ binding domain (CaM) with two EF-hand motifs, but differ in the order and number of these domains. Alpha-Actinins additionally possess a rod domain consisting of spectrin repeats.

It is important to note that the force needed to perform endocytosis depends on the actin cytoskeleton together with myosin motors [121-123]. Thus, our findings also expand previous reports that Proline-rich-domains (PRD) in ATXN2 interact with SH3 motifs within proteins of the endocytosis apparatus (such as SH3GL2, SH3GL3, SRC, GRB2) to slow the internalization of growth factor receptor tyrosine kinases (RTK). This interactions modulates growth/stress signalling via phosphorylation cascades, probably including the mTORC1 complex that controls cell size [56, 72, 73, 124-129]. ATXN2 may therefore control such plasma membrane uptake events at two sites, on the one hand for ultrafast clathrin-independent endocytosis during vesicle neck formation and scission via Endophilin-A [130-132], on the other hand for clathrin-dependent endocytosis during the slow internalization process and scission via ACTN1 with T-Plastin as modifiers of actin stabilization [133-135]. However, it is important to note that the ATXN2 interaction with ACTN1 might not only modulate actin-interactions, since some ABD motifs were reported to have roles as microtubule-binding site and as signalling scaffold [136, 137]. There is also an ongoing debate about the physiological role of the poorly conserved N-terminal region of ATXN2 upstream from the polyQ domain encoded within exon-1. One start codon initiates the ATXN2 reference sequence that includes a N-terminal PRD motif (aa 118-123), but an alternative start codon exists just before the polyQ sequence. The Lsm/LsmAD motifs encoded by exons 3-8 and the Pam2 motif encoded by exon-16 are constitutively expressed. In contrast, the second PRD motif (aa 588-593) is encoded by the alternatively spliced exon-10 [125, 138]. The functional consequences of these isoforms of ATXN2 remain to be elucidated.

Although clear evidence was observed for the physiological occurrence of the ATXN2/ACTN1 protein complex, the GST-pull-down experiments failed to demonstrate an obvious impact of the polyQ expansion on this association. It is therefore crucial to discuss recent observations during the characterization of our *Atxn2*-CAG100-KnockIn mouse mutant, which represents an authentic SCA2 model [53, 56, 61]. The global transcriptome profile in spinal cord at incipient versus terminal disease stages consistently showed expression dysregulations in opposite directions for two main surveillance mechanisms: Firstly, the expression upregulations concerned PABPC1 and other RNA toxicity control factors, together with activations of innate immunity, microgliosis and lysosomal activation. Secondly, the expression downregulations reflected metabolic deficits, lipid depletion and demyelination. Automated bioinformatics analyses revealed significant enrichments mainly for the pathway "regulation of actin cytoskeleton" (with dysregulation of several members of the FGF family and their receptors, prominent upregulation of *Myl1*, upregulations also for the ACTN1-interactors *Vcl* and *Gsn*, as well as downregulations for *Myh10* and *Limk1*, downstream *Ras*, and several components of Pi3k signalling with *Mapk6*), pathway "EGFR1 signaling", pathway "Insulin signaling", pathway "Focal adhesion-Pi3k-Akt-mTOR-signals", pathway "MAPK signalling", and pathway "Calcium regulation" (Suppl. Tables S6/S7 in [61]). This was accompanied by significant downregulations of clathrin-dependent RTK endocytosis factors such as *Ntrk2*, *ErbB3*, *Fgfr2*, *Fgfr3*, *Fgf12*, *Insig1*, *Aak1*, *Ttbk1*, *Ttbk2* and the ACTN1 interactor mRNA *Pdlim1* (Suppl. Tables S5 in [61]). Thus, at least in mouse nervous tissue with prolonged observation over years, a polyQ expansion to 100Q in ATXN2 appears to affect the pathways that are connected to ACTN1 function.

The most prominent pathology of SCA2 appears as progressive rarefaction in the dendritic trees of the large cerebellar Purkinje neurons [23, 139], whose main afferents are glutamatergic from the local granule neurons. In addition, the SCA2 disease process is quite similar to ALS in affecting spinal motor neurons and their glutamatergic monosynaptic input from cortical motor neurons

[39-41, 43, 44, 140]. In both preferentially affected regions, glutamatergic presynapses modulate the trophic state of postsynaptic spines. It is therefore noteworthy that the subunit GluR4 of the glutamatergic AMPA receptor interacts directly with ACTN1 and is regulated in a phosphorylation dependent manner [141, 142]. In addition, ACTN1 interacts with the metabotropic glutamate receptor mGluR5b and regulates its surface expression and function [143]. Finally, ACTN1 modulates the interactions of CAMK2, the crucial factor for postsynaptic spine plasticity in glutamatergic synapses [144-146]. Other alpha-actinins were reported to play an important role in the nervous system for synaptic plasticity and spine morphology [147, 148] via regulation of the NMDA receptor [149, 150]. Alpha-actinin-4 was shown to be crucial when the activation of growth factor receptor tyrosine kinases (RTK) triggers actin-remodelling and macropinocytosis in a calcium-dependent process called circular ruffling [112, 151-154].

The role of alpha-actinins in other neurodegenerative diseases has already been substantiated. In polyQ-expansion triggered Huntington's disease, decreased abundance of ACTN2 and specific subunits of the glutamatergic NMDA receptor were observed in hippocampal tissue [155], and microscopy as well as proximity ligation assays demonstrated the expanded disease protein Huntingtin to associate with ACTN1 / VCL (Vinculin) in stress fibers, membrane ruffles and lamellar protrusions [156]. Protein aggregates of actin and alpha-actinins have been described under the name of "Hirano bodies" in patients with age-associated neurodegenerative disorders, such as ALS, FTLN (aka Pick's disease), Parkinsonism-Dementia complex, Alzheimer's disease and Creutzfeld-Jakob disease [157, 158]. It is interesting to note that alpha-actinin dysfunction also has an impact on the trophic state of muscle tissue after immobilization and denervation [159], given that SCA2 patients early on suffer from muscle cramps and preterminally suffer from massive muscle atrophy.

Altogether, in view of the known interactions of ATXN2 with Endophilins A1/A3 as well as T- and L-Plastin, our new observations suggest that ATXN2 may modulate the slow endocytic internalization of growth factor receptors, neurotransmitter receptors and ion channels in dendritic spines via ACTN1 binding and actin filament remodelling.

4. Materials and Methods

Mouse breeding

The animal experiments were revised by the Regierungspräsidium Darmstadt with approval code V54-19c20/15-FK/1083. Breeding was performed as previously reported [160-162].

Cell culture

Monkey COS-7 (CRL-1651 from ATCC, Manassas, VA, USA) and human HEK-293 cells (CRL-1773 from ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (all Gibco/Invitrogen, Waltham, MA, USA) at 37 °C and 5% CO₂. Prior to passaging the cells were washed with Phosphate-buffered-saline (PBS) prewarmed to 37 °C. To detach the cells, a solution with 0.25% (w/v) Trypsin and 0.53 mM EDTA was used.

Transient transfection of COS-7 and HEK-293 cells.

Cells were grown to an optical confluency of 75% and then transfected with recombinant tag-constructs of ATXN2 [73] and ACTN1 (provided by Erich Wanker, Berlin) using Lipofectamine 2000, according to the manufacturer's instructions.

Rat hippocampal neurons

Day 18 embryonic Sprague/Dawley rat hippocampal neurons were purchased from Genlantis (San Diego, CA, USA). The neurons were cultured in Neurobasal medium, B27 serum-free

supplement and glutamax (Invitrogen, Carlsbad, CA, USA) on poly-D-lysine (Sigma, St. Louis, MI, USA) coated coverslips.

Preparation of Protein Extracts from transfected cells.

Transfected cells were washed in ice-cold PBS and lysed for 20 min in a lysis buffer containing 50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Igepal CA-630, protease inhibitor cocktail, 1 mM PMSF, 1 mM Na_3VO_4 , and 1 mM NaF. Cell debris and insoluble material was removed by centrifugation at 20000 xg and 4 °C. The supernatant was stored at -80 °C for co-immunoprecipitation experiments.

Immunocytochemistry.

Either COS-7 cells or rat hippocampal neurons were grown on 12 mm glass coverslips in 24 well plates. Optional transfection of COS-7 cells was carried out with Lipofectamine 2000 according to the manufacturer's instructions. Either 24 hr post transfection (COS-7) or after seven days in culture (rat hippocampal neurons) cells were fixed at room temperature in 4% paraformaldehyde for 20 min. Cells were washed three times with PBS and incubated with 0.5% TritonX-100 and 5% Chemiblocker (Chemicon, Temecula, CA, USA) to permeabilize the cells and to block unspecific binding. Incubation with the primary antibody was 1 hr at room temperature followed by three washing steps. Myc-ATXN2 was detected using anti-Myc Ig (Clontech/Takara, Heidelberg, Germany, 1:1000), while HA-ACTN1 was detected using anti-HA Ig (Clontech/Takara, Heidelberg, Germany, 1:1000). The endogenous proteins were detected with a mouse antibody against ATXN2 (BD Biosciences, San Jose, CA, USA, titer 1:100) and a rabbit antibody against ACTN1 (Santa Cruz, Dallas, TX, USA, titer 1:50). The secondary antibody was applied for 1 hr under light protection. Alexa 546-conjugated anti-mouse immunoglobulin (Ig) (Molecular Probes, Eugene, OR, USA) and Alexa 488 conjugated anti-rabbit Ig (Molecular Probes, Eugene, OR, USA) were used (titer 1:1000). After three washing steps, the cells were mounted using ProLong Antifade reagent (Molecular Probes/Invitrogen, Eugene, OR, USA). DAPI was from Invitrogen (Carlsbad, CA, USA). Pictures were taken on a Zeiss LSM 510 confocal microscopy System.

Preparation and purification of GST-fusion proteins.

Glutathion-S-transferase (GST) -fusion proteins were transformed into BL21-Gold *E.coli* (Stratagene, La Jolla, CA, USA). 10 ml of starter culture were inoculated into 250 ml 2xYT medium and grown to an OD_{600} of 0.6-0.8. Induction was carried out with 0.25 mM IPTG for 4 hr at 37 °C. Cells were harvested at 3000 xg in a swinging bucket centrifuge. The pellets were resuspended in TEN (50 mM Tris 7.5; 0.5 mM EDTA; 0.3 mM NaCl). Then 10 mg lysozyme and 4 mM DTT were added and the suspension was incubated on ice for 15 min. Subsequently, 200 μl of 10% NP-40 were added and the suspensions were stored overnight at -80%. Finally, 15 ml NaCl-Mg (1.5 M NaCl; 12 mM MgCl_2) and 100 μg DNase were added, followed by incubation over 1 hr on ice. Debris and membranes were pelleted at 20,000 xg with 4 °C in a Sorvall-centrifuge. The supernatants were analysed by SDS-PAGE with Western blotting and stored at -80 °C.

In vitro Protein Binding Experiments.

Glutathione-sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) was loaded with GST-fusion proteins for 1 hr at 4° with end-over-end rotation. Protein loaded beads were washed with binding buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2 mM MgCl_2 , 1 mM DTT, 5% glycerol, 0.01% Igepal CA-630, protease inhibitor cocktail, 1 mM PMSF, 1 mM Na_3VO_4 , 1 mM NaF). Cell lysates were added and incubated at 4° C for 4 hr with end-over-end rotation. Following the incubation, five washing steps with 5 volumes of binding buffer were made and bound proteins were eluted by boiling in SDS sample buffer. The samples were analysed by SDS-PAGE with Western blots. The GST-antibody was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Differential fractionation of mouse brain tissue.

Freshly dissected mouse brain was disrupted with a Dounce homogeniser in 5 µl homogenisation buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, protease inhibitor cocktail, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF) per mg wet weight. Separation of cellular organelles by differential velocity centrifugation followed exactly a reported protocol [163] as shown in figure 4A, which yielded fractions that predominantly contained nuclei, mitochondria/heavy membranes, light membranes/polysomes, and cytosol. The cytoplasm fraction was concentrated using a centrifugal Amicon Ultra 5.000 MWCO (Millipore) at 4 °C. The light membrane fraction was resuspended in binding buffer (25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 5% glycerol, 0.01% Igepal CA-630, protease inhibitor cocktail, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF) and then centrifuged at 16,000 xg for 15 min at 4 °C. The supernatant was stored at -80 °C for co-immunoprecipitation assays.

Co-immunoprecipitation.

Protein extracts were incubated with appropriate amounts of antibodies for at least 12 hr at 4 °C with end-over-end rotation. Antibody-protein complexes were precipitated with Protein G-PLUS agarose (Santa Cruz Biotechnology, Heidelberg, Germany) at 4° C overnight. The agarose beads were sedimented by centrifugation at 1500 xg and extensively washed with ice-cold lysis buffer. The bead sediment was boiled in SDS sample buffer or non-reducing sample buffer (Pierce, Rockford, IL, USA), and the supernatant was analysed by SDS-PAGE and Western blotting.

5. Conclusions

(i) Previously reported yeast-two-hybrid findings on direct protein-protein-interaction, (ii) current data on the co-sedimentation, co-localization and co-immunoprecipitation in mammalian cells, and (iii) recent literature on pathomechanisms in a SCA2 mouse model are consistent and indicate that the association of the mutant N-terminus of ATXN2 with the actin-binding-domain of ACTN1 contributes to the trophic deficits of dendritic spines in affected neurons.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

°C	degree Celsius (temperature)
22Q	22 consecutive glutamines
A2BP1	Ataxin-2-binding protein 1 (aka RBFOX1)
aa	amino acids
<i>Aak1</i>	mouse mRNA for AP2-Associated Protein Kinase 1
ABD	Actin-Binding-Domain
ACTN1	Alpha-Actinin-1
AD	Alzheimer’s disease
ADORA2A	Adenosine receptor A 2 alpha
Akt	Proto-Oncogene C-Akt (aka Protein Kinase B)
ALS	Amyotrophic Lateral Sclerosis

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (agonist at one glutamate receptor type)
anti-	antibody against
ATCC	American Tissue Culture Collection
ATXN2	human Ataxin-2 gene or mRNA
<i>Atxn2</i>	mouse Ataxin-2 gene or mRNA
ATXN2	human Ataxin-2 protein
ATXN2L	human Ataxin-2-Like protein
Ca	Calcium
Ca ²⁺	divalent Calcium ion
CaM	Calmodulin-like domain
CAMK2	Calcium/Calmodulin-dependent kinase II
CANX	Calnexin
cDNA	Complementary Deoxyribo-Nucleic-Acid
CH1/2	Calponin-Homology-Domain 1/2
c-Kit	Proto-Oncogene C-Kit (named after sequence Δ gag-kit- Δ pol- Δ env)
CO ₂	Carbon Dioxide
COS-7	$\overline{\text{C}}$ V-1 in O origin with $\overline{\text{S}}$ V40 gene cell line 7 derived from CV1 African green monkey fibroblasts
DAPI	4',6-diamidino-2-phenylindole, a fluorescent stain of DNA
DDX6	DEAD-box helicase 6
<i>D.</i>	<i>Drosophila</i>
Datx2	<i>Drosophila</i> fly Ataxin-2
DMEM	Dulbecco's Modified Eagle Medium
DRPLA	Dentatorubro-Pallidoluysian Atrophy
DTT	Dithiothreitol
EDTA	EDTA
EDTA	Ethylene-Diamine-Tetraacetic Acid
EEA1	Early Endosome-Associated Protein P162
EFh	EF-hand motif to bind calcium
EGFR1	Epidermal Growth Factor Receptor 1
<i>Erbb3</i>	mouse mRNA for Epidermal Growth Factor Receptor 3
FGF	Fibroblast Growth Factor
<i>Fgf12</i>	mouse mRNA for Fibroblast Growth Factor 12
<i>Fgfr2</i>	mouse mRNA for Fibroblast Growth Factor Receptor 2
<i>Fgfr3</i>	mouse mRNA for Fibroblast Growth Factor Receptor 3
FTLD	Fronto-Temporal Lobar Dementia
g	gravity unit
GluR4	Glutamate Ionotropic Receptor AMPA Type Subunit 4, encoded by <i>GRIA4</i> gene
GRB2	Growth Factor Receptor Bound Protein 2
GSH	Glutathione
<i>Gsn</i>	mouse mRNA for Gelsolin
GST	Glutathione-S-Transferase
HA	Human influenza hemagglutinin-antigen-derived tag
HD	Huntington's disease
HEK-293	Human embryonic kidney 293 cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
<i>Insig1</i>	mouse mRNA for Insulin Induced Gene 1
IP:	immunoprecipitation with:
IPTG	Isopropyl β - d-1-thiogalactopyranoside
ITPR1	Inositol-trisphosphate receptor 1
kDa	kiloDalton
LCP1	Lymphocyte Cytosolic Protein 1 (aka L-Plastin)
<i>Limk1</i>	mouse mRNA for LIM Domain Kinase 1
Lsm	Like "Smith-antigen" motif

LsmAD	Like “Smith-antigen” motif-associated domain
M	Molar
MAPK	Mitogen-Activated Protein Kinase
<i>Mapk6</i>	mouse mRNA for Mitogen-Activated Protein Kinase 6
mem.	membrane
mGluR5b	Metabotropic Glutamate Receptor 5, encoded by <i>GRM5</i> gene
min	minutes
mito.	mitochondrial
mitoch.	mitochondrial
mg	milligram
ml	milliliter
mm	millimeter
mM	milliMolar
mTORC1	mechanistic Target of Rapamycin Complex 1 (main growth signaling kinase complex)
Myc	Sequence tag derived from c-Myc Proto-Oncogene
<i>Myl1</i>	mouse mRNA for Myosin light chain 1
<i>Myh10</i>	mouse mRNA for Myosin heavy chain 10
µg	microgram
µl	microliter
NMDA	N-methyl-D-aspartate (agonist at one glutamate receptor type)
<i>Ntrk2</i>	mouse mRNA for Neurotrophic Receptor Tyrosine Kinase 2
OD ₆₀₀	Optical Density at wavelength 600 nanometer
OPCA	Olivo-Ponto-Cerebellar Atrophy
ORF	Open Reading Frame
PABPC1	Poly(A)-binding protein cytosolic 1
Pam2	Poly(A)-binding protein-interacting motif 2
PBS	Phosphate-Buffered Saline
<i>Pdlim1</i>	mouse mRNA for PDZ And LIM Domain 1
Pi3k	Phospho-inositide-3-kinase
PLS1	Plastin-1 (aka I-Plastin or Fimbrin)
PLS3	Plastin-3 (aka T-Plastin), member of the Fimbrin family
PMSF	Phenyl-Methyl-Sulfonyl Fluoride
PRD	Proline-Rich-Domain
PSP	Progressive Supranuclear Palsy (aka Parkinson plus)
polyQ	polyglutamine
RNA	Ribo-nucleic acid
<i>Rras</i>	mouse mRNA for Ras Family Small GTP Binding Protein R-Ras
RTK	Receptor Tyrosine Kinase
SCA1	Spinocerebellar ataxia type 1
SCA2	Spinocerebellar ataxia type 2
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3GL2	SH3 Domain Containing GRB2 Like 2 (aka Endophilin A1)
SH3GL3	SH3 Domain Containing GRB2 Like 3 (aka Endophilin A3)
SH-SY5Y	Neuroblastoma cell line, thrice-cloned sub-line of bone marrow biopsy-derived line SK-N-SH
SMA	Spino-Muscular Atrophy
SN	supernatant
SPEC	Spectrin repeat domain, which gives rise to linear proteins
SRC	V-Src Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog
<i>Ttbb1</i>	mouse mRNA for Tau Tubulin Kinase 1
<i>Ttbb2</i>	mouse mRNA for Tau Tubulin Kinase 2
<i>Vcl</i>	mouse mRNA for Vinculin
WB:	Western blot (aka immunoblot) detection with:
w / v	weight per volume

YTA Yeast Tryptone medium with Ampicillin
xg multiple of earth gravity

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