dAtaxin-2 Mediates Expanded Ataxin-1-Induced Neurodegeneration in a Drosophila Model of SCA1

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Spinocerebellar ataxias (SCAs) are a genetically heterogeneous group of neurodegenerative disorders sharing atrophy of the cerebellum as a common feature. SCA1 and SCA2 are two ataxias caused by expansion of polyglutamine tracts in Ataxin-1 (ATXN1) and Ataxin-2 (ATXN2), respectively, two proteins that are otherwise unrelated. Here, we use a Drosophila model of SCA1 to unveil molecular mechanisms linking Ataxin-1 with Ataxin-2 during SCA1 pathogenesis. We show that wild-type Drosophila Ataxin-2 (dAtx2) is a major genetic modifier of human expanded Ataxin-1 (Ataxin-1[82Q]) toxicity. Increased dAtx2 levels enhance, and more importantly, decreased dAtx2 levels suppress Ataxin-1[82Q]-induced neurodegeneration, thereby ruling out a pathogenic mechanism by depletion of dAtx2. Although Ataxin-2 is normally cytoplasmic and Ataxin-1 nuclear, we show that both dAtx2 and hAtaxin-2 physically interact with Ataxin-1. Furthermore, we show that expanded Ataxin-1 induces intranuclear accumulation of dAtx2/hAtaxin-2 in both Drosophila and SCA1 postmortem neurons. These observations suggest that nuclear accumulation of Ataxin-2 contributes to expanded Ataxin-1-induced toxicity. We tested this hypothesis engineering dAtx2 transgenes with nuclear localization signal (NLS) and nuclear export signal (NES). We find that NLS-dAtx2, but not NES-dAtx2, mimics the neurodegenerative phenotypes caused by Ataxin-1[82Q], including repression of the proneural factor Senseless. Altogether, these findings reveal a previously unknown functional link between neurodegenerative disorders with common clinical features but different etiology.

Introduction

Inherited ataxias are a genetically heterogeneous group of neurodegenerative diseases characterized by loss of motor coordination and balance. They can be caused by loss-of-function or gain-of-function mechanisms; some ataxias are triggered by missense mutations, while others by triplet repeat expansions, which may occur either in coding or non-coding sequences. Furthermore, the gene products implicated in the different ataxias do not share obvious functional or structural relationships to each other. In spite of this genetic heterogeneity, many ataxias show striking similarities. In particular, it is often difficult to distinguish between Spinocerebellar ataxias (SCAs) based only on clinical and pathological observations, and their differential diagnosis often requires genetic testing. In addition, a common neuropathological feature of SCAs is the atrophy of the cerebellar module (reviewed in [1–3]). These similarities suggest that SCAs, and perhaps other ataxias, may also share common mechanisms of pathogenesis. In support of this hypothesis a recent study reported a network of physical protein-protein interactions among many factors associated with ataxia and Purkinje cell degeneration in humans and mice [4]. However, no specific molecular mechanisms are known that can account for the clinical and neuropathological similarities among SCAs and other ataxias.

SCA1 is caused by the expansion of a CAG repeat encoding a polyglutamine tract in the protein Ataxin-1 that induces a toxic gain of function [5]. The expanded protein accumulates in neuronal nuclear inclusions (NIs) that also contain transcription factors, chaperones, proteasome subunits, and other components of the protein quality control/degradation machinery like CHIP or Ataxin-3 [6–11]. Abnormally long polyglutamine tracts are the common cause of pathogenesis in at least five other SCAs (SCA2, 3, 6, 7 and 17) and three additional neurodegenerative diseases including Huntington’s disease (HD) [1,12]. Protein quality control machinery as well as transcriptional dysregulation are general mechanisms that have been implicated in the pathogenesis of these polyglutamine disorders [13–15]. Although the polyglutamine expansion triggers the toxicity...
of Ataxin-1, experiments in Drosophila and mouse SCA1 models have shown that protein context plays a key role in expanded Ataxin-1-induced neurodegeneration (reviewed in [15]). The nuclear localization signal[16] and phosphorylation[17] influence the toxicity of expanded Ataxin-1. In addition, certain interacting partners of unexpanded Ataxin-1 are critical to expanded Ataxin-1 toxicity [9,18,19]. In this context, expanded Ataxin-1 was recently found to induce a decrease in the levels of Senseless (Sens) and its murine orthologue growth factor independent 1 (Gfi1) [18]. These are transcription factors that interact with unexpanded Ataxin-1 and are necessary for Purkinje cell survival in mice [18] and for sensory organ development in Drosophila [20]. The importance of the protein framework has also been shown in models of other polyglutamine diseases [15,21].

Genetic screening in Drosophila models of neurodegenerative diseases is a powerful approach to identify modifier genes and pathways implicated in pathogenesis [22–24]. We found that Ataxin-1 and Ataxin-2 physically interact, and that mutant Ataxin-1 forces Ataxin-2 to accumulate in the nucleus instead of the cytoplasm. Most importantly, using an animal model, we discovered that the Drosophila Ataxin-2 gene is a strong suppressor of Ataxin-1-induced neurotoxicity. Thus, neuronal degeneration may take place through common mechanisms in different SCAs. These findings open the possibility of future common therapies for these neurodegenerative disorders for which there is no effective treatment.

**Results**

Increased/Reduced Levels of Ataxin-2 Enhance/Suppress Expanded Ataxin-1 Toxicity in the Drosophila Eye

Expression of Ataxin-1[82Q] in the eye of SCA1[82Q] flies causes external and internal abnormal phenotypes [25].

Externally, the eyes of these animals show severe ommatidial disorganization as well as interommatidial bristle loss when compared with control eyes (Figure 1, compare A and A′ with B and B′). Internally, examination of the retina reveals tissue loss and shortened and curved photoreceptor neurons (Figure 1, compare F with G). In a screen for genetic modifiers of Ataxin-1[82Q]-induced toxicity we recovered EP(3)3145 as an enhancer of the eye phenotype (data not shown). This is an insertion of an EP transposable element [37] in the 5′ end of dAtx2, the Drosophila orthologue of human Ataxin-2. The Drosophila and human proteins share 23% amino acid identity and 36% amino-acid similarity over the entire protein with the most conserved sequences corresponding to the ATX2-N and ATX2-C domains (43% and 62% identity, respectively) [33]. Molecular analysis revealed that the EP element is inserted 3121 bp upstream of the ATG and in the same orientation as the dAtx2 transcription unit (data not shown and [33]). These data suggested that EP(3)3145 over-expresses the dAtx2 transcription unit to enhance the SCA1[82Q] eye phenotype. As described below, this possibility was confirmed using a transgene that over-expresses the dAtx2 cDNA.

Co-expression of a wild-type dAtx2 transgene (dAtx2[2OE]) at low levels enhances the Ataxin-1[82Q]-induced eye phenotype. Externally, the eyes of SCA1[82Q]/dAtx2[2OE] animals show no bristles and increased ommatidial disorganization when compared with the eyes of SCA1[82Q] controls (compare Figure 1D and D′ with B and B′). Internally, photoreceptor cells are considerably shorter (compare Figure 1I with G). Expression of the same low levels of dAtx2 alone in the eye causes relatively mild external disorganization and reduction of the retinal width (Figure 1E, E′ and J). Overexpression of dAtx2 from EP(3)3145 and UAS-dAtx2 also aggravates the phenotypes of other fly models of neurodegenerative diseases besides SCA1 [38,39]. However, since overexpression of dAtx2 causes an eye phenotype by itself (Figure 1E, E′ and J) and it is toxic in many other tissues [33], it is difficult to make strong conclusions about the specificity of these genetic interactions.

To test the specificity of the genetic interaction, we investigated if decreasing the levels of endogenous dAtx2 modifies expanded Ataxin-1-induced toxicity. For this, we used a 1.4 kb deletion in the dAtx2 locus (dAtx2[27]) that removes part of the dAtx2 promoter, the ATG codon and extends into the first intron [33]. We find that flies expressing Ataxin-1[82Q] and heterozygous for the dAtx2[2X] mutant allele show a strong suppression of the eye phenotype, with much improved arrangement of the ommatidia and bristles compared to eyes from flies expressing Ataxin-1[82Q] with normal dAtx2 levels (compare Figure 1C and C′ with B and B′). This suppression is also evident in the retinas of SCA1[82Q]/ dAtx2[2X] flies that show elongated photoreceptors and very little tissue loss (compare Figure 1H with G). To further test the specificity of this interaction, and to exclude potential genetic background artefacts, we asked whether adding back dAtx2 to SCA1[82Q]/dAtx2[2X] flies eliminates the suppression effect. Figure S1 shows that SCA1[82Q]/dAtx2[2X]/dAtx2[2OE] flies show an eye phenotype that is very similar to the phenotype of SCA1[82Q] flies. The effects of the dAtx2[2OE] and dAtx2[2X] alleles decreasing/increasing dAtx2 levels are demonstrated in Figure S2.

Since dAtx2 is an RNA binding protein, we investigated if the observed suppression of Ataxin-1[82Q] toxicity was the...
Reduced Levels of Ataxin-2 Suppress Expanded Ataxin-1-Induced Neuronal Dysfunction in Drosophila

To verify that the genetic interaction between Ataxin-1 and dAtx2 is not limited to the eye, we analyzed the effect of altering dAtx2 levels on expanded Ataxin-1-induced neurodegeneration. The motor performance of flies as a function of age can be quantified using a climbing assay [40]. This assay has been used to analyze the effects of toxic proteins on neurons in other Drosophila models of neurodegenerative diseases [41,42]. Control flies show no significant decrease in their motor performance until late in life. Figure 2A shows that 74% of control flies still climb after thirty-six days (black triangles). Flies expressing Ataxin-1[82Q] specifically in the nervous system (using nrv2-GAL4) display a progressive impairment of their motor performance (Figure 2A, blue circles). In the context of the Drosophila life span, this is a late onset and progressive phenotype compared to the performance of control flies in the same period of time. We then analyzed the

Figure 1. dAtx2 Levels Modulate Ataxin-1[82Q]-Induced Eye Neurotoxicity
(A–E, A’–E’) Scanning electron microscopy (SEM) eye images and (F–J) retinal paraffin sections of eyes from the genotype combinations indicated on the top. Arrows indicate photoreceptor length. Transgenes are expressed from the gmr-GAL4 eye driver.

(A, F) Control eyes show regularly arranged ommatidia, and evenly distributed interommatidial bristles (A). Control retinas have long, straight photoreceptors with no gaps (F).

(B, G) SCA1[82Q] eyes have disorganized ommatidia and interommatidial bristles are unevenly distributed or missing (compare B and A). SCA1[82Q] retinas show fewer photoreceptors that are shortened and curved (compare G with F).

(C, H) Suppression of external and retinal degeneration in SCA1[82Q] flies with decreased dAtx2 levels (SCA1[82Q]/dAtx2X1). Note the regular arrangement of the ommatidia in SCA1[82Q]/dAtx2X1 animals when compared to SCA1[82Q] eyes with normal levels of dAtx2 (compare C with B). Note also the improved retinal organization with longer straight photoreceptors (compare H with G).

(D, I) Eyes co-expressing Ataxin-1[82Q] and wild type dAtx2 (SCA1[82Q]/dAtx2OE) have more disorganized ommatidia when compared to SCA1[82Q] alone. Note the absence of bristles (compare D with B). In addition, photoreceptors are shorter and more degenerated (compare I with H).

(E, J) Eyes overexpressing low levels of dAtx2 (dAtx2OE) show mild ommatidial disorganization, with few missing bristles (compare E with A). Internally, photoreceptor cells are shortened (compare J with F). A–E flies were raised at 27°C and F–J at 25°C.

(K) Semiquantitative RT-PCR revealing the levels of SCA1[82Q] mRNA in flies with different levels of dAtx2.

(L) Western blot analysis revealing the levels of Ataxin-1[82Q] protein in flies with different levels of dAtx2.

Genotypes: (A–J): Control: yw/+; gmr-GAL4/UAS-LacZ. SCA1[82Q]: UAS-SCA1[82Q] [F7]/yw; gmr-GAL4/+; SCA1[82Q]/dAtx2X1: SCA1[82Q]/yw; gmr-GAL4/+. dAtx2OE: gmr-GAL4/UAS-dAtx2 [4]. (A–E) SEM scale bar=100 μm. (A’–E’) are 30 μm X 30 μm. (F–J) scale bar=10 μm.
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Figure 2. Specificity of the dAtx2/Ataxin-1 Interaction

(A, B) Reduced levels of dAtx2 also suppress Ataxin-1[82Q]-induced motor dysfunction and shortened life span.

(A) Motor performance quantification in flies of the indicated genotypes measured as climbing ability as a function of age. Control animals expressing just GFP perform well in the motor assay for 36 days (nrv2->GFP, black triangles), as do flies that are heterozygous for the dAtx2X1 allele (nrv2->GFP/dAtx2X1, green diamonds). Flies expressing Ataxin-1[82Q] in the nervous system show progressive motor dysfunction when compared with controls (nrv2->SCA1[82Q], blue circles). All nrv2->SCA1[82Q] flies stop climbing after day 23. Flies expressing Ataxin-1[82Q] in the nervous system but with decreased dAtx2 levels show improved motor performance (nrv2->SCA1[82Q]/dAtx2X1, red squares). Error bars represent standard deviation. Flies were raised at 27°C.

(B) Survivorship in flies of indicated genotypes. Control flies (nrv2->GFP, black triangles). Expression of Ataxin-1 [82Q] in the eye leads to retinal degeneration and loss of photoreceptors and other cell types (compare D with control shown in C). This eye phenotype is not altered in animals with reduced levels of dAtx2 (compare D and E). Expression of N-Htt[128Q] in the eye leads to progressive motor dysfunction (nrv2->N-Htt[128Q]/dAtx2X1, red), and decreasing the levels of dAtx2 does not significantly suppress this phenotype (nrv2->N-Htt[128Q]/dAtx2X1, blue).

Genotypes: (A–B): nrv2->GFP; nrv2-GAL4/UAS-eGFP; nrv2->GFP/dAtx2X1; nrv2->GAL4/UAS-eGFP; dAtx2X1/+. nrv2->SCA1[82Q]; nrv2-GAL4/+. UAS-SCA1[82Q]/M6/+. nrv2->SCA1[82Q]/dAtx2X1; nrv2->GAL4/+. UAS-SCA1[82Q]/M6/dAtx2X1. (C) yw; gmr-GAL4/UAS-GFP. (D) yw; gmr-GAL4/+. UAS-N-Htt[128Q]/+. (E) yw; gmr-GAL4/+. UAS-N-Htt[128Q]/dAtx2X1. (F) nrv2->N-Htt[128Q]; nrv2->GAL4/+. UAS-N-Htt[128Q]/+. nrv2->N-Htt[128Q]/dAtx2X1; nrv2->GAL4/+. UAS-N-Htt[128Q]/dAtx2X1.
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Effect of decreased levels of dAtx2 on the climbing phenotype caused by Ataxin-1[82Q]. As shown in Figure 2A (red squares) in SCA1[82Q] flies also heterozygous for the dAtx2X1 mutation climbing performance is significantly improved compared to flies with normal dAtx2 levels (p<0.0001 for repeated measures ANOVA (rma) between genotypes). Figure 2A shows that while all SCA1[82Q] animals fail to climb after 26 days, SCA1[82Q]/dAtx2X1 flies continue to climb until later in life. Thus the impairments in motor performance caused by neuronal expression of Ataxin-1[82Q] are suppressed by decreased dAtx2 levels.

We also studied the effect of Ataxin-1[82Q] expression in a life span assay. Figure 2B shows that expression of Ataxin-1[82Q] in the nervous system leads to premature death in SCA1[82Q] flies in comparison to GFP controls (Figure 2B, compare blue circle with black triangles). While SCA1[82Q] animals do not survive past 30 days, this early lethality phenotype is suppressed in SCA1[82Q]/dAtx2X1 animals (Figure 2B, red squares).

Decreased Levels of dAtx2 Do Not Suppress Neurodegeneration Caused by Expanded Huntingtin

To investigate whether dAtx2 also modulates neurodegeneration in other models of polyglutamine disease, we tested the effect of altering the dAtx2 levels in a Drosophila model of Huntington’s disease [7,43]. Adult flies expressing an expanded N-terminal fragment of human huntingtin (N-Htt[128Q]) in the eye show a progressive retinal degeneration which becomes obvious at day 5 after eclosion [7]. N-Htt[128Q] retinas show disorganized and missing photoreceptors (Figure 2D, compare with control in 2C). N-Htt[128Q] flies also overexpression dAtx2 present a more degenerated retina than flies expressing N-Htt[128Q] with normal levels of dAtx2 (data not shown). However, since overexpression of dAtx2 is...
sufficient to cause retinal degeneration (Figure 1J), this result is not conclusive by itself. Therefore, we tested the effect of decreasing dAtx2 levels on the N-Htt128Q induced retinal degeneration. As shown in Figure 2D-E, decreasing the levels of dAtx2 (N-Htt128Q/dAtx2X1) does not obviously alter degeneration induced by N-Htt128Q in the *Drosophila* eye.

We also investigated a possible genetic interaction between dAtx2 and N-Htt128Q in the motor performance assay described above. Like with Ataxin-1[82Q], expression of N-Htt128Q in the nervous system leads to motor performance impairments (Figure 2F) where N-Htt128Q animals stop climbing before day 20. The climbing performance of animals expressing N-Htt128Q with decreased levels of dAtx2 (N-Htt128Q/dAtx2X1) is not significantly different from that of animals expressing N-Htt128Q with normal levels of dAtx2 (Figure 2F). Therefore decreasing the levels of dAtx2 fails to suppress N-Htt128Q induced degeneration both in the retina and in the nervous system.

**Ataxin-2 Levels Modulate Expanded Ataxin-1-Induced Loss of Mechanoreceptors Caused by Loss of Senseless Protein**

Senseless (Sens) is a proneural factor that is expressed and required in the sensory organ precursor (SOP) cells of the peripheral nervous system [20]. Expression of high levels of Ataxin-1[82Q] in the thoracic SOPs using *seabrous-GAL4* (seca-GAL4) leads to a reduction in Sens protein levels in these cells and loss of large mechanoreceptors (macrochaetae) in the thorax of adult flies [18]. Therefore, scoring adult thoracic macrochaetae compared to Ataxin-1[82Q] alone (Figure 3E, 1–4, p < 0.0001, Tukey-Kramer HSD; see Figure 3M). Reducing the levels of endogenous dAtx2 with the heterozygous dAtx2X1 mutation partially rescues the Ataxin-1[82Q]-induced bristle phenotype. Loss of macrochaetae in SCA182Q/dAtx2X1 animals is approximately half of that seen in animals with normal levels Ataxin-2 (Figure 3L, compare columns 2 and 3 p < 0.005, Tukey-Kramer HSD, and see Figure 3N).

**Physical Interaction between Human Expanded Ataxin-1 and Ataxin-2 Proteins**

To further characterize the interactions between Ataxin-1 and Ataxin-2, we investigated possible protein-protein interactions. Lysates from cells expressing *Drosophila* or human Ataxin-2 and GST-Ataxin-1[82Q] were subjected to co-affinity purification (co-AP) glutathione-S-transferase (GST) pull-down assays. As shown in Figure 4A (lanes 1 and 2) and Figure 4B (lanes 1 and 4), GST-Ataxin-1[82Q] is able to pull down the *Drosophila* and human Ataxin-2 proteins, which indicates that both proteins are able to physically interact. We also asked whether this interaction is polyglutamine dependent. As shown in Figure 4B lanes 2–4 we did not detect significant differences in the interactions between GST-Ataxin-1 with 2, 30 or 82 glutamines and human Ataxin-2 in this co-AP assay. Ataxin-1[82Q] is phosphorylated at Serine residue 776 on the C-terminal portion of the protein, and a (Ser776Ala) mutation inhibits the toxicity of Ataxin-1[82Q] in mice [17]. We investigated the importance of Ser776 for the Ataxin-1[82Q]-dAtx2 interaction. As shown in (Figure 4A lane 3) the interaction of dAtx2 with Ataxin-1[82Q]S776A is weaker than with normal Ataxin-1[82Q], suggesting that this interaction is phosphorylation dependent.

In addition, we investigated if Ataxin-1 can pull down endogenous hAtx2. We find that unexpanded Ataxin-1 is able to precipitate endogenous hATX2 from human cells, suggesting that the two proteins may be functional interactors in vivo (Figure 4C).

We also investigated whether the interaction between Ataxin-1 and Ataxin-2 is cytoplasmic or nuclear. We carried out co-AP assays with Ataxin-1 and dAtx2 after nuclear/cytoplasmic fractionation of cultured cells. Figure 4D shows that we were not able to detect differences in protein interactions between these cellular compartments using this assay.

Lastly, we investigated whether specific domains of the hAtaxin-1 protein are responsible for the interaction with hAtaxin-2. Co-AP experiments were carried out with lysates...
Figure 3. dAtx2 Levels Modulate Ataxin-1(82Q)-Induced Loss of Mechanoreceptors

(A) Percentage of missing thoracic macrochaetae (large mechanoreceptor bristles) over a total of 26 in flies of the indicated genotypes raised at 25°C. Columns-1 and 2, no macrochaetae loss is detected in control flies or flies over-expressing dAtx2 (dAtx2OE) in the thoracic sensory organ precursor (SOP) cells respectively with the scabrous-GAL4 driver. Column-3, mild decrease in thoracic macrochaetae number caused by expression of Ataxin-1(82Q) at low levels in the SOP cells (p<0.0001). Column-4, ~80% decrease in the number of thoracic macrochaetae in animals co-expressing Ataxin-1(82Q) and dAtx2 (SCA182Q/dAtx2OE) in the SOPs (p<0.0001). Error bars=s.e.m.

(B–E) Images of the macrochaetae present in the posterior region of the notum and scutellum (white rectangle in B) of flies with same genotypes as (A). (B) Control thoraxes have eight macrochaetae in the selected area (white rectangle). (C–E) Over-expression of dAtx2 enhances Ataxin-1(82Q) induced macrochaetae loss. Black arrow indicates missing bristle in C and white arrow in E points to the only bristle present in the scutellum of an SCA182Q/dAtx2OE animal. Flies grown at 25°C.

(F) Anti-Sens immunofluorescence revealing the normal pattern of Sens distribution in the wing disc. White rectangle highlights the wing margin SOP and bristle precursor cells.

(G–J) Close-up of the wing margin region from: control animals (G), animals expressing Ataxin-1(82Q) alone (H), wild-type dAtx2 (I) or coexpressing Ataxin-1(82Q) and dAtx2 (J). (K) Quantification of anti-Sens signal in the wing margin of animals of the genotypes indicated on the bottom. 20 wing discs were used per genotype and experiments carried out at 25°C (see materials and methods for more details). Bars=Std. Dev.

(L) Percentage of lost macrochaetae in flies expressing Ataxin-1(82Q) with normal or decreased levels of dAtx2. Flies grown at 27°C to boost expression levels of the GAL4/UAS system. Column-1, control animals do not show macrochaetae loss. Column-2, ~20% of thoracic macrochaetae are lost after expression of Ataxin-1(82Q) at high levels in the SOP cells (p<0.0001). Column-3, partial rescue of Ataxin-1(82Q)-induced macrochaetae loss in animals heterozygous for the dAtx2[4] mutant (p<0.005). Bars=s.e.m. (M–N) Images of the macrochaetae present in the posterior notum and scutellum (white rectangle in B) of flies with same genotypes as L. Arrows indicate missing bristles. Flies grown at 27°C.

Genotypes: control: sca109–68-GAL4/UAS-GFP. dAtx2OE: sca109–68-GAL4/ UAS-dAtx2[4]. SCA182Q: UAS-SCA182Q[F7]/+. UAS-SCA182Q/dAtx2OE: UAS-SCA182Q[F7]/+; SCA182Q/dAtx2OE. doi:10.1371/journal.pgen.0030234.g003
Figure 4. The Ataxin-1[82Q] and Ataxin-2 Proteins Physically Interact

(A) GST Co-AP pull-down experiments between dAtx2 and GST-constructs carrying human Ataxin-1[82Q] or Ataxin-1[82Q] with S776A mutation. 
(B) GST Co-AP experiments between human Ataxin-2 (Myc-hATXN2) and GST-Ataxin-1 with different polyglutamine lengths. 
(C) Interaction between GST-Ataxin-1[2Q] and endogenous hATXN2 in cell culture. 
(D) Co-AP pull down experiments between GST-Ataxin-1 and Flag-dAtx2 after nuclear/cytoplasmic fractionation. Tubulin is used as cytoplasmic marker and Mecp2 as nuclear marker. 
(E) Comparative analysis of the interaction of hAtaxin-2 with different domains of the Ataxin-1 protein. Lane-1, GST alone does not pull down Myc-hAtaxin2. Lane-2, GST-ATXN1[82Q] pulls down Myc-hAtaxin2. Lane-3 expanded N-terminal Ataxin-1 (GST-ATXN1N-term[82Q]) lacking the AXH domain (aa# 1–575) also pulls down Myc-hAtaxin-2 with high affinity. Lanes-4 and 5 show that both the C-terminus portion of Ataxin-1 containing the AXH domain (aa# 529–816; lane-4) or the AXH domain alone (aa# 558–700; lane-5) pull down Myc-hAtaxin2. These interactions are weaker than that of the N-terminus part (compare lanes 4 and 5 with lane 3). Cell lysates for co-AP were run through Glutathione conjugated beads, and blots stained with anti-GST or anti-Myc. Immunoblots for dAtx2, Myc-hAtaxin2 and GFP carried out on cell lysates before the co-AP pull-down reveal similar levels of both proteins between samples.
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from cells expressing Myc-hAtaxin-2 and one of the following hAtaxin-1 fragments tagged with GST: polyglutamine expanded N-terminal (aa# 1–575, Figure 4E lane-3), C-terminus Ataxin-1 containing the AXH domain (aa# 529–816; Figure 4E lane-4) or the AXH domain alone (aa# 558–700; Figure 4E lane-5). All three fragments pull-down Myc-hAtaxin-2, indicating that each Ataxin-1 fragment can interact independently with hAtaxin-2 (Figure 4E lanes 3–5). However, the interaction of hAtaxin-2 is stronger with the N-terminal Ataxin-1 fragment (Figure 4E, lane-3) as compared to the C-terminal or AXH portions (Figure 4E lanes 4 and 5 respectively), since less N-terminal peptide pulls down more hAtaxin-2.

Ataxin-2 Accumulates in the Nucleus of Expanded Ataxin-1-Expressing Drosophila Cells and Human Neurons

The co-AP assays with expanded hAtaxin-1 and hAtaxin-2 indicate that the two proteins are able to interact. However, Ataxin-1 normally localizes to the nucleus in Drosophila and many human cell types, while Ataxin-2 is a cytoplasmic protein. To address whether the interaction observed in cultured cells is relevant in vivo, we monitored the localization of Ataxin-2 in Drosophila cells expressing Ataxin-1[82Q]. Since Ataxin-1[82Q]-induced toxicity is suppressed by dAtx2 loss of function in the Drosophila eye; we first analyzed the localization of dAtx2 in retinal cells. dAtx2 is not normally detected in the nuclei of retinal cells from either control eyes or eyes overexpressing dAtx2 (Figure 5A and C respectively). In contrast, we find that endogenous dAtx2 localizes to the nuclei of retinal cells expressing Ataxin-1[82Q]. Furthermore, nuclear dAtx2 signal is detected both diffusely in the nucleoplasm as well as in nuclear inclusions (NIs) (Figure 5B). To confirm this unexpected result, we examined the localization of dAtx2 in other Ataxin-1[82Q]-expressing neurons. Similar to the results obtained in the retina, endogenous dAtx2 normally localizes to the cytoplasm.
Nuclear Accumulation of Ataxin-2 Is Induced by Pathogenic Ataxin-1[82Q], but Not Unexpanded Ataxin-1[2Q] and Ataxin-1[30Q]

SCA1 pathogenesis is triggered by polyglutamine expansion in Ataxin-1 beyond 39–44 residues [5]. To investigate if nuclear accumulation of Ataxin-2 is specific to the pathogenic Ataxin-1 form, we analyzed dAtx2 localization in Drosophila VNC neurons expressing human Ataxin-1 with different polyglutamine lengths: Ataxin-1[2Q], Ataxin-1[30Q] and Ataxin-1[82Q]. The Ataxin-1[2Q] line used has higher levels of protein expression than the Ataxin-1[30Q] and [82Q] lines, both of which have comparable expression levels (Western blot data not shown). Figure 7B-D shows that no nuclear dAtx2 signal is detected in control neurons (Figure 7A), or neurons expressing Ataxin-1[2Q] or [30Q] (Figure 7B and C). In contrast, accumulation of dAtx2 is detected in the nucleus of Ataxin-1[82Q]-expressing neurons (Figure 7D). Although this result does not rule out an interaction between wild-type Ataxin-1 and dAtx2, it indicates that abundant nuclear accumulation of Ataxin-2 is specific to the expanded Ataxin-1 form responsible for SCA1 pathogenesis.

Nuclear Accumulation of Ataxin-2 Causes Severe Eye Toxicity

The observations that expanded Ataxin-1 induces nuclear accumulation of Ataxin-2 and that decreasing the levels of endogenous Ataxin-2 suppresses toxicity in SCA1[82Q] flies, suggest that nuclear accumulation of Ataxin-2 may lead to neurotoxicity. To test this hypothesis, we generated dAtx2 transgenic flies with an exogenous nuclear localization signal (NLS) engineered on its C-terminus end (dAtx2NLS) (Figure 7F–G), and compared their toxicity (Figure 7B–D). Ten transgenic lines were recovered for each dAtx2 construct, with a wide range of expression levels for each transgene. We selected transgenic lines expressing wild-type dAtx2 (dAtx2OE), dAtx2NLS or dAtx2NES at similar levels (Figure 7D compare lanes 2–4), and compared their toxicity in the eye. As shown in Figure 7E–H, dAtx2NLS is more toxic than wild-type dAtx2 or dAtx2NES. While both wild-type dAtx2 and dAtx2NES induce a relatively mild eye phenotype compared to controls (Figure 7E–G), expression of dAtx2NLS results in strong eye toxicity (Figure 7H). Eyes of dAtx2NLS flies show a severely disorganized ommatidial lattice and a
complete absence of interommatidial bristles (Figure 7H).
These observations were consistent in several lines for each of
the dAtx2 constructs at similar levels of expression (data not
shown).

In summary, expression of dAtx2NES and wild-type dAtx2
in the eye cause similar mild phenotypes. This is consistent
with dAtx2 being mainly cytoplasmic, and with observations
of SCA2 pathogenesis in the cytoplasm[44,45]. Interestingly,
increasing the levels of Ataxin-2 in the nucleus is sufficient to
cause a much more severe eye phenotype. These observations
suggest that the toxicity of expanded Ataxin-1 is mediated in
part, by the nuclear accumulation of Ataxin-2.

Nuclear Ataxin-2 Decreases Sens Protein Levels and
Induces Loss of Mechanoreceptor Bristles

To further test the hypothesis that nuclear Ataxin-2
contributes to expanded Ataxin-1-induced toxicity, we
analyzed the effect of expressing the different dAtx2 trans-
genes on Sens distribution in the wing margin SOPs.
Expression of Ataxin-1[82Q] in the antero-posterior com-
partment boundary (using dpp-GAL4) induces a cell autono-
mous decrease of Sens only in the dpp-GAL4 expressing area
of the wing margin (Figure 8A, A’, and B’, arrowhead).

This provides a molecular readout of Ataxin-1[82Q]-induced
neurotoxicity.

Expression of dAtx2NES from dpp-GAL4 does not reduce the
levels of Sens, whose distribution in imaginal wing discs is
unchanged (Figure 8C and C’, arrowhead). Next, we tested the
effect of dAtx2 with a nuclear localization signal (dAtx2NLS).
Like Ataxin-1[82Q], expression of dAtx2NLS induces loss of
Sens in the wing margin SOPs (Figure 8D and D’, arrowhead).

Salivary gland cells express Sens at high levels [20],
which localizes to the nucleus (Figure 8E-H). Salivary gland
cells expressing dAtx2NES show no detectable change in Sens
levels or distribution (Figure 8I-L). In contrast, expression of
dAtx2NLS causes a dramatic decrease in the amount of Sens
(Figure 8M-P). The nuclei of dAtx2NLS cells are still present
and their morphology is similar to controls, indicating that
Sens loss is unlikely a consequence of cell death. Therefore,
nuclear dAtx2 mimics Ataxin-1[82Q] in causing loss of Sens
protein accumulation.

Expression of Ataxin-1 in the thoracic SOPs (using seag-
GAL4) leads to loss of macrochaetae in the adult thorax
(Figure 3 and ref.[18]). Therefore, we also investigated the
consequences of nuclear or cytoplasmic Ataxin-2 accumu-
Figure 8. Nuclear dAtx2 Induces Decreased Levels of Senseless (Sens) Protein and Loss of Mechanoreceptors

(A–D’) Immunofluorescence staining revealing Sens endogenous pattern in the wing margin SOPs on animals of genotypes indicated on top. (A and A’) Distribution of Sens in wing discs expressing a neutral NLS-DsRed protein (RedStinger) in the dpp territory (red in A’). Sens is detected as two parallel rows of cells (SOPs of the dorsal and ventral wing margins) uninterrupted in the area where they cross the dpp territory (arrowhead). (B, B’) Ataxin-1[82Q] eliminates Sens signal in the wing discs in a cell autonomous manner. Notice the gap in Sens signal when it crosses the dpp territory (arrowhead). (C, C’) Expression of dAtx2NES (red in C’) in the dpp territory does not affect Sens signal (arrowhead). (D, D’) Expression of dAtx2NLS (red in D’) in the dpp territory reduces Sens signal in the SOPs, note the gap in Sens pattern (arrowhead).

(E–P) Immunofluorescence staining revealing Sens (red, anti-Sens) and the dAtx2NES or dAtx2NLS proteins (green, anti-Flag), in salivary gland cells of the indicated genotypes. Nuclei are visualized using anti-Lamin (blue). (E–H) Control salivary gland cells showing robust Sens signal. Asterisks in G indicate the position of the nuclei. (I–L) dAtx2NES-expressing salivary gland cells have normal Sens levels when compared to controls. (M–P) Expression of dAtx2NLS in salivary gland cells causes a decrease of Sens protein. Sens signal (red) is dramatically decreased in dAtx2NLS-expressing cells (compare N with F and J). Asterisks in N indicate the position of the nuclei.

(Q–S) Effect of dAtx2NES or dAtx2NLS on adult thoracic macrochaetae formation after SOP specific expression. (Q) SOP-specific expression of dAtx2NES causes no macrochaetae loss. (R) Expression of dAtx2NLS in the SOP cells causes significant macrochaetae loss in the same conditions as Q. Arrows point out missing macrochaetae. (S) Quantification of lost macrochaetae in adult thoraxes of the same genotypes as Q and R. Column-1 shows no decrease in the number of macrochaetae per thorax in flies expressing dAtx2NES. Column-2 reveals increased loss of macrochaetae following SOP specific expression of Atx2NLS (p<0.0001). 20 animals were used per genotype; data was analyzed using Students t; error bars represent s.e.m. Experiments in Q–S were performed at 25°C.

Genotypes: (A, A’) dpp-GAL4/ UAS-RedStinger. (B, B’) UAS-SCA1[82Q]F2; dpp-GAL4/+. (C, C’) dpp-GAL4/UAS-dAtx2NES. (D, D’) UAS-dAtx2NLS/+. (E–H) UAS-dAtx2NLS/+. (Q–S) dAtx2NES+/+. dAtx2NLS+/+. dpp-GAL4/+. UAS-dAtx2NES+/+. dpp-GAL4/+. UAS-dAtx2NLS/+. 

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lation on the development of adult macrochaetae. As with wild-type dAtx2 (Figure 3), expression of dAtx2NES in the thoracic SOP cells causes no visible change in the number of macrochaetae in the adult thorax (Figure 8Q and Figure 8S column-1). In contrast, expression of dAtx2NLS induces a significant decrease in the number of macrochaetae, with an approximate twenty percent reduction in comparison to control animals (Figure 8R and Figure 8S column-2). These results indicate that increasing the levels of nuclear dAtx2 mimics expanded Ataxin-1 in inducing loss of mechanoreceptors and reducing the levels of Sens protein.

Discussion

Here we report functional interactions between the proteins causing two distinct Spinocerebellar ataxias. We use a Drosophila model of SCA1 to show that wild-type dAtx2 (the fly homolog of the protein that when expanded causes SCA2) mediates, at least in part, neuronal degeneration caused by expanded Ataxin-1 (the protein triggering SCA1). Ataxin-1[82Q]-induced toxicity is worsened by increasing the levels of dAtx2. More significantly, decreasing the levels of dAtx2 suppresses expanded Ataxin-1-induced neuronal degeneration as shown in several independent assays. The suppression of Ataxin-1[82Q] phenotypes by partial loss of function of dAtx2 argues against a possible mechanism by which sequestration and depletion of Ataxin-2 contributes to expanded Ataxin-1-induced neurodegeneration. This is further supported by lack of cerebellar or other neuronal abnormalities in mice that are deficient for Ataxin-2[46].

We find that the human expanded Ataxin-1 interacts with the dAtx2 and human Ataxin-2 proteins in co-AP assays. Furthermore, overexpressed Ataxin-1 pulls down endogenous hAtaxin-2 in cultured cells. These results suggest that Ataxin-1 and Ataxin-2 may be functional interactors in vivo. Consistent with this, we find that expanded Ataxin-1 induces accumulation of Ataxin-2 in the nucleus, where the two proteins localize in NIs both in Drosophila neurons and SCA1 human brain tissue. These are surprising observations since Ataxin-2 is normally a cytoplasmic protein both in humans and Drosophila. Interestingly, wild-type Ataxin-1 can cause neurotoxicity when overexpressed, although to a much lesser extent than expanded Ataxin-1[25]. However, nuclear accumulation of dAtx2 is triggered by pathogenic but not wild-type forms of Ataxin-1, at least in detectable amounts. Taken together these data suggested that accumulation of Ataxin-2 in the nucleus contributes to the exacerbated toxicity of expanded Ataxin-1, and is an important mechanism of pathogenesis in SCA1. To investigate this hypothesis we targeted dAtx2 to the nucleus by means of an exogenous NLS signal. We find that dAtx2NLS is sufficient to cause a dramatic increase of its toxicity, when compared to either wild-type dAtx2 or dAtx2 with an exogenous nuclear export signal (dAtx2NES) expressed at similar levels.

To further test the hypothesis that nuclear accumulation of Ataxin-2 contributes to neurodegeneration caused by expanded Ataxin-1 we investigated Sens levels. Sens and its murine orthologue Gfi1 are proneural factors whose levels are decreased in the presence of expanded Ataxin-1[18]; thus providing a molecular readout for the neurotoxicity of Ataxin-1. In Drosophila, reduction of Sens levels leads to the loss of mechanoreceptors [18], so we monitored Sens in the context of flies expressing either dAtx2NLS or dAtx2NES but not carrying the Ataxin-1[82Q] transgene. We find that nuclear targeted, but not cytoplasmic, dAtx2 mimics both the Sens reduction and mechanoreceptor loss phenotypes caused by Ataxin-1[82Q].

Expanded Ataxin-2 accumulates both in the cytoplasm and the nuclei of SCA2 postmortem brains [2,47–49]. In mouse and cell culture models of SCA2, expanded Ataxin-2 accumulates in the cytoplasm and its nuclear accumulation is not necessary to induce toxicity [44,45]. However, nuclear accumulation of expanded Ataxin-2 also occurs in cultured cells [45], and is consistently observed in human SCA2 postmortem brainstem neurons [2,47–49]. These observations suggest that both nuclear and cytoplasmic mechanisms of pathogenesis contribute to neurodegeneration in SCA2, as it is known to occur in other polyglutamine diseases like HD and SCA3 [50–52]. One possibility is that Ataxin-2 shuttles between the nucleus and the cytoplasm although the protein is normally detected only in the cytoplasm. Our data show that accumulation of dAtx2 in the nucleus is more harmful than in the cytoplasm. Thus, neurons with nuclear Ataxin-2 in SCA2 patients may be relatively more compromised than neurons where Ataxin-2 accumulates in the cytoplasm. In agreement with this possibility, expanded Ataxin-2 is found in the nuclei of pontine neurons of SCA2 brains, one of the neuronal groups and brain regions with prominent degeneration in SCA2 [2,47–49].

Reducing Ataxin-2 levels suppresses expanded Ataxin-1 toxicity, strongly arguing against a mechanism of pathogenesis by loss of function of Ataxin-2 in the cytoplasm. Studies of the normal function of Ataxin-2 and its yeast [29,30], C. elegans [31], and Drosophila [32,33] homologs suggest a role in translational regulation. Thus, an attractive possibility is that Ataxin-1[82Q] requires dAtx2 to impair Sens translation and induce the loss of mechanoreceptors. Consistent with this hypothesis is the finding that partial loss of function of dAtx2 suppresses the loss of mechanoreceptors phenotype caused by expanded Ataxin-1.

The data described here uncover unexpected functional interactions between proteins involved in two different SCAs. Nuclear accumulation of Ataxin-2, normally a cytoplasmic protein, is a common denominator of SCA1 and SCA2, and leads to reduced levels of at least one important proneural factor; i.e. Sens, whose mammalian orthologue Gfi1 is required for Purkinje cell survival [18]. Thus neuronal degeneration may take place through common mechanisms in different ataxias, and one of these mechanisms may involve the abnormal accumulation of Ataxin-2 in neuronal nuclei.

Methods

Drosophila strains. The cDNA GH27029 containing dAtx2 was obtained from the BDGP repository. SV40 nuclear localization signal and PKI nuclear export signal were engineered on the 3’ end of dAtx2 cDNA by PCR. Both constructs were then subcloned first in pGEM-T (Promega) and then in a previously generated pUAST-FLAG expression vector[53,54]. The Drosophila transgenic lines UAS-dAtx2NES and UAS-dAtx2NLS were obtained by injecting both constructs following standard procedures. EP(3)3143 was obtained from the Szeged Drosophila Stock Center in Hungary. The wild type UAS-dAtx2 (dAtx2OE) and mutant dAtx2-X1 lines [33] were kindly provided by Dr. Pallanck, L.J.. The UAS-SCA182Q, UAS-SCA130Q and UAS-SCA12Q lines have been previously described [7,25]. N-Htt128Q flies have been previously described [7,43]. All other Drosophila strains...
were obtained from the Bloomingtom Drosophila Stock Center at Indiana University.

**Scanning electron microscopy and retinal paraffin sections.** We used previously published procedures [7,25].

**Scoring of macrochaetae in adult thoraces.** Flies were raised at either 25°C for low Ataxin-1 [82Q] expression levels or 27°C for high Ataxin-1 [82Q] expression levels. Flies were collected at day 1 and the number of macrochaetae per thorax of same sex flies was counted for 20 animals per genotype. The percentage of lost macrochaetae over a total of 26 was calculated, and the average per genotype was plotted in the chart.

**Motor performance assay.** Between 25 and 30 adult females per genotype are collected for periods no longer than 24 hours. Flies are transferred to vials containing new food every day. The assay is carried out in an empty vial. The vial is tapped so all flies fall to the bottom then we score flies that climb past a line 5cm high in 18 seconds, and this procedure is repeated ten times for each day shown in the chart.

**Immunoblotting.** Eye imaginal discs from ten larvae per genotype were dissected in cold PBS, homogenized in 30 μl of Laemmli buffer (Bio-Rad) using a pellet pestle motor ( Kontes) and loaded on a 7.5% Tris-HCl Ready Gel (Bio-Rad). Membranes were stained with primary antibodies rabbit anti-dAtx2 (1:5000, L.J. Pallack) and mouse anti-tubulin (Hybridoma Bank, 1:1000). Horse-radish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:5000). Bio-Rad were used and membranes were developed using ECL Western blot detection kit (Amersham Biosciences).

### Supporting Information

**Figure S1.** Co-Expression of dAtx2 Reverts the Suppression of Ataxin-1 [82Q]-Induced Degeneration Observed in SCA1 [82Q] X dAtx2 [*X1*] Animals

(A-D') SEM images of eyes from flies of the genotypes indicated on top. (A, A') normal shape and arrangement of ommatidia in control animals (UAS-SCA1 [82Q]; gmr-GAL4/+). (B, B') Ataxin-1 [82Q] expression causes distortion of ommatidia and loss of inter-ommatidial bristles in SCA1 [82Q] animals (UAS-SCA1 [82Q]; gmr-GAL4/+). (C, C') Suppression of the phenotype induced by Ataxin-1 [82Q] in flies with only one functional copy of dAtx2 (UAS-SCA1 [82Q]; gmr-GAL4/dAtx2 [*X1*]+). Note in C' improved arrangement of ommatidia, and more interommatidial bristles. (D, D') Expression of low levels of dAtx2 in Ataxin-1 [82Q] flies that are also heterozygous for the dAtx2 [*X1*] allele (UAS-SCA1 [82Q]; gmr-GAL4/UAS-dAtx2 [*X1*]); gmr-GAL4/+). dAtx2 [*X1*] reverses the suppression observed in SCA1 [82Q] dAtx2 [*X1*] flies. Note that the eye phenotype observed in D and D’ is more similar to B and B’ than to C and C’ both at the level of ommatidial disorganization, as well as bristle loss. 

**Figure S2.** Levels of dAtx2 Protein in Control and Mutant Flies Carrying the Alleles Used in This Work

(A) Comparison between dAtx2 in wild-type and homozygous mutant dAtx2 [*X1*]/dAtx2 [*X1*] ovaries. Note the absence of dAtx2 signal in dAtx2 [*X1*]/dAtx2 [*X1*] ovaries. (B) Increased levels of dAtx2 are observed when dAtx2 is overexpressed in the eye with the dAtx2 [*X2*] line, in comparison with the levels observed in wild-type flies.

Genotypes: (A) Control: w; dAtx2 [*X1*]/HS-Flip; FRT, dAtx2 [*X1*]/FRT, dAtx2 [*X2*]. (B) Control: w; gmr-GAL4; +, dAtx2 [*X1*]; w; gmr-GAL4/UAS-dAtx2 [*X2*].

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**Author contributions.** I. Al-Ramahi, J. Lim, S. M. Pulst, H. Y. Zoghbi, and J. Botas conceived and designed the experiments. I. Al-Ramahi, A. M. Pérez, J. Lim, M. Zhang, R. Sorensen, and J. Branco performed the experiments. I. Al-Ramahi, A. M. Pérez, J. Lim, S. M. Pulst, H. Y. Zoghbi, and J. Botas analyzed the data. M. de Haro, S. M. Ramahi, A. M. Pérez, J. Lim, M. Zhang, R. Sorensen, and J. Branco contributed reagents/materials/analysis tools. I. Al-Ramahi and J. Botas wrote the paper.

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