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The AXH Domain of Ataxin-1 Mediates Neurodegeneration through Its Interaction with Gfi-1/Senseless Proteins

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Summary

Spinocerebellar ataxia type 1 (SCA1) is a neurodegenerative disease caused by an expanded glutamine tract in human Ataxin-1 (hAtx-1). The expansion stabilizes hAtx-1, leading to its accumulation. To understand how stabilized hAtx-1 induces selective neuronal degeneration, we studied *Drosophila* Atx-1 (dAtx-1), which has a conserved AXH domain but lacks a polyglutamine tract. Overexpression of hAtx-1 in fruit flies produces phenotypes similar to those of dAtx-1 but different from the polyglutamine peptide alone. We show that the *Drosophila* and mammalian transcription factors Senseless/Gfi-1 interact with Atx-1's AXH domain. In flies, overexpression of Atx-1 inhibits sensory-organ development by decreasing Senseless protein. Similarly, overexpression of wild-type and glutamine-expanded hAtx-1 reduces Gfi-1 levels in Purkinje cells. Deletion of the AXH domain abolishes the effects of glutamine-expanded hAtx-1 on Senseless/Gfi-1. Interestingly, loss of *Gfi-1* mimics SCA1 phenotypes in Purkinje cells. These results indicate that the Atx-1/Gfi-1 interaction contributes to the selective Purkinje cell degeneration in SCA1.

Introduction

Spinocerebellar ataxia type 1 (SCA1) is a neurodegenerative disease caused by the expansion of a translated CAG repeat encoding glutamine in the ataxin-1 (Atx-1) protein. This autosomal-dominant disorder typically has its clinical onset in adulthood, when patients exhibit a progressive loss of balance and coordination and gradually develop swallowing and breathing difficulties (Zoghbi and Orr, 2001). The primary sites of neurodegeneration in SCA1 include cerebellar Purkinje cells (PCs), brain-stem nuclei, the inferior olive, and the spinocerebellar tracts. At least eight other inherited neurodegenerative diseases, including Huntington's disease (HD), are caused by a similar mutational mechanism (Taroni and DiDonato, 2004; Zoghbi and Orr, 2000). A common feature of polyglutamine (polyQ) diseases is the loss of specific subsets of neurons in spite of broad expression of the mutant gene product (Zoghbi and Orr, 2000). For example, both Atx-1 and huntingtin are expressed in PCs and striatal medium spiny neurons, yet in SCA1, PCs are the prominent sites of pathology, whereas in HD, the spiny neurons are predominantly affected. Furthermore, although mutant Atx-1 is expressed throughout brain development, the clinical symptoms of SCA1 appear during adulthood, suggesting a temporal restriction of the pathology despite prolonged protein expression.

The mechanisms mediating SCA1 pathogenesis are still not fully understood, but some general principles have emerged. Genetic studies in mice and *Drosophila* support a toxic gain-of-function mechanism. Mice lacking Atx-1 do not develop ataxia or PC pathology, arguing against a loss-of-function mechanism (Matilla et al., 1998). In contrast, overexpression of mutant Atx-1 with an expanded polyQ tract causes neurodegeneration in mice and flies (Burright et al., 1995; Fernandez-Funez et al., 2000). Interestingly, even human Atx-1 with a nonpathogenic repeat of $(CAG)_{14}$CAT-CAG-CAT$(CAG)_{15}$, hereafter hAtx-1[30Q], causes neuronal degeneration if expressed at sufficiently high levels in either mice or *Drosophila* (Fernandez-Funez et al., 2000), suggesting that protein domains other than the expanded polyQ tract might contribute to SCA1 pathogenesis. Also, a single serine (S776) is critical for Atx-1-induced degeneration, as neuronal dysfunction is dramatically dampened in mice expressing a glutamine-expanded hAtx-1[82Q] in which S776 is replaced by alanine (Emamian et al., 2003). Phosphorylation of Atx-1 at S776 allows the 14-3-3 protein to bind to both wild-type (wt) and mutant Atx-1 (Chen et al., 2003). Glutamine-expanded Atx-1 binds more strongly to 14-3-3 than wt Atx-1, and this in turn leads to the accumulation of mutant Atx-1 and enhances neurotoxicity in the SCA1 *Drosophila* model. These data indicate that regions outside the polyQ expansion are critical for SCA1 pathogenesis.

Several reports have suggested that Atx-1 may play a role in the regulation of gene expression. First, genetic studies in mice have shown that nuclear localization of mutant Atx-1 is critical to the course of the disease (Klement et al., 1998). Second, alterations in gene expression occur very early in *SCA1*-transgenic mice, prior to the onset of any detectable neurological or pathological changes (Lin et al., 2000; Serra et al., 2004). Third, heterozygosity for loss-of-function alleles of several genes encoding transcriptional coregulators, including *Sin3A*, *Rpd3*, *dCtBP*, and *dSir2*, enhances the neurodegeneration in a SCA1 *Drosophila* model (Fernandez-Funez et al., 2000). Lastly, several reports have suggested that Atx-1 may interact with or modulate the function of transcriptional coregulators, including polyQ binding protein 1 (PQBP1) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) (Okazawa et al., 2002; Tsai et al., 2004). How these in-

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teractions might contribute to the disease process and how they might cause toxicity in only a subset of neurons in SCA1 is not fully understood.

To gain insight into how Atx-1's function contributes to SCA1 pathogenesis, we studied the *Drosophila* Atx-1 homolog (dAtx-1), which lacks a polyQ tract, and compared its in vivo effects and interactions to those of the human protein. Here we show that overexpression of hAtx-1 induces phenotypes similar to those of dAtx-1 overexpression but distinct from those observed upon overexpression of polyQ chains in flies. Furthermore, we identify the *Drosophila* zinc-finger transcription factor Senseless (Sens) and its mammalian homolog growth factor independence-1 (Gfi-1) as physical and genetic interactors of *Drosophila* and mammalian Atx-1, respectively. We propose that the Sens/Gfi-1 interaction provides a mechanism for SCA1 pathogenesis and the selective vulnerability of PCs in this disease.

Results

dAtx-1 and hAtx-1 Induce Similar Phenotypes When Overexpressed in Flies

A 110 residue region of hAtx-1 shows sequence homology (28% identity, 54% similarity) with a transcription factor called HMG box containing protein-1 (HBP1) (Yee et al., 2004). This domain of homology has been termed the AXH (*a*t*a*xin-1/*H*BP1) domain (SMART Database accession number SM00536) (Figure 1A) (Chen et al., 2004). The closest homolog of hAtx-1 in *Drosophila* (dAtx-1; 44% identity) is a 230 amino acid protein encoded by *CG4547*. dAtx-1 contains the AXH domain but not a polyQ tract (Figure 1A). Phylogenetic-tree analysis indicates that dAtx-1 shows higher sequence homology with hAtx-1 and its paralog Atx-1-like (H.-K.C. and H.Y.Z., unpublished data) than with HBP1 or any other human protein (data not shown). There is also a small protein corresponding to the AXH domain in *C. elegans*, suggesting that this domain serves as an independent functional unit (Figure 1A).

To test the function of the AXH domain, we overexpressed dAtx-1 in flies using the GAL4/UAS system (Brand and Perrimon, 1993) and compared its effects to those of hAtx-1. Overexpression of dAtx-1 by *Rhodopsin1(Rh1)-GAL4*, which drives expression in the differentiated R1–R6 photoreceptor cells (Mollereau et al., 2000; O'Tousa et al., 1985), results in neurodegeneration in the eye, as does overexpression of hAtx-1[82Q]. Although at 2 days after eclosion, overexpression of either Atx-1 does not show obvious morphological changes in the photoreceptor cells (data not shown), both genotypes show many large holes and loss of cell integrity at 28 days (Figures 1B–1D). Overexpression of dAtx-1 using the *GMR-GAL4* driver also induces eye abnormalities. The external structures of the eyes that overexpress dAtx-1 show disorganized ommatidia and loss of interommatidial bristles (Figure 1F), and these eyes exhibit loss of rhabdomeres and tissue integrity (Figure 1J). In both cases, dAtx-1 phenotypes are similar to but milder than hAtx-1. We also found that dAtx-1 and hAtx-1 induce similar phenotypes in other tissues. Although the *GMR-GAL4* driver is mainly eye specific (Freeman, 1996), it also induces a broad low-level expression in the wing during the early pupal stage (Figure 1L). Interestingly, expression of dAtx-1 and hAtx-1[82Q] using *GMR-GAL4* generates extra wing-vein tissue (Figures 1N and 1O). Similar to the eye phenotypes, dAtx-1 overexpression causes a milder phenotype.

To examine the wing phenotypes induced by Atx-1, we overexpressed dAtx-1 in the wing pouch using *C5-GAL4* (Yeh et al., 1995) and in the wing margin using *C96-GAL4* (Gustafson and Boulianne, 1996). Overexpression of dAtx-1 using the wing-pouch driver at 27°C causes several vein abnormalities (Figure 1R). Overexpression of hAtx-1[82Q] at 27°C has a more severe phenotype and causes an atrophic wing (Figure 1S). When flies overexpressing hAtx-1 are incubated at 23°C to lower the level of hAtx-1[82Q] expression, we observe a phenotype similar to that of dAtx-1 (inset in Figure 1S). With the wing-margin driver, both dAtx-1 and hAtx-1[82Q] cause bristle abnormalities including patterning defects (Figures 1V and 1W, arrows) and bristle loss (Figure 1W, brackets). We compared the effects of five dAtx-1-, two hAtx-1[30Q]-, and three hAtx-1[82Q]-transgenic lines, using the *GMR-GAL4*, the wing-pouch, or the wing-margin drivers. Even the strongest dAtx-1 transgenes show milder phenotypes than the weakest human Atx-1[30Q] (data not shown) or hAtx-1[82Q] transgenes (Figure 1). Consistent with our previous report (Fernandez-Funez et al., 2000), the phenotypes of hAtx-1[30Q] are also milder than those of hAtx-1[82Q] (data not shown).

The polyQ chain alone has been shown to cause cytotoxicity (Kazemi-Esfarjani and Benzer, 2000; Marsh et al., 2000). To confirm that the Atx-1-induced toxicity is AXH-domain mediated and distinct from the polyQ tract, we compared phenotypes induced by a polypeptide comprised of 127 glutamines (127Q) (Kazemi-Esfarjani and Benzer, 2000) to those induced by Atx-1. Although overexpressing 127Q using *GMR-GAL4* results in a more severe eye phenotype than dAtx-1 overexpression (Kazemi-Esfarjani and Benzer, 2000) (Figure 1H), flies overexpressing 127Q do not show extra veins in the wing (Figure1P). Overexpression of 127Q using the wing-pouch driver does not cause significant vein abnormalities, although it induces a mild wing atrophy at 29°C (Figure 1T). Furthermore, overexpression of 127Q with the wing-margin driver does not cause patterning defects as observed with Atx-1 overexpression (Figure 1X). Because hAtx-1 induces phenotypes similar to dAtx-1 and since these phenotypes are not induced by the polyQ tract, we conclude that the polyQ tract is not necessary to generate the gain-of-function phenotype of Atx-1 in flies.

Drosophila and Mammalian Ataxin-1 Physically Interact with Sens and Gfi-1

Based on the similar phenotypes of dAtx-1 and hAtx-1 in *Drosophila* (Figure 1), we hypothesized that some of the aberrant signaling may be conserved between dAtx-1 and hAtx-1. We focused on the bristle phenotypes. Bristles are mechanosensory organs of the peripheral nervous system (PNS) that develop from epidermal cells through the coordinated activities of proneural proteins and Sens, a zinc-finger transcription factor required for the generation of sensory organs in

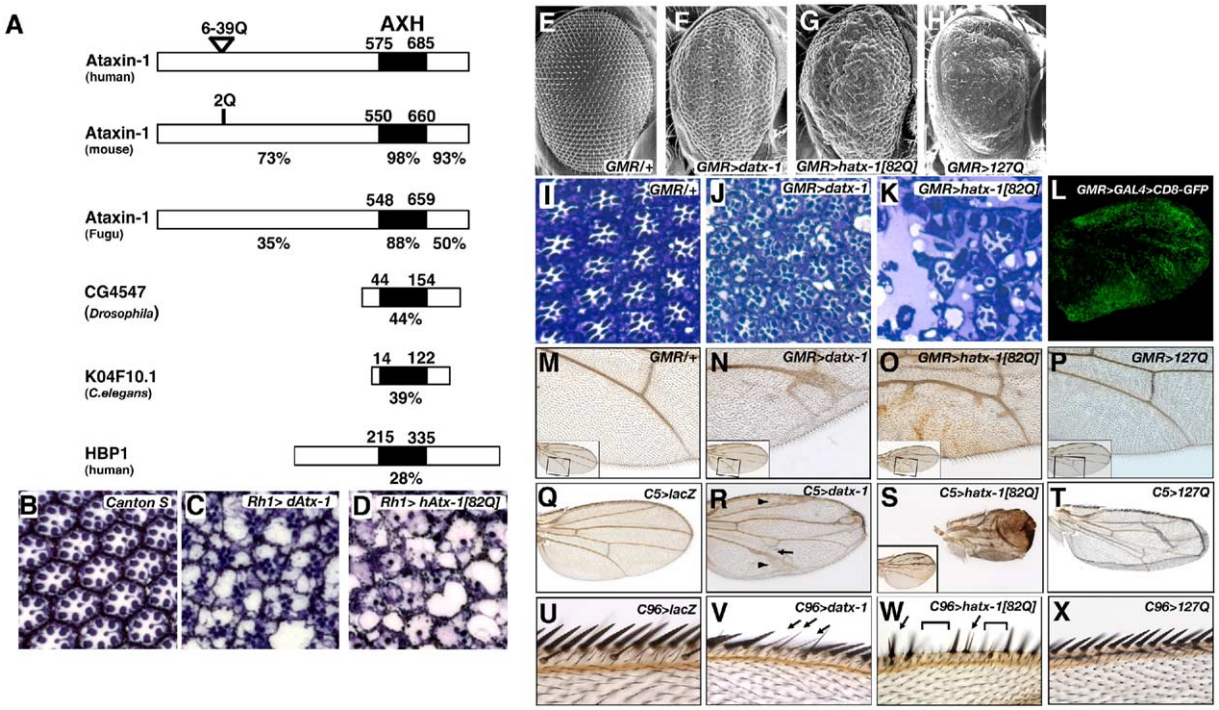


Figure 1. *Drosophila* and Human Atx-1[82Q] Overexpression Phenotypes

(A) Proteins of the Atx-1 family and HBP1 show high-percentage amino acid identity to hAtx-1 in the AXH domain (solid boxes).

(B–D) Overexpression of Atx-1 using *Rh1-GAL4* causes degeneration in photoreceptor cells. Toluidine-blue-stained eye sections of *Canton S (CS)* (B), *Rh1-GAL4/+; UAS-datx-1/+* (C), *Rh1-GAL4/+; UAS-hatx-1[82Q]/+* (D) at 28 days posteclosion are shown. In flies overexpresing dAtx-1, five or six photoreceptor cells remain out of the eight found in normal eyes, whereas in flies overexpressing hAtx-1[82Q] only one or two photoreceptors remain.

(E–K) Expression of Atx-1 or 127Q driven by *GMR-GAL4* leads to eye abnormalities. (E)–(H) are SEM eye images; (I)–(K) are internal eye structures. *GMR-GAL4/+ control* (E and I), *GMR-GAL4/+; UAS-datx-1/+* (F and J), *GMR-GAL4/+; UAS-hatx-1[82Q]/+* (G and K), *GMR-GAL4/+; UAS-127Q/+* (H).

(L) GFP fluorescence in the wing of *GMR-GAL4/+; UAS-GAL4/+; UAS-CD8-GFP* during the early pupal stage. *UAS-GAL4* was used to increase the GAL4 protein levels.

(M–P) Expression of dAtx-1 and hAtx-1[82Q] in the wing under the control of *GMR-GAL4* causes wing-vein abnormalities, whereas that of 127Q does not. Wings of *GMR-GAL4/+* control (M), *GMR-GAL4/+; UAS-datx-1/+* (N), *GMR-GAL4/+; UAS-hatx-1[82Q]/+* (O), and *GMR-GAL4/+; UAS-127Q/+* (P) are shown. Regions of magnification are shown in insets.

(Q–T) Atx-1 expressed by a wing-pouch driver (*C5-GAL4*) shows wing-vein abnormalities, including extra veins (arrowheads) and truncation of normal veins (arrow). Expression of 127Q using *C5-GAL4* does not cause wing-vein abnormalities. Wings of flies overexpressing LacZ (Q), dAtx-1 (R), hAtx-1[82Q] (S), and 127Q (T) raised at 27°C are shown. Wing of a fly overexpressing hAtx-1[82Q] at 23°C is shown in the inset in (S).

(U–X) Overexpression of Atx-1 but not 127Q causes patterning defects (arrows) and loss of bristles (brackets) in the wing margin. Wing margins of flies overexpressing LacZ (U), dAtx-1 (V), hAtx-1[82Q] (W), and 127Q (X) are shown. In all cases, hAtx-1[82Q] overexpression causes a more severe phenotype than dAtx-1 overexpression.

flies (Nolo et al., 2000; Bertrand et al., 2002). Interestingly, we found that dAtx-1 interacts with Sens in a yeast two-hybrid screen aimed at identifying Sens-interacting proteins (Figure 2A). To determine which domain of dAtx-1 can bind Sens, we performed two-hybrid interaction assays using dAtx-1 deletion constructs. As shown in Figures 2A and 2C, residues 100 through 154, a part of the AXH domain, of dAtx-1 are necessary and sufficient for the interaction with Sens. To confirm the interaction, we performed coimmunoprecipitation (CoIP) assays using FLAG-tagged dAtx-1 and HA-tagged Sens in COS7 cells. Monoclonal anti-FLAG or anti-HA antibody immunoprecipitates Sens or dAtx-1, respectively, only when FLAG-dAtx-1 and HA-Sens are coexpressed (Figure 2D). Together, these data indicate that dAtx-1 physically interacts with Sens.

As shown in Figure 2A, hAtx-1 also interacts with Sens in yeast. Given the structural and functional similarities between Sens and its vertebrate homolog Gfi-1 (Jafar-Nejad and Bellen, 2004), we then asked whether hAtx-1 binds Gfi-1 (Gilks et al., 1993). As shown in Figure 2B, Gfi-1 interacts with hAtx-1[2Q] or [82Q] based on a yeast two-hybrid assay as well as CoIP studies in transfected cells (data not shown). The AXH subdomain of hAtx-1 (residues 631–685 corresponding to residues 100–154 in the fly) is necessary and sufficient to interact with Gfi-1 (Figures 2B and 2C). To confirm the direct interaction between Atx-1 and Gfi-1, we assessed the ability of GST-hAtx-1 to pull down Gfi-1 protein. As shown in Figure 2E, while GST-hAtx-1[2Q] and GST-AXH domain can pull down Gfi-1 protein, GST-hAtx-1[2Q] lacking AXH domain fails to interact with Gfi-1, sug-

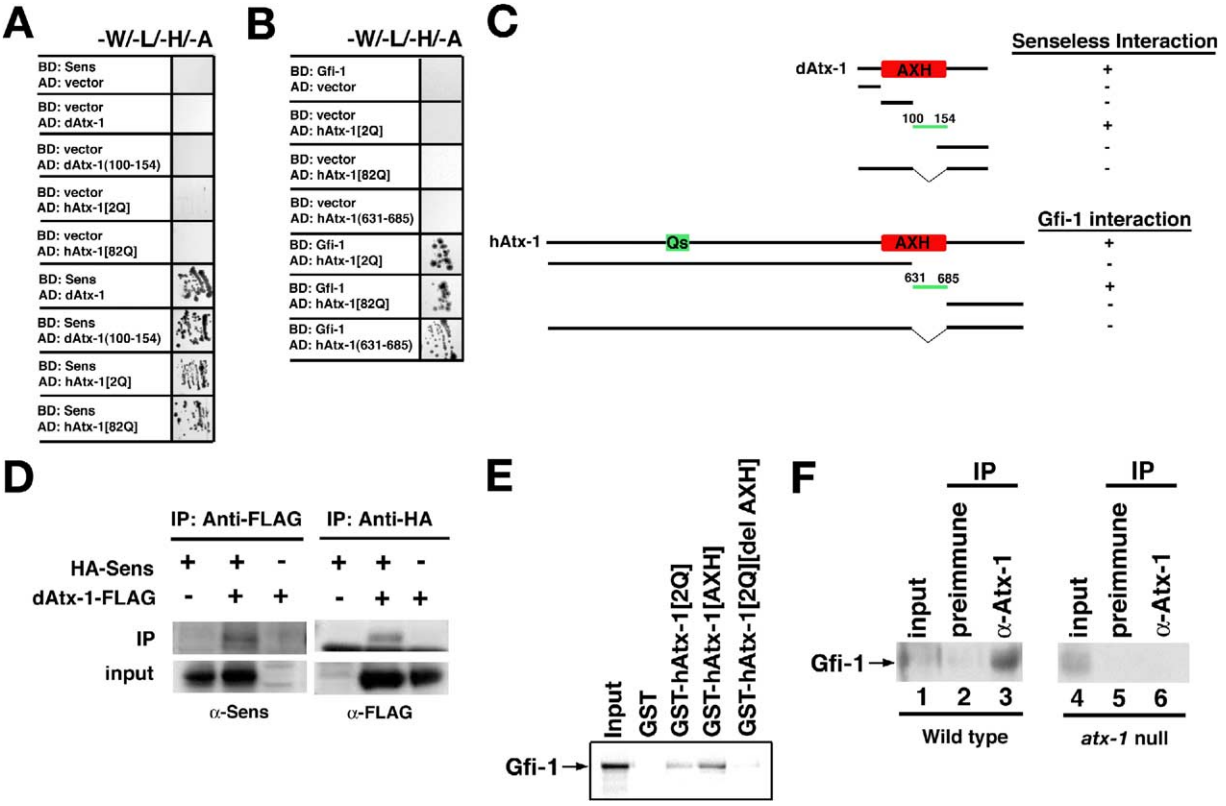


Figure 2. dAtx-1 and hAtx-1 Interact with Sens and Gfi-1, Respectively

(A–C) Sens and dAtx-1 (A) and Gfi-1 and hAtx-1 (B) interaction in a yeast two-hybrid assay. BD, DNA binding domain; AD, activation domain. Double transformants were selected on -Trp/-Leu/-His/-Ade (-W/-L/-H/-A) media. (C) shows a schematic diagram of Atx-1 constructs and their interaction with Sens and Gfi-1.

(D) FLAG-tagged dAtx-1 and HA-tagged Sens coimmunoprecipitate from transfected COS7 cells. IP, immunoprecipitate.

(E) GST pull-down assays show that Gfi-1 can bind hAtx-1[2Q] and AXH domain but not hAtx-1[2Q] lacking the AHX domain.

(F) Mouse Atx-1 and Gfi-1 coimmunoprecipitate from cerebellar extracts of wt mice (left) but not *atx-1* knockout mice. The immunoprecipitation was done with anti-Atx-1 and immunoblotting with anti-Gfi-1 antibody.

gesting that the AXH domain mediates the direct interaction between the two proteins. To find the reciprocal domain of Sens/Gfi-1 that binds Atx-1, we tested deletion constructs for their ability to bind dAtx-1 or hAtx-1. As shown in Figures S1A and S1B in the Supplemental Data available with this article online, the amino-terminal regions, not the zinc-finger domain of Sens or Gfi-1, are sufficient for the interaction with dAtx-1 or hAtx-1, respectively. Lastly, to confirm the interaction in vivo, we performed CoIP assays using mouse brain extracts. The anti-Atx-1 antibody coimmunoprecipitates Gfi-1 from brain extracts of wt mice but not from extracts of *atx-1* null mice (Matilla et al., 1998) (Figure 2F), indicating that the two proteins interact in vivo. In summary, our data indicate that dAtx-1 and hAtx-1, via the AXH domain, can bind to Sens and Gfi-1, respectively.

Sens and Gfi-1 Are Coexpressed with Atx-1 Homologs in *Drosophila* and Mice

The findings that fly Atx-1 and Sens as well as mammalian Atx-1 and Gfi-1 physically interact prompted us to examine if Atx-1 and Sens/Gfi-1 are coexpressed in vivo. In situ hybridization and Northern analyses show that *datx-1* is expressed in embryonic stages (Figures 3A–3D and data not shown). The expression of *datx-1* is first observed in the dorsolateral region in the stage 5 embryos (Figure 3A). During gastrulation, *datx-1* is expressed in the dorsolateral ectoderm that encompasses the peripheral neuroectoderm (Figure 3B). *sens* mRNA is first expressed in presumptive sensory organ precursor (SOP) cells at stage 10 (Nolo et al., 2000). We found that *sens* is expressed in a subset of cells within the region of *datx-1* expression (Figures 3C and 3D). In mice, Gfi-1 is expressed in many areas that give rise to neuronal cells during embryonic development (Wallis et al., 2003). However, our data show that, in the adult cerebellum, Gfi-1 expression is mainly confined to PCs, where Atx-1 is most abundant (Figures 3E–3G) (Banfi et al., 1996).

Ataxin-1 Inhibits Sens Function during SOP Formation

To address the relevance of the biochemical interactions between Atx-1 and Sens, we examined the functional relationship between these proteins in vivo. Sens

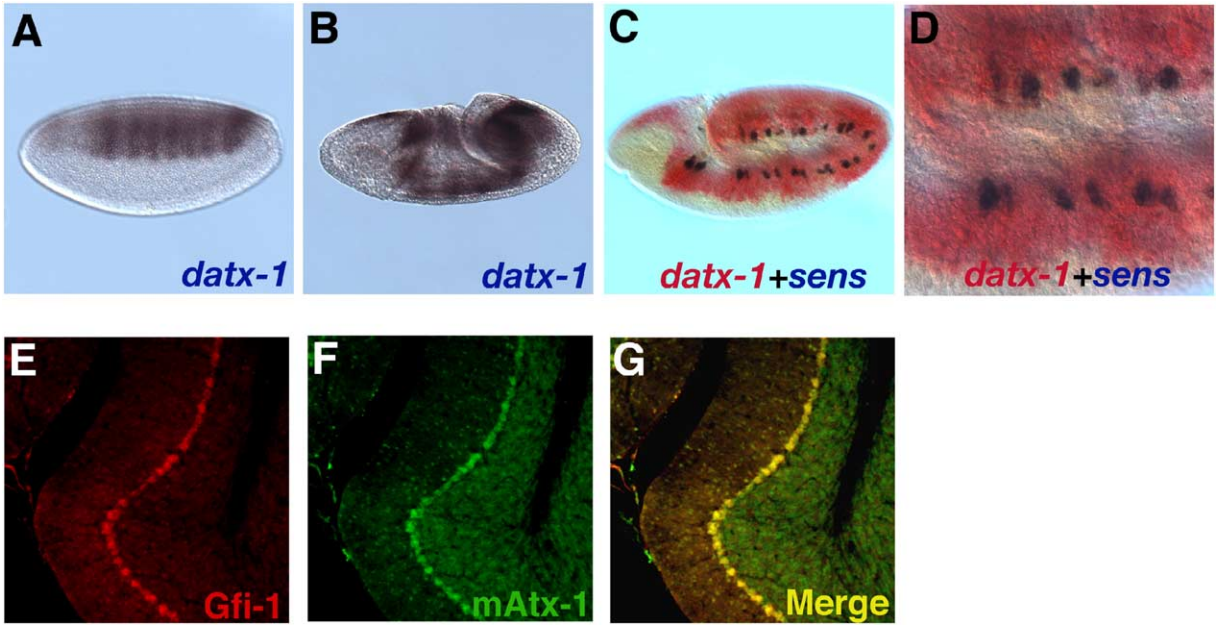


Figure 3. Fly and Mouse Atx-1 Colocalize with Sens and Gfi-1 in Certain Cell Types

(A–D) Temporal and spatial expression of *datx-1* analyzed by whole-mount in situ hybridization. (A) shows blastula stage, lateral view; (B) shows gastrula stage, lateral view; (C) and (D) show germband elongation stage, lateral view. (D) is a higher magnification of (C).

(E–G) Gfi-1 and Atx-1 are coexpressed in PCs. Double staining of a cerebellar section from an 8-week-old mouse with anti-Gfi-1 (E) and anti-Atx-1 (F) antibodies. (G) is the merged image.

is directly activated by the proneural proteins in the presumptive SOPs, where it acts as a transcriptional activator and synergizes with proneural proteins (Jafar-Nejad et al., 2003). Overexpression of Sens, using the *Scabrous* (*C109-68-GAL4*) driver (Abdelilah-Seyfried et al., 2000), in the SOPs and a few cells around them causes extra bristle formation—microchaetae and macrochaetae—in the thorax of the adult fly (Figure 4D). In contrast, overexpression of either dAtx-1 or hAtx-1 using the *Scabrous* driver causes loss of thoracic bristles (Figures 4B and 4C), with human Atx-1[82Q] causing a more severe phenotypes (Figures 4B, 4C, and 4G). When dAtx-1 and Sens are coexpressed in SOPs, Sens overexpression abolishes the phenotype of dAtx-1 overexpression (Figures 4E and 4G). Similarly, Sens overexpression suppresses the loss-of-bristle phenotype induced by hAtx-1[82Q], although the number of extra bristles is decreased compared to Sens overexpression alone (Figures 4F and 4G). These data suggest that Atx-1 overexpression phenotype may result from an alteration in Sens expression or function.

Sens synergizes with Achaete (Ac) to promote bristle formation in the thorax. Previously, an in vitro transcription assay, using the proximal *ac* promoter as the reporter, was established that recapitulates the in vivo activity of Sens in SOPs (Jafar-Nejad et al., 2003). Sens alone does not activate the *ac* promoter (data not shown). Cotransfection of Ac and Daughterless (Da) expression constructs activates the promoter about 50-fold. Adding Sens leads to a further activation of the *ac* promoter (150-fold), indicating synergism between Sens and these proneural proteins (Figure 4J). To refine the molecular basis of Atx-1's effect on bristle formation, we examined its activity in this transcription assay. Adding dAtx-1 does not have a significant effect on the *ac* promoter directly (Figure 4H) or on Ac/Da activity (Figure 4I). However, dAtx-1 suppresses the synergism between Sens and Ac/Da in a dose-dependent manner (Figure 4J). Furthermore, this effect on Sens activity is more potent with the mammalian Atx-1 homologs. As shown in Figure 4K, lesser amounts of hAtx-1[2Q] or [82Q] are required to inhibit Sens activity when compared to dAtx-1 (Figure 4J). Together, our in vivo and in vitro data strongly suggest that overexpression of Atx-1 inhibits the activity of Sens in the SOP formation.

Atx-1 Decreases the Level of Sens Protein in a Cell-Autonomous Manner

To explore the mechanism by which Atx-1 suppresses Sens function, we evaluated the levels of Sens protein in wing imaginal discs, where it is expressed in the SOPs (Figure 5A) (Nolo et al., 2000). Using the *Scabrous* driver, we found that overexpression of either fly or human Atx-1 decreases the level of endogenous Sens protein (Figures 5A–5C). Consistent with the bristle phenotypes, overexpression of hAtx-1[82Q] has a more severe effect on Sens levels than dAtx-1 (Figures 5B and 5C). Overexpression of Atx-1 using *GMR-GAL4* also suppresses Sens protein levels in the R8 photoreceptor cells (data not shown), where Sen is expressed (Frankfort et al., 2001).

We then assessed whether Atx-1 exerts its activity on Sens in a cell-autonomous manner. In the wing margin, Sens is expressed in several rows of cells flanking

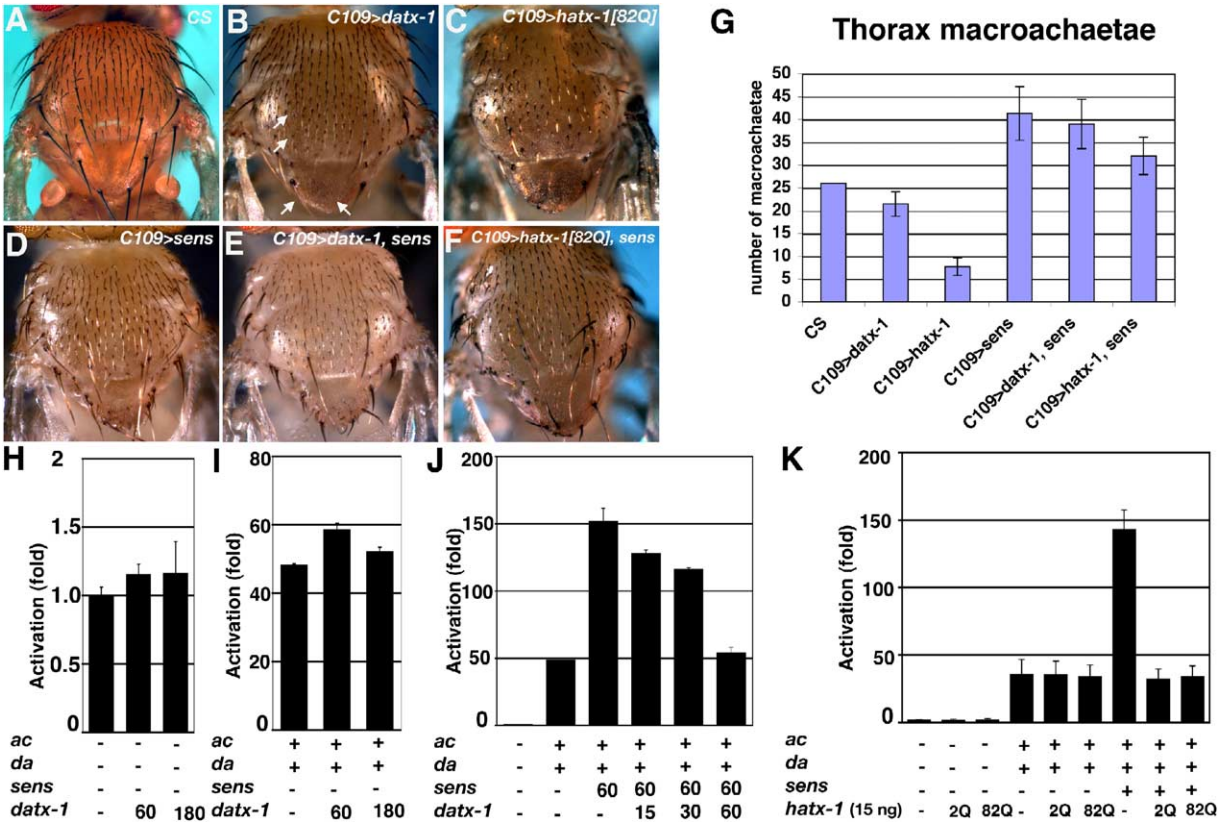


Figure 4. Atx-1 Inhibits the Function of Sens In Vivo and In Vitro

(A–F) Atx-1 inhibits bristle formation.

(A) Thorax of a *CS* fly.

(B–D) Thoraxes of flies expressing dAtx-1 (B), hAtx-1[82Q] (C), or Sens (D) under the control of the *C109-68-GAL4* driver. Atx-1 causes loss of macrochaetae (arrow) and microchaetae, whereas Sens induces extra bristles.

(E and F) The effect of Sens overcomes those of dAtx-1 (E) and hAtx-1[82Q] (F).

(G) Quantification of the number of macrochaetae shown in panels (A)–(F). Error bars indicate 95% confidence interval.

(H–K) Transcription assay in S2 cells using an enhancer/promoter fragment of the *ac* gene to drive luciferase. Error bars indicate standard error of the mean.

(H and I) The effects of dAtx-1 on the *ac* promoter in the absence (H) or presence of Ac/Da (I). Note that even 180 ng of *datx-1* does not have a significant effect in the absence of Sens.

(J) dAtx-1 suppresses the synergism of Sens and Ac/Da in a dose-dependent manner.

(K) 15 ng of hAtx-1[2Q] or [82Q] suppresses the activity of Sens. Note that the effect of 15 ng of either hAtx-1 is comparable to that of 60 ng dAtx-1.

the dorsoventral margin, where the Wingless gradient is at its peak (Figures 5E–5G). Sens expression in these cells, which will give rise to wing-margin SOPs, is thought to be dependent on Wingless (Parker et al., 2002). As shown in Figures 5J and 5L, overexpression of hAtx-1[82Q] using the *dpp-GAL4* driver causes a loss of Sens protein, but the cells flanking the hAtx-1 overexpression domain are not affected (Figures 5I–5L). Furthermore, Wingless expression in the wing margin is not decreased in the cells in which hAtx-1 is overexpressed (Figure 5K and arrow in Figure 5L). These data demonstrate that the effects of Atx-1 on Sens are cell autonomous. Overexpression of dAtx-1 using the *dpp-GAL4* driver also decreases the level of Sens (data not shown). Furthermore, the effect of hAtx-1[82Q] on Sens levels is not merely due to the expanded polyQ tract, as overexpression of 127Q or hAtx-1[82Q] lacking AXH domain has no significant effects on Sens levels (Figures 5N–5Q).

To determine whether the loss of Sens in the Atx-1 overexpression domain is due to cell death or decreased Sens transcription, we examined the level of *sens* RNA in the wing margin with or without Atx-1 overexpression. In situ hybridization for *sens* showed that Atx-1 overexpression does not affect *sens* mRNA levels (Figure 5M), suggesting an effect at the protein level and ruling out cell death as a cause. The direct physical interaction between Atx-1 and Sens raises the possibility that Atx-1 might cause the Sens protein to become unstable.

Gfi-1 Expression Is Decreased in *SCA1*-Transgenic Mice in the Absence of Notable Cell Death

Does the in vivo effect of Atx-1 in fly SOPs have a functional correlate in mammals? To answer this question, we determined the consequences of Atx-1 overexpression on Gfi-1 levels in PCs. We examined Gfi-1 levels in mice that overexpress hAtx-1[82Q] only in PCs.

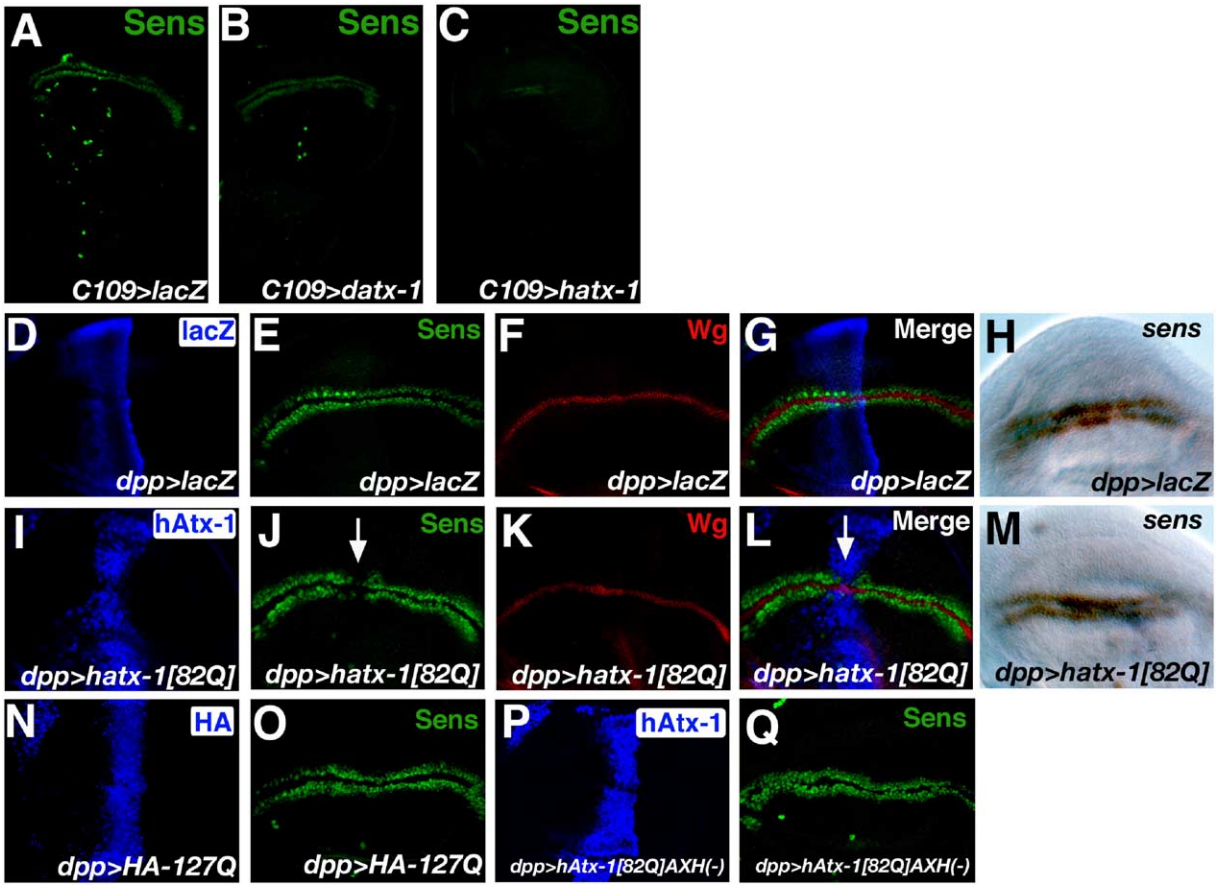


Figure 5. Atx-1 Overexpression Decreases Sens Protein Levels in a Cell-Autonomous Manner

(A–C) Atx-1 expression driven by *C109-68-GAL4* suppresses endogenous Sens protein. Wing imaginal discs from flies expressing LacZ (A), dAtx-1 (B), and hAtx-1[82Q] (C) stained with anti-Sens antibody are shown.

(D–M) hAtx-1[82Q] decreases Sens protein, not mRNA. *dpp-GAL4* was used to drive the expression of LacZ (D–H) and hAtx-1[82Q] (I–M) along the anterior-posterior boundary in the wing imaginal disc. Staining with anti-β-gal (D), anti-Atx-1 (I), anti-Sens (E and J), and anti-Wingless (F and K); merged images (G and L); and whole-mount in situ hybridization for *sens* mRNA expression (H and M) are shown. Overexpression of hAtx-1[82Q] using the *dpp-GAL4* driver decreases Sens protein (arrow in [J] and [L]) but not Wingless protein (K and L) or *sens* mRNA levels (M).

(N–Q) Overexpression of 127Q or hAtx-1[82Q] lacking AXH domain does not have significant effects on Sens expression. *dpp-GAL4/+; UAS-HA-127Q/+* (N and O). *dpp-GAL4/+; UAS-hAtx-1[82Q]AXH(-)/+* (P and Q). Staining is with anti-HA (N), anti-hAtx-1 (P), and anti-Sens (O and Q).

These mice develop ataxia after 5 weeks of age and show significant loss of PCs after 6 months (Burright et al., 1995; Clark et al., 1997). We observed a marked decrease in Gfi-1 protein levels in PCs of these mice compared to wt mice (Figures 6B and 6E). The decrease in Gfi-1 protein levels occurs at a time when there is no significant loss of PCs, as determined by calbindin staining (Figure 6D). Lastly, to confirm that the effect of hAtx-1 on Gfi-1 is indeed mostly due to sequences other than the glutamine tract, we evaluated Gfi-1 levels in mice overexpressing wt hAtx-1[30Q]. These mice, when bred to homozygosity and aged, develop Purkinje cell degeneration. Here again, we find Gfi-1 levels to be decreased (Figures 6J–6L), suggesting that the functional and physical interactions of Atx-1 and Sens/Gfi-1 are conserved between *Drosophila* and mice.

Atx-1 Destabilizes Gfi-1 by Enhancing Its Degradation via the Proteasome

To explore the mechanism by which Atx-1 reduces Gfi-1 levels, we examined the effect of Atx-1 on the degradation of Gfi-1. We used pulse-chase radiolabeling analysis to evaluate the stability of endogenous Gfi-1. About 72% of the labeled Gfi-1 remains in control cells after 2 hr. However, in cells which express dAtx-1 or hAtx-1[82Q], Gfi-1 is less stable (46% and 31% remaining, respectively). Gfi-1's stability is not affected in cells that expressed hAtx-1[82Q] lacking the AXH domain (78% remaining) (Figures 6M and 6N). These data suggest that Atx-1 enhances the degradation of Gfi-1 upon interaction via its AXH domain.

To determine if Gfi-1's degradation is dependent on the proteasome, we examined the effect of proteasome inhibition on the stability of Gfi-1. Whereas mock-treated cells have no Gfi-1 immunofluorescence 72 hr after transient transfection of Gfi-1 expression vector, proteasome-inhibitor-treated cells accumulate Gfi-1 (Figure S2D). Moreover, accumulated Gfi-1 colocalizes with the 20S proteasome (Figures S2D–S2F), suggesting that Gfi-1 degradation is via the proteasome pathway. We next examined whether inhibiting the proteasome can interfere with the effect of Atx-1 on Gfi-1 stability. Treatment of cells with proteasome inhibitor

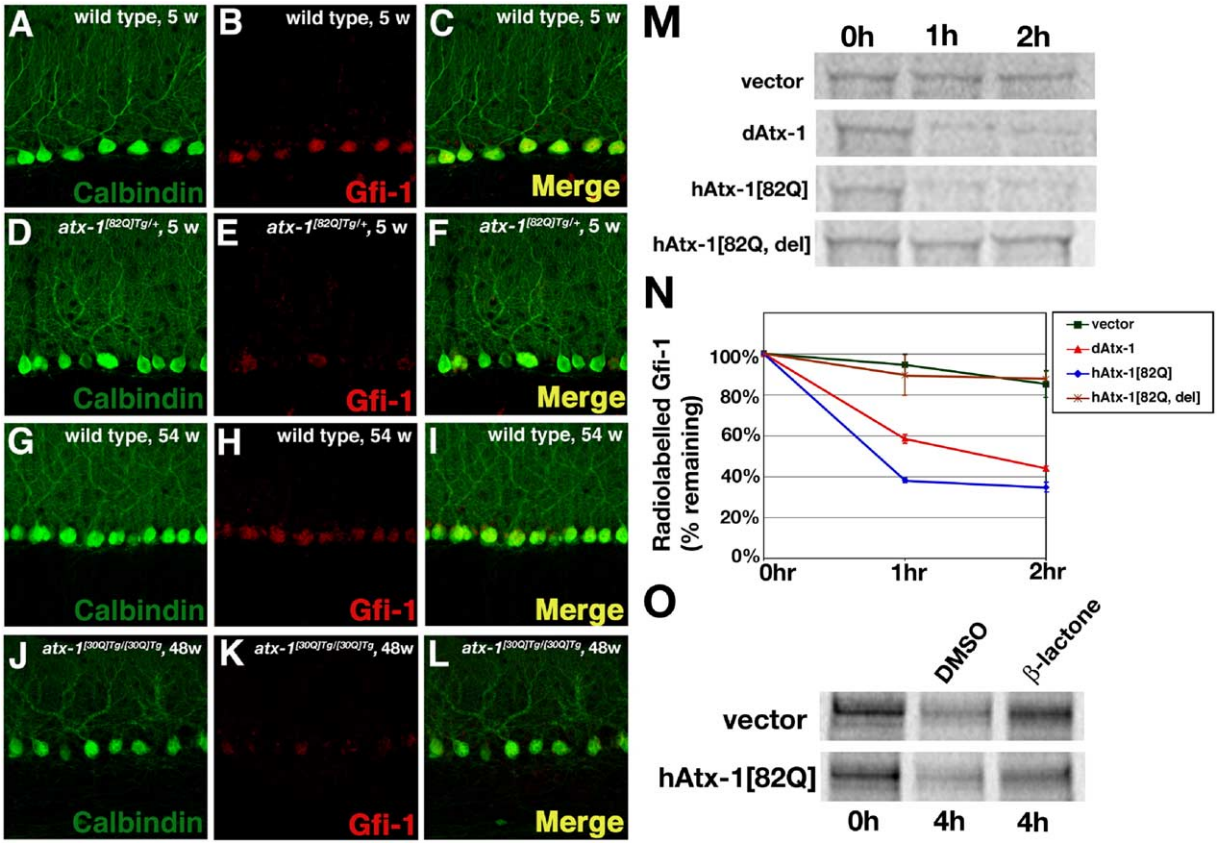


Figure 6. The Effects of Atx-1 on Gfi-1 Level In Vivo and In Vitro

(A–L) Gfi-1 levels are markedly decreased in both Atx-1[82Q]- and Atx-1[30Q]-transgenic mice (*atx-1*$^{[82Q]Tg/+}$ and *atx-1*$^{[30Q]Tg/[30Q]Tg}$) even in the absence of cell loss. Cerebellar slices from a 5-week-old wt mouse (A–C), a 5-week-old Atx-1[82Q]-transgenic mouse (D–F), a 54-week-old wt mouse (G–I), and a 48-week-old Atx-1[30Q]-transgenic mouse (J–L) are shown. Staining is with anti-calbindin (A, D, G, and J) and anti-Gfi-1 (B, E, H, and K) antibodies; (C), (F), (I), and (L) are the merged images.

(M–O) The pulse-chase assays show that Atx-1 destabilizes Gfi-1 in the BE-2 human neuroblastoma cell line.

(M) Representative autoradiographs of ^{35}S-labeled endogenous Gfi-1 protein in BE-2 cells transiently transfected with empty vector, dAtx-1, hAtx-1[82Q], or hAtx-1[82Q] lacking AXH domain.

(N) Amount of radiolabeled Gfi-1 remaining after the indicated time points in the pulse-chase assays. Gfi-1 is less stable in cells that express dAtx-1 and hAtx-1[82Q] but not in cells that express hAtx-1[82Q] lacking the AXH domain. Error bars indicate standard error of the mean.

(O) Autoradiographs of ^{35}S-labeled endogenous Gfi-1 protein in BE-2 cells after a 4 hr incubation period in unlabeled medium with the presence of β-lactone or DMSO. β-lactone increases the radiolabeled Gfi-1 in cells transfected with Atx-1[82Q] by 56% compared with DMSO.

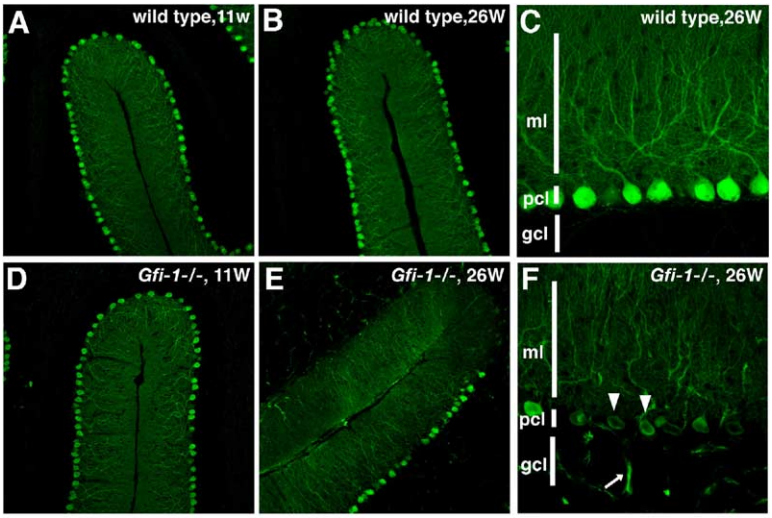
leads to the accumulation of Gfi-1 in the hAtx-1[82Q] transfected cells, suggesting that hAtx-1 enhances Gfi-1 degradation via the proteasome pathway (Figure 6O).

Loss of Gfi-1 Leads to Purkinje Cell Degeneration

Gfi-1 heterozygous mice are viable and fertile (Hock et al., 2003) and display no overt cerebellar phenotypes based on the accelerating-rotarod analysis and cerebellar histochemistry (Wallis et al., 2003 and data not shown). *Gfi-1* null mice display loss of the cochlear hair cells and aberrant differentiation of vestibular hair cells, and they exhibit several behavioral abnormalities, including severe ataxia (Wallis et al., 2003). Since either vestibular or cerebellar defects can cause ataxia, we evaluated the cerebellar phenotype of *Gfi-1* null mice by immunohistochemistry. The overall gross morphology of the cerebellum is normal in adult *Gfi-1* null mice (data not shown). Calbindin staining of cerebellar sections from 11-week-old *Gfi-1* null mice reveals a normal number of PCs (Figure 7D). However, at 6 months of age, *Gfi-1* null mice display a loss of PCs (Figure 7E). In addition, most of the surviving PCs exhibit abnormal nuclear and cytoplasmic morphology (arrowheads in Figure 7F) in the soma and aberrant and disorganized dendrites (Figure 7F). The axons of the PCs have focal swellings (arrow in Figure 7F), similar to what has been reported in SCA1 patients (Zoghbi and Orr, 2001). The observation that loss of PCs appears several months postnatally argues against a developmental defect and indicates that Gfi-1 contributes to survival of PCs. In summary, the cerebellar pathology in *Gfi-1* mutant mice strongly suggests that alterations in the level of Gfi-1 may account in part for the PC-specific phenotype of SCA1.

Loss of One Copy of *Gfi-1* Enhances the Pathogenesis of SCA1

hAtx-1[82Q]-transgenic mice display progressive dendritic and somatic atrophy of PCs prior to cell death



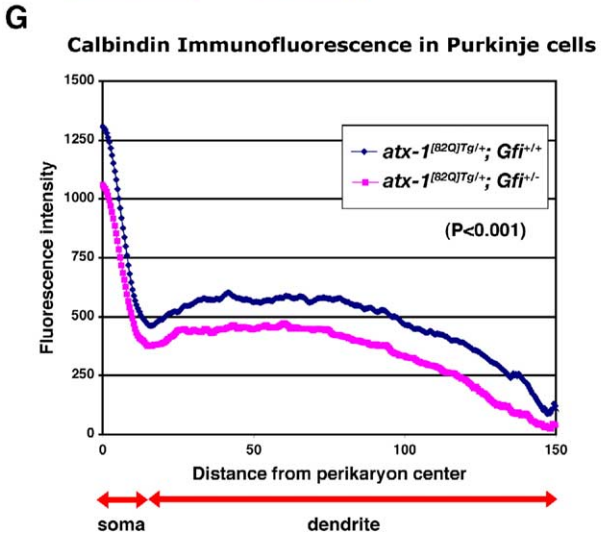


Figure 7. Gfi-1 Loss Contributes to Purkinje Cell Degeneration

Calbindin staining in PCs from wt (A–C) and *Gfi-1* null mice (D–F), 11 weeks of age (A and D) and 26 weeks of age (B, C, E, and F), is shown. wt (A) and *Gfi-1* null mice (D) have similar PC morphology at 11 weeks of age. By 26 weeks, *Gfi-1* null mice (E and F) show abnormal nuclear and cytoplasmic morphology (arrowheads), disorganized dendritic arborization, swollen axons (arrows), and loss of PCs. The molecular layer (ml), PC layer (pcl), and granule-cell layer (gcl) are indicated in (C) and (F). As shown in (G), calbindin staining is decreased in PCs of hAtx-1[82Q] lacking one allele of *Gfi-1* ($p < 0.001$). Quantitative analysis of calbindin immunofluorescence is an indirect measure of PC soma and dendritic integrity.

(Clark et al., 1997). To examine the role of Gfi-1 in SCA1 pathogenesis, we evaluated the effects of heterozygosity for a loss-of-function allele of *Gfi-1* in PCs in which Atx-1 is overexpressed. Interestingly, hAtx-1[82Q]-transgenic mice that lack one functional copy of *Gfi-1* show a worsening of their PC pathology at 8 weeks of age as quantified by measuring calbindin immunofluorescence in the soma and dendrites of PCs (Figure 7G). Furthermore, these mice perform more poorly on the accelerating-rotarod apparatus at 4 weeks of age as compared to mice having the hAtx-1[82Q] transgene in a wt *Gfi-1* background (data not shown). The dosage-dependent genetic interaction between *atx-1* and *Gfi-1*, together with the decreased level of Gfi-1 in the hAtx-1[82Q]-transgenic mice, strongly suggests that decreasing the level of Gfi-1 by mutant Atx-1 mediates or contributes to SCA1 pathogenesis.

Discussion

The expanded glutamine tract is clearly the initiating event in the pathogenesis of polyQ neurodegenerative disorders. In SCA1 and related disorders, the length of the glutamine tract correlates inversely with the age of onset and directly with the severity of the phenotype. Overexpression of a polyQ peptide in cell-culture, invertebrate, and mouse models is quite toxic and causes progressive dysfunction and cell death. However, there are several pieces of data that argue that the expanded glutamine tract is not the sole factor that induces pathology and determines disease severity. For example, several genetic studies support the idea that sequences outside of the glutamine tract are critical for causing neurodegeneration phenotypes in SCA1. First, overexpression of wt hAtx-1[30Q] in flies and mice causes pathology (Fernandez-Funez et al., 2000). Second, replacing serine 776 with an alanine in Atx-1[82Q] dampens SCA1 pathology (Emamian et al., 2003). Also, a CAG repeat length of 45 causes severe disease in SCA2, mild disease in SCA1, and no symptoms in SCA3 (Zoghbi and Orr, 2000). These genotype-phenotype correlation studies argue that protein sequences outside of the glutamine tract as well as protein-protein interactions are critical for pathology.

Our data indicate that hAtx-1 induces phenotypes similar to (but more severe than) those of dAtx-1 and distinct from those induced by overexpression of a polyQ peptide. The effects of hAtx-1[82Q] on Sens/Gfi-1 protein levels are abolished in the absence of the AXH domain in both flies and mammalian cells. Furthermore, overexpression of wt hAtx-1[30Q] in mice decreases Gfi-1 levels and induces degeneration. This is noteworthy given that the 30Q allele does not contain 30 consecutive Qs but is comprised of two stretches of 14 and 15 Qs interrupted by two histidine residues. These data collectively argue that the AXH domain but not the expanded polyQ tract is necessary to generate the Atx-1 gain-of-function phenotype in flies or mice. The findings that glutamine-expanded hAtx-1 produces more severe phenotypes than wt hAtx-1 and dAtx-1 suggest that the polyQ tract enhances the activity of the AXH domain in a quantitative and/or qualitative manner.

Because the AXH domain is the likely mediator of the effects of Atx-1 overexpression, the prediction would be that this domain might mediate the biochemical and genetic interactions that modulate or contribute to the SCA1 phenotypes. Indeed, our data on the genetic and biochemical interactions of fly and hAtx-1 proteins with *Drosophila* Sens and its mammalian homolog Gfi-1 strongly support this prediction. The finding that the AXH domain of dAtx-1/hAtx-1 is necessary and sufficient for physical interactions with Sens/Gfi-1 underscores the cross-species functional conservation of this domain and the potential role of Sens/Gfi-1 in mediating Atx-1-induced phenotypes. In support of Atx-1's specific effect on Sens, we find that (1) Atx-1 has direct effects on Sens protein (not RNA) levels through the AXH domain; (2) Atx-1 antagonizes the activity of Sens, not that of Ac or Da, in a transcription assay; and (3) Sens overexpression suppresses the Atx-1-induced bristle phenotypes in flies. Similarly, hAtx-1 and Gfi-1 also genetically interact in mice, as evidenced by the worsening of PC pathology in a background heterozygous for a *Gfi-1* loss-of function allele. Moreover, hAtx-1 reduces Gfi-1 protein levels in the absence of cell loss. Exactly how Atx-1 promotes the degradation of Sens/Gfi-1 is not known at this time, but our data suggest that Atx-1 enhances the proteasome-mediated degradation of Gfi-1. We propose that Atx-1 and Sens/Gfi-1 interactions alter the conformation, modification, or other protein interactions of Sens/Gfi-1, rendering them less stable.

Gfi-1 Is Necessary for the Survival of Purkinje Cells

Given the decline of Gfi-1 levels in PCs of *SCA1*-transgenic mice, a key question is whether this decrease contributes to SCA1 pathogenesis. Indeed, we find that loss of Gfi-1 phenocopies SCA1 and causes PC degeneration after several months of normal PC development. Furthermore, loss of one allele of *Gfi-1* enhances the motor-incoordination phenotype of *SCA1* mice at 4 weeks of age, well before the PCs of these mice develop obvious morphological changes (Burright et al., 1995; Clark et al., 1997). These data, together with the finding that Gfi-1 and Atx-1 physically interact, highlight the important role of Gfi-1 in PC degeneration and SCA1 pathogenesis. The finding that a transcriptional regulator like Gfi-1 is a major modifier of SCA1 phenotypes and an interactor of Atx-1 is interesting given the proposal that Atx-1 might function in the regulation of gene expression (Lin et al., 2000; Serra et al., 2004).

Pathogenic Mechanisms in SCA1: Relevance to Other Neurodegenerative Diseases

Several neurodegenerative diseases are caused by a gain-of-function mutational mechanism, which renders the host protein toxic to neurons. Examples include mutations in amyloid precursor protein (APP) causing Alzheimer's disease (AD) (Mattson, 2004), mutations in tau causing frontotemporal dementia (FTD) (Hutton et al., 1998), and mutations in α-synuclein causing Parkinson's disease (PD) (Vila and Przedborski, 2004). It is interesting that an extra dose of wt APP in Down's syndrome causes early onset AD-like changes (Mrak and Griffin, 2004) and that duplications as well as triplications of wt α-synuclein cause PD (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Singleton et al., 2003). Our finding that overexpression of wt Atx-1 causes neurodegeneration is reminiscent of these observations. In fact, accumulation of wt Atx-1 has been noted in patients with neuronal intranuclear inclusion disease in the absence of a CAG expansion of the *SCA1* gene (Lieberman et al., 1999). These data collectively suggest that higher than wt levels of proteins such as Atx-1 or α-synuclein are toxic to specific groups of neurons. We propose that expansion of the glutamine tract in Atx-1 (and possibly other polyQ proteins) will stabilize them and/or enhance their interaction with other proteins. Thereby, enhancement of wt functions results in the neurodegeneration.

To date, several Atx-1-interacting proteins have been identified, including leucine-rich acidic nuclear protein, PQBP1, and SMRT (Matilla et al., 1997; Okazawa et al., 2002; Tsai et al., 2004). Gfi-1 is the first protein to fulfill the criteria to be a mediator of the SCA1 pathogenesis: it interacts physically and genetically with Atx-1 (as do the fly orthologs), loss of Gfi-1 copies the SCA1 phenotype in PCs, and Gfi-1 and SCA1 show a dose-dependent interaction. The molecular mechanisms described in the current study underscore the importance of studying the wt function and interactors of proteins involved in neurodegenerative diseases.

Experimental Procedures

For details on GST pull-down assays, IP, Western blotting analysis, and yeast two-hybrid assays, see Supplemental Experimental Procedures.

Fly Strains

Fly strains used were *y w*, *Canton S* (Bloomington Stock Center), UAS-*human atx-1[82Q]* (F7, N35Y, M6), *[30Q]* (F1, F7) (Fernandez-Funez et al., 2000), *UAS-127Q* (Kazemi-Esfarjani and Benzer, 2000), *UAS-sens* (C12) (Nolo et al., 2000), *Rh1-GAL4* (Mollereau et al., 2000), *GMR-GAL4* (Freeman, 1996), *C5-GAL4* (Yeh et al., 1995), *C96-GAL4* (Nolo et al., 2001), *C109-68-GAL4* (Abdelilah-Seyfried et al., 2000), *dpp-GAL4/TM6B* (G. Mardon, Houston), *UAS-lacZ* (Nolo et al., 2001), *UAS-GAL4* (Hassan et al., 2000), and *UAS-mCD8-GFP* (Dubruille et al., 2002).

Immunohistochemistry

Primary antibodies were as follows: Guinea pig anti-Sens (1:800) (Nolo et al., 2000), mouse anti-β-gal (1:1000) (Promega), rabbit anti-Atx-1 (117650VII or 11NQ) (Matilla et al., 1998; Skinner et al., 1997), Guinea pig anti-Gfi-1 (1:1000) (Wallis et al., 2003), mouse anti-Wingless (1:10) (Developmental Studies Hybridoma Bank), anti-calbindin (1:1000) (Sigma), and anti-20S proteasome (1:500) (Affiniti Research). Secondary antibodies were as follows: Alexa 488-anti-Guinea pig (1:500) (Molecular Probes) and Cy3-anti-Guinea pig (1:500), Cy3-anti-rabbit (1:500), Cy3-anti-mouse (1:500), and Cy5-anti-rabbit (1:500) (Jackson ImmunoResearch Laboratories). Confocal images were captured using a Zeiss 510 microscope. For details on calbindin immunofluorescence quantification, see Supplemental Experimental Procedures.

Cell Culture and Protease-Inhibitor Treatment

S2 cell transfection and luciferase assay were performed as described previously (Jafar-Nejad et al., 2003). BE-2 human neuroblastoma cells were plated the day before transfection at 1×10^5 cells per well in 24-well plates. The following day, a HA-tagged Gfi-1 construct was transfected into BE-2 cells using Lipofectamine plus (Invitrogen). Sixty to sixty-two hours later, the proteasome was inhibited using *clasto*-lactocystin β-lactone (20 μM; Calbiochem) for 10–12 hr.

Pulse-Chase Experiments

Ataxin-1[82Q] lacking AHX domain was generated and cloned into pxFLAG-CMV10 vector (Sigma). Other constructs and transfection into the BE-2 cells are explained above. Metabolic labeling was carried out by first starving the cells in methionine-free medium. Thirty minutes later, this medium was removed, and cells were pulsed in the same medium containing 50 μCi/ml L-[^{35}S]methionine for 1 hr. Cells were washed twice and incubated in medium supplemented with 150 μg/ml unlabeled methionine. Cells were then chased in the presence or absence of 20 μM *clasto*-lactocystin β-lactone and collected at various times. Whole-cell extracts were obtained in TST buffer (50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.5% Triton X-100) containing protease inhibitors and subjected to immunoprecipitation with Gfi-1 antibody.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/4/633/DC1/.

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