Loss of *Drosophila* Ataxin-7, a SAGA subunit, reduces H2B ubiquitination and leads to neural and retinal degeneration

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The Spt–Ada–Gcn5–acetyltransferase (SAGA) chromatin-modifying complex possesses acetyltransferase and deubiquitinase activities. Within this modular complex, Ataxin-7 anchors the deubiquitinase activity to the larger complex. Here we identified and characterized *Drosophila* Ataxin-7 and found that reduction of Ataxin-7 protein results in loss of components from the SAGA complex. In contrast to yeast, where loss of Ataxin-7 inactivates the deubiquitinase and results in increased H2B ubiquitination, loss of Ataxin-7 results in decreased H2B ubiquitination and H3K9 acetylation without affecting other histone marks. Interestingly, the effect on ubiquitination was conserved in human cells, suggesting a novel mechanism regulating histone deubiquitination in higher organisms. Consistent with this mechanism in vivo, we found that a recombinant deubiquitinase module is active in the absence of Ataxin-7 in vitro. When we examined the consequences of reduced Ataxin-7 in vivo, we found that flies exhibited pronounced neural and retinal degeneration, impaired movement, and early lethality.

*Keywords: SAGA complex; H2B ubiquitination; spinocerebellar ataxia*

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The Spt–Ada–Gcn5–acetyltransferase (SAGA) chromatin-modifying complex is a highly conserved 2-MDa protein complex comprised of ~20 subunits [Helmmlinger et al. 2004; Lee and Workman 2007; Weake and Workman 2012]. The complex is arranged in a modular fashion and contains two enzymatic activities: an acetyltransferase activity associated with the GCN5/Pcaf subunit and a deubiquitinase activity associated with the yUbp8/dNon-stop/hUsp22 subunit [Lee et al. 2011]. In yeast, the ataxin-7 homolog is Sgf73, and studies have shown that it anchors the deubiquitinase module [which includes Sgf73, Sgf11, Sus1, and Ubp8] to the SAGA complex [Lee et al. 2009, 2011; Weake and Workman 2012]. Crystal structures and biochemical analysis of the yeast deubiquitinase module have shown that the N terminus of Sgf73 extends deep into the deubiquitinase module, intertwining between the components of the module to ensure an active conformation for the deubiquitinase. Without Sgf73, the deubiquitinase is inactive [Kohler et al. 2010; Samara et al. 2010]. The carefully orchestrated addition and removal of ubiquitin on H2B are important regulators of transcription. H2B monoubiquitination is a prerequisite for di- and trimethylation of H3K4 and H3K79, modifications associated with transcriptionally active chromatin [Lee et al. 2007].

Here, we identify the gene product of CG9866 as the *Drosophila* homolog of ataxin-7 (Ataxin-7). Biochemical analysis, including affinity purification, multidimensional protein identification technology (MudPIT) proteomic analysis, and gel filtration chromatography, establish that Ataxin-7 is a stable component of the SAGA chromatin remodeling complex. Analysis of SAGA from Ataxin-7-deficient flies revealed the loss of components from the SAGA complex, consistent with a role for Ataxin-7 in anchoring the deubiquitinase module to the complex. In
contrast to the increased H2Bub observed upon loss of ataxin-7 in yeast, we observed a decrease in H2B ubiquitination in Drosophila with no associated changes in histone methylation and a reduction in the levels of H3K9 acetylation but not K3K14 acetylation. This surprising change in H2B ubiquitination was confirmed in human cells in which knockdown of human Ataxin-7 also resulted in decreased H2B ubiquitination. We hypothesize that this decrease reflects the release of an active deubiquitinase module from SAGA and, consequently, loss of SAGA-associated regulation of the deubiquitinase activity. Consistent with this model, we found that the deubiquitinase is enzymatically active when the complex is reconstituted in vitro even in the absence of Ataxin-7. An examination of flies with reduced expression of Ataxin-7 showed that loss of Ataxin-7 results in neural and retinal degeneration, impaired movement, and decreased life span.

Results

CG9866 is the Drosophila homolog of Ataxin-7
During characterization of the Drosophila SAGA complex through iterative complex purification coupled to MudPIT total protein identification, we consistently observed copurification of novel peptides that were the product of the uncharacterized gene CG9866 (Fig. 1A; Washburn et al. 2001; Weake et al. 2009). To determine whether the CG9866 protein has homology with known SAGA components, we performed in silico analysis of the primary amino acid sequence using the position-specific iterated basic local alignment search tool [PSI-BLAST]. This analysis revealed similarity to human Ataxin-7, a component of the human SAGA complex [Helmlinger et al. 2004]. More detailed examination by direct ClustalW alignment and Box Shade amino acid comparison analysis showed widespread conservation of amino acid sequence and similarity [Supplemental Fig. 1]. Further alignment using the amino acid sequence “blocks” suggested by Helmlinger et al. [2004] showed that CG9866 shared similarity to their proposed “Ataxin-7 signature” in all three blocks. However, the order of blocks 2 and 3 are reversed in Drosophila [Fig. 1B]. Due to sequence conservation along with biochemical and phenotypic data described below, CG9866 is hereafter referred to as Ataxin-7.

Ataxin-7 is a component of SAGA
To determine whether Ataxin-7 is indeed a bona fide member of Drosophila SAGA, we analyzed the subunit composition of Ataxin-7-containing complexes. S2 cells were stably transfectected with pRMHα3-Ataxin-7-Flag(x2)-HA(x2) [Ataxin-7-HF], and low-expressing clones were selected to generate a stably transfectected cell line with the minimal amount of overexpression. We prepared nuclear extracts from these cells and purified Ataxin-7-HF-containing complexes using sequential Flag and HA immunoprecipitations. The purified complexes were fractionated by SDS-PAGE, and silver staining revealed that the complex profile was similar to that seen during purification of other SAGA components (Fig. 2A; Guelman et al. 2006). Western blotting confirmed the presence of the bait protein as well as major SAGA complex components, including acetyltransferase Gcn5 and SAGA-specific protein Ada2B (Fig. 2B). Analysis of purified Ataxin-7-associated proteins by MudPIT revealed subunits corresponding to the entire SAGA complex, which were not present in mock purifications (Fig. 2C). This composition was comparable with the composition obtained upon MudPIT analysis of the SAGA complex purified by immunoprecipitation of other SAGA subunits [Fig. 1A; Weake et al. 2009].

To show that Ataxin-7 is a stable component of SAGA, we performed gel filtration chromatography. The purified complex was applied to a Superose 6 gel filtration column and fractionated by size. Fractions were collected and immunoblotted to track elution of Ataxin-7 and SAGA-specific Ada2B. The majority of Ataxin-7 co-eluted with Ada2B [Fig. 2D]. Together, these data indicate that Ataxin-7 is a stable component of the SAGA complex and suggest that Ataxin-7 is predominantly associated with SAGA.

A P-element insertion reduces the level of Ataxin-7 protein
The Ataxin-7 gene produces four transcripts [A, B, C, and D] that differ in their 5′ untranslated regions [UTRs] but encode identical protein products. Large-scale transcriptome analyses available on Flybase.org indicate that transcripts are provided maternally to the embryo and are later detected at moderate levels in most stages of development and in a variety of tissues [Berkeley Drosophila Genome Project, http://insitu.fruitfly.org]. To investigate the biological importance of Ataxin-7 function in Drosophila, we used a fly stock in which a P-element had been inserted within the 5′ UTR of the gene [Fig. 3A]. The majority of these homozygous mutant flies survive to wandering third instar larvae but die prepupation. Analysis of transcripts by RT–PCR showed that the P-element was transcribed along with the Ataxin-7 gene and then spliced into the A and D transcripts, leaving a portion of the P-element but removing a portion of the 5′ UTR [Supplemental Figs. 2, 3]. Analysis of transcripts B and C also showed defective splicing [Supplemental Fig. 3]. In order to determine whether these changes in transcript architecture affected protein levels, we raised an antibody against full-length Ataxin-7. This antibody recognizes the full-length antigen expressed in bacteria [data not shown] as well as the full-length Ataxin-7 protein and a truncated form of Ataxin-7 expressed in baculovirus [Supplemental Fig. 4]. Immunoblotting of whole-cell extracts prepared from wandering third instar larvae homozygous for the P-element insertion showed that Ataxin-7 protein levels were drastically reduced [Fig. 3B]. We further confirmed the absence of Ataxin-7 by analyzing polytene chromosome preparations. In homozygous mutant flies, no staining was observed with the Ataxin-7 antibody, while strong signals that colocalized with RNA polymerase II [Pol II] were seen on wild-type chromosomes [Fig. 3C].
In addition to the P-element insertion (mutant) fly stock, RNAi hairpin constructs designed to knock down Ataxin-7 by RNAi were also tested. The RNAi 1 stock was obtained from the Drosophila genome project (Keleman et al. 2009). A second RNAi fly line was generated using the Valium20 vector (RNAi 2) (Ni et al. 2008). The RNA hairpin design for RNAi 2 was predicted to target all four Ataxin-7 transcripts with no off-targets.

Figure 1. The putative Drosophila homolog of Ataxin-7 is CG9866. [A] Analysis of Drosophila SAGA complexes by MudPIT mass spectrometry consistently identified the protein product of the uncharacterized gene CG9866 (Ataxin-7 hereafter). A comparison of complex normalized spectral abundance factor (cNSAF) values for the SAGA subunits found in purifications performed with the indicated bait proteins is shown (Weake et al. 2009). [B] Human and Drosophila Ataxin-7 share a high degree of primary sequence homology in the Ataxin-7 signature blocks defined by Helmlinger et al. (2004). Amino acid sequences from human and Drosophila Ataxin-7 were aligned using ClustalW and shaded using Box Shade. Identical residues are shown with a black background and white text; similar residues are shown with a light-gray background and black text. The complete alignment can be found in Supplemental Figure S1.

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Figure 2. Ataxin-7 is a member of the SAGA chromatin-modifying complex. (A) Ataxin-7-containing complexes were affinity-purified from S2 cells stably transfected with an expression vector for Ataxin-7-HF and selected to express low levels of recombinant protein. Silver staining shows the protein profile of the purified complex, including the expected position of bait proteins. Complexes purified through another SAGA subunit (HA-Flag-WDA) are included for comparison. (B) Immunoblotting of purified Ataxin-7 complexes confirmed the presence of SAGA complex members in the purification. (C) MudPIT proteomic analysis verified that the entire SAGA complex was present in the purification. The predicted molecular mass of each protein is indicated. (D) Purified Ataxin-7-containing complexes were fractionated by size on a Superose 6 gel filtration column. Eluted fractions were analyzed by immunoblotting with the indicated antibodies to detect Ataxin-7 (αHA) and SAGA-specific complex member Ada2B (αAda2B). The majority of Ataxin-7 stably interacted with SAGA, coeluting with Ada2B.
Ataxin-7 deficiency alters SAGA complex composition and H2B ubiquitination

Human Ataxin-7 and yeast Sgf73 are known members of the highly conserved SAGA complex, a modular complex comprised of ~20 members and ~2 MDa in size. Yeast Sgf73, which shares 16% similarity with Ataxin-7 (Helmlinger et al. 2004), serves to connect the acetyltransferase and deubiquitinase modules of the complex (Lee et al. 2009, 2011). To determine the effect of Ataxin-7 reduction on SAGA complex composition, we fractionated nuclear extracts prepared from Ataxin-7 mutant wandering third instar larvae as above. The elution profile of SAGA was tracked by immunoblotting for Ada2B and revealed a reduced size, suggesting that SAGA complex members were lost in the absence of Ataxin-7 (Fig. 4A).

To show that loss of Ataxin-7 released the deubiquitinase module tethered to SAGA, Sgf73 plays a critical role in ensuring the proper conformation of Non-stop for full enzymatic function of the deubiquitinase (Kohler et al. 2010; Samara et al. 2010). Consequently, disruption of Sgf73 reduces SAGA-mediated H2Bub deubiquitination, resulting in global increases in this critical histone modification (Kohler et al. 2008; Lee et al. 2009). To determine the effect of Ataxin-7 loss on H2Bub levels in Drosophila, we collected mutant wandering third instar larvae and acid-extracted histones for immunoblotting with histone modification-specific antibodies. H4 immunoblots served as loading controls. Surprisingly, in contrast to the effect of Sgf73 loss in yeast, we found a global decrease in the levels of H2Bub upon reduction of Ataxin-7 (Fig. 5B). We also found a decrease in H3K9 acetylation but not H3K14 acetylation. Since H2Bub facilitates H3K4 and H3K79 methylation, one might anticipate that methylation at these sites might decrease (Lee et al. 2012). To address this possibility, we examined Ataxin-7 mutants for such changes but found no significant decrease in either H3K4 di- or trimethylation or H3K79 trimethylation (Fig. 5B). To confirm that the increase in fluorescence as the AMC fluorophore is released from ubiquitin. We found that these endogenous Non-stop-containing complexes were indeed enzymatically active [Fig. 4C]. To determine whether these small active DUB modules were associated with chromatin in the absence of Ataxin-7, we analyzed polytene chromosomes prepared from wild-type or Ataxin-7 mutant larvae and found that Non-stop was chromatin-bound in both cases [Fig. 5A].

In addition to serving as an anchor to keep the deubiquitinase module tethered to SAGA, Sgf73 plays a critical role in ensuring the proper conformation of Non-stop for full enzymatic function of the deubiquitinase (Kohler et al. 2010; Samara et al. 2010). Consequently, disruption of Sgf73 reduces SAGA-mediated H2Bub deubiquitination, resulting in global increases in this critical histone modification (Kohler et al. 2008; Lee et al. 2009). To determine the effect of Ataxin-7 loss on H2Bub levels in Drosophila, we collected mutant wandering third instar larvae and acid-extracted histones for immunoblotting with histone modification-specific antibodies. H4 immunoblots served as loading controls. Surprisingly, in contrast to the effect of Sgf73 loss in yeast, we found a global decrease in the levels of H2Bub upon reduction of Ataxin-7 [Fig. 5B]. We also found a decrease in H3K9 acetylation but not H3K14 acetylation. Since H2Bub facilitates H3K4 and H3K79 methylation, one might anticipate that methylation at these sites might decrease [Lee et al. 2012]. To address this possibility, we examined Ataxin-7 mutants for such changes but found no significant decrease in either H3K4 di- or trimethylation or H3K79 trimethylation [Fig. 5B]. To confirm that the increase in
ubiquitinated H2B was due to the P-element-mediated disruption of Ataxin-7 expression, we examined whether genetic mobilization of the P-element rescued wild-type Ataxin-7 function (Supplemental Fig. S6). Indeed, upon precise excision of the P-element insert (Supplemental Fig. S6), acid-extracted histones from wandering third instar hop-out larvae exhibited wild-type levels of H2Bub (Fig. 5B).

Finally, to address whether the detrimental effects of Ataxin-7 loss were dependent on the Non-stop deubiquitinase, we examined whether a reduction in Non-stop suppressed lethality in Ataxin-7 mutant flies. Normally, homozygous mutant Ataxin-7 flies do not survive past the third instar larval stage of development [Fig. 5C]. However, loss of one copy of Non-stop resulted in partial suppression of the Ataxin-7 mutant lethality, resulting in survival of adult flies despite loss of both wild-type copies of Ataxin-7. These data suggest that the lethality associated with Ataxin-7 mutants is at least in part due to unregulated deubiquitinase activity. As expected, Non-stop homozygous mutants displayed a Non-stop phenotype and did not live past larval stages [Fig. 5C].

These surprising observations regarding H2B ubiquitination differ from those predicted from yeast experiments and suggest that regulation of the SAGA ubiquitin protease module differs in higher eukaryotes. To explore this hypothesis, we examined the effect of human Ataxin-7 knockdown in a tissue culture model. Ataxin-7 protein levels were reduced by transient siRNA-mediated knockdown in HeLa cells, and whole-cell extracts were prepared from these cells. Upon fractionation of these extracts by SDS-PAGE and immunoblotting with an antibody to H2Bub, we found a decrease in H2B ubiquitination upon decrease in Ataxin-7 protein levels (Fig. 6A). In humans, knockdown of hSgf11 specifically inactivates the SAGA deubiquitinase [Lang et al. 2011]. To verify that the observed reduction in H2Bub was indeed dependent on release of an active SAGA deubiquitinase module, we knocked down hSgf11 and found that the effect of Ataxin-7 knockdown was eliminated (Fig. 6B). Thus, in contrast to the increase in H2Bub observed upon loss of Sgf73, a decrease in either Drosophila or human Ataxin-7 results in decreased H2Bub.

The SAGA deubiquitinase module is active in the absence of Ataxin-7

To investigate the deubiquitinase function of SAGA further, we first verified that SAGA purified by immunoprecipitation...
of tagged Ataxin-7 was enzymatically active using the ubiquitin-AMC assay. From these studies, we conclude that SAGA purified via Ataxin-7 is indeed enzymatically active and can deubiquitinate H2B in a dose-dependent manner over time (Fig. 7A). Furthermore, we fractionated Ataxin-7-containing complexes and tested the fractions for deubiquitinase activity (Fig. 7B). We found that the peak deubiquitinase activity corresponded to the fractions containing SAGA, again indicating that the majority of Ataxin-7 is associated with the intact, enzymatically active SAGA complex (Fig. 7C).

Since Sgf73 is known to tether the SAGA deubiquitination module to the acetyltransferase core, we hypothesized that loss of Ataxin-7 may result in separation of the acetyltransferase and deubiquitinase modules of SAGA. However, in contrast to the loss of deubiquitinase activity caused by this separation in yeast, we hypothesized that it may free an enzymatically active deubiquitinase module from SAGA-mediated regulation. To test this idea in vitro, we assayed whether a deubiquitinase module assembled from recombinant proteins had deubiquitinase activity in the absence of Ataxin-7. Recombinant proteins were expressed using the baculovirus system, and lysates containing the overexpressed deubiquitinase module subunit were combined to allow the module to assemble in vitro. After extensive washing with high salt, the purified deubiquitinase module was assayed for activity using the ubiquitin-AMC assay. As shown in Figure 7D, the isolated deubiquitinase module is indeed enzymatically active on its own and in the absence of Ataxin-7 [Fig. 7D, left panel]. Immunoblotting analysis of an aliquot from the deubiquitinase reactions verified the presence of the components of the deubiquitinase reaction as expected [Fig. 7D, right panel]. The slightly lowered level of deubiquitinase activity of the module in the absence of Ataxin-7 might be due to slightly lower levels of the Non-stop deubiquitinase in these particular extracts. Ataxin-7 may serve to hold the module together tightly but be dispensable for ensuring optimal enzyme conformation in Drosophila.

To explore this possibility, we tested a deubiquitinase module reconstituted from proteins purified to isolation prior to assembly (Supplemental Fig. 6, top panels). Combining the purified Non-stop, E(y)2, and Sgf11 proteins also resulted in an enzymatically active deubiquitinase module, independent of Ataxin-7 (Supplemental Fig. 6, bottom panel). Using this method, each reaction contained identical levels of enzyme and showed similar levels of activity.

Loss of Ataxin-7 results in neurodegeneration

In humans, expansion of the N-terminal Ataxin-7 polyglutamine region causes spinocerebellar ataxia 7 (SCA7) disease, resulting in neural degeneration as well as deterioration of the retina and macula [David et al. 1998; Del-Favero et al. 1998]. Similar effects can be seen upon overexpression of polyglutamine-expanded human Ataxin-7 in Drosophila [Latouche et al. 2007]. Polyglutamine-expanded human
Ataxin-7 is resistant to proteasomal degradation and accumulates in cells, forming intranuclear inclusions. At the same time, there is only one copy of wild-type Ataxin-7 remaining, which would be subject to normal proteolytic degradation (Yvert et al. 2001). This imbalance might make it difficult to distinguish the effects of polyglutamine-expanded Ataxin-7 from reductions in the levels of wild-type Ataxin-7.

Considering the effects of perturbing normal Ataxin-7 function in humans, we were interested in determining whether loss of Ataxin-7 expression in flies results in defects in the fly nervous system. As noted earlier, the majority of Ataxin-7 mutant flies die before pupation. By separating homozygotes from their heterozygote counterparts, however, we were able to get a very small number of "escapers" from which we were able to generate a sickly but stable stock. We isolated heads from these mutant flies, subjected them to histological sectioning, and visualized tissues by hematoxylin and eosin (H&E) staining. Lesions were widely observed throughout neural and retinal structures (Fig. 8A). We confirmed that these defects were due to P-element-mediated disruption of Ataxin-7 expression by analyzing the P-element hop-out "rescue," which restored the wild-type gene structure of the Ataxin-7 gene (Supplemental Fig. S6). In humans, neural degradation results in ataxia. To address whether Ataxin-7 mutant flies had difficulty moving, we used the negative geotaxis assay, which monitors the ability of flies to scale the side of the vial, and found that mutant flies were unable to climb like their wild-type counterparts [Fig. 8D]. Notably, mutant flies also had a greatly reduced life span [Fig. 8E].

We further confirmed that these defects were due to reduction in Ataxin-7 levels by knocking down Ataxin-7 expression using two independent RNAi lines specific for Ataxin-7 [RNAi 1 and RNAi 2, discussed above]. Ubiquitous knockdown using the Actin-GAL4 driver resulted in lesions throughout neural and retinal structures that were very similar to those seen in mutant flies [Fig. 8A]. We also knocked down Ataxin-7 expression in the retina and lamina using the tissue-specific driver GMR-GAL4. While the eyes of these adults were normal upon eclosion from the pupal case, they had a pronounced rough-eye phenotype by 14 d. These results suggest that the cells were degrading over time, a phenotype commonly seen upon overexpression of polyglutamine-expanded proteins [Fig. 8B]. As expected, histological analysis of these heads revealed neural and retinal lesions throughout the regions targeted by the GMR-GAL4 driver but not in adjacent regions in which the Ataxin-7 RNAi was not expressed [Fig. 8C]. While targeting specific tissues for knockdown of Ataxin-7 using GMR-GAL4 resulted in degeneration of those tissues, we noted that in the P-element fly, the pattern of degeneration was not identical. In particular, it appeared that specifically targeting the retina and lamina resulted in a much smaller retina than that observed in the mutant or upon ubiquitous expression of RNAi. We suspected that this difference was simply a consequence of disrupting the complex system of communication used by the Drosophila brain to establish and maintain neural organization and connectivity. Close re-examination of the effects of Ataxin-7 mutant and ubiquitous Ataxin-7 knockdown histological sections showed that this was indeed the case [Fig. 8A].

**Discussion**

Here we presented the first study of Drosophila Ataxin-7. Ataxin-7 shares primary amino acid sequence conservation with human Ataxin-7 and, accordingly, is also a member of the SAGA chromatin-modifying complex. We found that SAGA subunits are lost in the absence of Ataxin-7, resulting in a global decrease in the levels of H2B ubiquitination and H3K9 acetylation without affecting H3K14ac, H3K4me2/3, or H3K79me3. Because Ataxin-7 associates with the intact S AGA complex and loss of Ataxin-7 results in fragmentation of the complex, we explored whether decreased levels of H2B ubiquitination were due to release of an active deubiquitinase module from SAGA. Indeed, the deubiquitinase module assembled in vitro from purified Non-stop, E[y]2, and Sgf11 is enzymatically active and is unaffected by the...
Figure 7. The SAGA deubiquitinase module is enzymatically active in the absence of Ataxin-7. (A) SAGA complex purified through Ataxin-7 was assayed for deubiquitinase activity using the reporter substrate ubiquitin-AMC. Increasing amounts of Ataxin-7-containing complex were incubated with an excess of ubiquitin-AMC, and release of AMC from ubiquitin was measured by indirect fluorescence. Time- and dose-dependent deubiquitination was observed. (B) Schematic of the experimental flow for analysis of deubiquitinase-containing complexes: SAGA complex purified through Ataxin-7 was separated by size on a gel filtration column, and fractions were assayed for enzymatic (deubiquitinase) activity. (C) Purified complex was fractionated by size, and fractions were assayed for enzymatic activity. Peak activities detected by ubiquitin-AMC activity assay are outlined by dotted lines, indicating that Ataxin-7 associates with intact and enzymatically active SAGA complex. (D, left panel, inset) A recombinant deubiquitinase module was isolated by coinfecting SF9 cells with baculovirus expression vectors for Flag-HIS-Sgf11, HA-E(y)2, HA-Non-stop, and HA-CG9866 (1–200), as indicated. (Right panel) The recombinant deubiquitinase module was then purified from whole-cell lysates through the Flag epitope present on Flag-HIS-Sgf11, and the components of the reaction were verified by immunoblotting to ensure complex integrity. (Left panel) These complexes were assayed for enzymatic activity using the ubiquitin-AMC assay.
presence or absence of Ataxin-7. In vivo, disruption of Ataxin-7 expression leads to severe neural and retinal degeneration, limited life span, and defective locomotion. These defects are at least in part due to elevated deubiquitinase activity, since loss of one copy of the Non-stop deubiquitinase suppresses the lethality of Ataxin-7 mutants.

These results suggest a more elaborate mode of regulation of histone ubiquitination in higher eukaryotes than that found in yeast. Studies in *Saccharomyces cerevisiae* showed that the deubiquitinase module comprised of Sgf73, Ubp8, Sgf11, and Sus1 is arranged so that each member of the module is in contact with the other three, and these contacts establish an enzymatically active module. Truncation of the Sgf73 N terminus led to an enzymatically inactive module (Kohler et al. 2010; Samara et al. 2010). In contrast, in *Drosophila* and in human cells, the presence of Ataxin-7 is not necessary for deubiquitinase activity, and, instead, loss of Ataxin-7 results in increased deubiquitination and reduced levels of H2B ubiquitination [Fig. 9].

Interestingly, we found that this decrease in H2B ubiquitination does not coincide with a decrease in H3K4me2/3 or H3K79me3. Previously, it was shown that H2B ubiquitination was necessary for recruitment of methyltransferases to place these marks, and reduction of the *Drosophila* Bre1 E3 ubiquitin ligase results in loss of both H2B ubiquitination and H3 methylation (Sun and Allis 2002; Lee et al. 2007; Nakanishi et al. 2009; Mohan et al. 2010). If indeed H2B ubiquitination is required for H3 methylation, then the mark would have been placed and then removed post-methylation by the mistargeted deubiquitinase module. This suggests that methylation may not be affected by the loss of Ataxin-7 because the deubiquitinase acts after ubiquitination-dependent methylation has occurred. It is also possible that a high level of H2B ubiquitination is not required to target methyltransferases. Recently, it was shown in yeast that loss of Chd1 results in reductions in H2B ubiquitination without a corresponding loss of H3K4 or H3K79 trimethylation, suggesting that very low levels of H2B ubiquitination are enough to target methyltransferases (Lee et al. 2012). Alternatively, it is possible that another mechanism exists for targeting methyltransferases when levels of ubiquitinated H2B are low or absent. During muscle differentiation, the RNF20 ubiquitin ligase is depleted in differentiated

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**Figure 8.** Loss of Ataxin-7 results in neural and retinal degeneration, reduced locomotion, and shortened life span. (A) The majority of (P)Ataxin-7 mutants do not survive to adulthood, but a small number of escapers survive under careful culture conditions. We analyzed the heads of these mutants by histological sectioning followed by hematoxylin and eosin (H&E) staining and analysis by bright-field microscopy. Widespread lesions were apparent throughout the mutant head. Similarly, knockdown of Ataxin-7 by actin-Gal4-directed ubiquitous expression of RNAi resulted in lesions throughout the head, similar to those seen in mutants. The mutant was rescued by excision of the P-element, restoring neural and retinal integrity. (B) Targeted knockdown of Ataxin-7 within the retina (R) and lamina (L) by GMR-Gal4-driven Ataxin-7-specific RNAi resulted in a progressive age-dependent rough-eye phenotype. Eyes were visualized by electron microscopy. (C) Two weeks post-eclosion, fly heads from GMR-GAL4-driven RNAi knockdown were analyzed as in A. Morphological analysis showed severe deterioration of the targeted tissues but normal physiology in untargeted areas. (D) The surviving homozygous (P)Ataxin-7 flies displayed locomotor defects in a negative geotaxis assay. In contrast to the rapid geotaxis behavior of wild-type flies, these mutant flies were unable to scale the side of a vial within 20 sec, and mobilization of the P-element restored climbing ability. (E) Survival curves show that mutant flies have a shortened life span compared with wild-type counterparts, and normal life span was restored by mobilization of the P-element.
myotubes, resulting in the absence of H2B ubiquitination, yet trimethylation of H3K4 and H3K79 is detected on chromatin lacking H2B ubiquitination (Vethantham et al. 2012). Recently, it was shown that transcription factors such as p53 along with p300 can recruit the SET1 histone methyltransferase complex independent of H2B ubiquitination (Tang et al. 2013).

Mutation of different SAGA subunits does not necessarily result in changes in expression of the same sets of genes (Weake et al. 2008). Disrupting expression of subunits in the acetyltransferase module versus the deubiquitinase module affects a curiously divergent set of genes, indicating that different genes have varying requirements for each catalytic activity of the complex (Weake et al. 2008, 2011). A dissociated active deubiquitinase module may play a role in this sophisticated regulation, since a free ubiquitin protease module could act on ubiquitinated chromatin independent of SAGA recruitment and regulation (Fig. 7). In yeast lacking Sgf73, Sus1 is released from SAGA but is still recruited to genes, albeit at a reduced level [Pascual-Garcia et al. 2008]. Moreover, early reports identified USP22 as an H2Aub deubiquitinase acting on polycomb-regulated genes [Zhang et al. 2008]. In principal, SAGA may release subcomplexes to participate in diverse functions. It was recently shown in S. cerevisiae that the proteasome is capable of pulling the enzymatically active DUB module, including Sgf73, Sgf11, and Sus1, from SAGA, indicating that separation of SAGA may normally occur [Lim et al. 2013]. Further investigation into the modularity of SAGA and its implications for transcriptional regulation in vivo may aid in understanding how this complex might provide a sophisticated mechanism for chromatin modification and gene regulation. In addition to direct chromatin modification, Non-stop and its homologs have also been shown to act on nonhistone proteins, and it is possible that nonhistone targets play a significant role in SCA7 pathology [Atanassov et al. 2009, Atanassov and Dent 2011].

SCA7 and other polyglutamine expansion diseases have divergent pathologies. These differences suggest that the function of the expanded protein is critical to the etiology of each disease [Orr 2012a,b]. Unfortunately, there is currently a limited understanding of the molecular functions of the wild-type proteins and what role loss or gain of function might play in the characteristic patterns of neural degeneration found upon polyglutamine expansion. In initial studies examining the wild-type function of other SCA proteins, loss of the wild-type protein did not entirely phenocopy the polyglutamine expansion disease. For example, Ataxin-1 knockout mice do not show cerebellar or brainstem degeneration but do exhibit cognitive defects and ataxia [Matilla et al. 1998]. Our observations suggest that loss of Ataxin-7 function may play a role in SCA7 disease progression. Polyglutamine-expanded Ataxin-7 is resistant to proteolysis, accumulating in cells. At the same time, the wild-type protein is only produced from one genomic copy, and the resulting protein is subjected to normal proteolysis. Furthermore, the Ataxin-7 N terminus extends into the deubiquitinase module, and this region is subject to polyglutamine tract expansion. Polyglutamine-expanded Ataxin-7 is present in the larger SAGA complex, but the deubiquitinase module was not specifically analyzed in this model [McMahon et al. 2005; Palhan et al. 2005]. It will be interesting to examine whether the deubiquitinase module is also recruited to the complex under these conditions and, if so, whether it is enzymatically active. If the deubiquitinase module does not form around the expanded Ataxin-7, tracking the localization of this module may be insightful. It will also be interesting to examine...
the potential regulation of the deubiquitinase module independent of SAGA under wild-type conditions. Understanding what might trigger release of the module from the larger complex will be critical to understanding the role of SAGA in neural and retinal stability.

Materials and methods

Sequence analysis

The human and Drosophila Ataxin7 protein sequences were aligned using the ClustalW algorithm. ClustalW was performed using the accurate alignment method with the Gonnet series weight matrix and a gap open penalty of 10 and gap extension penalty of 0.1. The aligned sequences were then shaded using the Box Shade algorithm with a similarity threshold of 0.8. Completely conserved residues are shown with a black background with white text; similar residues, as defined by the Box Shade default similarities, are shown with a light-gray background with black text.

Antibodies

Full-length histidine-tagged Ataxin-7 was expressed in BL21 cells and purified to homogeneity using nickel resin. Purified protein was sent to Pocono Rabbit Farm and Laboratory for injection into rabbits. Serum was collected, and antibody specificity was tested against the antigen as well as whole-cell extracts prepared from SF9 cells infected using the baculovirus system to express full-length Ataxin-7 and an N-terminal fragment comprising of residues 1–200 (Supplemental Fig. 4). Ataxin-7 antibody toward human Ataxin-7 was purchased from Abcam (ab90907). Ubiquitinated H2B antibody was purchased from Millipore (05–1312). H3K4me2 antibody was purchased from Active Motif (39141). H3K4me3 antibody was purchased from Upstate Biotechnology (07–473). H3K79me3 antibody was purchased from Epigentek (A-4054–025). H3 antibody was purchased from Upstate Biotechnology (05–499). H3K9ac antibody was purchased from Abcam (ab4441). H3K14ac antibody was purchased from Abcam (ab1191). Other antibodies have been described previously [Weake et al. 2008, 2011; Suganuma et al. 2010].

Knockdown

siRNAs were purchased from Qiagen [Atxn7 siRNA1 [SI00308273], Atxn7 siRNA2 [SI04357297], control [SI03650325], and hSgf11 [SI04322276]]. Six-well plates were seeded with $8 \times 10^5$ HeLa cells per well and transfected using Dharmafect 1 according to the manufacturer’s instructions. Cells were harvested 2 d post-transfection by scraping, and the cell pellet was lysed directly in $60 \mu$L of 2× Laemmli buffer.

Chromatography

Chromatographic separation of nuclear extracts and purified complexes has been described before [Kusch et al. 2003; Suganuma et al. 2010].

Ubiquitin-AMC assays

Ubiquitin-AMC assays were performed as described before [Kohler et al. 2010, Samara et al. 2010].

Fly stocks

The CG9866 P-element fly line [y1] w[67c23], P[w+mDint2]/w[BR.REF]=SUPor-P[CG9866]/KG02020] obtained from the Bloomington Drosophila Stock Center (14255). The deficiency fly line [w[1118], Df[2L]Exel7011, P–PBac[XPS.WH5]Exel7011/CyO] was obtained from the Bloomington Drosophila Stock Center (7011). The RNAi fly line [P[KK110634/VIE-260B]RNAi 1 described in the text] was obtained from the Vienna Drosophila RNAi Center stock [v102078]. The GMR-Gal4 driver[w[+]; P[w+mC]=GAL4-ninaE.GMR12] was obtained from the Bloomington Drosophila Stock Center (1104). The Non-stop P-element fly line [P[ry[+7.2]=PZ; not[02069] ry[506]/TM6B, ry[CB] Tb[+]] was obtained from the Bloomington Drosophila Stock Center (25708). Wild type refers to OregonR flies.

The lethality of the P-element insertion was confirmed by the lack of complementation by the deficiency fly line noted above. The CG9866 P-element was excised by standard protocols using the Δ2–3 transposase and confirmed to be a precise excision by DNA sequencing.

The RNAi fly stock designed to target Ataxin-7 (RNAi 2) was made using the Vivaldi20 vector according to Transgenic RNAi Project [TRIP] protocols, with oligo 5’-tagctcgatGTATATGTT TATACACTACCTgattatcaagcataGGTATTGTATAAACATATAAACGcgg-3’ [Ni et al. 2011]. This RNA hairpin was predicted to target all four Ataxin-7 transcripts with no off-targets.

Histology

Fly heads were collected from flies anaesthetized by CO2 and fixed immediately with 4% paraformaldehyde in PBS overnight at 4°C. Heads were embedded in paraffin wax, and 5-μm sections were made by microtome sectioning. Sections were then mounted on slides and stained by H&E staining. Sections were imaged on a Leica Axioplan 206 microscope using a 5× 0.15 NA Plan-Neofluar objective. Scanning electron microscope [SEM] was performed on fly heads that were fixed in glutaraldehyde/paraformaldehyde, washed, and then fixed again with 1% osmium tetroxide. Samples were then dehydrated in gradually increasing concentrations of ethanol with a final dehydrant of hexamethyldisilazane and air-dried before being imaged on a Hitachi TM-1000 tabletop scanning electron microscope.

Life span determination

Flies were collected post-eclosion and distributed into 10 vials of 10 flies each. They were passaged to new food daily, and the number of remaining flies was counted. A minimum of 100 flies were assayed.

Negative geotaxis assay

Flies were collected immediately post-eclosion and aged in vials for 12 d. Flies were gently tapped to the bottom of the vial, and the number of flies able to climb the vial was counted. A minimum of 100 flies were assayed—(at least 10 vials of 10 flies each).

Nuclear/whole-cell extracts and complex purifications

Preparation of nuclear extracts and complex purifications were performed as previously described [Suganuma et al. 2010]. Whole-cell extracts were made by lysing cells/tissues directly in Laemmli buffer.

Nucleic acid preparations and quantitative PCR (qPCR)

Tissues were processed in Trizol according to the manufacturer’s directions to extract nucleic acids. cDNA was generated from
Loss of Ataxin-7 reduces H2B ubiquitination

Table 1. Primers used to detect the CG9866 transcript

| Primer location (see Supplemental Fig. S3) | Forward 3′–5′ | Reverse 3′–5′ |
|-------------------------------------------|--------------|--------------|
| Control RP39                              | ATGTGATTCCCGACCATGTTACAAGA | AAGAAGCGCAACACAGACTTCA |
| CG9866 region 1–7                         | CAAAATCCCGATTTATCCACGGTATAGAGT | CGTTCGATTGTCCTGTGAT |
| CG9866 region 2–7                         | GCTATACCAAACCCCAACA | CAGTTCGATTGTCCTGTGAT |
| CG9866 region 3–7                         | GTGAGCTTCTGGCGAAGTACCCCGCTGAG | GATCAGACGAAGACGCGCATCAAGTCGCTG |
| CG9866 region 6–13                        | CGTTCGCCATCACCTGCTACAA | AAGGAGCTTCCTCCTCTCATCGG |
| CG9866 region 6–14                        | CACAGCTTCTATCCGGAATGCCCCTGAAC | GATCAGACGAAGACGCGCATCAAGTCGCTG |
| CG9866 region 5–7                         | GATTTTAACATGGGCATTTCGTAGGGATTC | GATCAGACGAAGACGCGCATCAAGTCGCTG |

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