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Drosophila models reveal novel insights into mechanisms underlying neurodegeneration

Ryan D Mohan[1], Jerry L Workman[1], and Susan M Abmayr[1,2,*]

[1]Stowers Institute for Medical Research; Kansas City, MO USA; [2]Department of Anatomy and Cell Biology; University of Kansas Medical Center; Kansas City, KS USA

The SAGA chromatin modifying complex functions as a transcriptional coactivator for a large number of genes, and SAGA dysfunction has been linked to carcinogenesis and neurodegenerative disease. The protein complex is comprised of approximately 20 subunits, arranged in a modular fashion, and includes 2 enzymatic subunits: the Gcn5 acetyltransferase and the Non-stop deubiquitinase. As we learn more about SAGA, it becomes evident that this complex functions through sophisticated mechanisms that support very precise regulation of gene expression. Here we describe recent findings in which a Drosophila loss-of-function model revealed novel mechanisms for regulation of SAGA-mediated histone H2B deubiquitination. This model also yielded novel and surprising insights into mechanisms that underlie progressive neurodegenerative disease. Lastly, we comment on the utility of Drosophila as a model for neurodegenerative disease through which crucial and conserved mechanisms may be revealed.

Keywords: Ataxin-7, CAG repeat, chromatin, Drosophila, H2Bub, H2B ubiquitination, neurodegenerative disease, Non-stop, polyglutamine expansion, PolyQ, retinal and macular degeneration, SAGA complex, SCA7, spinocerebellar ataxia 7, transcription, transcriptional coactivator, USP22

*Correspondence to: Susan M Abmayr; Email: SMA@Stowers.org

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Introduction

Maintenance of genomic DNA as chromatin facilitates its compaction and suppresses stochastic gene activation. Gene expression is regulated in chromatin through sequential recruitment of individual proteins and large multiprotein complexes to gene regulatory regions. These proteins and complexes can then function as coactivators and corepressors to aid or repress gene expression, respectively. Specificity is achieved by sequence specific transcription factors that bind to consensus DNA sequences found within gene regulatory regions. Transcriptional coactivators often possess chromatin modifying activities that promote accessibility of the underlying DNA sequences and facilitate assembly of the transcription apparatus. The evolutionarily conserved SAGA chromatin modifying complex functions as a major transcriptional coactivator. SAGA is comprised of approximately 20 subunits and bears 2 enzymes – the Gcn5 acetyltransferase and the Non-stop deubiquitinase. Combined deletion and proteomic analysis performed in Saccharomyces cerevisiae has revealed that the complex is arranged in a modular fashion.[1] The 4 modules are comprised of functionally related subunits with the Spt module interacting with transcription factors, the Taf module interacting with the basal transcription apparatus, and the acetyltransferase and deubiquitinase modules serving to modify chromatin and non-chromatin substrates.[1,2]

Here we will focus on the deubiquitinase module, which is comprised of the Non-stop deubiquitinase, ENY2, Sgf11, and Ataxin-7 subunits. The amino (N)-terminus of Ataxin-7 extends into the deubiquitinase module, while the carboxy (C)-terminus extends into the larger SAGA complex and anchors the deubiquitinase module to the complex.[3] In humans, intergenerational expansion of CAG repeats in the Ataxin-7 gene results in expanded polyglutamine stretches in the N-terminus of the Ataxin-7 protein.[4] This expansion leads to spinocerebellar ataxia type 7 (SCA7) disease, which is characterized by progressive degeneration of the brain and the retina.[5] The wild-

type protein has 10 CAG trinucleotides, and disease symptoms are observed when the repeat length extends beyond 34, although repeat lengths greater than 400 have been reported. As repeat length increases, the age at which symptoms are first diagnosed is reduced, and the severity of the symptoms is increased.[6]

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

Nearly 2 decades ago, our group purified the SAGA chromatin modifying complex from the yeast Saccharomyces cerevisiae.[7] Since then, results from our group and many others have shed light on the principles regulating SAGA's ability to coactivate gene expression.[8] Increasingly, however, evidence has indicated that regulation of SAGA in multicellular organisms involves more sophisticated mechanisms than in their unicellular counterparts.[9,10]

In order to gain more insight into SAGA function in multicellular eukaryotes, we turned to Drosophila. This model provided powerful genetic and biochemical tools in which to explore principles underlying SAGA-mediated gene regulation. At that time, the identities of the subunits comprising the Drosophila SAGA complex were largely unknown. To characterize this complex biochemically, we used cell lines stably expressing known SAGA subunits fused to dual FLAG and HA epitope tags. The complex was purified through these tagged subunits and shotgun proteomics used to determine its composition.

Though this method we identified a putative complex member encoded by the uncharacterized gene CG9866. In silico analysis indicated that this protein shared sequence similarity with human Ataxin-7, known as Sgf73 in yeast. Closer analysis of the amino acid sequence of the CG9866 protein revealed conservation of a proposed "Ataxin-7 signature" identified by Helmlinger et al.[11] The presence of this conserved sequence, suggested that we had indeed identified Drosophila Ataxin-7 and that it may be a subunit of the SAGA complex.

Since human and yeast Ataxin-7 were found to be bona fide members of SAGA, we performed a series of biochemical studies to demonstrate that the putative Drosophila Ataxin-7 is also a component of SAGA. Reciprocal purification of Ataxin-7 followed by Multidimensional Protein Identification Technology (MudPIT) analysis showed that it was able to co-purify SAGA.[12] To establish that Ataxin-7 is a stable component of the complex and not simply a strongly interacting protein, we separated putative protein complexes by size exclusion chromatography. This analysis revealed that nearly all of the Ataxin-7 co-fractionated with SAGA, providing strong support for the conclusion that Ataxin-7 is a stable component of the Drosophila SAGA complex. Moreover, the observation that virtually all Ataxin-7 protein migrated with SAGA suggested that this is its primary function.

The modular model of SAGA organization predicted that Ataxin-7 would anchor the deubiquitinase module to the larger SAGA complex. This model predicts that, in the absence of Ataxin-7, the module would be released from the larger complex.[1] To test the validity of this hypothesis, we performed gel filtration chromatography to measure the apparent sizes of wild-type SAGA versus SAGA from flies lacking Ataxin-7. We found that SAGA from Ataxin-7-mutant flies does indeed have a reduced mass compared to that of its wild-type counterpart. This finding implied that the deubiquitinase module is released from SAGA in Ataxin-7 mutants. Immunoprecipitation using an antibody against the SAGA deubiquitinase, Non-stop, showed that Non-stop is stably associated with SAGA in wild-type 3rd instar larvae, but not in Ataxin-7 mutant larvae. Thus, the deubiquitinase module is separated from the remainder of the SAGA complex in Ataxin-7 mutants,.

Biochemical and structural analysis of the yeast deubiquitinase module, considered to include the yeast orthologs of Ataxin-7, ENY2, Sgf11, and Non-stop, showed that the deubiquitinase is held in an enzymatically active conformation by the presence of all members of the module. The absence of any member of the module inactivated the yeast deubiquitinase.[13,14] Surprisingly, deubiquitinase assays revealed that the deubiquitinase module purified in the absence of Ataxin-7, and therefore separated from SAGA, remains enzymatically active. To confirm that the Non-stop deubiquitinase module could remain enzymatically active in the absence of Ataxin-7, we reconstituted this module using purified recombinant proteins. We found that it did, indeed, retain deubiquitinase activity.

The above finding showed that the released Non-stop-containing deubiquitinase module is enzymatically active when purified from Ataxin-7 mutants and when reconstituted in vitro. However, these studies did not provide insight into the behavior and localization of this module in cells lacking Ataxin-7. We took advantage of polytene chromosome spreads from the nuclei of third instar salivary glands to show that the deubiquitinase module still associated with chromatin. This raised the possibility that it would act enzymatically without constraint from SAGA regulation. Indeed, analysis of bulk histones acid extracted from mutant third instar larvae showed reduced levels of H2Bub.

Under standard culture conditions, Ataxin-7 homozygous mutants die prepupation, at the late 3rd instar stage of development. In light of the above molecular and biochemical results, we hypothesized that this lethality was due to the action of the overactive deubiquitinase. If this theory is correct, one might predict that reducing the level of the deubiquitinase would rescue loss of Ataxin-7. Indeed, genetically reducing the level of Non-stop in an Ataxin-7 mutant fly by introducing one copy of a mutant allele suppressed the Ataxin-7 lethality and allowed some mutants to survive to adulthood. This result strongly suggests that loss of Ataxin-7 results in a toxic gain of activity of the Non-stop deubiquitinase.

Since these findings deviated from predictions made from studies performed in yeast, we looked to human tissue culture models to determine whether our results were representative of SAGA function in multicellular organisms. In human HeLa cells, as in Drosophila, we found a reduction in H2Bub upon knockdown of Ataxin-7. Moreover, this effect can be rescued by inactivating the human deubiquitinase USP22 through knock-down of the deubiquitinase module subunit Sgf11.[15]

Thus, while required in yeast, the presence of Ataxin-7 is dispensable for deubiquitinase module activity in higher eukaryotes.

Considering the phenotype associated with polyglutamine expansion of Ataxin-7, we examined the phenotypic consequences of Ataxin-7 loss in flies. Although the majority of Ataxin-7 homozygous mutants die in the third instar larval stage of development, adult escapers can be obtained through careful culture conditions. Upon analyzing these escapers, we noted that these mutant flies exhibited neural and retinal degeneration, reduced mobility, and shortened life span. Importantly, this phenotype is strikingly similar to that found upon exogenous expression of the polyglutamine-expanded human Ataxin-7 in Drosophila.

The promise of Drosophila models for uncovering new mechanisms underlying neural instability caused by expansion of polyglutamine repeats.

Polyglutamine expansion as a cause of neurodegenerative disease was uncovered over 20 y ago when SCA17 was shown to be caused by polyglutamine expansion of in the androgen receptor (AR).[16] Since then, 9 genes have been identified to be subject to CAG expansion, causing 9 distinct diseases. Incredibly, the functions of many of these genes are only partially known, or completely unknown, and effective therapeutics for treatment of polyglutamine expansion diseases are not currently available. In order to proceed toward new discoveries, we must first determine which pathways mediate progression of these neurodegenerative diseases, and then identify novel components for intervention.

We believe that Drosophila models will be fruitful in the search for mechanisms contributing to neurodegeneration. Many seminal discoveries about PolyQ disease etiology have already come from Drosophila models. For example, the Gitler and Bonini groups have discovered modifiers of the Amyotrophic lateral sclerosis (ALS)-associated 43 kDa TAR DNA binding protein (TDP-43) using a combination of yeast and Drosophila models.[17] They found that Ataxin-2, the polyglutamine-expanded protein associated with SCA2 disease, strongly enhanced the toxicity of TDP-43. The Ataxin-2 and TDP-43 proteins, along with RNA, exist in a complex that is mislocalized in both SCA2 and ALS.[18] This surprising result was the first to suggest a link between polyglutamine disease and risk for ALS. This potential link prompted examination of ALS patient samples for the prevalence of polyglutamine expanded Ataxin-2, which showed that intermediate polyglutamine expansion of Ataxin-2 was present in nearly 5% of ALS cases. The next best predictor of ALS is mutant superoxide dismutase (SOD), which occurs in about 2% of ALS cases.[18] Thus, insights from a Drosophila model represented a leap forward in our understanding of the pathways that contribute to ALS and polyglutamine disease. To further illustrate that point, these groups went on to use Drosophila models to discover small molecule inhibitors suitable for modulating TDP-43-associated toxicity.[19]

The Orr, Westbrook, Botas, and Zoghbi laboratories combined efforts in a strategy that utilized Drosophila and cell-based screens to identify proteins that modulate the levels of neurodegenerative disease-driving proteins. This approach proved to be powerful in identifying modifiers of polyQ expanded Ataxin-1,

Table 1. Drosophila orthologs of human genes expanded to cause polyQ disease. There are 9 human genes which undergo CAG expansion, resulting in PolyQ-expansion in their protein products. Here, to the best of our knowledge, we list the Drosophila orthologs of the human proteins. We also note whether the effect of PolyQ-expansion of the native Drosophila protein has been examined. References are: Zala et al. 2013[23]; Napoletano et al. 2011[24]; Zhai et al. 2008[25]; Lessing and Bonini 2008[26]; Gu et al. 2009[27]; Mohan et al. 2014[28]; Hoey et al. 1990[29]; and Nisoli et al. 2010[30]

Polyglutamine expansion diseases

Disease name	Products of expanded gene - Human	Drosophila ortholog	Reference	Studies performed with expanded Drosophila ortholog*	Reference
Huntington disease (HD)	Huntingtin	CG9995, htt, dhtt	Zala et al. 2013	No	———–
Spinal and bulbar muscular atrophy (SBMA)/Kennedy's disease	Androgen receptor	Unknown	———–	No	———–
Dentatorubral-pallidoluysian atrophy	Atrophin 1	CG6964, Gug, Grunge, Atro, atrophin, l(3)03928	Napoletano et al. 2011	Yes	Nisoli et al. 2010
SCA1	Ataxin-1, alt-ATXN1	CG4547, Atx-1, dAtx-1	Zhai et al. 2008	No	———–
SCA2 and amyotrophic lateral sclerosis (ALS)	Ataxin-2	CG5166, Atx2, Ataxin-2, dAtx2, SCA2, dSCA2, l(3)06490	Lessing and Bonini 2008	No	———–
SCA3, Machado-Joseph disease	Ataxin-3	Unknown	———–	No	———–
SCA6	a1A voltage-dependent calcium channel subunit, and a1ACT trascription factor	CG43368, cac, cacophony, Dmca1A, nbA, CG1522, l(1)L13, 13, CG15928, cac1	Gu et al. 2009	No	———–
SCA7	Ataxin-7	CG9866, Ataxin-7, Atxn7	Mohan et al. 2014	No	———–
SCA17	TATA box binding protein (TBP)	CG9874, Tbp, dTBP, TFIID, TFIIDτ, dTFIID	Hoey et al. 1990	No	———–

which causes SCA1disease. They chose to focus on mutants which led to a reduced level of polyQ Ataxin-1 protein because this also led to reduction in disease severity. This strategy was very effective, and they were able to show that down regulation of RAS-MAPK-MSK1 pathway components results in reduced levels of Ataxin-1. Importantly, pharmacological inhibitors of this pathway also reduced neurodegeneration in Drosophila and in mice, raising the possibility that these inhibitors might also be effective for treatment of patients.[20]

In our study we have identified a novel, conserved, function for Ataxin-7 in regulating H2B ubiquitination levels. These observations, along with previous findings by our group and others, suggest that the SAGA complex is an important regulator of neural development. Mutants of the Drosophila SAGA subunits Non-stop, Sgf11, and Ada2B all result in aberrant targeting of neural connections from the retina to the optic lobe of the brain.[9] Gcn5 mutant mice exhibit decreased brain size, severe cranial neural tube closure defects and exencephaly.[21,22] Now, we have provided evidence that SAGA complex member Ataxin-7 is also necessary for regulating H2B ubiquitination and maintenance of neural stability. It is tempting to predict that polyglutamine expansion of Ataxin-7 will affect H2B ubiquitination, and that removal of this histone mark might be linked directly to neurodegeneration.

Moving forward, D. melanogaster will certainly continue to be a driving force in the discovery of mechanisms contributing to neurodegenerative disease. To our knowledge, Drosophila orthologs for 7 out of the 9 genes subject to polyQ expansion have now been identified. Of these, polyglutamine expansion studies have been performed on just 1 (**Table 1**). Thus, expanded use of Drosophila to uncover novel therapeutic entry points for treatment of neurodegenerative disease is both warranted and promising.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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