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SAGA-mediated H2B deubiquitination controls the development of neuronal connectivity in the *Drosophila* visual system

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Nonstop, which has previously been shown to have homology to ubiquitin proteases, is required for proper termination of axons R1–R6 in the optic lobe of the developing *Drosophila* eye. Herein, we establish that Nonstop actually functions as an ubiquitin protease to control the levels of ubiquitinated histone H2B in flies. We further establish that Nonstop is the functional homolog of yeast Ubp8, and can substitute for Ubp8 function in yeast cells. In yeast, Ubp8 activity requires Sgf11. We show that in *Drosophila*, loss of Sgf11 function causes similar photoreceptor axon-targeting defects as loss of Nonstop. Ubp8 and Sgf11 are components of the yeast SAGA complex, suggesting that Nonstop function might be mediated through the *Drosophila* SAGA complex. Indeed, we find that Nonstop does associate with SAGA components in flies, and mutants in other SAGA subunits display *nonstop* phenotypes, indicating that SAGA complex is required for accurate axon guidance in the optic lobe. Candidate genes regulated by SAGA that may be required for correct axon targeting were identified by microarray analysis of gene expression in SAGA mutants.

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Introduction

The compound eye of *Drosophila melanogaster* provides a powerful system to study the mechanisms regulating the target layer selection of neurons. There are eight different neuronal cells (R cells; R1–R8) within the ~800 ommatidia of the compound eye. Each of these neurons projects growth cones to distinct targets within the optic ganglia (Clandinin and Zipursky, 2002). R1–R6 cells project to the lamina, forming the lamina plexus. R7 and R8 axons continue through the lamina and terminate within the medulla. Screens for mutations affecting axon targeting identified Nonstop, a ubiquitin protease, as an essential protein required for correct axon targeting in the developing visual system of *Drosophila* (Martin *et al*, 1995). Mutations in *nonstop* affect the number of glial cells located within the lamina plexus. The lack of glial cells causes mistargeting of the R1–R6 axons (Poeck *et al*, 2001).

The function of Nonstop as an ubiquitin protease was thought to indicate a role for protein degradation in this axon targeting mechanism (Poeck *et al*, 2001). Many of the ubiquitin proteases that have been identified in flies have roles in protein degradation (Chen and Fischer, 2000). Polyubiquitination commonly targets proteins for degradation via the 26S proteasome. However, we find that the closest ortholog of Nonstop in yeast is the H2B deubiquitinase, Ubp8. Moreover, monoubiquitination of particular substrates such as histones is more generally involved in transcriptional regulation than in protein degradation (Osley, 2006). In particular, the C terminus of human histone H2B is subject to the dynamic addition and removal of a single ubiquitin moiety at Lys-120. A balance in the level of this modification is important for transcription activation and elongation. Monoubiquitination is catalyzed by the Rad6/UbcH6 E2 conjugase in conjunction with the Bre1 E3 ligase (Jentsch *et al*, 1987; Robzyk *et al*, 2000; Wood *et al*, 2003a; Zhu *et al*, 2005). Rad6 and Bre1 associate with elongating RNA polymerase II in a PAF-dependent manner, and this interaction is required for Lys-4 methylation on histone H3 (Wood *et al*, 2003b; Xiao *et al*, 2005).

The role of H2B deubiquitination in development of higher eukaryotes had not been characterized, and we were interested in whether the axon-targeting defect was due to the lack of removal of this histone modification. Hence we sought to determine whether Nonstop is a functional homolog of Ubp8 and acts as an H2B deubiquitinase in flies, and if it functions in axon guidance as a component of the *Drosophila* SAGA complex.

Results

Nonstop and CG13379 are orthologs of proteins required for H2B deubiquitination in yeast

To gain insights into the potential functions of Nonstop, we carried out a phylogenic analysis of the ubiquitin proteases from *Saccharomyces cerevisiae* and *D. melanogaster* and identified yeast Ubp8 as the ubiquitin protease most closely related to Nonstop (Figure 1B). Nonstop shares 20% identity with Ubp8, and has 31% similarity (Figure 1A). Furthermore, there is considerable conservation between Ubp8 and Nonstop within the zinc finger, and cysteine and histidine boxes. Ubp8 requires Sgf11 to deubiquitinate

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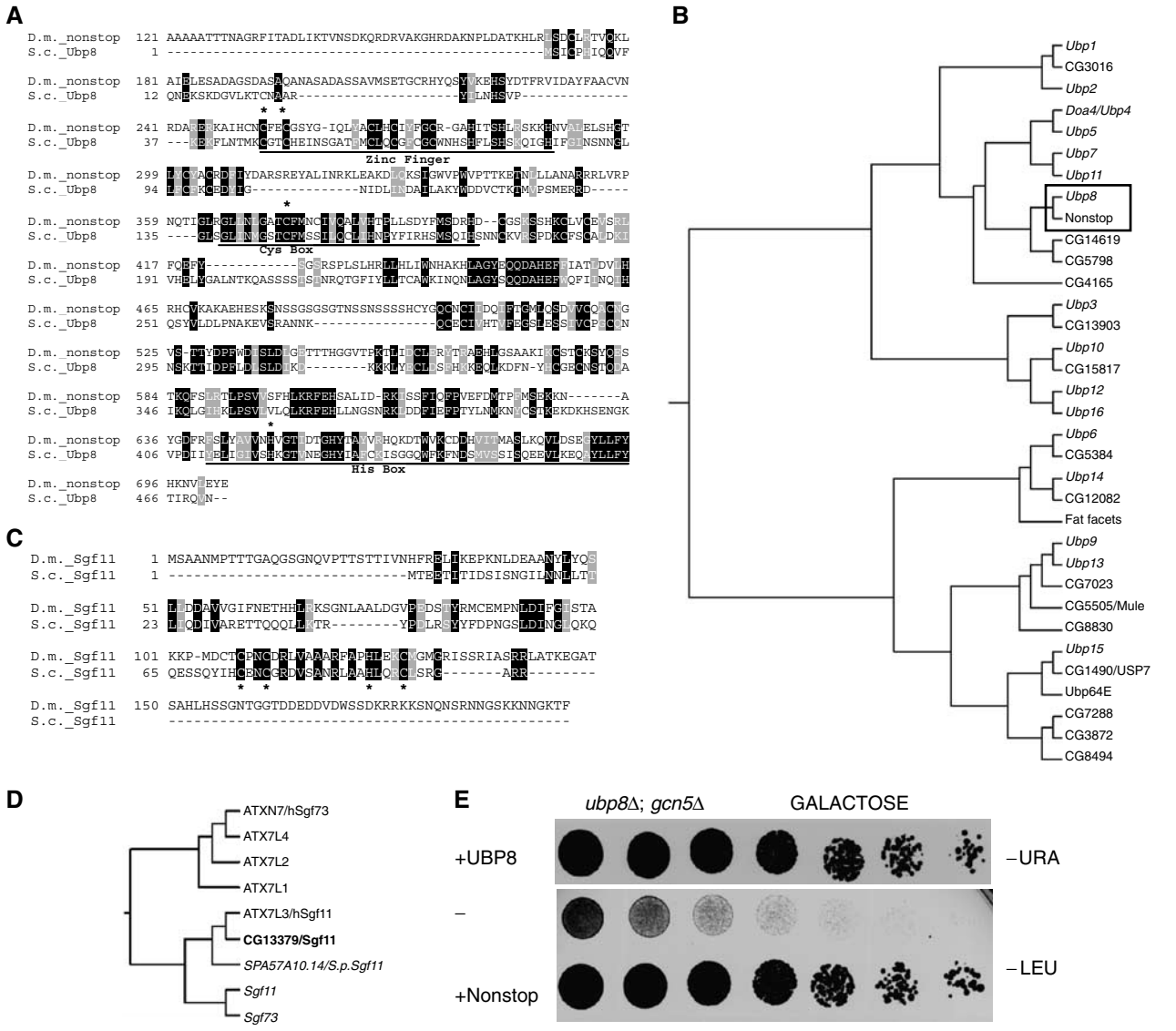

Figure 1 Orthologs of the proteins required for H2B deubiquitination in yeast were identified in *Drosophila*. (**A**) Alignment of *D. melanogaster* Nonstop and *S. cerevisiae* Ubp8. The conserved zinc finger, Cys-box and His-box domains are indicated. (**B**) Dendrogram of the 16 yeast and 18 *Drosophila* ubiquitin proteases. The yeast Ubps are indicated in italics. Nonstop and Ubp8 form a single clade (box). (**C**) Alignment of *D. melanogaster* Sgf11/CG13379 and *S. cerevisiae* Sgf11. Key cysteine and histidine residues are indicated by asterisks. (**D**) Dendrogram showing the relationship between human ATAXIN, *S. cerevisiae* Sgf11 and Sgf73, *S. pombe* Sgf11 and *D. melanogaster* CG13379/Sgf11. (**E**) Four-fold serial dilutions of *ubp8*Δ*gcn5*Δ yeast were grown at 30°C for 2 days on galactose plates. The growth defect can be rescued by the introduction of yeast UBP8 or *Drosophila* Nonstop.

H2B in yeast (Henry *et al*, 2003; Ingvarsdottir *et al*, 2005; Lee *et al*, 2005). BLAST searches identified an Sgf11 ortholog, CG13379, in *Drosophila*, which has 36% similarity and 28% identity when aligned with yeast Sgf11 (Figure 1C). CG13379 will henceforth be referred to as Sgf11. *Drosophila* Sgf11 and yeast Sgf11 are similar to human ATX7L3 (Helmlinger *et al*, 2004), and to the yeast SAGA component Sgf73 (Figure 1D).

Nonstop is functionally equivalent to Ubp8

Although phylogenetic analysis indicated that Nonstop is the potential ortholog of Ubp8, there are 18 additional ubiquitin proteases that have been identified in the *Drosophila* genome (Chen and Fischer, 2000). Therefore, it was necessary to determine whether Nonstop is functionally equivalent to Ubp8. To test if Nonstop could complement the function of Ubp8 within yeast SAGA, we generated yeast expression constructs containing galactose-inducible Nonstop. We asked whether Nonstop could rescue the phenotypes associated with *ubp8*Δ (Figure 1E). In yeast, the UBP8; GCN5 double deletion exhibits a growth defect on galactose, which can be rescued by the introduction of a plasmid-expressing yeast Ubp8 (Henry *et al*, 2003; Lee *et al*, 2005). Introduction of Nonstop into this double deletion strain also rescues this growth defect, indicating that Nonstop can functionally replace Ubp8 in yeast. Two other fly ubiquitin proteases, USP7 and CG5384, cannot rescue the growth defect of the UBP8; GCN5 double deletion on galactose, indicating that this rescue is specific to Nonstop (Supplementary Figure 1).

Nonstop and sgf11 mutants exhibit defects in axon targeting

nonstop was originally identified in a screen for mutations that affect photoreceptor connectivity in the *Drosophila* visual system (Martin *et al*, 1995). This leads us to hypothesize that Sgf11, and potentially H2B deubiquitination, may also be required for neural development in flies. To test this, we identified a mutation in *sgf11* caused by the insertion of a *piggyBac* transposon in the promoter region of *sgf11* (Figure 2A). This insertion is homozygous lethal during the late larval/early pupal stage and no transcripts for *sgf11* were detected in mutant larvae by RT–PCR (Figure 2B).

In the developing visual system of *Drosophila*, the eye imaginal disc is connected to the optic ganglia by the optic stalk. There are eight different classes of neuronal cells, R1–R8, each of which project growth cones to distinct target regions in the optic lobe (Clandinin and Zipursky, 2002). The axons of R1–R6 terminate in the lamina, while R7 and R8 terminate within the medulla. The wild-type axon projection pattern can be visualized using a marker specific for those photoreceptors that terminate in the lamina (R2–R5; *ro-τlacZ*; Figure 2C). A triple layer of glial cells (anti-repo; Figure 2F) in the lamina specifies the termination point for R1–R6 axons. Mutations in *nonstop* cause a loss of glial cells from the target region of R1–R6 in the lamina, resulting in the misprojection of R1–R6 axons into the medulla (Figure 2E) (Poeck *et al*, 2001). We asked whether this targeting defect in the *nonstop* mutant reflected its potential role in H2B deubiquitination. To test this hypothesis, we visualized photoreceptor projections in *sgf11* larvae (Figure 2D). As in *nonstop* larvae, many *sgf11* photoreceptor axons fail to terminate in the lamina and instead project into the medulla. This axon-targeting defect in *sgf11* is associated with a disruption in the organization of the lamina glial cells that is identical to that observed in *nonstop* by Poeck *et al. nonstop* glial cells fail to migrate from the dorsal and ventral glial precursor cell areas to form their characteristic layers along the lamina. Instead, an increased number of repo-positive glial cells are observed at the dorsal and ventral margins of the R-cell projection field in the *nonstop* mutant (Poeck *et al*, 2001). We observed a similar increase in the number of glial cells at these margins in *sgf11* relative to the wild type (Figure 2F). This is accompanied by a decrease in the overall number of glial cells present along the lamina in the *sgf11* mutant. Repo-positive glial cells were counted in *nonstop*, *sgf11* and wild-type optic lobes along a particular length of the lamina to control for differences in the size and orientation of the R-cell projection field (Figure 2G and H). This shows that there is at least a two-fold decrease in the number of glial cells along the lamina in the *sgf11* and *nonstop* mutants relative to the wild type. The similarity of the axon-targeting defect with regard to both the R-cell projection pattern and glial cell migration observed in the *nonstop* and *sgf11* mutants indicated that these may function together for neural development in flies.

Nonstop associates with Sgf11

To determine if Nonstop and Sgf11 associated *in vivo*, we generated constructs to express C-terminally tagged versions of these proteins in S2 cells. These constructs express C-terminally V5-tagged Sgf11 and FLAG-HA-tagged Nonstop. We prepared whole-cell extracts from S2 cells transiently transfected with those constructs and immunoprecipitated Nonstop using anti-FLAG antibodies. Nonstop-HA_2FL_2 co-immunoprecipitated Sgf11-V5 (Figure 3A, lane 3). Nonstop was not detectable in the input material, when co-expressed with Sgf11-V5 because two-fold less DNA was used for this co-transfection relative to that of Nonstop alone (compare Figure 5B, lane 1). This result indicates that Nonstop and Sgf11 interact *in vivo*.

Nonstop and Sgf11 are required for deubiquitination of H2B in vivo

Ubp8 and Sgf11 deubiquitinate histone H2B at Lys-123 in yeast (Henry *et al*, 2003; Ingvarsdottir *et al*, 2005; Lee *et al*, 2005). We therefore asked whether Nonstop and Sgf11 are also required for H2B deubiquitination in flies (Lys-120; Supplementary Figure 2). Histones were acid-extracted from OregonR, *nonstop* or *sgf11* third instar larvae and analyzed by western blotting using antibodies against histone H2B (Figure 3B). Histone preparations were normalized to the level of histone H3 (data not shown). A higher molecular weight band corresponding to monoubiquitinated H2B (ubH2B) is detected using an antibody against H2B in histone extracts from *nonstop* and *sgf11*. This band corresponding to ubH2B is also detected at lower levels in wild-type histone extracts (data not shown). The level of ubH2B was increased greater than seven- and four-fold in the *nonstop* and *sgf11* mutants, respectively, in comparison to the wild-type control. These findings show that Nonstop and Sgf11 are required for deubiquitination of histone H2B *in vivo*.

Nonstop and Sgf11 coregulate a large subset of genes

The similar axon targeting and histone modification defects in the *nonstop* and *sgf11* mutants indicated that these proteins may function together to regulate gene expression. To test this, we examined patterns of gene expression in *nonstop* and *sgf11* mutant larvae using microarray analysis. Homozygous mutant third instar larvae of each genotype were compared to their heterozygote siblings or OregonR (wild type). There appears to be a large degree of overlap between the effects of the *nonstop* and *sgf11* mutations, as evidenced by the spectrum of genes with greater than two-fold differences in transcript levels relative to the heterozygote (Figure 3C). This observation was confirmed mathematically by the calculation of Pearson correlation coefficients for the mutants relative to the heterozygotes. The *nonstop* and *sgf11* mutants have a correlation of 0.68 with each other. Similar results are obtained for gene expression analyses from each pair of mutants relative to the wild type. The high correlation between the *nonstop* and *sgf11* mutants is strikingly similar to results obtained in yeast (Ingvarsdottir *et al*, 2005). This expression analysis indicates that Nonstop and Sgf11 function together to deubiquitinate H2B and regulate transcription at a subset of genes.

Does loss of H2B deubiquitination affect global H3 acetylation?

In yeast, Ubp8 and Sgf11 function as part of the SAGA histone acetyltransferase (HAT) complex (Henry *et al*, 2003; Ingvarsdottir *et al*, 2005; Lee *et al*, 2005). Mutations in components of dSAGA, such as *ada2b* and *wda*, reduce global levels of acetylated histone H3 Lys-9/Lys-14 (Kusch *et al*, 2003; Pankotai *et al*, 2005; Guelman *et al*, 2006b). We therefore asked whether mutations affecting H2B deubiquitination

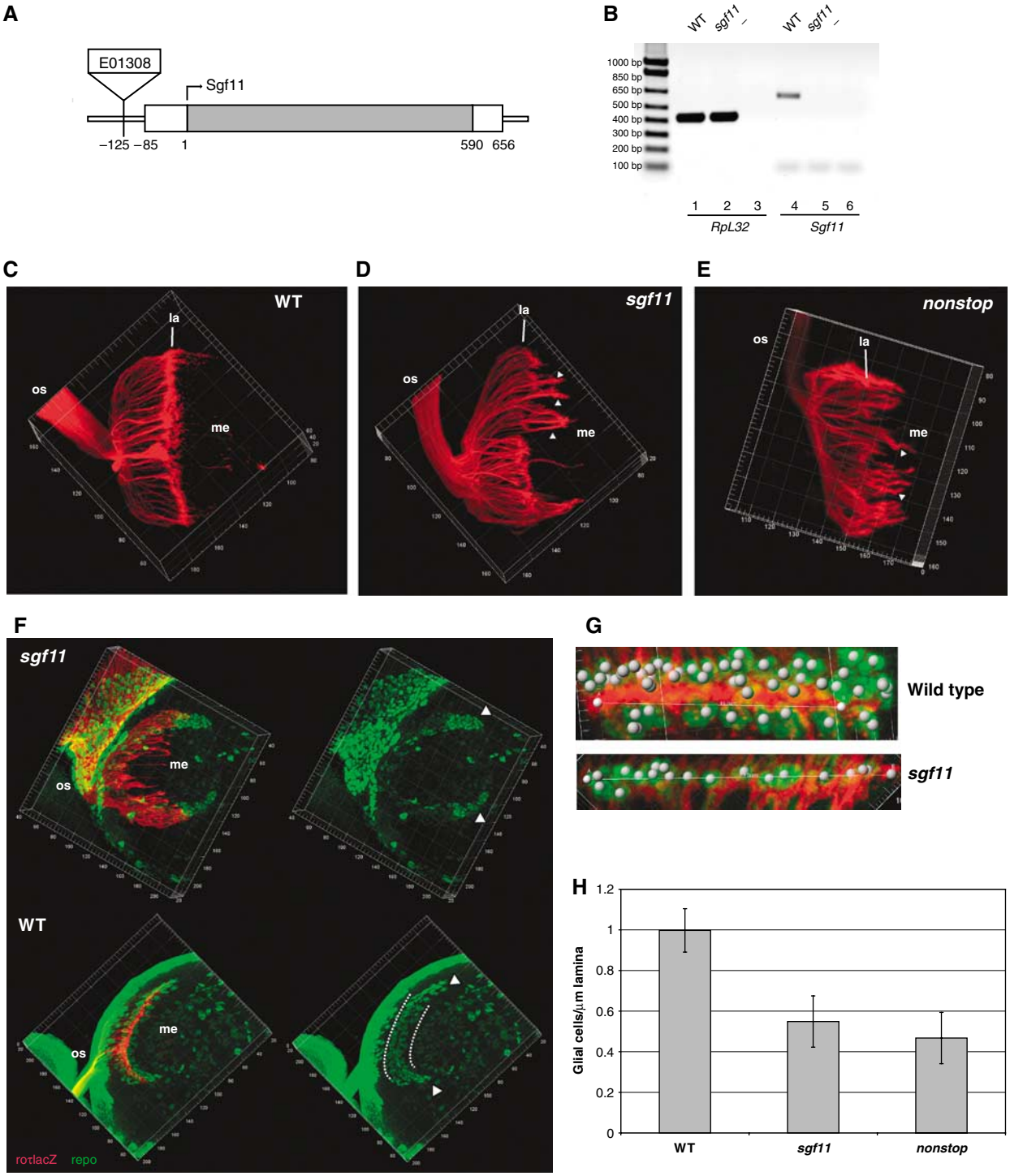

Figure 2 Axon targeting is disrupted in the *sgf11* mutant. (**A**) Schematic representation of the *sgf11* (*CG13379*) locus, showing the position of the *piggyBac* E01308 transposon (insertion). The single exon is represented by a box. Translated sequences are filled with gray, and 5′ and 3′ untranslated regions are open boxes. (**B**) RT–PCR from WT (lanes 1 and 4) or *sgf11* (lanes 2 and 5) third instar larvae with primers specific for the constitutively expressed gene *RpL32* (lanes 1–3) or for *sgf11* (lanes 4–6). Lanes 3 and 6 correspond to the negative PCR control. (**C**–**F**) In third instar larvae, photoreceptor cells from the eye disc extend axons through the optic stalk (os) into the optic lobe. R1–R6 axons terminate in the lamina (la) in wild type, while R7–R8 project through the lamina into the medulla (me). The projection pattern of R2–R5 was visualized in wild type (C), *sgf11* (D) and *nonstop* (E) larval optic lobes using the *ro-τlacZ* marker (red). In wild-type larvae, R2–R5 axons terminate in the lamina. However, in *nonstop* and *sgf11* larvae, many R2–R5 axons fail to terminate in the lamina (arrowheads), and instead project into the medulla. (F) A triple layer of glial cells, visualized using anti-repo (green), is present at the lamina in wild type (dotted lines) where R2–R5 growth cones terminate. These glial cells migrate from regions at the dorsal and ventral margins of the lamina (arrowheads) into the lamina. In the *sgf11* mutant, an increased number of glial cells accumulate at the edges of the lamina (arrowheads) and fewer glial cells are present along the lamina. (C–F) Scale bars, 20 μm. (**G**, **H**) The number of repo-positive glial cells (green) along a given length of the lamina (*ro-τlacZ*, red) was compared in wild-type, *nonstop* and *sgf11* mutants.

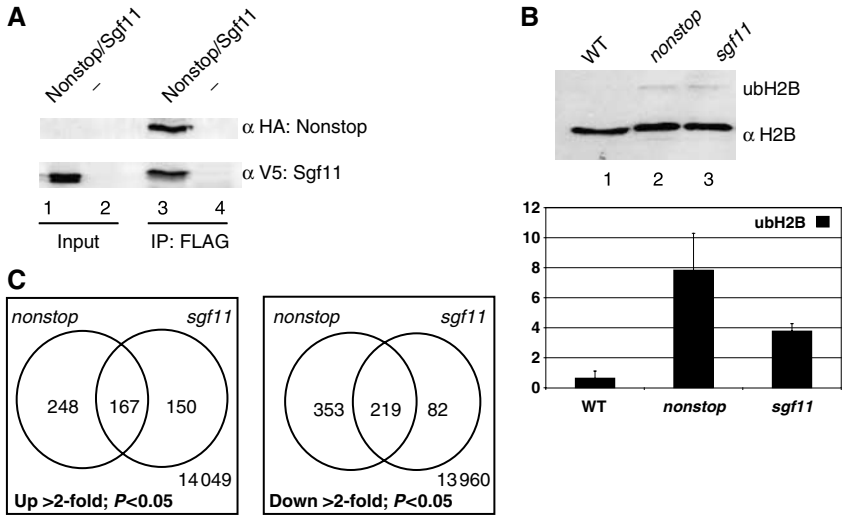

Figure 3 Nonstop and Sgf11 associate and are both required for H2B deubiquitination and regulation of gene expression. (**A**) Extract from S2 cells transfected with pRmHa3-Nonstop-HA_2FL_2 and pMT-Sgf11-V5 or untransfected S2 cells (−) was incubated with FLAG-M2-agarose beads. The immunoprecipitated material (IP, lanes 3 and 4) was analyzed by western blotting relative to 4% input (lanes 1 and 2). (**B**) Monoubiquitinated H2B levels increase in the *nonstop* and *sgf11* mutants. Histones were acid-extracted from OregonR (WT), *nonstop* or *sgf11* third instar larvae nuclei and analyzed by western blotting using antibodies against H2B. Mean results from three separate experiments are graphed normalized to H2B levels. (**C**) Nonstop and Sgf11 coregulate a large subset of genes. The number of overlapping genes with increased or decreased transcript levels greater than two-fold in *nonstop* and *sgf11* homozygous mutant third instar larvae compared to their heterozygote siblings ($P<0.05$ for $\geqslant 2$ biological replicates).

in flies would affect global levels of acetylated histone H3. To address this question, histones were acid-extracted from OregonR, *nonstop*, *sgf11* or *ada2b* third instar larvae and analyzed by western blotting using antibodies against histone H2B and acetylated H3 Lys-9 (Figure 4A). Histone preparations were normalized to the level of histone H3 (data not shown). The level of acetylated H3 Lys-9 is equivalent to that of the wild type in the *nonstop* and *sgf11* mutant larvae, but is significantly decreased in the *ada2b* mutant larvae. These findings show that while Nonstop and Sgf11 are required for deubiquitination of histone H2B *in vivo*, they are not essential for the acetyltransferase activity of dSAGA.

Mutations affecting dSAGA acetyltransferase activity have overlapping but distinct effects on gene expression when compared to nonstop and sgf11

Nonstop and Sgf11 are required for H2B deubiquitination, but have no effect on global acetylated H3 levels relative to mutations in other components of dSAGA. We therefore asked how these H2B deubiquitinating proteins function with regard to gene expression relative to Ada2b, which is required for dSAGA HAT activity (Figure 4A). Patterns of gene expression in *nonstop*, *sgf11* and *ada2b* mutant larvae were examined using microarray analysis. Homozygous mutant third instar larvae of each genotype were compared to their heterozygote siblings or OregonR (wild type). Identification of genes showing increased or decreased transcript levels greater than two-fold in the mutants relative to the heterozygote indicates that there is only a small degree of overlap between the *nonstop* and *sgf11* mutants compared to *ada2b* (Figure 4B). The *nonstop* and *sgf11* mutants have correlations with *ada2b* of 0.03 and 0.07, respectively, relative to the heterozygote. Slightly higher correlations are obtained for gene expression analyses from each pair of mutants relative to the wild type (data not shown). The high correlation between the *sgf11* and *nonstop* mutants, and their lack of

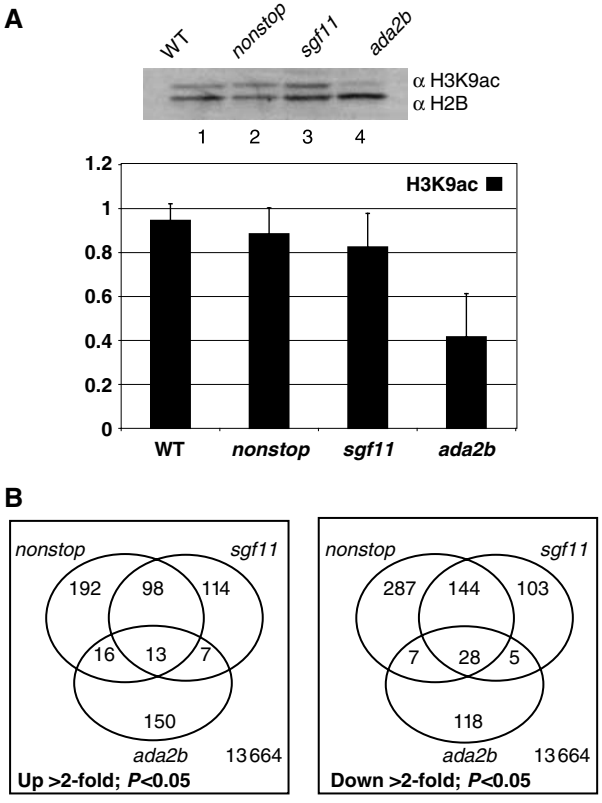

Figure 4 Mutations in *nonstop* and *sgf11* do not affect H3K9 acetylation and have distinct but overlapping effects on gene expression when compared to a dSAGA mutation. (**A**) Histones were acid-extracted from OregonR (WT), *nonstop*, *sgf11* or *ada2b* third instar larvae nuclei and analyzed by western blotting using antibodies against H2B and acetylated H3 Lys-9. Mean results from three separate experiments are graphed normalized to H2B levels. (**B**) The number of overlapping genes with increased or decreased transcript levels greater than two-fold in *nonstop*, *sgf11* and *ada2b* homozygous mutant third instar larvae compared to their heterozygote siblings ($P<0.05$ for $\geqslant 2$ biological replicates).

A

	Nonstop		Sgf11 (CG13379)	
	Total peptides	% coverage	Total peptides	% coverage
dAda1-HA_2FL_2	29	26.4	13	37.2
WDA-HA_2FL_2	11	7.8	5	31.4
Ada2b-HA_2FL_2	10	7.3	6	13

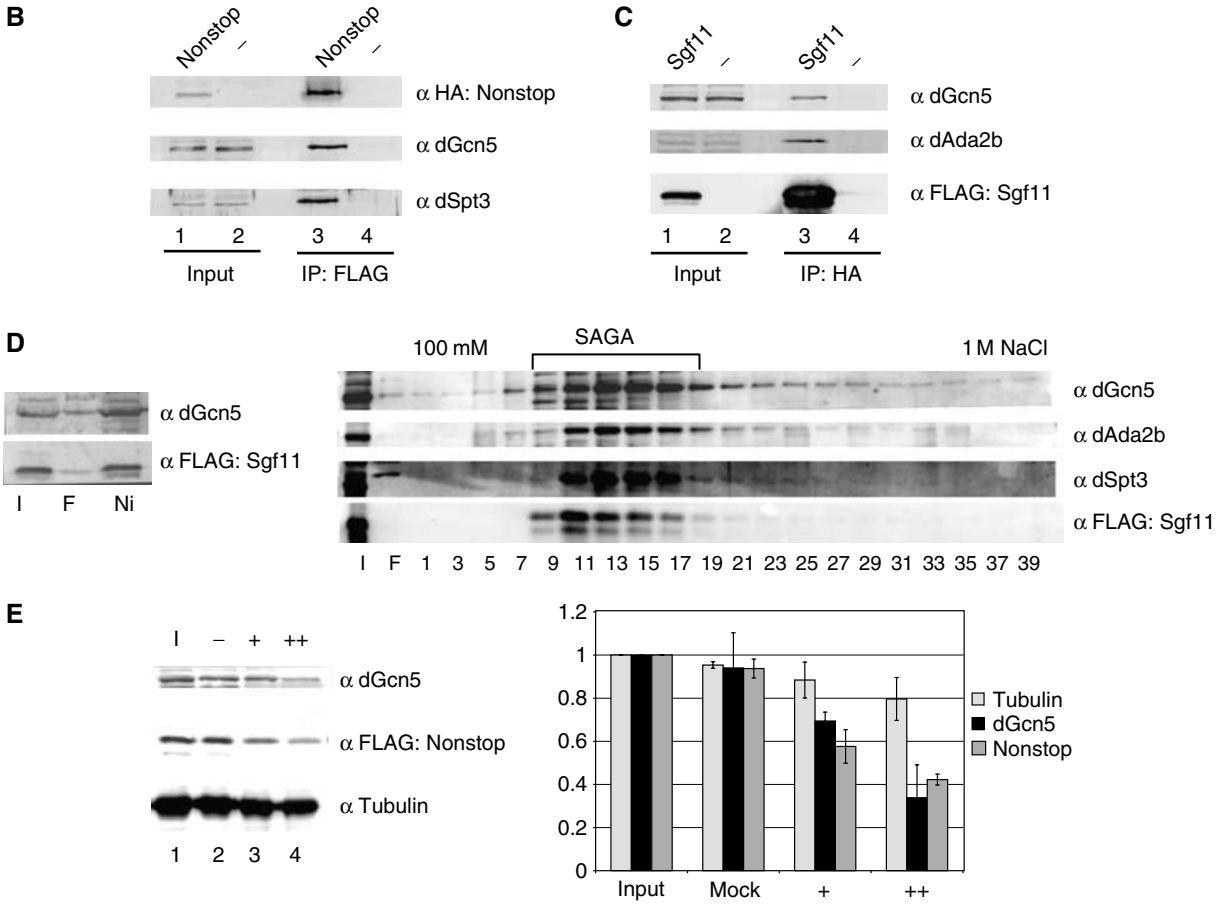

Figure 5 Nonstop and Sgf11 are associated with dSAGA. (**A**) MudPIT analysis of dSAGA, affinity purified from cells expressing tagged dAda1, WDA or dAda2b identified Nonstop and CG13379/Sgf11 as putative components of dSAGA. The table shows the number of non-redundant spectra for each protein (total peptides) and the amino-acid sequence coverage (% coverage). (**B**) Extract from S2 cells transfected with pRmHa3-Nonstop-HA_2FL_2 was immunoprecipitated as described in Figure 3. Immunoprecipitated material (IP, lanes 3 and 4) was analyzed by western blotting relative to 1% input (lanes 1 and 2). (**C**) Extract from S2 cells transfected with pRmHa3-Sgf11-HA_2FL_2 was incubated with HA-agarose beads, and the immunoprecipitated material analyzed as in (B). (**D**) Sgf11-HA_2FL_2 Ni-agarose HAT-enriched S2 cell nuclear extract was applied to a Mono-Q column and the elution profiles of dGcn5 (dKAT2), Ada2b, Spt3 and Sgf11 compared by western blotting. I, input; F, unbound; Ni, eluted from Ni-agarose. (**E**) Antibodies against dGcn5 (dKAT2) were added to 1 mg of Nonstop-HA_2FL_2 extract. Equal amounts of input (I), mock depletion (−) and immunodepleted extract (+, ++) were analyzed by western blotting for the presence of dGcn5 (dKAT2), Nonstop and tubulin.

similarity to *ada2b* is similar to deletion analysis in yeast (Ingvarsdottir *et al*, 2005). This expression analysis indicates that proteins required for H2B deubiquitination in flies have distinct regulatory effects on transcription that are separable from proteins required for HAT activity.

Nonstop and Sgf11 are components of dSAGA

Nonstop and Sgf11 have distinct effects on histone modifications and gene expression relative to other SAGA components, such as Ada2b. We therefore asked whether these proteins indeed constitute part of the deubiquitination module of the dSAGA complex in flies. Affinity purification of dSAGA enabled us to identify complex-specific subunits, such as dAda1, WDA and Ada2b (Kusch *et al*, 2003; Guelman *et al*, 2006b). Using mass spectroscopy analysis (using Multi-Dimensional Protein Identification Technology; MudPIT) of affinity-purified dSAGA, we identified Nonstop and Sgf11 as novel proteins that associate specifically with the complex. MudPIT results from the dADA1, WDA and Ada2b affinity purifications identified peptides specific for Nonstop and Sgf11 (Figure 5A). In *Drosophila*, dGcn5 (dKAT2) is a component of both the dSAGA and ATAC HAT complexes (Kusch *et al*, 2003; Muratoglu *et al*, 2003; Allis *et al*, 2007). No peptides for Nonstop or Sgf11 were identified in MudPIT analysis of affinity purifications of ATAC (Guelman *et al*, 2006a) (data not shown) or in mock purifications from untransfected S2 cell nuclear extract. These data indicate that Nonstop and Sgf11 associate specifically with dSAGA

and are not present in additional dGcn5 (dKAT2)-containing complexes. Supporting the association of Nonstop and Sgf11 with dSAGA, purified dSAGA shows moderate deubiquitination activity on H2B *in vitro* (Supplementary Figure 3, lane 2).

To confirm that Nonstop and Sgf11 associate with dSAGA, we generated constructs to express C-terminally tagged versions of these proteins in S2 cells. We prepared whole-cell extracts from S2 cells transiently transfected with these constructs and immunoprecipitated Nonstop using anti-FLAG antibodies. Nonstop-HA_2FL_2 co-immunoprecipitated dGcn5 (dKAT2) and dSpt3, both of which are stable components of dSAGA (Figure 5B, lane 3). Similarly, immunoprecipitation of Sgf11-HA_2FL_2 co-immunoprecipitated dGcn5 (dKAT2) and Ada2b (Figure 5C, lane 3). These results indicate that Nonstop and Sgf11 are both associated with dSAGA.

Since these observations provided evidence that Nonstop and Sgf11 stably associate with dSAGA, we wanted to determine if Sgf11 co-fractionated with dSAGA components by anion-exchange chromatography. First, nuclear extract from S2 cells stably expressing Sgf11-HA_2FL_2 was applied to Ni-agarose to enrich for dGcn5 (dKAT2)-containing complexes prior to anion-exchange chromatography. Both Sgf11 and dGcn5 (dKAT2) bind Ni-agarose (Figure 5D), indicating that the majority of Sgf11 is associated with this HAT-enriched fraction. This Ni-agarose HAT-enriched extract was applied to a Mono-Q column, and the elution profiles of dGcn5 (dKAT2), Ada2b, dSpt3 and Sgf11-HA_2FL_2 were compared by western blotting (Figure 5D). Sgf11 elutes within the same range of the salt gradient as other dSAGA subunits, indicating that the majority of Sgf11 is associated with the complex. The broader elution profile of dGcn5 (dKAT2) is consistent with its association with additional HAT complexes in flies.

Since this analysis indicated that the majority of Sgf11 co-fractionates and is thus associated with dSAGA, we then asked whether the same was true for Nonstop. Whole-cell extract was prepared from S2 cells transiently transfected with Nonstop-HA_2FL_2 and immunodepleted for dGcn5 (dKAT2) (Figure 5E). The level of Nonstop and dGcn5 (dKAT2) in the immunodepleted and mock-depleted extracts was analyzed by western blotting relative to the input material. A two-fold decrease in the level of dGcn5 (dKAT2) was observed with the highest amount of antibody added, corresponding to a similar decrease in the level of Nonstop. Nonstop was not completely depleted due to the overexpression of the tagged protein in these transiently transfected cells, which still contain endogenous Nonstop. This analysis indicates that the majority of Nonstop is associated with dGcn5 (dKAT2).

ada2b and gcn5 mutants exhibit defects in axon targeting in the optic lobe

Although our evidence showed that Nonstop and Sgf11 are components of dSAGA, the distinct effects on histone

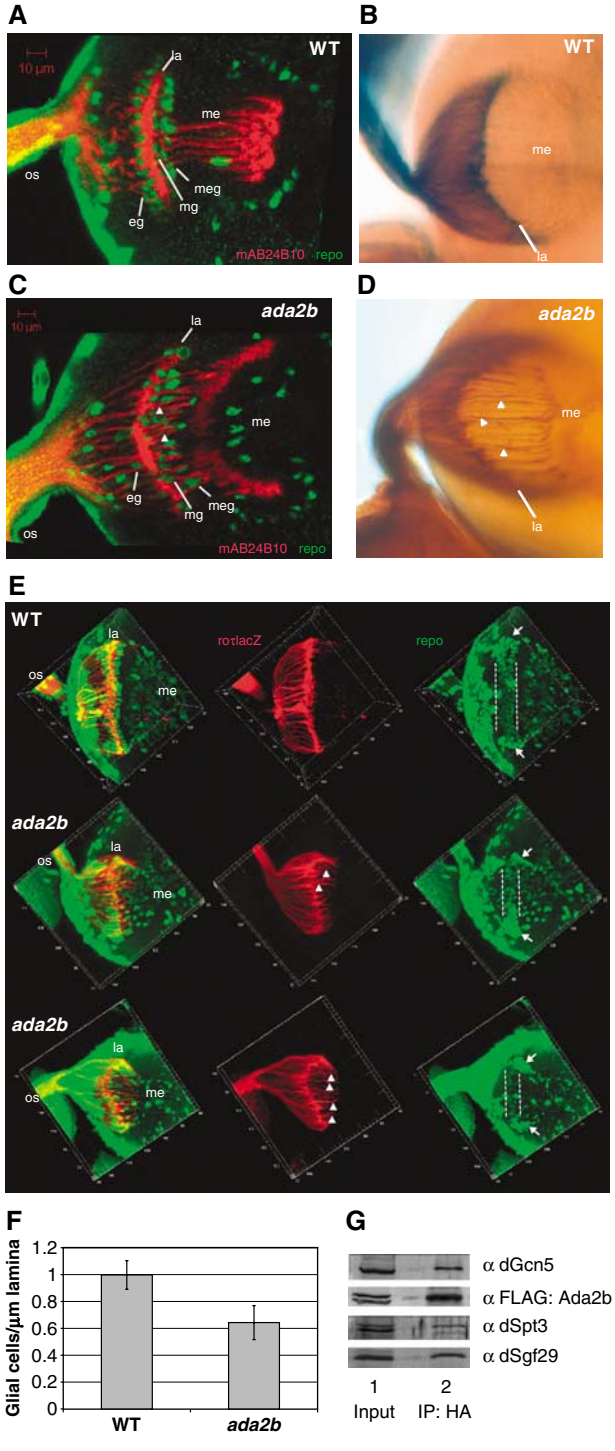

Figure 6 Axon targeting is disrupted in the *ada2b* mutant. (**A**–**E**) The R-cell projection pattern in eye–brain complexes from wild-type and *ada2b* larvae were examined using mAB24B10 (R1–R8, red; A, C) or *ro-τlacZ* (R2–R5; B, D, E) relative to the glial cell position (anti-repo, green; A, C, E). A single plane is shown in A–D, and 3D reconstructed images in E. In the wild type, R1–R6 axons terminate in the lamina (la) between rows of epithelial glial cells (eg), and marginal (mg) and medulla (meg) glial cells. R7–R8 project through the lamina into the medulla (me). In the *ada2b* mutant, some R1–R6 cells project inappropriately into the medulla (arrowheads), and there are gaps in the lamina plexus accompanied by a reduction in glial cell number. (E) More glial cells are present at the ventral and dorsal margins (arrows) of the lamina (dotted lines) in *ada2b* relative to the wild type. Two different images representing the variability in axon mistargeting in *ada2b* are shown in (E). Scale bars, 20 μm. (**F**) The number of glial cells along a given length of the lamina was compared in wild type and *ada2b* as described in Figure 2. (**G**) Extract from *UAS-Ada2b-HAFL$_2$/gcm-GAL4* larval CNS/eye-antennal disc complexes was incubated with HA-agarose beads. Immunoprecipitated material (IP, lane 2) was analyzed relative to 1% input (lane 1) by western blotting.

modifications and gene expression caused by mutations in *nonstop* and *sgf11* relative to *ada2b* suggested that the axon-targeting defect observed in *nonstop* and *sgf11* might be independent of dSAGA. If this axon-targeting defect is due to the role of Nonstop and Sgf11 within dSAGA, then mutations in other components of dSAGA should result in a similar phenotype. To test this hypothesis, we examined R-cell projection patterns in the *ada2b* and *gcn5* mutants.

R-cell projections in *ada2b* larvae were visualized using a marker for all R cells (mAB24B10; Figure 6A and C) or a marker specific for R2–R5 (*ro-τlacZ*; Figure 6B, D and E). In the *ada2b* mutant, many axons project inappropriately through the lamina into the medulla (arrowheads; Figure 6C–E), as observed for *sgf11* and *nonstop*. We asked whether this targeting defect is accompanied by a loss of glial cells from the lamina region, as it is in the *nonstop* and *sgf11* mutants. Glial cells were counted as described previously over a given length of the lamina in *ada2b* and wild-type optic lobes (Figure 6F). A reduced number of glial cells are present along the lamina in *ada2b* relative to wild-type (compare Figure 6A and C), and slight increases in the number of glial cells at the margins of the lamina region (arrowheads; Figure 6E) are observed. A*da2b* mutants exhibit some variability in the severity of the axon-targeting defect, and examples of a less severely (middle panel; Figure 6E) and more severely affected (lower panel; Figure 6E) *ada2b* optic lobe are shown for comparison.

Mutations in the catalytic HAT subunit of dSAGA, dGcn5 (dKAT2), result in a severe disruption to the R-cell projection pattern (*ro-τlacZ*; Supplementary Figure 4). The presence of dGcn5 in multiple protein complexes in *Drosophila* could explain the increase in the severity of this phenotype compared to *ada2b*, *nonstop* and *sgf11*. We observed that *gcn5* eye discs are much smaller in size than those of wild-type or *ada2b* larvae (Supplementary Figure 5). This observation is consistent with the defects in cell proliferation and reduction in the size of other imaginal tissues, such as the wing disc in the *gcn5* mutant (Carre *et al*, 2005). Preliminary observations indicate that photoreceptor organization may also be disrupted in *gcn5* eye discs, and thus the defects observed in axon targeting may represent a secondary phenotype in the *gcn5* mutant. Our results suggest that these particular eye disc defects observed in *gcn5* may be independent of dSAGA, because there is no apparent reduction in eye disc size in the *ada2b* mutant.

The axon-targeting defect observed in *sgf11* and *ada2b* is consistent with a role for dSAGA components within glial cells, as previously determined for *nonstop* (Poeck *et al*, 2001). To determine if dSAGA subunits associate within these cells, we asked if a FLAG-HA-tagged component of dSAGA, Ada2b, could co-immunoprecipitate other dSAGA subunits when expressed in glial cells of the optic lobe. To this end, we generated transgenic flies expressing C-terminally FLAG-HA-tagged Ada2b under the control of the UAS/GAL4 system and induced expression in glial cells, committed glial precursor cells, and in lamina neurons and their precursors using the *gcm-GAL4* driver (Chotard *et al*, 2005). Whole-cell extract was prepared from partially dissected CNS/eye–antennal disc complexes from ~500 *UAS-Ada2b-HAFL$_2$/gcm-GAL4* wandering third instar larvae and incubated with HA-agarose. The immunoprecipitated material was analyzed by western blotting for the presence of dSAGA subunits, such as dGcn5 (dKAT2), dSpt3 and dSgf29 (Figure 6G). Tagged Ada2b associates with these dSAGA subunits in glial cells and lamina neurons, indicating that dSAGA components associate within this group of cells.

Taken together, these findings demonstrate that dSAGA is required for axon targeting in the developing visual system, and implicate dSAGA in the regulation of key pathways required for neural development.

dSAGA is required for expression of ecdysone-response genes, including broad

The finding that dSAGA is required for axon targeting in the developing visual system of *Drosophila* led us to ask which of its targets might be important for this particular role. Our examination of the transcriptional defects associated with mutations in the catalytic modules of dSAGA had revealed that among the genes coregulated by both activities, there are a large number involved in the ecdysone-regulated transcriptional program. For example, many of the genes involved in the ecdysone-stimulated secretion of glue proteins by the

Table I Genes in the salivary gland glue protein battery (upper panel) and early ecdysone response genes (lower panel) showing decreased transcript levels in the *nonstop*, *sgf11* and *ada2b* mutant genotypes compared to the wild type

Genes	*nonstop*		*sgf11*		*ada2b*	
	Log_2 ratio	*P*	Log_2 ratio	*P*	Log_2 ratio	*P*
Sgs1	−3.64016	1.01E−05	−3.27933	2.66E−05	−2.44607	0.001506
Sgs3	−4.35852	1.37E−09	−3.91908	4.24E−09	0.111747	0.708226
Sgs4	−4.58538	4.66E−07	−4.21553	1.08E−06	−3.19541	9.45E−05
Sgs5	−5.06071	3.61E−06	−4.72412	6.98E−06	−2.16852	0.01253
Sgs7	−7.18653	1.8E−12	−5.90535	1.52E−11	−0.30724	0.261427
Sgs8	−5.04108	3.25E−08	−5.17932	2.45E−08	−1.35371	0.012536
CG7587	−5.19894	1.44E−06	−3.88678	2.29E−05	−0.44163	0.52952
Eig71Ee	−3.67483	4.9E−07	−3.74932	4E−07	−1.27376	0.013171
sage	−2.13898	0.000227	−1.40228	0.001054	−0.37554	0.326419
CG12715	−5.82437	1.07E−07	−4.74204	8.57E−07	0.023168	0.969318
CG13560	−0.83379	9.44E−08	−1.45838	2.58E−10	0.944577	2.1E−07
CG11300	−2.32197	2.2E−08	−1.46392	2.37E−06	0.064045	0.756583
EcR	−0.78515	0.031676	−0.50897	0.139052	−0.42534	0.299746
Eip74EF	−0.83973	0.023857	−1.05929	0.007029	−0.27353	0.500419

Log_2 ratios and *P*-values are shown for three biological replicates.

Table II Genes coregulated in all three mutant genotypes

Genes	*non-stop*	*sgf11*	*ada2b*	GO:molecular function	GO:biological process
Fbp1	−7.02	−6.28	−1.07	Protein transporter activity	Storage protein import into fat body
CG7160	−4.36	−4.03	−1.75	Structural constituent of cuticle	
CG11086	−4.11	−4.90	−1.82		
Sgs4	−4.07	−4.06	−1.71	Structural molecule activity	Puparial adhesion
CG15756	−3.47	−4.11	−1.65		
CG6995	−3.25	−3.18	−1.36	DNA binding; mRNA binding	Regulation of alternative nuclear mRNA splicing, via spliceosome
CG9866	−3.22	−4.34	−1.53		
Est-P	−3.00	−1.65	−2.17	Carboxylesterase activity; receptor binding	Neuromuscular synaptic transmission; signal transduction
CG12534	−2.30	−2.21	−1.13	Flavin-linked sulfhydryl oxidase activity	Electron transport; oxidative phosphorylation
Lcp65Ae	−2.08	−1.09	−2.34	Structural constituent of larval cuticle (sensu Insecta)	
Takr86C	−2.07	−4.08	−1.78	Neuropeptide receptor activity; tachykinin receptor activity; neuropeptide Y receptor activity	G-protein-coupled receptor protein signaling pathway; tachykinin signaling pathway; transmission of nerve impulse
CG6277	−2.06	−1.48	−1.23	Triacylglycerol lipase activity; phospholipase A1 activity	Lipid metabolism
CG40374	−1.99	−2.32	−1.32		
CG1773	−1.89	−1.78	−1.86	Trypsin activity; chymotrypsin activity	Proteolysis
CG14752	−1.78	−1.31	−1.49		
Mcm7	−1.68	−3.59	−1.04	Chromatin binding; ATP binding; DNA replication origin binding; DNA helicase activity; DNA-dependent ATPase activity	DNA replication initiation; pre-replicative complex formation and maintenance
CG31686	−1.67	−2.23	−1.23		
CG18146	−1.63	−1.57	−2.20	Receptor binding; structural molecule activity	Cell communication; cell–matrix adhesion; signal transduction
br	−1.58	−1.39	−1.09	DNA binding; specific RNA polymerase II transcription factor activity; transcription factor activity; hydrolase activity, acting on ester bonds	Autophagy; catabolism; cell death; central nervous system remodeling (sensu Insecta); ecdysone-mediated induction of salivary gland cell autophagic cell death; eclosion
CG3818	−1.49	−3.73	−1.32	Structural constituent of pupal cuticle (sensu Insecta)	
CG4500	−1.48	−1.01	−1.23	Long-chain fatty-acid-CoA ligase activity	Fatty-acid metabolism; mesoderm development
CG11893	−1.40	−1.02	−1.81		
CG8502	−1.28	−1.28	−1.13	Structural constituent of larval cuticle (sensu Insecta)	
Acer	−1.23	−3.91	−1.08	Peptidyl-dipeptidase A activity; zinc ion binding	Proteolysis
CG7342	−1.20	−1.29	−1.43	Organic cation porter activity; carbohydrate transporter activity	Cation transport; extracellular transport
sec13	−1.19	−3.45	−1.06		RNA localization; exocytosis; intracellular protein transport; vesicle budding
CG11509	−1.09	−1.25	−1.03		
CG12951	−1.03	−1.41	−1.38	Serine-type endopeptidase activity; chymotrypsin activity; trypsin activity	Proteolysis

Genes showing decreased transcript levels greater than two-fold in the *nonstop*, *sgf11* and *ada2b* genotypes compared to the heterozygotes. Log_2 ratios are shown for two biological replicates ($P<0.05$ for all genes shown).

salivary gland (Li and White, 2003) are strongly repressed in the dSAGA mutants (Table I). This observation is consistent with the previous finding that there is a strong reduction in the size of the early ecdysone puffs in salivary gland polytene chromosomes from *gcn5* mutants (Carre *et al*, 2005).

One early-ecdysone response transcription factor, *broad*, is repressed in all three mutant genotypes (Table II). *broad* encodes a family of zinc finger isoforms that regulate a variety of developmental processes, including morphogenetic furrow progression and photoreceptor specification in the developing eye (Brennan *et al*, 2001). Studies on mushroom body neuronal remodeling in flies indicate that ecdysone is required to stimulate glial cell infiltration of neurons during axon pruning (Lee *et al*, 2000; Awasaki and Ito, 2004). Although loss of the EcR-B isoform alone does not appear to affect the R-cell projection pattern in larvae, mutations inactivating all EcR isoforms are embryonic lethal (Schubiger *et al*, 1998). A second candidate gene that might influence the differentiation or migration of glial cells within the optic ganglia is *Takr86C* (*NKD/CG6515*), which encodes a receptor for tachykinin similar to the mammalian NK1–3 receptors (Monnier *et al*, 1992; Johnson *et al*, 2003). *Takr86C* is also repressed in all three mutant genotypes (Table II). However, it is likely that dSAGA may function as a coactivator required by more than one genetic pathway affecting eye development. Furthermore, none of the mutations identified thus far in

dSAGA are viable beyond the late larval/early pupal stage, indicating that dSAGA is essential for development.

Discussion

In this study, we have identified a novel role for the coactivator complex dSAGA in *Drosophila* neural development. The Gcn5 (KAT2) HAT acts as the catalytic subunit of the yeast SAGA, SLIK, ADA and A2 multi-subunit protein complexes (Grant *et al*, 1997; Lee and Workman, 2007). Although many studies in multicellular organisms have focused on the HAT activity of the Gcn5 (KAT2) complexes, SAGA itself possesses a second catalytic activity. In addition to its HAT activity, yeast SAGA contains an H2B deubiquitinating enzyme, Ubp8 (Henry *et al*, 2003; Daniel *et al*, 2004). Ubp8 functions as part of a modular subunit domain within yeast SAGA that contains two additional proteins, Sgf11 and Sus1 (Ingvarsdottir *et al*, 2005; Lee *et al*, 2005; Kohler *et al*, 2006). Previous studies on histone deubiquitination have focused on its role in transcription in yeast (Henry *et al*, 2003; Lee *et al*, 2005). In this study, we have characterized a role for histone deubiquitination in gene regulation in *Drosophila*.

We demonstrate in this study that Nonstop and Sgf11 constitute the H2B deubiquitination module within dSAGA. Moreover, both Nonstop and Sgf11 are required for correct axon targeting in the developing visual system. As in yeast, the two catalytic functions of SAGA are separable (Ingvarsdottir *et al*, 2005; Lee *et al*, 2005). However, mutations that differentially affect the two catalytic activities of dSAGA have overlapping but distinct effects on gene expression. Despite the differences in activity, both catalytic modules of dSAGA are required for correct axon targeting in the optic lobe. This implicates dSAGA in the regulation of pathways essential for neural development in higher eukaryotes.

Our analysis of the axon-targeting defects in the *nonstop*, *sgf11* and *ada2b* mutants indicates that the R-cell misprojection phenotype is associated in all three cases with a loss of glial cells from the lamina region of the optic lobe, and an increase in the number of glial cells at the dorsal and ventral margins of the lamina. Clonal analysis indicates that *nonstop* glial cells fail to migrate from these dorsal and ventral regions into the lamina plexus (Poeck *et al*, 2001). This suggests that dSAGA may be required within glial cells to regulate pathways important for their migration. It is possible that the requirement of dSAGA may be due to its role in the ecdysone response as indicated by our microarray analysis of genes downregulated in *nonstop*, *sgf11* and *ada2b* mutant larvae. However, further studies on the role of dSAGA in these glial cells will be required to determine which pathway(s) are primarily responsible for the axon-targeting defect observed in these dSAGA mutants.

Mutations that affect the deubiquitination activity of dSAGA, such as *nonstop* and *sgf11*, appear to result in a more severe axon-targeting defect than those that affect the acetylation activity, such as *ada2b*. However, there is some variability in the degree of expressivity of the phenotype in all three mutant genotypes, and some *ada2b* mutants show phenotypes very similar to *nonstop* and *sgf11*. It is evident from studies on yeast SAGA that both enzymatic activities are required for optimal transcription upon gene induction (Henry *et al*, 2003). The variability in the *ada2b* mutant may be due to the large maternal contribution of Ada2b (Kusch *et al*, 2003), and it is notable that the *ada2b* mutant larvae show considerably less developmental delay in comparison to *nonstop*, *sgf11* and *gcn5*. Thus far, mutations in other components of dSAGA, such as *wda* and *nipped-A*, have not been examined for this phenotype because these do not reach the third instar larval stage of development (Gause *et al*, 2006; Guelman *et al*, 2006b).

Although in this study we observed a specific effect of the dSAGA deubiquitination module on histone H2B, it remains likely Nonstop and Sgf11 are also required for deubiquitination of other target proteins within the cell. Poeck *et al* (2001) observed an increase in the level of three ubiquitinated proteins of 29, 55 and 200 kDa in extracts from *nonstop* third instar larval tissue relative to wild-type extracts. The smallest of these may correspond to ubiquitinated H2B, but the others may correspond to as yet unidentified potential targets of the deubiquitination module within dSAGA.

It is likely that the role of SAGA and H2B deubiquitination in neural development may be conserved in mammalian systems, as there is a striking degree of similarity between Sgf11 and ATXN7L3/ATXN7 in humans. ATXN7 is a subunit of the human STAGA and TFTC complexes (Helmlinger *et al*, 2004). Polyglutamine expansions in the *Spinocerebellar ataxia type 7* (*sca7*) gene, encoding ATXN7, result in a dominant neurodegenerative disorder that affects the retina (David *et al*, 1997). There is increased recruitment of STAGA/TFTC containing this polyQ-expanded ATXN7 at certain promoters in a mouse SCA7 model, resulting in hyperacetylation of H3 (Helmlinger *et al*, 2006). Interestingly, despite this increased promoter recruitment and hyperacetylation, these genes show decreased levels of transcription. However, in other studies incorporation of polyQ-expanded ATXN7 into STAGA reduces the acetyltransferase activity of the complex (Palhan *et al*, 2005). The effect of the polyQ expansions in ATXN7 on the putative deubiquitination activity of mammalian STAGA/TFTC has not yet been examined.

The Verrijzer group has demonstrated that another ubiquitin protease in flies, USP7, specifically deubiquitinates histone H2B *in vitro* (van der Knaap *et al*, 2005). However, RNAi of USP7 had little effect on global levels of ubiquitinated H2B, and we find that USP7 cannot functionally replace UBP8 in yeast. Yeast Ubp15, the putative homolog of USP7, also deubiquitinates H2B *in vitro*, but the biological role of this activity is presently unknown (Supplementary Figure 3). In addition to Ubp8 in yeast, Ubp10 is also required for deubiquitination of H2B at the telomeres and at the rDNA locus (Emre *et al*, 2005). It is unknown whether the putative homolog of Ubp10 in flies, CG15817, is also required for H2B deubiquitination and gene silencing (Figure 1B). It is intriguing that both Ubp10 and USP7 (via an interaction with the Polycomb complex) function in regulating heterochromatin structure, while Ubp8/Nonstop are required for active transcription (van der Knaap *et al*, 2005). It remains to be determined whether alternative mechanisms for regulating histone ubiquitination at distinct chromatin environments exist within the genomes of higher eukaryotes.

Taken together, our results advance the understanding of the role of histone deubiquitination in transcription, while demonstrating a novel role for SAGA in regulating neural development in the visual system of *Drosophila*. These findings have implications for the use of *Drosophila* as a model system to understand the underlying mechanisms of neurodegenerative diseases.

Materials and methods

Phylogenetic analysis

Alignments were performed using web-based CLUSTALW (http://align.genome.jp), followed by the Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). The aligned sequences were then subjected to phylogenetic tree evaluation using standard N-J tree generator available through CLUSTALW.

DNA constructs

The LD43147 and IP07752 cDNAs were cloned into the S2 cell expression vectors pRmHa3-CHA_2FL_2 (Guelman *et al*, 2006a) and pMT-V5His (Invitrogen). Ada2b was cloned into pUAST-$CHAFL_2$. LD43147, *USP7* (*CG1490*) and *CG5384* were cloned into pRS415. Additional details are provided in Supplementary data.

Co-immunoprecipitations and western blots

Co-immunoprecipitations and affinity purifications of dSAGA using anti-FLAG or anti-HA antibodies were performed as described previously (Guelman *et al*, 2006a). S2 cells were transfected using Effectene (Qiagen) and induced with 0.5 mM $CuSO_4$ prior to preparation of whole-cell extracts for immunoprecipitation. The following antibodies were used in western blots: dGcn5 (dKAT2) (rabbit, 1:3000); dSpt3 (rabbit, 1:1000); Ada2b (guinea pig, 1:1000); dSgf29 (rabbit, 1:500); tubulin (mouse, 1:5000; Developmental Studies Hybridoma Bank (DSHB)); HA-HRP (mouse, 1:5000; Sigma); FLAG-HRP (mouse, 1:5000; Sigma); V5 (mouse, 1:10 000; Sigma); H3 (rabbit, 1:3000; Abcam); acetylated H3 Lys-9 (rabbit, 1:1000; Abcam) and H2B (rabbit, 1:1000; Upstate).

Genetics

The *nonstop*, *sgf11* and *gcn5* fly stocks used in this study were provided by the Bloomington Drosophila Stock Center at Indiana University. Stock numbers are indicated after each genotype. w^{1118}; $PBac\{w^{+mC}=RB\}CG13379^{e01308}$/*TM6B*, Tb^1 (17941) contains a *piggyBac* insertion in the promoter region of *sgf11*. Excision of the *piggyBac* transposon by crossing *sgf11* to w^{1118}; *CyO*, $P\{Tub\text{-}PBac\}2/wg^{Sp-1}$ (8285) completely restores viability. $P\{ry^{+t7.2}=PZ\}not^{02069}$ ry^{506}/*TM6B*, $r^{CB}Tb^{+}$ (11553) corresponds to the not^2 allele (Martin *et al*, 1995; Poeck *et al*, 2001). w^{1118}; $Pcaf^{E333st}P\{w^{+mW.hs}=FRT(w^{hs})\}2A$ $e^1/TM3$, $P\{w^{+mC}=ActGFP\}JMR2$, Ser^1 (9333) corresponds to the $gcn5^{E333st}$ allele (Carre *et al*, 2005). The $ada2b^1$ mutant flies were kindly provided by Matthias Mannervik (Qi *et al*, 2004). The *sgf11*, *nonstop* and *ada2b* mutants were crossed to the stock w^{1118}; $Dr^{mio}/TM3$, $P\{w^{+mC}=GAL4\text{-}twi.G\}2.3$, $P\{UAS\text{-}2xEGFP\}AH2.3$, Sb^1Ser^1 (6663) to generate EGFP balanced stocks. Homozygous mutant embryos were identified as described previously (Guelman *et al*, 2006b) and populations sorted using the CoPas Plus (Union Biometrica). The *gcm-GAL4* flies were kindly provided by Iris Salecker.

Immunohistochemistry

CNS/eye–antennal disc complexes from wandering third instar larvae were stained using the following antibodies: anti-chaoptin (mAb24B10, mouse, 1:100; DSHB) (Fujita *et al*, 1982); anti-Elav (9F8A9, mouse, 1:100; DSHB) (O'Neill *et al*, 1994); anti-repo (rabbit, 1:500; Joachim Urban) (Halter *et al*, 1995); anti-β-galactosidase (mouse, 1:5000; Promega); anti-histone H3 (phospho S10) (ab5176, rabbit, 1:100; Abcam); phalloidin Alexa 594 (1:250; Molecular Probes); goat anti-mouse Alexa Fluor 568 (1:300; Molecular Probes); goat anti-mouse Alexa 647 (1:300; Molecular Probes); goat anti-rabbit Alexa Fluor 488 (1:300; Molecular Probes); anti-mouse poly-HRP (ImmunoVision). Laser-scanning confocal imaging was performed using a Zeiss (Thornwood, NY) LSM 510 META attached to a Zeiss Axiovert 200M inverted microscope. Confocal images were collected using either a $\times 40/1.2$ NA water immersion C-Apochromat objective or a $\times 40/1.3$ NA oil immersion Plan-Neofluar objective at variable scanning zoom. Confocal images are presented either as single planes generated in Zeiss AIM software or as 3D reconstructed images using Imaris (Bitplane). Brightfield imaging was performed using a $\times 40/1.3$ NA oil immersion Plan Neofluar objective on a Zeiss Axiovert 200M upright microscope equipped with a Zeiss AxioCam HRc r1.4 digital color camera connected by a $\times 0.63$ coupler. Repo-positive glial cells with a 2.5 μm minimum diameter were identified and counted digitally using Imaris in 11 samples of each genotype along a measured length (40–100 μm) of the lamina plexus.

Gene expression analysis

RNA was isolated in triplicate from separate collections of late third instar larvae of the appropriate genotype using TriZol (Invitrogen) and mRNA enriched using Oligotex beads (Qiagen). Arrays consist of 14 593 unique 70-mer oligonucleotides corresponding to version 1.1 of the *D. melanogaster* set (Operon). Microarray data are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-TABM-383.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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