Enzymatic modules of the SAGA chromatin-modifying complex play distinct roles in *Drosophila* gene expression and development

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The Spt–Ada–Gcn5–acetyltransferase (SAGA) chromatin-modifying complex is a transcriptional coactivator that contains four different modules of subunits. The intact SAGA complex has been well characterized for its function in transcription regulation and development. However, little is known about the roles of individual modules within SAGA and whether they have any SAGA-independent functions. Here we demonstrate that the two enzymatic modules of *Drosophila* SAGA are differently required in oogenesis. Loss of the histone acetyltransferase (HAT) activity blocks oogenesis, while loss of the H2B deubiquitinase (DUB) activity does not. However, the DUB module regulates a subset of genes in early embryogenesis, and loss of the DUB subunits causes defects in embryogenesis. ChIP-seq (chromatin immunoprecipitation [ChIP] combined with high-throughput sequencing) analysis revealed that both the DUB and HAT modules bind most SAGA target genes even though many of these targets do not require the DUB module for expression. Furthermore, we found that the DUB module can bind to chromatin and regulate transcription independently of the HAT module. Our results suggest that the DUB module has functions within SAGA and independent functions.

[*Keywords*: SAGA complex; deubiquitinase module; embryogenesis; transcription]

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Histone modifications, such as acetylation and ubiquitination, play a key role in facilitating a number of nuclear events, including transcriptional regulation. Acetylation of histones is largely associated with relaxing chromatin structures in order to support the entry of transcriptional machinery to the genomic loci for activation, while histone ubiquitination has been linked to both gene activation and repression (Brownell et al. 1996; Weake and Workman 2008). The histone-modifying enzymes that catalyze these post-translational modifications are often integrated into large complexes to facilitate their enzymatic activity, substrate specificity, and genomic localization.

SAGA (Spt–Ada–Gcn5–acetyltransferase) is a 2-MDa transcription coactivator protein complex that contains several distinct modules of subunits. These include a transcription activator interaction module, a TATA-binding protein (TBP) interaction module, and two enzymatic modules: the histone acetyltransferase (HAT) module and the deubiquitinase (DUB) module. Given the role of SAGA in transcription regulation, it is not surprising that some SAGA subunits are essential for development, especially subunits of the enzymatic modules. Gcn5 is the acetyltransferase subunit of SAGA and ADA (yeast) and ATAC (metazoans) complexes (Grant et al. 1997; Martinez et al. 1998, 2001; Ogryzko et al. 1998; Guelman et al. 2006; Demeny et al. 2007). *Drosophila* Gcn5 is required for metamorphosis and oogenesis (Carre et al. 2005). In mice, GCN5-null embryos die during embryogenesis (Xu et al. 2000), and GCN5 HAT activity is critical for cranial neural tube closure (Bu et al. 2007). The SAGA-specific HAT module subunit Ada2b, which is required for the HAT activity, is also essential for *Drosophila* viability (Kusch et al. 2003; Muratoglu et al. 2003; Qi et al. 2004; Pankotai et al. 2005; Zsindely et al. 2009).

The DUB module subunits also control developmental processes. In *Drosophila*, the Non-stop (DUB) and Sgf11

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function together in neural development (Weake et al. 2008). The mouse DUB USP22 is critical for the early embryogenesis (Lin et al. 2012) and has also been shown to play a role in cell differentiation and lineage specification (Kosinsky et al. 2015).

Several studies have investigated the structural features of SAGA and how SAGA subunits integrate into the whole complex. The HAT module subunits were lost from SAGA when purified from an *ada2*Δ strain, suggesting that the Ada2 is essential to anchor the HAT module (Lee et al. 2011). Similarly, Sgf73 anchors the DUB module into SAGA (Lee et al. 2009). These results are confirmed by separate groups using electron microscopy (EM) analysis and cross-linking studies (Han et al. 2014; Setiaputra et al. 2015). Moreover, the N terminus of Sgf73 is necessary for DUB module activation and its recruitment to SAGA. (Kohler et al. 2008, 2010). Notably, the DUB module can disassociate from SAGA under some conditions and remain stable. In yeast, a functional DUB module can be separated from SAGA by the proteasome regulatory particle (Lim et al. 2013). In *Drosophila*, loss of the Sgf73 homolog Ataxin-7 leads to disassociation of an enzymatically active DUB module (Mohan et al. 2014). In humans, the DUB module is still stable in SPT20 knockdown cells. Thus, the integrity of the DUB module appears to be independent of SAGA, suggesting that it may be able to exist as a free form (Nagy et al. 2009).

In this study, we took advantage of genetic approaches in *Drosophila* to remove the maternal contribution and investigated the requirement for SAGA HAT and DUB modules in early development. We found that perturbation of the HAT module caused defects in oogenesis. In contrast, DUB activity was expendable during oogenesis, and at least some early zygotic genes were transcriptionally active. Nevertheless, it was essential for normal cellularization in these early embryos and their survival through embryogenesis. It was also essential for wild-type levels of transcription. We identified binding sites for several SAGA subunits genome-wide in early embryos and found coincident binding at most sites. Notably, the DUB module bound to chromatin with other SAGA subunits even when it was not required for transcription of the associated genes. More interestingly, we identified sites in which DUB module subunits bound chromatin independently of the core SAGA modules, where it regulated transcription.

Results

The HAT module is required for oogenesis, whereas the DUB is expendable

Since the SAGA complex contains distinct functional modules, we sought to investigate whether all of the modules of SAGA are required coordinately or whether different modules play distinct roles during development. We focused on the two enzymatic modules, which contain the HAT activity and the DUB activity. We first evaluated whether both the HAT activity and the DUB activity were required at the earliest stages of development, during oogenesis. We used the *FLP/FRT/ovoD* system (Chou and Perrimon 1996) to delete subunits of the DUB and HAT modules in the germline cells of the germline clone (GLC) females' ovaries. This analysis was carried out with null alleles of SAGA-specific subunits Ada2b, which is required in the HAT module for its activity (Kusch et al. 2003; Muratoglu et al. 2003; Qi et al. 2004); Ataxin-7, which anchors the DUB module to the remainder of SAGA; and the DUB subunit Non-stop (Supplemental Fig. S1).

We found that oogenesis is blocked in *Ada2b* GLC females, which did not lay eggs. This result is consistent with previous data that *Ada2b* germ cells arrest at an early stage of oogenesis (Qi et al. 2004). To determine the stage at which Ada2b was required, we examined the morphology of dissected ovaries. Overall, *Ada2b* mutant egg chambers developed to stage 10, when nuclei began to exhibit degeneration (Fig. 1A), and apoptotic cell death was observed (Supplemental Fig. S2). Differential interference contrast images also revealed degeneration of the oocyte prior to maturity (Supplemental Fig. S3). In contrast with the *Ada2b* mutants, oogenesis proceeded normally in both the *Ataxin-7* and *non-stop* GLC females, suggesting that oogenesis is not dramatically affected by disrupting the DUB activity of SAGA. Moreover, nuclei in these developing oocytes were normal (Fig. 1A), and the females laid eggs. Whole dissected ovaries show the dramatic impact

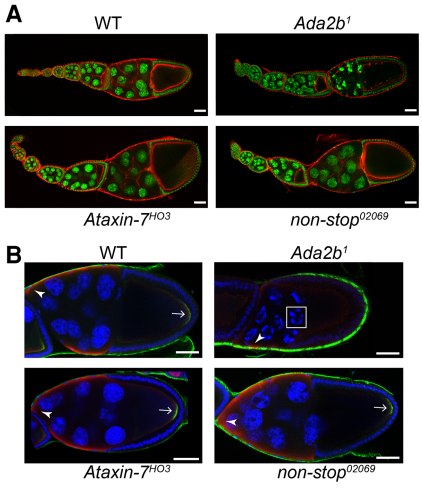

Figure 1. The HAT module is required for oogenesis, whereas the DUB is expendable. (*A*) Whole ovaries were stained with phalloidin (red) for actin and DAPI (green) for nuclei. Posterior is to the right in all panels. (*B*) Vasa protein was observed in the anterior end of stage 10 oocytes for all genotypes (red; arrowhead). Staufen was observed at the posterior end of wild-type, *Ataxin-7*^{HO3}, and *non-stop*^{02069} GLC oocytes (green; arrow). The muscle sheath that surrounds the ovariole shows prominent but likely nonspecific staining with the Staufen antibody. DAPI (blue) revealed degenerating germline nurse cell nuclei in *Ada2b*^{1} egg chambers (highlighted by a white box). The genotypes of GLC ovaries were as follows: (WT) Oregon R; (*Ada2b*^{1}) *hs-Flp*/+; *ada2b*^{1}, *FRT*^{82B}/*FRT*^{82B}, *ovo*$^{D1-18}$; (*non-stop*^{02069}) *hs-Flp*/+; *non-stop*^{02069}, *FRT*^{2A}/*FRT*^{2A}, *ovo*$^{D1-18}$; (*Ataxin-7*^{HO3}) *hs-Flp*/+; *Ataxin-7*^{HO3}, *FRT*^{40A}/*FRT*^{40A}, *ovo*$^{D1-18}$. Bars: *A*,*B*, 50 µm.

of loss of Ada2b and the normal state of the *non-stop* and *Ataxin-7* GLCs (Supplemental Fig. S4). Egg laying rates for GLC females are shown in Supplemental Table S1.

To examine these mutant ovaries in more detail and begin to assess requirements for gene expression, we stained ovaries with the germline markers Vasa and Staufen, which play critical roles in germ plasm assembly during oogenesis (Brendza et al. 2000; Johnstone and Lasko 2001; Wilkie et al. 2003). Interestingly, we detected weak localized Vasa protein but no localized Staufen protein in the degenerating *Ada2b* germline cells (Fig. 1B). However, Staufen was detected and localized to the posterior end of earlier stage oocytes (Supplemental Fig. S5). These results suggest that *vasa* and *staufen* mRNA transcription still occurs in the absence of Ada2b. Not surprisingly, in *Ataxin-7* and *non-stop* GLC females' ovaries, those two proteins were still expressed and localized at the right places. Taken together, we conclude that the HAT module is required for normal oogenesis but not for transcription of all genes. However, oocytes lacking Non-stop or Ataxin-7 in the DUB module develop normally.

The HAT module plays a critical role in transcriptional regulation in the ovary

Although clearly some transcription occurred in ovary germ cells lacking Ada2b, as evidenced by the expression of Staufen and Vasa, the degeneration of these oocytes prompted us to investigate transcription defects genome-wide using RNA sequencing (RNA-seq). We hypothesized that comparison of gene expression in oocytes lacking Ada2b with those lacking Ataxin-7 or Non-stop would allow us to identify genes requiring the SAGA HAT activity but not the DUB activity for their expression. The ovaries include both the germline-derived oocytes and overlying somatic follicle cells. The latter are wild type for these SAGA subunits in the GLCs. We were therefore unable to evaluate changes in expression of some genes in the oocytes if their expression in follicle cells was higher. However, this analysis did reveal those genes that differ between the HAT and the DUB modules in the ovary germline cells. Consistent with the severe phenotype, >1000 genes changed expression in *Ada2b* GLC ovaries. In contrast, expression of only a few hundred genes was affected by absence of Ataxin-7 or Non-stop (Fig. 2A). We also found that very few genes overlapped between the three mutants, suggesting the possibility that the DUB module is exclusively required for the expression of some genes. Because of the presence of the overlying wild-type follicle cells, we are unable to identify genes that do not require the HAT module in the ovary. Nevertheless, these studies reveal different requirements for the DUB and the HAT modules in developing oocytes, with the latter playing a greater role.

To determine whether specific gene networks and pathways have a common requirement for the HAT activity during oogenesis, we performed gene ontology (GO) term analysis (Fig. 2B). Genes involved in DNA replication, eggshell formation, and chromosome organization were significantly down-regulated in the absence of Ada2b. Specifically, genes involved in DNA repair were down-regulated in *Ada2b* GLC ovaries, which may explain the nuclear degeneration phenotype (Supplemental Table S2). Similar analysis in *Ataxin-7* or *non-stop* GLC ovaries revealed no significant enrichment. These data

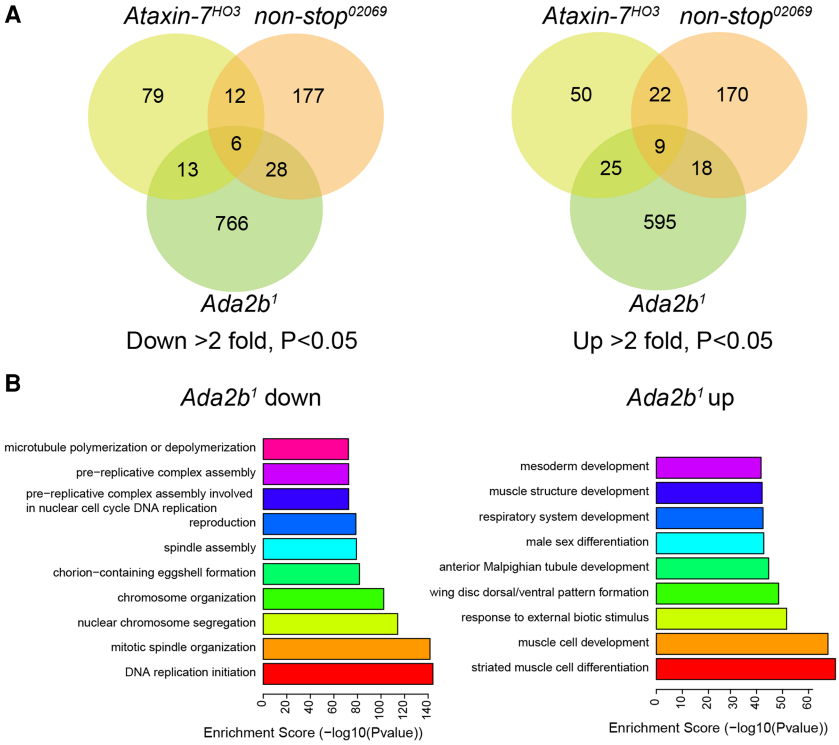

Figure 2. RNA-seq analysis reveals that the HAT module plays a critical role in transcriptional regulation in the ovary. (*A*) Venn diagrams showing the overlapping genes with decreased or increased transcript levels in *Ada2b*^{1}, *Ataxin-7*^{HO3}, and *non-stop*^{02069} GLC ovaries. Fold change > 2; $P < 0.05$; FPKM [fragments per kilobase per million mapped fragments] > 1. (*B*) Gene ontology (GO) term analysis of the biological process (BP) of genes down-regulated or up-regulated in *Ada2b*^{1} GLC ovaries. A complete list of enriched GO terms with an adjusted *P*-value of <0.05 is in Supplemental Table S2.

correspond well with the different phenotypes shown earlier and support our earlier observations that nuclei degenerate in *Ada2b* oocytes and that the DUB module is expendable for oogenesis.

Early patterning still occurs in embryos lacking Ataxin-7 or non-stop

To investigate whether the DUB module plays a role in embryogenesis, we examined the fertilized embryos from *Ataxin-7* and *non-stop* GLC females. We focused on stage 5 embryos and confirmed from RNA-seq analysis that no maternal load or zygotic transcripts of *Ataxin-7* or *non-stop* were yet detectable (Supplemental Fig. S1C). Therefore, we inferred that the presence of a wild-type zygotic copy from the male upon fertilization did not impact analysis at this early stage. We then examined the anterior–posterior (A–P) and dorsal–ventral (D–V) axes of these embryos. The pair rule gene *even-skipped* (*eve*) contributes to axis specification, and its zygotic expression is visible in a seven-stripe pattern along the A–P axis (Macdonald et al. 1986). The basic helix–loop–helix (bHLH) factor *twist* is one of the earliest zygotically active genes along the D–V axis, and its expression along the ventral side of the embryos specifies mesoderm (Thisse et al. 1987). As shown in Figure 3A, Eve was apparent in a seven-stripe pattern in *Ataxin-7* and *non-stop* GLC embryos in a pattern that is reminiscent of wild type, suggesting both that *eve* is expressed and that the A–P axis forms in the absence of DUB module subunits. We also found that *twist* was expressed in the presumptive mesoderm and localized on the ventral side, indicating formation of an early D–V axis. We therefore conclude that transcription of some early zygotic genes occurs in early embryogenesis independent of the DUB module of SAGA.

Loss of DUB module subunits affects cellularization and gene expression

Although expression of some zygotic genes was initiated and early patterning occurred, we did observe defects in cellularization. Normally, the newly fertilized embryo undergoes several rounds of nuclear divisions without cytokinesis and remains syncytial. The nuclei then migrate to the periphery of the embryo, at which time they become surrounded by individual cell membranes (Mazumdar and Mazumdar 2002). In wild-type embryos, nuclei at the surface of the embryos formed an organized pattern (Fig. 3B, C). However, in *Ataxin-7* and *non-stop* GLC embryos, nuclear Twist staining revealed a disorganized pattern, with the absence of nuclei in some locations. Expression of the membrane marker Discs-large (Dlg) revealed misshapen membranes in these regions. DAPI staining of all nuclei indicated that these defects were not limited to the ventral side (Fig. 4A). Notably, we were unable to distinguish the male chromosome provided to the egg upon fertilization by heterozygous mutant males and the genotype of the resulting embryos at this stage ($m^{-/-}$, $z^{-/-}$ vs. $m^{-/-}$, $z^{-/+}$). However, the above defects were observed in all embryos

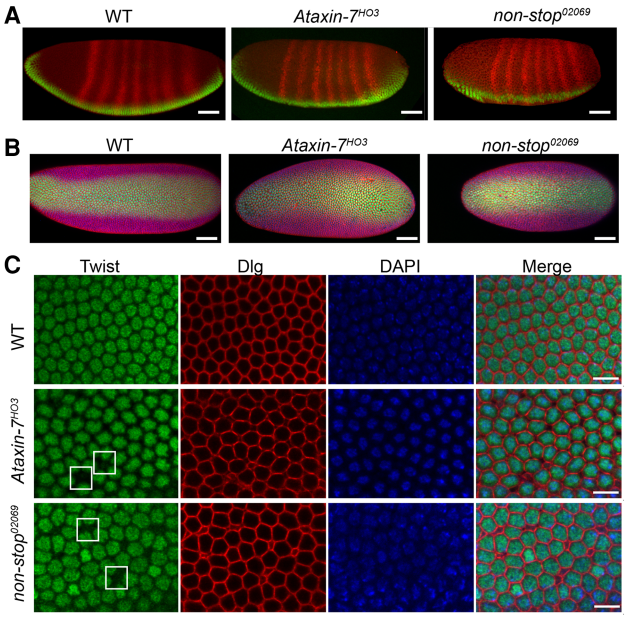

Figure 3. A–P patterning, segmentation, and mesoderm specification occur in *Ataxin-7* and *non-stop* GLC embryos, but these embryo exhibit defects in cellularization. (*A*) Eve (red) staining was observed in the expected seven-stripe pattern in stage 5 GLC embryos, and Twist staining (green) revealed specified mesoderm on the ventral side of these embryos. Anterior is to the left and ventral is to the bottom. (*B*) Stage 5 GLC embryos were stained with Discs-large (Dlg; red) to mark membranes, Twist (green) to mark mesodermal nuclei, and DAPI (blue) to mark all nuclei. A ventral view with anterior to the left is shown. Disruptions in the organization of Twist-expressing nuclei are apparent at this low magnification. (*C*) High magnification of stage 5 embryos shows defects in organization of the migrated nuclei at the time of cellularization. Staining was as in *B*. Boxes denote positions in which nuclei on the surface appear to be absent. The embryos were collected from crosses as follows: (WT) Oregon R; (*Ataxin-7^{HO3}*) *Ataxin-7* GLC females crossed to *Ataxin-7^{HO3}/Cyo*; (*non-stop^{02069}*) *non-stop* GLC females crossed to *non-stop^{02069}/TM3*. Bars: *A*,*B*, 50 µm; *C*, 10 µm.

independent of the zygotic copy provided by the male. Last, the ovary and embryonic phenotypes of *Ataxin-7^{HO3}* were confirmed with *Ataxin-7^{2A-1}* (Supplemental Fig. S6). Thus, we conclude that these defects are a consequence of the absence of maternal Ataxin-7 or Non-stop.

Interestingly, we noted the presence of mislocalized nuclei in the interior of the embryo underneath the distorted regions (Fig. 4A). Time-lapse imaging revealed that these nuclei were actually able to migrate to the periphery but did not maintain this position and fell back into the interior of the embryos (Supplemental Movie S1). Despite the ability of most nuclei to remain at the periphery, these nuclei also exhibited defects in cellularization. Tubulin and Enabled revealed that cell membrane invagination was defective in GLC embryos (Fig. 4A). Real-time PCR showed that genes known to be involved in cellularization and nuclear anchoring (*kuk*, *slam*, *bnk*, *Sry-α*, and *nullo*) (Ibnsouda et al. 1993; Schejter and Wieschaus 1993;

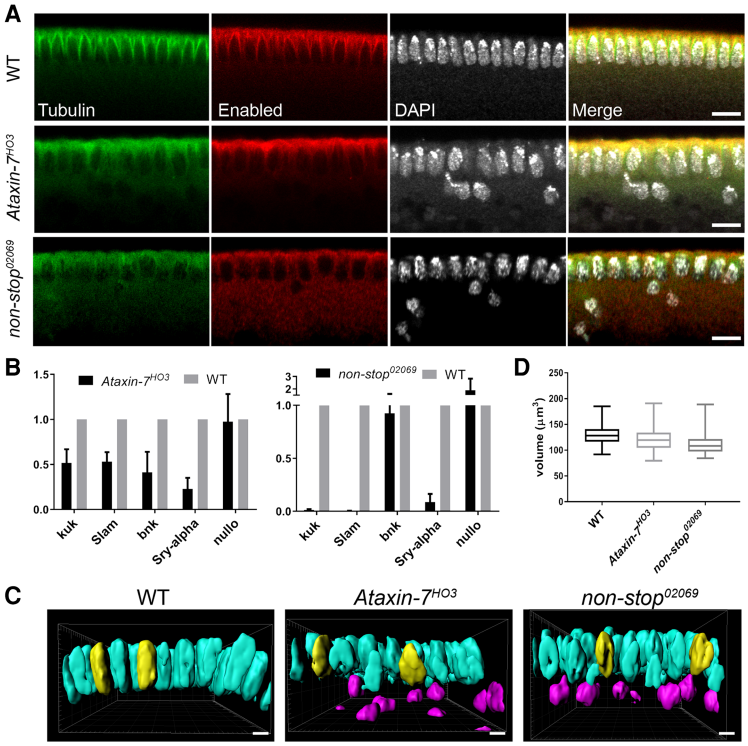

Figure 4. Similar defects in cellularization, gene expression, and nuclear shape are observed upon loss of the DUB module subunits Ataxin-7 and Non-stop. (*A*) Stage 5 embryos were stained with α-Tubulin (green) and Enabled (red) to visualize invaginating membranes during cellularization. DAPI (gray) marked nuclei. (*B*) Real-time PCR was performed on cDNA isolated from stage 5 wild-type, *Ataxin-7*^{HO3}, and *non-stop*^{02069} GLC embryos, and expression of genes associated with cellularization was assessed. *Actin5C* was used as normalization control. (*C*) Screenshots of confocal data analyzed in three dimensions using Imaris to determine shape and volume for Twist-expressing nuclei. The embryo surface is at the top, and interior is at the bottom. Arbitrary coloring highlights nuclei at the surface (cyan), mislocalized nuclei (pink), and differences in nuclear shape (yellow). Three-dimensional movies are provided in Supplemental Movies S2–S4. (*D*) Quantification of nuclear volume of stage 5 embryos for each genotype, obtained from the Imaris analysis in *C*. Genotypes in *A–D* were the same as in Figure 3. Bars: *A*, 10 µm; *C*, 5 µm

Pritchard and Schubiger 1996; Stein et al. 2002; Brandt et al. 2006; Pilot et al. 2006; Chen et al. 2013) depend on the DUB module and change expression in *non-stop* and *Ataxin-7* GLC embryos. In conjunction with similar loss-of-function phenotypes for *kuk*, *non-stop*, and *Ataxin-7*, we conclude that cellularization and nuclear anchoring are abnormal as a consequence of these decreases in expression. As shown in Figure 4B, transcript levels of these genes changed in the absence of Ataxin-7 or Non-stop (primer sequences are in Supplemental Table S3). Therefore, we conclude that the DUB module plays an important role in regulating transcription prior to cellularization.

We examined the muscle, central nervous system, H3K9ac, and H3K14ac in stage 16 *Ada2b* zygotic mutant embryos. The levels of H3K9ac and H3K14ac were reduced, indicating that the HAT module affects acetylation during embryogenesis, as shown previously (Qi et al. 2004). However, decreased acetylation at this stage does not result in phenotypic defects, possibly indicating that maternal Ada2b is sufficient for early transcription and specification (Supplemental Fig. S7A,B). We also identified Ada2b knockdown conditions that allowed oogenesis to proceed and examined the phenotype of the resulting embryos. The embryos exhibited defects in cellularization, indicating a role for Ada2b in early embryogenesis and suggesting that reduction in Ada2b has consequences similar to those of loss of Ataxin-7 and Non-stop (Supplemental Fig. S7C,D).

We also noted that most nuclei in *Ataxin-7* and *non-stop* GLC embryos were misshapen. To better visualize these shapes and quantitate their volumes, we used the surfacing function of Imaris software. It revealed that a large number of nuclei at both the surface and interior did not adopt the elongated shape characteristic of wild type (Fig. 4C). The defective nuclei in GLC embryos appeared larger than wild type (Fig. 3C), but Imaris surfacing indicates that they are only modestly smaller (Fig. 4D). Given that all *Ada2b* GLC oocyte nuclei appeared to degenerate, we then asked whether these misshapen embryo nuclei in *Ataxin-7* and *non-stop* GLCs were also degenerated. We examined the DNA damage marker γH2Av in *Ataxin-7* GLC embryos, but this marker revealed no damaged nuclei (data not shown). Consistent with this finding, the early defects in embryos lacking the DUB module subunits could be rescued by zygotic expression of a wild-type copy. This rescue was apparent in the muscle pattern of *Ataxin-7* ($m^{-/-}$, $z^{-/+}$) embryos despite their early defects (Supplemental Fig. S8A). In fact, 25% of these embryos survived to first instar larvae (Supplemental Fig. S8B). Thus, we conclude that the distorted shape and defective cellularization described above are not associated with degeneration of the nuclei and, moreover, that the embryo must be able to accommodate or correct these defects.

Despite the modest impact of the absence of DUB module subunits in early development, Ataxin-7 and Non-stop are important for embryogenesis. Notably, 75% of *Ataxin-7* ($m^{-/-}$, $z^{-/+}$) embryos and 100% of *non-stop* ($m^{-/-}$, $z^{-/+}$) embryos died during embryogenesis (Supplemental Fig. S8B). More significantly, zygotic expression is essential, since embryos lacking both maternal and zygotic expression exhibited dramatic defects later in embryogenesis (Supplemental Fig. S8A), suggesting that the presence of the DUB module subunits is critical for embryogenesis.

Altogether, these data demonstrate the importance of the DUB module for wild-type levels of gene expression in the early embryo and overt embryogenesis.

The DUB module regulates a subset of genes in early embryogenesis

Based on the known role of the DUB module in deubiquitination of H2B and the effect of its mutation on expression of several genes above, we wished to explore whether dependence on the DUB module at this stage extended beyond genes associated with cellularization. RNA-seq revealed expression of ~6000 genes at stage 5, as expected (RPKM [reads per kilobase per million mapped reads] > 1), but only 40% of those genes changed expression in *Ataxin-7* and *non-stop* GLC embryos (Fig. 5A). These data support our phenotypic results that while defects were observed, initial patterning of the early embryos was relatively normal.

The similar cellularization defects in *Ataxin-7* and *non-stop* GLC embryos shown above suggests that these proteins function together within the DUB module to regulate transcription. To address this hypothesis, we compared the patterns of gene expression in these GLC embryos and found that ~50% of those genes that changed expression are similarly affected in both mutants (Fig. 5A; Supplemental Fig. S9). We then performed GO term analysis of the genes that are commonly changed. As shown in Figure 5B and Supplemental Table S4, down-regulated genes were enriched in several pathways, primarily those involved in embryonic development and tissue morphogenesis, indicating that these processes were highly dependent on the DUB module. Only genes associated with metabolic processes were enriched in the commonly up-regulated genes. As suggested by the GO term analysis (Supplemental Table S5), some of the up-regulated genes may be secondary effects of down-regulated repressors in the DUB mutants. Combined with the results of our phenotypic analysis, these findings demonstrate that the DUB module of SAGA regulates a subset of genes during early embryogenesis.

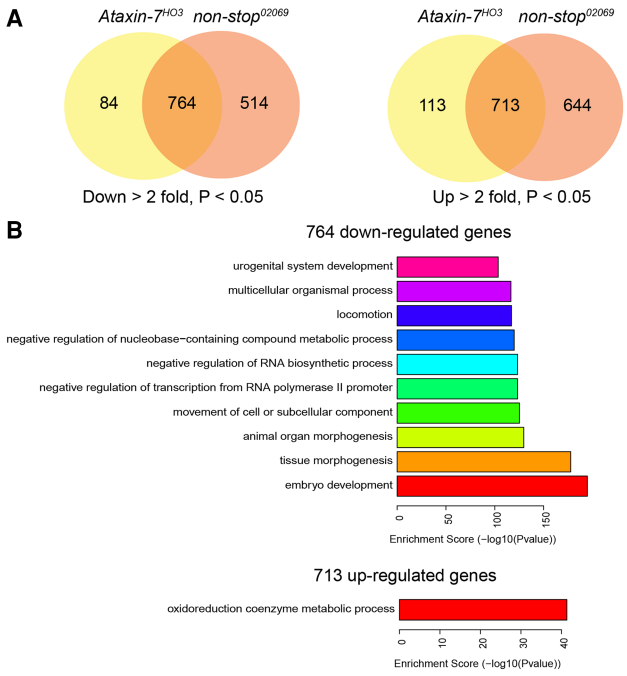

Figure 5. RNA-seq analysis reveals that the DUB module regulates expression of a subset of early genes in embryogenesis. (*A*) Venn diagrams showing the overlapping genes with decreased or increased transcript levels in stage 5 *Ataxin-7*^{HO3} and *non-stop*^{02069} GLC embryos. Fold change > 2; $P < 0.05$; FPKM > 1. A large portion of genes was similarly affected in both mutants. (*B*) GO term analysis of biological process (BP) of genes commonly changed in both GLC embryos. A complete list of enriched GO terms with an adjusted *P*-value of <0.05 is in Supplemental Table S4.

Previous studies have suggested that loss of DUB module subunits does not impair HAT activity and the integrity of the rest of SAGA (Weake et al. 2008; Lee et al. 2011; Mohan et al. 2014). To examine the HAT activity in *Ataxin-7* GLC embryos, we performed ChIP-seq (chromatin immunoprecipitation [ChIP] combined with high-throughput sequencing) of H3K9ac and H3K14ac and found no significant change in *Ataxin-7* GLC embryos. Moreover, Ada2b-binding sites did not change in *Ataxin-7* GLC embryos (Supplemental Fig. S10). We also examined H3K9ac and H3K14ac in the germline cells of *Ataxin-7* and *non-stop* GLC ovaries and observed levels similar to those seen in wild type (Supplemental Fig. S11).

Identification of SAGA-bound genes in early embryos

To establish which genes were direct targets of SAGA, ChIP-seq assays were performed to determine occupancy in wild-type embryos. We chose subunits from each of three different SAGA modules: Ada2b in the HAT module, Spt3 in the SPT module, and Sgf11 in the DUB module. Multiple biological replicates were carried out, and peaks were called only if present in at least two replicates. We identified 3033 Ada2b peaks, 2382 Spt3 peaks, and 5425 Sgf11 peaks. To identify sites that likely bound the intact SAGA, we compared the binding patterns of these three factors for overlap and found good congruence (Fig. 6A). Here, we refer to the 1650 common peaks as SAGA peaks. Next, we assigned these peaks to the nearest transcription start sites (TSSs) and identified 1998 genes in which SAGA bound within 500 base pairs (bp) of the TSSs. Note that the number of target genes is greater than the number of peaks due to the fact that binding can occur within 500 bp of the TSSs of one, two, or, in rare cases, three genes. As shown in Figure 6B and Supplemental Figure S9C, only some of the differentially expressed genes were bound by SAGA. These indirect targets may be secondary effects of down-regulated transcriptional activators in DUB mutants (Supplemental Table S5). Of the SAGA-bound targets, some showed expression changes in the DUB mutant (differentially expressed in DUB mutants; referred to here as DUB DE), but, interestingly, many others did not change (Fig. 6B). Thus, the DUB module binds to SAGA targets as a part of SAGA

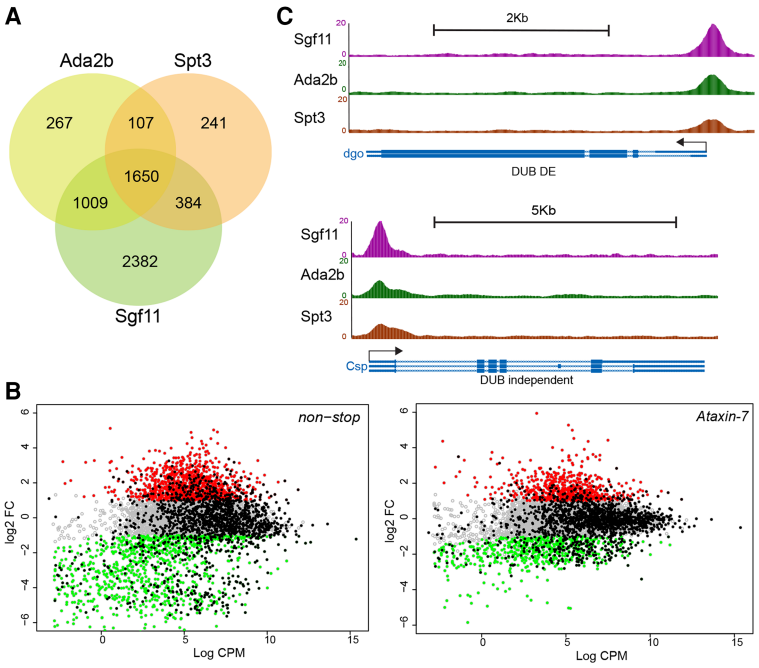

Figure 6. ChIP-seq analysis reveals that the DUB module binds to SAGA targets as a part of the whole complex but is required by only a subset of these genes for their expression. (*A*) Venn diagram showing the overlapping ChIP-seq peaks between Sgf11, Ada2b, and Spt3. The overlapped peaks are referred to as SAGA peaks. (*B*) MA plots showing gene expression in *non-stop* and *Ataxin-7* GLC embryos (genes with FPKM < 1 in wild type have been removed). Fold changes >2 are marked in red, and fold changes less than −2 are marked in green. Genes bound by SAGA are marked in black. (*C*) Example genes show that the DUB module binds to SAGA targets even though it is not always required for their expression. Binding profiles for Sgf11 (purple), Ada2b (green), and Spt3 (orange) are shown at the *dgo* (DUB DE gene) and *Csp* (DUB-independent gene) loci.

even though it is not always required for the expression of the target genes (Fig. 6C). We conclude that SAGA binds to many target genes as a whole complex in early embryos, but the DUB module is required by only a subset of these genes for their expression.

The DUB module binds and regulates gene expression independent of the HAT module

As noted above, some studies suggest that a stable DUB module exists independent of SAGA under some conditions. However, these studies did not address whether this module can bind to genomic chromatin independent of other SAGA modules under wild-type conditions. Whereas most of the Sgf11 peaks overlapped with Ada2b and Spt3, we noticed 2382 target sites where only Sgf11 was bound (Fig. 6A). We re-evaluated peaks using highly stringent criteria, including only those sites in which Ada2b and Spt3 binding was absent in all biological replicates. This analysis identified 647 Sgf11 peaks independent of Ada2b and Spt3 (Fig. 7A). MEME analysis showed that these sites include forkhead domain and homeobox domain motifs and are particularly enriched for zinc finger (Znf)-binding motifs (Supplemental Table S6). To address whether these Sgf11-bound sites reflected binding of the entire DUB module, we carried out ChIP analysis of Non-stop binding and found that ~90% of all Sgf11 peaks were also bound by Non-stop (Supplemental Fig. S12). Since Non-stop was bound at 80% of the unique Sgf11 peaks, we inferred that these peaks represent SAGA-independent binding of an intact DUB module.

To examine the role of the DUB module in regulating transcription of novel targets, we assigned the 647 Sgf11-only peaks to the nearest TSS. Like SAGA, we found a similar binding preference of the DUB module near the TSS, with 345 of these sites within 500 bp of a TSS (Fig. 7B). Of the 345 DUB target genes (as identified by Sgf11 ChIP-seq), 56 genes required DUB subunits for their expression (Fig. 7C). Although the DUB module prefers to bind to TSS like SAGA does, it binds to genes with different types of promoters (Discussion; Supplemental Fig. S13A).

To examine the correlation of ubH2B and gene expression in the DUB mutants for the DUB targets and SAGA targets, we performed ChIP-seq of ubH2B in wild-type and *Ataxin-7* GLCs. As shown in Supplemental Figure S14, down-regulated SAGA target genes have slightly decreased ubH2B in the coding regions in the absence of Ataxin-7, while up-regulated SAGA target genes have increased ubH2B in both promoters and coding regions (Supplemental Fig. S14B). In contrast, down-regulated DUB target genes showed decreased ubH2B in the coding regions in the absence of Ataxin-7, whereas up-regulated genes showed increased ubH2B in their promoters (Supplemental Fig. S14A). Thus, loss of Ataxin-7 leads to (1) increased ubH2B at up-regulated genes bound by either SAGA or DUB module subunits and (2) decreased ubH2B at down-regulated genes bound by either SAGA or DUB module subunits. This finding is consistent with an overall activating role of ubH2B. The different distribution of ubH2B in up-regulated genes in the DUB module targets (promoters) versus the SAGA targets (promoters and gene bodies) suggests that the DUB module plays distinct roles at those two sets of target genes.

We noticed throughout our analysis that a large portion of SAGA or DUB module target genes did not require the DUB module for their expression. We therefore wished to determine whether the SAGA target genes and the DUB target genes that did not require DUB for expression nevertheless had RNA polymerase II (Pol II)

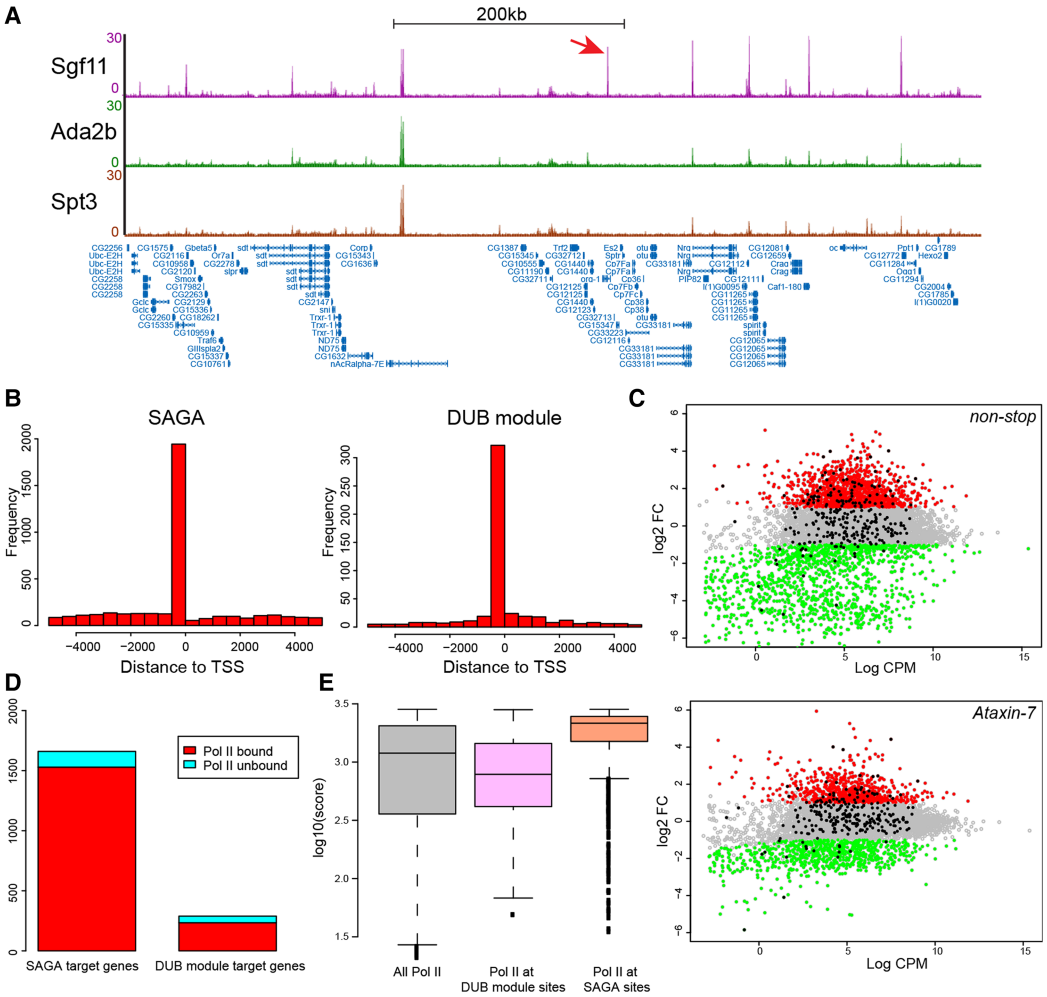

Figure 7. The DUB module binds and regulates gene expression independent of the HAT module. (*A*) Genome browser of ChIP-seq tracks at a representative region revealed that the DUB module binds to some genes without the HAT or SPT modules. The red arrow points to the Sgf11-only peak. (*B*) Histograms of distance (from –5000 bp to +5000 bp) to the TSSs for SAGA peaks and the DUB module peaks. (*C*) MA plots showing gene expression in *non-stop* and *Ataxin-7* GLC embryos (genes with FPKM < 1 in wild type have been removed). Fold changes >2 are marked in red, and fold changes less than –2 are marked in green. Genes bound by the DUB module are marked in black. (*D*) Bar plot showing that the SAGA target genes or the DUB module target genes that did not require the DUB module at this stage are bound by RNA polymerase II (Pol II). (*E*) To compare the Pol II levels at different sites, the $\log_{10}$ of Pol II peaks of expressed genes with an FPKM > 1 was plotted.

bound. The majority of these genes was indeed bound by Pol II (Fig. 7D). To further characterize the difference of the DUB DE and DUB-independent genes, we performed promoter analysis (Chen et al. 2013). As shown in Supplemental Figure S13A, for both SAGA targets and DUB targets, DUB DE genes and DUB-independent genes have distinct promoter types, indicating that different promoter types of genes have different requirements of the DUB module. DUB DE DUB targets are enriched for DPE and Inr promoter classes, while DUB-independent DUB targets are enriched for DRE and Ohler6. DUB DE SAGA targets are enriched for TATA, Inr, and MTE, while DUB-independent SAGA targets are enriched for DRE and Ohler1. Moreover, GO term analysis of SAGA targets suggested that dynamically expressed patterning genes are more likely to require the DUB module, while metabolic process genes tend to be independent of the DUB module (Supplemental Fig. S15). Using intensity of H2B binding to monitor nucleosome occupancy between different classes of genes, we found no difference between DUB DE and DUB-independent targets for either DUB-specific or SAGA targets (Supplemental Fig. S13B).

Last, we examined the level of Pol II binding at expressed genes (Fig. 7E). Compared with all Pol II peaks of expressed genes (Fig. 7E, gray box), much higher levels of Pol II were present at SAGA-bound sites (Fig. 7E, orange box), consistent with previous findings (Weake et al. 2011). Pol II binding was not elevated at robustly expressed DUB target genes (Fig. 7E, pink box). However, the DUB module clearly facilitates Pol II binding genome-wide, possibly reflecting a role for the DUB module

in the regulation of paused Pol II and elongation (Supplemental Fig. S16).

In summary, we conclude that the DUB module binds to chromatin as a part of SAGA and also independently of SAGA. Its presence is essential for transcription of some of these genes.

Discussion

The Drosophila *HAT and DUB module subunits are required at different stages of development*

Our results reveal the developmental stages at which the DUB and HAT modules of the SAGA chromatin-modifying complex are first required. Whereas the HAT module is required during oogenesis, the DUB module is not required for this process, and ovaries lacking Non-stop or Ataxin-7 are normal. Moreover, the D–V and A–P axes form in these embryos. The first defect observed in embryos lacking these DUB module subunits is at the stage of cellularization.

Numerous studies have explored the consequences of mutations in SAGA DUB and/or HAT module subunits at other stages of *Drosophila* and in other organisms. In some cases, loss of the two enzymatic modules has the same phenotypic impact, while in other cases, their loss has different consequences. In yeast, DUB module subunits Ubp8 and Sgf73 contribute to centromere stability, while the HAT module subunits Gcn5 and Ada3 do not (Canzonetta et al. 2015). An RNAi screen found that mouse GCN5 regulates reprogramming initiation in embryonic stem cells, whereas the DUB module appears to play no role in this process (Hirsch et al. 2015). In contrast, loss of the *Drosophila* Non-stop, Sgf11, and Ada2b causes similar defects in axon guidance in the eye imaginal disc (Weake et al. 2008). Loss of Gcn5 blocks oogenesis and metamorphosis (Carre et al. 2005), and zygotic Ada2b is also essential for viability (Qi et al. 2004; Pankotai et al. 2005). Although their mutant phenotypes have not been compared, the mouse *USP22*-, *GCN5*-, or *ADA3*-null embryos all die during embryogenesis (Xu et al. 2000; Lin et al. 2012; Mohibi et al. 2012). Additionally, GCN5 HAT activity is critical for cranial neural tube closure (Bu et al. 2007). Interestingly, loss of the *Drosophila* Ataxin-7 in the eye and adult brain results in neural degeneration (Mohan et al. 2014). Similarly, mammalian Ataxin-7 plays a primary role in the neurodegenerative disease spinocerebellar ataxia type 7 (David et al. 1997, 1998; Del-Favero et al. 1998; Johansson et al. 1998), and some genes that are regulated by Ataxin-7 contribute to the pathology (Chen et al. 2012).

It is important to note in many of the above analyses that several SAGA subunits are present in multiple complexes. For example, Gcn5 is also present in the ADA (yeast) and the ATAC (metazoans) complexes. Thus, the greater severity of Gcn5 mutants may result from the loss of these other complexes. Our studies with SAGA-specific subunits allow direct comparison of their requirement in regard to genetic phenotype, expression profiles, and target genes.

DUB and HAT module subunits control expression of a subset of genes

Many studies show that loss of SAGA subunits affects expression of a subset of genes and that different subunits seem to regulate distinct genes. In yeast, SAGA predominates at more regulated nonhousekeeping genes (de Jonge et al. 2017) and are preferentially used by those with TATA boxes, including those associated with stress (Basehoar et al. 2004; Huisinga and Pugh 2004). A more comprehensive genome-wide analysis of all viable SAGA deletion mutants suggests that different modules regulate different sets of genes (Helmlinger et al. 2011). Spt3 and Spt20 regulate 3% and 10% of yeast genes, respectively. Gcn5 has been reported to regulate between 1.1% and 4%, most of which are distinct from those regulated by Spt3 or Spt20 (Lee et al. 2000; Helmlinger et al. 2008). Bonnet et al. (2014) suggested that SAGA is required for all transcribed genes based on Pol II recruitment and nascent mRNA synthesis at select genes. Gene expression profiling has also been reported for later stages in the *Drosophila* life cycle. Loss of zygotic Ada2b influences the expression of only ~600 genes in larvae and ~900 genes in pupa (Zsindely et al. 2009; Pankotai et al. 2013; Pahi et al. 2015). Similar to yeast, many genes in *Drosophila* larvae require SAGA for their expression, but some are dependent on the DUB module, while others require the HAT module (Weake et al. 2008). Our results suggest that genes transcribed in the *Drosophila* ovary also have distinct requirements for the two enzymatic modules. Many genes require Ada2b in the ovary, while only a small number are affected by the loss of DUB module subunits.

Loss of these subunits alters transcription of many genes, while others exhibit robust expression that is unaffected. As noted above, we observed a reduction in expression of a subset of genes that are important for cellularization, which is likely responsible for the mutant phenotype. Our analysis showed that many genes are influenced by loss of only one of the subunits, but, consistent with their similar embryonic phenotypes, ~50% of those genes impacted by the loss of Ataxin-7 or Non-stop are similarly affected in both mutants (Fig. 5A).

The possibility that other DUBs can substitute for Non-stop remains a formal possibility. There appear to be 23 candidate DUBs in *Drosophila*, several with significant homology with Non-stop. Scrawny and USP7 have been reported to have activity on ubH2B (van der Knaap et al. 2005; Buszczak et al. 2009). Scrawny encodes the *Drosophila* homology of yeast Ubp10, which has been reported to deubiquitinate ubH2b in the coding sequence (Schulze et al. 2011). It remains to be determined whether any of these can substitute in SAGA in the absence of Non-stop or whether another complex might provide this activity.

The DUB module binds to chromatin both as a part of the HAT-containing SAGA complex and independent of HAT module subunits

Previous genome-wide studies have used binding of a single SAGA subunit to infer targets of the entire complex.

For example, Ada2b binding identified putative SAGA targets in *Drosophila* larva muscle and neurons (Weake et al. 2011), and SPT20 was shown to bind to a subset of transcribed genes in human cell lines (Krebs et al. 2011). In contrast to our analysis, these studies did not evaluate binding differences between modules.

We used three SAGA-specific subunits for three different modules in our whole-genome analysis of early embryos. Interestingly, we found that the DUB module subunits bound with HAT, and SPT module subunits bound to SAGA targets even though it was not always required for their expression. In addition, the DUB module bound to novel targets without the HAT or SPT modules in wild-type embryos. While we were unable to eliminate the possibility that the unique Sgf11 peaks reflect a robust antibody and are not actually devoid of HAT components, we note that different promoter types are enriched in SAGA targets and DUB-specific targets. This finding supports our view that the DUB targets are distinct from those of SAGA.

The mechanism by which the DUB module is recruited to chromatin is an interesting question. Sgf11 is a Znf protein and can bind to nucleosomal DNA (Koehler et al. 2014). Moreover, we found that Znf-binding sites are enriched in the Sgf11-specific targets (Supplemental Table S6). These findings support the hypothesis that DUB binds directly to DNA in the absence of the HAT and SPT modules via Sgf11. Sgf11-specific target sites are also enriched for forkhead domain and homeobox domain protein-binding motifs, supporting the possibility that this module binds to chromatin indirectly through non-SAGA proteins. Relevant to this issue, studies have already reported that the DUB module can interact with non-SAGA complexes. Under wild-type conditions in yeast, the proteasome chaperones interact with the Ubp8 and Sgf73 and separate them from SAGA (Lim et al. 2013). The yeast DUB module subunit Sus1 is also present in the mRNA export complex TREX-2 (Rodriguez-Navarro et al. 2004). The *Drosophila* Sus1 homolog E(y)2 is also required for mRNA export (Kurshakova et al. 2007). These results suggest that the DUB module interacts with non-SAGA proteins and may have functions independent of SAGA. Further purification studies should address these various options and which proteins interact with the SAGA DUB module independent of the HAT module.

Materials and methods

Fly strains and culture

Drosophila melanogaster was grown on standard medium at 25°C unless stated otherwise. For GLC analyses, larvae were heat-shocked for 2 h at 37°C for 2 d starting from the second day after hatching. Females were conditioned with yeast for 2–3 d before ovary dissection. Embryos were collected using standard apple juice plates with yeast. The *non-stop*^{02069} stock (*P*{*ry*$^{+t7.2}$ = *PZ*} *not*^{02069}*ry*^{506}/*TM6B*, *r*^{CB} *Tb*$^{+}$) was provided by the Bloomington *Drosophila* Stock Center (BL11553) (Martin et al. 1995; Poeck et al. 2001). The *ada2b*^{1} fly stock was provided by Matthias Mannervik (Qi et al. 2004). The *Ataxin-7*^{HO3} stock was generated by imprecise excision of the P element of Bloomington stock *y*^{1}*w*^{67c23}; *P*{*SUPor-P*}*Atxn7*^{KG02020} (BL14255) (details are in the Supplemental Material)

Immunostaining

Dechorionated embryos were mixed vigorously in fixative (2% formaldehyde, 0.1 M PIPES at pH 6.9, 2 mM $MgSO_4$, 1 mM EDTA) with an equal volume of heptane for 18 min at room temperature.

Ovaries were dissected in Shields and Sang M3 insect medium and fixed in PBS with 4% formaldehyde for 15 min at room temperature.

Primary antibodies included Dlg (1:20), Eve (1:10), Enabled (1:500), and Fasciclin II (1:500) from Developmental Studies Hybridoma Bank; Twist (1:500); α-Tubulin (1:200; F2168) from Sigma-Aldrich; Vasa (1:200; sc-30210) and Staufen (1:200; sc-15823) from Santa Cruz Biotechnology; and H3K9ac (ab4441) and H3K14ac (ab52946) from Abcam. Alexa fluor 647 phalloidin (1:20; Thermo Fisher Scientific, A22287) was used to detect F-actin. The myosin heavy chain monoclonal antibody (1:1000) was obtained from D. Kiehart. Alexa fluor secondary antibodies (1:200) were obtained from Invitrogen. Colorimetric detection used biotinylated anti-mouse secondary antibodies (1:200; Vector Laboratories) and the VectaStain ABC elite kit (Vector Laboratories). Cell nuclei were stained using 1 µg/mL DAPI.

Images were taken using Zeiss LSM 510 VIS and LSM 700 Falcon confocal microscopes. Some embryo and ovary images were generated by maximum projection of selected confocal slices using ImageJ. Channel colors were altered in ImageJ for optimal viewing. Brightness and contrast were adjusted in ImageJ when necessary. Shape and volume were determined for Twist-stained nuclei using the surface function of Imaris.

RNA purification and analysis

Stage 5 embryos were collected and hand-sorted. Ovaries were dissected in Shields and Sang M3 insect medium. Total RNA was extracted using the RNeasy Plus minikit (Qiagen).

Real-time PCR was performed from biological triplicates using a 7900HT real-time system. For RNA-seq analysis, biological triplicates were carried out for each genotype. Reads generated were 51-bp single-end poly-A-selected directional using the Illumina protocol. Resulting reads per gene were analyzed in R with the edgeR package using default methods. Differentially expressed genes were identified using the cutoff of $P < 0.05$ and FPKM (fragments per kilobase per million mapped fragments) > 1. The cutoff of fold change >2 was applied for identifying down-regulated genes and up-regulated genes.

ChIP-seq analysis

Stage 4–6 embryos were collected at 25°C, and embryo stage was confirmed microscopically. Embryos were fixed in 1.8% formaldehyde for 15 min at room temperature (details are in the Supplemental Material). ChIP was performed as described previously (Huang et al. 2014).

The antibodies used for ChIP included 10 µg of rabbit α-Sgf11, 20 µg of rabbit α-Ada2b, 20 µg of rabbit α-Spt3, 20 µg of rabbit α-Non-stop (details are in the Supplemental Material), 3 µg of mouse α-Pol II (4H8) (Abcam, ab5408), 5 µg of mouse α-ubH2B (EMD Millipore, 17-650), 5 µg of rabbit α-H2B (Abcam, ab1790), 5 µg of rabbit α-H3K9ac (Abcam, ab4441), 5 µg of rabbit α-H3K14ac (Abcam, ab52946), and 5 µg of rabbit α-H3 (Abcam, ab1791).

Reads of 51 bases from an Illumina HiSeq 2500 were aligned to version dm3 of the *Drosophila* genome from University of California at Santa Cruz using Bowtie using parameters –best –strata -k 1 -m 3. The resulting BAM files were analyzed in R using Bioconductor to generate coverage and normalize the data in reads per million. Locations of enrichment for each protein were identified using MACS2 with default parameters.

ChIP-seq and RNA-seq data are available in Gene Expression Omnibus under accession number GSE98865.

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