Establishment and maintenance of histone acetylation levels are critical for metazoan development and viability. Disruption of the balance between acetylation and deacetylation by treatment with chemical histone deacetylase (HDAC) inhibitors results in loss of cell proliferation, differentiation and/or apoptosis. Histone deacetylation by the SIN3 complex is essential in Drosophila and mice, as loss of the scaffolding factor SIN3 or the associated HDAC results in lethality. The objective of this study is to elucidate contributions of SIN3 complex components to these essential processes. We used the Drosophila model organism to carry out a systematic functional analysis of the SIN3 complex. We find that SIN3 associated proteins are essential for viability and cell proliferation during development. Additionally, tissue specific reduction of SIN3 complex components results in abnormal wing development. Interestingly, while knockdown of each factor resulted in similar phenotypes, their individual effects on recruitment of SIN3 to polytene chromosomes are distinct. Reduction of some factors leads to large changes in the morphology of the chromosome and/or greatly reduced SIN3 binding. These findings suggest that while individual SIN3 complex components work through distinct molecular mechanisms, they each make a substantial contribution to the overall function of this highly conserved histone deacetylase complex.
interacts with a subset of SIN3 complexes\(^\text{11}\). In studies performed by independent research groups using different organisms, a number of accessory factors including BRMS1, PF1, FAM60A, ING1/2, MRG15, RbAp46/48, RBP1, SAP180, SAP130, SAP30, SAP18 and SDS3 have been reported as members of a SIN3 complex, in addition to the two enzymatic components\(^\text{12,13}\). In an earlier proteomic study to identify isoform-specific SIN3 complexes, we found that the following proteins interact with each of the two major Drosophila SIN3 isoforms: HDAC1, CG14220 (SDS3), hat-trick (htk) (ARID4B), CG7379 (ING1/2), Sap130, CG3815 (PF1) and BRMS1\(^\text{14}\). Additionally, we determined that Caf1-55, dKDM5/LID and CG15356 (EMSY) are factors that predominantly interact with the largest and most widely expressed SIN3 isoform, SIN3 220. Apart from the enzymatic activity of HDAC1 and dKDM5/LID, little is understood about the role of the accessory factors in the context of SIN3 complex function.

To address this lack of comprehensive understanding, the objective of this study was to perform a systematic analysis of a defined SIN3 complex in Drosophila melanogaster. We sought to identify common and unique phenotypes resulting from reduced expression of complex components, either individually or in combination with reduction of the SIN3 scaffolding factor. We also set out to determine which factors participate in recruitment and/or stabilization of SIN3 onto chromatin, which is believed to be a pre-requisite for complex regulatory activity. Our data indicate that while reduced expression of the majority of the subunits results in highly similar biological phenotypes, only a subset of the factors impact chromatin recruitment. These findings highlight functional specialization of SIN3 complex components.

**Results and Discussion**

To examine functions of SIN3 complex subunits, we measured a number of phenotypic traits in flies with reduced levels of SIN3 complex components compared to wild type controls. The complex components investigated included factors previously determined to associate with the largest isoform of SIN3\(^\text{14}\) and are listed in Table 1. To generate flies with reduced protein levels, we used RNA interference (RNAi) to knock down expression of the desired target. Flies carrying a transgene comprised of a UAS regulatory element upstream of a sequence to express double stranded (ds) RNA were crossed to driver flies that express the GAL4 activator. In the progeny of the cross, GAL4 binds the UAS element and activates expression of the dsRNA\(^\text{15}\). This dsRNA is processed by the RNAi machinery and will lead to degradation of the endogenously expressed target mRNA; ultimately resulting in reduced protein expression\(^\text{16}\). For these studies, we utilized a variety of GAL4 driver lines to express GAL4, and thus induce RNAi knock down, in different tissues. For all experiments, we compared the knockdown fly with a control that was the progeny of the same GAL4 driver crossed to either a control UAS-GFP\(^\text{RNAi}\) or UAS-mCherry\(^\text{RNAi}\) fly. These control flies have expression of GAL4 and induction of the RNAi pathway, but no specific Drosophila gene is targeted for knock down. As an additional control, to reduce the possibility that observed phenotypes are due to an off-target effect, when available, we utilized more than one RNAi line to target different regions of the targeted RNA. To validate the efficiency of knockdown, we performed RT-qPCR analysis (Supplementary Fig. S1). The assay was performed using RNA isolated from wing imaginal discs, in which only a subset of cells express the dsRNA targeting the gene of interest. The driver for these studies, and for the wing assay described below, is Ser-Gal4. This driver contains a portion of the regulatory region for the serrate (Ser) gene upstream of the GAL4 coding sequence. Ser has a complex expression pattern with prominent expression in the dorsal compartment of

| Gene       | Stock Name (UAS-GOI) | Act5C-Gal4/UAS-GOI | CyO/UAS-GOI | Act5C-Gal4/UAS-GOI | CyO/UAS-GOI |
|------------|----------------------|--------------------|-------------|--------------------|-------------|
| GFP        | GFP\(^\text{RNAi}\)  | 53                 | 51          | 61                 | 66          |
| SmiA       | SmiA\(^\text{RNAi}\) | 0                  | 172         | 0                  | 189         |
| HDAC1      | HDAC1\(^\text{RNAi}\) | 0                  | 132         | 0                  | 144         |
|            | HDAC1\(^\text{TRIP}\) | 0                  | 158         | 0                  | 152         |
| Caf1-55    | Caf1-55\(^\text{RNAi}\) | 0                  | 182         | 0                  | 162         |
|            | Caf1-55\(^\text{TRIP}\) | 0                  | 154         | 0                  | 167         |
| hd         | hd\(^\text{RNAi}\)   | 0                  | 122         | 0                  | 237         |
|            | hd\(^\text{TRIP}\)   | 0                  | 239         | 0                  | 334         |
| htk (ARID4B)| htk\(^\text{RNAi}\)   | 0                  | 114         | 0                  | 111         |
| Sap130     | Sap130\(^\text{RNAi}\) | 0                  | 126         | 0                  | 154         |
| CG15356    | CG15356\(^\text{RNAi}\) | 0                  | 182         | 0                  | 199         |
| CG3815     | CG3815\(^\text{RNAi}\) | 0                  | 181         | 0                  | 188         |
| CG7379     | CG7379\(^\text{RNAi}\) | 0                  | 249         | 0                  | 237         |
|            | CG7379\(^\text{TRIP}\) | 0                  | 167         | 0                  | 182         |
| CG14220    | CG14220\(^\text{RNAi}\) | 0                  | 110         | 0                  | 175         |
| Brms1      | Brms1\(^\text{RNAi}\) | 0                  | 108         | 0                  | 151         |
|            | Brms1\(^\text{TRIP}\) | 0                  | 99          | 0                  | 126         |

Table 1. SIN3 complex components are required for Drosophila viability. The values represent total number of adult flies produced from three independent trials. GOI, gene of interest; KD, knockdown; TRiP, Transgenic RNAi Project at Harvard Medical School; KK, F31 Transgenic RNAi Library from Vienna Drosophila Research Center (VDRC); GD, P-element Transgenic RNAi Library (VDRC).
the wing imaginal disc.17,18 We previously determined that this selected driver results in expression of GAL4 in the wing imaginal disc throughout larval development in a pattern that is largely consistent, however that is somewhat broader than, the reported expression of Ser29. We typically observed RNA levels reduced to about 60-70% of the level in wild type wing discs in these heterogeneous cell populations.

**SIN3 complex components are essential for viability and wing development.** We first tested whether each of the complex components is essential for Drosophila viability. We used the Act5C-Gal4 driver line for ubiquitous expression of GAL4. Progeny of the cross of this driver to the UAS-targeted30 line results in ubiquitous knockdown of the targeted complex component. Knockdown of each gene coding for a SIN3 complex component resulted in no adult progeny (Table 1). Thus, each tested protein in the complex is encoded by an essential gene. These results are fully consistent and validate previous studies indicating that ubiquitous RNAi knockdown of Sin3A, lid and Caf1-55 results in lethality14,20,21. Additionally, previous studies indicated that flies carrying homozygous genetic mutations of HDAC126 and Brms123 are not viable. Data generated in our assay demonstrate that the rest of the genes encoding proteins of the complex, including htk (ARID4B), Sap130, CG15356 (EMSY), CG3815 (PF1), CG7379 (ING1/2) and CG14220 (SDS3), are also required for viability.

Because ubiquitous RNAi knockdown results in loss of viability, we utilized a conditional knockdown system to further investigate SIN3 complex components. The Drosophila wing is a nonessential tissue and has been used by us and others to investigate factors involved in development and cell cycle control16-22. We previously determined that SIN3 and dKDM5/LID are necessary for development of a normal wing9,21. For this study, we have repeated those experiments to allow for direct comparison with knockdown of the other complex components. As in those prior studies, here we used the Ser-Gal4 driver to induce RNAi knockdown of the SIN3 complex components in wing imaginal discs and observed the resulting adult wing morphology.

We first tested whether the individual complex components were necessary for wing development. Individual reduction of HDAC1, Caf1-55 or CG3815 (PF1) resulted in severely malformed wing phenotypes (Fig. 1, Table 2, Supplementary Fig. S2). The wing tissue is blistered and blackened. This phenotype is more severe compared to that resulting from knockdown of either Sin3A or lid. HDAC1 and Caf1-55 are each found in additional complexes apart from SIN3. HDAC1 is a component of other HDAC complexes including NuRD and CoREST9. Caf1-55 associates not only with HDACs, but also with KATs and chromatin assembly factors26. It is possible that the loss of each of these factors impacts the function of not only the SIN3 complex but also the other chromatin organization complexes in which these factors are found. The severe disruption of wing morphology is thus likely due to effects on multiple developmental pathways. The other factor found to yield the blistered phenotype upon knockdown is CG3815 (PF1). Little is known about PF1 in flies and work in other organisms has focused on the role of this factor as a component of the SIN3 complex. In triple negative breast cancer cells, disruption of the interaction between PF1 and SIN3A altered the expression of cancer-related genes and importantly, decreased the metastatic potential of cancer cells in a mouse tumor model27. These authors additionally found that knockdown of PF1 phenocopies the effects of disruption of the PF1 and SIN3A interaction, indicating that a main role for PF1 is as part of the SIN3 complex. The data from the fly wing development assay suggests that, in flies, PF1 might function apart from SIN3. Interestingly, the blistered wings are only found in the male CG3815 (PF1) knockdown flies. The wings of the female CG3815 (PF1) knockdown flies are curved, similar to those of the Sin3A knockdown flies (Fig. 1). These data, as well as a few additional sex specific discrepancies in observed phenotypes as noted below, suggest that when compared to females, males are more sensitive to a reduction in SIN3 complex activity. We observed that the rest of the tested SIN3 complex subunits, including htk (ARID4B), Sap130, CG15356 (EMSY), CG7379 (ING1/2), CG14220 (SDS3) and Brms1, are necessary for formation of a normal straight adult wing (Fig. 1, Table 2). For these flies, besides the curvature of the wing, no additional wing defects were noted upon visual inspection. While the majority of adult CG14220 (SDS3) and Brms1 knockdown female flies had curved wings, some had straight wings supporting the idea that females are less sensitive to reduced SIN3 complex function as compared to males. Like wild type strains, progeny of the control cross had straight wings. When multiple RNAi lines were tested for an individual gene, the resulting phenotypes were similar across the lines. These results suggest the phenotypes are not due to off-target effects but rather are due to reduced expression of the targeted gene.

We note that sex specific phenotypic differences comparing flies with mutation or reduced expression of SIN3 complex components to wild type have been reported previously. For example, we previously observed that over-expression of dKDM5/LID in the context of Sin3A wing specific knockdown suppresses the curved wing phenotype in female flies by not in males31. Additionally, male flies that carry a mutation in lid have shorter life spans and a higher sensitivity to the presence of paraquat-induced reactive oxygen species as compared to the females with the mutation29. Mutations in HDAC1 have also been found to yield sex specific phenotypic variation. Flies carrying certain specific alleles of HDAC1 survive at significantly lower rates as compared to their genetically identical females counterparts29. We do not have a definitive answer as to the mechanism controlling the sex specific differential sensitivity to mutation in SIN3 complex components. It is possible that the differences are related to the epigenetic variation linked to the presence of the heterochromatic Y chromosome in male flies and/or the single X chromosome, which is subject to dosage compensation controlled in part by histone acetylation29. In addition to the possible impact of dosage compensation mechanisms, male and female flies have differences in their genome-wide chromatin landscape. It is possible that the SIN3 histone modifying complex controls some of these sex specific differences, which then differentially affect genes important for normal wing morphology.

An additional observation is that although we determined efficiency of knockdown in the wing imaginal discs at approximately 60 and 70% of control levels (Supplementary Fig. S1), to our knowledge, no wing abnormalities have been observed in the flies heterozygous for genetic mutations, and thus with perhaps 50% reduction, in the SIN3 complex factor. As noted above, the GAL4 expression pattern controlled by the Ser-Gal4 driver is complex and thus the amount of knockdown is likely variable across the wing imaginal discs cells, with some cells affected very little and others a lot.
We next examined the effect of reducing levels of SIN3 along with a second component of the complex. We previously determined that dual knockdown of Sin3A and lid resulted in a more severe phenotype compared to knockdown of either factor alone. For the current study, we used a fly line with constitutive knockdown of Sin3A

Figure 1. SIN3 complex components are necessary for normal wing development. (a) Ser-Gal4 or Sin3 KD/ CyO-Ras (Serrate-Gal4 - > UAS-SIN3 RNAi, which is balanced over chromosome II balancer CyO-Ras, marked by a curly wing and a rough eye phenotype) are crossed to each of the complex component RNAi lines, as well as to mCherry RNAi, as a control. Progeny are scored for wing phenotype caused by single or double knockdown as shown. (b) Micrographs of flies or wings with either individual knockdown (left panels) or with the gene of interest along with Sin3A knockdown (right panels). mCherry: control flies expressing mCherry dsRNA. Scale bar (whole flies) 1 mm. Scale bar (wings) 100 μm.
Components of the SIN3 complex are necessary for cell proliferation or cell viability during development. Early studies indicated that SIN3 and some complex components, including HDAC1 and CAF1-55, are required for proliferation of the Drosophila S2 cultured cell line. It was subsequently determined that SIN3, HDAC1, the histone demethylase dKDM5/LID and CAF1-55 are also critical for cell proliferation and cell survival during Drosophila development. To investigate if other components of the complex are necessary for cell proliferation during development, we performed clonal analysis in wing imaginal disc cells. For these studies, we utilized the heat shock “flip-out” GAL4 driver (Actin > GAL4) to activate expression of a UAS-linked RNAi target along with a GFP marker in random clones of cells via the heat-shock induction of FLP recombinase at a precise time point in larval development. Once activated, cell clones will continue to have reduced target gene expression and the effect of this reduced expression on cell proliferation can be measured by the size and number of the GFP positive cells. Reduction of all SIN3 complex components resulted in fewer GFP positive cells. In some instances, the clones are smaller in size and for others, the clones are fewer in number, relative to the clones produced in the mCherry RNAi controls (Fig. 2). The low yield of GFP positive cells in discs with reduced expression of a complex component is consistent with slower growth and progression through the cell cycle as compared to wild type cells. It is also possible that cells with RNAi reduced expression of a particular factor undergo apoptosis. In our previous studies with RNAi knockdown of Sin3A, HDAC1, Caf1-55 and lid in S2 cultured cells, however, we did not observe an increase in the number of dead cells in the population following treatment with the dsRNA to target the individual gene.

Table 2. SIN3 complex components are essential for wing development. Results are from at least three independent biological replicates. For specific phenotypes, refer to Fig. 1. *57% with blistered wings, 43% were curved.

| Stock Name (UAS-GOI) | Ser-Gal4/UAS-GOI | SIN3 KD/UAS-GOI |
|----------------------|------------------|------------------|
|                      | # Scored | % with phenotype | # Scored | % with phenotype | # Scored | % with phenotype | # Scored | % with phenotype |
| HDAC1RNAi-GD     | 97      | 100              | 116      | 100              | 92       | 100              | 115      | 100              |
| HDAC1RNAi-KK     | 192     | 93               | 213      | 76               | 176      | 94               | 226      | 94               |
| Caf1-55RNAi-GD   | 50      | 100              | 75       | 100              | 19       | 100              | 90       | 100              |
| Caf1-55RNAi-KK   | 156     | 100              | 216      | 95               | 69       | 100              | 140      | 100              |
| htkRNAi-GD       | 87      | 100              | 127      | 100              | 101      | 100              | 136      | 100              |
| htkRNAi-KK       | 120     | 100              | 149      | 100              | 51       | 100              | 207      | 100              |
| HtKRNAi-KK       | 146     | 100              | 204      | 87               | 189      | 100              | 242      | 79               |
| Sap130RNAi-GD    | 160     | 100              | 197      | 98               | 196      | 100              | 266      | 100              |
| CG1535RNAi-RGD   | 180     | 100              | 155      | 100              | 177      | 100              | 176      | 100              |
| CG3815RNAi-RGD   | 187     | 100              | 87       | 100              | 108      | 100              | 81       | 100              |
| CG7379RNAi-GD    | 212     | 100              | 245      | 100              | 222      | 100              | 203      | 100              |
| CG7379RNAi-KK    | 170     | 93               | 182      | 97               | 189      | 100              | 159      | 100              |
| CG14220RNAi-KK   | 100     | 100              | 202      | 60               | 114      | 100              | 138      | 100              |
| Brm11ENS-KK      | 263     | 78               | 478      | 55               | 145      | 100              | 162      | 100              |
| Brm11ENS-KK      | 180     | 100              | 110      | 100              | 92       | 100              | 117      | 100              |
The human homologs of a number of SIN3 complex factors, including ARID4B, EMSY, PF1, ING1/2 and BRMS1, are known to function in cell proliferation and the level of their expression has been linked to growth of cancer cells. Elevated expression of ARID4B was found to promote metastasis in a mouse model of human breast cancer. EMSY was first identified in a screen to look for factors associated with BRCA2 and breast cancer. In more recent work, researchers analyzed RNA-seq data available in the TCGA Research Network and found that EMSY, along with the demethylase KDM5A as well as SIN3B, is overexpressed in primary tumors. As noted above, disruption of the interaction between PF1 and SIN3B limits the metastatic potential of breast cancer cells in a mouse tumor model. ING1 and ING2 are members of a family of proteins involved in cell proliferation. In flies, the single gene CG7379 is approximately 40% similar to both human ING1 and ING2 (Supplementary Fig. S3). ING1 was first isolated in a screen to discover novel tumor suppressor genes. ING proteins are generally thought to be tumor suppressors, but have been found to be overexpressed in some cancer types. BRMS1 was identified in a screen for factors important for suppression of human breast cancer carcinoma metastasis and growth.
subsequently found to serve a similar role in other tumor types. It is interesting to note that some SIN3 complex factors, including SIN3, HDAC1, KDM5B, ARID4B, EMSY and PF1, are generally required for cell proliferation, while expression of ING2 and BRMS1 limits growth of many cancer cell types. Data generated from the clonal analysis in Drosophila wing imaginal discs indicates that the cell proliferation function of these factors, and indeed of the SIN3 complex, is conserved. RNAi knockdown of CG7379 (ING1/2) and Brms1 led to reduced cell proliferation in the developing wing tissue. This finding suggests that their function in these cells is similar to that of other complex components; they are important for cell proliferation rather than functioning as tumor suppressors.

Multiple components in the SIN3 complex can recruit SIN3. SIN3 is described as corepressor as it does not contain a DNA-binding domain for gene targeting. To recruit the complex to promoters where it functions to regulate gene expression, sequence-specific DNA-binding transcription factors interact with SIN3 and/or components of the complex. We asked if any of the complex components are critical for recruitment of SIN3 to chromatin. For this analysis, we used polytene chromosomes prepared from Drosophila larval salivary glands. We utilized the feb36-GAL4 driver line to induce RNAi and reduce the level of the SIN3 complex factor in salivary glands. Chromosome preparations from third instar larval salivary glands were probed with antibody to SIN3 and also stained with DAPI to visualize the DNA.

Unlike with the assays described above, knockdown of individual SIN3 complex factors resulted in different phenotypic effects. Knockdown of only a limited set of factors impacted SIN3 binding. Additionally, overall chromatin structure was affected by knockdown of some complex components and not others (Fig. 3). First, RNAi of HDAC1 led to disruption of polytene chromatin structure. The salivary glands were smaller and overall DAPI staining of nuclei was low compared to wild type controls. The chromosomes lack integrity; the banding pattern is disrupted and less distinct as compared to control chromosomess. SIN3 binding is apparent along the chromosome arms, but due to the disrupted morphology, we were unable to quantify the level of staining. We conclude from these data that HDAC1 is important for formation or stability of normal structure of the polytene chromosome but may not be essential for recruitment of SIN3. Knockdown of three factors, Caf1-55, Brms1, and Sap130, resulted in strongly diminished SIN3 recruitment (Fig. 3). The effect of reduction of dKDM5/LID led to a more subtle effect on SIN3 staining levels. The reduction of overall SIN3 binding was apparent, but not statistically significant (Fig. 3). Other tested components of the complex, including htk (ARID4B), CG15356 (EMSY), CG3815 (PF1), CG7379 (ING1/2) and CG14220 (SDS3), are not critical for SIN3 chromosomal recruitment as no effect on SIN3 binding was observed along polytene chromosomes prepared from salivary glands with reduced levels of these factors (Fig. 3 and Supplementary Fig. S4).

It is interesting to note that reduction of multiple individual factors of the complex led to such a strong effect on SIN3 recruitment. Perhaps Caf1-55, BRMS1 and Sap130 form a sub-complex. One possibility is that loss of one of these three factors affects assembly of the other two of the three into the sub-complex. This assembled sub-module may be key for recruitment of SIN3 and the other components of the complex. Caf1-55 has been demonstrated to interact with histones and this interaction may be critical for either recruitment or stability of SIN3 to chromatin. The data support the idea that certain factors are important for SIN3 chromatin recruitment. The lack of staining on polytene, however, could possibly be because the chromatin landscape itself is altered such that SIN3 or the complex is prevented from binding. For example, loss of a factor could lead to binding of an activator that prevents a transcription factor involved in recruiting SIN3 from binding. Future work will be necessary to examine the mechanism of how various SIN3 complex components affect SIN3 chromatin binding.

Summary

In this study, we have analyzed the contributions of components of the SIN3 complex to key developmental processes including viability, tissue development and cell proliferation. We found that all tested complex components are essential for viability and critical for cell proliferation and development of adult tissue. Unlike these shared requirements, only a limited set of the factors is necessary for chromatin recruitment of the SIN3 scaffolding complex. Reduction of each Caf1-55, BRMS1 and Sap130 largely diminishes the binding of SIN3 to chromosomes compared to wild type. Further work will be necessary to dissect protein-protein interactions of the complex to determine how loss of some factors and not others impacts SIN3 binding. Additionally, we note that while loss of only a few factors affects SIN3 chromatin binding, all work in similar biological processes necessary for cell proliferation and development to adulthood. The complex thus likely works as an integrated module to regulate gene activity, with each component making a substantial contribution to function.

Methods

Drosophila stocks. Drosophila melanogaster stocks were maintained at 25 °C, and crosses were performed at 27 °C, according to standard laboratory procedures. The stocks that were used can be found in Table 3. SIN3 KD (knockdown) flies were created by crossing CG7379 (ING1/2) and Brms1 led to reduced cell proliferation in the developing wing tissue. This finding suggests that their function in these cells is similar to that of other complex components; they are important for cell proliferation rather than functioning as tumor suppressors.

Reverse transcription PCR assay. Total RNA was extracted from 20 to 30 wing discs isolated from wandering third instar larvae using the RNeasy mini kit (Qiagen). cDNA was generated from total RNA using the ImProm-2 Reverse Transcription System (Promega) with random hexamers. The cDNA was used as template in a quantitative real-time PCR (qPCR) assay. The analysis was performed using Absolute SYBR Green ROX master mix (Fisher Scientific) and carried out in a Stratagene Mx3005P real-time thermocycler. Primers used for analysis are given in Supplemental Table S1. Taf1 and Pkg were used to normalize cDNA amounts in the 2−ΔΔCt comparative analysis.
Imaging flies. Whole flies were imaged at 30x magnification using an Olympus DP72 camera coupled to an Olympus SZX16 microscope. Wings were imaged at 80x magnification using a SPOT RT color camera coupled to a Leica MZ125 microscope.
shock at 37 °C for two hours. After returning to 27 °C, wandering 3rd instar larvae were dissected and immunostained 120 hours after egg laying19. Antibody against GFP (1:1000; Abcam, ab1218) followed by sheep anti-mouse from the protocol outlined here49. A primary antibody against SIN3 (1:1000) 50 was followed by a secondary anti-

 pixels in the DAPI-stained disc 48.

Clonal analysis. hsFLP;Act5C > CD2 > Gal4, UAS-EGFP virgin females were crossed to mCherry RNAi-TRiP males or UAS-SIN3 complex component RNAi to generate random GFP positive clones via the heat shock flip-out system. Embryos were collected for four hours and then after 48–52 hours, 2nd instar larvae were subjected to heat shock at 37 °C for two hours. After returning to 27 °C, wandering 3rd instar larvae were dissected and immunostained 120 hours after egg laying49. Antibody against GFP (1:1000; Abcam, ab1218) followed by sheep anti-mouse Alexa Fluor 488 (1:2000; Life Technologies, A11001) was used for staining. Visualization and imaging was done using Olympus BX53 compound microscope with a DP72, 12.8 megapixel camera. Images were processed using Olympus CellSens software. Clones were analyzed in a minimum of 20 discs per genotype using Photoshop CS

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Chromosomes were imaged using an Olympus BX53 compound microscope with a DP72, 12.8 megapixel camera. Images were processed using

All significance values were calculated by the two sample Student’s t test using GRAPHPAD software. http://www.grapphpad.com/quickcalcs/index.cfm.

Data Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

References

1. Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. Cell Res 21, 381–395, https://doi.org/10.1038/cr.2011.22 (2011).
2. Allfrey, V. G., Faulkner, R. & Mirsky, A. E. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of RNA Synthesis. Proc Natl Acad Sci USA 51, 786–794 (1964).

Table 3. Fly stocks used. *Original VDRC stock is balanced over TM3, Sb; we rebalanced over TM6, Tb for scoring 3rd instar larvae for wing disc dissection.

Polytene chromosome staining. Polytene chromosome preparation and staining methods were modified from the protocol outlined here49. A primary antibody against SIN3 (1:1000) 50 was followed by a secondary antibody Alexa Fluor 594 (1:400) (Life Technologies). Chromosome spreads were prepared from a minimum of three independent parental crosses and representative images are shown. A minimum of four slides of each control and experimental genotype were prepared at the same time and photographed using identical exposure times.

Chromosomes were imaged using a minimum of 20 discs per genotype using Photoshop CS to count the GFP positive pixels for each immunostained disc and comparing that number to the total number of pixels in the DAPI-stained disc48.

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

References

1. Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. Cell Res 21, 381–395, https://doi.org/10.1038/cr.2011.22 (2011).
2. Allfrey, V. G., Faulkner, R. & Mirsky, A. E. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of RNA Synthesis. Proc Natl Acad Sci USA 51, 786–794 (1964).
44. Andrews, H. K., Zhang, Y. Q., Trotta, N. & Broadie, K. *Drosophila* sec. 10 is required for hormone secretion but not general exocytosis or neurotransmission. *Traffic* 3, 906–921 (2002).
45. Siegmund, T. & Korge, G. Innervation of the ring gland of *Drosophila melanogaster*. *J Comp Neurol* 431, 481–491 (2001).
46. Martinez-Balbas, M. A., Tsukiyama, T., Gdula, D. & Wu, C. *Drosophila* NURF-55, a WD repeat protein involved in histone metabolism. *Proc Natl Acad Sci USA* 95, 132–137 (1998).
47. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408 (2001).
48. Kagey, J. D., Brown, J. A. & Moberg, K. H. Regulation of Yorkie activity in *Drosophila* imaginal discs by the Hedgehog receptor gene patched. *Mech Dev* 129, 339–349, https://doi.org/10.1016/j.mod.2012.05.007 (2012).
49. Pile, L. A. & Wassarman, D. A. Localizing transcription factors on chromatin by immunofluorescence. *Methods* 26, 3–9 (2002).
50. Pile, L. A. & Wassarman, D. A. Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. *The EMBO Journal* 19, 6131–6140 (2000).
51. Bugga, L., McDaniel, I. E., Engie, L. & Armstrong, J. A. The *Drosophila melanogaster* CHD1 chromatin remodeling factor modulates global chromosome structure and counteracts HP1a and H3K9me2. *PloS One* 8, e59496, https://doi.org/10.1371/journal.pone.0059496 (2013).

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**Author Contributions**

V.L.B., K.A.L. and L.A.P. conceived and designed the experiments. V.L.B., K.A.L. and M.P. performed the experiments and along with L.A.P., analyzed the data. V.L.B. supervised the work of a number of undergraduate student researchers who performed many of the genetic crosses to test for viability, wing morphology and prepared polytene chromosomes for staining. V.L.B., K.A.L. and L.A.P. wrote and edited the manuscript.

**Additional Information**

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