1-1-2014

The Phenotypic Analysis Of The Knockdown Of The Sin3a Complex Components And Their Role In Recruitment And Cell Proliferation

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THE PHENOTYPIC ANALYSIS OF THE KNOCKDOWN OF THE SIN3A COMPLEX COMPONENTS AND THEIR ROLE IN RECRUITMENT AND CELL PROLIFERATION

by

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THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2014

MAJOR: BIOLOGICAL SCIENCES

Approved By:

_____

Advisor Date

ACKNOWLEDGEMENTS

I would first like to thank all of my lab members for their help and support over the years. Thanks to my advisor Lori Pile for all of her guidance and help with this project, I could not have made it through without you. Thank you Valerie Barnes for letting me collaborate with you on the clonal analysis project. Thank you to all of my graduate course instructors for providing me with the knowledge necessary to complete my degree. A special thanks to all of the students I have had the privilege to instruct over the years. Thank you to the Meller lab for allowing me to use your microscope whenever I needed it, this work would not be possible without you. Thanks to Drs. Athar Ansari and Victoria Meller for being on my thesis committee and for all of your help and understanding. Finally, to my family and friends, thank you for all of your love, support and understanding, which helped me make it to where I am today.

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CHAPTER 1 INTRODUCTION

Transcription and the organization of eukaryotic DNA

An important aspect of gene regulation and expression is transcription. This process is controlled by different activators and repressors as well as co-activators and co-repressors. The protein complexes that can modify histones have an essential role in how transcription is able to take place. The potential modifications that can take place on the histone amino (N)-terminal tails include acetylation, methylation, phosphorylation and ubiquitination (Bannister and Kouzarides, 2011). Although these modifications are reversible by other histone modifying complexes, any kind of misregulation of these processes can have deleterious effects on an organism's ability to develop normally. The modifications that regulate transcriptional activation and repression are necessary for proper gene regulation.

Eukaryotic DNA requires multiple levels of compaction in order for it to fit into the nucleus of a cell. DNA is wrapped around a histone octamer, which consists of a pair of each histone H2A, H2B, H3 and H4. There is one histone H1 that is bound to linker DNA. The nucleosomes are organized into 10 nm chromatin and further supercoiled into the more compact 30 nm fiber (Fig 1). This chromatin is further compacted into additional loops in order to fit into the nucleus of the cell. The N-terminal tails of histones stick out of the nucleosome, which allows histone modifications to take place because of the action of histone modifying enzymes (Felsenfeld and Groudine, 2003). The histone modifications affect chromatin packaging and whether or not heterochromatic or euchromatin regions are formed. Euchromatin regions consist of less dense chromatin packaging and are known to be gene rich, while heterochromatin is highly dense

chromatin and has limited transcription occurring (James and Elgin, 1986). There is transcription repression in heterochromatic areas because of the tight packaging of DNA. Euchromatin is found to be transcriptionally active since the loose DNA in these regions is more accessible to transcription machinery.

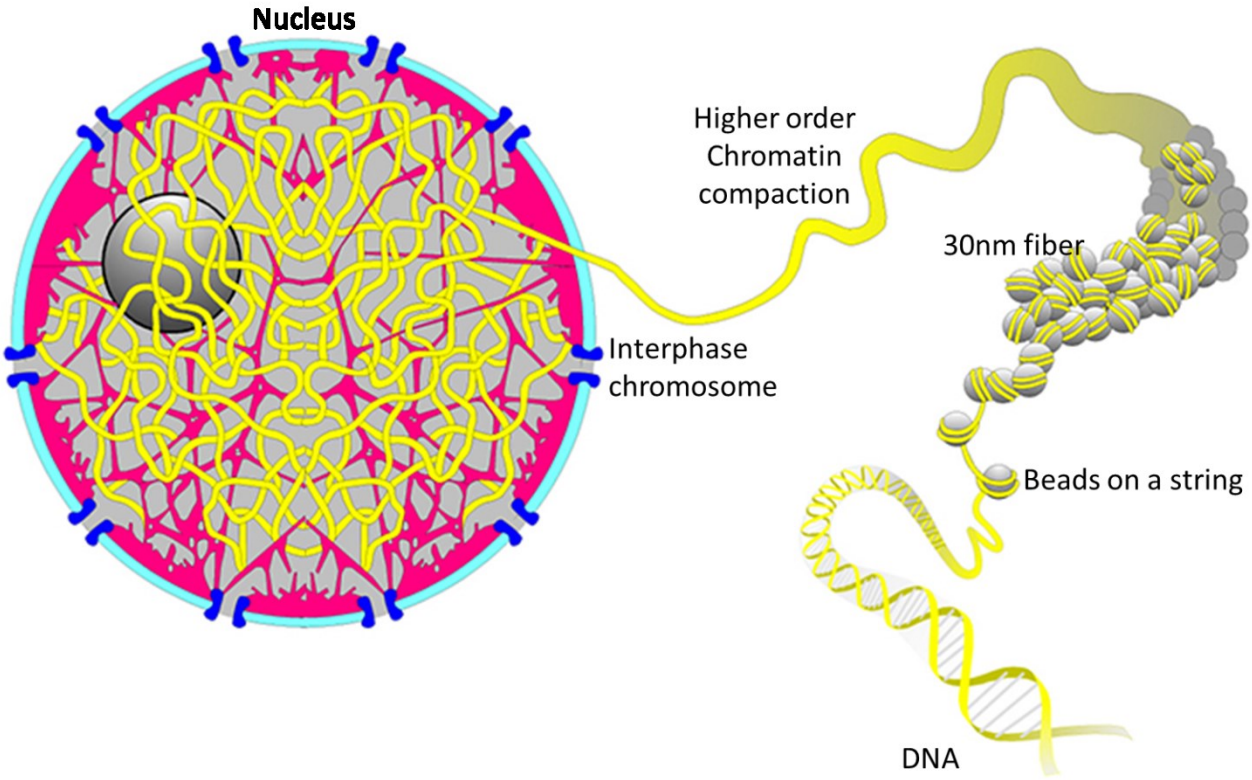


Figure 1. Organization of eukaryotic DNA

Adapted from http://www.biology.emory.edu/research/Corces/Research2.html

SIN3A and its essential role in *Drosophila melanogaster*

The SIN3A-RPD3 complex is a histone deacetylase (HDAC) complex that is conserved in many species including yeast, *Drosophila* and human (Silverstein and Ekwall, 2005). Although SIN3A is believed to be a transcriptional repressor of eukaryotic genes, it is also believed to play a role in activation (Icardi *et al*., 2012). In polytene chromosome analysis in *Drosophila melanogaster* salivary glands, SIN3A and RPD3 were found to bind throughout the genome but in less condensed euchromatin (Pile and Wasserman, 2000). SIN3A and RPD3 colocalize along most of the chromosome arms but there are some differences in their binding patterns. The SIN3A-RPD3 complex does not bind onto DNA directly but instead is able to bind through interaction with DNA binding proteins, which allow SIN3A to be targeted to specific genes through protein-protein interactions (Knoepfler and Eisenman, 1999).

Sin3A is an essential gene in *Drosophila melanogaster*. A null mutation of *Sin3A* causes lethality at some point in the embryonic stage of development with very few embryos being able to transition into the first larval instar stage (Neufeld *et al*., 1998; Pennetta and Pauli, 1998). SIN3A has also been implicated in cell cycle progression and is necessary for transition from the second growth phase into mitosis in the cell cycle (Pile *et al*., 2002). SIN3A was also found to be essential for cell proliferation in *Drosophila melanogaster* larval wing discs (Swaminathan and Pile, 2010). The loss of SIN3A in the developing wings of the fruit fly causes a curved wing phenotype with overall smaller wings showing that SIN3A is essential for normal development in adult flies.

SIN3A has a role in hormone signaling, which is one of the ways in which SIN3A plays a role in the developmental process of *Drosophila melanogaster* (Tsai *et al*., 1999; Sharma *et al*., 2008). An essential hormone for *Drosophila* developmental progression is ecdysone. It is known to control the metamorphosis of the fly by way of the ecdysone receptor (EcR), which activates vital transcription processes (Riddiford *et al*., 2001). The protein SMRTER, which is corepressor known to interact with EcR, has been shown to colocalize and associate with SIN3A (Tsai *et al*., 1999; Pile and Wasserman, 2000). Interestingly, the level of chromosome binding of SIN3A decreased when ecdysone activation of transcription took place and increased when there was repression of transcription, further confirming the role of SIN3A in transcription repression and development (Pile and Wasserman, 2000; Pile *et al*., 2002).

SIN3A isoforms and complexes

SIN3A has three isoforms, SIN3 187, SIN3 190 and SIN3 220, that differ in their carboxyl (C) - terminal ends (Fig 2) (Pile and Wasserman, 2000; Sharma *et al*., 2008). SIN3 190 does not have a homolog in other insect species and was only found to be expressed in early embryos and adult females, which is why it was not examined as closely as the two other isoforms (Sharma *et al*., 2008). Both SIN3 187 and 220 have functional differences; they were found to bind to similar and unique areas in the *Drosophila melanogaster* genome based on polytene chromosome analysis (Spain *et al.*, 2010). During the development of *Drosophila melanogaster* the SIN3A isoforms are differentially expressed. SIN3 187 was found to be expressed in differentiated adult tissues while SIN3 220 was expressed more in highly proliferating cells of developing tissues (Sharma *et al*., 2008).

SIN3A is thought to act as a scaffold protein, which allows for the assembly of its complex components and targets the complex to its specific promoter sites (Silverstein and Ekwall, 2005). The members of the SIN3A complex were identified by the coimmunoprecipitation of SIN3 220 and SIN3 187 isoforms from *Drosophila* S2 cells and embryo extracts (Spain *et al*., 2010). The proteins that were found to interact with the individual isoforms were identified through liquid chromatography tandem mass spectrometry (LC/MS/MS). Associating with both isoforms, RPD3, ARID4B, and SDS3 were at similar levels and therefore are believed to be present in both complexes (Fig 3 A and B). In the SIN3 220 complex higher levels of SAP130, BRMS1, ING1 and p55 were found relative to levels associated with SIN3 187. LID and EMSY were also found to be a unique part of SIN3 220 (Fig 3 B).

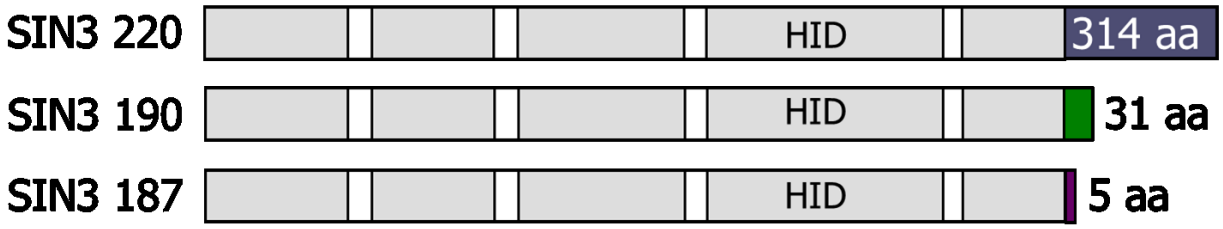


Figure 2. SIN3A isoforms. White boxes represented paired amphipathic helixes, which are known protein-protein interaction domains. HID, Histone interaction domain. Blue box amino acids of the C-terminal tail for SIN3 220, green box amino acids of the C-terminal end of SIN3 190, purple box amino acids of the C-terminal end of SIN3 187.

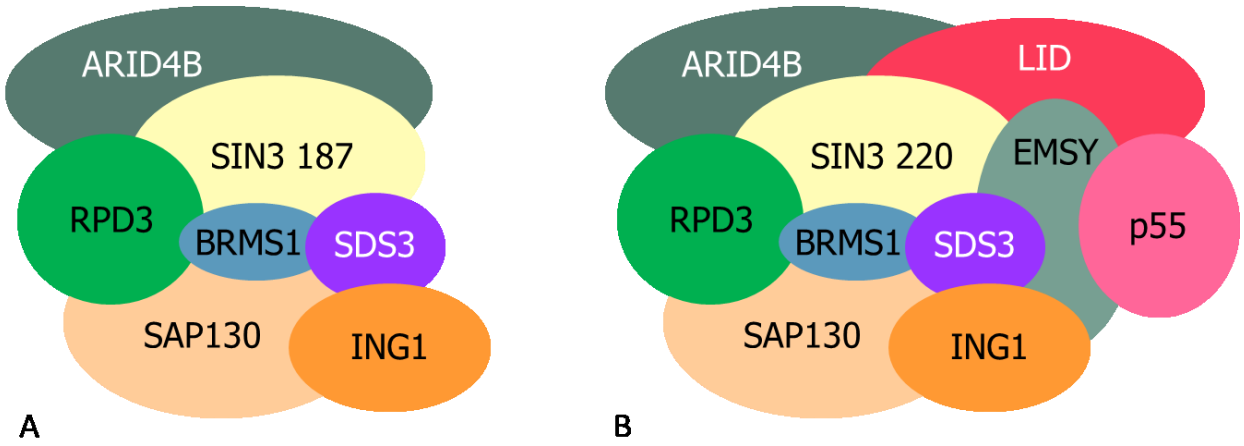


Figure 3. Cartoon of the SIN3 187 and 220 complexes. SIN3 187 (A) and SIN3 220 (B) complex and their known members.

The members of the SIN3A-RPD3 complex in *Drosophila melanogaster* have been identified but it is still not fully understood how these complex components affect SIN3A activity. Since the SIN3A-RPD3 complex is known to bind DNA indirectly, it was of interest to know whether any of the SIN3A complex components are playing a role in the binding of SIN3A to chromatin. This analysis was done with salivary gland polytene chromosomes of *Drosophila melanogaster*. Polytene chromosomes allow for the analysis of transcription and binding activity for chromatin of polyploid interphase cells (Hill *et al*., 1987). SIN3 220 was found to be the predominant isoform in *Drosophila melanogaster* salivary gland extracts (Pile and Wasserman, 2000). It was also found to associate with specific proteins that are involved in chromatin recruitment and histone modification (Spain *et al*., 2010). Therefore, components of the SIN3 220 complex were used to examine their effect on SIN3A binding; these included SDS3, ING1, SAP130, BRMS1, CAF1/p55, LID and ARID4B. SIN3 220 has also been found to be more predominantly expressed in developing tissues (Sharma *et al*., 2008). This suggests

that these complex components may also play a role in development, more specifically in cell proliferation. This can be analyzed through clonal analysis, which was used previously to show SIN3A effects on cell proliferation in third instar wing imaginal discs (Swaminathan and Pile, 2010).

CG34422, also known as *Arid4b* and *CG7274*, is a part of the ARID (AT-rich interaction domain) family of DNA binding proteins (Kortschak *et al*., 2000). The specific roles for this protein are not fully understood but it has been implicated in having both a positive and negative role in transcription regulation and may even be involved in modifying the structure of chromatin. In addition to being a member of the SIN3A complex, ARID4B was also found in an RNAi screen to be required for phagocytosis of *Candida albicans* by *Drosophila melanogaster* (Stroschein-Stevenson *et al*., 2006). ARID4B has protein-protein interactions with both SIN3A and BRMS1, another member of the SIN3A complex (Mintseris *et al*., 2009; Spain *et al*., 2010).

Brms1 is the *Drosophila melanogaster* homolog of the human gene *Brmsl-1*, breast cancer metastasis suppressor 1-like. BRMS1 has been found to have many protein-protein interactions including CG34433 (ARID4B), CG7379 (ING1), CG14220 (SDS3), RPD3, SAP130 and SIN3A, which are all known members of the SIN3A complex (Mintseris *et al*., 2009; Spain *et al*., 2010). One of the more recent discoveries was the role of BRMS1 as an essential gene in *Drosophila melanogaster*, playing roles in ecdysone signaling that is required for metamorphosis and normal fly development (Song *et al*., 2013).

CAF1/p55 is also known as chromatin assembly factor 1. It is one of the more well known members of the SIN3A complex and is a member of many other complexes

in *Drosophila melanogaster*. p55 is a subunit of the NURF complex, which is associated with polytene chromosomes and impacts the assembly of different protein complexes onto chromatin (Martinez-Balbas *et al*., 1998). It has also been predicted to be important for SIN3A complex recruitment and stabilization to target genes (Spain *et al*., 2010).

CG7379, also known as *Ing1*, is a member of the inhibitor of growth family of proteins. It is a chromatin modifying protein that has a similar C-terminal sequence to other ING family members and contains PHD finger domains, which are involved in transcription regulation (Loewith *et al*., 2000). A study done in human 293T cells found that loss of ING2, which is found to be part of the human SIN3B complex, interrupts SIN3B binding onto specific promoters (Smith *et al*., 2010). ING1 has been found to have protein-protein interactions with BRMS1, RPD3, SAP130 and SIN3A (Mintseris *et al*., 2009; Spain *et al*., 2010).

Little imaginal discs (*lid*) encodes a histone demethylase that demethylates lysine 4 of histone H3 and is associated with actively transcribed genes (Secombe *et al*., 2007). When *lid* is mutated there is an increase in the levels of H3K4me3. It is believed that LID contributes to the functional differences between SIN3 187 and SIN3 220 since LID is found in the SIN3 220 complex (Spain *et al*., 2010). Mutation of *lid* has also been found to affect chromatin organization by affecting promoters that control heterochromatin spreading past heterochromatin-euchromatin boundaries (Di Stefano *et al*., 2011).

Rpd3 belongs to the histone deacetylase 1 family. It functions as a transcriptional corepressor (Miotto *et al*., 2006). RPD3 is present in a variety of protein complexes, which was determined by numerous physical interactions (Tie *et al*., 2003; Lewis *et al*.,

2004; Thompson and Travers, 2008). RPD3 has many protein-protein interactions including with BRMS1 and SIN3A (Mintseris *et al*., 2009; Spain *et al*., 2010). RPD3 can function independently of SIN3A. It is a member of the Mi-2-NURD complex, which interacts with other *Drosophila melanogaster* repressors such as Hunchback and RPD3 also interacts with the corepressor Grouncho (Kehle *et al*., 1998; Ayer, 1999; Chen *et al*., 1999).

Sin3A-associated protein 130 (SAP130) may function in the assembly or enzymatic function of the SIN3A complex (Fleischer *et al*., 2003). It may also control the interaction that the SIN3A complex has with promoters and other complexes. An RNAi screen preformed on *Drosophila melanogaster* S2 cells identified SAP130 as being required for mitotic spindle organization (Goshima *et al*., 2007). SAP130 has several protein-protein interactions including BRMS1, ING1 and SIN3A (Mintseris *et al*., 2009; Spain *et al*., 2010).

CG14220 (*Sds3*) has a conserved region found in Sds-like family of proteins, which are believed to be involved in transcription repression by histone deacetylases that contain co-repressor complexes (Nikolaev *et al*., 2004). SDS3 was found to interact with BRMS1 and SIN3A through protein-protein interactions (Mintseris *et al*., 2009; Spain *et al*., 2010). The human ortholog of SDS3 is *Suds3*, suppressor of defective silencing 3. The yeast homolog of SDS3 was found to have an important role regulating the ability of in SIN3A to repress transcription in an HDAC dependent manner (Alland *et al*., 2002).

Project summary

To investigate the effect that the SIN3A-RPD3 complex components have on SIN3A binding, RNA interference (RNAi) was used to reduce the expression of the complex members. This allowed for the knockdown of an individual complex component of interest and allowed the visualization of SIN3A binding onto chromatin using an antibody against SIN3A. When lines containing UAS-RNAi transgenes are crossed with a GAL4 driver, there is formation of a hairpin loop of RNA of the gene of interest, which subsequently targets the mRNA for degradation (Duffy, 2002). Two different GAL4 drivers were utilized for this work, Feb36-GAL4 and eyeless-GAL4, which are both expressed in the salivary glands of *Drosophila melanogaster* (Andrews *et al*., 2002; Hazelett *et al*., 1998; Corona *et al*., 2007). This allowed for a more detailed comparison and confirmation of the results. Taken together, this study of the components of the SIN3A-RPD3 complex indicates that some, but not all, factors have an effect on SIN3A binding onto chromatin.

To demonstrate the effects caused by the knockdown of the SIN3 complex components on cell proliferation, clonal analysis was performed on wing imaginal discs of *Drosophila melanogaster*. GFP positive clones were randomly generated using a heat shock flip out system (Hyun *et al*., 2005). Reduced clonal growth in the mutant wing discs indicates a requirement of the complex component for cell proliferation. The percentage of GFP positive clones were quantified for each complex component and compared to controls preformed with w^{1118} and mCherry RNAi lines. Taken together, the results indicate that some of the SIN3A complex components have an affect on cell cycle progression in *Drosophila melanogaster*.

CHAPTER 2 MATERIALS AND METHODS

Stocks

Gene	Stocks
Sin3A	UAS-RNAi-SIN3-17 (Sharma *et al*., 2008)
CG14220 (Sds3)	VRDC v105162 P{KK102695}VIE-260-B
CG7379 (Ing1)	VDRC v27988 w^{1118}; P{GD12222}v27988
Sap130 (CG11006)	VDRC v31394 w^{1118}; P{GD7168}v31394
Caf1 (p55/CG4236)	VDRC v105838 P{KK102930}VIE-260B
Brms1 (CG4400)	VDRC v105494 P{KK108153}VIE-206-B
lid (CG9088)	Bloomington 29844 y^1 v^1; P{TRIP.HM05155}attP2
CG34422 (ARID4B)	Bloomington 31754 y^1 v^1; P{TRIP.HM04064}attP2
Rpd3 (CG7471)	VDRC v46930 w^{1118}; P{GD17233}v46930/TM3
mCherry	Bloomington 35785 y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=VALIUM20-mCherry}attP2
hsFLP	Gift from the Bohmann lab at U of Rochester
EGFP	Bloomington 6658 y[*] w[*]; P{w[+mC]=UAS-2xEGFP}AH3
eyeless-GAL4	Bloomington 8220 y[1] w[1118]; P{w[+mC]=ey3.5-GAL4.Exel}2
Feb36-GAL4	Bloomington 29968 w[*]; P{w[+mW.hs]=GawB}Feb36

Table 1. *Drosophila melanogaster* stocks.

System to Induce RNAi Knockdown in Larval Development

To target certain genes and alter their expression, the UAS/GAL4 system in *Drosophila melanogaster* was utilized. When an upstream activating sequence (UAS) is combined with an RNA-mediated interference (RNAi) sequence, this allows for controlled reduction of expression of specific genes. When lines containing these transgenes are crossed with a GAL4 driver, which “drives” the expression of the activator GAL4 under the control of a specific regulatory element, there is a formation of a hairpin loop of RNA that targets mRNA of the gene of interest causing degradation (Duffy, 2002). The GAL4 fly lines that are used in this work are tissue specific drivers that promote expression in the salivary glands of *Drosophila melanogaster*.

The goal of the initial set of crosses was to examine GAL4 expression levels in the salivary glands of early and late third instar larvae. Flies containing the prothoracic

gland driver (Feb36-GAL4) and the eyeless-GAL4 driver were separately crossed to flies containing UAS-EGFP, a transgene that encodes enhanced green florescent protein activated by the UAS element. The parents were put into vials containing instant fly food (Carolina Biologicals) mixed with 0.05% bromophenol blue and laid embryos onto the food. The progeny of the crosses were raised on this blue food. Early third instar larvae will have more blue pigmentation in their digestive track while late third instar will have little or no blue pigmentation because they are closer to the prepupal stage of development (Maroni and Starmey, 1983). Early and late third instar animals were selected based upon the amount of blue pigmentation observed in the gut and their salivary glands were dissected. The glands were immediately observed under a fluorescence microscope. Images were collected at 200x using Qcapture to determine the levels of GFP at the different stages of development.

Polytene Chromosome Preparation

Polytene chromosome preparation and staining methods were modified from the protocol outlined in Pile and Wasserman (2002). *Drosophila melanogaster* fly lines were raised using standard laboratory protocols at 27°C until the progeny reached the third instar stage of larval development. The salivary glands from the larvae were dissected in 1 X PBS (phosphate-buffered saline) and transferred into 15 µl of fixative (45% acetic acid, 3.7% formaldehyde in deionized distilled water) on a siliconized coverslip for one min. The glands and coverslip were then transferred to a superfrost glass slide and any excess fixative was removed. Using a spoonula spatula, the glands were squashed ten times to burst the nuclei. The tip of a pencil eraser was tapped approximately 50 times over the coverslip to spread the polytene chromosomes. An inverted microscope was

used to confirm the proper form of the polytene chromosomes. The slides were then submerged into liquid nitrogen to fix the polytene chromosomes onto the glass slide and the cover slip was removed. The slides were stored in 95% ethanol until further processed.

Polytene Immunostaining and Imaging

Slides were removed from 95% ethanol storage and washed in a slide chamber in 1 X PBS two times for 30 min. During this time the boundary of the polytene chromosome spread was marked with a PAP pen (Scientific Device Laboratory) to allow for any reagents added to the slides to be concentrated over the spread. The slides were then incubated with blocking buffer BTP (0.5% bovine serum albumin (BSA), 0.1% Tween in 1 X PBS) in a humid chamber for 30 min. The block was removed and a primary antibody against SIN3 (1:1000) (Pile and Wassermann, 2000) was added and the slides incubated at room temperature in the humid chamber for one hour. The primary antibody was removed from the slides by rinsing with wash buffer (0.01% Tween in 1 X PBS). The slides were placed in a slide staining chamber with fresh wash buffer two times for 10 min. After the wash was completed the slides were transferred back into the humid chamber. A secondary antibody Alexa Flour 594 (1:400) (Life Technologies) was added to each slide and the polytenes were incubated for 30 min while covered. The secondary antibody was removed by rinsing wash buffer over the slides followed by one 10 min wash in a slide staining chamber with fresh wash buffer. The slides were removed from the wash buffer, dried and place on a stack on paper towels lined with Kimwipes. A drop of Vectashield mounting medium with DAPI (Vector Laboratories, Inc) was added to each slide and a clean non-siliconized coverslip was

added. The slides were then covered with Kimwipes and more paper towel and a heavy book (about five pounds) was placed on top for one hr. The polytene chromosome spreads were then stored at 4°C in a light tight slide holder until imaged. Polytene were imaged using a compound microscope by Zeiss and Qcapture analysis at 400x. All stainings were done with experimental slides included control slides, which contained polytenes prepared from one of the parents of the cross. The control and experimental slides were all imaged at the same offset and exposure levels. Polytenes were prepared from a minimum of three independent parental crosses and representative images are shown.

Clonal Analysis

hsFLP;Act5C > CD2 > GAL4, UAS-EGFP flies were crossed to UAS-mCherry or UAS-complex component RNAi fly lines. The hsFLP;Act5C > CD2 > GAL4, UAS-EGFP transgene allows for the development of random GFP positive clones. Embryos were collected from 0-4 hr on apple juice agar plates, placed on molasses food and incubated at 27°C. When the larvae reached second instar larval stage at 48-52 hr after egg laying (AEL) they were heat shocked at 37°C for 2 hr. The wing discs from wandering third instar larvae (approximately 120 hr AEL) were dissected and immunostained with antibodies against GFP as described below.

Immunostaining Cloned Wing Discs

The wing discs from wandering third instar larvae were dissected in 1 X PBS. About 20-30 discs were fixed in 4% formaldehyde in 1 X PBS and stained using the protocol described in Swaminathan and Pile (2010). A primary antibody against GFP (1:1000) (Abcam) and secondary sheep anti-mouse Alexa 488 (1:2000) (Invitrogen)

were used to stain the wing discs. The discs were mounted onto glass slides using Vectashield mounting medium with DAPI (Vector Laboratories, Inc.) and a clean cover slip was added. Wing discs were imaged using a Zeiss microscope with a Qcapture imaging system at 400x.

CHAPTER 3 RESULTS AND DISCUSSION: Phenotypic Analysis of SIN3A Complex Components

Section 3.1: GAL4 drivers and expression of target genes in third instar larvae

To investigate the effects that the SIN3A-RPD3 complex components have on SIN3A recruitment onto chromatin, the individual complex components were knocked down using the UAS/GAL4 system. RNAi allows for the inhibition of a genes expression by the degradation of mRNA (Saudi, 2012). This occurs following formation of double standed RNA (dsRNA), which contains a complementary sequence to the gene of interest. The dsRNA activates the RNAi pathway. The enzyme Dicer cleaves the dsRNA into short fragments of small interfering RNAs (siRNA), which is further degraded into single-stranded RNAs (ssRNA). One of the ssRNA stands is integraded into RNA-induced silencing complex (RISC). This allows agronaute, a part of RISC, to cleave the target mRNA that is complementary to the siRNA, for degradation.

The RNAi knockdown of *Sin3A* in *Drosophila melanogaster* causes death in the embryonic stage of development (Sharma *et al*., 2008). Unpublished data from our laboratory has demonstrated that the individual members of the SIN3A complex are all essential for viability when knocked down using the Actin–GAL4 driver, which provides ubiquitous expression of the GAL4 activator (Barnes *et al*.). This made it necessary to use a tissue specific driver, instead of one that knocks down the protein throughout the whole fly. For the purposes of this work salivary gland specific drivers were used.

The driver Sgs3-GAL4 was originally used to test for knockdown of the SIN3A complex components in the salivary glands. This driver has been shown in previously published work to allow for knockdown in salivary glands of *Drosophila melanogaster* (Yurlova *et al*., 2009). In that work the authors found the Sgs3 regulatory element to be

most active in mid-third instar larvae, when most of the replication in the salivary glands has stopped. This small window when the driver is most active was problematic when isolating salivary glands and examining the level of SIN3A in the polytene chromosome spreads. There were inconsistencies between crosses and spreads depending on the age of the larvae that were used in the preparations (data not shown). Due to the problems with this driver, others had to be chosen based on their level of GAL4 expression in the salivary gland and when GAL4 was expressed during third instar larval development.

The next driver tested was Feb36-GAL4, which will be referred to as the prothoracic gland driver in this work. This driver has been shown to promote GAL4 expression in the salivary glands, ring gland, trachea, cells in the midgut and malphigian tubules (Andrew *et al*., 2002). To determine the level of GAL4 expression in early and late third instar larvae, a fly containing the GAL4 transgene was crossed with a fly that carried a transgene for enhanced green fluorescent protein (EGFP). To better determine the stage of third instar development in which the progeny of this cross were at, the larvae were fed food containing bromophenol blue. Early third instar larvae have a greater amount of blue visible in their gut compared with late third instar larvae (Fig 4 A and B). For the prothoracic gland driver, salivary glands that were dissected out of early and late third instar larvae had approximately equal levels of GFP expression (Fig 4 A' and B').

The eyeless-GAL4 driver was chosen to compare the results obtained using the prothoracic gland driver. This particular driver has been shown in several published papers to have expression in the salivary glands (Hazelett *et al*., 1998; Corona *et al*,.

2007). When this driver was crossed with EGFP, it did not have as great of a level of GAL4 expression as the prothoracic gland driver, as indicated; GFP expression, however, was approximately equal in both early and late third instar larvae (Fig 4 A'' and B''). This finding indicates that the larvae of both early and late third instar can be used in this study to see the effect of SIN3A binding onto the polytene chromosomes of *Drosophila melanogaster* when SIN3A complex components are knocked down.

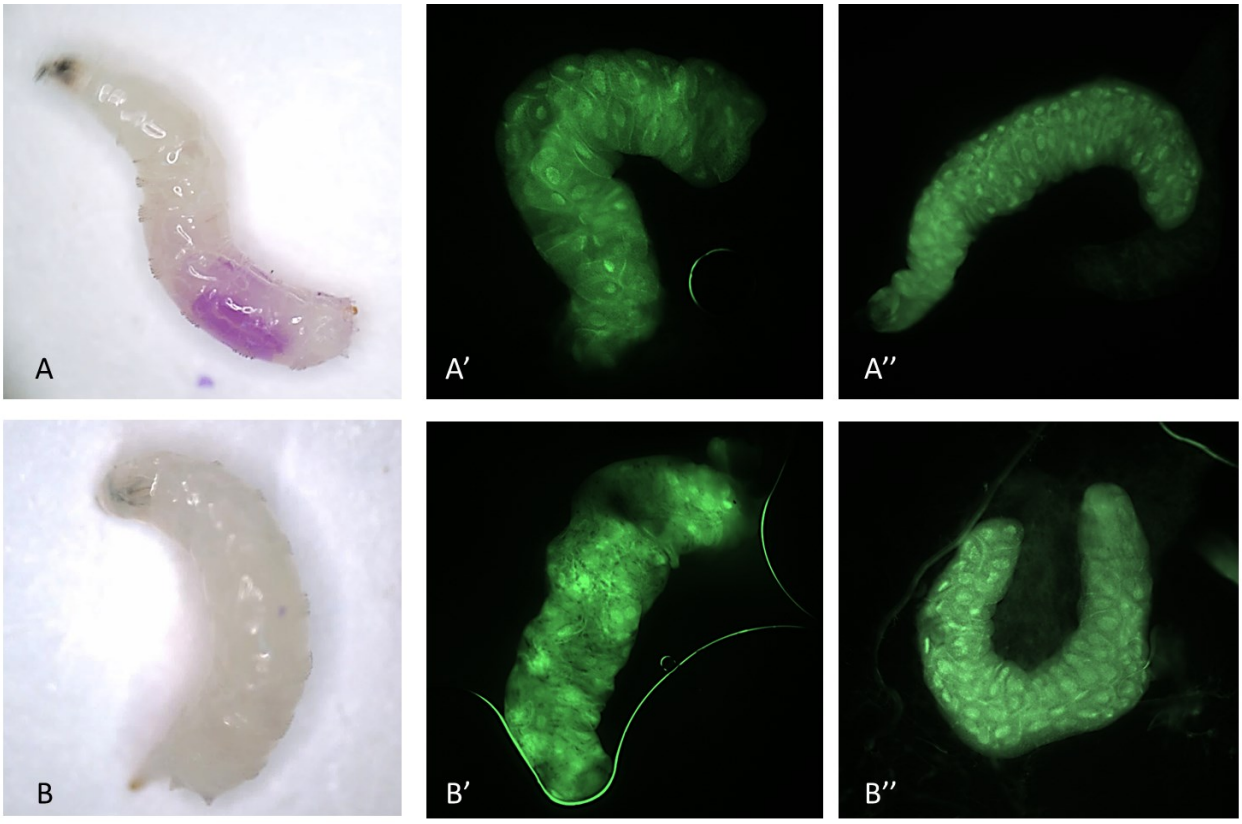


Figure 4. GAL4 drivers expression in early and late third instar larvae. (A) Early third instar larva with bromophenol blue visible in the gut. (B) Late third instar larva. Salivary glands dissected from early third instar (A') and late third instar (B') progeny of prothoracic gland driver crossed to EGFP. Salivary glands dissected from early third instar (A") and late third instar (B") progeny of eyeless-GAL4 crossed to EGFP.

Section 3.2: The level of RNAi knockdown of *Sin3A*

It was known that the GAL4 drivers used activate GAL4 expression in salivary glands but the drivers needed to promote enough GAL4 expression to be able to target mRNA for degradation to properly knockdown the complex components. The level of *Sin3A* knockdown using the two different GAL4 drivers was tested first because an antibody against SIN3A was available. A fly with a transgene containing UAS-RNAi-SIN3A (SIN3A KD1) was crossed to both drivers separately. Salivary glands were dissected from progeny in the wandering third instar larvae stage of development and polytene chromosomes were prepared and stained for SIN3A. All experimental knockdown slides were stained at the same time as control slides. The level of reduced expression for the complex components was verified in wing imaginal discs through qRT-PCR analysis of mRNA, data not shown (Barnes *et al*.).

The polytene chromosomes for these knockdown crosses and others to follow were stained along with a control polytene chromosome spread made from the GAL4 drivers stocks. This allowed for the direct comparison of differences in the levels of SIN3A staining on the polytene chromosomes when RNAi knockdown is present. The levels of SIN3A normally found on polytene chromosomes are represented in Fig 5 A' and C'. SIN3A was bound throughout the *Drosophila melanogaster* genome, consistent with previously published results (Pile and Wasserman, 2000). DAPI staining was performed to confirm proper morphology of the polytene chromosomes. DAPI stains double stranded DNA and binds to A-T rich regions of DNA (Kubista *et al*., 1987). SIN3A was found to bind in less condensed euchromatin regions (Fig 5 A' and C').

When *Sin3A* was knocked down using the eyeless-GAL4 and prothoracic gland drivers, there was a very clear reduction in the amount of SIN3A staining on the polytene chromosomes (Fig 5 B' and D') compared with the controls (Fig 5 A' and C'). The knockdown of *Sin3A* showed little or no SIN3A staining for both the prothoracic gland driver (Fig 5 B and B') and the eyeless-GAL4 driver preparations (Fig 5 D and D'). This gave another confirmation, along with the GFP expression shown in Fig 4, that the GAL4 drivers are able to induce RNAi knockdown in polytene chromosomes.

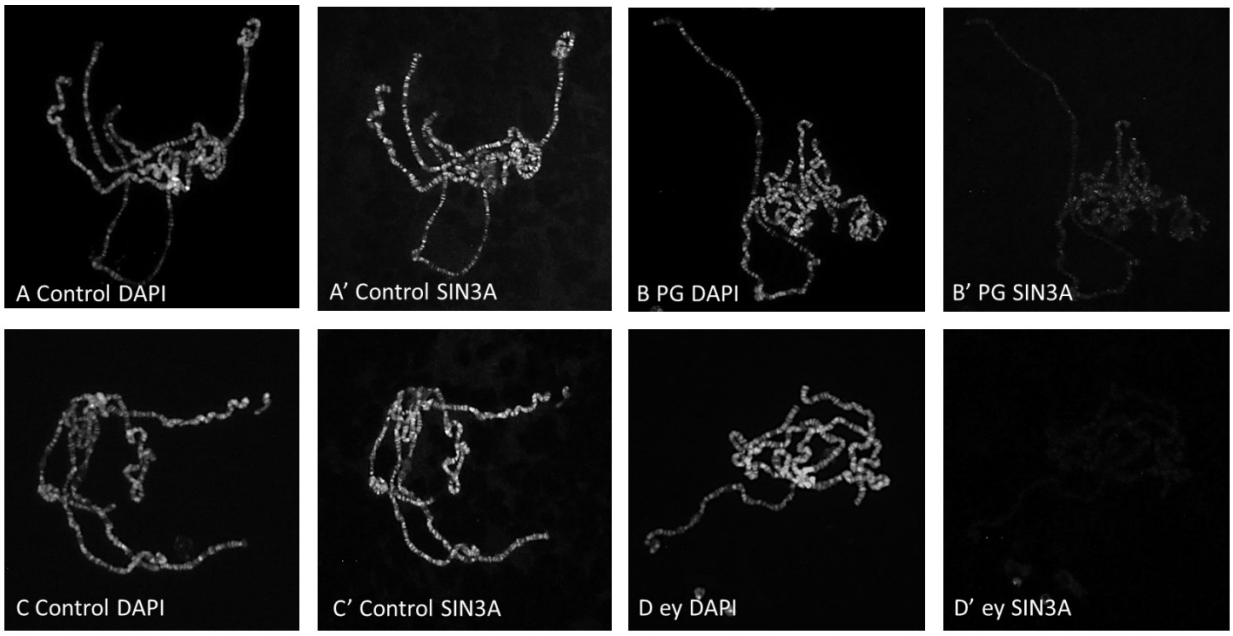


Figure 5. *Sin3A* control and knockdown polytene chromosomes. Controls for the level of SIN3A staining on the polytene chromosome prothoracic gland driver(A') and eyeless-GAL4 (C'). SIN3A KD1 crossed with the prothoracic gland driver (B, B') shows reduced levels of SIN3A staining when knocked down (B'). SIN3A KD1 crossed with eyeless-GAL4 (D, D') shows reduced levels of SIN3A staining when knocked down (D'). DAPI staining (A, C, B, D). SIN3A staining (A', B', C', D')

Section 3.3: The effect of SIN3A complex members on SIN3A binding onto polytene chromosomes

SIN3A binds indirectly onto DNA through an interaction with DNA binding proteins. The members of the SIN3A complex have been previously identified as being ARID4B, BRMS1, SDS3, SAP130, RPD3, ING1, Lid and Caf1/p55 (Spain *et al*., 2010). These are the members of the SIN3 220 complex and SIN3 220 is found to be the predominantly expressed isoform in the salivary glands of *Drosophila melanogaster* (Pile and Wasserman, 2000). The SIN3A complex components were individually knocked down through UAS-RNAi induction. Polytene chromosomes were prepared and stained for SIN3A to see the effect on SIN3A binding onto chromatin.

ING1 was looked at first based on inconsistencies in previously published data. Work done with the human homolog of ING1, ING2, affected the ability of SIN3B to bind to specific promoters (Smith *et al*., 2010). More recently published data from Cheng *et al*., (2014) showed that ING1 alone is not responsible for the recruitment of SIN3A to chromatin. Rather E2F4, a factor known to recruit SIN3A to DNA, is believed to be playing a role allowing SIN3A to continue to bind to DNA even in the absence on ING1. It is possible that ING1 on its own is not able to recruit SIN3A to DNA but requires one or more factors in order for this to take place in *Drosophila melanogaster*. It was curious as to which one of these findings were true for ING1 in *Drosophila melanogaster*. When flies containing a transgene with UAS-RNAi for ING1 were crossed to the prothoracic gland driver, there was no distinct differences seen between the polytenes with ING1 knockdown (Fig 6 A', B', C') and control polytenes (Fig 5 A'). The same RNAi fly line for ING1 was crossed to the eyeless-GAL4 driver. There was also no noticeable difference

between these polytenes (Fig 6 D', E', F') compared with the control (Fig 5 C'). It is possible that ING1 on its own is not able to recruit SIN3A to DNA but requires one or more factors in order for this to take place in *Drosophila melanogaster*.

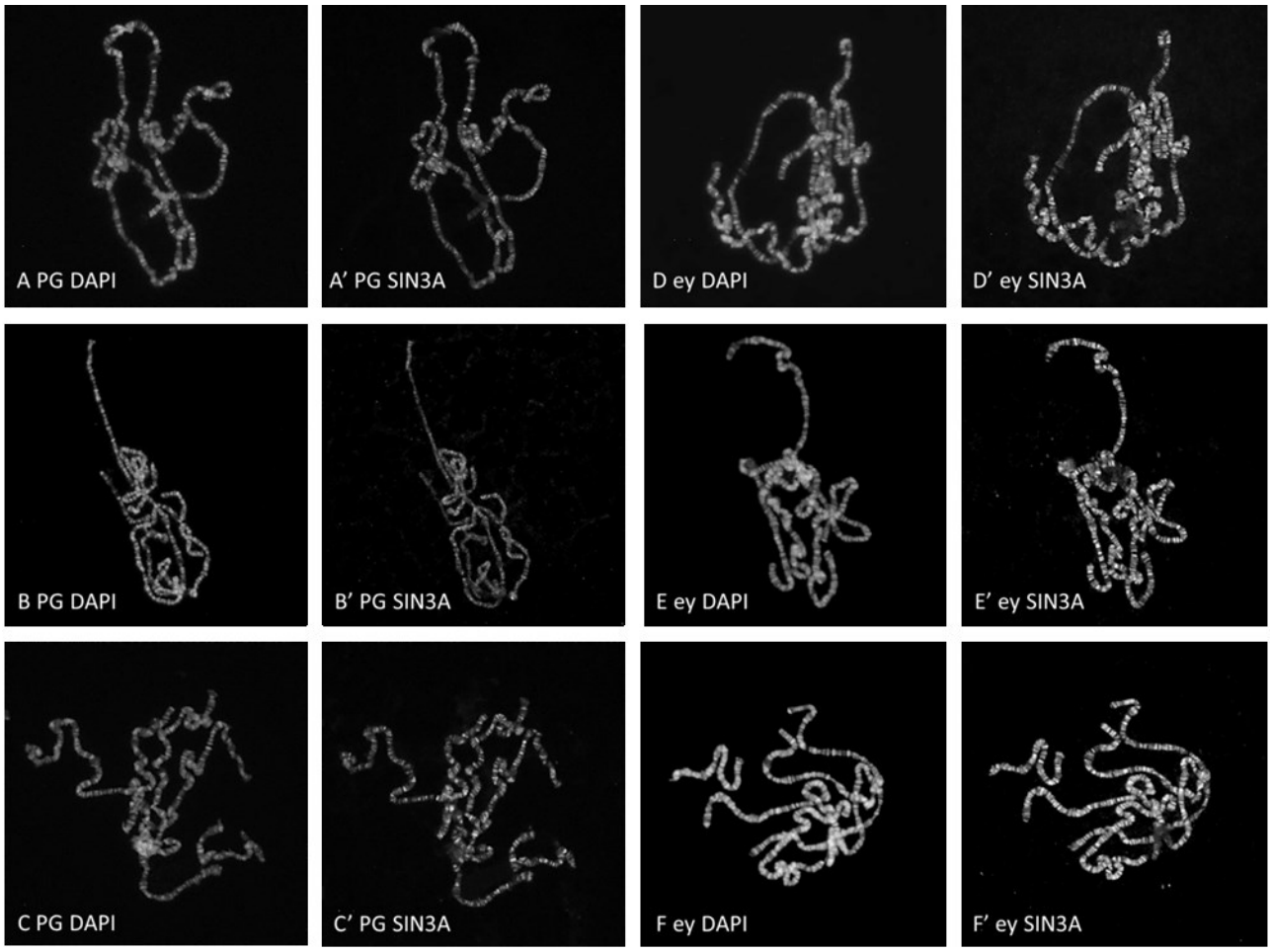


Figure 6. *Ing1* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of UAS-RNAI-ING1 crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-ING1 crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross.

Another member of the SIN3A complex, LID, was next examined to see the effect its knockdown has on SIN3A recruitment to chromatin. Reduced levels of LID have been shown in unpublished work from the Pile laboratory to have phenotypic similarities to those resulting from reduction of SIN3A (Gajan *et al*.). When the RNAi fly line for LID was crossed to the prothoracic gland driver the results showed a noticeable difference in SIN3A staining (Fig 7 A', B', C') compared to a control (Fig 5 A'). There is

an obvious change in the amount of SIN3A staining along with its localization on chromatin. The normal binding pattern of SIN3A also changes when LID is knocked down. There is reduced binding in some areas, along with areas of increased brightness. The same can be seen when the LID RNAi fly line was crossed to eyeless-GAL4 (Fig 7 D', E', F') compared to the control (Fig 5 C'). There is a change in the binding pattern of SIN3A and the level of SIN3A staining. This finding shows a role for LID in the recruitment and proper localization of SIN3A to chromatin.

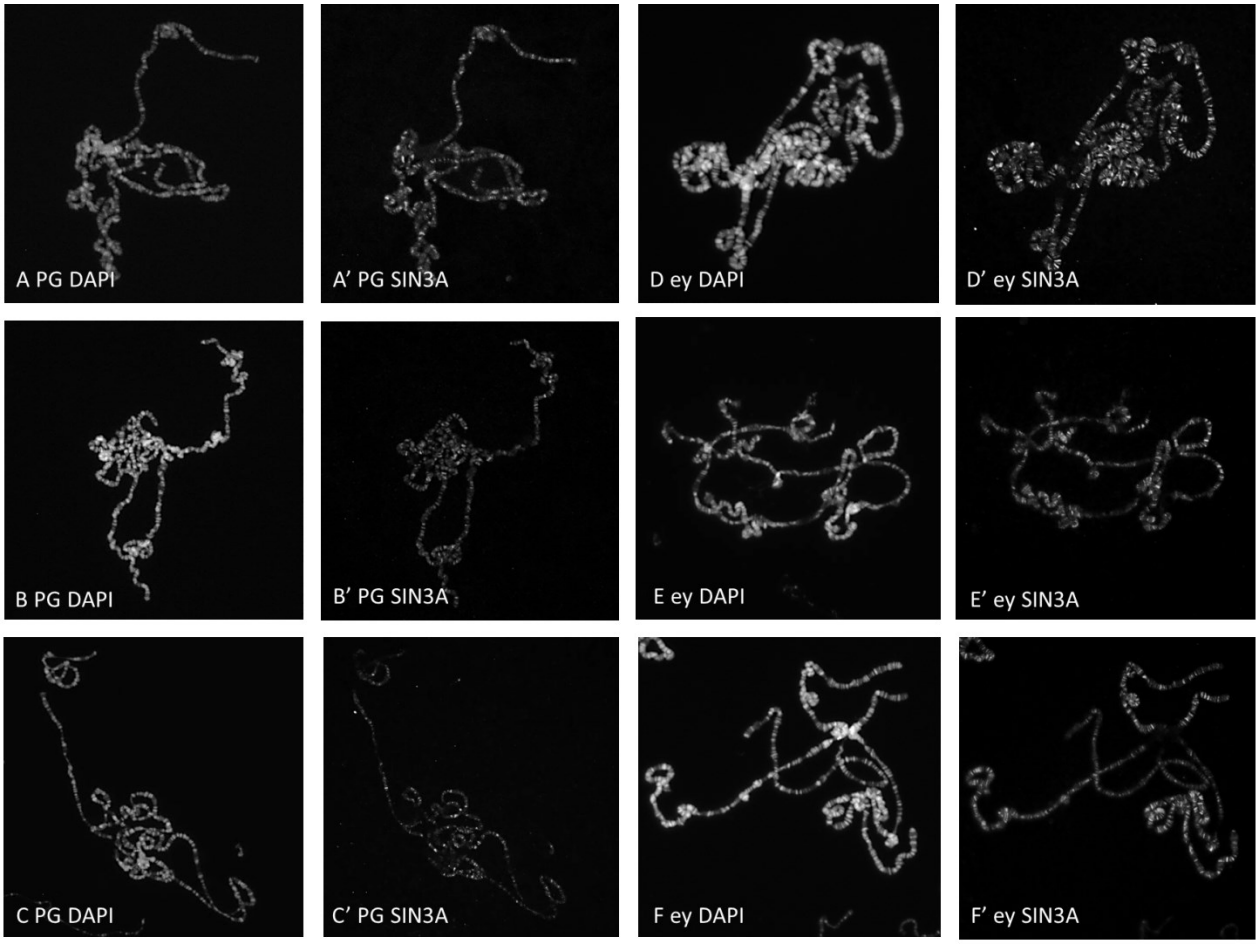


Figure 7. *Lid* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of UAS-RNAi-LID crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-lid crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross.

RPD3, which has deacetylase activity, was knocked down to determine whether or not it affects the ability of SIN3A to bind to DNA. SIN3A and RPD3 colocalize throughout the *Drosophila melanogaster* genome but they have some differences in binding patterns (Pile and Wasserman, 2000). The RPD3 RNAi line that was used in this experiment was heterozygous and required the use of a balancer. This allowed for progeny with RNAi to be separated from the wild type control. Since this work was done with larvae, a tubby balancer was used. The progeny of these crosses have tubby larvae, which will not carry the *Rpd3* RNAi transgene and have no knockdown; these were used as a control. Larvae without the tubby body phenotype have *Rpd3* knockdown since they carried a UAS-RNAi transgene. When *Rpd3* was knocked down with the prothoracic gland driver there was an obvious phenotypic abnormality seen in the salivary glands of the knockdown larvae compared with the control tubby larvae (Fig 8 A and B). Control larvae had normal sized salivary glands but in the knockdown larvae, the salivary glands were considerably smaller in size with less condensed nuclei that had less DAPI staining. When polytene chromosomes were prepared from the knockdown larvae there was a severe phenotypic abnormality (Fig 8 D and D') compared to the polytenes isolated from the control tubby larvae (Fig 8 C and C'). The polytene chromosomes were over fixed and broken up from the preparations. In order for a more complete spread to be made, the fixation time was reduced from 1 min to 20 s. This allowed for a better polytene spread although the chromosomes were still broken up. The level of SIN3A staining on the knockdown polytenes (Fig 8 D') was similar to the level of SIN3A in the control tubby polytenes (Fig 8 C'), even with the abnormalities of the polytene chromosomes. This finding was further confirmed using the eyeless-

GAL4 driver. The severe phenotypes observed using the prothoracic gland driver did not occur when using the eyeless-GAL4 driver (data not shown). This may be due to RPD3 having a role in the hormone signaling from the prothoracic gland, which controls the growth and development of the salivary glands of the fly (Pile and Wasserman, 2000; Riddiford *et al*., 2001). The tubby and knockdown larvae had normal salivary gland phenotypes. The knockdown larvae carrying the eyeless-GAL4 transgene have comparable levels of SIN3A chromatin binding (Fig 8 F and F') to the tubby controls (Fig 8 E and E'). This shows that even though the repressive activity of SIN3A is mostly dependent on the histone deacetylase activity of RPD3, RPD3 is not required for SIN3A binding to chromatin.

CAF1/p55 has been predicted to be involved in the recruitment of SIN3A onto chromatin because of its ability to bind directly to histones (Song *et al*., 2008; Spain *et al*., 2010). p55 is found in several different complexes in *Drosophila melanogaster,* including the NURF and NuRD complexes (Martinez-Balbas *et al*., 1998; Marhold *et al*., 2004). When a fly line for CAF1/p55 RNAi was crossed with the prothoracic gland driver, the salivary glands were smaller in size (data not shown) but normal polytene chromosome spreads were prepared from the glands. There was little or no SIN3A staining of the polytene chromosome spreads when *Caf1/p55* was knocked down with the prothoracic gland driver (Fig 9 A', B', C'). The same observed when using the eyeless-GAL4 driver, the polytenes had very little SIN3A staining (Fig 9 D', E', F'). These data support previously published data indicating that Caf1/p55 is likely to be a major factor for the recruitment of SIN3A onto chromatin.

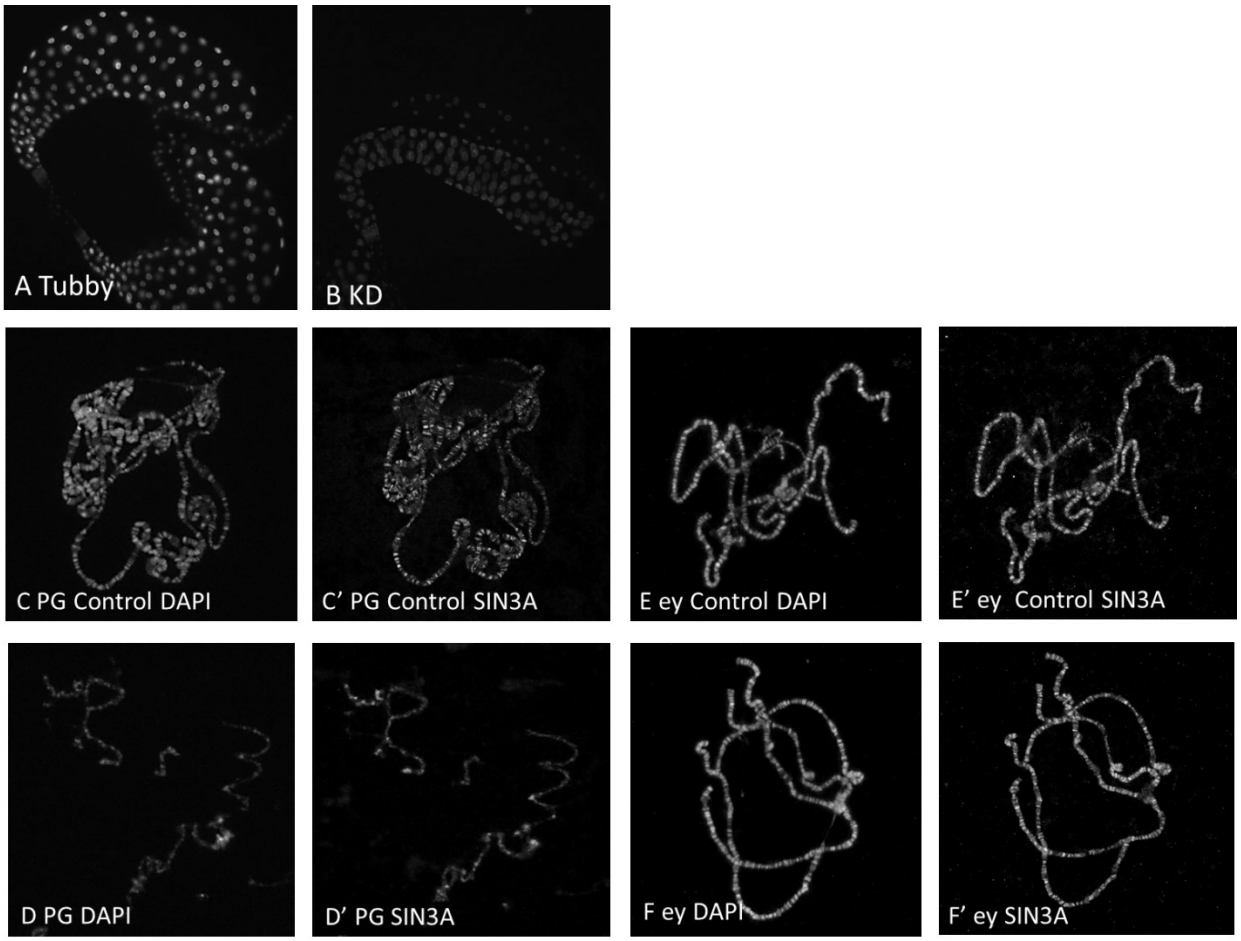


Figure 8. *Rpd3* knockdown and its effect on SIN3A binding onto chromatin. (A) Salivary glands from control larvae (B) Salivary glands dissected from *Rpd3* knockdown larvae generated using the prothoracic gland driver. Polytene chromosomes prepared from tubby control larvae (C and C'). Polytene chromosomes prepared from *Rpd3* knockdown larvae generated using prothoracic gland driver (D and D'). Polytene chromosomes prepared from control larvae (E and E'). Polytene chromosomes prepared from *Rpd3* knockdown larvae generated using eyeless-GAL4 driver (F and F'). (A, B, C, D, E, F) DAPI staining. (C', D', E', F') SIN3A staining.

BRMS1 has direct protein-protein interaction with several members of the SIN3A complex including ARID4B, ING1, SDS3, RPD3, SAP130 and SIN3A (Mintseris *et al*., 2009; Spain *et al*., 2010). This made it an interesting factor regarding whether or not these interactions may affect the binding of SIN3A to chromatin. When *Brms1* was knocked down using the prothoracic gland driver, there is a reduction in the amount of SIN3A staining on the polytene chromosomes (Fig 10 A', B', C'). When inducing knockdown of *Brms1* with the eyeless-GAL4 driver the same overall reduction in SIN3A

staining was observed (Fig 10 D', E', F'). The localization of SIN3A along the chromosome arms was not affected in any obvious way. BRMS1 thus does have some effect on the overall level of SIN3A binding to chromatin. This might be due to the numerous interactions with members of the SIN3A complex.

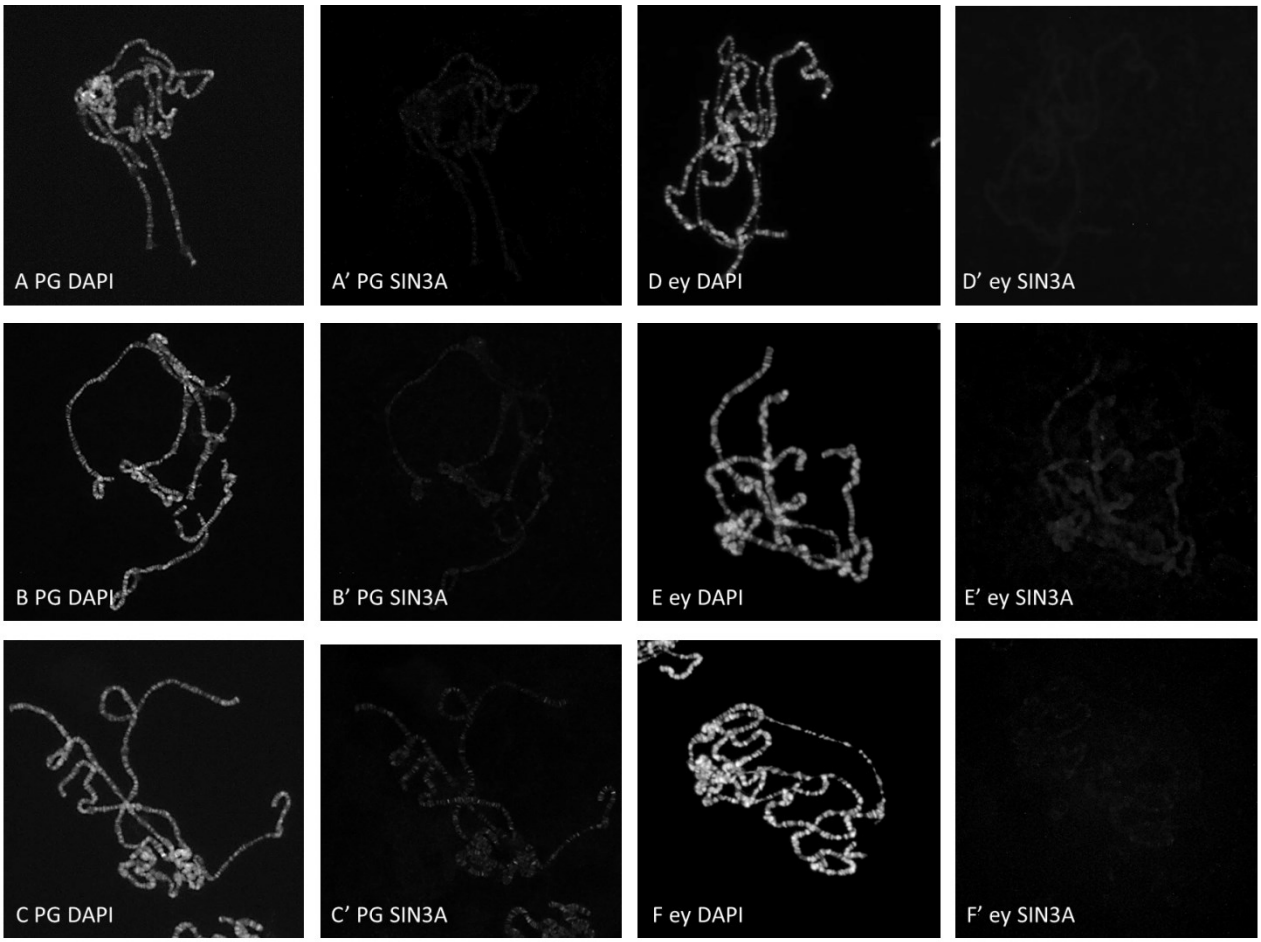


Figure 9. *Caf1/p55* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of UAS-RNAi-Caf1/p55 crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-p55/Caf1crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross.

ARID4B, is a member of ARID/BRIGHT DNA-binding domain containing family of proteins (Kortschak *et al*., 2000). Based on the properties of this domain it is likely to have a role in the modification of chromatin structure. While there were not structural abnormalities in the chromosomes when *Arid4b* was knocked down, there were differences in SIN3A staining and localization. When *Arid4b* was knocked down using

the prothoracic gland driver, there was not a dramatic decrease in SIN3A staining but there were areas on the polytene chromosomes that had increased bright spots indicated by white arrows (Fig 11 A', B', C') compared to controls (Fig 5 A'). *Arid4b* knockdown with eyeless-GAL4 overall led to a reduction in SIN3A staining and also affected binding in that the same areas of increase brightness in staining were observed as indicated by white arrows (Fig 11 D', E', F'). Thus, there appears to be a role for ARID4B in the recruitment and localization of SIN3A to chromatin.

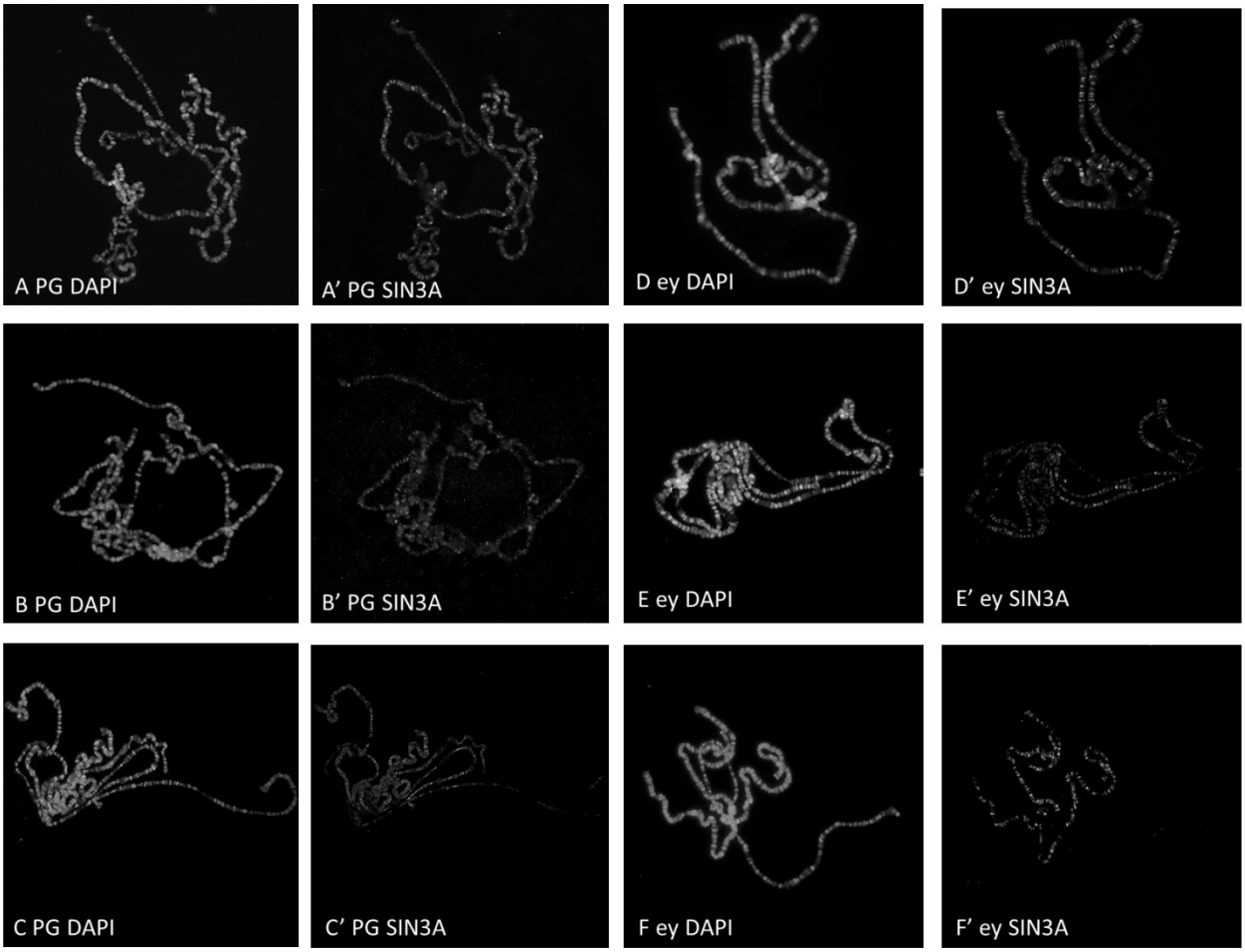


Figure 10. *Brms1* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of BRMS1 crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-BRMS1 crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross.

Because of the lack of research done with SDS3 in Drosophila melanogaster, we were unable to predict the effect on SIN3A binding to chromatin. When the prothoracic gland driver was used to knockdown *Sds3*, the SIN3A staining (Fig 12 A', B', C') was equivalent to control staining (Fig 5 A'). Similar results were found when using the eyeless-GAL4 driver (Fig 12 D', E', F') compared to the control (Fig 5 C'). This result shows that *Sds3* knockdown did not to affect the binding of SIN3A to chromatin.

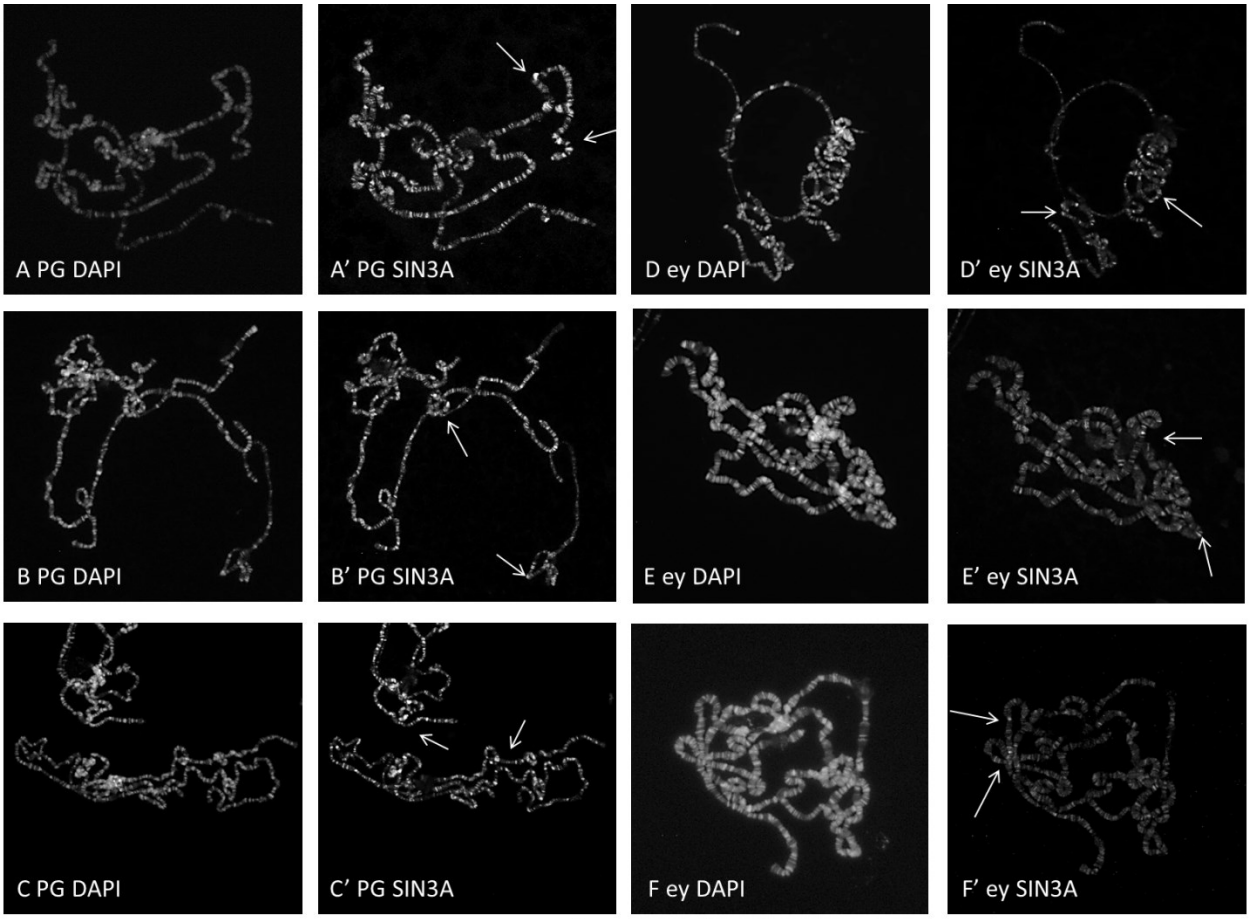


Figure 11. *Arid4b* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of UAS-RNAi-ARID4B crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-ARID4B crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross. White arrows indicate examples of bright spots in SIN3A staining.

There has not been a lot of research done on SAP130, except for its possible role in mitotic spindle organization (Goshima *et al*., 2007). When *Sap130* was knocked

down with the prothoracic gland driver, there was a reduced level of SIN3A staining and the normal binding pattern was no longer present (Fig 13 A', B', C'). The polytene chromosomes had a dull staining appearance compared with the control (Fig 5 A and A'). The same was seen when knocking down with the eyeless-GAL4 driver. There was a reduction in staining along with a dull banding pattern (Fig 13 D', E', F') compared to the control (Fig 5 C and C'). Thus, SAP130 does play some type of a role in SIN3A recruitment onto polytene chromosomes.

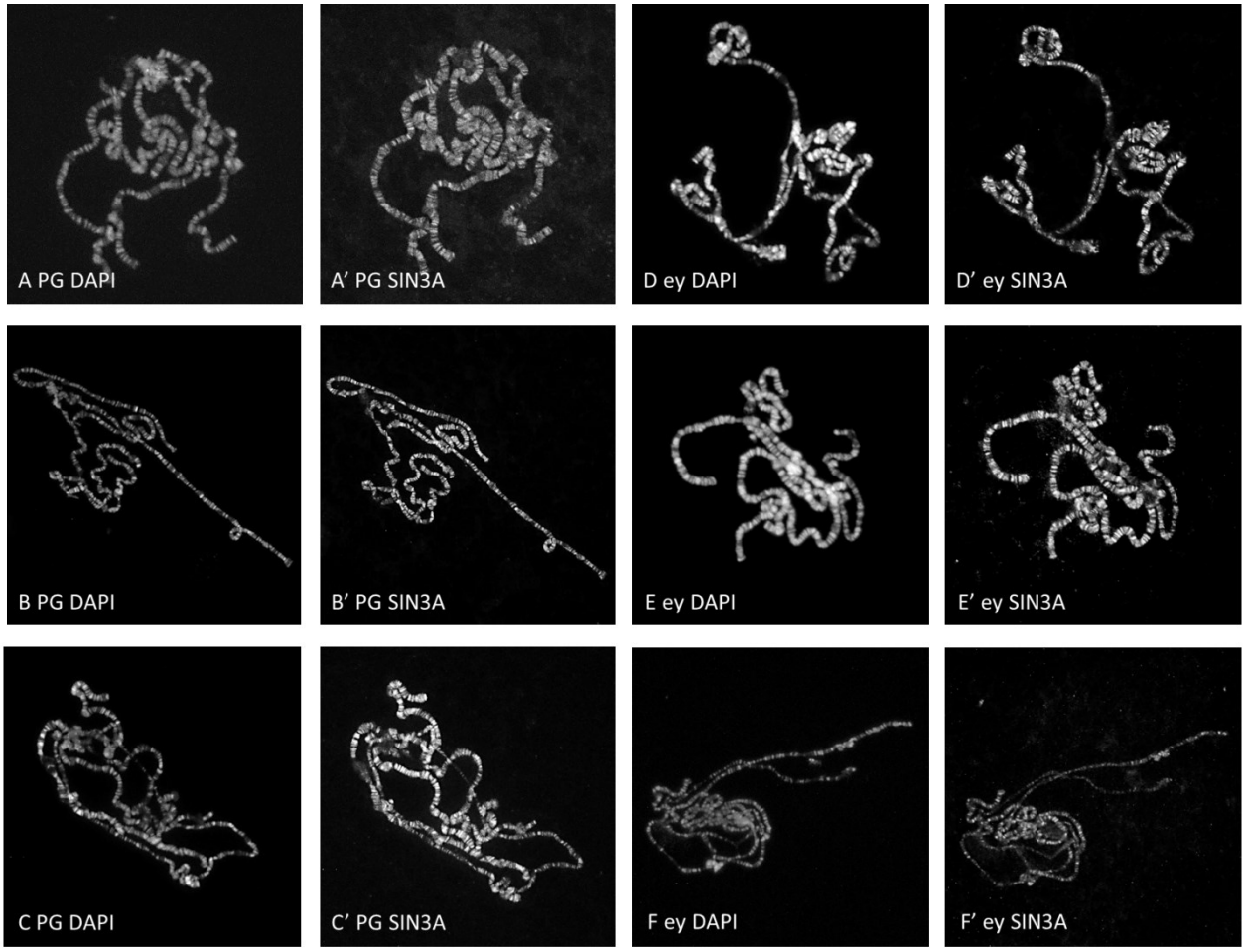


Figure 12. *Sds3* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of UAS-RNAi-SDS3 crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-SDS3 crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross.

Overall SIN3A recruitment to chromatin in *Drosophila melanogaster* is dependent on several members of the SIN3A complex. SAP130, BRMS1, CAF1/p55, ARID4B and LID have varying effects in the regulating the ability of SIN3A to bind to chromatin. ING1, SDS3 and RPD3 had no obvious effect on SIN3A binding or localization. Whether or not these complex components are affecting SIN3A in an independent manner or in connection with other proteins or complexes remains to be seen. Further analysis is needed to determine if there are any other factors that might be affecting the SIN3A complex and how it is able to bind onto chromatin.

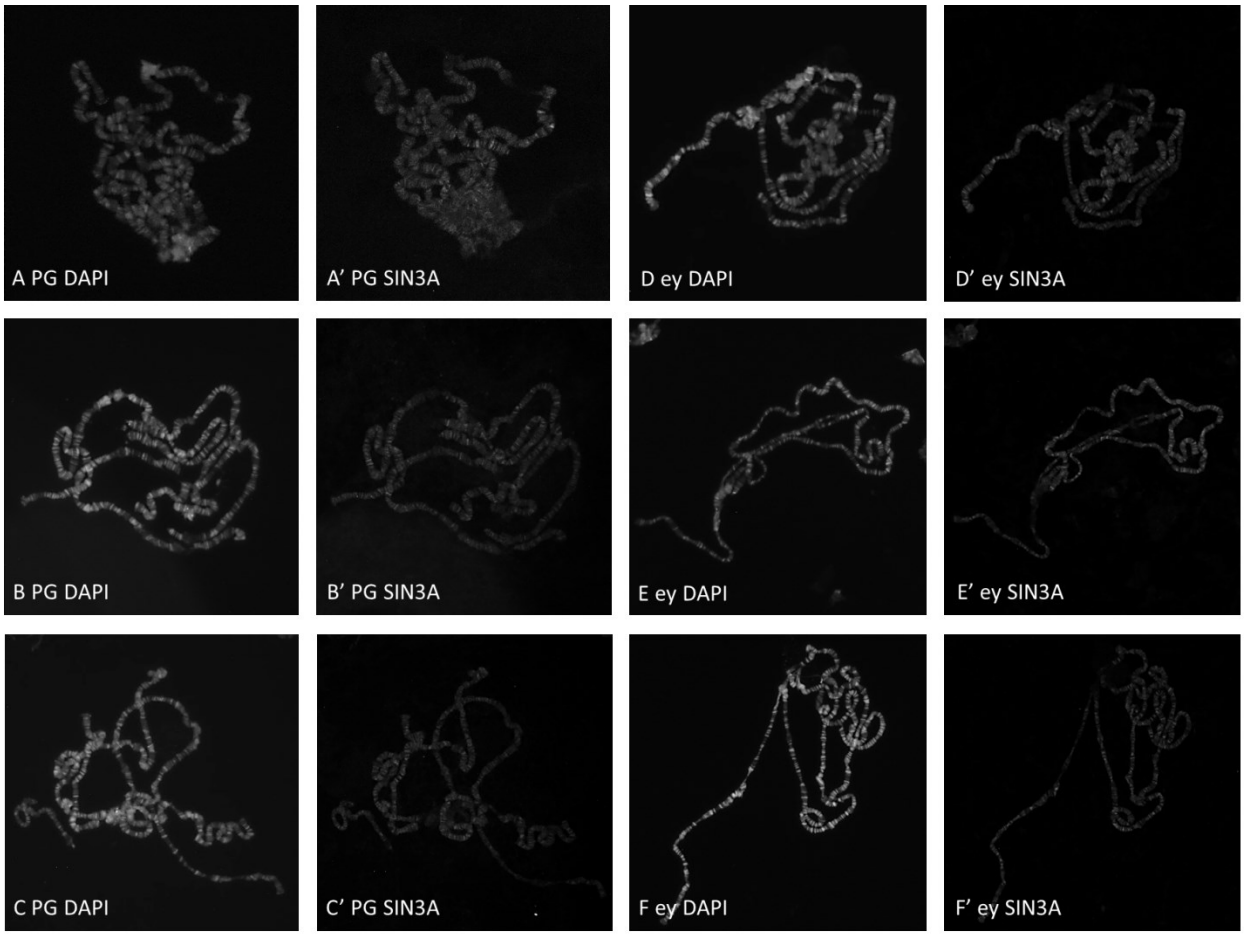


Figure 13. *Sap130* knockdown and its effect on SIN3A binding onto chromatin. Polytene chromosomes prepared from progeny of UAS-RNAi-SAP130 crossed to the prothoracic gland driver (A-C'). Polytene chromosomes prepared from progeny of UAS-RNA-SAP130 crossed to eyeless-GAL4 driver (D-F'). DAPI staining (A, B, C, D, E, F). SIN3A staining (A', B', C', D', E', F'). Each set of panels show a polytene spread prepared from a distinct parental cross.

Section 3.4: Clonal analysis of the SIN3A complex components

SIN3A has previously been shown to affect cell cycle progression in *Drosophila melanogaster* (Pile *et al*., 2002). It was also shown to be necessary for proper cell proliferation in imaginal wing discs using clonal analysis (Swaminathan and Pile, 2010). This method uses the hsFLP;Act5C>CD2>GAL4, UAS-EGFP fly stock. Wherever hsFLP activates Act5C-GAL4 random clones of cells having RNAi mutation of an individual member of the SIN3A complex and expression of EGFP will be generated. If a complex component is necessary for cell proliferation in developing imaginal disc cells, there will be less EGFP staining present in the disc because of the decrease in cell proliferation. As controls for this work, w^{1118} and mCherry-RNAi lines were used. w^{1118} carries no transgenes and therefore will not have any UAS turned on by Act5C-GAL4 (Fig 14 A). mCherry is a gene that is not found in *Drosophila melanogaster*. This fly still carries a transgene allowing RNAi activation in the cells but no knockdown will take place. This allows for more relevant control because the effects of RNAi are accounted for (Fig 14 B).

As previously shown in Swaminathan and Pile (2010), SIN3A has an effect on cell proliferation in the imaginal wing discs, indicated by the small amount of EGFP positive clones generated (Fig 14 C). When the individual members of the SIN3A complex were knocked down by hsFLP;Act5C>CD2>GAL4, UAS-EGFP, almost all of the mutant discs showed a reduction in the amount of EGFP clones. Knockdown of *Sds3* had the least effect on cell proliferation, compared to the rest of the complex components and the controls (Fig 14 K). Knockdown of all of the other complex components resulted in low amounts of EGFP positive clones. Similar effects to

reduced SIN3A levels on cell proliferation were observed (Fig 14 C-J). To quantify the analysis of the effect of each complex component, the average amount of GFP positive clones observed in the RNAi knockdown discs for each complex component was determined and compared to the controls (Fig 15). Overall every member of the SIN3A complex had some effect on cell proliferation in larval development in *Drosophila melanogaster*.

Acknowledgement: The clonal analysis was conducted in collaboration with Ms. Valerie Barnes, the research technician of the Pile laboratory.

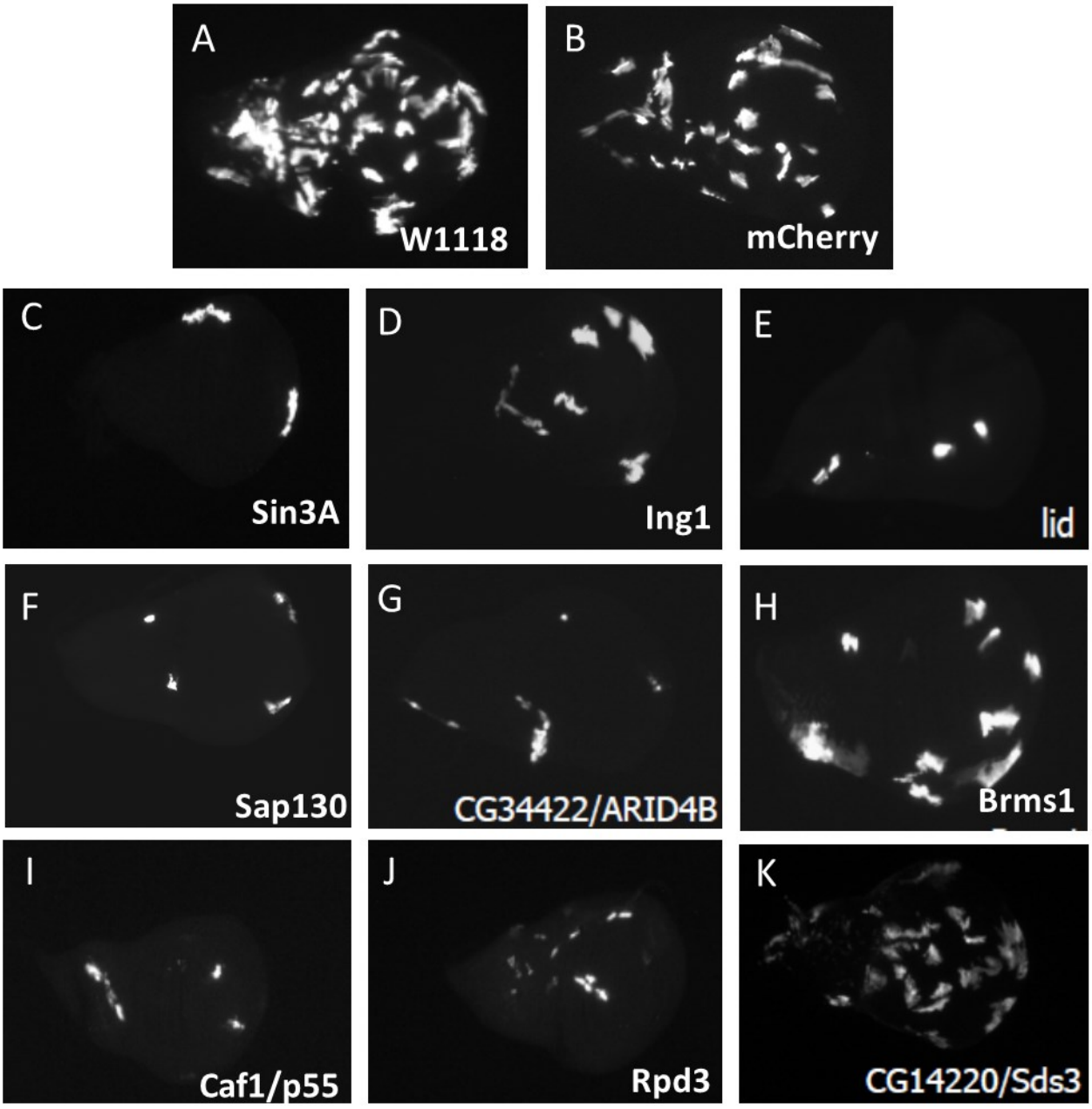
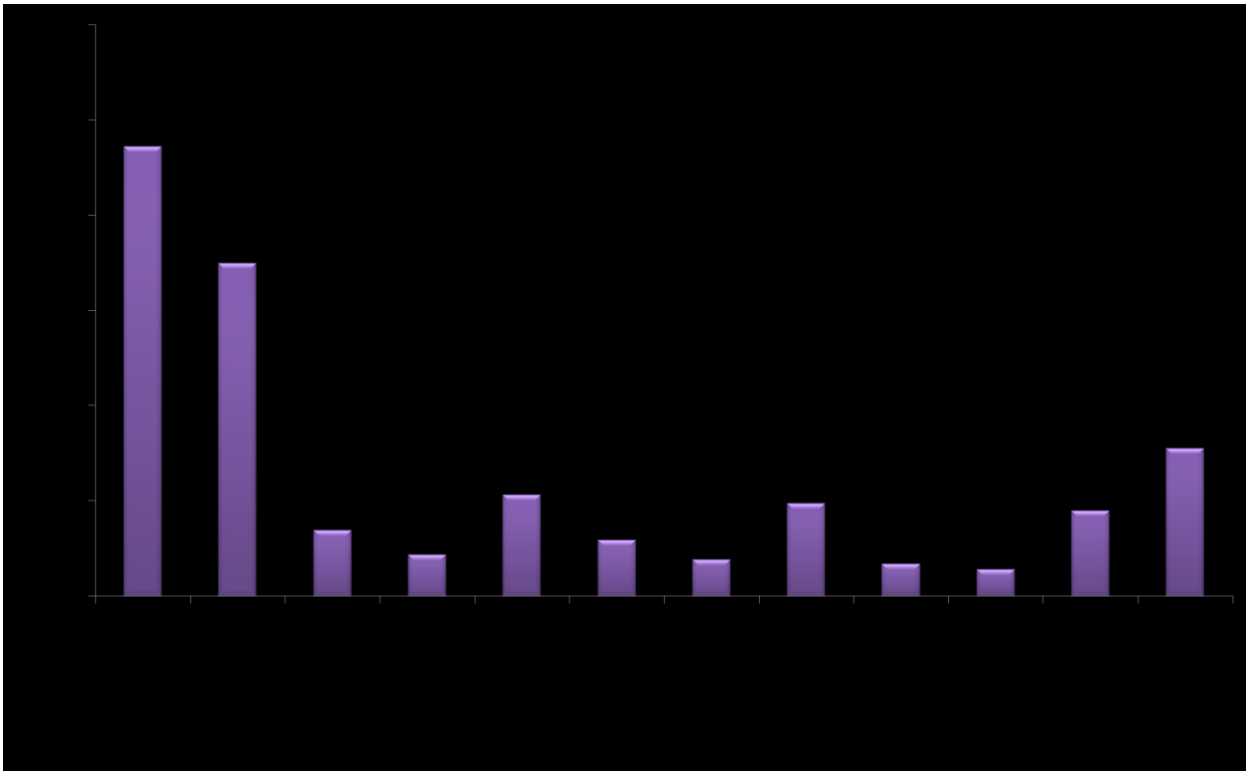


Figure 14. Clonal analysis of SIN3A complex components in *Drosophila melanogaster* wing discs. (A-K) Cell proliferation was marked in wings discs by GFP staining, which was induced using a heat shock flipase MARCM method as described in the materials and methods section.



Section 3.5: Summary

This study has shown a larger, more defined role for the members of the SIN3A complex. SIN3A does not directly bind to chromatin in *Drosophila melanogaster*; it requires interactions with DNA binding proteins to be recruited it to target areas (Knoepfler and Eisenman, 1999). Since SIN3A is part of complex, it is possible that some of the members of this complex are involved in this process. Several members of the SIN3A complex were shown to have an effect on SIN3A recruitment and binding onto chromatin. BRMS1 and CAF1/p55 both showed a decrease in the amount of SIN3A staining levels on polytene chromosomes. ARID4B, LID and SAP130 not only had an effect on the level of SIN3A, but also altered the binding pattern of SIN3A to chromatin. ING1, SDS3 and RPD3 did not have any noticeable effect on the level of SIN3A staining on polytene chromosomes and therefore have no effect on SIN3A recruitment to polytene chromosomes. Whether or not the absence or presence of these proteins in the SIN3A complex in combination affects SIN3A recruitment and binding to chromatin is not yet known. There might be other factors at play that are influencing how SIN3A is binding to chromatin. Additionally, some factors might be influencing the stability of the SIN3A complex as a whole. When certain members of the complex are knocked down, the complex may disassemble thus affecting the binding ability of SIN3A onto chromatin.

The knockdown of *Sin3A* lowers the amount of cell proliferation that is taking place during the early development of *Drosophila melanogaster (*Swaminathan and Pile, 2009). The members of the SIN3A complex were hypothesized to possibly have the same effect on cell proliferation. This study showed that when the individual members of

the complex components were knocked down in imaginal wing discs, there was a decrease in the level of proliferating cells when compared to controls. ARID4B, SAP130, LID, ING1, CAF1/p55 and BRMS1 all had significantly less cell proliferation and therefore are involved in proper cell proliferation. SDS3 had the least effect on cell proliferation but has involvement in cell proliferation when compared to controls.

Interestingly, all SIN3A complex components are required for normal cell proliferation of imaginal wing disc cells but only a subset are important for SIN3A binding to chromatin. This finding suggests that possibility that the SIN3A complex components not required for binding affect another aspect of complex activity. Possibly the other components modulate the HDAC activity of RPD3 in the complex. Future experiments will be done to analyze how SIN3A and the associated protein complex might be working in connection to regulate development and cell proliferation of *Drosophila melanogaster*.

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ABSTRACT

THE PHENOTYPIC ANALYSIS OF THE KNOCKDOWN OF THE SIN3A COMPLEX COMPONENTS AND THEIR ROLE IN RECRUITMENT AND CELL PROLIFERATION

by

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August 2014

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The SIN3A-RPD3 complex components have previously been identified in *Drosophila melanogaster*. The role of these components in SIN3A function and recruitment was not known. Polytene chromosome analysis following RNAi knockdown was performed to determine if any of the complex members affect the ability of SIN3A to bind to chromatin. The complex components effect on cell proliferation was also examined through clonal analysis of imaginal wing discs. The results of this work implicate a role of several members of the SIN3A complex for proper recruitment and localization to chromatin. All of the SIN3A complex members had some varying effect on cell proliferation, much like that of SIN3A. This study provides a better understanding of SIN3A-RPD3 complex members regarding how they might be influencing SIN3A function in *Drosophila melanogaster*.

AUTOBIOLOGRAPHICAL STATEMENT

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Born and raised in Michigan. I grew up in the Utica/Shelby Township area of Macomb County where I attended Utica Community School District.

I received my Bachelors of Science degree from Madonna University in Livonia, Michigan where I majored in forensic science. I also earned a certificate in DNA analysis.

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