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ANALYZING THE EFFECTS OF LOSS OF SIN3 IN *DROSOPHILA MELANOGASTER*

by

AISHWARYA SWAMINATHAN

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

For the degree of

DOCTOR OF PHILOSOPHY

2010

MAJOR: BIOLOGICAL SCIENCES

Approved by:

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Advisor Date

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CHAPTER 1

INTRODUCTION

Transcription regulation is a vital step in controlling gene expression. One of the ways of achieving transcription regulation is through the control of the activity of the appropriate transcription activators and repressors in the right space and time. Histone modifying complexes play a vital role as transcription coactivators or corepressors. They are recruited to specific genes by transcription activators or repressors respectively (Emre and Berger, 2006; Torchia et al., 1998). Histone modifying complexes are large multisubunit protein complexes with enzyme activity. Histone modifications that have been linked to transcription activity include acetylation (Allfrey et al., 1964; Racey and Byvoet, 1972), phosphorylation (Langan, 1969), methylation (Allfrey et al., 1964) and ubiquitination (Levinger and Varshavsky, 1982) on the amino (N)-terminal tails of histones H3 and H4. All of these modifications are reversible and the reverse reactions are also carried out by histone modifying enzyme complexes.

The SIN3 complex is one such histone modifying complex. It deacetylates the lysine residues on the N-terminal tails of histones H3 and H4 and plays an important role in transcription repression (Wolffe, 1996). Null mutations in *Sin3A* have been shown to be lethal in the early stages of *Drosophila* development (Neufeld et al., 1998b; Pennetta and Pauli, 1998). The genes regulated by the SIN3 complex during development and in adult *Drosophila,* therefore, are largely unknown. Elucidating the role of the SIN3 complex in development is essential

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for understanding the key regulatory events that control all stages of *Drosophila* development.

Chromosome organization

Eukaryotic DNA is packaged into chromatin. Chromatin consists of DNA wrapped around an octamer of basic, conserved proteins called histones (Germond et al., 1975) (Fig. 1.1). The octamer consists of two molecules of each histone H2A, H2B, H3 and H4. One unit of nucleosome consists of 146 base pairs (bp) of DNA wrapped around the histone octamer (Noll, 1974). A continuous string of nucleosomes gives rise to the first order of chromosome organization called the 11 nm fiber (Kornberg, 1974; Kornberg and Lorch, 1999; Kornberg and Thomas, 1974). DNA that is present between nucleosomes is called linker DNA. Linker DNA length can vary from 0 to 80 bp and at the entry and exit points on the nucleosome, is bound by histone H1 (Noll and Kornberg, 1977). The N-terminal tails of histones protrude out of the nucleosome structure and are subject to various modifications (Jenuwein and Allis, 2001). Higher order packaging is achieved when nucleosomes are further wound in a coiled fashion (McGhee et al., 1980) or in a zigzag fashion (Schalch et al., 2005) to give rise to a 30 nm fiber or when associated with scaffold proteins (Paulson and Laemmli, 1977).

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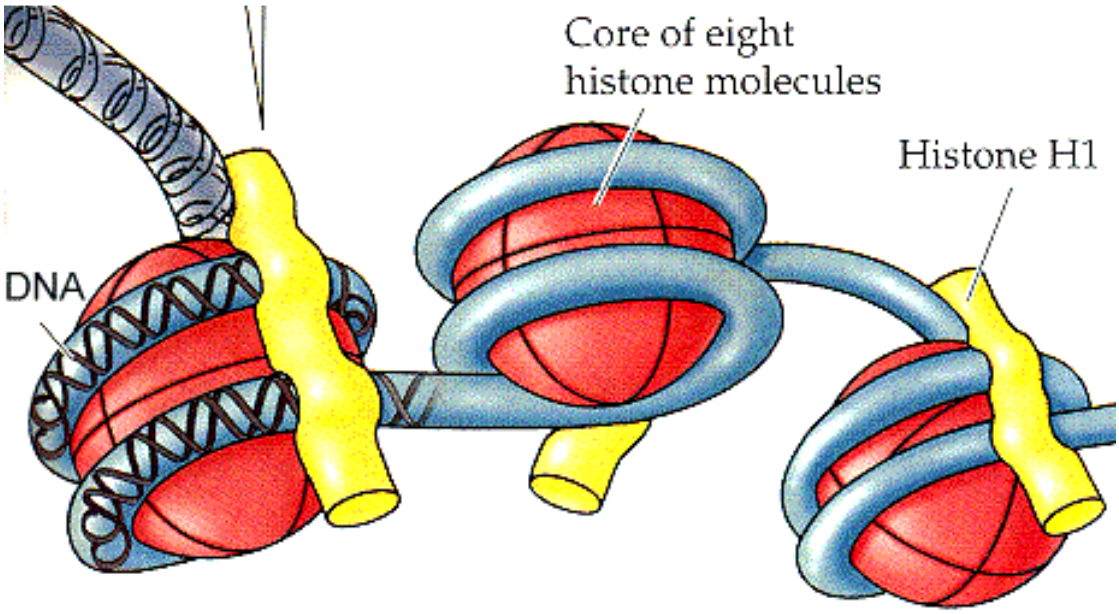

Figure 1.1: Structure of a nucleosome. DNA (blue) wraps around an octamer of histones (red) to give rise to the first order of chromosome organization that is called the 11 nm fiber. Linker DNA is usually associated with histone H1 (yellow) which binds DNA at the DNA entry and exit points on the nucleosome.

http://www.palaeos.com/Eukarya/Images/Nucleosome.gif

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Chromatin packaging, histone acetylation and transcription regulation

Packaging of DNA along the chromosome is not uniform, i.e. some regions are more tightly packed than others. The relatively loosely packed regions are referred to as euchromatin while the regions that are tightly packed throughout the cell cycle are called heterochromatin. Euchromatin tends to be gene rich and hyperacetylated while heterochromatin is gene poor and hyopacetylated (Dillon, 2004). This packaging is dynamic. The dynamic packaging is partly responsible for regulating gene expression (Felsenfeld, 1992). When DNA is tightly packaged it is less accessible to the transcription machinery. This often causes transcription repression of genes that are present in this tightly packaged region. Conversely, when the DNA is loosely packaged it becomes accessible to the transcription machinery and can now be actively transcribed (Felsenfeld, 1993). Early *in vivo* studies showed that depletion of nucleosomes via histone loss resulted in the activation of many genes in *Saccharomyces cerevisiae* (Han and Grunstein, 1988). Later studies showed that depletion of histone H4 from the promoter resulted in the activation of the PHO5 gene even under repressive, high inorganic phosphate conditions (Han et al., 1988). In *Drosophila*, studies with the *white* gene show that when this gene is placed in or near a heterochromatic region, it is transcriptionally repressed (Wallrath and Elgin, 1995). Thus, incorrect packaging results in altered gene activity.

Histone acetylation has been implicated in regulating gene expression, in part through modulation of chromatin structure. *In vitro* studies show that

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acetylated histones antagonize the propensity of chromatin fibers to fold into highly compact structures (Garcia-Ramirez et al., 1995; Tse et al., 1998). In a *Drosophila* embryo-derived cell-free system, chromatin reconstituted with hyperacetylated histones shows increased DNase I sensitivity (Krajewski and Becker, 1998). This indicates an increase in accessibility of DNA to proteins with which it interacts. At the chicken β-globin locus, the transcriptionally active domains displayed higher levels of acetylation and DNase I hypersensitivity (Hebbes et al., 1994). The DNase I resistant domains flanking the hypersensitive sites were transcriptionally inactive and contained histones with lower levels of acetylation. Activation of the chicken β-globin locus is associated with a dramatic increase in acetylation of both histones H3 and H4 (Litt et al., 2001). In *Drosophila*, acetylation of H4K16 by MOF is involved in transcription activation during dosage compensation (Akhtar and Becker, 2000). These results suggest that acetylation of histones affects gene expression, possibly by making large chromatin domains more readily accessible to trans-acting factors that regulate transcription.

In addition to affecting chromosomal domains, localized histone acetylation has been implicated in activation of specific genes, while histone deacetylation is generally associated with transcription repression (Fig. 1.2) (Chahal et al., 1980; Ginder et al., 1985; Li et al., 1999; Marushige, 1976; Mizzen and Allis, 1998). One mechanism that potentially explains this phenomenon is that acetylation of histones affects their interaction with transcription factors and components of the transcription machinery. This in turn affects gene expression.

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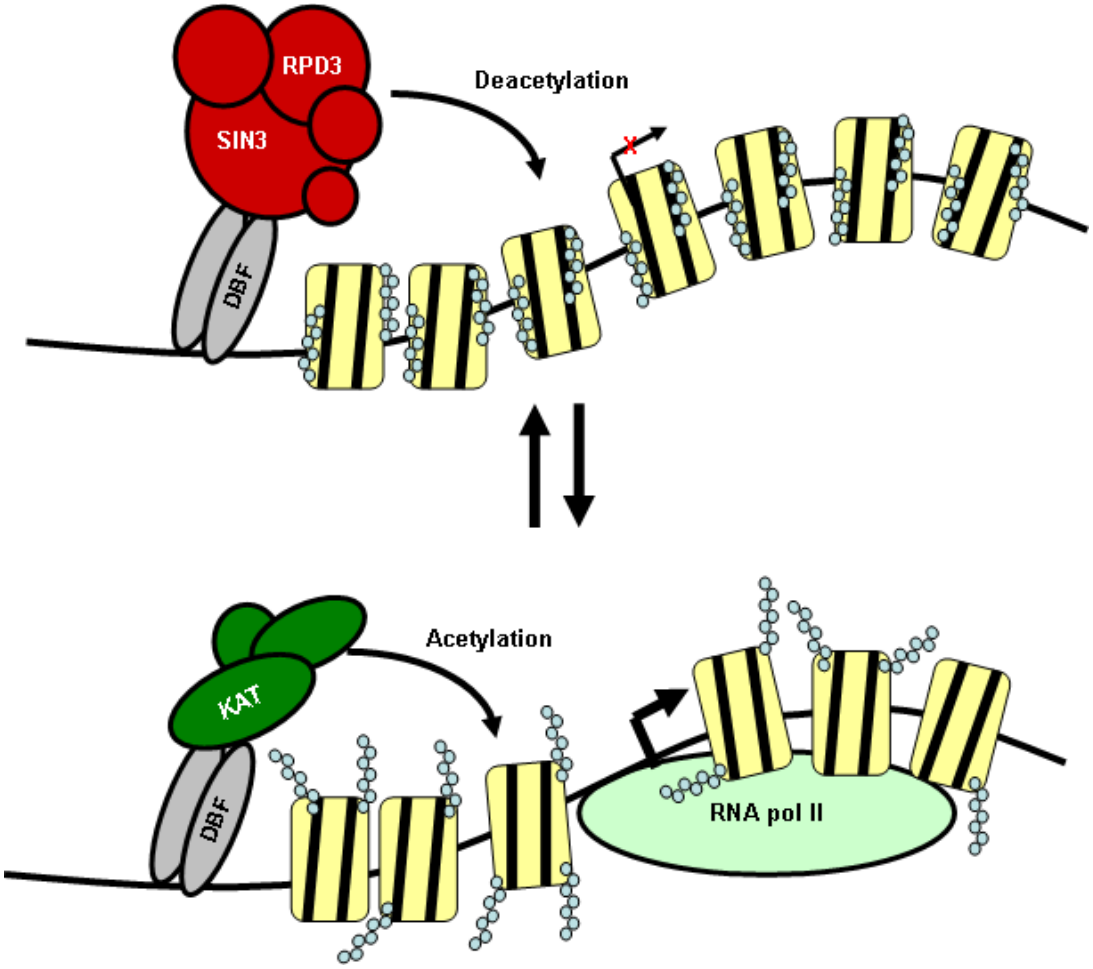

Figure 1.2: Transcription regulation by chromatin modification. Histone acetyltransferases (KATs) and deacetylases (HDACs), including the SIN3 complex, are recruited to promoters through interactions with sequence specific DNA-binding factors (DBF). The KATs and HDACs modify lysine residues on the N-terminal tails of histones and facilitate either activation or repression.

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In vitro studies have shown that acetylated histone H4 has high affinity for transcription activators (Vettese-Dadey et al., 1996). In this study, two yeast transcription factors USF and GAL4-AH were found to have greater affinity for reconstituted chromatin consisting of hyperacetylated nucleosomes. The nucleosomes consisted of DNA containing a single USF or GAL4-AH binding site. Association with the transcription factor was greater when the nucleosome cores consisted of acetylated H4 than when the nucleosome cores consisted of acetylated H3. Acetylation of nucleosomes by the histone lysine acetyltransferase (KAT) Gcn5 was shown to form a recognition site for CBP-RNA polymerase II at the promoter which in turn recruits chromatin remodeling complex SWI/SNF to the promoter and facilitates activation of transcription (Agalioti et al., 2000). Another such factor is the TBP associated factor TAF1. Crystallography data of human TAF1 suggest that the bromodomain module of TAF1 has a structure suited to recognize and therefore preferentially bind to diacetyl histone H4 tail peptides (Jacobson et al., 2000). These results suggest that acetylated histones may act as docking sites for proteins required to bring about transcription activation.

Classical models postulated that histone modifications may influence histone-DNA or histone-histone interactions to affect transcription. But in 2000, the histone code hypothesis was proposed (Strahl and Allis, 2000). According to this idea, the occurrence of one modification on a histone affects a subsequent modification or a combination of modifications that may serve as a recognition site for various transcription factors and hence influence transcription. For

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example, it has been shown that during transcription activation of c-fos by epidermal growth factor, histone H3 is sequentially phosphorylated and then acetylated (Cheung et al., 2000). Thus various modifications by different enzymes govern gene expression. Chromatin packaging affects gene expression presumably by influencing accessibility of transcription factors and/or the RNA polymerase complex to genes. Histone acetylation can modulate gene expression either by affecting local chromatin structure or by facilitating recruitment of specific transcription factors to genes.

SIN3 and its role in transcription regulation

SIN3 was first identified in genetic screens in *S. cerevisiae* as a global regulator of transcription (Nasmyth et al., 1987; Sternberg et al., 1987). A mutation in *SIN3* or in a second gene called *RPD3*, the gene that we now know encodes the deacetylase component of the SIN3 complex, could bypass the requirement of Swi5p in activating the HO endonuclease during mating-type switching in yeast. A mutation in *SIN3* led to the improper expression of meiosis-specific genes like *SPO11*, *SPO13* and *SPO16* during vegetative growth in yeast (Strich et al., 1989). Both *SIN3* and *RPD3* were identified in a yeast genetic screen to identify genes that imparted reduced potassium dependency to the cell (Vidal et al., 1990). Again, in another yeast genetic screen, mutations in *SIN3* and *RPD3* were identified as genes that led to the improper expression of an INO1-LacZ reporter construct during repressing conditions (Hudak et al., 1994). These mutations led to the constitutive expression of phospholipid biosynthesis

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genes. These studies establish the role of SIN3 and RPD3 in regulating transcription of a wide variety of genes.

Subsequent studies led to the characterization of Rpd3p as an integral part of the yeast SIN3 complex (Kasten et al., 1997). The SIN3 complex is a histone deacetylase that enzymatically modifies the N-terminal tail of histones H3 and H4 (Wolffe, 1996). The two major components of this complex are the SIN3 and RPD3 proteins. RPD3 possesses histone deacetylase activity and catalyzes the removal of the acetyl group from lysine residues on the N-terminal tail of histones H3 and H4 (Rundlett et al., 1996). SIN3 on its own lacks DNA binding activity. It recruits the entire complex to the promoter of the target gene by associating with DNA binding factors some of which belong to the Mad-Max family of transcription repressors (Ayer et al., 1995; Kasten et al., 1996; Wang and Stillman, 1990). In yeast, the SIN3 complex was shown to be recruited to promoters of genes through its association with Ume6p (Kadosh and Struhl, 1998). This recruitment leads to deacetylation of histones H3 and H4, preferentially H4K5 and H4K12, over a highly defined region of one to two nucleosomes. As discussed above, deacetylation of histones is correlated with transcription repression.

In mammals, SIN3 has been shown to bring about transcription repression via its association with the Mad-Max family of transcription repressors. Mouse Sin3 associates with the Mad-Max heterodimeric complex to repress a reporter construct consisting of a Myc-Mad and Mad-Max binding site cloned upstream of the thymidine kinase gene (Ayer et al., 1995). Mutations in the paired

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amphipathic helix 2 (PAH2) domain of mSin3A or mSin3B disrupt the Mad-Sin3 interaction and blocks Mad mediated repression of this reporter construct. In a yeast-two-hybrid system, it was determined the presence of SIN3 inhibits the activation of a LexA-LacZ reporter construct by the interaction of LexA-Mad and VP16-Max (Kasten et al., 1996). These findings suggest that the interaction of Mad with Sin3 is important for Mad to function as a transcription repressor. Transcription factors belonging to the Sp-1 family also bring about repression via their interaction with SIN3 through a similar mechanism (Ellenrieder et al., 2002; Zhang et al., 2001).

The role of SIN3 in transcription repression has been established in many model systems. In *S. cerevisiae*, *SIN3* has been shown to repress genes involved in iron, glucose and phospholipid metabolism and regulation of various stages of the cell cycle, and to activate many genes involved in gluconeogenesis, transcription activation, genes that provide resistance against various toxins and some genes with unidentified function (Bernstein et al., 2000; Fazzio et al., 2001; Watson et al., 2004). In mammals, two homologs of Sin3 exist that are encoded by the SIN3A and SIN3B genes (Ayer et al., 1995). mSin3A regulates genes involved in cell proliferation during embryogenesis (Cowley et al., 2005; Dannenberg et al., 2005) while mSin3B regulates genes involved in exiting the cell cycle (David et al., 2008). Sin3 associates with the Mnt-Max heterodimer to antagonize Myc mediated activation of genes involved in cell proliferation (Hurlin et al., 1997). In mouse fibroblasts, Sin3 interacts with Mnt to repress cell cycle progression genes like cyclin D2 (Popov et al., 2005). Mouse Sin3 is also

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involved in repressing cell cycle genes that are E2F4 targets through its association with RBP2 (van Oevelen et al., 2008). There is a coordinated binding of E2F4 and Sin3 immediately downstream of the transcription start site. In *Drosophila,* the SIN3 complex is estimated to regulate about 3% of all genes in tissue culture (Pile et al., 2003). Microarray analysis of *Drosophila* tissue culture (S2 and KC167) cells shows that SIN3 represses a wide variety of genes involved in diverse cellular processes including signal transduction, gene regulation, cell division, glycolysis, oxidative phosphorylation and mitochondrial physiology (Pile et al., 2003). These results suggest that SIN3 plays a role in regulating genes involved in a wide variety of processes.

Isoforms of *Drosophila* SIN3

Multiple isoforms of SIN3 exist in *Drosophila*. These are splice variants of the same pre-mRNA (Neufeld et al., 1998b; Pennetta and Pauli, 1998). They differ only in the carboxy (C)-terminus and are 187 kD, 190 kD and 220 kD in size (Fig. 1.3). They are differentially expressed during development and are hypothesized to have different functions (Sharma et al., 2008). SIN3 220 is the major isoform that is expressed in most tissue. Its expression increases during embryogenesis and peaks from stage 12-16 of embryogenesis and decreases at stage 17 while the expression of SIN3 187 remains constant throughout. Larval imaginal discs show greater expression of SIN3 220, brain has equal expression of both isoforms whereas SIN3 187 is the major isoform expressed in adults. SIN3 190 has a unique expression pattern in that it is exclusively expressed in

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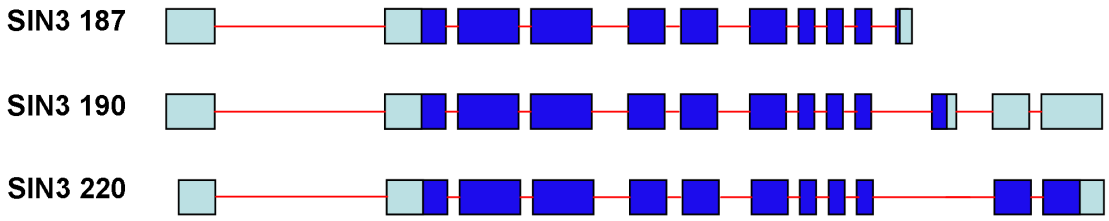

Figure 1.3: The intron-exon map of the SIN3 isoforms. The three different isoforms of *Drosophila* SIN3 are 187 kD, 190 kD, 220 kD. Exons are indicated by boxes and introns by red lines. Untranslated regions are indicated light blue and protein-coding regions in dark blue. The transcript structures are based on cDNAs described by Neufeld et al., 1998 (SIN3 187, GenBank AF024604; SIN3 190, GenBank AF024603) and Penneta and Pauli, 1998 (SIN3 220, GenBank AJ007518).

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embryos and adult females, presumably in the ovaries. The differential expression suggests that the different isoforms have different roles in the organism and possibly during development.

SIN3 complex in development

Whereas SIN3 is dispensable for viability in *S. cerevisiae*, it is vital for viability in *Schizosaccharomyces pombe* (Dang et al., 1999) and early embryonic development in *Drosophila* (Neufeld et al., 1998b; Pennetta and Pauli, 1998). In *Xenopus*, the SIN3 complex is important for post-embryonic development and metamorphosis (Sachs et al., 2001). In mouse, null mutations in SIN3A result in embryonic lethality (Cowley et al., 2005; Dannenberg et al., 2005). Mouse embryonic fibroblasts and T-cells that are homozygous null SIN3A show a decrease in doubling time indicating a defect in proliferative potential (Cowley et al., 2005). mSin3B is also essential during embryogenesis (David et al., 2008). SIN3B null embryos die during late gestation and the embryos show growth retardation by stage E14.5. Furthermore, these fetuses have a marked increase in erythroid progenitors in the liver, immature nucleated erythrocytes in the blood stream and significantly low levels of hemoglobin. These phenotypes indicate a defect in erythroid differentiation. This suggests that mSin3B is important for hematopoietic cells to exit the cell cycle and undergo differentiation (David et al., 2008). SIN3 has also been implicated in some signaling pathways that govern development. In mouse, the SIN3 complex is recruited to Gli-binding element containing promoters via its association with Su(Fu) (Cheng and Bishop, 2002).

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This results in the repression of hedgehog responsive genes. Recruitment of the SIN3 complex prevents Gli mediated activation of hedgehog pathway that controls tissue patterning during development. mSin3A also associates with HERP, a downstream effector of the notch pathway to bring about repression of target genes and controlling cell fate decisions (Iso et al., 2001).

SIN3 has been implicated in diverse processes in *Drosophila* as well. Elimination of expression of SIN3 in *Drosophila* tissue culture cells affected their ability to proliferate (Pile et al., 2002). FACS analysis showed that SIN3-deficient cells were arrested in the G2/M phase of the cell cycle. SIN3 also plays a role in regulating development via the ecdysone pathway. SMRTER, a steroid hormone corepressor brings about transcription repression through its association with SIN3 (Tsai et al., 1999). SIN3 colocalizes with SMRTER on *Drosophila* polytene chromosomes (Pile and Wassarman, 2000). The recruitment of SIN3 to chromatin was reduced at loci that undergo ecdysone-induced activation. Recruitment was restored when the expression of these genes was reduced. These results suggest a role for SIN3 in regulating genes in response to hormone signaling. SIN3 also plays a role in *Drosophila* eye development via the MAP-kinase pathway (Neufeld et al., 1998b). In that study, SIN3 was identified as an enhancer of the rough eye phenotype caused by a mutation in *sina*, a gene required for R7 photoreceptor specification in the developing *Drosophila* eye. These findings suggest that SIN3 not only regulates the cell cycle, as has been shown in many model systems, but also specific genes involved in development.

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Project outline

Although SIN3 has been implicated in some developmental pathways, we do not yet know its specific gene targets or the mechanism by which SIN3 controls these processes. We have chosen to address these questions using the well-studied and characterized *Drosophila* model system. *Drosophila* have a short generation time. Well-developed tools like online databases, transgenic and mutant fly lines and DNA clones are readily available. Several other reagents required for observing cellular and molecular alteration are also available that make *Drosophila* a good model system for this study.

The requirement of SIN3 for viability of the organism or mitotic clones has precluded our ability to study its role in *Drosophila in vivo*. To this end we have created a system whereby we can knock down the expression of SIN3 by RNA interference (RNAi) in a temporal and/or spatial manner to further understand its functions. Due to the advantage of the conditional RNAi system, we have identified a role for SIN3 not only during embryogenesis but also during larval development. The system and results are described in Chapter 2. Loss of SIN3 during embryogenesis and early stages of larval development results in lethality before adulthood. We also find that the two major isoforms of SIN3 play different roles in supporting viability of the adult.

We next set out to understand the mechanism by which SIN3 regulates the cell cycle in a developing tissue such as the wing disc. Results of these analyses are given in Chapter 3. We find that SIN3 controls cell proliferation in the wing discs. Loss of SIN3 results in misregulation of an important G2 regulator

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called String which is at least partly responsible for the cell cycle defects observed upon loss of SIN3.

Finally, we set out to identify the role of SIN3 in various pathways by identifying genes that show a genetic interaction with SIN3. Results of a genetic screen are given in Chapter 4. We find that genes involved in a variety of processes and pathways are able to interact with SIN3, suggesting that this protein plays a role in regulating genes involved in diverse processes.

This study has generated a suitable assay to analyze the function of SIN3 in the cell. Data from these studies provide initial insight into the role of SIN3 during development. The results of this investigation have also lead to the generation of some interesting questions, the answers to which will further unravel the various functions of SIN3. Additional analysis is therefore likely to provide important information and may suggest mechanistic links to many key cellular processes involved at different developmental stages. Some of these questions are summarized in Chapter 5.

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CHAPTER 2

DROSOPHILA SIN3 IS REQUIRED AT MULTIPLE STAGES OF DEVELOPMENT

This chapter has been published:

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and

Spain, M.M, J.A. Caruso, A. Swaminathan and L.A. Pile. 2010. *Drosophila* SIN3 isoforms interact with distinct proteins and have unique biological functions. *J Biol Chem.* 285(35):27457-67.

ABSTRACT

SIN3 is a component of a histone deacetylase complex known to be important for transcription repression. While multiple isoforms of SIN3 have been reported, little is known about their relative expression or role in development. Using a conditional RNA interference (RNAi) system we knocked down SIN3 at various stages of development. We find that SIN3 is required for embryonic and larval periods. Furthermore, not all of the SIN3 isoforms can support viability on their own. Taken together, the data suggest that SIN3 is required for multiple developmental events during the *Drosophila* life cycle.

INTRODUCTION

Development of multicellular organisms requires coordinate spatial and temporal regulation of gene expression. Levels of gene expression have long been correlated with the amount of histone acetylation (Allfrey et al., 1964). Overall acetylation levels are regulated by the opposing activities of histone

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lysine acetyltransferases (KATs) and histone deacetylases (HDACs) (Kuo and Allis, 1998). Mutations in KATs and HDACs lead to developmental defects and/or lethality in a variety of metazoans, likely due to altered expression of genes encoding key developmental regulators (Lin and Dent, 2006). For example, *Drosophila melanogaster* reared on food containing the HDAC inhibitor trichostatin A (TSA) exhibited a developmental delay at low concentrations and lethality at the highest concentrations tested (Pile et al., 2001).

Multiple HDACs are present in *Drosophila*. SIN3 is a component of one multisubunit HDAC complex conserved from yeast to human (Silverstein and Ekwall, 2005). SIN3 is believed to serve as a scaffold protein for assembly of the complex and has been shown to be the major subunit that targets the complex to specific promoters (Silverstein and Ekwall, 2005). Null mutations in *Drosophila Sin3A* result in embryonic lethality with only a few animals surviving to the first larval instar stage (Neufeld et al., 1998b; Pennetta and Pauli, 1998). For this reason, investigating the role of SIN3 in the regulation of specific developmental pathways has proven difficult. SIN3 is believed to be involved in various biological processes linked to development and cell cycle progression. For instance, SIN3 has been implicated in eye development as *Drosophila Sin3A* was isolated in a screen to identify factors involved in modulation of the rough eye phenotype caused by ectopic expression of seven in absentia (Neufeld et al., 1998b). SIN3 has also been linked to developmental regulation through hormone signaling. SIN3 has been shown to associate with SMRTER, a 20-hydroxyecdysone (ecdysone) steroid hormone corepressor (Tsai et al., 1999).

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SIN3 colocalizes with SMRTER on polytene chromosomes isolated from *Drosophila* third instar larvae salivary glands (Pile and Wassarman, 2000). SIN3 binding to ecdysone steroid hormone regulated genes was shown to be dynamic and coincident with a developmental expression pattern of these genes in response to hormone signal. SIN3 has also been shown to be important for cell proliferation. Knock down of SIN3 in *Drosophila* tissue culture cells by RNA interference (RNAi) resulted in a G2 phase delay in cell cycle progression (Pile et al., 2002). Furthermore, comparison of gene expression profiles from wild type and RNAi-induced SIN3-deficient cells revealed differences in expression of genes encoding proteins that control multiple cellular processes, including cell cycle progression, transcription, and signal transduction (Pile et al., 2003). Taken together, the phenotypes of the *Drosophila* mutants and the tissue culture knockdown cells, along with the links to hormone signaling, suggest that SIN3 is a critical regulator of development and cell cycle progression.

Drosophila Sin3A is represented by a single gene. Multiple cDNAs that correspond to alternatively spliced transcripts, however, have been isolated (Neufeld et al., 1998b; Pennetta and Pauli, 1998). These transcripts are predicted to produce distinct protein isoforms that differ in amino acid sequence only at the carboxy (C)-terminus of each protein. An antibody raised against a region of the SIN3 protein common to all predicted isoforms recognized proteins of approximately 200 and 220 kD in embryonic extracts (Pile and Wassarman, 2000). These protein sizes are consistent with the predicted molecular weights of the isoforms of 187, 190, and 220 kD. Accordingly, we have named the

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different SIN3 isoforms SIN3 187, SIN3 190 and SIN3 220. Mammals have two SIN3 genes, SIN3A and SIN3B. Multiple alternatively spliced transcripts from mouse and human SIN3A and SIN3B have been reported (Alland et al., 2002; Yang et al., 2000). Interestingly, similar to the finding in *Drosophila*, the isoforms differ at the C-terminal region of the predicted proteins.

To advance our understanding of the role of SIN3 in development, we have determined the consequences of elimination of SIN3 expression at various stages of the *Drosophila* life cycle. To assess the effect of loss of SIN3 at later stages of development, we have established a conditional SIN3 knockdown transgenic line that circumvents the embryonic requirement for SIN3. Loss of SIN3 during post-embryonic development leads to lethality, indicating that SIN3 is required at multiple stages of *Drosophila* development.

MATERIALS AND METHODS

Western blot analysis

Western blot analysis was performed in accordance with standard protocols (Russell, 2001). Protein extracts from adult *Drosophila* were prepared by homogenizing approximately 10 flies in 200 µl of Laemmli sample buffer (Bio-Rad). Protein concentration was determined using the DC protein assay reagent (Bio-Rad) according to the manufacturer's protocol. Protein extract (15 to 20 µg) was fractionated by sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membrane, PVDF (Pall), and probed with IgG purified polyclonal rabbit antibodies against

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SIN3 pan (1:2000) and RPD3 (1:1000) (as a loading control), followed by donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G, IgG, (1:3000) (GE Healthcare) and detected with enhanced chemiluminescence reagents (GE Healthcare).

Drosophila stocks

Drosophila melanogaster stocks were maintained and crosses were performed according to standard laboratory procedures. The following stocks were used: w^{1118}, Sco/CyO (#335), CxD/TM3-Sb (Bloomingtom #3607) Act-GAL4 (Bloomington #4414), Hsp70-GAL4 (Bloomington #1799) and tub-GAL4 (Bloomington #5138), ey-GAL4 (Bloomington #8228), $Sin3A^{08269}$ (Bloomington #12350), $Sin3A^{e374}$(gift from Dr. David Wassarman).

Embryo collection

Embryos were collected on apple juice/agar plates supplemented with yeast paste for 2 hours and allowed to age according to standard protocol.

Cloning of the UAS-SIN3^{RNAi} and UAS-190,220^{RNAi} construct

To create the UAS-SIN3^{RNAi} construct a 730 bp region of the third exon of Sin3 was generated by PCR using genomic DNA isolated from *Drosophila* Kc167 tissue culture cells as template with the following primers (oriented 5' to 3') TAAATCTAGAGTGGCTTCGATACAGGCTGG and ATTGTCTAGATGATGG-CGATATGTCCGGCAC. This PCR product was inserted into the pWiz vector

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(obtained from the *Drosophila* Genomics Resource Center) at each of the AvrII and NheI restriction sites, in opposite orientations (Lee and Carthew, 2003). The UAS-190,220^{RNAi} construct was created similarly using the following primers (oriented 5' to 3') CAGTTCTAGAGCGTAACTCAGGCGAAATAC and CAGTTCTAGACGTCGA-GGAACTGGTATCAC. The clones were confirmed by sequencing.

Generation of transgenic flies carrying the UAS-SIN3^{RNAi} and UAS-190,220^{RNAi} construct

w^{1118} embryos were injected with the UAS-SIN3^{RNAi} or UAS-190,220^{RNAi} construct at Model System Genomics, Duke University, according to standard protocol (Rubin and Spradling, 1982). Homozygous lines for each single site transgene insertion were generated.

Generation of recombinant flies overexpressing SIN3 187, SIN3 220 or both

UAS-187HA and UAS-220HA constructs were first cloned into pUASp and pUAST vectors respectively and injected into *w^{1118}* embryos at Model System Genomics, Duke University to generate the transgenic flies. To create recombinants that constitutively overexpress SIN3 187HA, UAS-187HA/tub-GAL4 females were crossed to CXD/TM3-Sb males. SIN3 220HA were similarly generated by crossing UAS-220HA/Act-GAL4 females to Sco/CyO males. Balanced recombinants were selected on the basis of eye color. Recombinants were verified by performing a western blot analysis on whole cell protein extracts

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from adult flies. The blot was probed with antibodies against the HA tag to confirm overexpression of the transgene. UAS-187HA and UAS-220HA were also recombined onto the second chromosome carrying the *Sin3A*^{08269} or *Sin3A*^{e374} allele. Recombinants were selected on the basis of eye color and their inability to rescue lethality in the presence of the *Sin3A*^{08269} or *Sin3A*^{e374} allele. These recombinants will express the transgene only when crossed to a GAL4 driver.

RESULTS AND DISCUSSION

Generation of SIN3 conditional mutants

Expression pattern analyses indicated that the SIN3 187 and 220 isoforms are expressed throughout *Drosophila* development (V. Sharma). Because SIN3 is required for embryogenesis, characterization of the role of SIN3 in larval and pupal development has not been possible (Neufeld et al., 1998a; Pennetta and Pauli, 1998). Therefore, to determine whether SIN3 is also required for post-embryonic development, we designed a conditional knock down transgenic fly. In *Drosophila*, conditional knock down can be achieved by developmental stage specific induction of RNA interference (RNAi) using the GAL4-UAS system (Duffy, 2002; Lee and Carthew, 2003). We constructed a transgene (UAS-SIN3^{RNAi}) designed to target the degradation of all SIN3 isoforms. Tubulin, actin and heat shock GAL4 driver lines were used to knock down SIN3 expression in all tissues. Progeny resulting from the cross of UAS-SIN3^{RNAi} lines to GAL4 driver lines are referred to as SIN3-deficient flies.

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SIN3 is required for post-embryonic development

To induce ubiquitous loss of SIN3, we crossed heterozygous tub-GAL4 or Act-GAL4 driver males to homozygous UAS-SIN3^{RNAi} females (test crosses) (Fig. 2.1A). One half of the progeny are expected to be SIN3-deficient. Nine independent UAS-SIN3^{RNAi} lines were tested. As a control, both UAS-SIN3^{RNAi} females and GAL4 driver males were crossed to *w*^{1118} males and females respectively (Fig. 2.1 and data not shown). Progeny of all crosses were allowed to develop to adulthood. Three independent test and control crosses were set up for each UAS-SIN3^{RNAi} line. The minimum number of adults that were scored was 54. In eight out of nine test crosses, no SIN3-deficient flies survived to adulthood. Flies from the single test cross that survived to adulthood showed no obvious phenotype. Western blot analysis revealed that these adult flies expressed SIN3 at levels comparable to control animals, suggesting that SIN3 is not being effectively knocked down in that single viable cross (data not shown). The finding that ubiquitous loss of SIN3 resulting from RNAi leads to lethality is consistent with previous reports demonstrating that SIN3 is essential during early stages of development (Neufeld et al., 1998b; Pennetta and Pauli, 1998).

To determine the stage of development during which SIN3-deficient flies die, we followed the development of embryos from the control and test crosses (Fig. 2.1B). In the control crosses, 100% of embryos survived to adulthood. In

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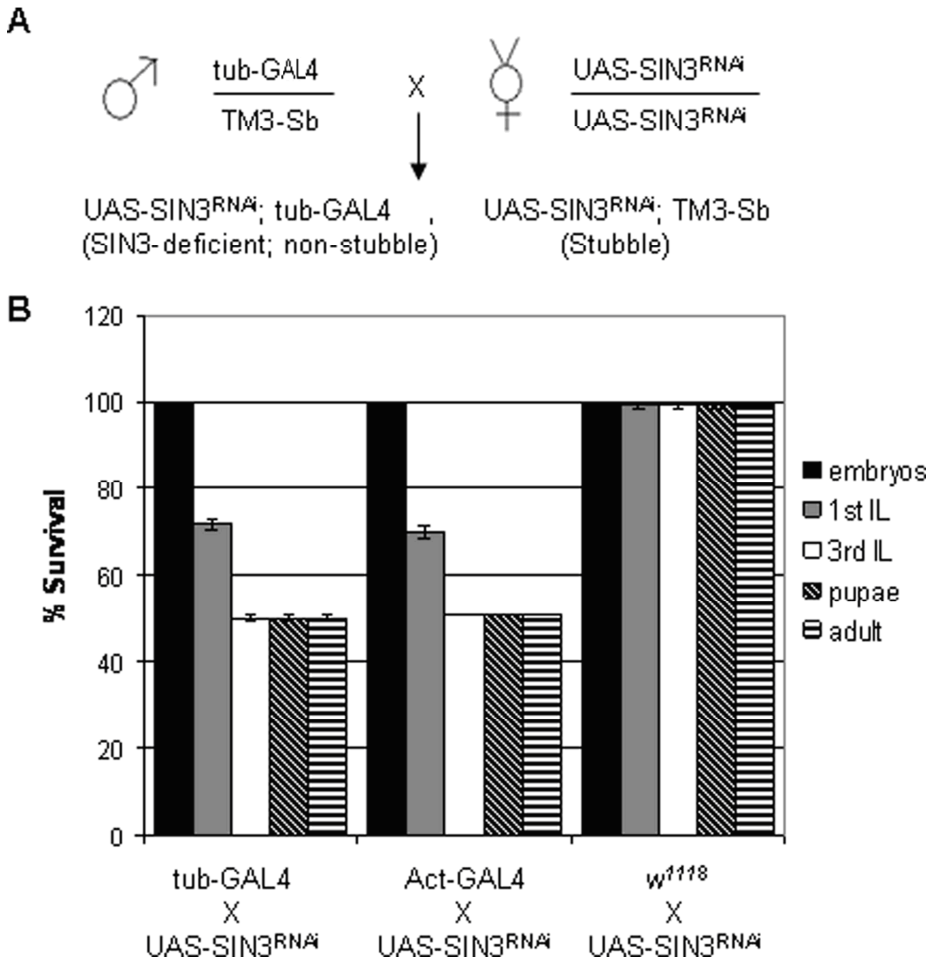

Figure 2.1: SIN3 is required for embryonic and larval development. (A) Schematic of the test cross to assay for viability. (B) 2-4 hour embryos resulting from the test and control crosses were counted, and plated, and the number of surviving animals in each developmental stage was determined each day until they reached adulthood. The results were obtained from three independent experiments. The total number (n) of embryos from these three experiments is as follows: tub-GAL4 x UAS-SIN3^{RNAi}, n = 504; Act-GAL4 x UAS-SIN3^{RNAi}, n = 499; *w*^{1118} x UAS-SIN3^{RNAi}, n = 538. All adults obtained from the test crosses had stubble bristles, indicating that they were not SIN3-deificent. IL – Instar larvae. Error bars represent the standard deviation.

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the test cross, 72% of embryos hatched into first instar larvae, but only 51% developed into wandering third instar larvae (consistent with the predicted ratio of progeny that will be SIN3-deficient). All surviving wandering third instar larvae developed into adults. All surviving adults in the test cross had stubble bristles, indicating that they were not SIN3-deficient flies. These results indicate that SIN3-deficient embryos die during embryonic and first, second, or early third instar larval development.

In order to verify the knock down of SIN3, and to analyze the SIN3-deficient embryos, embryos from the test cross were immunostained for SIN3 using the SIN3 pan antibody, and DNA using DAPI. Initially we analyzed a pool of 0-20 hour embryos. Loss of SIN3 upon induction of RNAi is inferred by the decrease in SIN3 staining intensity in 37% of these embryos (n = 94). This number is smaller than the predicted 50%. RNAi-induced loss of SIN3 expression is a consequence of degradation of transcribed RNA by the introduction of double stranded RNA (dsRNA) and of degradation of existing protein by normal cellular turnover. Depending on the stability of SIN3, the RNAi-induced effect may be delayed long after induction of the dsRNA from the UAS-$SIN3^{RNAi}$ transgene. In order to allow for protein turnover, we collected embryos for two hours, and allowed these embryos to age 18 hours. This pool of 18-20 hour embryos was immunostained with the SIN3 pan antibody. In this aged population, 48% of the embryos (n = 75) had little to no SIN3 staining (Fig. 2.2). DNA staining of the 0-20 hour collection with DAPI revealed that the SIN3-deficient embryos fell into different phenotypic categories. Some of the SIN3-

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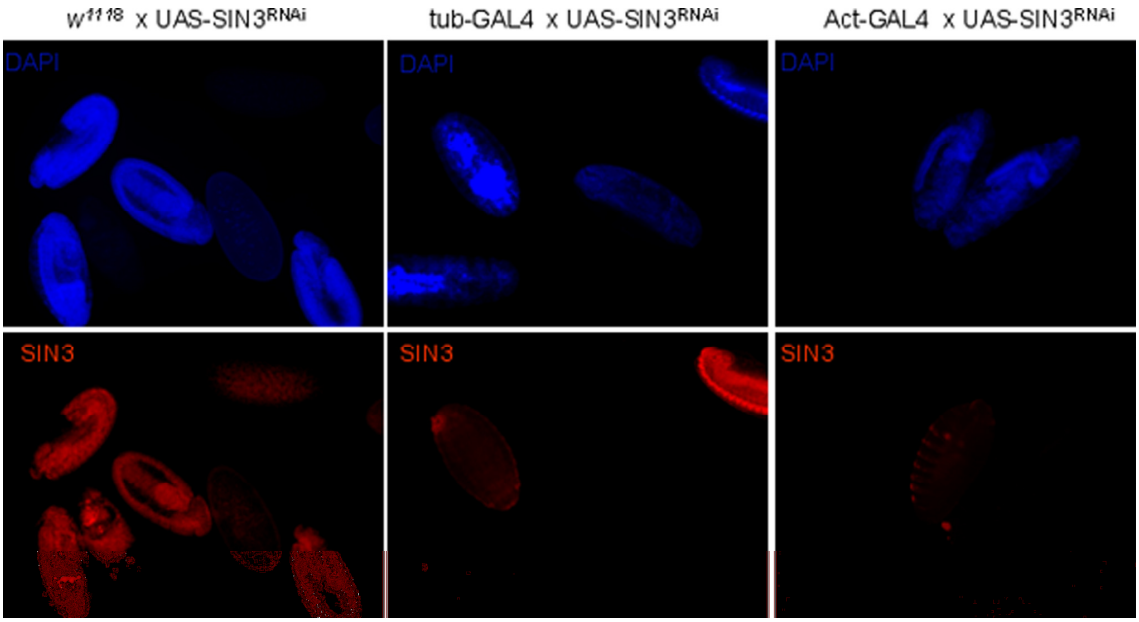

Figure 2.2: SIN3-deficient embryos show decreased immunostaining with α-SIN3 pan antibody. A pool of 0-18 hour embryos from the indicated crosses were immunostained with the SIN3 pan antibody and counter stained with DAPI. Approximately 50% of the embryos from the test cross are SIN3-deficient. 37% of embryos in the test cross show little to no staining for SIN3. n = 94

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deficient embryos had wild type morphology and the stage of development could thus be determined. 29% of the SIN3-deficient embryos were in stages 9-11, 26% in stages 12-14, and 11% in stage 15. The remaining 34% of the SIN3-deficient embryos had poor DNA staining by DAPI, and in some, the cells of the embryo appeared to pull away from the periphery, suggesting embryo degeneration. Due to loss of recognizable cellular structure, a stage of development for these embryos could not be assigned. As no SIN3-deficient embryos at stage 16 or later were identified, ubiquitous loss of SIN3 by RNAi appears to allow development for about 13 hours, to stage 15. The finding that some embryos develop to stage 15 is likely due to the presence of maternally deposited SIN3 that is not targeted by the RNAi pathway (Pennetta and Pauli, 1998). That some SIN3-deficient animals survive to larvae is possibly due to the presence of a low level of SIN3 that allows development to that stage. Eventually SIN3 is reduced to lethal levels in all animals having both the GAL4 driver and the UAS-SIN3^{RNAi} transgenes. The SIN3-deficient larvae appeared phenotypically normal, but failed to continue to develop into wandering third instar larvae. Loss of SIN3 by RNAi in *Drosophila* tissue culture cells resulted in loss of cell proliferation, likely due to a G2 cell cycle block (Pile et al., 2002). Homozygous null SIN3 clones in the developing eye resulted in scars across the eye consistent with a role for SIN3 in cell survival or proliferation (Neufeld et al., 1998b; Pennetta and Pauli, 1998). Given these previous findings, we hypothesized that lethality following RNAi induced loss of SIN3 in developing *Drosophila* results either from loss of cell proliferation or cell viability

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To determine the effect of loss of SIN3 on post-embryonic development, we induced SIN3 RNAi at different stages of larval development by crossing hsp70-GAL4 males to UAS-SIN3^{RNAi} females (Fig. 2.3). Control crosses were set up as mentioned above. Embryos were collected and subjected to initial heat shock at different stages of development to induce SIN3 RNAi. The developing larvae were subjected to heat shock by incubating at 37^{o}C for one hour. The larvae were subjected to heat shock each day, with a 24 hour recovery period at room temperature between heat shock treatments, until the larvae either died or developed into adults. The number of animals that survived to wandering third instar, pupae, and adulthood was determined. Induction of SIN3 RNAi in first or second instar larvae caused lethality prior to the wandering third instar, while inducing loss of SIN3 in wandering third instar larvae or pupae had no detectable effect on fly viability (Fig. 2.3B and Fig. 2.4). Inspection of the SIN3-deficient dead larvae revealed no gross phenotypic abnormalities, and the surviving adults appeared phenotypically normal (data not shown).

The transitions from larva to prepupa, and from prepupa to pupa, are each driven by pulses of the steroid hormone ecdysone (Riddiford, 1993). Induction of loss of SIN3 in first, second or early third instar, prior to the ecdysone pulse at the end of the third instar larval stage, results in lethality, while induction during or following the time frame of this pulse has no effect on fly viability. SIN3 has been found to bind to ecdysone-regulated genes on polytene chromosomes isolated from third instar larval salivary glands (Pile and Wassarman, 2000). It is possible that the larval lethality is due to aberrant

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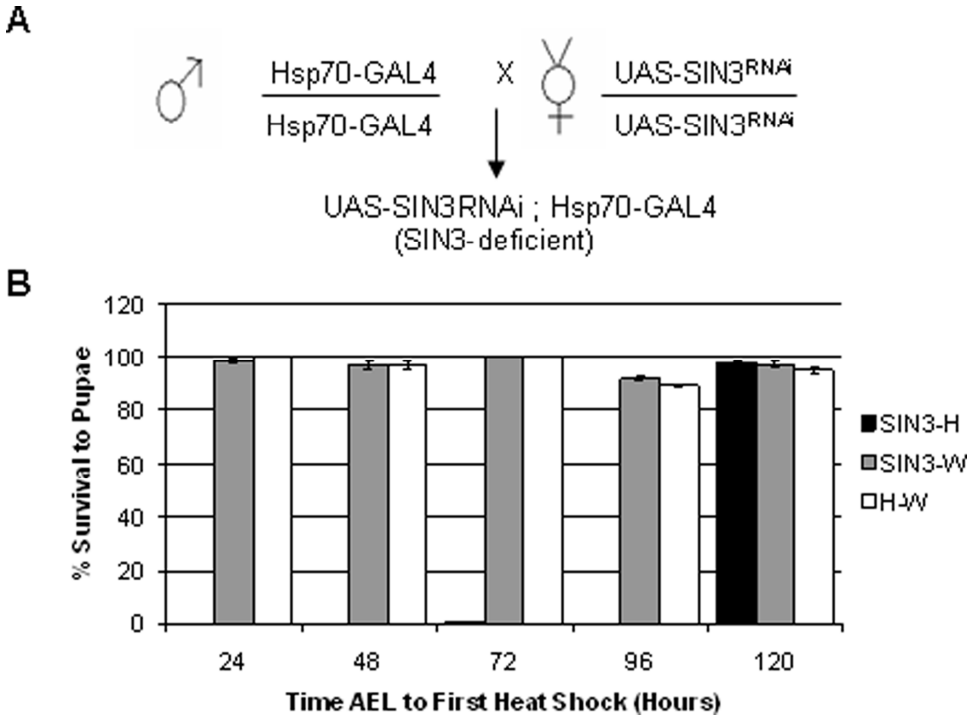

Figure 2.3: SIN3 is required during the first and second larval stages. (A) Schematic of the test cross for this assay. (B) 2-4 hour embryos resulting from the test and control crosses were counted, plated and aged for the times indicated before the first heat shock. The animals were subsequently heat-shocked at 37°C for one hour every 24 hours and allowed to recover at 25°C. The percent survival to the pupal stage was determined by counting the number of viable animals. The results were obtained from three independent experiments. The average total number of embryos tested for each time interval was 112. The range was from 70 to 216. SIN3-H: UAS-SIN3^{RNAi} x Hsp70-GAL4; SIN3-W: UAS-SIN3^{RNAi} x w^{1118}; H-W: Hsp70-GAL4 x w^{1118}. Error bars represent the standard deviation.

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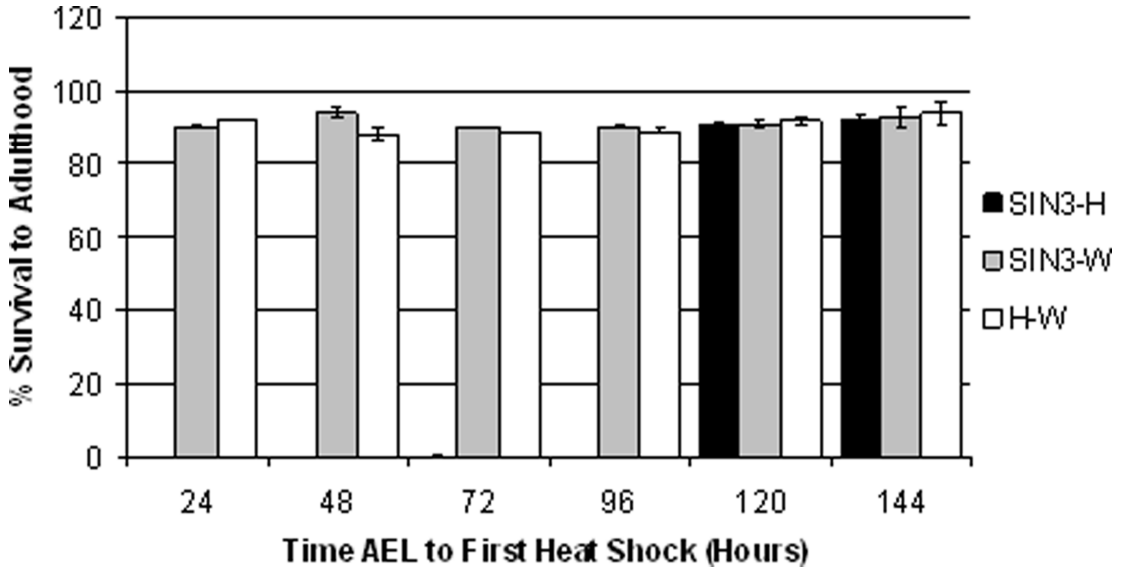

Figure 2.4: SIN3 is required at the first and second larval stages in order for the animals to develop into adults. 2-4 hour embryos resulting from the test and control crosses were counted, plated and aged for the times indicated before the first heat shock. The animals were subsequently heat-shocked at 37°C for one hour every 24 hours and allowed to recover at 25°C. The percent survival to adulthood was determined by counting the number of viable animals. The results were obtained from three independent experiments. The average total number of embryos tested for each time interval was 112. The range was from 70 to 216. SIN3-H: UAS-SIN3^{RNAi} x Hsp70-GAL4; SIN3-W: UAS-SIN3^{RNAi} x w^{1118}; H-W: Hsp70-GAL4 x w^{1118}. Error bars represent the standard deviation.

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expression of ecdysone responsive genes. Loss of SIN3 might lead to premature activation or lack of down regulation of ecdysone target genes, resulting in altered expression of genes required for morphogenesis. Through the use of a conditional knockdown transgenic fly system, we have established that SIN3 is required for both embryonic and early larval development. Results from our experiments have not detected a role for SIN3 in late larval, pupal or adult development. We do not, however, rule out the possibility that SIN3 functions in these late stages of development, when, as in the early stages, expression is detected. Again, depending on the stability of SIN3, the RNAi-induced effect may occur after induction of the dsRNA from the UAS-SIN3^{RNAi} transgene. Thus, even though we induced RNAi during the late larval and pupal stages, SIN3 protein levels may have remained at levels sufficient to allow development to the adult stage. It is also possible that in the latter stages of *Drosophila* development, other proteins are able to compensate for SIN3-deficiency.

SIN3 187 is not able to compensate for other SIN3 isoforms

To confirm that the embryonic and larval lethality is a consequence of SIN3-deficiency and not due to an RNAi off target effect, we introduced a UAS-187HA transgene (UAS driven expression of cDNA for the SIN3 187 isoform containing an HA tag at the C-terminus) into flies that are SIN3-deficient. Since the RNAi effect has been shown to be dose dependent, the expression of a SIN3 transgene should be able to rescue the RNAi-induced lethality (Yang et al., 2005). To test this hypothesis, we first generated flies carrying both the tub-

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GAL4 and UAS-187 trangenes on the third chromosome, over the TM3-Sb balancer chromosome. One half of the progeny from the cross of tub-GAL4, UAS-187/TM3-Sb X UAS-SIN3^{RNAi}/UAS-SIN3^{RNAi} would carry only the UAS-SIN3^{RNAi} construct (identified by the presence of stubble bristles), while the other half would simultaneously express UAS-SIN3^{RNAi} and over express SIN187 under the influence of tub-GAL4. Expression of SIN3 187 from UAS-187 increased survival of SIN3-deficient flies from 0 to 66% in males and 0 to 31% in females (Fig. 2.5A). Surviving adults appeared phenotypically normal. The rescue of lethality by the SIN3 187 transgene supports the idea that the dsRNA produced from the UAS-SIN3^{RNAi} transgene specifically targets SIN3 RNA for degradation and that the RNAi-induced lethality is the result of SIN3-deficiency.

We next carried out a similar experiment to test rescue by SIN3 220. We introduced a UAS-220HA transgene (UAS driven expression of cDNA for the SIN3 220 isoform containing an HA tag at the C-terminus) into flies that are SIN3-deficient. Interestingly, while SIN3 220 is also able to rescue SIN3 RNAi-induced lethality, the amount of rescue is not the same as that of SIN3 187. Expression of SIN3 220 from UAS-220 increased survival of SIN3-deficient flies from 0 to 53% in males and 0 to 55% in females. The difference in the ability of the individual isoforms to suppress the lethal phenotype is unlikely due to differences in the amount of expression from the two transgenes as the protein level of SIN3 187 and SIN3 220 in extracts prepared from adult females were similar (Fig. 2.5B). In contrast, the level of SIN3 220 in males compared to

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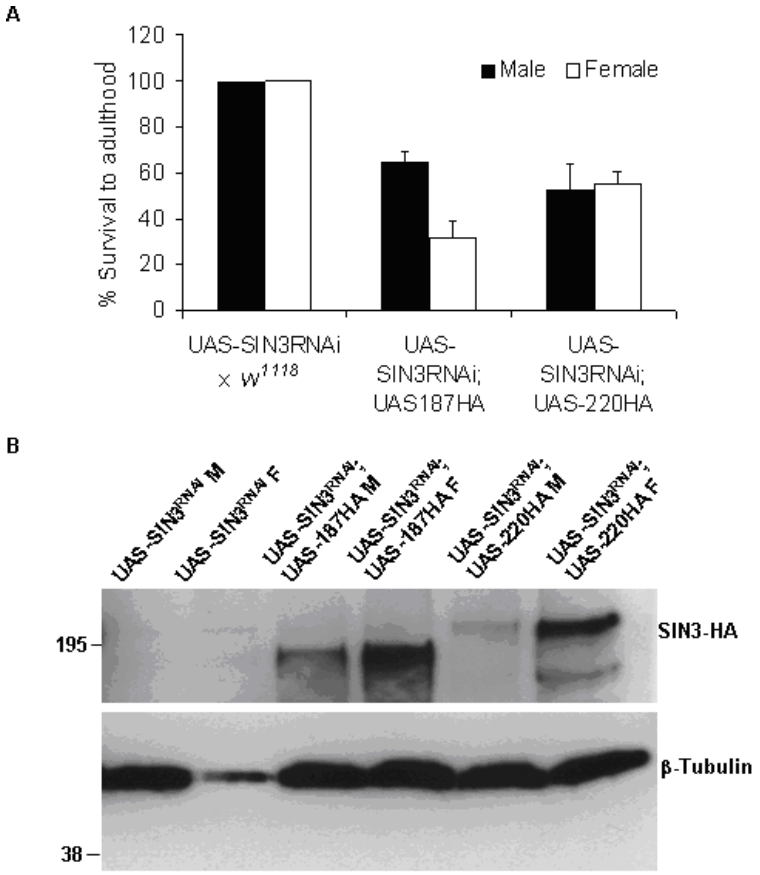

Figure 2.5: SIN3 187 and 220 transgenes can rescue lethality. (A) Homozygous UAS-SIN3 RNAi flies were crossed to *w*^{1118} or recombinant tub→187HA/Sb or Act→220HA/CyO flies to monitor rescue by these transgenes. (B) Western blot assays of whole cell extracts prepared from adults of either SIN3-deficient or rescued flies as indicated. The SIN3 isoforms expressed from the transgenes contain an HA tag. The blot was probed with antibody specific for HA (Sigma) and second antibody specific for β-tubulin (Amersham) as a loading control. Protein molecular weight markers (kD) are indicated on the left of the blot. M-Male, F-Female. Error bars indicate standard deviation.

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females was quite dissimilar. Like the SIN3 187 transgenic flies, surviving SIN3 220 transgenic adults appeared phenotypically normal. The finding that SIN3 187 or 220 can individually rescue the lethal phenotype due to knock down of expression of all SIN3 proteins suggests that SIN3 187 or 220 can partially substitute for the other isoforms. That the extent of rescue is different in males and females indicates that there may be sex specific roles of the isoforms that cannot be compensated for by another isoform.

Null mutation in *Sin3A* results in embryonic lethality (Neufeld et al., 1998b; Pennetta and Pauli, 1998). The above data suggests that SIN3 187 and SIN3 220 differ in their abilities to support *Drosophila* viability, but due to the nature of the experimental system, those results are somewhat inconclusive. RNAi targets degradation of mRNA. Overexpression of a cDNA encoding SIN3 187 or SIN3 220 from a transgene was able to overcome the RNAi degradation system to allow expression of some level of SIN3 protein. Even though the transgene was specific for one isoform or the other, the RNA produced from the transgene serves as a competitor for the endogenous SIN3 mRNA. It is entirely possible that endogenous SIN3 mRNA for the other isoforms will be expressed upon overexpression of a single SIN3 isoform cDNA. To definitely determine if SIN3 isoforms differentially support viability, we tested if expression of SIN3 187 or SIN3 220 alone could rescue the lethality caused by a homozygous null mutation in *Sin3A*. We overexpressed either SIN3 187 or SIN3 220 in two different *Sin3A* null backgrounds, $Sin3A^{e374}$, an EMS allele or $Sin3A^{P08269}$, a P-element insertion allele. We generated flies that carry the SIN3 187HA or 220HA transgene on the

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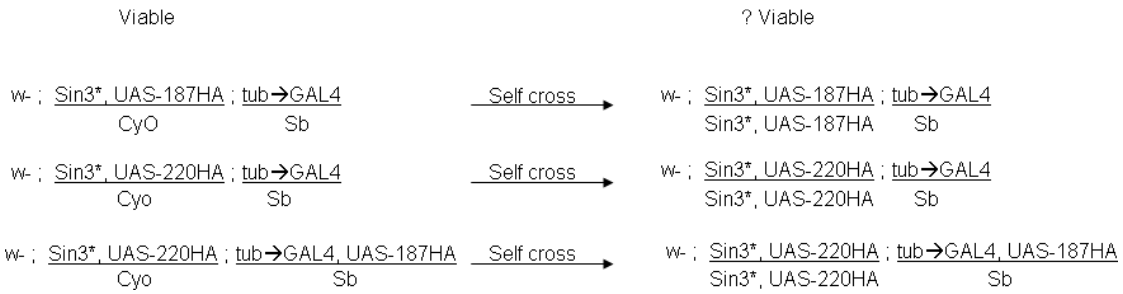

Figure 2.6: Scheme of crosses to monitor rescue of lethality caused by loss of SIN3. Recombinants carrying the 187HA or 220HA overexpression constructs were generated in a heterozygous *Sin3A*^{e374} or *Sin3A*^{P08269} background. The alleles are indicated by *. Refer to materials and methods for details. Expression of these transgenes was induced by tub-GAL4. In a self cross of these flies 25% of the progeny will be homozygous for the sin3 chromosome and therefore die unless expression of the transgene can rescue lethality. These rescued flies will have straight wings, whereas all other genotypes generated in this cross will have curly wings.

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same chromosome as one of the two SIN3 mutants. We then crossed these flies to the tub-GAL4 driver for ubiquitous expression of the tagged isoform (Fig. 2.6). We observed very few flies of the genotype *Sin3A-/-,* SIN3 187HA+, indicating that flies that express only SIN3 187 are essentially non-viable (Table 2.1). In contrast, flies of the genotype *Sin3A-/-,* SIN3 220HA+ were observed, indicating that expression of SIN3 220 alone can support viability. Interestingly, flies that express both SIN3 187 and 220 were observed in the highest numbers suggesting that 220 cannot completely compensate for essential functions of SIN3 187. Western blot analysis of rescued adult files shows that these flies express the appropriate tagged SIN3 isoform (Fig. 2.7). These data show that SIN3 187 has a distinct function from SIN3 220.

To confirm that SIN3 187 alone does not support fly viability, we performed RNAi knockdown in transgenic flies to eliminate expression of two of the three SIN3 isoforms. The transgene UAS-SIN3 190,220^{RNAi} drives expression of an inverted repeat of the SIN3 transcript designed to target both SIN3 190 and 220. To verify that the expressed double stranded RNA (dsRNA) resulted in knockdown of SIN3 190 and 220, we used a driver specific for eye imaginal disc expression. Western blot analysis of whole cell extracts prepared from SIN3 190,220 knockdown larval eye discs indicated a decrease in SIN3 220 and a small increase in SIN3 187 expression, demonstrating the specificity of the dsRNA (Fig. 2.8). The lower molecular weight signal is specific to SIN3 187, as we have previously shown that SIN3 190 expression is not detectable in larvae (Sharma et al., 2008). Next, the Act-GAL4 driver line was used to knock down

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***Sin3A* allele**	**Percent Survival**		
	Sin3A-/-	*Sin3A*+/-, 187HA+	*Sin3A*-/-, 187HA+
P-element	0	100	6 ± 1
EMS	0	100	6 ± 2
	Sin3A-/-	*Sin3A*+/-, 220HA+	*Sin3A*-/-, 220HA+
P-element	0	100	74 ± 3
EMS	0	100	66 ± 11
	Sin3A-/-	*Sin3A*+/-, 187HA+, 220HA+	*Sin3A-/-*, 187HA+, 220HA+
P-element	0	100	88 ± 8
EMS	0	100	81 ± 3

Table 2.1: SIN3 isoforms vary in their ability to rescue lethality of genetic *Sin3A* loss of function alleles. Flies carrying one of two *Sin3A* alleles, either the UAS-187HA, UAS-220HA or both transgenes and the tub-GAL4 transgene were generated and self crossed. Please refer to Experimental Procedures for details. The number of progeny that were genetic null for *Sin3A* and that expressed neither, one or both of the SIN3 tagged isoforms was determined. The percent survival was calculated by setting the value for the number of *Sin3A* heterozygous flies expressing the indicated isoform(s) to 100% and comparing the number to that of the rescued *Sin3A* homozygous mutant flies expressing the indicated isoform(s). The results are the average of three independent experiments. Standard deviation is indicated.

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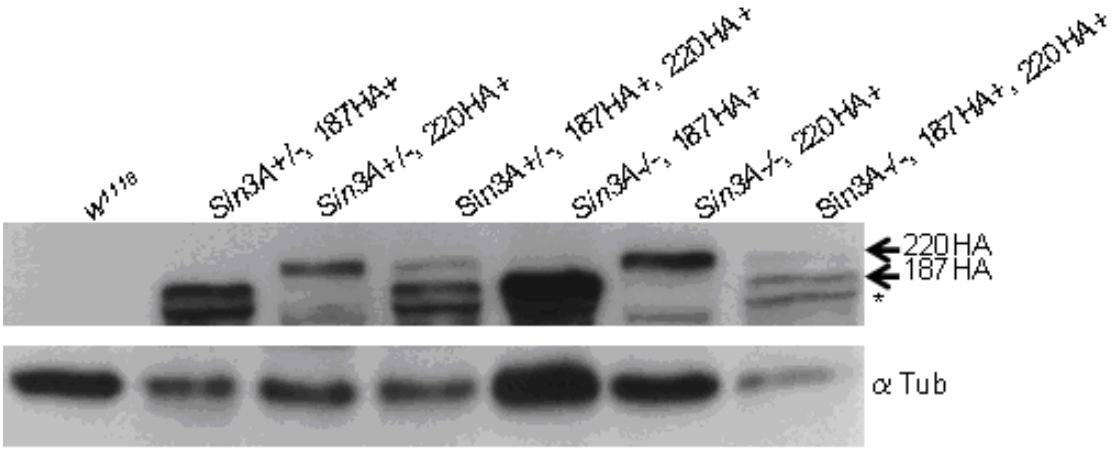

Figure 2.7: SIN3 187HA and 220HA are expressed in *Sin3A* mutant flies. Western blot analysis of whole cell extracts from adults flies of the indicated genotypes. The blot was probed with antibody specific for HA (Sigma) and second antibody specific for α-tubulin (αTub) (GE Healthcare) as a loading control. * indicates a likely degradation product.

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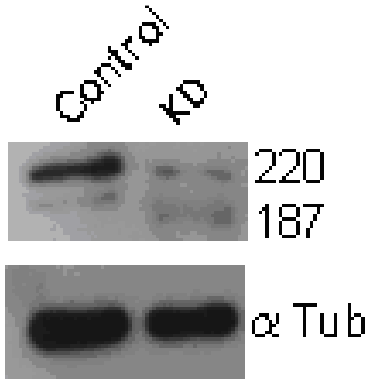

Figure 2.8: Expression from the UAS-190,220^{RNAi} construct leads to specific knockdown of SIN3 220. Western blot analysis of total protein extracted from eye imaginal discs of control *w*^{1118} or progeny of the ey-GAL4 crossed to UAS-190,220^{RNAi} (KD) line. The blot was probed with an antibody to all SIN3 isoforms and to α-tubulin (αTub) as a loading control.

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RNAi line	Number of Adults Observed	
	UAS-190,220^{RNAi}/ tub-GAL4	UAS-190,220^{RNAi}/Sb
UAS-190,220^{RNAi} #4	0	362
UAS-190,220^{RNAi} #16	0	447
UAS-190,220^{RNAi} #17a	0	374
UAS-190,220^{RNAi} #18	0	575

Table 2.2: Simultaneous knockdown of SIN3 190 and SIN3 220 results in lethality. Multiple independent UAS-190,220^{RNAi}/UAS-190,220^{RNAi} fly lines were crossed to the tub-GAL4/Sb driver line and the progeny analyzed and counted. All adult progeny had stubble bristles, indicating that they did not express the GAL4 activator required for SIN3 knockdown. The number of flies reported represents the total number from two independent parental crosses for each line.

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SIN3 190 and 220 expression in all tissues. We did not observe any viable SIN3 190/220 knockdown adult flies (Table 2.2). The RNAi knockdown results, as well demonstrate that native expression of SIN3 187 cannot compensate for loss of the other isoforms. In contrast, expression of SIN3 220 on its own supports viability. These data suggest that whereas SIN3 220 is necessary and sufficient for viability, SIN3 187 is not.

CONCLUSION

It is well established that regulation and maintenance of histone acetylation levels are important for normal development (Lin and Dent, 2006). In this study, we have investigated the expression and post-embryonic requirement of SIN3, one component of a multisubunit HDAC complex. Vishal Sharma performed an extensive analysis of isoform expression patterns and found that SIN3 isoforms are expressed throughout development. The different isoforms have distinct patterns of expression. SIN3 187 has prominent expression in differentiated tissue such in the final stage 17 embryos and in adults. SIN3 220 expression is low in those differentiated tissues and high in proliferating cell such as larval imaginal discs and embryonic tissue culture cells. SIN3 190 has most restricted pattern of the three, as it was detected only in embryos and adult females. The distinct expression patterns and the finding that SIN3 187 and SIN3 220 rescue SIN3-deficiency and null mutations in *Sin3A* to different levels suggest that SIN3 187 can not compensate for the loss of SIN3 220. The results lead to the possibility that these proteins regulate distinct genes that are required

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for specific developmental events. A second possibility is that the complexes have distinct activities. For example, they may have non-histone targets. Consistent with their expression throughout development, elimination of SIN3 during embryonic and first, second, and early third instar larval development results in lethality. Further analysis of phenotypes resulting from SIN3-deficiency in specific tissues is anticipated to reveal the role of SIN3 in regulating specific developmental pathways.

ACKNOWLEDGEMENTS

We thank Blake Walker for assistance in counting flies for the SIN3 190, 220 knockdown experiment.

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CHAPTER 3

REGULATION OF CELL PROLIFERATION AND WING DEVELOPMENT BY *DROSOPHILA* SIN3 AND STRING

This chapter has been published:

Swaminathan, A., and L.A. Pile. 2010. Regulation of cell proliferation and wing development by *Drosophila* SIN3 and String. *Mech Dev*. 127(1-2):96-106.

ABSTRACT

The transcriptional corepressor SIN3 is an essential gene in metazoans. In cell culture experiments, loss of SIN3 leads to defects in cell proliferation. Whether and how SIN3 may regulate the cell cycle during development has not been explored. To gain insight into this relationship, we have generated conditional knock down of *Drosophila* SIN3 and analyzed effects on growth and development in the wing imaginal disc. We find that loss of SIN3 affects normal cell growth and leads to down regulation of expression of the cell cycle regulator gene String (STG). A SIN3 knock down phenotype can be suppressed by overexpression either of STG or of Cdk1, the target of STG phosphatase. These data link SIN3 and STG in a genetic pathway that affects cell cycle progression in a developing tissue.

INTRODUCTION

Histone acetylation levels are regulated by the opposing activities of histone lysine (K) acetyltransferases (KATs) and histone deacetylases (HDACs). The SIN3 complex is one of two major class I containing HDAC complexes

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present in cells (Ayer, 1999). The corepressor SIN3 and the HDAC RPD3 (HDAC1 and 2 in mammals) are two important components of the multisubunit complex (Silverstein and Ekwall, 2005). Mutations in either SIN3 or RPD3 result in lethality in both *Drosophila* and mouse model systems (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008; Neufeld et al., 1998b; Pennetta and Pauli, 1998). Accordingly, establishment and/or maintenance of histone acetylation levels are critical for metazoan development and viability.

SIN3 has been shown to be important for cell proliferation. In *Drosophila* tissue culture cells, reduction of SIN3 protein expression by RNA interference (RNAi) resulted in a G2 phase delay in cell cycle progression (Pile et al., 2002). A comparison of gene expression profiles from wild type and RNAi-induced SIN3 knockdown cells revealed differences in expression of genes encoding proteins that control multiple cellular processes, including cell cycle progression, transcription, mitochondrial activity and signal transduction (Pile et al., 2003). Expression of two genes critical for the G2/M transition of the cell cycle, String (STG) and cyclin B (CycB), was reduced in the SIN3 knock down tissue culture cells. STG is the *Drosophila* homolog of *S. pombe* Cdc25, a conserved protein phosphatase that dephosphorylates and activates the cyclin dependent kinase, Cdk1 (also known as DmCdc2), which is critical for entry into M phase (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990; Moreno and Nurse, 1991). CycB interacts with Cdk1 and promotes the G2/M transition (Edgar et al., 1994; Nigg, 1995; O'Farrell et al., 1989).

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In mouse, knock out of either SIN3 gene, *mSin3a* or *mSin3b,* by gene disruption revealed links to cell cycle regulation. Analysis of SIN3-deficient mouse embryonic fibroblasts (MEFs) indicated that mSin3A is important for cell proliferation (Cowley et al., 2005; Dannenberg et al., 2005). The mSin3A-deficient MEFs exhibited reduced proliferative capacity relative to their wild type counterparts. Analysis of the DNA content the MEFs indicated a reduction in the number of cells in S phase with an increase in the number of cells in the G2/M phase of the cell cycle. Although *mSin3b* is highly similar to *mSin3a,* the proteins are non-redundant as loss of either gene by targeted gene disruption resulted in embryonic lethality (David et al., 2008). Furthermore, mSin3B-deficient, but not mSIN3A-deficient, MEFs, proliferated similarly to the wild type cells under standard culture conditions (David et al., 2008). Upon serum starvation, however, wild type cells ceased to proliferate while the mSin3B-deficient cells continued to cycle, indicating that mSin3B is necessary for cell cycle exit at the start of differentiation (David et al., 2008).

Null mutations in *Drosophila Sin3A* result in embryonic lethality with only a few animals surviving to the first larval instar stage (Neufeld et al., 1998b; Pennetta and Pauli, 1998). Using an RNAi conditional mutant, we determined that SIN3 is also necessary for post-embryonic development (Sharma et al., 2008). To study the role of SIN3 during the process of cellular proliferation and differentiation, we utilized an RNAi conditional mutant to eliminate SIN3 in wing imaginal disc cells. We analyzed SIN3 knock down cells during larval and adult stages of development. Loss of SIN3 resulted in fewer cells in the wing blade

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and a curled wing phenotype in the adult. The curly wing phenotype was partially suppressed by overexpression of the cell cycle regulator STG and its target Cdk1. These data suggest that SIN3 and G2 to M regulators work in a similar pathway to affect cell cycle progression.

MATERIALS AND METHODS

Drosophila stocks

Drosophila melanogaster stocks were maintained and crosses were performed according to standard laboratory procedures. The following stocks were used: w^{1118}, Ser-GAL4 (Bloomington #6791), Bx-GAL4 (Bloomington #8696), □S-GAL4 (Bloomington #8142), Vg-GAL4 (Bloomington #82222), A9-GAL4 (Bloomington #8761) and en-GAL4 (Bloomington #6356), *Pcaf* Df (Bloomington #4507), *Rpd3* Df (Bloomington #3686), UAS-STG(II) (Bloomington #4447), UAS-STG(III) (Bloomington #4778), UAS-Cdk1 (Bloomington #6642), UAS-GFP (Bloomington #7374), $cdk1^{c03495}$ (Bloomington #11302), $Stg^{EY12338}$ (Bloomington #20349), $Pcaf^{Q186st}$ (Bloomington #9334), $Pcaf^{C137T}$ (Bloomington #9335), $Pcaf^{\Delta T280\text{-}F285}$ (Bloomington # 9336), ED(3L)4483 (Bloomington #8070), ED(3L)215 (Bloomington #8071), ED(3L)4486 (Bloomington #8072). CyO-Ras/Sco stock was generated by crossing Df(2R)vg-C/CyO,P{ry[+t7.2]=sevRas1.V12}psFK1 (Bloomington #754) to CyO/Sco (Bloomington #335) and selecting progeny with rough eye, curly wing, and scutoid bristle phenotypes. The hsFLP;Act5C>CD2>GAL4,UAS-EGFP stock was a kind gift from Dr. Dirk Bohmann.

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Cloning of the UAS-SIN3$^{RNAi-II}$ construct

The UAS-SIN3^{RNAi-I} construct was previously described (Sharma et al., 2008). The UAS-SIN3$^{RNAi-II}$ construct was cloned in the same way using forward and reverse primers (oriented 5' to 3') ATTTTCTAGATGGCGGGAACAGAACG and ATGCTCTAGAGCGAAGAAAAGGTCAG respectively.

Generation of transgenic flies carrying the UAS-SIN3$^{RNAi-II}$ construct

As with the UAS-SIN3^{RNAi-I} flies described in (Sharma et al., 2008), w^{1118} embryos were injected with the UAS-SIN3$^{RNAi-II}$ construct at Model System Genomics, Duke University, according to standard protocol (Rubin and Spradling, 1982). Homozygous lines for each single site transgene insertion were generated. SIN3 knock down recombinant flies were generated by crossing Ser-GAL4/UAS-SIN3^{RNAi-I} females to CyO-Ras/Sco males. Recombinant progeny were scored on the basis of eye color. These flies are referred to as SIN3 KD flies. Potential recombinants were verified by crossing to w^{1118} and monitoring the penetrance of the curly wing phenotype in the progeny.

Microscopy

Images of wings were taken at 80x magnification on a Leica MZ125 microscope.

Immunostaining

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Wing discs from wandering third instar larvae were dissected in 1 X PBS. 20 – 50 wing discs were fixed in 4% formaldelyde in 1 X PBS and stained as described previously (Sharma et al., 2008). Antibodies against SIN3 (1:500) (Pile and Wassarman, 2000) followed by donkey anti-rabbit Alexa 495 (1:1000) (Invitrogen) or histone H3 phospho-serine 10 (1:500) (Abcam) followed by sheep anti-mouse Alexa 488 (1:1000) (Invitrogen) or GPF (1:1000) (Abcam) followed by sheep anti-mouse Alexa 488 (1:2000) (Invitrogen) were used. Visualization and photography was done with a Zeiss Axioscope 2 fitted with an Axio-phot photography system.

Western blot analysis

Western blot analysis was performed in accordance with standard protocols (Russell, 2001). To prepare whole cell extracts, wing discs isolated from wandering third instar larvae were homogenized in Laemmli sample buffer (Bio-Rad). Protein concentration was determined using the DC protein assay reagent (Bio-Rad) according to the manufacturer's protocol. Protein extract (15 to 20 □g) was fractionated by sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membrane, PVDF (Pall), and probed with immunoglobulin G (IgG) purified polyclonal rabbit antibodies against SIN3 (1:2000) (Pile and Wassarman, 2000), followed by donkey anti-rabbit horseradish peroxidase-conjugated IgG (1:3000) (GE Healthcare) and detected with enhanced chemiluminescence reagents (GE Healthcare). The blots were subsequently probed with monoclonal mouse

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antibody against β-tubulin (1:1000) (Sigma), followed by sheep anti-mouse horseradish peroxidase-conjugated IgG (1:3000) (GE Healthcare) as a loading control.

GFP clonal analysis

hsFLP;Act5C>CD2>GAL4,UAS-EGFP flies were crossed to w^{1118} or UAS-SIN3^{RNAi-I} to generate random GFP positive clones. 0-4 hour embryos were collected and heat shocked 48-52 hours after egg laying. Wing discs from wandering third instar larvae (~120 hours after egg laying) were dissected and immunostained with anti-GFP as described above.

Determination of wing area

The area of wings was determined using ImageJ software.

Determination of cell number in the wing

Bristles were counted in a 2 x 2 cm^2 area of the dorsal side of the wings, at 80X magnification, between veins L4 and L5.

Reverse transcription PCR assay

Total RNA was extracted from wing discs isolated from wandering third instar larvae using the RNeasy mini kit (Qiagen). cDNA was generated from total RNA using the ImProm-II Reverse Transcription System (Promega) with random hexamers. The cDNA was used as template in a quantitative real-time PCR

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(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. The following primers (oriented 5' to 3') were used: STG F (AACACCAGCAGTTCGAG) and STG R (CCATAGCTGGCAGAATCTTC); TAF1 F (GTGGAGGAGCCAAGGGAGCC) and TAF1 R (TCCCGCTCCTTGTGCGAATG). *Drosophila* S2 cell culture and RNAi was carried out as previously described (Pile et al. 2002).

Statistical Analyses

The significance of all data was calculated using the student t-test from http://www.graphpad.com/quickcalcs/index.cfm.

RESULTS

The requirement of SIN3 for viability has hampered investigations into the role of this protein during post-embryonic development. To study the role of SIN3 in a developing organism, we generated a conditional knock down transgenic fly designed to reduce expression of SIN3 (Sharma et al., 2008). In *Drosophila*, conditional knock down can be achieved by developmental stage specific induction of RNAi using the GAL4-UAS system (Duffy, 2002; Lee and Carthew, 2003). The transgene UAS-SIN3^{RNAi} drives expression of an inverted repeat of the SIN3 transcript (Fig. 3.1A). The dsRNA produced by the inverted repeat targets degradation of endogenous SIN3 mRNA leading to loss of SIN3 protein (Fig. 3.2). To ensure that any observed phenotypes are due to loss of SIN3 and

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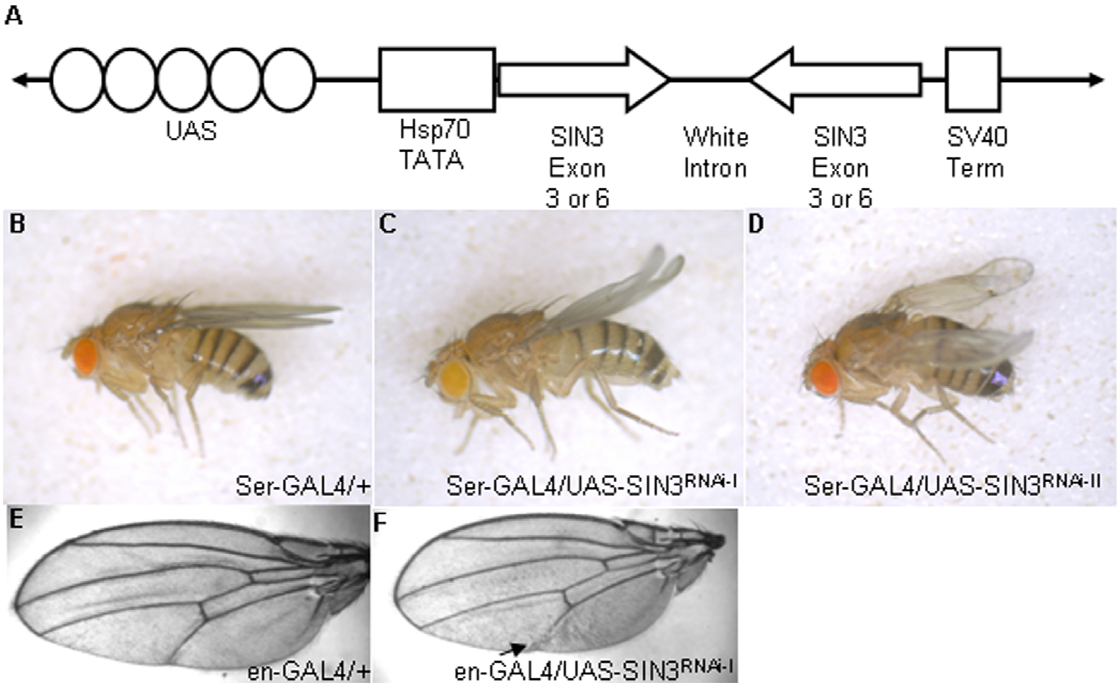

Figure 3.1: Loss of SIN3 results in a curly wing phenotype. (A) Schematic of the targeting construct UAS-SIN3^{RNAi}. Micrographs of flies (B-D) or wings (E, F) carrying indicated GAL4 driver and/or the UAS-SIN3^{RNAi} construct. Arrow indicates incomplete L5 vein.

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not due to an off target effect (Ma et al., 2006), we generated two SIN3 targeting constructs, each targeting distinct regions of the SIN3 transcript. Progeny resulting from the cross of UAS-SIN3^{RNAi} lines to GAL4 driver lines are referred to as SIN3 knock down flies. To investigate the role of SIN3 in cell cycle progression in the context of a developing tissue, we eliminated SIN3 specifically in the wing imaginal disc, a system well suited to the study of cell cycle regulators (Milan et al., 1996a; Milan et al., 1996b).

Mutations in regulators of histone acetylation affect wing development

To determine if SIN3 is required for normal wing development, several UAS-SIN3^{RNAi} transgenic lines were crossed to various driver lines that express GAL4 in the wing imaginal disc. The SIN3 knock down progeny of these crosses were screened for wing phenotypes. Loss of SIN3 in the wing imaginal disc produced SIN3 knock down progeny having curly wings (Fig. 3.1B-D). Using the engrailed (en) GAL4 driver, we observed the curly wing only in the posterior wing compartment, which reflects the expression pattern of *en*. This finding is consistent with the cell autonomous nature of RNAi in *Drosophila* (Roignant et al., 2003). The percentage of curly winged flies was similar regardless of which RNAi targeting construct was used, indicating that the curly wing phenotype was due to loss of SIN3 and not the result of an off target effect (Table 3.1). Variability in the penetrance of the phenotype observed using different driver lines as well as the different UAS-SIN3^{RNAi} lines (Table 3.1) is likely due to variability in amount of production of dsRNA.

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SIN3 RNAi line\Driver	A9-Gal4	Vg-Gal4	Ser-Gal4	ΔS-Gal4	Bx-Gal4
UAS-SIN3^{RNAi-I} #5	+	+	+	+	++
UAS-SIN3^{RNAi-I} #6	+++	+	++	++	+++
UAS-SIN3^{RNAi-I} #12	+	+	++	+	+++
UAS-SIN3^{RNAi-I} #15	++	++	+++	++	+
UAS-SIN3^{RNAi-I} #17	+++	+	+++	+++	+++
UAS-SIN3^{RNAi-I} #37	+	+	+	+	-
UAS-SIN3^{RNAi-I} #92	+	++	++	++	++
UAS-SIN3$^{RNAi-II}$	ND	ND	+++	ND	+++

Table 3.1: Loss of SIN3 in wing imaginal discs results in a curly wing phenotype. (-) no curly wings, (+) 1-33% penetrance (++) 34-66% penetrance and (+++) >66% penetrance of curly wing phenotype. Vg-vestigial; Ser-serrate; A9, ΔS, Bx-beadex are each derived from enhancer traps and have wing imaginal disc expression. ND, not determined.

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To verify knock down of SIN3 protein expression, wing imaginal discs isolated from larvae of a cross that produced high penetrance of the curly wing phenotype were immunostained with antibody to SIN3. The staining intensity of SIN3 knockdown wing discs was much lower than control wing discs suggesting efficient knock down of SIN3 (compare Fig. 3.2B to 3.2D and 3.2F). The SIN3 staining pattern reflected the GAL4 expression pattern as expected (Fig. 3.3). The Ser-GAL4 driver expressed GAL4 throughout the wing disc at different times in development, consistent with the reported expression pattern of serrate (Bachmann and Knust, 1998; Yan et al., 2004). Bx-GAL4 expressed GAL4 predominantly in the wing pouch area (Zeng et al., 1998). In addition, western blot analysis of whole cell extracts prepared from SIN3 knockdown wing discs indicates that there was a decrease of SIN3 protein upon RNAi induction (Fig. 3.2G). The results indicate that production of dsRNA against SIN3 in the wing imaginal disc resulted in less SIN3 protein and led to an altered phenotype in the adult wing.

We next tested if the SIN3 knock down wing phenotype could be modified by deficiencies in other proteins known to be important in regulation of histone acetylation. RPD3 is the catalytic subunit of the SIN3 complex responsible for HDAC activity (Hassig et al., 1997; Laherty et al., 1997; Zhang et al., 1997). PCAF is a KAT that carries out the reverse reaction to deacetylation (Yang et al., 1996). Mutations in the *Drosophila* PCAF homolog *dGcn5* affect cell proliferation in wing imaginal discs and lead to abnormal adult wing development (Carre et al., 2005). We asked if the SIN3 knockdown curly wing phenotype could be

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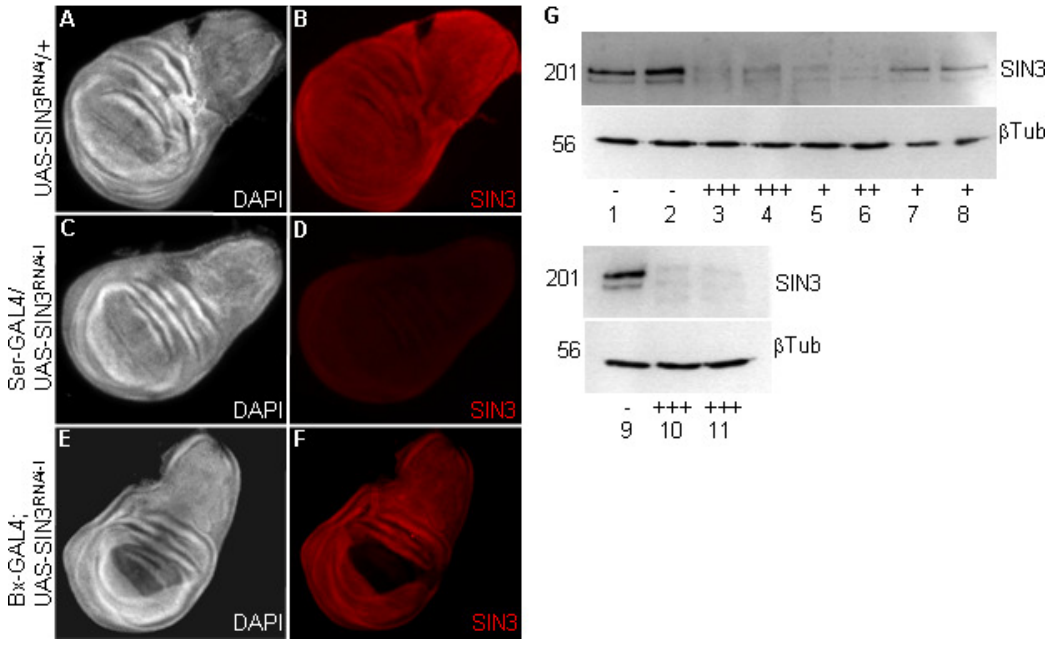

Figure 3.2: Induction of SIN3 RNAi leads to reduced expression of SIN3 protein. (A-F) Control or SIN3 knock down wing imaginal discs were stained with DAPI to visualize the DNA and antibody to SIN3. Driver lines are indicated. (G). Western blot analysis of total protein extracted from wing imaginal discs of control or SIN3 knock down larvae. The blots were probed with antibody to SIN3 or β-tubulin (βTub) as a loading control. Molecular weight markers are indicated to the left of the panel. Genotypes are as follows: Lane 1 – Ser-GAL4, 2 – UAS-SIN3^{RNAi-I} #17, 3 – Ser-GAL4 x UAS-SIN3^{RNAi-I} #17, 4 – Bx-GAL4 x UAS-SIN3^{RNAi-I} #17, 5 – Vg-GAL4 x UAS-SIN3^{RNAi-I} #17, 6 –Ser-GAL4 x UAS-SIN3^{RNAi-I} #92, 7 – Ser-GAL4 x UAS-SIN3^{RNAi-I} #5, 8 - Ser-GAL4 x UAS-SIN3^{RNAi-I} #37, 9 – UAS-SIN3$^{RNAi-II}$, 10 - Ser-GAL4 x UAS-SIN3$^{RNAi-II}$, 11 - Bx-GAL4 x UAS-SIN3$^{RNAi-II}$. The penetrance of the curly wing phenotype for each genotype is indicated by the (-) or (+), refer to Table 3.1.

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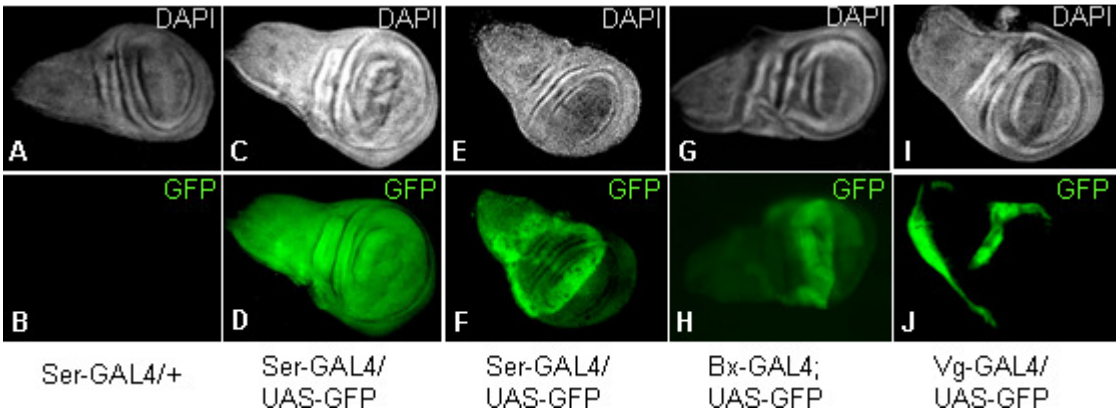

Figure 3.3: Characterization of expression patterns of different wing specific GAL4 drivers. The indicated wing driver males were crossed to UAS-GFP females and wings discs from early third instar larvae (C,D) or wandering third instar larvae (A,B,E-J) were immunostained with anti-GFP (B,D,F,H,J) and counter stained with DAPI to visualize DNA (A,C,E,G,I).

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modified by a 50% reduction in the amount of either RPD3 or PCAF by crossing SIN3 knockdown flies to flies carrying deficiencies of either of these histone acetylation regulatory factors. Progeny of these crosses are deficient for SIN3 and heterozygous for either RPD3 or PCAF. Reduction of RPD3 in a SIN3 knock down background did not further enhance the curly wing phenotype, which is already near 100%, nor did it lead to any additional wing phenotypes (Fig. 3.4). Reduction of PCAF suppressed the curly wing phenotype as only 63% of the flies had curly wings compared to 91% of the SIN3 knock down alone (Fig. 3.4). We tested three additional PCAF chromosomal deficiencies (Df(3L)ED4483, Df(3L)ED215, Df(3L)ED4486) as well as three specific alleles (Pcaf Q186st, Pcaf C137T and Pcaf ΔT280-F285). All but one of these PCAF mutations were able to suppress the SIN3 knock down curly wing phenotype (Fig. 3.4 and data not shown). These results suggest that SIN3 and regulated histone acetylation are critical for normal development of wing tissue.

SIN3-deficiency leads to smaller wings

To further investigate the role of SIN3 in wing development, we analyzed the overall size of both the larval precursor and adult tissue. Loss of SIN3 resulted in smaller wing imaginal discs and smaller adult wings. In flies carrying the Ser-GAL4 driver, SIN3 knock down discs are 5% smaller than wild type (wild type $n = 93$, SIN3 knock down $n = 121$, $p < 0.01$). We also measured the overall size of the adult wing blade in the progeny of crosses using different wing imaginal disc GAL4 drivers. SIN3 knockdown adult wings were reduced in size

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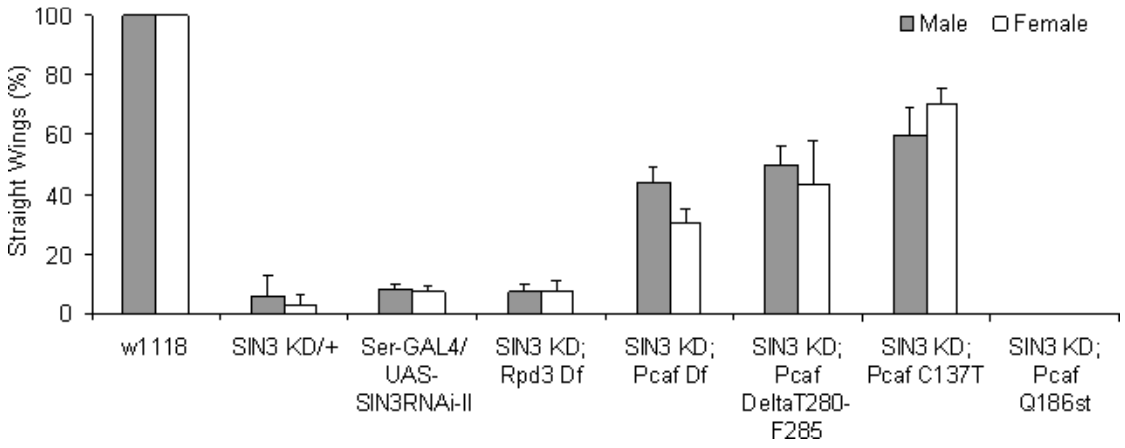

Figure 3.4: The SIN3 knockdown curly wing phenotype can be genetically modified. Adult flies of the indicated genotypes were scored for straight or curly wings. SIN3 KD represents flies having a recombined chromosome carrying both the Ser-GAL4 and UAS-SIN3^{RNAi-I} transgenes (refer to Experimental Procedures). The curly wing phenotype is suppressed by a reduction of *Pcaf*. The range of n values for each genotype is 87 to 883, average n = 254. Error bars represent standard deviation. $p \leq 0.01$ comparing SIN3 KD and Ser-GAL4/UAS-SIN3$^{RNAi-II}$ to control, and SIN3 KD; Pcaf Df to SIN3 KD.

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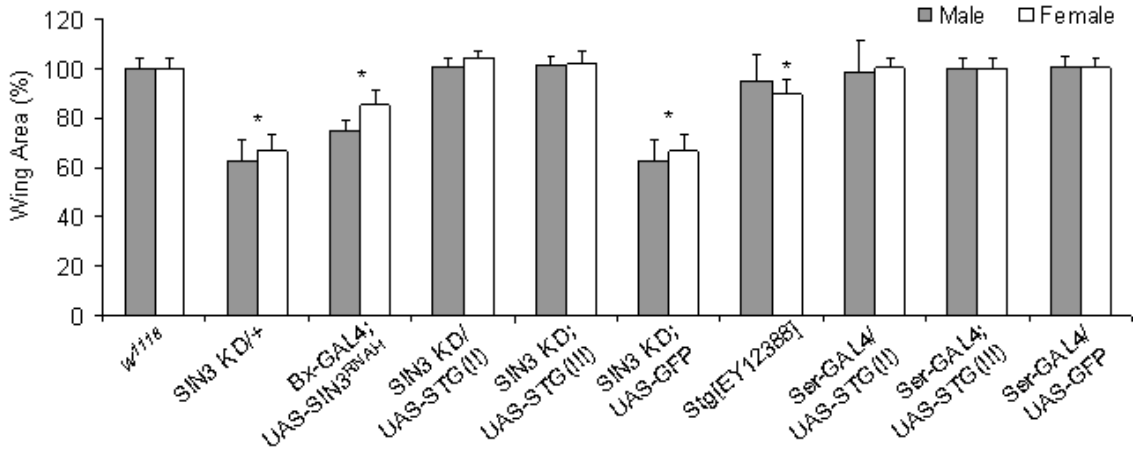
A



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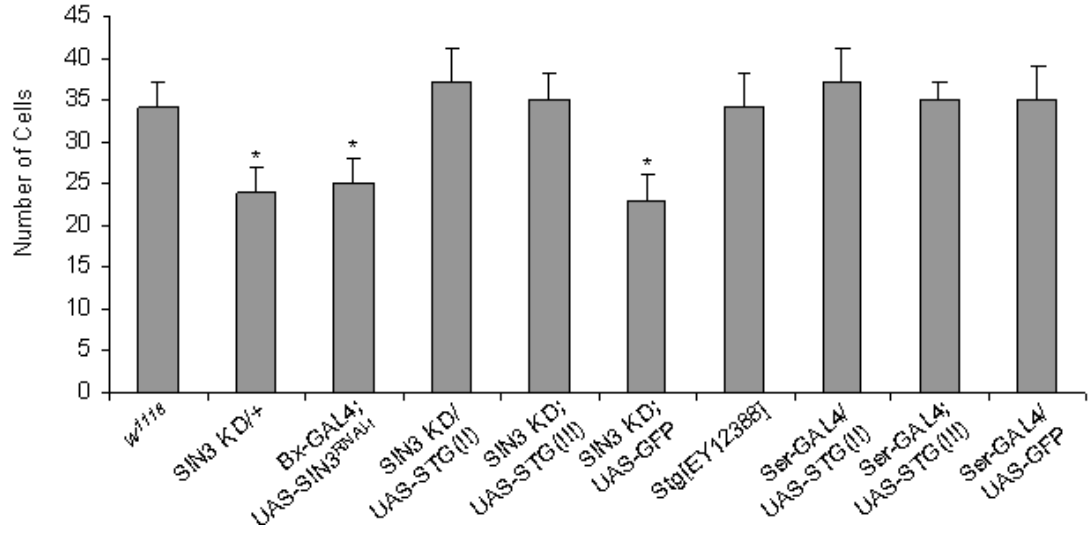

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Figure 3.5: Loss of SIN3 results in smaller adult wing size. SIN3 knock down resulted from induction of RNAi using two different GAL4 wing imaginal disc driver lines. (A) The wing area for each genotype was determined and compared to wild type wings using ImageJ software. The range of n values for each genotype is 140 to 200, average n = 170. (B) The number of bristles in a fixed area on the dorsal side of the wing was determined for all genotypes indicated. The range of n values for each genotype is 90 to 120, average n = 105. Error bars represent standard deviation. $p \leq 0.01$ (*) comparing indicated genotypes to w^{1118}. As in Fig. 3.4, SIN3 KD represents flies having a recombined chromosome carrying both the Ser-GAL4 and UAS-$SIN3^{RNAi\text{-}I}$ transgenes.

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relative to control wings (Fig. 3.5A). About 3% of the SIN3 knockdown wings also showed veination defects including thinner veins and an incomplete L5 vein (Fig. 3.1F).

To determine if the reduction in wing size was the result of fewer cells, we calculated the number of cells in a defined area of the wing. Each cell in the adult wing blade has a single bristle. We counted the number of bristles in a fixed area. The number of cells in the fixed area of the adult wing was reduced from an average of 34 in control to 24 in the SIN3 knock down wings (Fig. 3.5B). Loss of SIN3 thus results in fewer cells and overall smaller adult wings.

SIN3 knockdown wings show a cell cycle defect

Given our finding that the SIN3 knockdown larval wing discs and adult wings were smaller, we asked whether we could detect a cell cycle defect in the SIN3 knockdown wing discs. To assay cell proliferation, we first monitored whether EGFP-marked clones could be produced when the amount of SIN3 was reduced by RNAi. EGFP-marked clones were randomly generated using the heat shock Flip-out system (Struhl and Basler, 1993). The SIN3 knockdown clones were fewer in number and smaller in size than clones in control discs (Fig. 3.6). Furthermore, SIN3 knockdown clones were not found in discs isolated from larvae that were heat shocked early in development (data not shown). The lack of clones in the SIN3 knock down discs suggests that SIN3 is important for either cell proliferation or cell viability.

We next assayed the number of cells in mitosis by immunostaining wing

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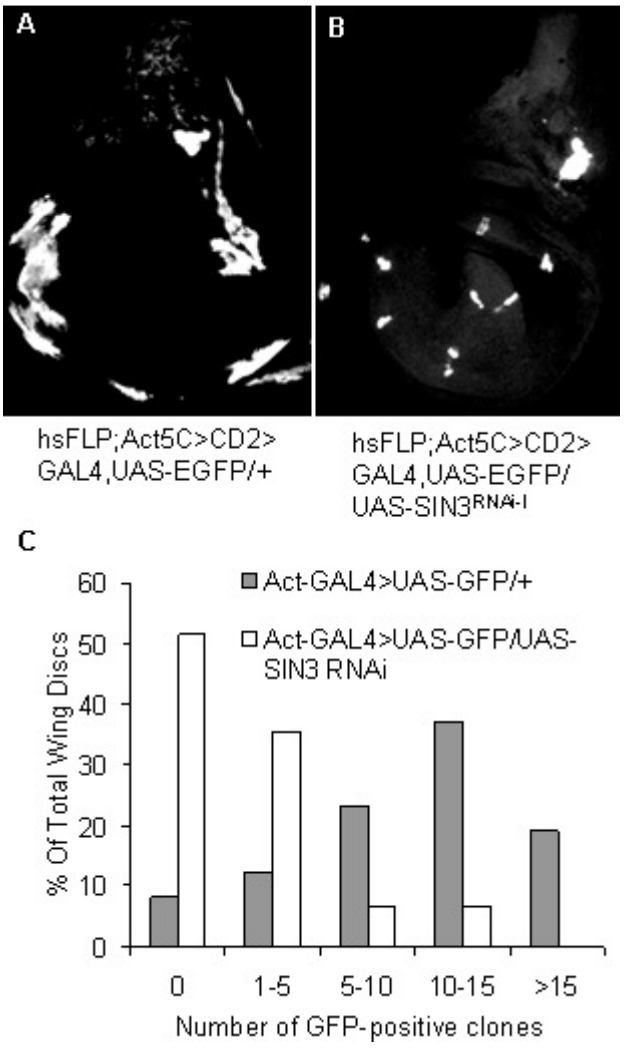

Figure 3.6: Loss of SIN3 results in reduced clonal growth in the wing imaginal discs. (A-B) Control and SIN3 knock down wing disc clones generated by the Flip-out GAL4 system were immunostained with antibody to GFP. (C) Graphical representation of the number of GFP positive clones in the control (n = 73) and SIN3 knock down (n = 62) wing discs.

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imaginal discs with antibody to phosphorylated serine 10 of histone H3 (H3PS10). Phosphorylation of serine 10 of histone H3 is a hallmark of initiation of mitosis (Hsu et al., 2000; Wei et al., 1999). Compared to control wing discs, SIN3 knockdown wing discs showed reduced staining with antibody to H3PS10 (Fig. 3.7A-D). We analyzed staining in discs using the Bx-GAL4 driver that leads to predominant SIN3 knockdown in the wing pouch area (Fig. 3.2F). We compared the staining intensity of the pouch and non pouch area the disc. Each area of an individual disc was assigned a score from 0 to 4 to indicate the overall level of H3PS10 staining (Fig. 3.7E). In the pouch, 24% of the SIN3 knockdown wing discs did not stain at all for this mark, whereas only 5% of control discs lacked pouch staining. 37% of control discs showed maximal staining compared to 0% of the SIN3 knock down discs. The staining in the rest of the disc was similar between control and SIN3 knockdown, although the staining in the SIN3 knockdown discs was somewhat reduced relative to controls. The difference in staining in the area outside of the pouch is likely due to the low level of GAL4 expression (and thus SIN3 RNAi induction) in areas outside of the pouch (Fig. 3.3H). These results indicate that loss of SIN3 leads to a reduction in the number of mitotic cells present in wing imaginal disc tissue. To determine if the number of cells in S phase was affected in the SIN3 knock down discs, we performed BrdU staining. We did not observe any difference in the BrdU staining pattern of control and SIN3 knock down discs (data not shown), indicating that the number of cells in S phase in not altered following loss of SIN3.

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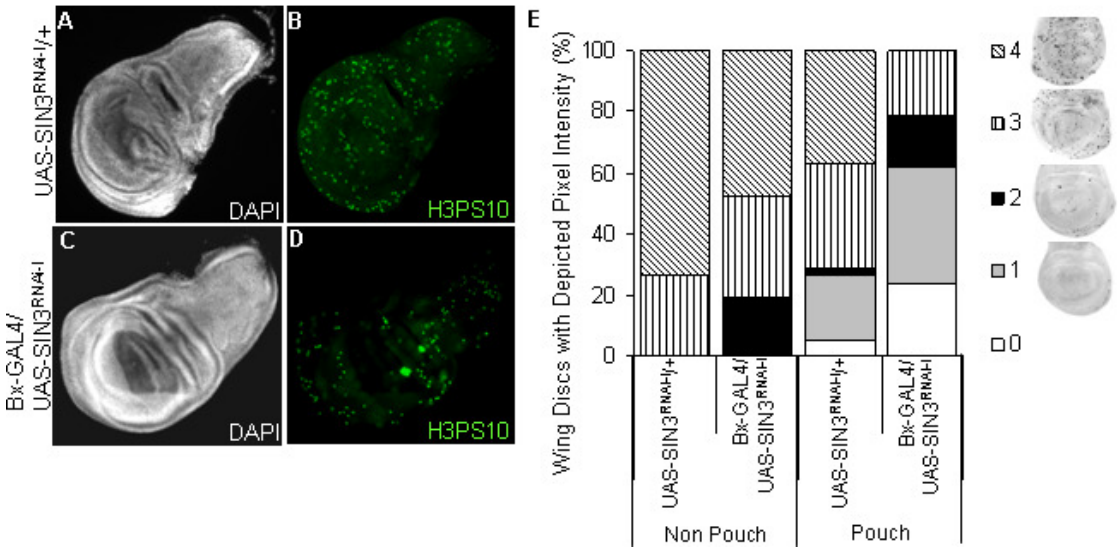

Figure 3.7: Loss of SIN3 results in fewer mitotic cells. (A-D) Control (UAS-SIN3^{RNAi-I}/+) or SIN3 knock down (Bx-GAL4 x UAS-SIN3^{RNAi-I}) wing imaginal discs were stained with DAPI to visualize the DNA and antibody to phosphorylated serine 10 of histone H3 (H3PS10). (E) A numeric value corresponding to the staining intensity was assigned to the pouch and to non pouch area of the disc for each wing disc (0-4). The graph represents the percentage of wing discs in each category. Control n = 38, Bx-GAL4; UAS-SIN3^{RNAi-I} n = 42.

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A key regulator of the cell cycle in wing imaginal disc cells is the protein STG (Neufeld et al., 1998a). STG is a protein phosphatase critical for the G2/M transition in the mitotic cell cycle (O'Farrell et al., 1989). We previously determined that loss of SIN3 in tissue culture cells results in reduction of *stg* mRNA (Pile et al., 2003). To determine the relative difference in *stg* expression between control and SIN3 knock down tissue culture cells, we analyzed gene expression by real time quantitative reverse transcription PCR (RT-qPCR) analysis. We further sought to determine whether a similar reduction of *stg* expression occurred in the SIN3 knockdown wing imaginal disc cells. To carry out these analyses, we compared the relative amounts of *stg* mRNA isolated from control and SIN3 knock down wing imaginal discs by RT-qPCR. We found that, relative to wild type, *stg* mRNA levels were reduced in SIN3 knockdown tissue culture cells and discs (0.6 fold) (Fig. 3.8A). Loss of SIN3 thus results in reduced expression of the critical cell cycle regulator STG.

We hypothesized that the observed reduction of cell proliferation upon loss of SIN3 in both tissue culture and wing imaginal disc cells is the result of decreased STG expression. To test this hypothesis, we forced expression of STG in the wing imaginal disc using two different UAS-STG transgenes. We observed a suppression of the curly wing phenotype in SIN3 knockdown flies that carry either UAS-STG construct (Fig. 3.8B). The suppression was specific to the UAS-STG transgenes as a UAS-GFP transgene did not suppress the SIN3 knockdown curly wing phenotype. We hypothesized that the difference in the amount of suppression observed using the two different UAS-STG constructs

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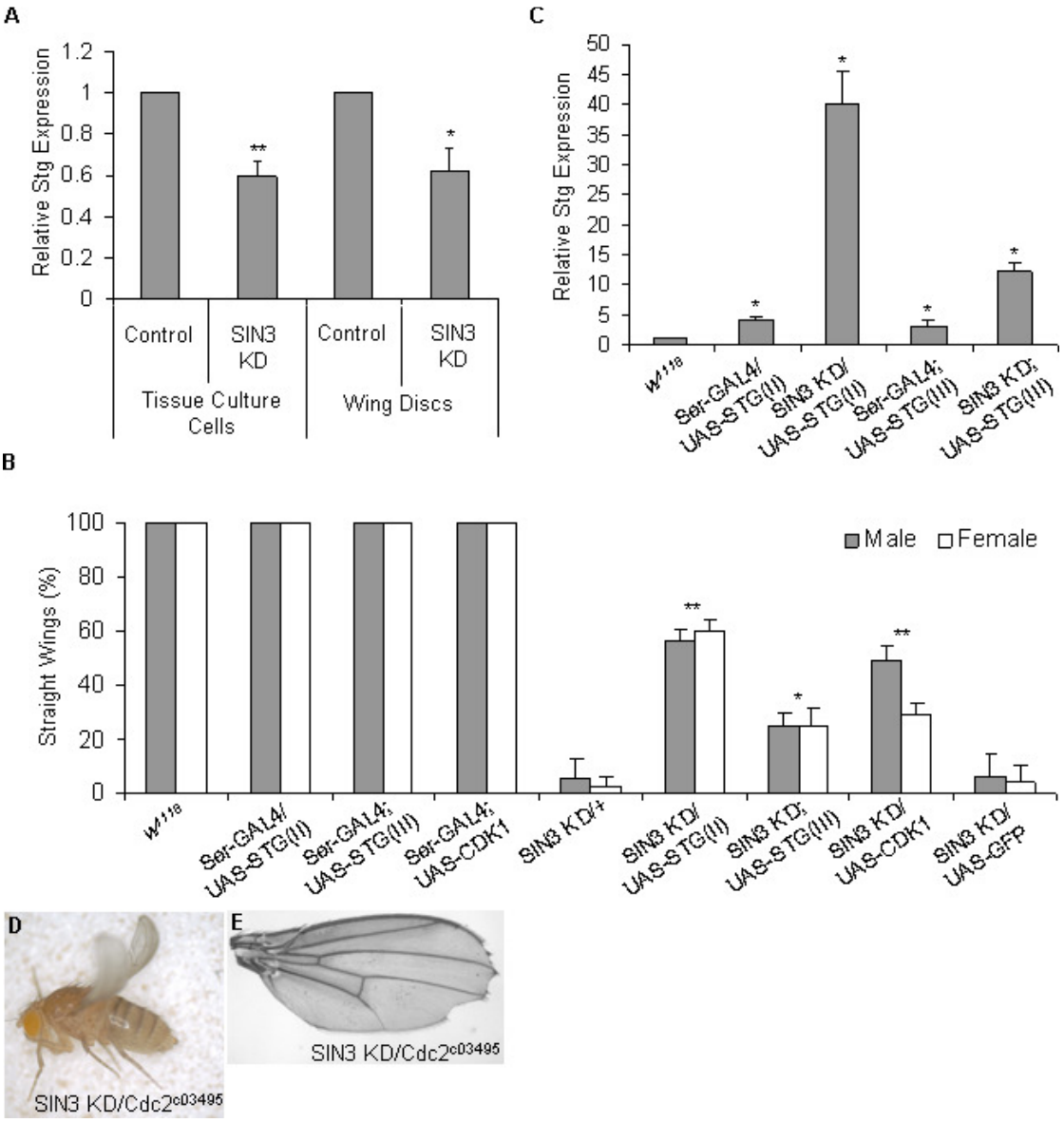

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Figure 3.8: STG expression affects the SIN3 knockdown phenotype. (A) Real time qPCR analysis of *stg* mRNA from control and SIN3 knock down tissue culture cells and wing discs. Total RNA was isolated from control and SIN3 knock down cells or wing discs and used to prepare cDNA template for qPCR. *stg* expression was normalized to TAF1. n = 4 for tissue culture and n = 6 for wing discs, p = 0.0016 (**) and 0.01 (*). (B) Recombinant SIN3 KD flies carrying both the Ser-GAL4 and UAS-SIN3$^{RNAi\text{-}I}$ transgenes were crossed to UAS-STG and UAS-Cdk1 flies. The progeny of the cross carrying the GAL4 and one of the two UAS constructs were scored for straight or curly wings. UAS-STG (II) and (III) represent flies carrying the construct on the second and third chromosomes respectively. The range of n values for each genotype is 145 to 193, average n = 176, p = 0.01 (*) and < 0.01 (**) comparing the indicated genotypes to SIN3 KD. (C) Similar analysis as in A with SIN3 knockdown wing discs overexpressing STG via two different UAS-STG constructs. n = 3 - 6, p < 0.001 (*). Error bars represent standard deviation. (D, E) Phenotypes observed upon introduction of a mutation in Cdk1 (Cdc2^{c03495}) into a SIN3 knock down background. (D) Micrograph of an adult fly with a strong curly wing phenotype (compare to Fig. 1C). (E) Micrograph of a wing that is straight but serrated.

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was due to variability in the amount of STG produced by each of the expression constructs. To test this idea, we analyzed the amount of *stg* mRNA isolated from wing imaginal discs of the various flies by RT-qPCR. In the flies carrying the UAS-STG transgenes we observed elevated *stg* mRNA levels (40 fold and 12 fold) (Fig. 3.8C). The amount of suppression correlated with the amount of STG overexpression from the two UAS-STG constructs. *Stg* expression in the SIN3 KD, UAS-STG flies was elevated relative to the Ser-GAL4, UAS-STG flies, indicating that SIN3 likely regulates transcription from either the Ser-GAL4 or UAS-STG transgene. The finding that elevation of STG levels did not fully suppress the curly wing phenotype is possibly due to the fact that the expression of STG from the transgenes is not coupled to the normal cell cycle control of STG expression. Alternatively, SIN3 may also regulate the cell cycle by STG-independent pathways. These data strongly suggest that the curly wing phenotype is, in part, the result of down regulation of STG upon loss of SIN3. This down regulation of STG may lead to cell cycle arrest of some of the wing imaginal disc cells.

We also tested the effect of STG overexpression on overall adult wing size. SIN3 knockdown wings are smaller than those of wild type flies (Fig. 3.5A). Wings of flies heterozygous for STG ($stg^{EY12388}$) were also found to be slightly smaller than wild type flies. Overexpression of STG in the context of the SIN3 knockdown wing imaginal disc cells resulted in restoration of the size of the adult wing blade to that of control wings as well as restoration of cell number (Fig. 3.5).

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Taken together, the data suggest that SIN3 works in concert with STG to regulate cell proliferation.

To further investigate the link between SIN3 and G2/M cell cycle progression, we overexpressed Cdk1 (Dmcdc2) in the background of the SIN3 knock down wing imaginal disc cells. Cdk1 is an important regulator of the G2 to M transition and is the target of the STG phosphatase (O'Farrell et al., 1989). Similar to overexpression of STG, overexpression of Cdk1 suppressed the curly wing phenotype in the SIN3 knock down flies (Fig. 3.8B). In addition, induction of SIN3 RNAi in the background of a fly that is heterozygous for Cdk1 (*cdc2*^{c03495}) resulted in a more aberrant wing phenotype than that of the SIN3 knock down flies alone (Fig. 3.8D,E). The SIN3 KD/*cdc2*^{c03495} flies had wings that can be categorized into three major groups. One group had wings that were comparable to the SIN3 KD flies (25%), another group had much more curly wings (30%, Fig. 2.8D), while the rest had straight wings with serrated edges (Fig. 3.8E). The observed genetic interactions between SIN3 and Cdk1 further support our hypothesis that SIN3 works with G2 to M cell cycle regulatory factors to promote cell proliferation.

DISCUSSION

Sin3A is an essential gene required for both embryonic and larval development (Neufeld et al., 1998b; Pennetta and Pauli, 1998; Sharma et al., 2008). To investigate the role of SIN3 in the context of proliferating tissue, we designed an RNAi-induced conditional knock down allele to reduce expression of

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SIN3 in a non-essential organ. Loss of SIN3 from wing imaginal disc cells resulted in a number of observable phenotypes, including smaller imaginal discs and smaller, curly adult wings. The SIN3 knock down curly wing phenotype could be modified by reduction in the level of PCAF, a KAT enzyme that carries out the opposing reaction to histone deacetylation. The curly wing phenotype was also partially suppressed by overexpression of the cell cycle regulatory factors STG and Cdk1.

SIN3 and proteins associated with the SIN3 complex have been linked to cell cycle regulation in multiple model systems. Loss of *Drosophila* SIN3 or RPD3 in tissue culture cells resulted in loss of cell proliferation (Pile et al., 2003). SIN3 has also been implicated in cell survival or proliferation during eye development, as generation of homozygous null SIN3 clones resulted in scars across the eye (Neufeld et al., 1998b). In mouse model systems, genetic knock out of *mSin3a* from embryonic fibroblasts resulted in loss of cell proliferation (Cowley et al., 2005; Dannenberg et al., 2005). Knock out of *mSin3b* from mouse embryonic fibroblasts resulted in loss of ability of the cells to exit the cell cycle at the start of differentiation (David et al., 2008). Recent work has indicated that *mSin3* is recruited to cell cycle regulated E2F4 target genes in terminally differentiated myoblasts to keep these genes in a repressed state (van Oevelen et al., 2008). In this study we observed that reduction of SIN3 in wing imaginal disc cells resulted in fewer mitotic cells in the wing disc and fewer cells in the adult wing (Figs. 3.7 and 3.5B). In addition, consistent with the previously performed genetic null clonal analysis, we found that conditional knock down of

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SIN3 by RNAi results in either no clones or fewer and smaller clones, depending on the time of induction of clone formation (Fig. 3.6). These results suggest that SIN3 is required for cell proliferation and/or cell survival in the context of a developing organism, as well as in tissue culture cells.

Loss of SIN3 in both tissue culture cells and wing imaginal disc tissue results in a decrease of *stg* mRNA expression (Fig. 3.8) (Pile et al., 2003). Overexpression of STG in the background of SIN3 knock down is able to partially suppress the small wing and curly wing phenotypes (Figs. 3.5 and 3.8). STG is a key regulator of the cell cycle, specifically of the G2 to M transition (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). Loss of STG in clones in wing imaginal discs resulted in loss of cell proliferation while overproduction of dE2F resulted in increased STG expression and accelerated cell proliferation, thus implicating dE2F as a transcriptional activator of *stg* (Neufeld et al., 1998a). *stg* has also been shown to be regulated at the level of transcription by the action of the activator Pointed and the repressor Tramtrack 69 (ttk69) (Baonza et al., 2002). Additional activators including eyes absent and Sine oculis were found to bind to the *stg* regulatory region in eye imaginal disc cells (Jemc and Rebay, 2007). Taken together, these results suggest that *stg* expression is likely regulated by the combinatorial action of multiple activators and repressors, the binding of which may vary with cell cycle stage and tissue (Lehman et al., 1999).

Because SIN3 is a transcriptional corepressor and loss of SIN3 leads to reduced *stg* expression rather than activation of *stg*, we hypothesize that the effect of SIN3 on *stg* gene expression is indirect. One possible model to explain

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this effect is that loss of SIN3 leads to an increase in expression of a repressor of STG. If this model is accurate, then loss of this repressor may be able to suppress the SIN3 knock down curly wing phenotype. A second possible model is that loss of SIN3 leads to increased acetylation of a transcription factor necessary for appropriate *stg* expression. Numerous transcription factors, including p53, have been found to be acetylated (Spange et al., 2009). Acetylation of these factors can affect protein stability, localization, interactions with other proteins and DNA binding activity (Spange et al., 2009). Experiments to test the possible models linking SIN3 and STG are currently underway.

We also observed genetic interactions between SIN3 and Cdk1, the substrate of STG and another important G2/M regulatory factor. Overexpression of Cdk1 suppressed the SIN3 knock down curly wing phenotype (Fig. 3.8B). A reduction of Cdk1 levels using the *cdc2*^{c03495} allele resulted in enhanced abnormal adult wing morphology as compared to the SIN3 mutants alone (Fig. 2.8D,E). Cdk1 must be dephosphorylated by STG in order for cells to pass from the G2 to M phase of the cell cycle (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990; Moreno and Nurse, 1991). Increasing the amount of the substrate for STG may permit formation of enough active cycB-Cdk1 complexes to drive cell proliferation in the SIN3 knock down cells. A similar suppression of a cell proliferation defect has been previously reported. In *Aspergillus nidulans*, introduction of an extra copy of cyclin B into a *cdc25* (STG homolog) mutant partially rescued the cell cycle defect of the *cdc25* mutant cells (O'Connell et al., 1992).

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Overexpression of STG does not fully suppress the SIN3 knock down phenotype, possibly because not all cells in larval wing imaginal discs are sensitive to ectopic STG expression (Haberland et al., 2009; Milan et al., 1996b). Consistent with a cell type specific response to STG, we found that STG overexpression in tissue culture cells is unable to suppress the strong RNAi-induced SIN3-deficient cell proliferation defect (data not shown). It is also possible that other factors interact with SIN3 to affect wing morphology. We are conducting experiments to identify other novel factors in the SIN3 regulatory network that may contribute to the role of SIN3 in development.

The SIN3 complex is one of the two major class I HDAC complexes conserved from *Drosophila* to human (Ayer, 1999). Our results have uncovered a genetic link between transcription repression by SIN3 and G2/M cell cycle progression by STG and Cdk1. Further investigation of this interaction is expected to shed light on the role that histone acetylation plays in the regulation of cell proliferation and differentiation.

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CHAPTER 4

IDENTIFICATION OF GENETIC SUPPRESSORS OF THE SIN3 KNOCKDOWN PHENOTYPE

ABSTRACT

The role of SIN3 in regulating different aspects of the cell cycle is established in various metazoans. Little is known, however, about the signaling pathways that trigger or are triggered by SIN3 function. To discover genes that work in similar or opposing pathways to SIN3 during development, we have performed an unbiased deficiency screen of the *Drosophila* third chromosome to identify genes that genetically interact with SIN3. Additionally we have performed a targeted loss of function screen to identify additional cell cycle genes that interact with SIN3. We have identified genes involved in regulation of gene expression, cell cycle and signaling pathways that can suppress the curly wing phenotype caused by the loss of SIN3. These data suggest that SIN3 plays a wide variety of roles in the cell.

INTRODUCTION

Histone acetylation levels are maintained by the opposing activities of KATs and HDACs. Modulation of acetylation levels that affect regulation of gene expression has been shown to be an important event during *Drosophila* development. Mutations in the KAT Pcaf result in defective oogenesis and morphogenesis (Carre et al., 2005). Growing larvae on low concentrations of the HDAC inhibitor Trichostatin A (TSA) results in delayed development and a notched wing phenotype in adults, suggesting that the deacetylase activity of

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HDAC complexes is important for regulating developmental events (Pile et al., 2001). The HDAC RPD3 has been shown to be important for thorax metamorphosis (Miotto et al., 2006). During thoraxic closure, inactivation of the JNK pathway results in the recruitment to and subsequent deacetylation of target genes by RPD3. RPD3 also represses genes involved in the immune response via the JNK pathway through its interaction with AP1 (Kim et al., 2005). SIN3, a corepressor component of HDAC complexes, is an essential gene in mouse and *Drosophila* (Cowley et al., 2005; Dannenberg et al., 2005; Neufeld et al., 1998b; Nicolas et al., 2007; Pennetta and Pauli, 1998). The SIN3 complex is hypothesized to regulate developmental processes via its association with RPD3.

SIN3 plays an important role in regulation of signaling pathways. In mouse, HERP, a notch effector, regulates gene expression by recruiting SIN3 to the target genes (Iso et al., 2001). SIN3 has also been implicated in the regulation of development via steroid hormone signaling. SMRTER, a corepressor that represses genes induced by the hormone ecdysone, brings about transcription repression by recruiting the SIN3 complex to target genes (Tsai et al., 1999). SIN3 colocalizes with SMRTER on polyene chromosomes (Pile and Wassarman, 2000). The recruitment of SIN3 to ecdysone responsive genes is reduced upon activation by the steroid hormone. SIN3 levels are restored at these genes when these are repressed. In addition SIN3 plays a role in *Drosophila* eye development through the MAP-kinase pathway (Neufeld et al., 1998b). Loss of SIN3 enhances the rough eye phenotype caused by a mutation in *sina*, a gene required for photoreceptor specification.

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SIN3 also plays an important role in regulating cell cycle. In *Drosophila*, SIN3 knockdown by RNA interference (RNAi) results in a G2 arrest in S2 cells (Pile et al., 2002). Ubiquitous knockdown of SIN3 results in embryonic lethality presumably due to defects in cell proliferation (Sharma et al., 2008). In wing discs loss of SIN3 results in a decrease in the number of mitotic cells leading to fewer cells in the adult wing (Cowley et al., 2005; Pile et al., 2002; Swaminathan and Pile, 2010). This wing phenotype is only partially suppressed by the overexpression of G2 phase regulators including String (STG) and CDK1. These results reinforce the role of SIN3 in the regulating the G2 phase, but also suggest that SIN3 may regulate other phases of the cell cycle. The mechanism by which this potential regulation is brought about is unknown.

Although SIN3 and histone acetylation have been implicated in various developmental processes, the direct gene targets of the SIN3 HDAC complex during development are unknown. To identify novel pathways in which SIN3 may function and genes that it regulates, we performed an unbiased screen of the third chromosome to find genes that interact with SIN3. We identified several genes that have been shown to play a role in various processes including cell division, regulation of transcription, negative regulation of Wnt signaling and imaginal disc growth. We have also performed a targeted screen to further understand the role of SIN3 in regulating the cell cycle in the developing wing disc. Results of the targeted screen indicate that SIN3 plays a role in regulating multiple phases of the cell cycle in the wing disc. These data provide us with a

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better insight into the role of SIN3 during development and potentially aid in identifying direct gene targets of the SIN3 complex.

MATERIALS AND METHODS

Drosophila stocks

Drosophila melanogaster stocks were maintained and crosses were performed according to standard laboratory procedures. The following stocks were used: UAS-SIN3^{RNAi-I} (Sharma et al., 2008), UAS-SIN3$^{RNAi-II}$, SIN3 KD I (Swaminathan and Pile, 2010) and SIN3 KD II (construction described below), CycJ allele and UAS-CYCJ (gift from Dr. Russell Finley).

Bloomington stock center: w^{1118}, Ser-GAL4 (#6791), , Isogenic/DrosoDel deficiency kit (Golic and Golic, 1996), Df(3R)Exel6146 (#7625), Df(3R)Exel6154 (#7633), Df(3R)Exel6155 (#7634), Df(3R)Exel6200 (#7679), Df(3R)Exel6201(#7680), Df(3R)Exel6205 (#7684), Df(3R)Exel6206 (#7685), Df(3R)Exel6208 (#7686), Df(3R)Exel6212 (#7690), Df(3R)Exel6263 (#7730), Df(3R)Exel9029 (#7981), Df(3L)BSC130 (#9295), Df(3R)BSC196 (#9622), Df(3R)BSC177 (#9692), Df(3R)BSC221 (#9698), Df(3R)BSC222 (#9699), Df(3R)BSC179 (#23146), Df(3R)BSC176 (#24334), Df(3R)BSC318 (#24344), Df(3R)BSC397 (#24421), Df(3R)BSC465 (#24969), Df(3R)BSC466 (#24970), Df(3R)BSC493 (#24997), Df(3R)BSC513 (#25017), Df(3R)BSC548 (#25076), Df(3L)BSC612 (#25687), Df(3R)BSC633 (#25724), Df(3R)BSC650 (#25740), Df(3R)BSC686 (#26538), Df(3R)BSC729 (#26581), elm^{EY07304} (#19816), sec23^{EY06757} (#19921), Nmdmc^{EY08061} (#20061), Fer2LCH^{DG24702} (#21752), Gld^{n2}

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(#2439), Gld^{n1} (#2440), pll^{2} (#3111), pll^{7} (#3112), spz^{2} (#3115), ash^{2} (#4584), tok^{1} (#4586), slo^{1} (#4587), $CycB3^{2}$ (#6635), $Snm1^{ZIII-2589}$ (#8461), $Rheb^{AV4}$ (#9690), $Cdc27^{L7123}$ (#10168), $Sas\text{-}4^{s2214}$ (#12119), $mia^{EY07883}$ (#16865), $CG1234^{e01488}$ (#17958), $CG11951^{f00339}$ (#18316), $CG12746^{EY10535}$ (#20182), $Bili^{MB01370}$ (#23079), $Bili^{MB07242}$ (#25639), $CRMP^{supl2}$ (#24173), $CG7910^{MB06548}$ (#25514), $neur^{11}$ (#2747), kkv^{1} (#3090), kto^{1} (#3618), skd^{2} (#5047), UAS-CDK2 (#6634), Rbf^{14} (#7435), UAS-CYCA (#6633), $CycA^{C8LR1}$(#6627), UAS-CYCB (#6626), $CycB^{2}$ (#6630), $Cdc16^{EY12544}$ (#20753), $Cdc23^{c06630}$ (#17775), $Cdc27^{L7123}$ (#10168), $CycB3^{2}$ (#6635), UAS-CYCB3 (#6628), $CycEk^{05007}$ (#10384), UAS-CYCE (#4781), $E2f^{07172}$ (#11717), $CycH^{KG02273}$ (#13200).

Vienna *Drosophila* Research Center: UAS-$CG5804^{RNAi}$ (#32587GD), UAS-$CG32023^{RNAi}$ (#108338KK), UAS-$CG32024^{RNAi}$ (#102205KK), UAS-hd^{RNAi} (#47309GD), UAS-kkv^{RNAi} (#100327KK), UAS-$RpII18^{RNAi}$ (#105937KK), UAS-$Mms19^{RNAi}$ (#11205GD), UAS-$retinophilin^{RNAi}$ (#28702GD), UAS-Hph^{RNAi} (#103382KK), UAS-$Snm1^{RNAi}$ (#37591GD), UAS-Mia^{RNAi} (#100313KK), UAS-$Ash2^{RNAi}$ (#7141GD), UAS-$Rpb10^{RNAi}$ (#102010KK), UAS-$Ets96B^{RNAi}$ (#30552GD), UAS-$polybromo^{RNAi}$ (#108618KK), UAS-$CG5804^{RNAi}$ (#32587GD), UAS-$HdacX^{RNAi}$ (#108098KK), UAS-$Bili^{RNAi}$ (#101424KK), UAS-$CG13651^{RNAi}$ (#11515GD), UAS-Ser^{RNAi} (#108348), UAS-DNApol-$\alpha 73^{RNAi}$ (#108579KK), UAS-$TfIIA\text{-}L^{RNAi}$ (#108355KK), UAS-woc^{RNAi} (#20995GD), UAS-$Art4^{RNAi}$ (#107009KK), UAS-$CG3909^{RNAi}$ (#104387KK), UAS-$CG9461^{RNAi}$ (#24039GD), UAS-$CG9467^{RNAi}$ (#45807GD), UAS-$CG34360^{RNAi}$ (#105014KK), UAS-$Mical^{RNAi}$ (#105837KK), UAS-$CG3309^{RNAi}$ (#15415GD), UAS-$mtTFB2^{RNAi}$ (#107086KK),

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UAS-nefrin-2^{RNAi} (#101434KK), UAS-Pbp95^{RNAi} (#33558GD), UAS-ImpE3^{RNAi} (#16403GD), UAS-Fer1^{RNAi} (#102406), UAS-Sas-4^{RNAi} (#106051KK), UAS-CG18012^{RNAi} (#20580GD), UAS-CG33547^{RNAi} (#39385GD), UAS-CG17801^{RNAi} (#29501GD), UAS-CG31246^{RNAi} (#10089KK), UAS-CG12347^{RNAi} (#106206KK), UAS-CG7357^{RNAi} (#10097), UAS-hdc^{RNAi} (#104322KK), UAS-Fer1HCH^{RNAi} (#102406KK), UAS-Mst85C^{RNAi} (#6493GD), UAS-neur^{RNAi} (#108239KK), UAS-CG11033^{RNAi} (#109295KK), UAS-Brd8^{RNAi} (#104879KK), UAS-Apc^{RNAi} (#151469GD), UAS-Apc2^{RNAi} (#100104KK), UAS-Axn^{RNAi} (#7748GD), UAS-CG10225^{RNAi} (#104432), UAS-crol^{RNAi} (#104313KK), UAS- Cyo310a1^{RNAi} (#100318KK), UAS-ft^{RNAi} (#108863KK), UAS-gro^{NAi} (#6316GD), UAS-pan^{RNAi} (#108679KK), UAS-sgg^{RNAi} (#101538KK), UAS-stan^{RNAi} (#107993KK), UAS-tum^{RNAi} (#106850KK).

Generation of SIN3 KD I and SIN3 KD II flies

Generation of constitutive wing imaginal disc SIN3 knockdown (SIN3 KD) recombinant flies is described in (Swaminathan and Pile, 2010). These flies are referred to as SIN3 KD I in this current study. SIN3 KD II recombinants were generated in a similar fashion by crossing Ser-GAL4/UAS-SIN3$^{RNAi\text{-}II}$ females to CyO-Ras/Sco males. Recombinant progeny were scored on the basis of eye color. Potential recombinants were verified by crossing to *w*^{1118} and monitoring the penetrance of the curly wing phenotype in the progeny. These stocks are maintained as heterozygotes, balanced on CyO-Ras, because they are healthier than the homozygous flies.

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Generation of flies for reverse transcription PCR assay

SIN3 KD I flies and flies carrying a loss of function (lof) allele or an RNAi construct for genes that genetically interact with SIN3 were generated in a yw background. SIN3 KD I flies were balanced on a CyO-y^+ balancer, the lof flies were balanced on TM6-Tb and the RNAi lines were maintained as homozygotes or balanced on CyO-y^+ or Tb as appropriate. SIN3 KD I females were then crossed to the lof or RNAi males. Larvae that were genotypically y and non-Tb were identified on the basis of yellow colored mouth hooks and wild type body length. These larvae are knocked down for SIN3 and have less than wild type dose of the suppressor. Wing discs from these larvae were used in the reverse transcription PCR assay.

Western blot analysis

Western blot analysis was performed in accordance with standard protocols (Russell, 2001). To prepare whole cell extracts, wing discs isolated from wandering third instar larvae were homogenized in Laemmli sample buffer (Bio-Rad). Protein concentration was determined using the DC protein assay reagent (Bio-Rad) according to the manufacturer's protocol. Protein extract (15 to 20 μg) was fractionated by sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membrane, PVDF (Pall), and probed with immunoglobulin G (IgG) purified polyclonal rabbit antibodies against SIN3 (1:2000) (Pile and Wassarman, 2000), followed by

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donkey anti-rabbit horseradish peroxidase-conjugated IgG (1:3000) (GE Healthcare) and detected with enhanced chemiluminescence reagents (GE Healthcare). The blots were subsequently probed with monoclonal mouse antibody against α-tubulin (1:1000) (Sigma), followed by sheep anti-mouse horseradish peroxidase-conjugated IgG (1:3000) (GE Healthcare) as a loading control.

Determination of wing area

The area of wings was determined using ImageJ software.

Determination of cell number in the wing

Bristles were counted in a 2 x 2 cm^2 area of the dorsal side of the wings, captured at 80X magnification, between veins L4 and L5.

Reverse transcription PCR assay

Total RNA was extracted from wing discs isolated from wandering third instar larvae using the RNeasy mini kit (Qiagen). cDNA was generated from total RNA using the ImProm-II 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 Table 4.1.

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Gene	Type	Primer sequence
Stg	Forward	AACACCAGCAGTTCGAG
	Reverse	CCATAGCTGGCAGAATCTTC
Taf1	Forward	GTGGAGGAGCCAAGGGAGCC
	Reverse	TCCCGCTCCTTGTGCGAATG
Cdk2	Forward	AGCGCGTAGCAACTCCAC
	Reverse	CGTCGAAGGAACACCCTC
Cdk8	Forward	GTGGGACGCGGAACATAC
	Reverse	ACATGGACAATCCGGTGC
Cdc16	Forward	CAATGAACACATCGACCTGG
	Reverse	AAAAAGCGCTGTGGAGTAGC
Arm	Forward	AGTTCACACGGAGGTCGC
	Reverse	CCACTGGGCTGCTGATCT
Ndk	Forward	CAAGATGTTGCGAAGGGC
	Reverse	CGAGGCAGTGGTCCTGGT
TCF	Forward	CCGCAAATGGGTATAGCG
	Reverse	TGTCACAATGCTGATCCGTT
Dm	Forward	GCGCCCTACAGTTCCAGA
	Reverse	TTGGCCACCGATTTCACT
Ubx	Forward	CATTCTACCCCTGGATGG
	Reverse	ATGCCGCCGTATTGTGTT
En	Forward	ATCCACCACCACAGAGGG
	Reverse	GTGGACGCTTCTCGTCGT
Ovo	Forward	AGCAAAGTCTTGCAGCGG
	Reverse	GGCCAGCGGGTTCTTAAT
Nemo	Forward	CTGACATCCGTGCAGCAG
	Reverse	GTGGATTGATGCACAGCG
Stripe	Forward	GAGCCACCGCCCATTACT
	Reverse	CCTGGGGTTCCAAAGACA
Wg	Forward	GTCAGGGACGCAAGCATAAT
	Reverse	GCGAAGGCTCCAGATAGACA
Bili	Forward	GGGAACACTGCAGTATAATCG
	Reverse	GCGACACTTCACATCCGT
Pgk	Forward	CCCCCCGGTGTCTTTGAG
	Reverse	GCCGTCCATGATGGACTTG
Act	Forward	CTGGGACGATATGGAGAAGA
	Reverse	CGCAGCTCATTGTAGAAGGT

Table 4.1: List of primers used in gene expression analysis. Forward and reverse primers (listed 5' to 3') for each of the genes indicated were designed using the Primer3 software (http://workbench.sdsc.edu/).

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RESULTS AND DISCUSSION

Knock down of SIN3 by RNAi in the wing discs results in a curly wing phenotype (Fig 3.1). This phenotype is a mild but completely penetrant phenotype that can be modified by mutations in or overexpression of genes that interact with SIN3 (Swaminathan and Pile, 2010). As the first step to identify genes that genetically interact with SIN3, we performed an unbiased screen of the third chromosome using the isogenic deficiency kit (Golic and Golic, 1996) to determine regions which when deleted can modify the curly wing phenotype (Fig 4.1). SIN3 KD I females balanced on CyO-Ras were crossed to males carrying a deletion on the third chromosome balanced on TM2-Ubx, TM3-Sb, TM3-Ser or TM6-Tb. The cross yields progeny with four different genotypes. One of these genotypes yields flies that are SIN3 knockdown only and therefore will have curly wings. These flies will also carry a third chromosome balancer and therefore will display the phenotype associated with the balancer. Another genotype yields flies that are SIN3 knockdown and heterozygous for a deletion on the third chromosome. If one or more of the genes within the deletion interact with SIN3 then this mutation could lead to modification the SIN3 knockdown curly wing phenotype. In the other two genotypes resulting from the parental cross, the presence of the dominant Ras mutation leads to a rough eye phenotype in the flies carrying the CyO-Ras balancer, allowing us to differentiate between flies that are curly due to loss of SIN3 as opposed to flies that are curly due to the presence of the balancer.

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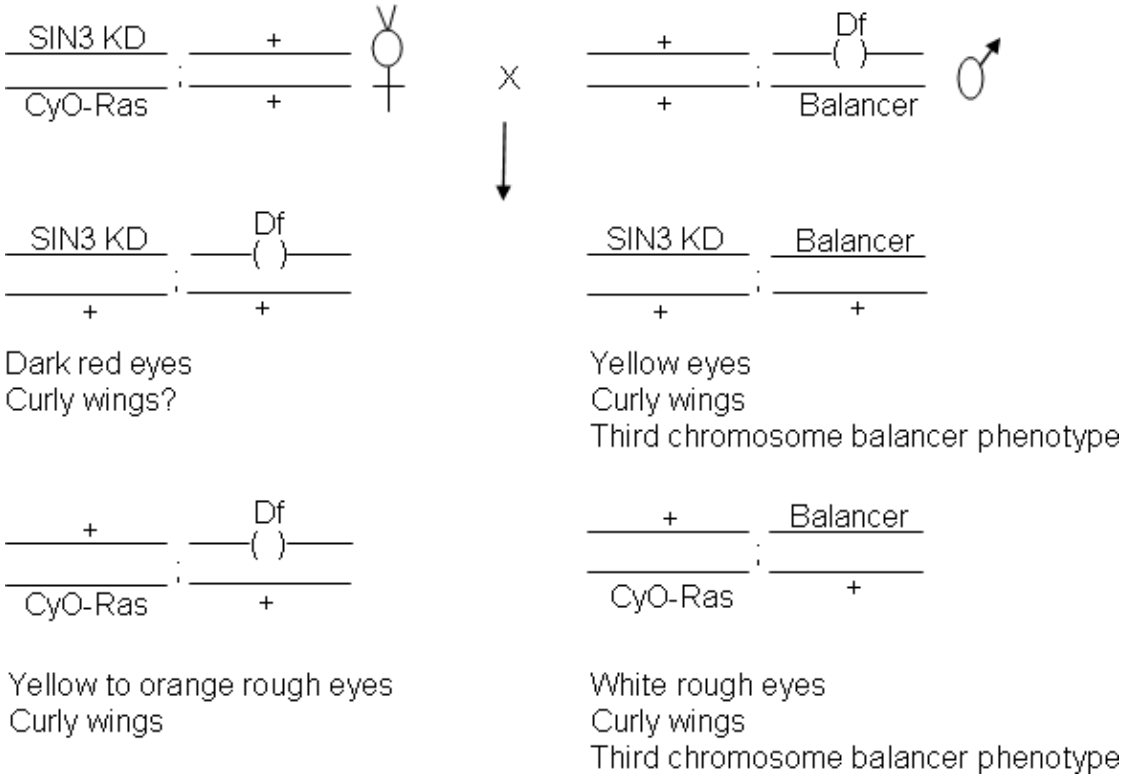

Figure 4.1: General scheme of crosses for the screen. SIN3 KD/CyO-Ras females were crossed to males heterozygous for either a deletion that removed multiple genes (phase I and II) or a lof allele of a single gene (phase III) balanced over a third chromosome balancer that was TM3-Sb, TM3-Ser, TM2-Ubx or TM6-Tb. The resulting progeny that are SIN3 knockdown and carry a third chromosome deletion were scored for the curly wing phenotype. In phase I of the screen, each deletion on the third chromosome was associated with a gene resulting in yellow to orange eye color. In the case of phase II, all the deletions tested were in a *w* background.

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The unbiased screen identified regions on the third chromosome that genetically interact with SIN3

The genetic interactions with SIN3 can be broadly categorized into two groups: (1) partial suppression of the penetrance but not the expressivity (i.e. a milder or stronger curl in the wing) of the curly wing phenotype. In this category any fly that showed suppression has completely straight wings, and (2) partial suppression of only the expressivity of the curly wing phenotype but not the penetrance. Scoring suppression of the expressivity was difficult and we were unable to assign a scale for the extent of suppression. Thus to accurately identify suppressors of the curly wing phenotype, we have taken into consideration only those regions that affect the penetrance of the curly wing phenotype and not those that modified its expressivity. In this first phase of the screen we found 21 out of a total of 148 deletions tested that suppressed the penetrance of the curly wing phenotype to varying degrees (Table 4.2). ED4415 and ED4413 represent identical deletions, while some of the remaining deletions are partially overlapping and generate a continuous region on the third chromosome that genetically interacts with SIN3. This narrows the number down to 14 unique regions or cytogenetic intervals on the third chromosome that genetically interact with SIN3 (Fig. 4.2). A control cross was also set up in which SIN3 KD/CyO-Ras females were crossed to w^{1118} males, and the progeny carrying the SIN3 KD chromosome were scored for curly wings. All the progeny of this cross carrying the SIN3 KD chromosome had curly wings. To ensure the genetic interaction observed is between SIN3 and a gene removed by the deletion, we performed a

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Isogenic deletion stock ID	% Straight wings in combination with SIN3 KD I
ED4191	33 ± 11
ED4415	17 ± 5
ED4413	21 ± 7
ED215	51 ± 2
ED4483	65 ± 8
ED4486	56 ± 1
ED5147	21 ± 4
ED5156	17 ± 8
ED10257	27 ± 10
ED5187	35 ± 2
ED7665	16 ± 3
ED5301	24 ± 6
ED5454	29 ± 8
ED5438	23 ± 7
ED5785	28 ± 2
ED6220	22 ± 6
ED6255	24 ± 7
ED6265	17 ± 8
ED6310	33 ± 1
ED6316	38 ± 6
ED6332	21 ± 5

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Table 4.2: The curly wing phenotype is suppressed by multiple deletions on the third chromosome. SIN3 KD I/CyO-Ras flies were crossed to flies from the isogenic kit in which every deletion was balanced over TM2-Ubx, TM3-Sb, TM3-Ser or TM6-Tb. The curly wing phenotype was scored in progeny of the cross that carried the SIN3 KD chromosome and the third chromosome balancer and compared to siblings carrying the SIN3 KD and deletion chromosomes. Refer to Figure 4.1 for details. The results are the average of three independent experiments. Standard deviation is indicated. n = 153 – 421.

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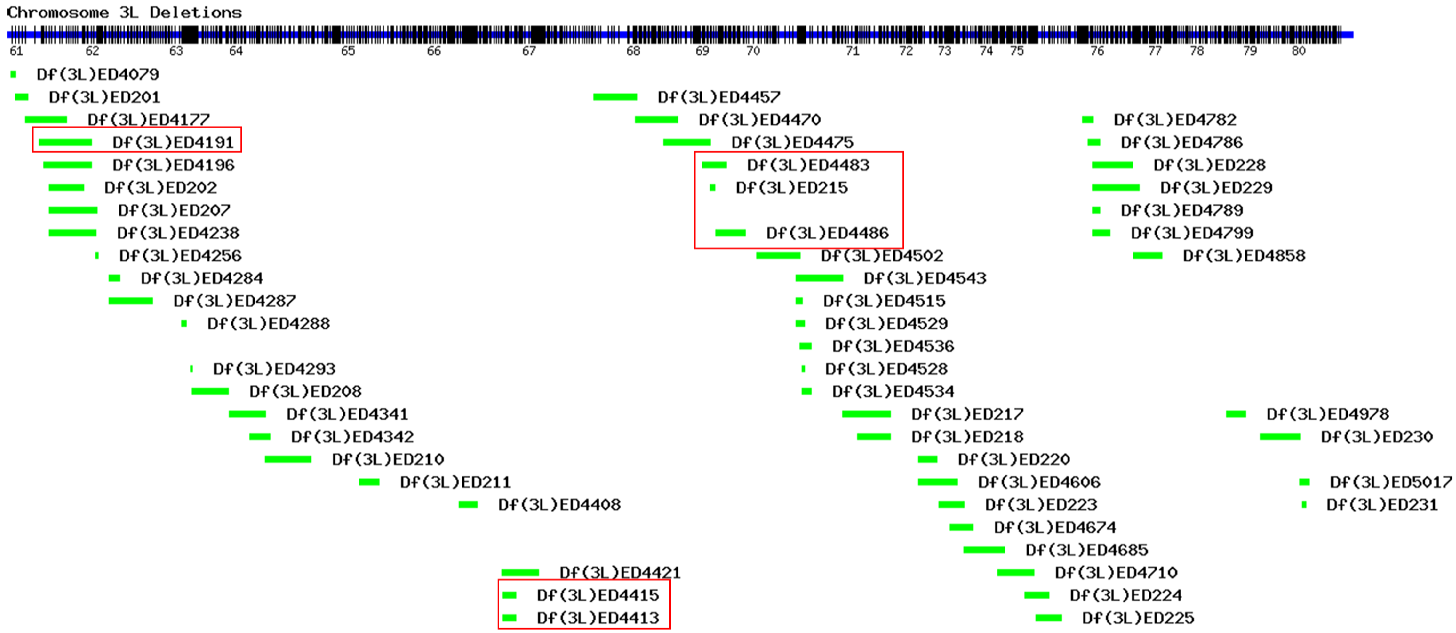

Figure 4.2: Diagrammatic representation of deletions tested. The blue bar represents chromosome arms 3L (this page) and 3R (next page). The numbers represent cytologenic intervals. The green bars represent the various deletions tested and the length of the bar represents the size of the deletion, drawn approximately to scale. Deletions boxed in red represent the ones that suppressed the curly wing phenotype. Modified from http://www.drosdel.org.uk/datagraph-3L-all-pre.php?submit=Chromosome+3L and http://www.drosdel.org.uk/datagraph-3R-all-pre.php?submit=Chromosome+3R

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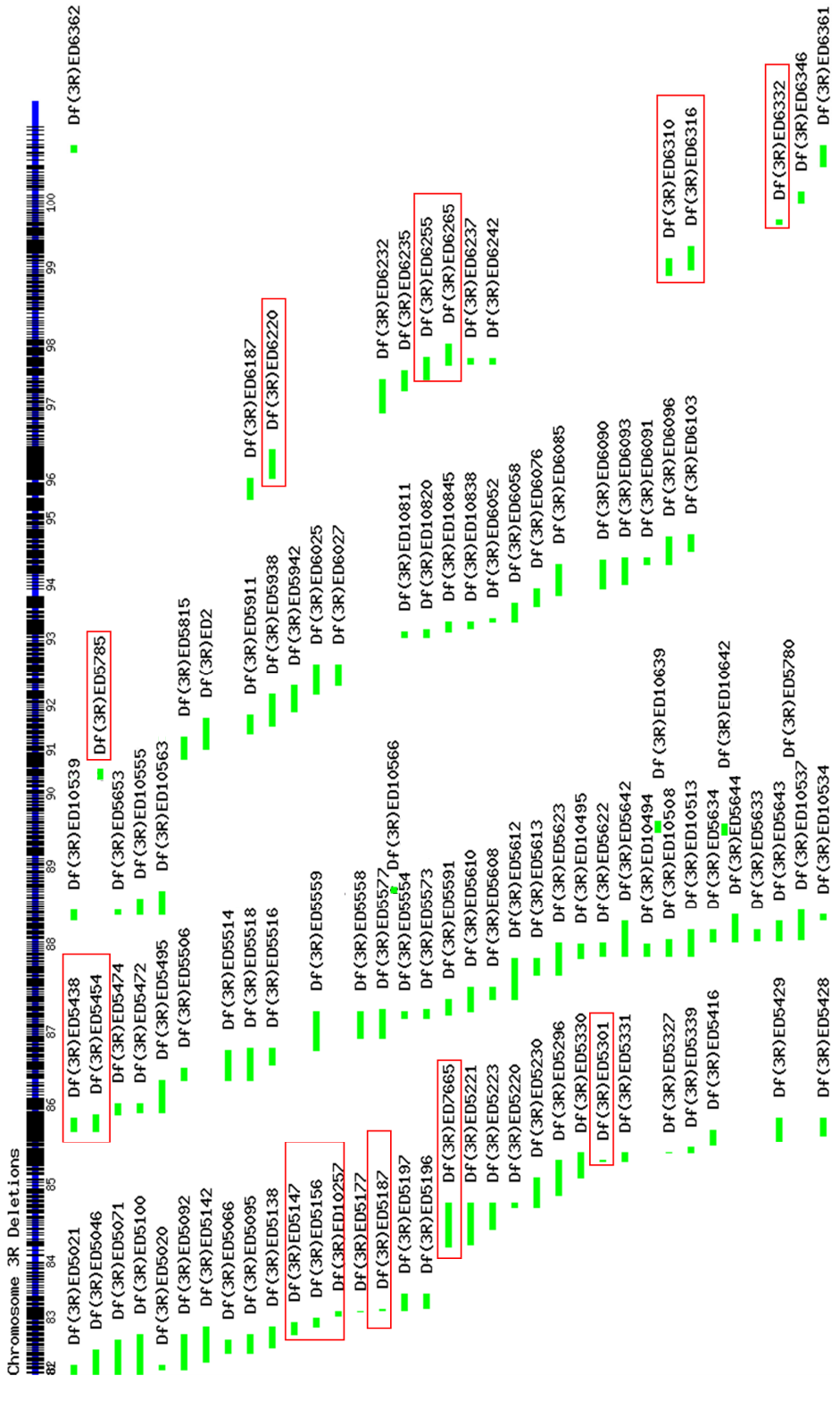

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second control in which we crossed the putative suppressors individually to Ser-GAL4 and UAS-SIN3^{RNAi-I}. The progeny of these crosses were scored for any wing aberrations. No wing phenotypes were observed in these progeny suggesting that the assay identified true suppressors of the SIN3 knockdown phenotype.

In phase II of the screen we attempted to narrow down the cytogenetic intervals that interact with SIN3 by using smaller deletions within the regions identified in phase I (Table 4.3). Deletions ED6332, ED5187 and ED5301 were relatively small and we proceeded to directly test single gene mutations in these regions that could suppress the curly wing phenotype. ED215, ED4338 and ED4486 encompassed Pcaf. We have previously shown a genetic interaction of Pcaf with SIN3 (Swaminathan and Pile, 2010). For the remaining ten cytological intervals we tested approximately 1-11 smaller deletions within these regions, depending on the size of the interval to narrow down the region(s) of the third chromosome that genetically interact with SIN3. Out of the 30 smaller deletions tested, 22 were able to suppress the curly wing phenotype. These 22 deletions represent at least one smaller region within each of the large cytological regions identified at the end of phase I. Table 4.3 summarizes these results. Inspection of the deletions allowed us to generate a list of 285 genes that could potentially modify the curly wing phenotype. To identify individual genes that interact with SIN3, we selected a subset of the genes present in the deletions for analysis. We chose individual genes based on the following criteria: (1) availability of a characterized lof allele or an RNAi line for the gene and (2) its molecular function,

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Isogenic deletion stock ID	Smaller deletions tested within the large region	% Straight wings in combination with SIN3 KD I	Approximate number of genes in smaller df	+ve / tested	Genes tested outside small df but inside big df (+ve / tested)
ED4191	BSC178	29 ± 1	19	0/4	0/1
ED4415 and ED	BSC612	31 ± 5	11	3/4	
	BSC130	0	25		
ED215	None	NA	11		
ED4483	None	NA	37	1/1	
ED4486	None	NA	94		
ED5147 and ED	BSC176	33 ± 6	8	0/4	0/1
	BSC177	17 ± 2	16	1/4	
	Exel9029	0	4	NA	
ED10257	BSC179	16 ± 1	10	1/5	
ED5187	None	NA	2	1/2	
ED7665	Exel6146	0	45	NA	
	Exel6263	29 ± 3	23	0/4	
	BSC196	24 ± 2	13	0/5	
	BSC221	31 ± 5	20	1/3	
	BSC222	0	34	NA	
	BSC465	0	125	NA	
	BSC466	17 ± 3	146	0/5	
	BSC513	0	36	NA	
	BSC548	22 ± 1	11	1/3	
	BSC633	14 ± 1	3	0/0	
	BSC729	19 ± 2	59	0/5	
ED5301	None	NA	6	1/4	
ED5454 and ED	Exel6154	18 ± 3	13	1/3	
	Exel6155	37 ± 1	25	1/5	
ED5785	BSC650	17 ± 4	7	0/7	
ED6220	BSC493	0	23	NA	

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Isogenic deletion stock ID	Smaller deletions tested within the large region	% Straight wings in combination with SIN3 KD I	Approximate number of genes in smaller df	+ve / tested	Genes tested outside small df but inside big df (+ve / tested)
	Exel6201	29 ± 6	3	1/2	
	BSC397	33 ± 5	30	4/7	
	Exel6200	49 ± 2	37	1/6	
ED6255 and ED	Exel6205	20 ± 6	17	0/0	
	Exel6206	0	18	NA	
	Exel6208	13 ± 1	20	2/5	1/2
	BSC686	14 ± 0	16	1/6	1/2
ED6310 and ED	Exel6212	17 ± 4	12	2/3	0/1
ED6332	None	NA	3	1/3	

Table 4.3: Narrowing down regions of the third chromosome that genetically interact with SIN3. Smaller deletions within the regions identified in phase I of the screen were tested for their ability to suppress the curly wing phenotype. Crosses were set up and analyzed as described in Figure 4.1. Results are an average of three independent experiments. Standard deviation is indicated. n = 169 – 322. NA – not applicable.

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CG number	Gene symbol	Contained within smallest deletion tested	Biological process	Molecular function	Localization	%Straight wings in combination with SIN3 KD I		%Straight wings in combination with SIN3 KD II	
						lof allele	RNAi	lof allele	RNAi
CG6677	ash2	BSC397	Chromatin-mediated maintenance of transcription	Histone methyltrasnferase activity	Nucleus	11 ± 6	66 ± 7	13 ± 4	44 ± 2
CG13628	Rpb10	BSC397	Transcription from RNA polymerase II promoter	DNA-directed RNA polymerase activity	Nucleus		13 ± 6		8 + 5
CG5930	TfIIA-L	Exel6208	Transcription initiation from RNA polymerase II promoter	RNA polymerase II transcription factor activity	Nucleus		22 ± 9		17 ± 5
CG10903	CG10903	BSC729, BSC446, Exel6263, BSC196	Metabolic process	S-adenosylmethionine-dependent methyltransferase activity	Unknown		11 + 3		0
CG4107	Pcaf	ED215, ED4483, ED4486	Regulation of histone acetylation	Histone acetyltransferase	Nucleus		64 ± 9		
CG11848	Bili	Exel6201	Negative regulation of Wnt	Binding	Cytoskeleton	28 ± 9	18 ± 6	19 ± 6	24 ± 3
CG5974	pll	Exel6208	Protein domain specific binding	Antifungal humoral response	Cytoplasm	23 ± 3		19 ± 5	
CG6134	spz	Exel6205	Toll binding	Response to stress; immune response	Extracellular region	39 ± 5		26 ± 3	
CG10061	Sas-4	BSC221	Centrosome organization	Unknown	Centriole	56 ± 7	88 ± 6	41 ± 8	56 ± 12
CG2669	hd	BSC177	DNA amplification; cell	Unknown	Nucleus		17 ± 6		33 ± 6
CG1081	Rheb	BSC179	G1/S transition of mitotic cell	GTPase activity	Cytoplasm	28 ± 5		22 ± 7	
CG6875	Asp	BSC397	Microtubule cytoskeleton organization	Myosin light chain binding protein kinase	Cytoplasm	9 ± 3	19 ± 5	5 ± 6	12 ± 2
CG1152	Gld	BSC548	Sperm storage	glucose dehydrogenase	Extracellular region	48 ± 6		33 ± 7	
CG1250	sec23	ED5187	larval chitin-based cuticle development	GTPase activator	ER/Golgi stack	24 ± 5		37 ± 6	
CG31445	CG31445	Exel6212	Proteolysis	Aminopeptidase	Integral to membrane	13 ± 8		7 ± 4	
CG11951	CG11951	Exel6212	Proteolysis	Aminopeptidase	Unknown	28 ± 4		15 ± 2	
CG5804	CG5804	BSC612	Cellular acyl-CoA homeostasis	diazepam binding, enzyme inhibitor activity; acyl-CoA binding	Unknown		54 ± 8		41 ± 9
CG32024	CG32024	BSC612	chitin metabolic process	Chitin binding protein	Extracellular region		50 ± 3		29 ± 7
CG12809	nefrin-2	Exel6155	Unknown	Zinc ion binding protein	Unknown		65 ± 8		41 ± 9
CG2216	Fer1HCH	ED6332	Cellular iron homeostasis	ferrous iron binding	Golgi apparatus		51 ± 6		36 ± 2
CG11993	Mst85C	ED5301	Unknown	Unknown	Unknown		21 ± 4		24 ± 3
CG32023	CG32023	BSC612	Unknown	Unknown	Unknown		40 ± 12		32 ± 6

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Table 4.4: List of genes that genetically interact with SIN3. Males that carry a heterozygous lof or UAS-RNAi construct for the listed genes were crossed to recombinant SIN3 KD I and SIN3 KD II females. The resulting progeny that were SIN3 knockdown and/or halpo-insufficient in or knocked down for the listed gene product were scored for straight wings. Empty cells indicate that no lof or RNAi allele of that gene was available. Refer to Figure 4.1 for general scheme. Results are an average of three independent experiments. Standard deviation is indicated. n = 217 – 381.

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CG number	Gene name	CG number	Gene name
CG42610	Fhos	CG3309	CG3309
CG12000	Prosβ7	CG3910	mtTFB2
CG2666	CG2666	CG1007	emc
CG1163	RpII18	CG2702	Pbp95
CG12005	Mms19	CG2723	ImpE3
CG10233	retinophilin	CG33323	Fer1
CG31543	Hph	CG2520	lap
CG2534	CG2534	CG42277	rn
CG10018	Snm1	CG1234	CG1234
CG10390	Mia	CG7910	CG7910
CG12746	CG12746	CG2747	CG2747
CG1411	CRMP	CG31247	tinc
CG10693	slo	CG18012	CG18012
CG6863	tok	CG33547	CG33547
CG6875	asp	CG17801	CG17801
CG6892	Ets96B	CG31246	CG31246
CG11375	polybromo	CG12347	CG12347
CG31119	HdacX	CG7357	CG7357
CG5814	CycB3	CG2216	Fer2LCH
CG13651	CG13651	CG16632	hdc
CG6172	Ser	CG2216	Fer1HCH
CG5923	DNApol-α73	CG2185	elm
CG5965	woc	CG11990	hyx
CG6338	Ets97D	CG11992	rel
CG5358	Art4	CG18466	Nmdmc
CG3909	CG3909	CG1988	neur
CG9467	CG9467	CG145614	Brd8
CG34360	CG34360	CG9181	CG9181
CG33208	Mical	CG9169	CG9169
CG32319	CG32319	CG11516	Protein tyrosine phosphatase 99A

Table 4.5: List of genes that do not genetically interact with SIN3. The experiment was set up and flies were scored as described in Table 4.4.

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for example, genes involved in regulating cell cycle, gene expression, wing development and signaling pathways. To this end we have tested lof alleles and/or RNAi lines for a total of 82 genes of which 22 were able to suppress the curly wing phenotype (Table 4.4). The remaining 60 genes that did not show an interaction with SIN3 are listed in Table 4.5.

Genetic suppressors do not affect the expression of SIN3

One possible mechanism by which the genetic interactors suppress the curly wing phenotype is by restoring SIN3 levels in the wing disc. This would imply that the suppressor is a direct or indirect regulator of SIN3 expression. To test this hypothesis we compared the SIN3 protein levels between wing discs that were SIN3 knockdown and SIN3 knockdown and haplo-insufficient for the suppressor by western blot. Our results indicate that suppressors do not restore SIN3 protein levels in the wing discs (Fig. 4.3). Two possibilities could lead to this result. One explanation is that the suppressors do not regulate expression of SIN3 and that the mode of suppression is through a different mechanism. Another possibility is that any change in SIN3 levels in the double mutants is not detectable as the flies showing suppression represent a small subset of the population. In either case, we have observed no change in SIN3 levels in the rescued wing discs.

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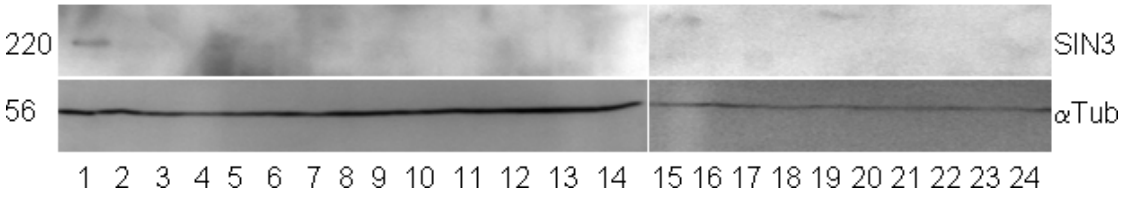

Figure 4.3: Suppression of the curly wing phenotype is not a result of upregulation of SIN3. Western blot analysis of total protein extracted from wing imaginal discs of the following genotypes: 1 - w^{1118}, 2 – SIN3 KD I, 3 - 24 represent genotypes that are SIN3 KD I and mutated for/knocked down in the genetic suppressor as indicated. 3 – $Rheb^{+/-}$, 4 - $asp^{+/-}$, 5 – $ash2^{+/-}$, 6 – $Bili^{+/-}$, 7 – $Rpb10^{RNAi}$, 8 – $pll^{+/-}$, 9 - $spz^{+/-}$, 10 –$TFIIA\text{-}L^{RNAi}$, 11 – $gld^{+/-}$, 12 - $Sas\text{-}4^{RNAi}$, 13 – $CG10903^{RNAi}$, 14 – $sec23^{+/-}$, 15 – $CG31445^{+/-}$, 16 –$CG11951^{+/-}$, 17 – $CG5804^{RNAi}$, 18 – $CG32023^{RNAi}$, 19 – $CG32024^{RNAi}$, 20 - hd^{RNAi}, 21 – $nefrin\text{-}2^{RNAi}$, 22 – $Fer1HCH^{RNAi}$, 23 – $Mst85C^{RNAi}$, 24 - Pcaf. The blots were probed with antibody to SIN3 or α-tubulin (αTub) as a loading control. Molecular weight markers are indicated to the left of the panel.

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***stg* levels are variable in the wing discs of the genetic suppressors**

Our previous work shows that loss of SIN3 results in downregulation of STG, a gene required for the G2/M progression of the cell cycle (Swaminathan and Pile, 2010). The curly wing phenotype can be partially rescued by overexpression of STG from a transgene. One possible mechanism by which the genetic interactors suppress the curly wing phenotype is by restoring STG levels in the wing disc. This would imply that the suppressor is a direct or indirect regulator of STG expression. To test this hypothesis we compared the *stg* mRNA levels between wing discs that were SIN3 knockdown and SIN3 knockdown and haplo-insufficient for the suppressor by quantitative reverse transcriptase-PCR (qRT-PCR). Our results indicate that a mutation in pll, ash2, Rpb10, Gld, sec23, Bili and TFIIA-L are unable to restore *stg* mRNA levels in a SIN3 knockdown background (Fig. 4.4). A mutation in the rest of the 17 genes is able to elevate *stg* mRNA levels to near wild type, but this increase is not statistically significant ($p = 0.069 – 0.91$). The lack of statistical significance is probably due to variability observed in the data. Additional trials will be performed to definitively determine *stg* levels in these genetic backgrounds. Overall, our results indicate that some of the suppressors do not affect *stg* mRNA levels in the wing discs (Fig. 4.4). As with the previous data on SIN3 levels, this result can mean one of two things. One explanation is that the suppressors do not regulate expression of STG and that the mode of suppression is through a different mechanism. It is also possible that any change in STG expression in the double mutants would not be detectable as many of the suppressors show mild suppression of the curly wing

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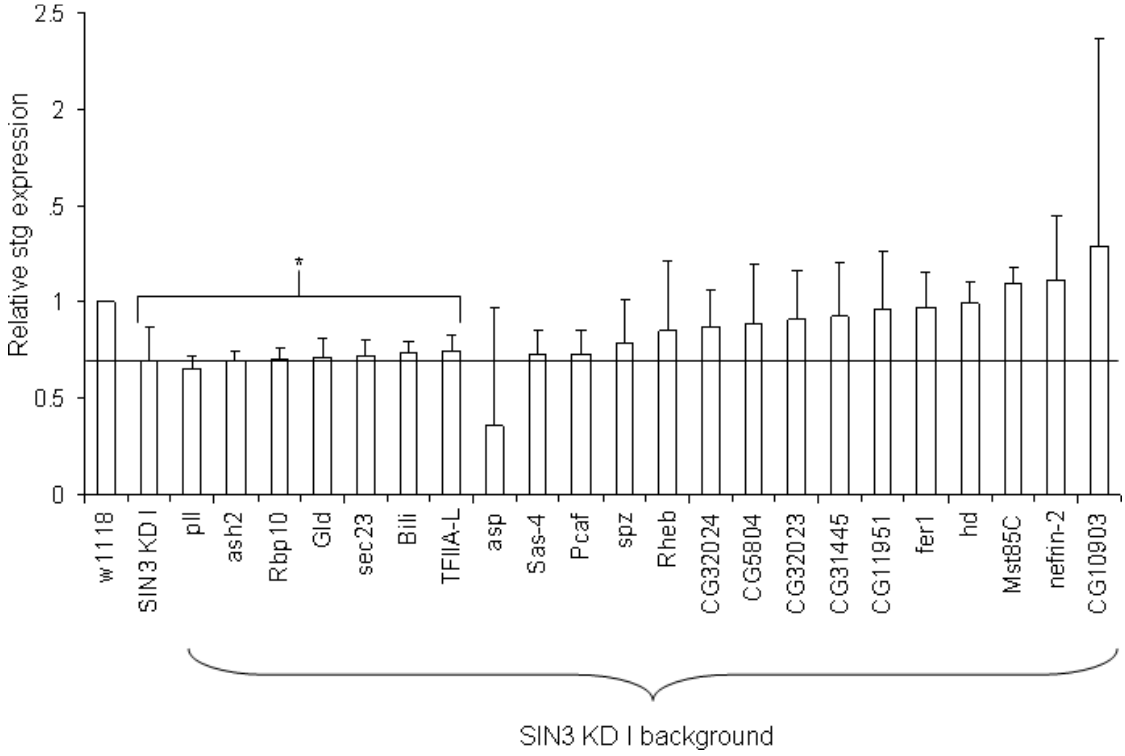

Figure 4.4: *stg* levels are variable in the wing discs of the genetic suppressors. qRT-PCR analysis of *stg* mRNAs from the indicated genotypes. Total RNA was isolated from control and SIN3 knockdown cells or wing discs and used to prepare cDNA template for qPCR. *stg* expression was normalized to TAF1 and PGK. n = 3 - 9. Error bars indicate standard deviation. (*) $0.008 \leq p \leq 0.036$

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phenotype. Thus, even if STG levels were restored in these wing discs they represent a small subset of the population. For this reason, we are unable to fully determine STG levels in the rescued wing discs.

SIN3 genetically interacts with genes that negatively regulate the Wnt pathway

The Wnt pathway has been implicated in cell division in the wing disc and development of the wing (Johnston and Edgar, 1998). Briefly, binding of the wingless (Wg) ligand to the Frizzled/low density lipoprotein (LDL) receptor-related protein (LRP) inhibits the degradation of Arm resulting in its accumulation in the cytoplasm and nucleus. Nuclear Arm interacts with TCF to influence transcription of Wnt responsive genes (Logan and Nusse, 2004). Bili, a gene that suppresses the curly wing phenotype (Table 4.4) acts as a negative regulator of the Wnt pathway by destabilizing the interaction between Wg and LRP5/6 such that the downstream signals can not be turned on, resulting in inhibition of the Wnt pathway (Kategaya et al., 2009). To determine if additional genes that encode negative regulators of the Wnt pathway can genetically interact with SIN3, we tested if knockdown of expression of known Wnt negative regulators by RNAi could modify the SIN3 knockdown curly wing phenotype. Using the QueryBuilder tool on FlyBase (www.flybase.org), we generated a list of genes that act as negative regulators of Wnt signaling. Five out of the twelve factors tested were able to suppress the curly wing phenotype (Table 4.6). Tumbleweed (twm) was unique amongst these genes in that loss of twm alone resulted in a

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Gene name (Gene symbol)	% Straight wings in combination with SIN3 KD I	% Straight wings in combination with SIN3 KD II
APC-like (Apc)	25 ± 3	16 ± 3
Apc2	0	0
Axin (Axn)	13 ± 4	16 ± 7
CG10225	48 ± 4	61 ± 3
crooked legs (crol)	0	0
Cyo310a1	0	0
fat (ft)	83 ± 10	71 ± 4
groucho (gro)	12 ± 4	17 ± 5
pangolin (pan)	0	0
Shaggy (sgg)	21 ± 2	14 ± 3
starry night (stan)	0	0
tumbleweed (tum)	0	0

Table 4.6: Genes involved in the negative regulation of the Wnt pathway genetically interact with SIN3. SIN3 KD/CyO-Ras females were crossed to males carrying a UAS driven RNAi construct for the indicated gene. Progeny of the cross that are knocked-down for SIN3 and the indicated gene were scored for straight wings. Results are an average of three trials. n = 112 - 144. Standard deviation is indicated.

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curly and wrinkled wing phenotype also seen in the SIN3 knockdown background (data not shown).

Negative regulators of the Wnt pathway interfere with signal transduction at various stages of the pathway ultimately resulting in downregulation of Wnt responsive genes. Thus loss of negative regulators of the Wnt pathway results in an upregulation in Wnt responsive genes. One hypothesis to explain the interaction between SIN3 and negative Wnt regulators is that knock down of SIN3 results in the downregulation of one or more Wnt target genes or components of the pathway itself, so that when a negative regulator Wnt is mutated, upregulation of the Wnt pathway occurs and the wings are restored to normal. To test this hypothesis, we assayed the gene expression of some Wnt pathway components, including targets and negative regulators by qRT-PCR in control and SIN3 knockdown wing discs. TCF, Dm, Nemo, Stripe and Bili are downregulated upon loss of SIN3 whereas the other genes are unaffected (Fig. 4.5). These results are consistent with our hypothesis, but suggest a novel role for SIN3 in gene activation in *Drosophila* whereby recruitment of SIN3 to Wnt targets results in upregulation. Alternatively, SIN3 may regulate negative regulators of the Wnt pathway such that loss of SIN3 results in upregulation of these genes. This in turn can result down regulation of the Wnt pathway. Although loss of SIN3 results in down regulation of Bili, we have not tested other negativbe regulators of the Wnt pathway.

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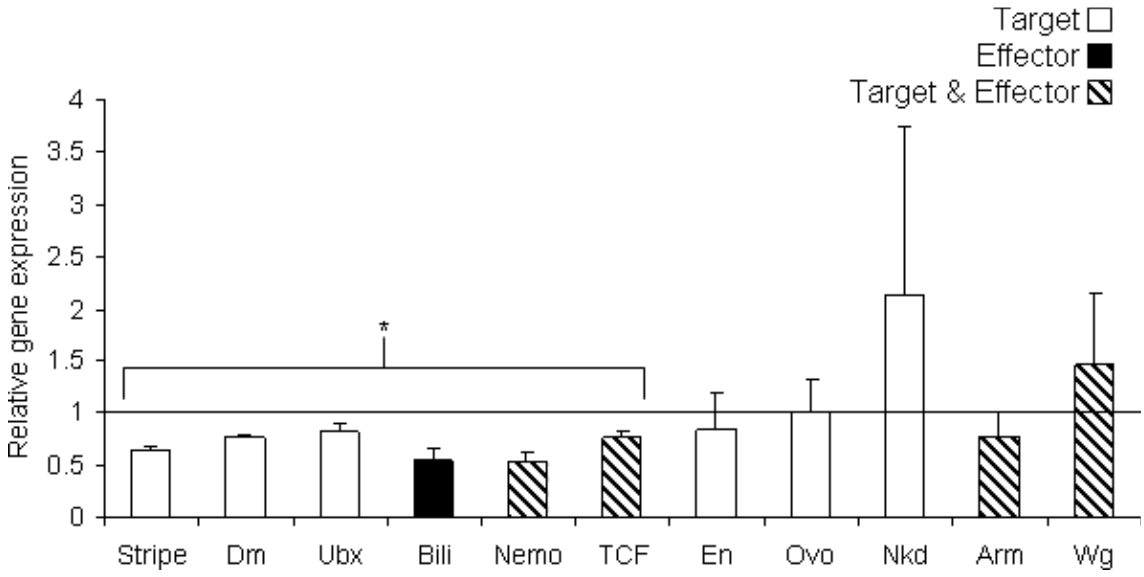

Figure 4.5: Loss of SIN3 results in down regulation of genes involved in the Wnt pathway. qRT-PCR analysis of the mRNAs of the indicated genes. mRNA from of control w^{1118} and SIN3 knockdown wing discs was reverse transcribed into cDNA to use as template in the PCR. Gene expression in SIN3 knockdown wing discs relative to w^{1118} is indicated. Expression was normalized to TAF1 and PGK expression. n = 3. Error bars indicate standard deviation. (*) $0.006 \leq p \leq 0.02$.

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Genes involved in the cell cycle genetically interact with SIN3

Previous work on *Drosophila* SIN3 suggests it plays an important role in regulating the cell cycle in tissue culture cells and the developing wing disc (Pile et al., 2002; Swaminathan and Pile, 2010). SIN3 genetically interacts with STG and its substrate CDK1, in that over expression of either of these genes important for the G2 to M transition suppressed the SIN3 knockdown curly wing phenotype albeit not completely (Pile et al., 2002; Swaminathan and Pile, 2010). To determine whether SIN3 interacts with genes involved in other phases of the cell cycle, we performed a targeted screen to test if mutations in, or overexpression of, known cyclins and/or cyclin dependent kinases (CDKs) could modify the curly wing phenotype. A lof mutation in CDC16, anaphase promoting complex/cyclosome (APC/C), important for mitosis, and overexpression of CDK2, a G1/S regulator, were able to suppress the curly wing phenotype (Table 4.7).

Next, we determined if the mutation in CDC16 that suppresses the SIN3 knockdown curly wing phenotype could also restore wing area and cell number that are reduced in the single SIN3 knockdown wings compared to wild type (Swaminathan and Pile, 2010). A mutation in CDC16 in the context of SIN3 knockdown was able to restore wing area to near wild type (Fig. 4.6A). Next, we sought to determine if the cell number in the wings were also restored. Each cell in the adult wing blade has a single bristle. We counted the number of bristles in a fixed area. Consistent with our previous report, the number of cells in the fixed area of the adult wing was reduced from an average of 37 in control to 26 in the SIN3 knockdown wings. A lof mutation in CDC16 (APC/C) in a SIN3 knockdown

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Gene name		% Straight wings in combination with SIN3 KD I	
	Phase	Allele	Overexpression
Cdk2	G1/S	0	31 ± 2
Rbf	G1/S	0	
Cyclin A	G2/M	0	0
Cyclin B	G2/M	0	0
Cdc16	Mitosis	21 ± 2	
Cdc23	Mitosis	0	
Cdc27	Mitosis	0	
Cyclin B3	Mitosis	0	0
Cyclin J	Mitosis	0	0
Cyclin E	G1/S	0	0
E2f	G1/S	0	
Cyclin H	Cell cycle	0	

Table 4.7: SIN3 interacts genetically with cell cycle regulators. SIN3 KD I/CyO-Ras females were crossed to males that carried a heterozygous lof mutation in or an overexpression construct of the indicated cell cycle regulator. Progeny of the cross carrying the SIN3 KD chromosome and the mutation in or overexpression of the indicated cell cycle regulator were scored for straight wings. Empty cells indicate that no fly lines of that type were available. Results are an average of three experiments. Standard deviation is indicated. n = 165 - 289.

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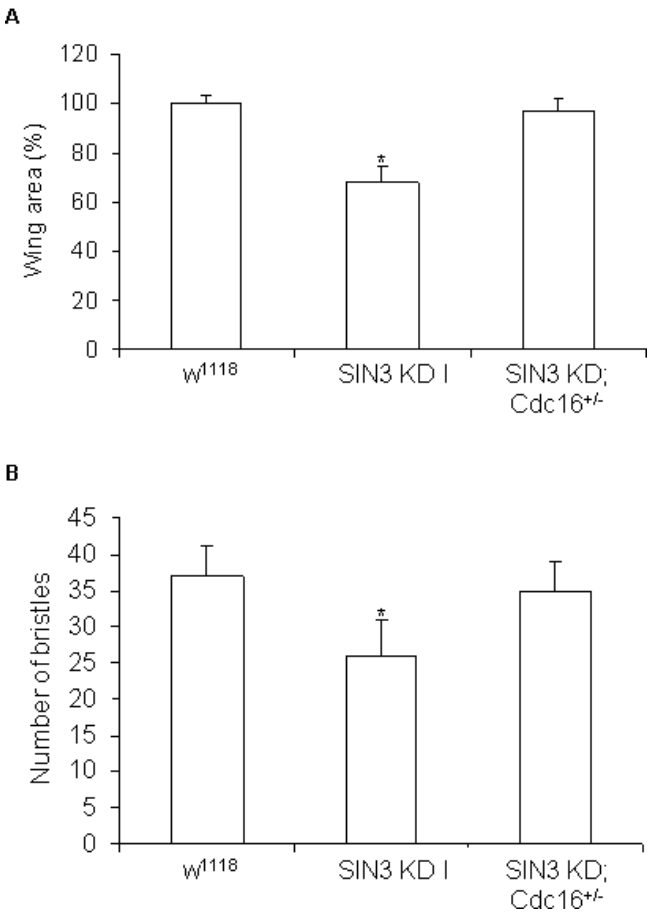

Figure 4.6: A mutation in Cdc16 restored cell number in the wings of SIN3 knockdown flies. (A) The wing area for each genotype was determined and compared to wild type wings using ImageJ software. (B) The number of bristles in a fixed area on the dorsal side of the wing was determined for all genotypes indicated. n = 103 - 118. Error bars represent standard deviation. (*) $p < 0.01$ comparing SIN3 KD I to w^{1118}.

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background was able to restore cell number in the adult wing to near wild type (Figure 4.6B). Taken together with our previously published report, this result suggests that SIN3 potentially plays a role in regulating multiple stages of the cell cycle including cell division itself. Cdc16 (APC/C) is important for metaphase-anaphase transition in mitosis (Zielke et al., 2008). Loss of CDC16 (APC/C) results in a metaphase arrest. It is responsible for the proteasome mediated degradation of Gemenin and A/B type cyclins to reduce the kinase activities of CDKs to facilitate separation of sister chromatids, disassembly of spindle, chromosome decondensation, cytokinesis and reassembly of the nuclear envelope (Acquaviva and Pines, 2006; Zielke et al., 2008).

In addition to mutation in the CDC16, we also found that overexpression of CDK2 partially suppresses the curly wing phenotype, suggesting that loss of SIN3 results in a defective transition through the S phase. Another possibility is that overexpression of CDK2 compensates for the defective progression through the G2/M phase of cells in the SIN3 knockdown wing. Although we observed the interaction between SIN3 and a G1/S regulator, SIN3 knockdown wing discs do not show decrease in BrdU staining (data not shown). Possible explanations for this are that loss of SIN3 acts prior to DNA synthesis or results in a small defect in S phase progression that is not visualized by BrdU staining. Alternatively, overexpression of a cell cycle regulator overcompensates for the loss of SIN3 even though it may not necessarily function in concert with SIN3.

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Expression of CDC16 and CDK2 are relatively unchanged following knockdown of SIN3

Loss of SIN3 results in downregulation of STG expression and accordingly overexpression of STG is able to partially rescue the curly wing phenotype (Swaminathan and Pile, 2010). To determine if the ability of the identified cell cycle regulators to modify the SIN3 knockdown curly wing phenotype is a direct effect of loss of SIN3 on their expression, we performed qRT-PCR analysis on mRNA isolated from these tissues. SIN3 knockdown does not affect the expression of either of the cell cycle genes tested (Fig. 4.7) suggesting that the effect is due to their involvement in the same process rather than a direct role for SIN3 in regulating these cell cycle genes.

SIN3 genetically interacts with components of the mediator accessory sub complex

Multiple CDKs are expressed in a cell, some of which are directly involved in regulating the cell cycle while others may have an indirect role. Some have also been implicated in regulating transcription. One such CDK is CDK8 which shares 32% homology with CDK1 (Leclerc et al., 1996; Lehner and O'Farrell, 1990). Since *Sin3A* genetically interacts with Cdk1 we wanted to test if it could also interact with Cdk8. We determined that the curly wing phenotype is suppressed by RNAi mediated downregulation of CDK8 (Table 4.8). CDK8 is a member of the transcription mediator sub complex. Other members of this complex are CYCC, KTO (MED12) and SKD (MED13). Similar to knock down of

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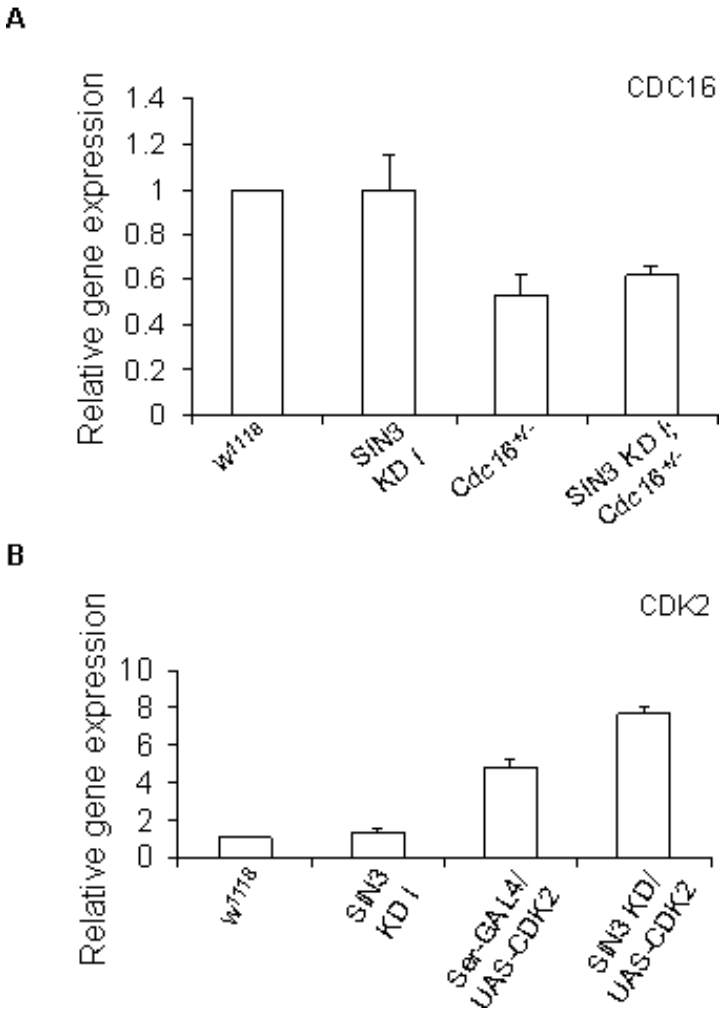

Figure 4.7: SIN3 does not regulate expression of CDK2 or CDC16. SIN3 KD I/Cyo-Ras females were crossed to males harboring a lof mutation in Cdc16 (A) or carrying an overexpression construct of CDK2 (B). The relative gene expression as determined by qRT-PCR analysis of mRNA from wing discs of the indicated genotypes is shown. n = 3. Error bars indicate standard deviation.

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CDK8, RNAi mediated downregulation of the other members of the sub complex was also able to suppress the curly wing phenotype (Table 4.8). RNA polymerase II dependent transcription depends on its association with the mediator complex that in turn associates with the mediator sub complex. The mediator sub complex is essential for the activation of Wnt target genes in the wing discs (Carrera et al., 2008). It is also essential for the development of external sensory organ on the notum which arises from the wing dics (Loncle et al., 2007). In that work, the researchers found that *skd* plays a role in adult cell specification and segment identity. Unlike other components of the mediator complex SKD and KTO are not required for cell proliferation or survival, rather they regulate the formation of boundaries in the eye disc. KTO and SKD have no effect on heat shock induced expression of Attacin A and Hsp26 whereas RNAi mediated depletion many other components of the mediator complex do affect expression. These results suggest that some members of the mediator complex regulate expression of specific genes whereas other components play a more general role.

One possible explanation for the interaction of SIN3 with these genes is that loss of SIN3 results in the upregulation of genes that are normally activated by the mediator accessory complex. A mutation in the complex components in a SIN3 knockdown background may restore the expression of these genes to near normal levels resulting in the suppression of the curly wing phenotype. The genetic interaction between SIN3 and KTO, SKD, CYCC and CDK8 suggests that the effect of loss of SIN3 on the cell cycle is not generally due to solely to

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Gene name	% Straight wings in combination with SIN3 KD I	
	lof allele	RNAi
Cdk8		75 ± 4
CycC		45 ± 6
kto	80 ± 4	61 ± 3
skd	84 ± 5	49 ± 6

Table 4.8: Components of the mediator accessory sub complex genetically interact with SIN3. SIN3 KD I/CyO-Ras females were crossed to males carrying a UAS driven RNAi construct for, or a heterozygous lof allele in the indicated gene. Progeny of the cross that are SIN3 knockdown and knocked down for the indicated gene were scored for straight wings. Empty cells indicate that no lof allele or RNAi line was available for the gene. Results are an average of three trials. n = 122 - 169. Standard deviation is indicated.

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defects in cell proliferation but also due to its role in regulating a specific set of genes involved in the process.

CONCLUSION

SIN3 has been implicated in development in various organisms including *Drosophila* (Neufeld et al., 1998b; Pennetta and Pauli, 1998), Xenopus (Sachs et al., 2001), chick (Bach et al., 1999).and mammals (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008). Data from many of these previous studies implicate SIN3 in regulating various aspects of the cell cycle that affect development. Evidence exists that SIN3 regulates development by functioning in signaling pathways possibly by regulating specific developmental genes. In this study we have attempted to identify novel genes of and signaling pathways in which SIN3 may function. We have found that SIN3 genetically interacts with genes involved in development including signaling pathway effectors, histone modifying enzymes, cell cycle regulators and components of the transcription machinery. This finding suggests that SIN3 plays a wide variety of roles in a developing tissue such as the wing disc and that its function is not limited to regulating the cell cycle. Loss of SIN3 results in misregulation of Wnt responsive genes (Fig. 4.5) suggesting that SIN3 plays a role in the Wnt pathway in the developing wing disc. Genes involved in regulating multiple stages of the cell cycle are able to suppress the curly wing phenotype suggesting that SIN3 not only regulates G2/M progression as has been previously described, but also other phases of the cell cycle including mitosis (Table 4.7). The genetic interaction between SIN3 and components of the mediator accessory sub

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complex suggest a role for SIN3 in counteracting the activation of genes by this complex. Further analyses of these interactions will shed light on the role in SIN3 in *Drosophila* development.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The role of histone acetylation in regulating gene expression during development is well established. SIN3, a component of the *Drosophila* SIN3 HDAC complex, is an essential gene that is important for embryonic development (Neufeld et al., 1998b; Pennetta and Pauli, 1998). This hinders our ability to determine the genes or specific developmental processes regulated by SIN3. To circumvent this issue, we have generated a GAL4-UAS based conditional RNAi system to knock down SIN3. Using this system we have determined that SIN3 is important for embryonic as well as post-embryonic development. Ubiquitous loss of all SIN3 isoforms during larval stages resulted in lethality before adulthood. We also found that conditional knockdown of SIN3 in the wing discs resulted in a curly wing phenotype in the adults. These phenotypes could be reproduced by expressing a second SIN3 RNAi construct that targets a different region of the SIN3 mRNA. Both these phenotypes can be rescued by overexpression of SIN3 187 or SIN3 220. These results suggest that the phenotypes observed upon induction of SIN3 RNAi are truly due to loss of SIN3 and not due to an off target effect of RNAi. Furthermore, we were able to employ the curly wing phenotype in a deficiency screen to identify genes that genetically interact with SIN3. Thus we have generated a system that can be used to dissect the role of SIN3 *in vivo* in the context of development.

SIN3 has been implicated in regulating the cell cycle in many model systems. Loss of SIN3 resulted in defective cell proliferation in *Drosophila* S2

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cells (Pile et al., 2002). In mouse, loss of SIN3A resulted in loss of cell proliferation in embryonic fibroblasts (Cowley et al., 2005; Dannenberg et al., 2005). Loss of SIN3B in embryonic fibroblast resulted in a failure to exit the cell cycle thus hindering cellular differentiation (David et al., 2008). The lethality caused by loss of SIN3 is also hypothesized to be a result of loss of cell proliferation or cell viability. We infer this from the fact that loss of SIN3 during embryogenesis leads to degeneration such that many of the embryos showed decreased DAPI staining of nuclei and lacked discernible structures beyond stage 11 (Fig. 2.2). SIN3 knockdown wing discs are defective in cell proliferation as SIN3 knockdown mitotic clones are smaller in size than wild type counterparts (Fig. 3.6). This in turn resulted in a decrease in the number of cells undergoing mitosis and fewer cells in the adult wing (Fig. 3.7). These results suggest that SIN3 is required for cell proliferation and/or survival in the wing disc. This is further supported by observation that changes in the dose of cell cycle regulators can modify the curly wing phenotype caused by loss of SIN3. Overexpression of STG, CDK1 and CDK2, and a heterozygous lof mutation in Cdc16 can suppress the curly wing phenotype (Fig 3.8 and Table 4.7). These data suggest that the curly wing phenotype is at least in part due to a defect in cell cycle progression.

SIN3 has previously been implicated in regulating multiple stages of the cell cycle. In mammals, Sin3 has been shown to interact with the tumor suppressor Rb and repress E2F-mediated transcription to regulate S-phase entry and exit of the cell cycle (Lai et al., 2001). SIN3 has also been shown to regulate G2 progression in *Drosophila* and mammals (Cowley et al., 2005; Dannenberg et

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al., 2005; Pile et al., 2002). Loss of SIN3 results in a 30% upregulation in the *Drosophila* homolog of p21, *Decapo* (data not shown), a gene involved in regulating cell cycle, senescence and differentiation (Campisi, 2000). Loss of Hdac1, the mammalian homolog of *Rpd3*, results in an upregulation of p21 and decreased cell proliferation in mouse embryonic stem cells, a phenotype that is rescued by knocking out p21 (Zupkovitz et al., 2010). One possibility is that the cell cycle defects observed upon loss of SIN3 may also be partly due to upregulation of p21. Our results have identified a genetic link between SIN3 and multiple genes that regulate the cell cycle. Further investigation of these interactions is expected to shed light on the role that histone acetylation plays in the regulation of cell proliferation and differentiation.

Apart from its role in governing the cell cycle, SIN3 has been implicated in developmental pathways from *Drosophila* to mammalian systems. We have identified genes that genetically interact with SIN3 in an attempt to find novel gene targets of and/or signaling pathways that SIN3 may regulate. We have identified genes involved in a variety of processes that genetically interact with SIN3 and suppress the curly wing phenotype (Tables 4.4, 4.6, 4.7 and 4.8). These results suggest that SIN3 not only controls the cell cycle in the wing disc but potentially other processes as well. Molecular and biochemical characterization of these genetic interactions will shed light on the role of SIN3 in the context of development outside the realm of cell cycle regulation.

The results described in this study reinforce the role of SIN3 in regulating the cell cycle in a developing organism. We have also identified signaling

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pathways that SIN3 is involved in during wing development. We do not yet completely understand the mechanism by which SIN3 regulates cell proliferation or wing development or know of all the direct gene targets of SIN3 that affect these processes. Listed below are some questions that will help to further our understanding of the role of SIN3 in these processes.

Does SIN3 directly regulate STG expression?

As described in Chapter 3 loss of SIN3 results in reduced levels of STG mRNA. This may potentially reflect a role for SIN3 in transcription activation which has not yet been shown in *Drosophila*. SIN3 has been implicated in activation of PHO5, SPO11 and SPO13 (Vidal et al., 1990), positively regulates the activities of GAL4 and HAP1 (Nawaz et al., 1994) and is required for transcription of STA1 and ADH2 (Yoshimoto et al., 1992). Deaceylation of histones has been associated with transcription activation in higher organisms (Qiu et al., 2006). If SIN3 does activate STG expression, this would be a novel finding but not completely unexpected. To test this hypothesis chromatin immunoprecipitation analysis can be performed on chromatin prepared from wing discs using antibodies specific to SIN3 to determine if SIN3 associates with the Stg promoter. Antibodies specific to acetylated H3K9, K3K14, H4K5 and H4K12 can be used on chromatin from SIN3 knockdown wing discs to determine what histone marks change at the Stg promoter upon loss of SIN3. Alternatively, the SIN3 complex could regulate levels of acetylated pools of STG in the cell. This can be tested by immunoprecipitating STG from extracts prepared from wing

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discs with or lacking SIN3 and performing a western blot analysis on the immunoprecipitates with an antibody that recognizes acetylated lysine.

Does loss of SIN3 result in cell death?

SIN3 knockdown wings show progressive, melanized blemishes with age (data not shown). This phenotype is characteristic of flies mutated in genes involved in programmed cell death (PCD) like dronc, rpr, grim and hid (Chew et al., 2004; Link et al., 2007; Muro et al., 2006). To determine if loss of SIN3 results in perturbed PCD SIN3 knockdown wing discs can be stained with acridine orange, a dye that stains the DNA of dying or dead cells green. Positive staining would suggest cell death upon loss of SIN3. Next the discs can be immunostained with an antibody that recognizes activated caspase-3 which is an indicator of apoptosis. Positive staining would suggest that cell death caused by loss of SIN3 is via apoptosis.

Does loss of SIN3 result in cell adhesion defects?

SIN3 knockdown wings are fragile and sometimes show the presence of a bubble in the wing. The bubble arises when, after the epithelium has retracted from between the two layers of the chitinous wing, the two layers fail to attach with one another. This in turn results in fragility. To test if SIN3 regulates genes involved in cell adhesion, we can test the expression of adhesion genes such as α-Catenin, Armadillo, C-Cadherin, Connectin, Contactin, Laminin B2 and α-Spectrin in control and SIN3 knock down wing discs by qRT-PCR. Alternatively it

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can be tested if the overexpression of one or more of these genes can rescue the fragile/bubble wing phenotype. If yes, then this finding indicates that SIN3 is important for the expression of adhesion molecules in the wing.

Do mutations in genes that genetically interact with SIN3 rescue the cell proliferation defect?

Loss of SIN3 results in proliferation defects resulting in smaller adult wings with fewer cells. Some of the genetic interactors that suppress the SIN3 knockdown phenotype by restoring straight wings also restore wing area and cell number. One hypothesis is that these suppressors ameliorate the cell proliferation defect. Loss of SIN3 also results in cell proliferation defects in *Drosophila* tissue culture cells. If the hypothesis is correct, then loss of the genetic interactor by RNAi in combination with SIN3 RNAi would suppress the proliferation defect in tissue culture cells. Cell proliferation can be assayed using a colorimetric kit called CellTiter 96® AQueous One Solution Cell Proliferation Assay. This kit contains a reagent, which is bioreduced by the live cells to form a chromophore. The intensity of the absorbance of this chromophore is a direct measure of the number of metabolically active cells and hence the rate of proliferation.

Does SIN3 regulate the genes with which it genetically interacts?

In Chapter 4, we describe many genes that genetically interact with and suppress the curly wing phenotype. One hypothesis to explain this mechanism of

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suppression is that loss of SIN3 results in the upregulation of these genes resulting in the curly wing phenotype. Thus a heterozygous loss of function mutation in these genes restores their mRNA levels to near wild type, in turn restoring normal wings. To test this hypothesis we can determine the expression level of genes that are genetic suppressors of the curly wing phenotype in SIN3 knockdown wing discs. This will help us identify more genes that are regulated by SIN3 and thus further our understanding of the role of SIN3 in wing development.

SIN3 is an important protein in all metazoans. This study has illuminated some of the various roles of SIN3 in the cell. This work has provided future researchers with the tools and preliminary data required to further elucidate the mechanism by which SIN3 controls cell division and development of an organism.

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ABSTRACT

ANALYZING THE EFFECTS OF LOSS OF SIN3 IN *DROSOPHILA MELANOGASTER*

by

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Sin3A has been previously shown to be an essential gene for *Drosophila* viability and is implicated in the regulation of cell cycle. In this study, we show that SIN3 is not only required for embryonic viability but also for post-embryonic development. Genetic analysis suggests that the different isoforms of SIN3 may regulate unique sets of genes during development. The developmental lethality occurring due to ubiquitous knock down of SIN3 is hypothesized to be to the result of defects in cell proliferation. Conditional knock down of SIN3 in the wing discs results in a curly wing phenotype in the adult fly. These wings are smaller and have fewer cells resulting from a defect in cell proliferation. This is visualized in the form of smaller SIN3 knockdown clones in the wing discs. Furthermore, loss of SIN3 results in a decrease in the number of mitotic cells in the wing discs. This is in part due to misregulation of the G2/M phase of the cell cycle. SIN3 genetically interacts with STG, a protein important for the G2/M phase of the cell cycle. Loss of SIN3 results in downregulation of STG whereas overexpression of

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STG in a SIN3 knockdown background is able to rescue the curly wing phenotype. SIN3 also genetically interacts with other genes involved in the cell cycle like Cdk2 and Cdc16 suggesting that SIN3 plays a role in multiple phases of the cell cycle. SIN3 also genetically interacts with genes involved in the Wnt and Toll signaling pathways, the mediator accessory sub complex, transcription regulation and chitin metabolism. These results suggest that SIN3 not only plays a role in regulating the cell cycle but also other processes during development.

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