

ABSTRACT

Gouge, Catherine A. SLD5, a Subunit of the Heterotetrameric GINS Complex is Necessary for Normal Cell Cycle Progression and Genomic Stability. (Under the direction of Timothy W. Christensen) Thomas Harriott College of Arts & Sciences, East Carolina University, Department of Biology, June 2010.

Sld5 is one component of the GINS heterotetrameric complex essential to DNA replication. Specifically, GINS is known for its integral role during the G1 to S phase transition in the cell cycle. The GINS complex is comprised of multiple subunits: Psf1, Psf2, Psf3 and Sld5, all of which are highly conserved in eukaryotes. During the initiation of S phase, GINS mediates the association of multiple proteins at replication origins. SLD5 plays a central role in the GINS complex through contact with both Psf1 and Psf2. Due to this pivotal role, Sld5 is the focus of our continuing investigation into the mechanisms of DNA replication and heterochromatin formation in *Drosophila*. Understanding Sld5 function has employed the use of several approaches. To recognize the range of protein interactions in which SLD5 participates we are using yeast two-hybrid analysis, confirming suspected interactions. In addition to interaction studies we are utilizing two recently identified mutant alleles of SLD5 to understand its function *in vivo*. These p-element insertion alleles result in the truncation of the Sld5 protein removing a large portion of the C-terminal beta domain in both mutants, a domain that is believed to play a role in facilitating interactions with other proteins. The arrest point determination of Sld5 was completed and shown to occur at the late embryo/early larval stage transition of the developing *Drosophila*. These homozygous lethal alleles of SLD5 are being used to understand the role of Sld5 in DNA replication through EdU incorporation assays. In addition, possible roles for Sld5 in chromosome biology are being examined. These methods include the analysis of the morphology of chromosomes in polytene tissues, larval brain tissues, and embryos. Roles of Sld5 within the cell cycle have been explored by quantitation of mitotic indexes using larval brain squashes with both alleles of Sld5 showing a marked increase in mitotic figures observed when compared to wild type. In addition, Embryo analysis has revealed severe mitotic defects including asynchrony, cell dropout, and anaphase bridges are present upon division. Exploration of the Sld5 subunit will further the understanding of the GINS complex and its role in DNA replication, along with its possible roles in chromosome biology and its role in genome maintenance.

**SLD5, a Subunit of the Heterotetrameric GINS Complex is Necessary for Normal
Cell Cycle Progression and Genomic Stability**

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Presented To

The Faculty of the Department of Biology
East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

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by

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“With your mind power, your determination,
your instinct, and the experience as well, you
can fly very high.”- Ayrton Senna

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CHAPTER 1: INTRODUCTION

DNA Replication Overview

Eukaryotic cells progress through the cell cycle in an ordered series of unidirectional specific events. Specifically, G1 (Gap 1 Phase), S (Synthesis), G2 (Gap 2 Phase) and M (Mitotic) phases (figure 1.1), in which the alteration of timing or length of any of these phases can influence growth and cell size (Malumbres, 2009). The act of DNA replication itself occurs during S phase and is responsible for copying the genetic code so that when the sister chromatids separate in anaphase the genetic material can be passed along from one cell to the next. Through an intricate dance of cellular signaling and protein interactions, the cell cycle progresses passing of the genetic material on to each of the newly dividing cells making up the varying tissues that comprise *Drosophila melanogaster*. Cancer can result as a consequence of the cell cycle misfiring in some way, such as uncontrolled proliferation of the cell beyond its life span.

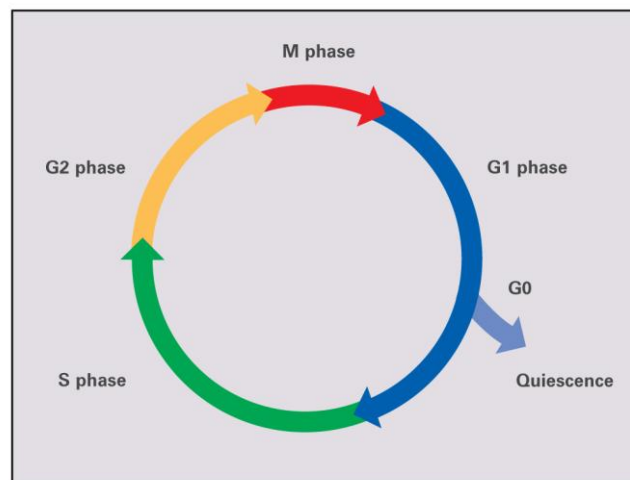


Figure 1.1 *The Cell Cycle (National Institute of General Medical Sciences, 2003)*. In all Eukaryotic cells the cell cycles progression is highly ordered and structured so that each phase is occurring one after another beginning with G1 progressing through Mitosis (M phase) with the finale resulting in cellular division and the passing of the copied genome to the daughter cell.

Cell cycle phases are pre-programmed to occur in a temporal fashion, occurring only once per cycle. As the events encompassing one complete cycle progress, the G₁ phase of the cell cycle corresponds to an intense period of cellular growth ensuring that the cells are ample in size before entering into S phase in which DNA replication takes place. Upon replicating the entire nuclear genome the cell enters G₂, yet another period of intense cell growth, although it is typically the shortest period in the cycle (Ninov, 2009). Once G₂ is complete, the mitotic or M phase occurs in eukaryotes. During M phase a mechanism ultimately resulting in sister chromatid separation and subsequent cytokinesis exists to ensure that the genome is partitioned equally between cells. These new daughter cells are identical to their parents. Each cycle length varies between the different tissue types housed within the organism in question, as well as between different species. Typically, a proliferating human cell progresses through an entire cell cycle in approximately 24 hours; with their S phase lasting approximately 10 hours (Lucas, 2004). In contrast, a full cell cycle in yeast takes roughly 90 minutes (Brewer, 1984) and a typical cell cycle in a *Drosophila* embryo can be as short as 8 minutes while a neuroblast cell may take as long as 50 minutes (Fichelson, 2005).

DNA replication is an amazing intricately coordinated process occurring solely during S phase. Paul Nurse said it best, “*There’s an industrial park’s worth of molecular machinery running the cell cycle.*” When considering just how much manpower and coordinated effort it takes to run an industrial park it becomes even more astonishing what an active normal cell can achieve in a 24 hour period of time. DNA replication machinery is constructed in a regulated sequential order leading up to the initiation of S phase (Costa, 2008). The first of these formed complexes is the Pre-Replication complex (Pre-RC). The multifactor Pre-RC assembles at

origins during the G₁ phase of the cell cycle. Multiple proteins come together to make up the Pre-RC including: ORC, Cdc6, Cdt1, and the MCM 2-7 complex (Arias, 2007).

The process of licensing a replication origin is initiated when ORC binds to chromatin. ORC is a hexameric complex (ORC 1-6) that binds in an ATP dependent manner upon scanning the DNA and recognizing A-T rich sequences, ultimately highlighting the origins (Bell, 1992). After ORC binding occurs licensing has been initiated and Cdc6 binds during the late M/early G₁ phase of the cell cycle (figure 1.2), resulting in a new ORC-Cdc6-DNA complex (Speck, 2007). After formation of the ORC-Cdc6-DNA complex Cdt1 (Cdt1's *Drosophila* homolog referred to as Double parked (Dup)) can then associate with the Mcm2-7 complex (figure 1.2). This marks the final step in the formation of the Pre-replication complex. Once all of these components have bound to the origin, licensing (ensures that replication only occurs once per cycle) is complete and replication can proceed.

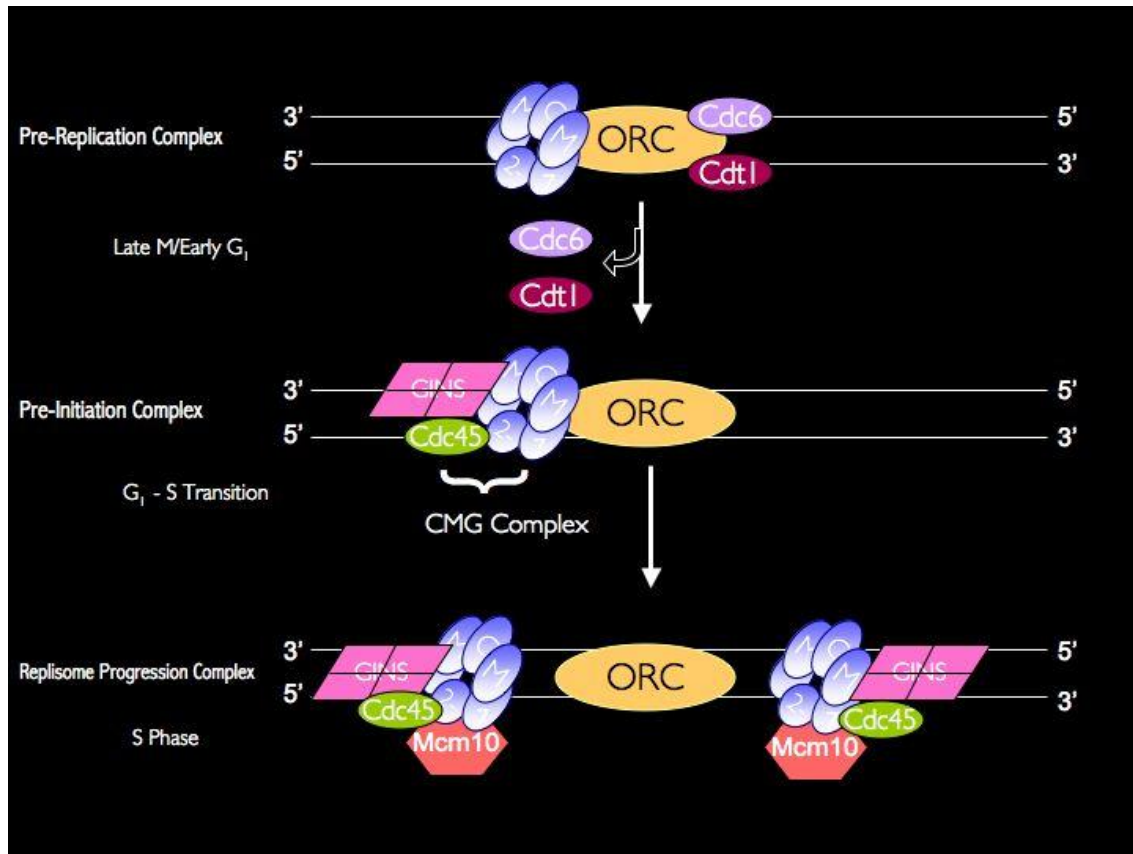


Figure 1.2 Formation of the Pre-Replication Complex through formation of the Replisome Progression Complex. ORC scans the double stranded DNA looking for A-T rich sequences to bind the chromatin. Once ORC binding occurs a cascade of events occur and formation of the Pre-Replication Complex completes resulting in an ORC-Cdc6-DNA complex completing the cycle through early G₁. During the G₁-S Transition the CMG complex has formed composed of GINS, Cdc45, and MCM2-7. During S phase the Replisome Progression Complex is formed consisting of multiple proteins with only GINS, Cdc45, Mcm10, and Mcm2-7 highlighted above. Through the intimate association of these proteins as well as several others replication occurs only once per cell cycle maintaining genomic integrity.

Licensing is essential to maintaining genomic integrity, as DNA must be precisely replicated once before cell division occurs. Each of the events leading to licensing of the origin occurs only once per cell cycle in a regulated manner, controlled via different regulatory proteins. Two such proteins, Geminin and CDK, act in separate ways to achieve regulation of the cell cycle. Geminin binds Cdt1 resulting in it being inactive until the Anaphase Promoting Complex (APC) degrades Geminin freeing the Cdt1 and allowing Mcm 2-7 to associate

(Wohlschlegel, 2000). Cyclin Dependent Kinase (CDK) is another regulatory protein that acts to degrade multiple pre-RC components to prevent re-replication (Nishitani, 1995). Cdt1 and Cdc6 facilitate the loading of the Mcm2-7 complex, which acts as the helicase within the Pre-RC to unwind the DNA double helix. Replication can then begin after the activation of the MCM helicase by phosphorylation via CDK and DDK. DDK phosphorylates the Mcm2 subunit of the Mcm 2-7 complex (Lei, 1997) and facilitates the loading of Mcm10 followed by Cdc45 (Walter, 2000). DDK has also been shown to activate the MCM helicase giving it the ability to unwind the DNA helix, which is essential for replication to proceed (Masuda, 2003). Cdc45 along with MCM facilitates the loading of the replisome along with the GINS complex.

Essential to initiation is a complex made up of Cdc45, Mcm 2-7, and GINS, referred to as the CMG complex (figure 1.2). Through the intimate association of each of the protein components of the CMG complex it has been shown to regulate initiation and the progression of replication (Bauerschmidt, Pollok et al. 2007). The CMG complex acts as the replicative helicase during replication and without its presence replication stalls.

Stage	Cell Cycle Phase	Proteins Involved	Function
Licensing/ Pre-RC Formation	G ₁	ORC	Highlights the DNA origins recruiting other replication proteins
		Cdc6	Required for assembly of Mcm2-7 complex at ORC, in conjunction with Cdt1
		Geminin	Binds to and inactivates Cdt1 regulating licensing/pre-RC formation
		Cdt1	Loads Mcm 2-7 complex on DNA at orc (regulated by Geminin)
		Mcm 2-7*	Catalytic core of the replicative helicase
Pre-RC Activation	G ₁ /S	Mcm10	Binds to initiation complex after Pre-RC formation and moves with replication fork during S Phase
		Cdc45*	Required for loading of various proteins for initiation and elongation
		GINS * PSF1 PSF2 PSF3 SLD5	CMG Complex component essential for elongation and normal replication fork progression
		CDK	Required for initiation
		DDK (Cdc7)	Required for initiation

Table 1.1: DNA Replication Proteins the Major Players. The table above highlights the stages for replication machinery assembly including their timing with the cell cycle, some of the proteins involved within each stage and their known/possible functions. *Highlights components of the CMG complex.

Discovery of the GINS Complex

With the study of DNA replication at the heart of cancer biology scientists are constantly seeking out new components that are required to maintain the replication machinery. As a result of early studies examining the role of DPB11, an essential replication protein, a new discovery was made. DPB11's dual roles have it play an important role. Its presence is not only necessary during active replication to load DNA pol epsilon at origins but it also acts at stalled forks by being recruited to activate Mec1p, an ortholog of human TopBP1. (In humans, TopBP1 plays a role in the rescue of stalled replication forks and checkpoint control.) Due to the importance and necessity of the DPB11 protein a genetic screen was designed to look for mutations that would be lethal in combination with the DPB 11 gene, ultimately resulting in the identification of what were termed SLD (Synthetic Lethal with DPB11) genes (Kamimura, 1998). In 2003 scientists unveiled four individual subunits (in three separate studies) dubbed the heterotetrameric GINS (Go – Ichi – Ni – San, Japanese for 5-1-2-3) complex fabricated from: SLD5, PSF1, PSF2, and PSF3. The first study performed in *Sacharomyces cerevisiae* examined the role of DNA Pol ε. The information highlighted therein resulted in information that DPB 11 was required for the loading of Pol ε at replication origins (Takayama et al, 2003). GINS was also recognized as a partner to DNA polymerase β an essential initiation factor along with Cdc45. Both Dup and Cdc45 are essential for the recruitment of DNA Pol α, δ, and ε. (Masumoto et al, 2000). The second study utilized a systematic screen with strains that housed a degron cassette to examine essential budding yeast cell cycle proteins of unknown function out of which a new set of proteins (Cdc105, Cdc101, and Cdc 102) were identified that coincided with three of the GINS subunits respectively (Sld5, Psf1, Psf3) (Kanemaki, 2003). The third study performed looked at immunodepletion of Sld5 from *Xenopus laevis* egg extracts and as a result co-depletion of the

Psf1-3 subunits was observed (Kubota et al, 2003). Depletion of GINS results in the inability of the nuclei to undergo replication highlighting the essential nature of GINS to chromosomal replication. GINS subunits were purified and were shown to occur in a 1:1:1:1 stoichiometric ratio as a stable complex, which would also suggest that each component is necessary for stability of the complex (Kamada, Kubota, et al. 2007).

Thus far there have been several proposed roles for the GINS complexes functionality in the replication process. Association of GINS with chromatin occurs during S phase and it travels along with the replication fork (Aparicio, 2009). Without GINS, Cdc45 will not one, associate properly with chromatin, stopping initiation and two, results in the stalling of the previously initiated replication fork (Takayama, 2003). This evidence shows that the GINS complex is essential in the establishment of the initiation complex as well as the normal progression of the replisome. Most recently, research has suggested that GINS plays a role in stabilization of the interaction between Cdc45 and the Mcm 2-7 hexamer (Aparicio, 2009). In addition, Human GINS has also been shown to bind and specifically stimulate human DNA polymerase α , which is responsible for the synthesis of an RNA primer on both the leading and lagging strand of replicating DNA (De Falco, 2009). All of the roles described are all important elements to the replication process.

In *Drosophila*, SLD5 encodes a protein of 228 amino acids that is composed of a highly evolutionarily conserved alpha and beta domain. Within the GINS complex Sld5 partners with Psf1 through the interaction of their alpha and beta domains, forming a heterodimeric complex, with Psf2 and Psf3 forming the base of the complex as heterodimers. The alpha domain coordinates and forms the central pore of the complex while the beta domain facilitates not only interactions with other proteins, but also helps to maintain the stability of the complex

subunits with one another. At the center of each domain are two conserved residues, arginine and glutamate forming a bidentate Hydrogen bond within the hydrophobic environment responsible for the stability of the complex. Further analysis of the biochemical data generated by Kamada highlights the importance of the B-domain of Sld5 in forming the core of the complex (Kamada, Kubota et al. 2007).

Due to the importance of the GINS complex in maintaining the integrity of the DNA replication process this project has aimed its attention on the SLD5 subunit, as it is highly conserved throughout eukaryotes (figure 1.1). *Drosophila and Homo sapiens* share a 41% identity within the conserved region. Sld5 is a protein-coding gene located within the *Drosophila* genome on chromosome 3R at position 21882213k to 21879955k.

To complete the examination of Sld5 in this study, two P-element insertion mutants were utilized that result in the truncation of the expressed SLD5 protein, cutting off a portion of the B Domain in each mutant reducing the size of the final protein from 228aa to 183aa (figure 1.3). The Sld5 subunit is comprised of two highly conserved domains referred to as the A and B domains. The A domain is composed of predominantly alpha helices while the B domain is largely beta sheets. The subunits interact with one another on a horizontal plain in which the subunits align alternating their A and B domains (Kamada, 2007). Both P-element insert constructs use the piggy bac transposase backbone, PBac{5HPw⁺}Dak1^{A462} and PBac{PB}Sld5[c010719] and are 6.938kb in length (figure1.4). The Piggy Bac transposable element consists of a short inverted repeat (Lobo, Li et al. 1999). When examining this truncation in relationship to the alpha helical/beta sheet structure of Sld5 a large portion of the B domain comprised of the β pleated sheets have been removed in both mutant lines (Figure 1.3).

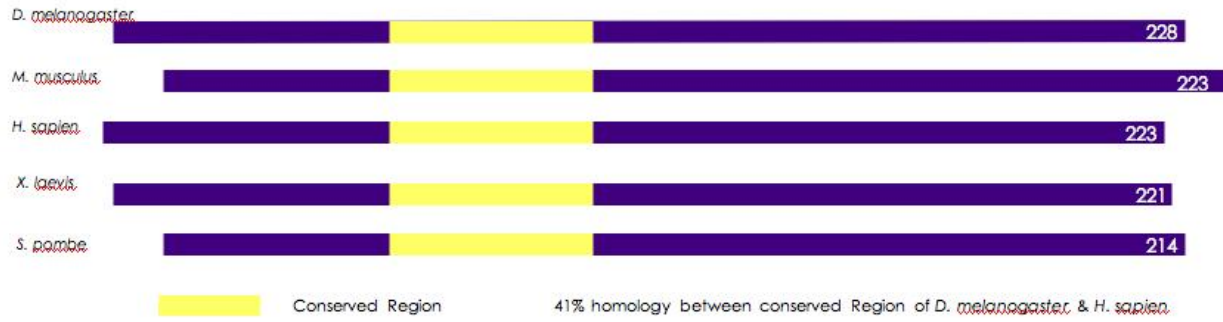


Figure 1.3: Conserved *Sld5* Genomic Sequences. *Sld5* is a GINS complex subunit that is conserved throughout multiple eukaryotic genomes including humans.

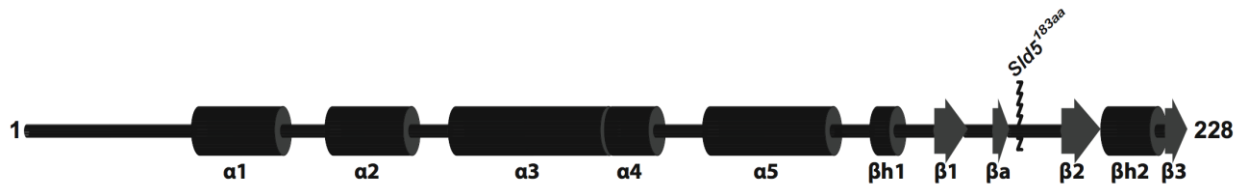


Figure 1.4: *Sld5* Predicted Secondary Structure. *Sld5* is made up of two conserved A and B domains. The figure shows the location of the alpha helical and beta sheet structure including the p-element insertion point.



Figure 1.5: *Sld5* P-element Insertion Site. *Sld5* has 3 exons in which p-element insertion site is highlighted in exon 2 for both mutant strains.

Highlighting the importance of this research stems is the fact that GINS is known to play an integral role during DNA replication, as DNA replication is essential for all Eukaryotes to flourish and develop normally. When there is a problem that arises within S phase of the cell cycle there are multiple replication defects that can arise. As a result of these replication defects

multiple diseases can occur. Some of the disease processes that are resultant from S phase defects documented as part of human disease include: Huntington's Disease, Tetralogy of Fallot and Cancer. With the presence of GINS in its functional state we know that DNA replication proceeds without fault, however in its absence the replication fork stalls (Chang, 2007). Thus, GINS is integral in the maintenance of the replication fork and for the genomes stability during S Phase. Without GINS presence the cell cycle of multi-cellular organisms would fail resulting in events detrimental to the survival of the organism.

Although there has been new evidence yielding a better picture of GINS function it is still poorly understood. An in depth analysis of the individual Sld5 subunit is yet another piece of the puzzle to deciphering how DNA replication maintains its ability to undergo replication without error and how proteins in S phase associate and coordinate with one another to replicate the genome. Through our examination and characterization of SLD5 we hope to gain more insight into the role that not only Sld5 plays within the GINS complex, but also potential insight in to what the entire GINS complex role is within DNA replication. By characterizing this Sld5 protein subunit new evidence may point ultimately towards new targets for drug therapies or other molecular therapies to deal with the understanding and treatment of DNA replication defects in human disease processes.

CHAPTER 2: DETECTION OF S PHASE CELLS IN MULTIPLE *DROSOPHILA* TISSUES UTILIZING THE EDU LABELING TECHNIQUE

INTRODUCTION

Examining cellular proliferation via fluorescent labeling is essential to the study of molecular genetics; specifically, especially for visualizing defects in the cell cycle. Techniques developed previously to examine cellular proliferation include tritiated thymidine incorporation visualized with autoradiography and 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry (Leif *et al.*, 2004). Over the course of the past few decades the BrdU immunohistochemistry labeling method has been standard for labeling of cells in S-phase (Gratzner, 1982; Dolbear, 1995), however a newly developed technique utilizing 5-ethynyl-2'-deoxyuridine (EdU) promises to revolutionize the ability to not only detect DNA synthesis in cells progressing through the cell cycle (Buck *et al.*, 2008; Warren *et al.*, 2009) but to also facilitate multiple labeling of the tissues (Figure 2.1) (Capella *et al.*, 2008).

The Click-iT EdU assay (Invitrogen, Carlsbad, CA, USA) utilizes EdU, a thymidine analogue which, like its predecessor BrdU, is easily incorporated during DNA synthesis. Unlike the required antibody detection in BrdU methods, EdU is detected chemically through a "Click" reaction with a fluorescent azide probe (Buck *et al.*, 2008). The Click reaction is based on a [3+2] Huisgen Copper (I)-catalyzed cycloaddition reaction (Figure 2.1) (Salic and Mitchison, 2008; Rostostev *et al.*, 2002). The small size of the azide molecule utilized in EdU labeling allows it to access the ethynyl group of the incorporated EdU with ease. This highlights one of the many advantages provided by EdU labeling. Typically with BrdU a harsh denaturation step using HCl is needed to open the DNA to provide the anti-BrdU antibody access, however because of its small size, EdU doesn't require this harsh denaturation step. This not only maintains the integrity of the DNA structure but the tissue structure as well (Bock *et al.*, 2006).

EdU labeling affords many advantages over the BrdU labeling method. These advantages include: a significant reduction in protocol time, a gentler cellular treatment, and increased sensitivity (Zeng *et al.*, 2010). EdU has already been shown to have the ability to label the same cells as its predecessor BrdU in multiple studies (Capella *et al.*, 2008; Zeng *et al.*, 2010).

Described in the following is an adapted method for utilizing EdU to label S phase cells simultaneously in multiple *Drosophila* tissues, including: neuroblasts, salivary glands, and wing discs. This adapted method also includes ways to incorporate this technique with other widely used methods including the ability to examine M phase indices and S phase indices.

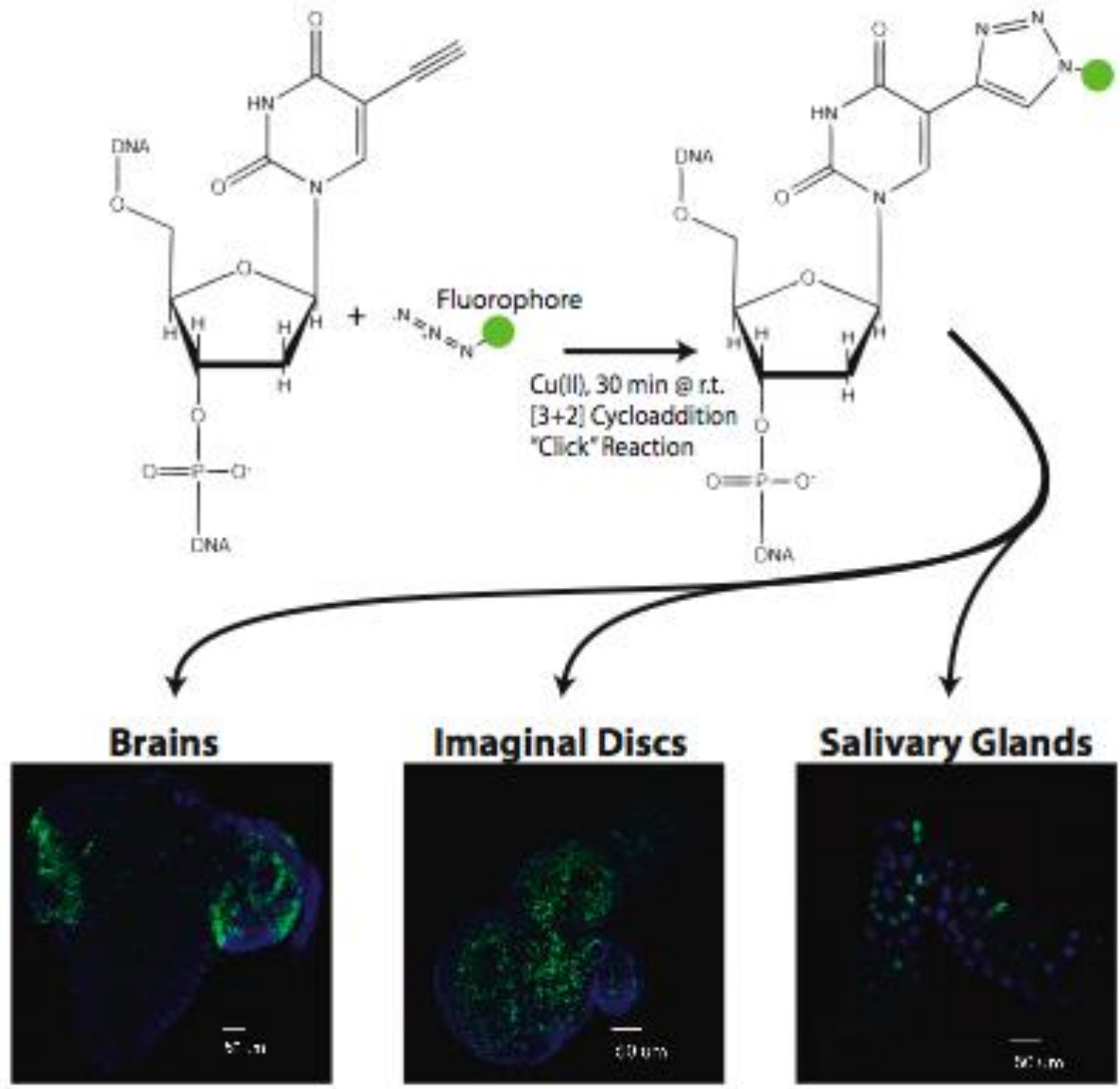


Figure 2.1: EdU reaction scheme and labeling of multiple *Drosophila* Tissues. (Gouge & Christensen, 2010)

EdU, a thymidine analog is incorporated into DNA during synthesis in multiple *Drosophila* tissues.. After incorporation of the EdU molecule a reaction utilizing CuSO₄ results in the 3+2 cycloaddition reaction depicted. The Alexa Fluor 488 fluorescent probe has been added and allows for the visualization of cells progressing through S phase before fixation. Below the reaction scheme various *Drosophila* tissues are shown where EdU has been incorporated (Green) and counterstained for DNA with Hoescht (Blue).

MATERIALS & METHODS

Drosophila Stocks

WT *Drosophila* stock was maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat # D9600-07B). The w¹¹⁸ line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326).

Tissue Acquisition

Wandering third in-star larva were selected and placed in a nine well plate containing 200µL, HyQ[®] Grace's Unsupplemented Insect Cell Culture Medium (Hyclone, Logan, UT, USA). No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA) were used to dissect and isolate the various tissues: brains, salivary glands, and wing imaginal discs. Upon dissection each tissue was isolated in a separate well containing 100µl of fresh Grace's media divided for each treatment group.

EdU Labeling

Recipes for solutions used in the EdU Protocol and the stepwise protocol can be found in Appendix A

A 2X (30µM) working solution was prepared in Grace's from the 10mM EdU stock solution (Click-iT[®] EdU Alexa Fluor Cell Proliferation Assay kit; Invitrogen, Carlsbad, CA, USA) and allowed to come to room temperature while isolating the desired tissue specimens. One half of the tissue was treated with Aphidicolin (Fischer Scientific, Pittsburgh, PA, USA) at 100µg/ml (diluted from a 1 mg/ml stock in DMSO) for 15 minutes and the other half treated with Grace's for the same 15 minute period. At the end of 15 minutes both solutions were removed from the wells housing the tissues and the tissues were washed twice with 200 µL 3% Bovine Serum Albumin (BSA) (Fisher Scientific, Fair Lawn, NJ) in 1X PBS. After the wash was

complete the BSA was removed and 100 μL of Fresh Grace's media was placed in each well followed by 100 μL of the 2X (30 μM) EdU solution (final EdU concentration 15 μM). Tissues were incubated for 30 minutes and the EdU solution removed. A 200 μL solution of 3% BSA in PBS was used to wash the tissues two times.

Tissue Fixation, Permeabilization & EdU Detection

A 3.7% formaldehyde fixative was added for 5 minutes, removed, and the tissues were washed with 200 μL 3% BSA in PBS. The solution was removed and 200 μL of 0.1% Triton-X-100 was added in 1X PBS to each well for 20 minutes. The Click-It[®] reaction cocktail was added containing 20 μL CuSO_4 , 430 μL of 1X Reaction Buffer, 50 μL 1X Buffer Additive, and 1.2 μL of the Alexa Fluor Azide while the tissue was incubating in the permeabilization buffer. After removal of the permeabilization buffer the tissues were washed with 200 μL of 3% BSA in PBS two times after which 200 μL of Click-iT[®] reaction cocktail was added to each well for 30 minutes, removed, and again washed with 200 μL 3% BSA in PBS.

DNA Labeling

To stain DNA a 1X (5 $\mu\text{g}/\text{mL}$) Hoescht33342 solution was added for 15 minutes. Each well was then washed with 1X PBS two times and the tissues were transferred to glass slides and mounted using 7 μL of Vectashield (Vector Laboratories, Burlingame, CA) mounting medium per tissue specimen. A Lifterslip[™] (Thermo Fisher Scientific, Portsmouth, NH # 25X60i-2-4789) was placed on top of the prepared tissue. Slides were then placed at 4[°]C until fluorescence could be visualized using an Olympus IX2-DSU Spinning Disc Confocal Microscope (Olympus America Inc., Center Valley, PA).

Brain Squashes

For brain squashes, after incubation in the 15 μ M 1X EdU solution the brains were incubated for exactly 10 minutes in 0.5% Sodium Citrate. These brains were then lightly fixed with an 11:11:2 acetic acid, methanol, and water solution for 30 seconds. The brains were transferred to individual slides each containing a 5 μ l dot of 1X PBS. A coverslip treated with SigmaCotetm (Sigma Diagnostics, # SL2-25ML) was placed over the tissue and a slide sandwich was created. The sandwich was prepared using a fresh slide, a piece of filter paper cut to the size of the slide placed in the middle, and the tissue specimen with the coverslip facing to the inside on the opposite side. The slide sandwich was then placed in a toolmaker vise (Wilton, Cat #: 11715 Penn Tools, Maplewood, NJ, USA) and a digital torque wrench (Gearwrench #85071) was used to apply 15N of force to the slide sandwich for two minutes. After removing the slide from the vise it was gently and slowly placed into a container of liquid nitrogen for approximately 5 seconds and removed. The coverslip was then popped off using a razor blade and the steps were continued as described above in the prior sections, Tissue fixation/permeabilization and cellular DNA labeling.

Calculation of the M phase & S phase index were performed using 10 brain squash preparations, examining 10 fields of view per brain, counting the total number of cells present, the number of cells positive for EdU incorporation (S phase), and the number of mitotic figures (M phase).

RESULTS

EdU labeling and detection are specific to cells undergoing DNA replication

In order to evaluate the specificity of EdU labeling and detection, dissected tissues were pre-incubated with Aphidicolin, a potent inhibitor of DNA replication, and compared to mock treated tissues (Raff and Glover, 1988) (Figure 2.2). In all cases observed, in multiple tissue types, EdU incorporation and subsequent detection only occurred in those treatments where DNA replication was allowed to proceed normally. In wandering 3rd instar brains (Figure 2.2, top panel) typical DNA replication patterns were observed as characteristic optic lobe proliferation centers stain positive for EdU incorporation. EdU incorporation in wing imaginal discs are also consistent with previous BrdU studies as incorporation occurs in disperse cells due to the fact that cells in this tissue undergo asynchronous cell cycles (Phillips and Whittle, 1993).

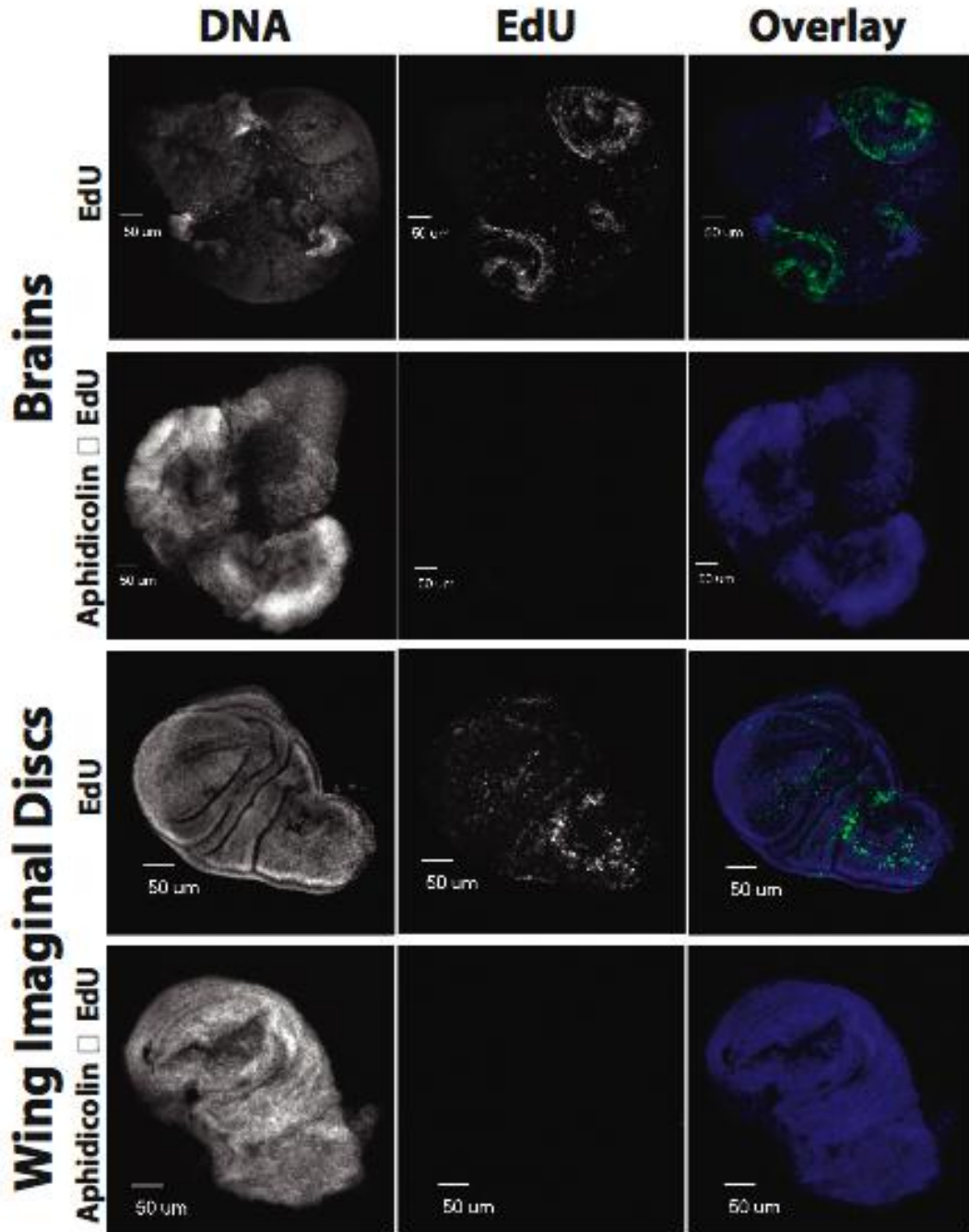


Figure 2.2: S Phase labeling of *Drosophila* Neuroblasts and Wing Discs. (Gouge & Christensen, 2010)

EdU and its detection are specific to cells undergoing DNA synthesis. Both *Drosophila* WT whole mount brain preps and Wing Imaginal discs were exposed to EdU with either a mock or pre-treatment with the DNA synthesis inhibitor Aphidicolin. DNA is stained with Hoescht 33342 and newly synthesized DNA that has incorporated EdU is visualized with a Alexa Fluor 488 probe. In all cases no appreciable detection of EdU is observed in tissue where DNA synthesis is blocked, whereas typical DNA replication patterns are observed in tissue where DNA replication is allowed to proceed.

EdU treatments may be used to generate S phase indices alongside M phase indices

The harsh acid treatment of squashed larval brains required for BrdU detection compromises the integrity of the tissue. As a result the reliable detection of mitotic chromosomes is hampered. Due to the fact that EdU detection is much less harsh, it is more feasible to simultaneously quantitate mitotic and S phase indices (Figure 2.3, top panel). Figure 2.3 (bottom panel) illustrates typical fields of view from brain squash preparations. Multiple fields of view from multiple brains squashes were used to quantify the fraction of cells in either M Phase (# of mitotic figures/total # of nuclei) and the fraction of cells in S phase (# of cells positive for EdU/total # of nuclei). For wild-type *Drosophila* under the condition tested S phase indices are $9.77 \times 10^{-2} \pm 1.9 \times 10^{-2}$ and M phase indices are $9.95 \times 10^{-4} \pm 2.70 \times 10^{-4}$

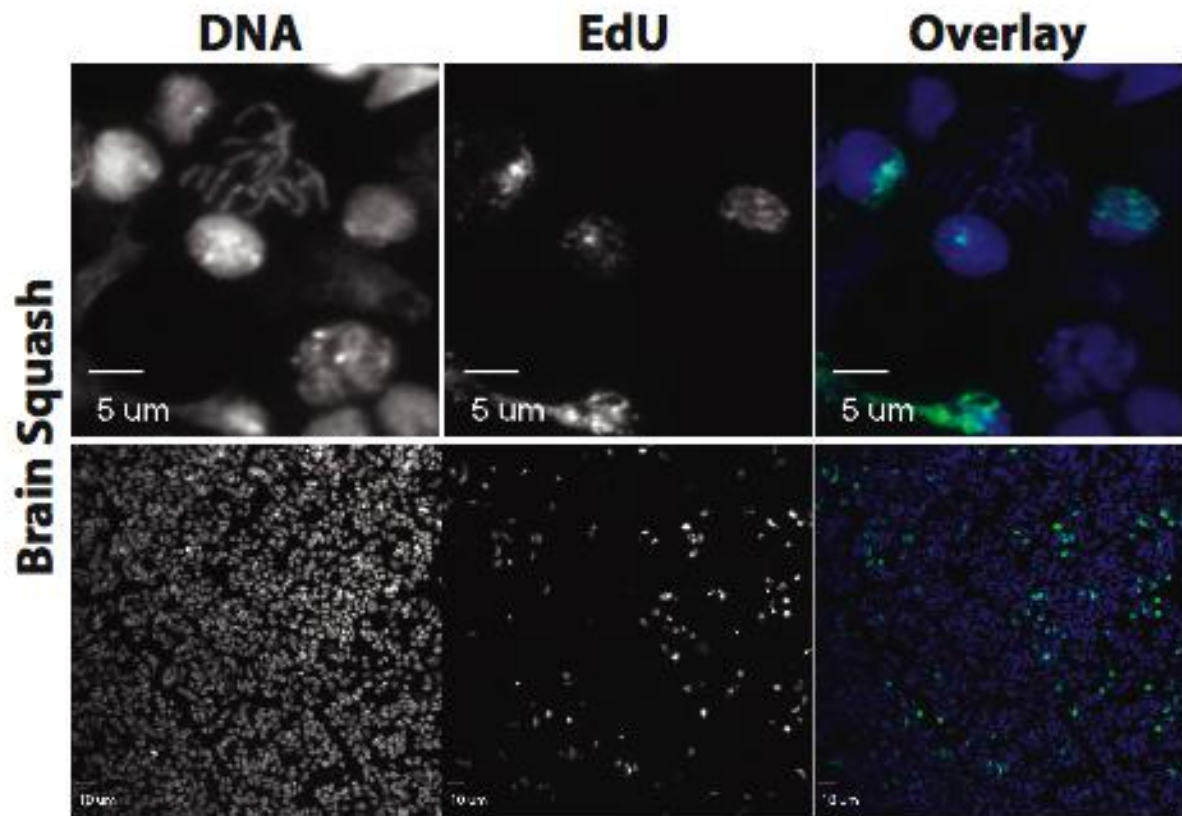


Figure 2.3: Visibility of Mitotic Figures in Conjunction with S Phase Labeling. (Gouge & Christensen, 2010) Examination of mitotic chromosomes and the quantitation of mitotic and S phase indices. Wandering 3rd instar larval brains were prepared as described and mitotic and S phase indices determined. Top panel demonstrates the ability of the method to maintain chromosome structure and the bottom panel shows a typical field of view used for measuring M and S phase indices.

DISCUSSION

In summary, utilizing the EdU labeling technique we have stained multiple tissues, all of which are routinely utilized when studying the cell cycle and DNA replication in *Drosophila*. Not only does this technique afford shorter incubation times and the preservation of the cellular structure, but it is also all completed *in vitro* without pulse feeding, as many of the BrdU assays employ. This new EdU assay is highly reproducible and cost effective compared to earlier techniques. Moreover, this technique is easily adapted to other research models where dissected tissues can be maintained for periods of time in culture media.

CHAPTER 3: SLD5 IS REQUIRED FOR NORMAL CELL CYCLE PROGRESSION AND GENOMIC STABILITY

INTRODUCTION

Eukaryotic chromosomal DNA replication is an essential, elegantly coordinated, and dynamic process essential for the survival of the organism. Genomic DNA responsible for carrying the genetic information must be replicated just once per cell cycle, in addition, it must be done faithfully and completely without error. Due to the importance of replication for survival of the genome, an error occurring can be detrimental to the cell and potentially to the entire organism. Multiple negative outcomes can result because of the genomic instability generated due to replication error, with cancer being the ultimate outcome.

For DNA replication to initiate, multiple essential and non-essential protein factors within the cell must facilitate the assembly and disassembly of the replication machinery at origins of replication (ORC). (Moyer, Lewis et al. 2006; Duncker, Chesnokov et al. 2009). As a result of the protein associations that occur, multiple complexes are formed at the origins, which result in the recruitment of other proteins to the origins. The majority of these complexes that are formed can be looked at as subcomplexes, such as MCM 2-7 and GINS, which form much larger complexes as part of the replication machinery.

One complex that assembles is referred to as the Pre-Initiation complex, also referred to as the CMG complex. Formation of CMG involves the recruitment of GINS and Cdc45, both critical DNA helicase components (Labib and Gambus 2007). Through the intimate association of Cdc45, Mcm 2-7, and GINS (CMG), the CMG complex has been shown to regulate initiation and the subsequent progression of replication (Bauerschmidt, Pollok et al. 2007). Due to its replicative helicase activity, its absence during replication results in stalling of the replication fork (Aparicio, Ibarra et al. 2006; Labib and Gambus 2007). After assembly is complete, DNA

Replication is slated to fire at the origin of replication site. While there have been several proposed roles for GINS functionality in the replication process it is still poorly understood. The most recent research has suggested that GINS plays a role in stabilization of the interaction between Cdc45 and the Mcm hexamer (Aparicio, 2009).

The ~ 100 kDa GINS complex has been shown to be an essential complex for both initiation and elongation, serving as a complex that aids in the progression of the MCM helicase along the replication fork (Marinsek et al, 2006). GINS is a heterotetrameric complex fabricated from: SLD5, PSF1, PSF2, and PSF3. The SLD5 protein subunit was originally identified in *Saccharomyces cerevisiae* as synthetically lethal with *dpb11*, thus *Sld5* is the gene that we have focused our investigation on (Kamimura, Masumoto et al. 1998).

In *Drosophila*, SLD5 encodes a protein of 228 amino acids that is composed of both highly evolutionarily conserved alpha and beta domains. *Sld5* spans a region on *Drosophila* chromosome 3R from 31:21882213 to 31:2187995, as shown in Figure 3.1A. Within the GINS complex, *Sld5* partners with *Psf1* through the interaction of their alpha and beta domains (Labib and Gambus 2007). The alpha domain coordinates and forms the central pore of the complex while the beta domain facilitates interactions with other proteins (Kamada, Kubota et al. 2007). GINS assembly is essential to maintaining the viability of the CMG complex and thus the continuation of replication.

In this work we have characterized two transposable p-element mutant lines for *Sld5*, both isolated from the Genomic mapping of the Exelixis third chromosomal p-element insertion gene disruption project (Thibault, Singer et al. 2004). The two mutant lines being examined are *Sld5*^{c010719} (PBac*Sld5*c01719) and *Sld5*^{A462} (*y*¹*w*¹¹¹⁸;PBac{5HPw+}Dak1A462/TM3,Sb¹Ser¹) (Kill, Bridger et al.). In both of the insertion mutants, *Sld5*^{c010719} and *Sld5*^{A462} the transposable p-

elements result in the truncation of the protein by 136aa and 108aa respectively (Figure 3.1B). When examining Sld5 mRNA it was recognized that Sld5 is a multicistronic-processed transcript along with Dak1 (Figure 3.1A) thus, concern arose that the observed phenotypes might not be solely due to Sld5. As a result of this discovery, a series of deletion mutants were utilized to examine whether or not the exhibited phenotypes were due to Sld5 and not a result of interference from the other gene present, Dak1. The deletion mutants utilized spanned beyond the entire Dak1, Sld5 region as well as a single deletion mutant that spanned the Dak1 region (Figure 3.1A).

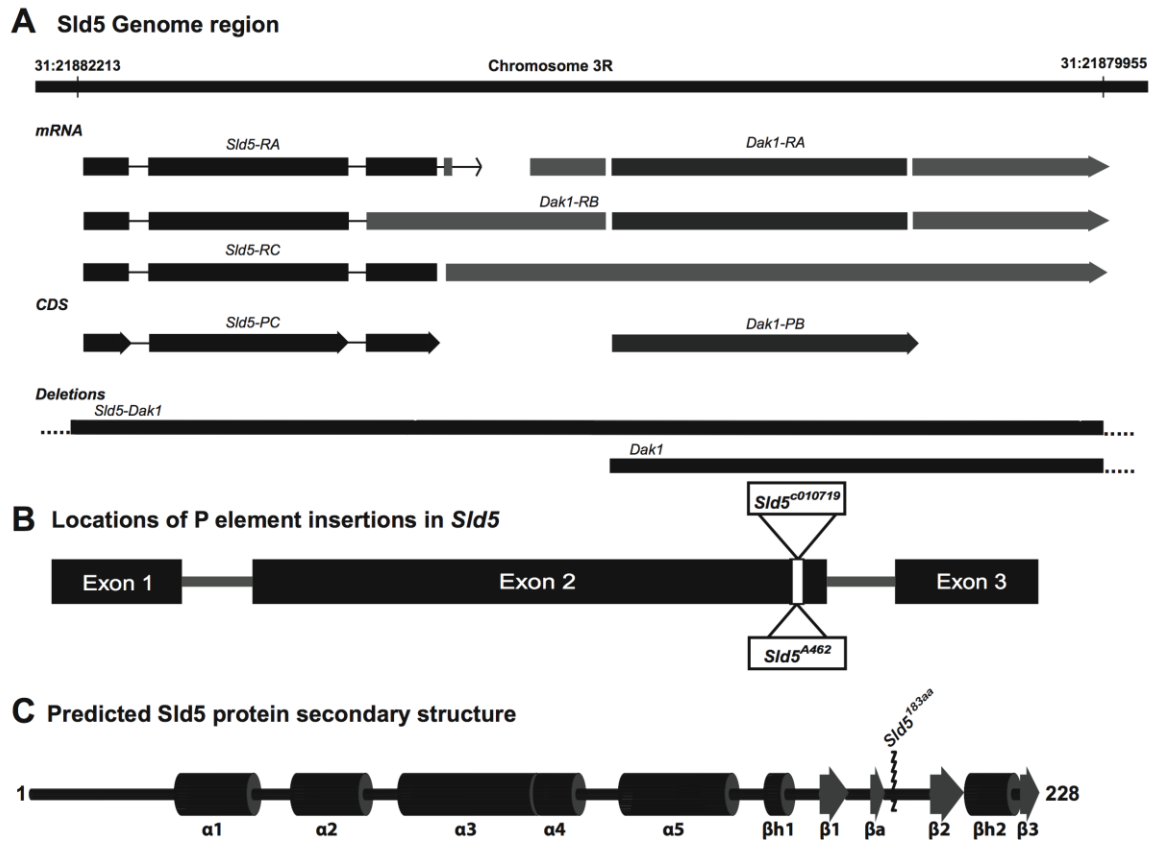


Figure 3.1: Gene Region, P-element insertion, and Protein layout. A) Sld5 gene is located on chromosome 3R from 2188213 to 21879955. Sld5 is a multicistronic-processed protein that codes for both Sld5 and Dak1. To show that the mutations were due to Sld5 we performed complementation testing utilizing deletion mutants with a mutant that spanned the entire Sld5, Dak1 region as well as a mutant that spanned only Dak1. B) Highlights the insertion site of the p-element in exon 2 for both mutants. C) Sld5 is comprised of both an A and B Domain, composed of multiple alpha helices and B sheet regions. When compared with B it is clear that the p-element insertion points occur in a portion of the B sheet region of Sld5 which is believed to be the region that facilitates interactions with other proteins that play a role in DNA replication.

Utilization of multiple experimental techniques in conjunction with the examination of various tissues was essential to the characterization of each of the p-element Sld5 mutants. *Drosophila* brains were prepared to allow for the examination of M & S Phase indexes in the neuroblast, while simultaneously examining the chromosomes looking for any observable defects. Embryos were examined to determine the arrest point and whether or not any other embryonic defects were observable. In addition, RNAi lines were utilized to knockdown expression of Sld5 in brains to examine the significance of a reduction in the amount of expressed Sld5 being present in varying tissues ultimately answering whether or not the *Drosophila* were viable.

MATERIALS AND METHODS

Drosophila Stocks

Wild type (yw¹¹¹⁸, Bloomington Stock Center, Bloomington, IN) and all mutant strains were maintained at 25°C on standard medium. Both the Sld5^{c010719}/sb¹,ser¹ (Sld5⁰¹⁰⁷¹⁹) and y¹w¹¹¹⁸;PBac(Duncker, Chesnokov et al. 2009){5HPw+}Dak1A462/TM3,Sb¹Ser¹ (Sld⁵⁴⁶²) (Bloomington Stock Center, Bloomington, IN) lines are both PBac insertional mutagenesis lines on the third chromosome from the Exelixis Collection. The RNAi lines utilized for the brain analysis included Brain P{GawB}167Y, w1118 and p[sld5], RNAi.

Nucleic Acid Procedures

Genomic DNA and cDNA were isolated via a simple genomic extraction (Sullivan, Ashburner, 2000) (Appendix A – Protocol 2) and amplified using Platinum Pfx[®] DNA Polymerase (Invitrogen, Carlsbad, CA).

Component	Volume	Final Concentration
10X Pfx Amplification Buffer	5.0 µl	1X
10mM dNTP Mixture	1.5 µl	0.3mM each
50mM MgSO ₄	1.0 µl	1mM
Primer 1 & 2 (10 uM each)	1.5 µl each	0.3 µM each
Template DNA	1.0	----
Platinum Pfx DNA Polymerase	0.5 µl	1.0 unit
Molecular Grade Water	to 50	----

Table 3.1 Platinum[®] Pfx PCR Reaction Mixture Components

The primers utilized were as follows:

Genomic Primers: 5'- CAC CAT TTA CCA GAA GGA TT GTT TGG A - 3'

5'- AAT TAG CTG CGC TTG GTT GTT TTG - 3'

cDNA: 5' - TTA AAT TAG CTG CGC TTG GTT GTT TTG - 3'

5'- CAA CAT GTC GGA TGT AGA AGA CGT G - 3'

After gel electrophoresis confirmed amplification of the desired genomic DNA and cDNA (Appendix B – Figure 1& 2) both products were cloned using the pENTRD/Topo[®] Cloning kit (Invitrogen, Carlsbad, CA). The desired genomic DNA product was cloned into the PTWM vector in competent ccdB *E. coli* with the desired insert being confirmed by enzymatic digest (BSRG1) and sequencing. The confirmed genomic Sld5 DNA cloned into the PTWM vector was then sent to Best Gene[®] Incorporated (Best Gene Inc, China Hills, CA) for the development of transgenic fly lines.

Reagent	Chemical Transformation
Fresh PCR Product	1.0 μ l
Salt Solution	1.0 μ l
Water	3.0 μ l
Topo [®] Vector	1.0 μ l
Final Volume	6.0 μ l

Table 3.2 Reaction mix for pENTRD/Topo[®] Cloning

Transgene Rescue

The returned transgenic fly lines were monitored and a line was selected that had the ability to lose the CyO (curly) balancer. A cross (Appendix C: Cross 1) was established to test whether or not the generated transgene had the ability to rescue the phenotype of the Sld5 p-element truncation lines.

Yeast 2 Hybrid Analysis

Drosophila cDNA was cloned using the pENTRD/Topo[®] Cloning kit recipe below (Invitrogen, Carlsbad, CA) and transformed into One Shot[®] chemically competent *E. coli*.

The isolated Sld5 cDNA was then cloned into three different vectors: pGBKT7GW, pGADT7GW and pGBKTetT7GW (Appendix A: Figure 1 & 2) for use in the yeast two-hybrid screening assay.

Yeast Two Hybrid Analysis begins by cloning the desired cDNA plasmid into the appropriate vector. For the general screen pGADT7GW and pGBKT7GW were utilized. First, the plasmid transformed into AH109 yeast transformed with pGADT7GW was used to transform in cDNA from the following list of plasmids: pGADT7GW Hp1, pGADT7GW Mcm10, pGADT7GW Psf1, pGADT7GW Psf2, pGADT7GW Psf3, pGADT7GW Cdc45, pGADT7GW Mcm2, pGADT7GW Mcm5, pGADT7GW Dup, and pGADT7GW Cdt1. AH109 yeast containing the pGBKT7GW vector was transformed with the confirmed Sld5 cDNA plasmid and plated. An attempt was also made to identify new interactors with Sld5 using an embryo derived cDNA library in conjunction with the pGBKTetT7GW vector and the pGADT7GW vector.

Arrest Point

Through crossing the Sld5 mutant fly line with a GFP balancer line and selecting for the glowing larvae, established an Sld5⁴⁶²/TM3 Sb, GFP fly line. Sld5⁴⁶²/TM3 Sb, GFP flies were isolated in a collection chamber and fed yeast paste for 24 hours. After removing the yeast paste a fresh grape plate with a film of yeast paste spread over its surface was introduced for the flies to deposit their embryos. Embryos were collected for 4-5 hours. The embryos were examined under an Olympus SZX7 Dissecting Stereo Microscope with X-Cite Series Q Epifluorescence illumination for GFP and separated based on their phenotype. Non-glowing flies were homozygous Sld5/Sld5 (experimental) and moderately glowing embryos were isolated as SLD5/GFP (control). Each set of embryos was aligned in a grid like fashion on two separate grape

plates and followed. As the Sld5/TM3 Sb, GFP embryos began to hatch the Sld5 homozygotes were examined to see if hatching occurred as normal and the arrest point was designated.

Brain Squash for M Phase Index

Brains were dissected in a 1% PEG 8000 in 1XPBS pH 7.2 solution and immediately transferred to a 0.7% Sodium Citrate Solution for 10 mins to allow the brains to swell. The brains were then transferred to a 11:11:2 Acetic Acid, Methanol, Water solution placed on a glass slide and a Sigmacote[®] (Sigma-Aldrich, St. Louis, MO) siliconized coverslip was added. The brains were squashed using a machinist vice and a digital torque wrench applying a force of 15 Nm for two minutes. After removing the brains from the vice the slides were dipped in liquid nitrogen and the coverslip was removed. The slides were treated in Ethanol, allowed to air dry, and were covered with 7 μ L of Vectashield (Burlingame Laboratories, Burlingame, CA) containing DAPI. A new coverslip was added and the slides were stored at 4^oC until imaging. Calculation of the M phase index was performed using 10 brain squash preparations, examining 10 fields of view per brain, counting the total number of cells present and the number of mitotic figures (M phase). (Appendix A: Protocol 7)

EdU Labeling Whole Mount Tissues

Tissues were dissected in Grace's Cell Culture medium at room temperature and incubated in a 1X 15 μ M EdU solution for 35 minutes in Grace's Cell Culture Medium. The tissue is washed two times with 3% BSA in 1X PBS. A 3.7% Formaldehyde fixative was applied for 10 minutes followed by two washes with 3% BSA in 1X PBS. A 0.5% PBT-X solution was added for 20 minutes followed by two washes in 3% BSA in 1X PBS. The Click-It[®] Reaction Cocktail was applied and the tissue incubated for 30 minutes in the dark followed by two washes with 3% BSA in 1X PBS. A 1X Hoescht 33342[®] (5 μ g/mL) solution in 1X PBS was added and

the tissue incubated for 20 minutes in the dark followed by two 1X PBS washes. The tissue was transferred to a microscope slide and 10 μ l of Vectashield mounting medium was added and a Liftersliptm coverslip was placed over the wholemount tissue. The coverslip was sealed with clear fingernail polish (Appendix A: Protocol 1).

EdU Labeling followed with brain squash for S phase index

Tissues were dissected in Grace's Cell Culture medium at room temperature and incubated in a 1X 15 μ M EdU solution for 35 minutes in Grace's Cell Culture Medium. The brains were then transferred to a well containing 0.5% Sodium Citrate Solution to allow the brains to swell for 10 minutes. The brains were then placed in an 11:11:2 Acetic Acid, Methanol and Water solution for 30 seconds. The brains were immediately transferred to a slide containing a dot (~5 μ l of 1X PBS) and a Sigmacote[®] coverslip was placed on top of the brain. The slide was then placed in a "sandwich" with a piece of filter paper separating it from another glass slide. This "sandwich" was then placed in a machinist vise and a torque wrench was used to apply a 15.0 Nm force to the slide for 2 minutes. The slide was then removed and a blue sharpie was used on the reverse side of the slide to circle the area that the brain encompassed. The slide was then gently dipped into liquid nitrogen. The coverslip was then popped off and the slide was washed with 3% BSA in 1X PBS. A 0.5% PBT-X solution was added for 20 minutes followed by two washes in 3% BSA in 1X PBS. The Click-It[®] Reaction Cocktail was applied and the tissue incubated for 30 minutes in the dark followed by two washes with 3% BSA in 1X PBS. A 1X Hoescht 33342 (5 μ g/mL) solution in 1X PBS was added and the tissue incubated for 20 minutes in the dark followed by two 1X PBS washes. After washing, 10 μ l of Vectashield (Vector Laboratories, Burlingame, CA) mounting medium was added and a coverslip was placed over the tissue and sealed with clear fingernail polish. Calculation of the S phase index was

performed using 10 brain squash preparations, examining 10 fields of view per brain, counting the total number of cells present and the number of cells positive for EdU incorporation (S phase). (Appendix A: Protocol 1).

Embryo Analysis

Drosophila of the desired genotype were isolated in a collection chamber and fed yeast paste for 24 hours. After removing the yeast paste a fresh grape plate with a film of yeast paste spread over its surface was introduced for the flies to deposit their embryos. Embryos were collected for 4-5hours, removed and another grape plate was introduced prepared as above. The embryo harvest was repeated for a twelve-hour period and the flies were again fed with yeast paste for 24 hours. After each collection the embryos were harvested from the grape plate and placed in a collection tube affixed with a mesh over the open end. The embryos were treated with a 50% bleach solution until the dorsal appendages disappeared signifying the removal of the embryos chorion. The embryos were immediately washed with 1X Embryo Wash Solution followed by a rinse with dH₂O. The embryos were placed into a glass vial where 500µl of Heptane was added followed by 250µl of Methanol. The embryos were shaken vigorously for 15s and the embryos were allowed to settle to the bottom of the tube for 1 minute. Those that sank to the bottom, of the vial were devitellinized. The upper heptane and methanol layers were removed, fresh methanol was added and the tubes were stored at 4°C overnight. The next day the methanol was removed from the embryos and they were rehydrated in PBTA for 15 mins on a rotator. After removal of the PBTA solution, 495µl of fresh PBTA was added along with 5µl of 100X DAPI in 1X PBS. The tubes were covered in tin foil and placed on a rotator for 5 mins. The staining solution was removed and fresh PBTA was added and immediately removed. After PBTA removal, 500 µl of fresh PBTA was added and the embryos were placed on the rotator for

one hour. The embryos were placed on a fresh microscope slide and covered with Vectashield (Vector Laboratories, Burlingame, CA) mounting medium and a Lifterslip[™] (Thermo Fisher Scientific, Portsmouth, NH # 25X60i-2-4789) was added. (Appendix A: Protocol 5)

RESULTS

Interaction with other GINS subunits confirmed

As has been shown previously the GINS complex interacts with multiple proteins, some of which are other members of the GINS complex, as well as other replication proteins (Gambus, Jones, et al. 2006). Yeast two-hybrid analysis utilizing *Drosophila* Sld5 cDNA yielded confirmation of the interaction of the SLD5 protein with other GINS complex subunits, Psf1 and Psf2. An interaction between SLD5 and Mcm10 was also observed via yeast two-hybrid analysis. All of the proteins exhibiting interactions with SLD5 are essential members of the replication machinery.

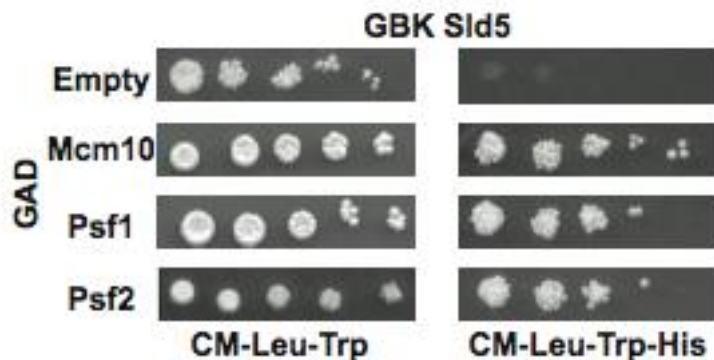


Figure 3.2: Yeast 2 Hybrid Confirms Interactors. Yeast two-hybrid utilizing serial dilutions highlights Psf1 and Psf2 as other members of the GINS complex, which interact with Sld5. Mcm10, another replicative protein, specifically a member of the Pre-initiation complex was also shown to interact with Sld5.

Embryonic Cell Cycle Delay and Arrest Point

To examine the effects of Sld5, the arrest point for Sld5 homozygotes was determined through the use of a GFP balancer line. Based on the observed arrest point in the developing *Drosophila* larvae, Sld5 is shown to be essential for maintaining viability in the developing larvae potentially pointing to a physiological dosage required for the fly to thrive and go through normal development. In addition, 4-5 hour syncytial embryos from both mutant lines were analyzed (Figure 3.3). Wild type embryos examined showed synchronous nuclei with no mitotic abnormalities. Further examination of the mutant lines Sld5^{A462}/TM3 Sb, ser and Sld5^{C010719}/TM3 Sb, Tb highlighted some interesting phenotypes. In Sld5^{A462}/Sld5^{A462}, anaphase bridging and asynchronous nuclei were seen (Figure 3.3, Panel B), whereas Sld5^{C010719}/Sld5^{C010719} highlighted what we believe to be cellular dropout occurring at the surface of the embryo or the inability of the cells to ever migrate to the outer edge of the embryo (Figure 3.3, Panel C).

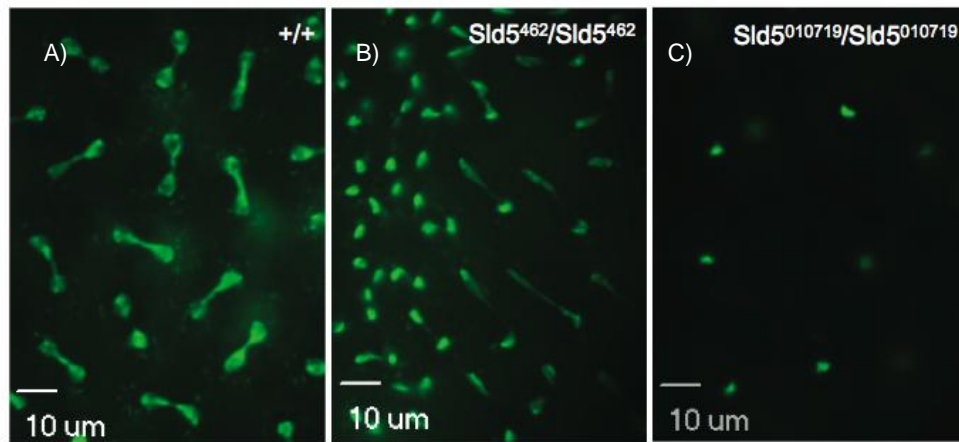


Figure 3.3 Observed Embryonic Defects in *Drosophila*. Early embryo analysis highlights cellular abnormalities stained with DAPI (green). A) Wild Type embryo B) Sld5^{A462} embryo and C) Sld5^{C010719} embryo.

M-Phase Delay and Chromosome Biology Defects Observed in Larval Brains

Mitotic Indexes were calculated from all lines and compared to Wild Type with each line having a delay in M-Phase. A higher degree of delay is observed in the $Sld5^{c010719}/TM3$ Sb, Tb line when compared to the $Sld5^{A462}/TM3$ Sb, ser and $Sld5^{RNAi}$ lines. In addition, when comparing each p-element line alongside WT there is an increase in Mitotic figures in each mutant line. For each line the M phase indices under the conditions tested are as follows: Wild type $9.95 \times 10^{-4} \pm 2.70 \times 10^{-4}$, $Sld5^{A462}$ $5.87 \times 10^{-3} \pm 1.0 \times 10^{-6}$, $Sld5^{c010719}$ $8.20 \times 10^{-3} \pm 1.1 \times 10^{-5}$, $Sld5^{RNAi}$ $7.51 \times 10^{-3} \pm 2.0 \times 10^{-5}$. Looking at these means closer it is easily seen that the $Sld5^{c010719}$ and $Sld5^{A462}$ line exhibit an increased mitotic delay compared to Wild type. Since an increase in mitotic figures was observed the mitotic figures were then analyzed for any visible defects at a higher magnification.

Mitotic figures were examined from each field of view at 100X, with Figure 3.4A highlighting several of the detected malformations. The most severe of which are exhibited in the $Sld5^{c010719}$ p-element line (Figure 3.4A: III-V). In addition, $Sld5^{c010719}$ shows telomeric fusions (Figure 3.4A: V) approximately 22.9% (Figure 3.4B) of the time, as well as polycentric chromosomes (Figure 3.4A: III & IV) 16.6% of the time. $Sld5^{A462}$ exhibits condensation defects being shown by the long arms of the chromosomes in comparison to wild type (Figure 3.4A: II). $Sld5^{RNAi}$ mitotic figures show anaphase bridging (Figure 3.4A: VI) 33% of the time (Figure 3.4B). Each of the observed mitotic defects in each of the mutant lines point toward genomic instability as a result of the truncation or the knockdown of *Sld5* in the developing *Drosophila* neuroblast.

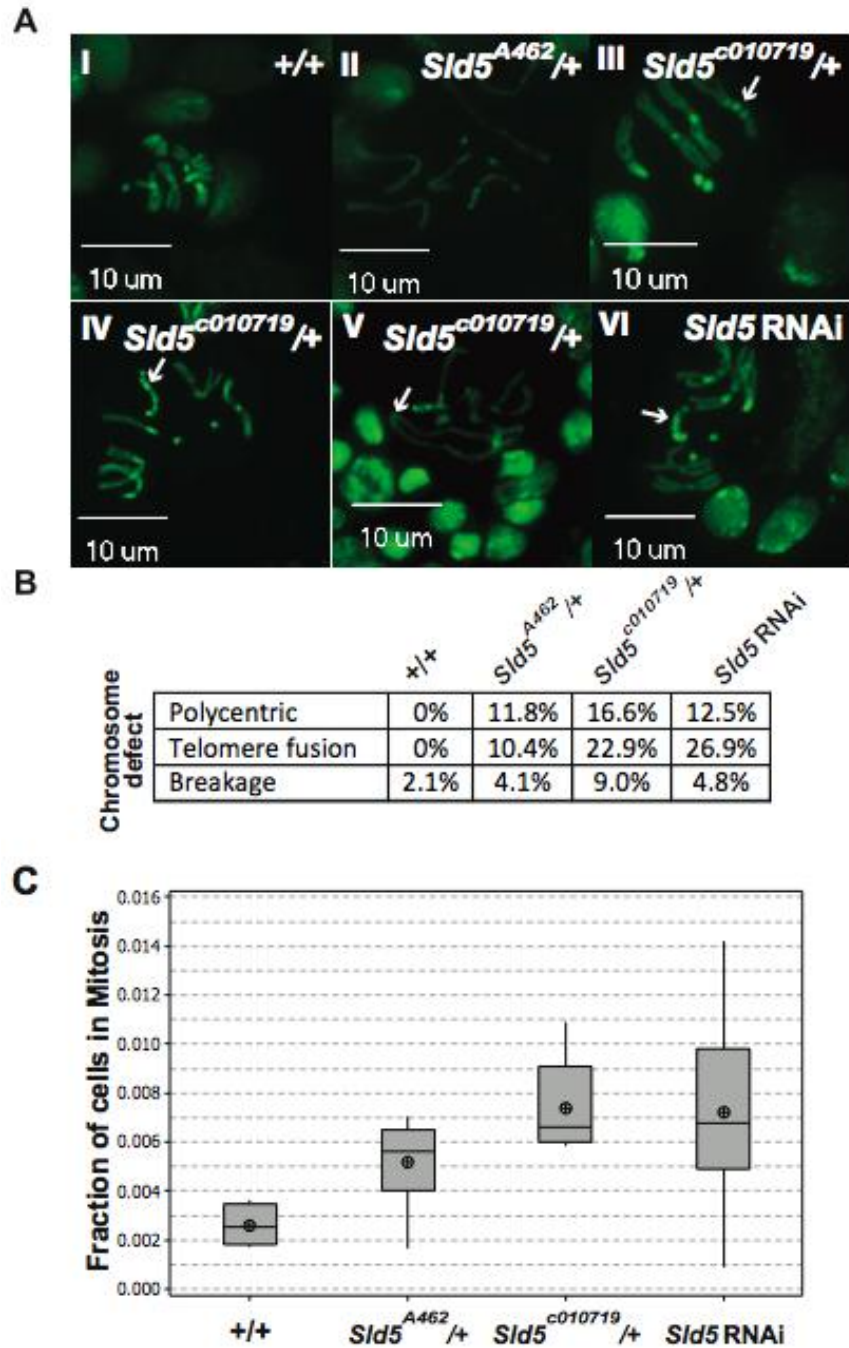


Figure 3.4: Observed Mitotic Defects and M-Phase Delay in the developing neuroblast. **A:** I) Wild type mitotic figures. II) *Sld5*^{A462} shows potential condensation defects in the chromosomes. III & IV) *Sld5*^{c010719} highlights the presence of Polycentric Chromosomes. V) *Sld5*^{c010719} also exhibits telomere fusions. VI) *Sld5*^{RNAi} consistently shows an X chromosomal abnormality. All of these phenotypic abnormalities highlight the importance of *Sld5* for maintaining normal cell cycle progression. **B:** Graph of the average % Mitotic Figures within the developing neuroblast from each line examined highlighting a delay in S phase in all of the mutant lines.

S-Phase Delay Observed in Larval Brains

Next we examined EdU incorporation in the *Drosophila* 3rd In-star larval brains (Figure 3.5) in order to determine whether or not the *sld5* mutants possessed defects in S phase progression. The brains were prepared and squashed to calculate an S phase index based on the fraction of EdU cells present. The subsequent calculation revealed a significant delay in S phase in the *Sld5*^{RNAi} brain with only mild delays being observed in the *Sld5*^{A462} and *Sld5*^{c010719} heterozygous lines. For Wild type *Drosophila* under the condition tested S phase indices were as follows: $9.77 \times 10^{-2} \pm 3.43 \times 10^{-2}$, for *Sld5*^{A462} $1.30 \times 10^{-1} \pm 2.8 \times 10^{-2}$, *Sld5*^{c010719} $1.19 \times 10^{-1} \pm 4.4 \times 10^{-2}$, *Sld5*^{RNAi} $1.76 \times 10^{-1} \pm 6.1 \times 10^{-2}$. The S Phase delay exhibited points toward a role for *Sld5* in maintaining replication through a potential required minimum physiologic dependent dose.

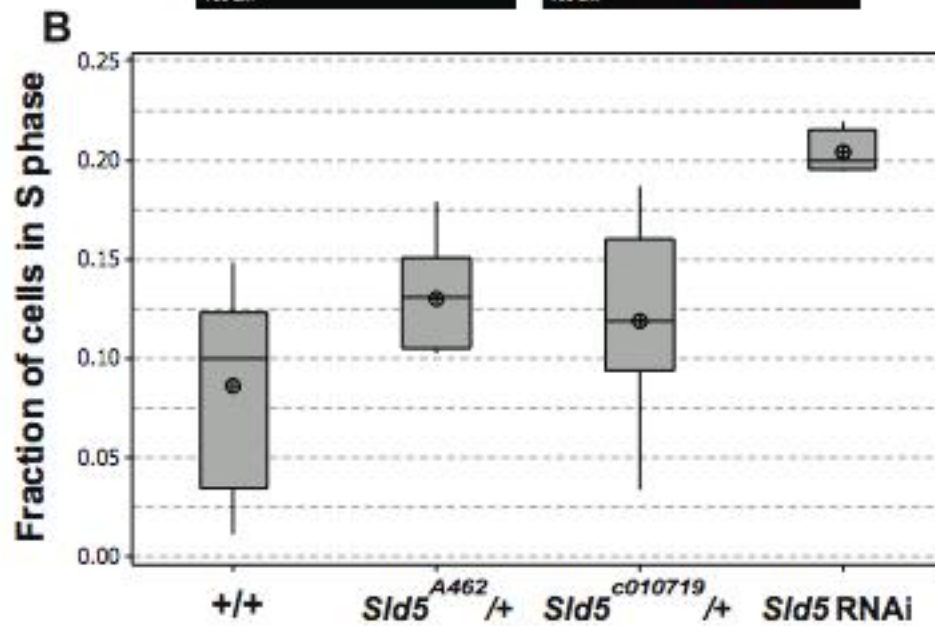
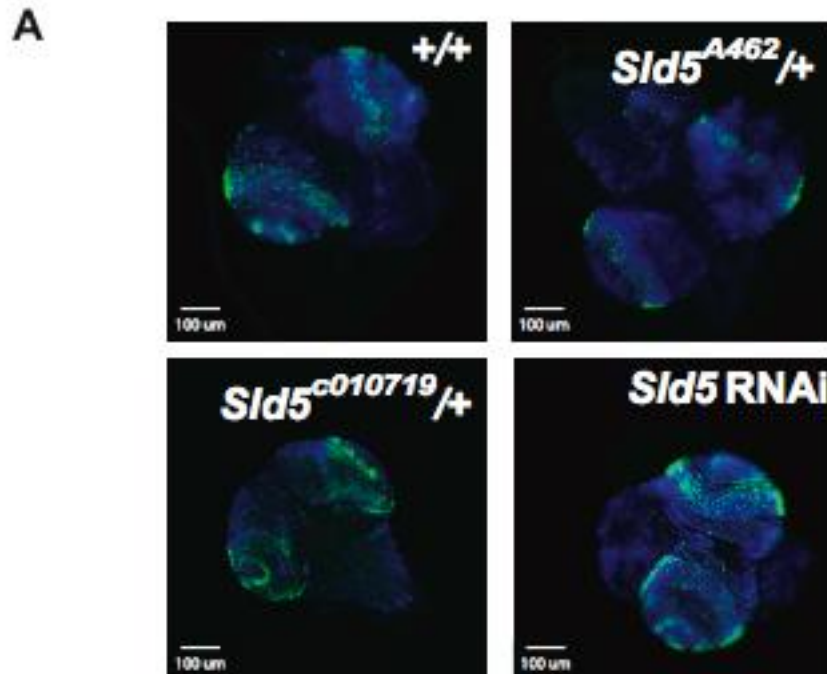


Figure 3.5: RNAi knockdown exhibits more cells in S-Phase. EdU incorporation was utilized to determine the S-Phase index in all fly lines. Gal4-UAS RNAi knockdown of the Sld5 protein in the neuroblast stained with EdU shows an increase in cells in S phase.

DISCUSSION

GIN5 has been shown to be an essential component to the replication machinery after having been isolated in 2003 (Kubota, Takase et al. 2003). As has been previously shown, the GIN5 complex interacts with multiple proteins involved at the replication fork as well as other GIN5 members. Specifically Sld5 interacts with the Psf1 and Psf2 subunits providing stability to the complex. Yeast two-hybrid analysis yielded confirmation of known Sld5 protein interactors including: Psf1, Psf2, and Mcm10 (Figure 3.2). Each of the interactors is either a member of the GIN5 complex or a major player in DNA replication. Next we wanted to examine what would happen to the GIN5 complex if a modified copy of the Sld5 protein were present. Through the use of p-element heterozygous insertion lines, Sld5^{A462} and Sld5^{C010719} we were able to analyze the phenotypes exhibited from these Sld5 truncations in hopes of gaining a better insight as to what an abnormality in Sld5 looks like.

Probing further, the function of Sld5 becomes more important. Starting with the arrest point we have isolated that there is a need for Sld5 to be present, possibly at some physiological dependent dosage as the embryo undergoes development. *Drosophila* homozygous early embryos revealed a late embryo/early larval arrest point (data not shown), highlighting the necessity of Sld5 presence during early development and replication. In *Drosophila*, maternal loading provides the young embryo with the means to survive until embryonic stage 10-12 at which time the embryo takes over producing its own proteins. Because of maternal loading handing off to the embryo in early development, more commonly called the mid-blastula transition (Weigel and Izaurralde 2006), we believe that the Sld5 protein is being used up or in effect being diluted. Since the embryo is a rapidly changing structure with the nuclei constantly dividing to form the adult fly, the amount of Sld5 present for replication origins would

effectively be decreasing with each division and ultimately causing the embryo to expire. In addition, examination of early embryos from both homozygotic $Sld5^{A462}$ and $Sld5^{C010719}$ truncation mutants exhibited multiple mitotic defects manifesting themselves as: anaphase bridges, nuclear asynchrony and cellular dropout (Figure 3.3). In these situations due to the $Sld5$ truncation we believe the GINS complex attempts to assemble are failing because of the amount of $Sld5$ available. A large portion of the $Sld5$ B domain has been removed in these p-element insertion mutants truncating the protein from 228aa to 183aa. In addition, based on what we know about the B domain and its function in maintaining the complex's stability we believe that while the GINS complex is trying to form it may not be able to maintain a stable confirmation. Thus the complex is falling apart resulting in its inability to participate in the replication of the genome in a normal fashion.

At this point we know that $Sld5$ exhibits multiple phenotypic defects but we have only brushed the surface. Not only were mitotic delays exhibited in the embryo but they were also visualized in the brains and expressed numerically with the calculation of the Mitotic Index for each line, highlighting an M phase delay in both mutant lines, as well as in RNAi. Along with the M phase delay severe defects were observed in chromosome biology, which revealed themselves in multiple ways: anaphase bridging, polycentric chromosomes, chromosome breakage, and telomere bridging. These individual Mitotic figure phenotypes were examined in Figure 3.4 highlighting several of the most common and severe malformations. The most critical of which are exhibited in the $Sld5^{C010719}$ p-element line (Figure 3.4A: III-V). In addition, $Sld5^{C010719}$ shows telomeric fusions (Figure 3.4A: V), as well as polycentric chromosomes (Figure 3.4A: III & IV). $Sld5^{A462}$ exhibits condensation defects being shown by the long arms of the chromosomes in comparison to wild type (Figure 3.4A: II). All of the observed mitotic

defects point toward genomic instability as a result of the truncation or the knockdown of Sld5 in the developing *Drosophila* neuroblast. In addition, the calculated mitotic delay in the RNAi line correlates with the mitotic delay in Sld5^{C010719} and Sld5^{A462} when compared to Wild Type. Based on the data herein in combination with the structural data available on the GINS complex it is possible that these truncations have removed a large portion of the essential interaction domain for not only the GINS subunits but also the ability of proteins to dock with Sld5 and interact with the replication machinery.

In 2009 a group examining human SiRNA treated HeLa cells generated data that resulted in the proposal for GINS to have two roles in both the initiation and elongation phases of replication (Aparicio, Guillou et al. 2009). Calculation of the S phase index utilizing EdU incorporation brought forth a moderate delay in S phase in both p-element lines, however a significant delay in S phase was observed in the RNAi line (Figure 3.5B). The S Phase delay exhibited in each of the mutant lines suggests that Sld5 is required to maintain normal cell cycle progression. Since Sld5 has a role as a subunit of the GINS complex we suspect that the GINS is unable to form adequately to allow replication to occur at the normal speed required for the neuroblast tissue. Due to the heterozygous mutant lines only having one good copy of Sld5 present we expect that the amount of Sld5 present is effectively half of what it would be in a Wild Type fly. The delay observed in S Phase in the developing neuroblast points toward a role for Sld5 in maintaining replication through a potential required minimum physiologic dependent dose.

The delays shown in the mutant lines in M phase show that there is a delay in the cell cycle potentially as a byproduct of the β -sheet truncation generating instability within the complex. To show that each of these exhibited phenotypes was due to Sld5 we utilized the

transgenic fly line that had the transgene inserted on chromosome 2. By utilizing this 2nd chromosome transgene we had hoped to see a rescue of the wild type phenotype. At this time we have not been able to achieve rescue utilizing the transgene, however we are confident based on complementation testing utilizing the Dak1 and Sld5 deficiency lines that we can make the argument that these p-element mutant lines are due to the Sld5 truncation and not as a result of some other mutation in the genome.

In conclusion, based on what is known from the GINS crystal structure and what is known from Sld5 we believe that the truncation of this β -sheet region in the B domain is severely affecting the functional capabilities of GINS. Looking at the S Phase and M Phase delays we believe that the GINS complex cannot form in adequate numbers to allow replication to proceed normally or at an adequate rate required by the described tissues, as a result of the decreased amount of Sld5 available. Each of the phenotypes observed throughout the varying tissues point to a physiological dependent dose of Sld5 being required for replication to occur. Lastly, we know that GINS is required during development due to the nature of the arrest point in the homozygous Sld5 embryo. All of the results described taken together show that Sld5 is an integral component of the GINS complex. Sld5 must be present in its native state to allow replication to proceed without pause and genomic instability results if a defect is present in Sld5. Genomic instability is the wild card, which can lead to cancer.

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APPENDIX A: SUPPLEMENTARY INFORMATION FOR EXPERIMENTAL METHODOLOGIES

Materials herein follow labeled with a protocol number which matches the in text notation.

Protocol 1:

Stepwise Experimental EdU Protocol

REAGENTS:

HyQ[®] Grace's Unsupplemented Insect Cell Culture Medium (Cat No. 30610.01, Hyclone, Logan, UT)

Click-It[™] EdU AlexaFluor[®] 488 Kit (Cat No. 10337, Invitrogen, Carlsbad, CA)

Bovine Serum Albumin (Cat. No. 9048-46-8, Fisher Scientific, Fair Lawn, NJ)

LIFTERSLIP Coverslips 25x60mm (25x60I-2-4789, Thermo Fisher Scientific, Portsmouth, NH)

Vectashield[®] Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)

Sigmacote[®] (Sigma Aldrich[™] Inc., St. Louis, MO)

EQUIPMENT:

Dissecting Scope equipped with oblique illumination setting.

Fluorescent capable microscope equipped with DAPI filter and GFP or other capable fluorescent filter dependent upon staining.

Wilton Toolmaker Vise, Cat #: 11715 Penn Tools, Maplewood, NJ, USA.

Torque Wrench with a visible readout that can apply 15N of force.

PROCEDURE:

EdU LABELING

1. Prepare a 2X (30 μ M) EdU solution from the 10mM EdU solution prepared from the Invitrogen kit in Grace's Cell Culture Medium (room temperature) and set aside.

* Add 1.5 μ L of 10mM EdU to 498.5 μ L of Grace's – scale down if you are only doing a couple of specimens.

2. Dissect out desired *Drosophila* tissue in Grace's in a 9 well plate or 2 well depression slide.
3. Transfer tissue using No. 5 tweezers to a holding well with 100 μ L of Grace's

**Helpful Hint: Judge the amount of Grace's you need based on the size of the well and the amount of tissue you are planning to stain, making sure to keep the appropriate concentration.*

4. After obtaining all of the tissue samples desired add an equal volume of the 2X EdU solution to the well containing the tissue, resulting in a 1X (15 μ M) EdU solution and incubate for 35 minutes.

i.e. 100 μ L EdU to 100 μ L Grace's and add brain

5. Pipette off the EdU solution

**ATTENTION if you plan to perform the brain squash proceed to step 21 **

6. Rinse two times with 3% BSA in 1X PBS

**Helpful Hint: Limit the amount of time from the start of the dissection of the tissues to the incubation with EdU to the tissues that can be dissected in 40 minutes.*

FIXATION/PERMEABILIZATION

7. Add a 3.7% Formaldehyde fixative for 10 minutes
8. Pipette off Formaldehyde fixative
9. Wash two times with 3% BSA or 1X PBS
10. Add a 0.5% Triton-X in 1X PBS for 20 minutes

11. While incubating in step 10 prepare the Click-It Reaction Cocktail recipe listed under recipes
12. Pipette off Triton-X solution
13. Wash two times with 3% BSA in PBS or 1X PBS

REACTION COCKTAIL

14. Add 200µl of the reaction cocktail per well and incubate for 30 minutes in the dark

ATTENTION: Protect the tissue from light throughout the remainder of the protocol.

15. Remove cocktail and wash two times with 3% BSA or 1X PBS

ATTENTION: At this point if you have another staining protocol you wish to perform then continue to that protocol. If not continue to step 16.

16. Add a 1X Hoescht 33342 (5µg/mL) solution in 1X PBS and incubate for 20 minutes in the dark.

17. Wash each well two times with 1X PBS

18. Pipette approximately 5µl of PBS on to a new clean microscope slide and transfer the tissue from the well to the center of the PBS on the microscope slide.

Helpful Hint: The PBS will prevent the whole mount specimens from drying out while transferring multiple specimens to the same slide.

19. After transferring the tissue specimens, pipette off excess liquid and add approximately 10µl of Vectashield or other mounting media to the slide
20. Place a Lifterslip[™] coverslip on top and seal around the edges with nail polish.

EdU BRAIN SQUASH

21. Remove the wash solution and add 200µl of 0.5% Sodium Citrate Solution
22. Remove Sodium Citrate Solution and add 200µl of 11:11:2 Acetic Acid, Methanol and Water to the well for 30 seconds.
23. While incubating pipette approximately 5µl of 1X PBS on to a slide
24. Remove the 11:11:2 fixative and wash with 1X PBS
25. Transfer each brain to a separate slide and add a Sigmacote[®] coverslip
26. Make a slide “sandwich” using a piece of paper cut to the size of the slide and another clean slide
27. Place the “sandwich” into the vise (and use a torque wrench to apply 15.0 N of force to the slide.
28. Remove the slide sandwich from the vise and remove the slide with the tissue specimen.
29. Carefully lower the slide into liquid nitrogen using forceps for approximately 5 seconds
30. Pull the slide out and use a razor blade to pop off the coverslip.

**Helpful Hint: To ensure the proper placement of the solution on to the tissue specimen we use a blue sharpie to draw a circle on the underside of the slide that circumnavigates the area the specimen occupies. It allows one to easily visualize the specimen when working with multiple.*

ATTENTION: Proceed back to step 9 and continue through to the end of the protocol with the only change being that the solutions will be pipetted directly on to the tissue specimen located on the slide.

EdU PROTOCOL SOLUTION RECIPES

.5% Sodium Citrate (100ml)
Sodium Citrate Dihydrate 0.5g

Add distilled H₂O to bring volume to 100ml

11:11:2 Acetic Acid, Methanol, and Water (100ml)

Acetic Acid	11ml
Methanol	11ml
Water	2ml

10X Phosphate Buffered Saline (1L)

NaCl	80.0 g	1.37 Molar
KCl	2.0g	26.8 mM
Na ₂ HPO ₄	14.4g	101.0 mM
KH ₂ PO ₄	2.4g	17.6 mM

Dissolve the above ingredients in 800mL of distilled H₂O and adjust the pH to 7.4. Adjust the volume to 1L and autoclave to sterilize.

30μM EdU Solution

5-ethynyl-2'deoxyuridine(EdU)	1.5μL
Cell Culture Media	498.5μL

Adjust amount of solution based on the amount of tissue to be stained.

Click-It Reaction Cocktail (500μL) (All components are a part of the Invitrogen kit C10337)

1X Click-It Reaction Buffer	430μL
CuSO ₄	20μL
Alexa Fluor Azide	1.2μL
Reaction Buffer Additive	50μL

1X Click-It[®] Reaction Buffer is prepared by using 43μL in 387μL of distilled H₂O.

Reaction Buffer Additive is prepared by using 5μL of buffer additive in 45 μL of distilled H₂O.

Note: Instead of diluting the reaction buffer as described in the kit we make a working solution from the stock for each set of staining.

Calculation of the S Phase Index:

S Phase indexes were determined by analyzing 10 fields of view from each brain, providing a total of 100 fields of view for each representative phenotype. The total number of EdU incorporated cells was quantitated versus the total number of DAPI stained nuclei per field of view. These numbers were used to calculate an average percentage (mean) of S Phase cells per brain. The sample data was then used to calculate the variance and from the variance we were then able to take the square root to determine the standard deviation from the mean. All values were reported with the mean \pm the standard deviation.

Protocol 2

Stepwise Quick Genomic DNA Prep

Materials:

2.5 ml Microcentrifuge tubes

Disposable tissue grinder

Microcentrifuge

Method:

1. Collect 30 anesthetized flies in a 1.5 ml microcentrifuge tube placed on ice.
2. Grind flies in 200 μ l of Buffer A with a disposable tissue grinder. Add an additional 200 μ l of Buffer A and continue grinding until only cuticles remain (~ 1-2 minutes, grinding by hand).
3. Incubate samples at 65°C for 30 minutes.
4. Add 800 μ l of Buffer B to each sample, mix well by inverting the tube multiple times, and incubate on ice for at least 10 minutes and up to a few hours.
5. Centrifuge at 12,000 rpm at room temp. for 15 minutes.
6. Transfer 1 ml of supernatant into a new microcentrifuge tube. Be careful not to transfer any floating precipitate. Discard the pellet.
7. Add 600 μ l of isopropanol to each sample, and mix well by inverting the tube several times.
8. Centrifuge at 12,000 rpm at room temperature for 15 minutes.
9. Discard the supernatant. Wash the pellet with 70% ethanol, air-dry, and resuspend in 150 μ l of molecular grade water.
10. Store at 4°C.

Solutions:
Buffer A

100mM Tris-HCl, pH 7.5
100mM EDTA
100mM NaCl
0.5% SDS

Protocol 3

Yeast 2 Hybrid Protocol

Destination/Entry Vector Construction for Yeast Two Hybrid Analysis:

Initially, a DNA preparation of *sld5* was prepared and amplified using the Platinum® Pfx PCR Kit. After confirmation that the desired product is amplified using gel electrophoresis the product is cloned into Top 10 competent *E. coli* host cells using the PentrD/TOPO® Cloning kit and plated on LB + Kanamycin plates. The colonies are grown overnight and screened using overnight LB cultures. These cultures are lysed and the plasmid DNA is extracted using a DNA mini-prep kit from Promega. The plasmid mini-prep is performed as follows:

- 5 ml of overnight culture is spun down for 5 minutes
- Supernatant is removed and the pellet is resuspended with 250µl of Cell Resuspension Solution
- 250µl of Cell Lysis Solution is added to sample and the sample is inverted 3 times to mix.
- 350µl of Neutralization solution is added and the sample inverted 3 times to mix.
- The centrifuge is used to spin the sample at top speed for 10 minutes at room temperature.
- The supernatant is extracted and added to a spin column collection tube
- The sample is spun at top speed for 1 minute and the flow through from the collection tube is discarded.
- 750µl of Column Wash is added and the column is spun for 2 minutes at top speed.
- The spin column is transferred to a new centrifuge tube and 50µl of Nuclease Free Water is added to elute the Plasmid DNA from the column.

- The Plasmid DNA is stored at -20 °C

After the Plasmid DNA is extracted it is sent to the core genomics facility for sequencing using M17 forward and reverse primers to ensure that the insert is in the correct orientation. Upon confirmation that the correct product was ascertained the entry clone has now been generated and is ready for use with the Gateway system. (See figure 11)

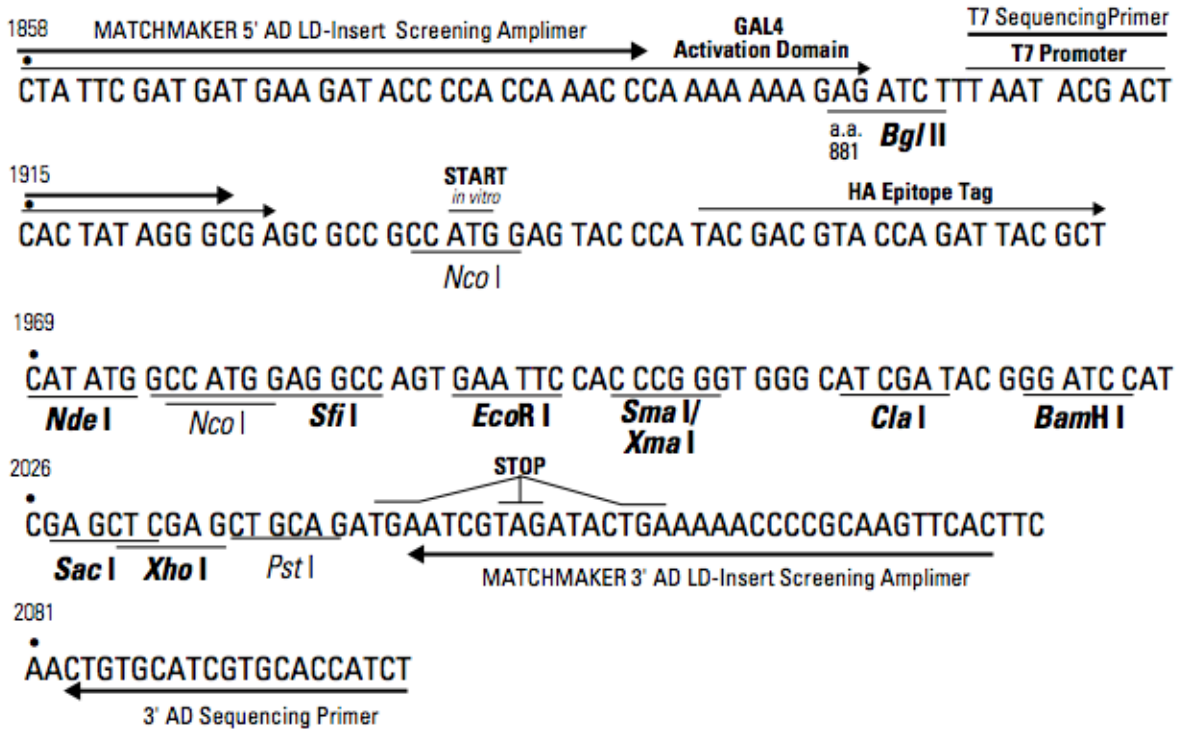
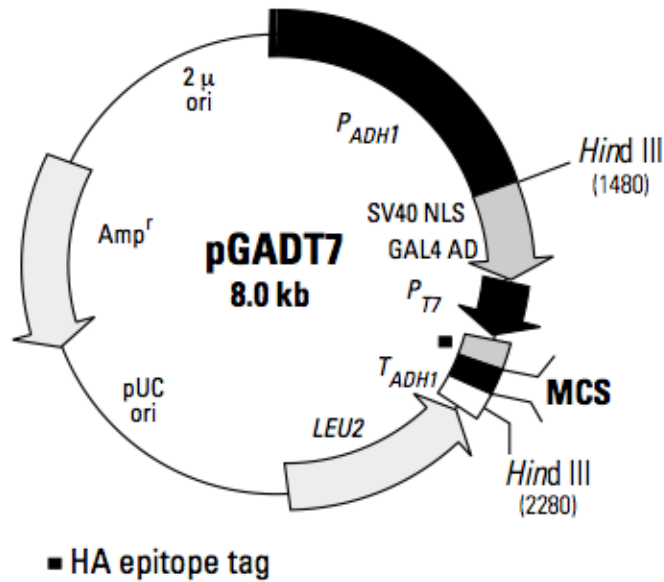
In addition to the entry clone, destination vectors are necessary; we will be using pGADT7GW and pGBKT7GW. These vectors are stored at -80 °C in glycerol stocks and are streaked out on LB+AMP plates, grown up overnight in a 37 °C incubator and a colony is used to inoculate overnight cultures. The overnight cultures are spun down and the Promega DNA plasmid prep kit is used to isolate the desired DNA. The Plasmid is isolated using the same protocol as detailed under the previous section Transgene Preparation.

After plasmid isolation the Lambda Recombinase, the driver behind the Gateway system, is ready to be performed. The Lambda Recombinase reaction catalyzes the reaction initiating recombination at the att sites. The difference here is that the destination vectors are different than that of the Transgene. The subsequent products that are generated from the LR reaction will be sent for sequencing to the core genomics facility utilizing the T7 primer. After sequence confirmation shows that the desired pGADT7GW and pGBKT7GW (Figure 4.2 Appendix: A&B) vectors contain the sld5 cDNA insert these products will be transformed into the AH109 strain of *Saccharomyces cerevisiae* for use in the Yeast Two Hybrid Screen.

Product	Company	Catalogue Number
Platinum [®] <i>Pfx</i> DNA Polymerase	Invitrogen	11708-013
pENTR [™] /D-TOPO [®] Cloning Kit	Invitrogen	K2400-20
Gateway [®] LR Clonase [®] enzyme mix	Invitrogen	11791-019
Wizard [®] <i>Plus</i> SV Minipreps DNA Purification System	Promega	A1460

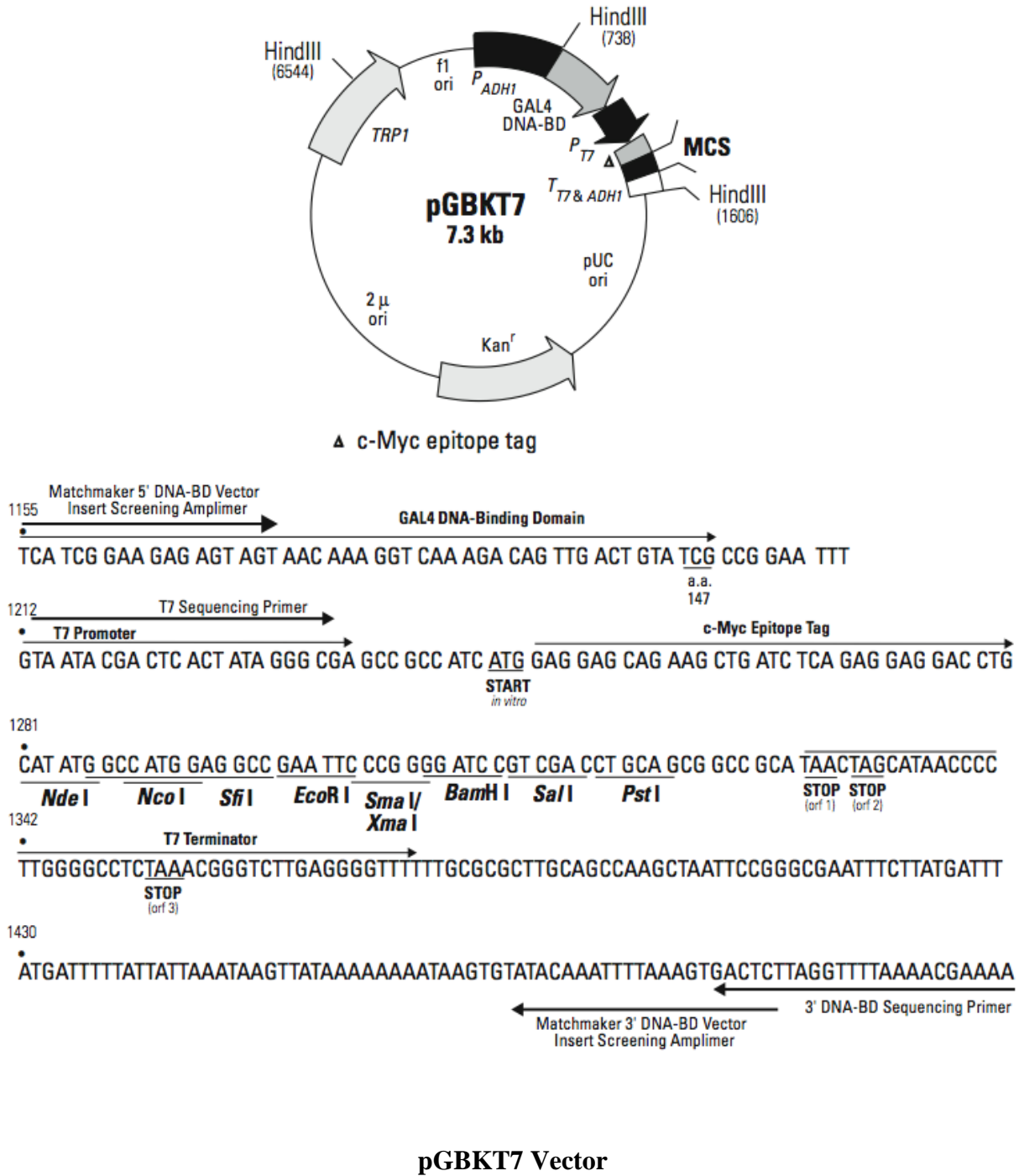
Table 1: Products Used to Prepare Constructs for Yeast Two Hybrid Screen and Transgenic Rescue Fly line.

Figure 1: pGADT7 Vector Map used for cloning in the Yeast two Hybrid System.



pGADT7 Vector

Figure 2: pGBKT7 Vector Map used for cloning in the Yeast two Hybrid System.



Protocol 4

cDNA Library Screen:

Recently, the lab has also developed a screening vector containing tetracycline resistance for use in both 3 hybrid analysis and cDNA screening. For this study the use of the new PGBKTetGW vector is being utilized to screen a cDNA library to confirm known interactors and potentially highlight new interactors if they are contained within the library. To perform the cDNA screen a yeast transformation using the cloned sld5 cDNA plasmid is performed with the PGBKTetGW vector. Due to the new tetracycline resistance all colonies that grow on the LB+Tet plates contain the desired sld5 insert. A colony was then isolated and used to inoculate an LB overnight culture and was placed in the 37°C incubator. An mini-prep was performed on the overnight culture and the PGBKTetGW + Sld5 cDNA plasmid was isolated. The plasmid was then transformed into the AH109 yeast strain. A colony was isolated to inoculate an overnight YPD culture and placed in the 30°C incubator overnight. The overnight yeast culture was then spun down and a yeast transformation was performed using the cDNA library (stored at -80°C). The transformation was plated on CM-Leu-His-Trp and all colonies that grew were then re-streaked on another CM-Leu-His-Trp plate. The colonies that grew were then assigned a number and used to inoculate overnight YPD cultures. The overnight culture was then spun down and a yeast plasmid prep was used to extract the yeast plasmid. The yeast plasmid prep protocol is as follows:

1. Spin down 1 mL of saturated yeast culture
2. Resuspend in 500 µl Y1 buffer (Y1 buffer: 1M Soribitol, 0.1 M EDTA, pH 7.4, add fresh β -mercaptoethanol (1µl/mL))
3. Add 50 µl of 5mg/mL Zymolyase

4. Incubate at 30°C for 1 hour
5. Spin down spheroplasts at 7.5kRPM for 10 minutes
6. Decant as much of the supernatant as possible

The spheroplasts that remain were mini-prepped using the normal plasmid mini-prep protocol as outlined previously. After isolating the desired unknown plasmids they were transformed into DH5 α competent *E. coli* cells. The transformation was then plated on LB + Amp plates and a colony was used to inoculate overnight cultures at 37°C. After culturing overnight a mini-prep was performed. The unknown isolated plasmids were sent for sequencing in the core genomics facility.

Protocol 5

Embryo Analysis

The *Drosophila* embryo as mentioned previously is a unique tool in that it can be used to highlight various stages within the cell cycle. For our purposes we will be examining embryos between 4-5 hours to look for the following:

- Cell dropout, meaning that there are literally spaces where a cell should be but is no longer present.
- Mitotic bridges – as the cells are going through cytokinesis and dividing mitotic bridges can be seen in cells that have an M phase defect and are seen as long string-like interconnections between the nuclei.
- Asynchrony – some cells have progressed farther than others or there is a smattering of varying stages seen in one field of view.

The embryos were prepared via the following protocol and imaged using the Olympus IX2-DSU Tandem Spinning-Disk Confocal Compound Light Microscope present in the Core Imaging Facility.

Embryo Protocol

Collection:

1. Isolate flies in a collection chamber and feed for 24hours using a grape plate smeared with a thick layer of yeast paste.
2. Exchange the feeding plate with a fresh grape plate after smearing a very faint film of yeast across it.
3. Wait 4-5 hours for embryos to reach the appropriate stage and remove plate from bottle

4. Cut a small square of mesh and place in screw cap to create the basket for harvesting embryos.
5. Gently add dH₂O to the egg plate and use a paint brush to remove the embryos.
6. Use a pipette to suck up embryos and place in collection tube.
7. Rinse embryos with dH₂O.
8. Dechorionate the embryos by placing the microcentrifuge tube in a Petri dish and add 50% bleach solution to cover the embryos, using a pipette to wash the embryos continuously.

Fixation:

1. Make sure that the embryos are in the center of the mesh, remove mesh and blot to remove excess liquid.
2. Place mesh with embryos face down into the 5ml glass vial.
3. Pipette 1ml of heptane to wash the embryos off of the mesh depositing them in the collection vial.
4. Add 1ml of methanol and shake vigorously for 15 seconds.
5. Let the glass vial stand at room temperature for 1 minute.
6. Embryos that sink to the bottom of the vial are now devitellinized.
7. Remove upper heptane layer and most of the methanol.
8. Add fresh methanol until 2/3 full and store at 4oC overnight.

Rehydration:

1. Transfer the embryos to a 1.5 ml tube removing as much methanol as possible.
2. Gently add 250 µl of methanol to the embryos followed by 250µl of PBTA solution. DO NOT SHAKE THE TUBE!

3. Add PBTA until 2/3 full. Invert 2-3x.
4. Remove solution and add 500 μ l of PBTA solution.
5. Allow embryos to rehydrate in this solution at room temperature for 15 minutes on rotator.

DAPI:

1. Allow rehydrated embryos to settle to the bottom of the tube and remove as much of the PBTA as possible.
2. Add 495ul of PBTA and 5 μ l of 100X DAPI to the embryos
3. Incubate on a rotator for 5 minutes avoiding light at this step.
4. Place on slides, add Vectashield Mounting Medium and cover slip.

Protocol 6

Transgene Preparation:

Isolated genomic DNA was cloned into a pTWM vector and sent for sequencing. The confirmed Sld5 genomic DNA cloned into the pTWM vector was sent to Best Gene Inc. for injection into embryos to generate a transgenic fly to examine complementation. The transgene affords us the ability to generate a fly that is homozygous viable for the rescue cross. (See Appendix C for cross)

Protocol 7

Supplementary Neuroblast Protocol

Drosophila Neuroblast Analysis (M-Phase):

Analysis of Drosophila neuroblasts will be performed on Wild Type, Sld5 heterozygote mutants, and an RNAi fly line. These brains will be used to calculate mitotic indexes for each genotype to determine an M phase defect. Simply put, if there are more mitotic figures present in the experimental line when compared to wild type one can assume an M phase delay is present. The data will be averaged and a box plot will be used to display the data. Each of the lines brains will be prepared using the following protocol:

Brain Protocol:

- Dissection of brains in PBS/PEG 8000 Solution
- Incubation for 10 minutes in Sodium Citrate
- Incubation for 30 seconds in 11:11:2 Acetic Acid, Methanol, Water Solution
- Place brain on Poly-lysine coated slides and cover with a Sigmacoate cover-slip
- Create a slide sandwich by placing a glass slide, a piece of paper, and the slide with the brain specimen on it together. Place the sandwich in a vice and tighten the torque wrench until achieving a pressure of 16nM for 2 minutes
- Remove the slide sandwich and lower the slide in liquid nitrogen
- Use a razor blade to pop off the cover slip and dip the slide in ethanol
- Allow the slide to dry and place 7uL of Vectashield with DAPI mounting medium on the slide
- Add a fresh cover slip and seal the edges with clear nail polish

Calculation of the Mitotic Index:

Mitotic indexes were determined by analyzing 10 fields of view from each brain, providing a total of 100 fields of view for each representative phenotype. The total number of mitotic figures and the total number of cells per field of view were quantitated by hand. These numbers were used to calculate an average percentage of mitotic figures per brain, which highlights the mean value for the sample group. The sample data was then used to calculate the variance. From the variance we were then able to take the square root to determine the standard deviation from the mean. All values were reported with the mean \pm the standard deviation.

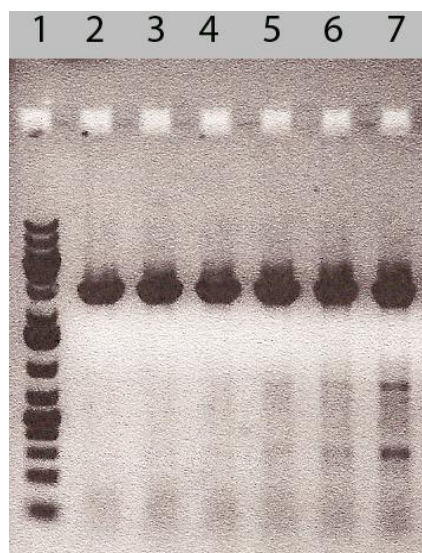
APPENDIX B: SUPPLEMENTARY GENOMIC DATA

Figure 1: PCR of Sld5 cDNA used for cloning. Image is the result of 0.7% gel electrophoresis of amplified Sld5 cDNA. Lanes are numerically labeled on each with a description below.



Lane Descriptions: (1) 1kb DNA Ladder (2) Sld5 cDNA amplicon (3) Sld5 cDNA amplicon

Figure 2: PCR of Sld5 genomic DNA used for cloning and transgenic fly preparation. Image is the result of 0.7% gel electrophoresis of amplified Sld5 genomic DNA. Lanes are numerically labeled on each with a description below.



Lane Descriptions: (1) 1kb DNA Ladder (2-4) Sld5 genomic DNA amplicon (5-7) Sld5 genomic DNA amplicon with undesired products

APPENDIX C: FLY CROSSES

Cross 1

Transgenic Fly Complimentation Cross

$$\frac{w ; \underline{ap}^{XaT(2;3)}}{Gla ; Sb} \quad x \quad \frac{\underline{p[sld5]} ; \pm}{p[sld5] \quad +}$$

$$\frac{\underline{p[sld5]} ; \pm}{p[sld5] ; Sb} \quad x \quad \frac{\underline{ap}^{XaT(2;3)}}{Hp1 ; Sb}$$

$$\frac{\underline{ap}^{XaT(2;3)}}{p[sld5] ; Sb} \quad x \quad \frac{\underline{ap}^{XaT(2;3)}}{p[sld5] ; Sb}$$

$$\frac{\underline{ap}^{XaT(2;3)}}{p[sld5] ; Sb} \quad x \quad \frac{\pm ; \underline{sld5}^{mut}}{+ \quad Sb}$$

$$\frac{\underline{p[sld5]} ; \underline{sld5}^{mut}}{+ ; Sb} \quad x \quad \frac{\underline{p[sld5]} ; \underline{sld5}^{mut}}{+ ; Sb}$$

Complimentation Cross with Dak1 and Sld5

The following complementation crosses were performed to determine whether the phenotypes visualized were due to Sld5 and not Dak1 due to the multicistronic nature of the processed transcript.

Cross 2 (line 42 x 199)

$$\frac{\text{Sld5}^{\text{A462}}}{\text{Sb, ser}} \quad \times \quad \text{w ; } \frac{\text{Df(3R)BSC140}}{\text{Tb}}$$

Cross 3 (line 103 x 199)

$$\frac{\text{Sld5}^{\text{C010719}}}{\text{Sb, Tb}} \quad \times \quad \text{w ; } \frac{\text{Df(3R)BSC140}}{\text{Tb}}$$

Crosses 2 & 3 above utilized a deletion mutant covering DAK1 and Sld5, crossing them with the original p-element mutant lines. The crosses were scored and the phenotypes examined for complementation.

Cross 4 (line 42 x 200)

$$\frac{\text{Sld5}^{\text{A462}}}{\text{Sb,ser}} \quad \times \quad \text{w ; } \frac{\text{Df(3R)BSC751}}{\text{Sb,cu}}$$

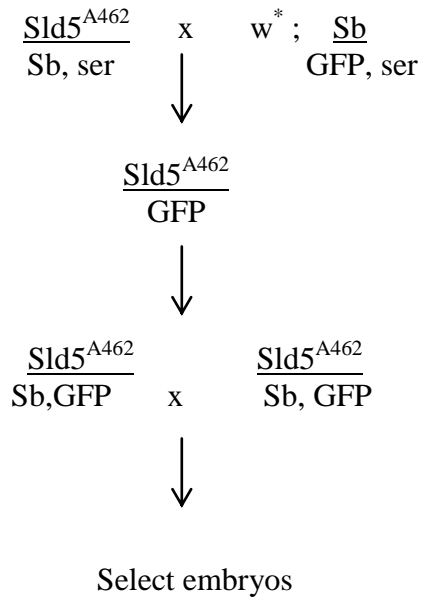
Cross 5 (line 103 x 200)

$$\frac{\text{Sld5}^{\text{C010719}}}{\text{Sb,Tb}} \quad \times \quad \text{w ; } \frac{\text{Df(3R)BSC751}}{\text{Sb,cu}}$$

Crosses 4 & 5 above utilized a deletion mutant covering DAK1 but not Sld5, crossing them with the original p-element mutant lines. The crosses were scored and the phenotypes examined for complementation.

Cross 6

Arrest Point Cross



This line allowed for the ability to select embryos of the following phenotypes:

$$\frac{Sld5^{A462}}{GFP} \quad \frac{Sld5^{A462}}{Sld5^{A462}} \quad \frac{GFP}{GFP}$$

APPENDIX D: OTHER EXPERIMENTAL DATA

Throughout the course of the study the *Sld5/Sb*, *Ser* line showed a curious phenotype that no one had seen before. Multiple flies from the stock were: missing a wing or both wings, had a malformed appendage in place of the wing, or had a scutelum that was not formed properly. A representative image of the phenotypes exhibited is shown below, as well as the points with which the adult wing measurements were taken. This information was not included in the body of the text because it is still inconclusive at this time; however, through the transgene rescue we have not yielded this phenotype suggesting that this wing malformation is due to *Sld5*.

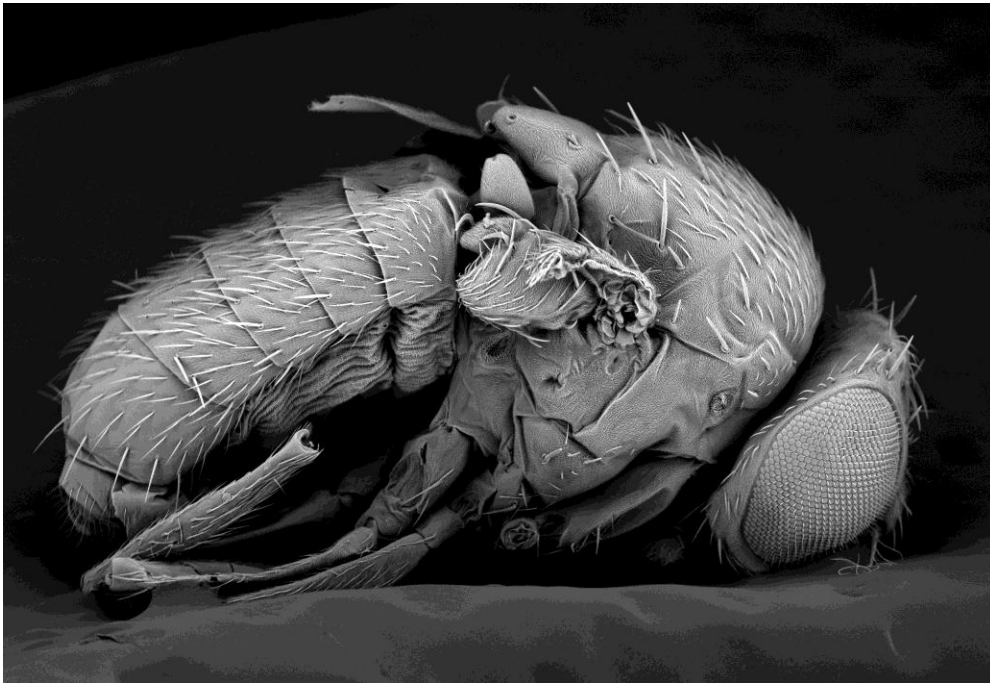


Figure 1: Wing Malformation

The fly above is a male from the *Sld5*^{A462}/*Sb*,*ser* line. Here the wing abnormality is visible as an added appendage that appears to be emerging from the location of the wing base but instead of wing tissue, the tissue appears to be more like that of the scutelum.

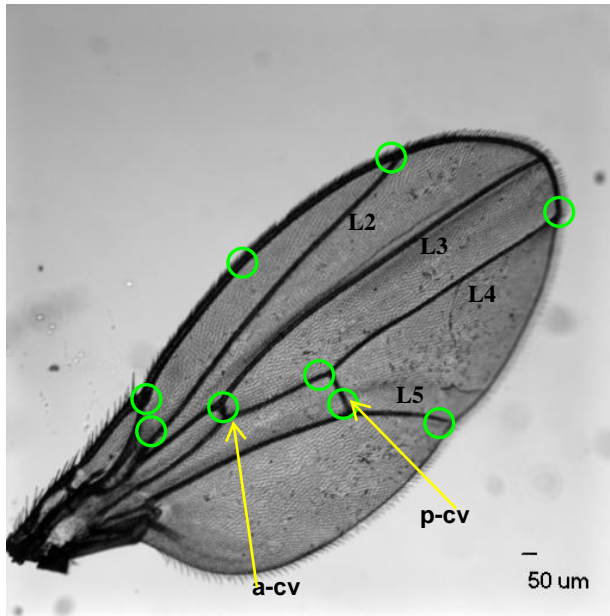


Figure 2: Adult *Drosophila* wing highlighting the measurement points for analysis of wing size.

To examine this abnormal phenotype in more depth we looked at the adult wings from both Wild Type flies and those from the *Sld5/Sb,GFP* line by cutting the adult wings off, imaging them using DIC, and measuring the surface area of the wings in μm^2 . The measurement region is highlighted in Figure 2. The measurements yielded no significant difference between those from Wild Type and the mutant *Sld5* line as shown in Figure 3.

	Female	Male
Missing One Wing	8.3%	4.3%
Missing Both Wings	0.97%	0.63%

Table 1: Percentage of Wings Missing in *Drosophila* adult flies from the *Sld5*^{A462} line. Adult flies were examined under a dissecting microscope and the wings or their absence thereof were scored and a simple percentage was calculated based on the scored data.

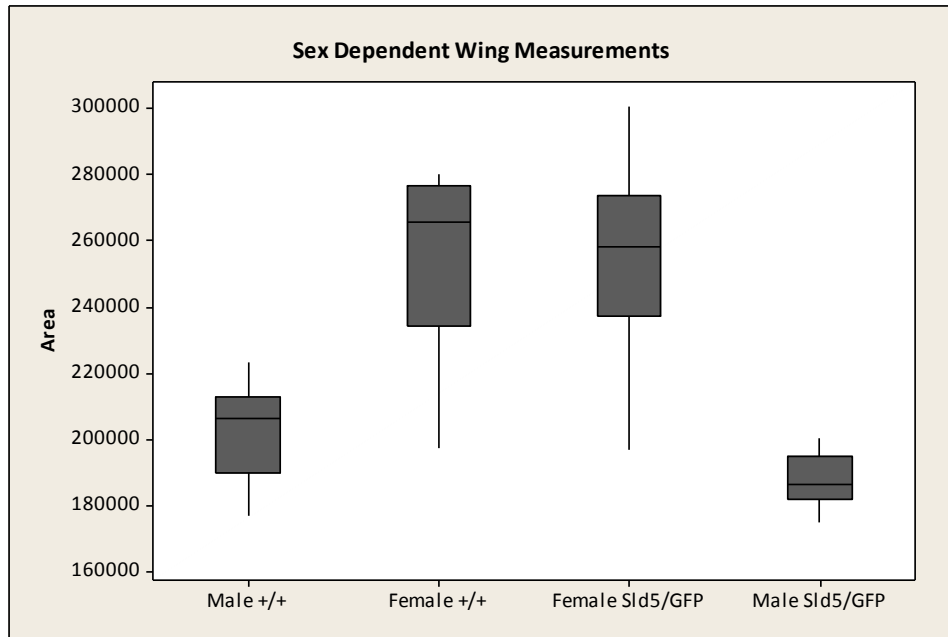


Figure 3. Sex Dependent Wing Measurements in *Drosophila* adults.

To examine other possible reasons for this observed phenotype we performed a simple cross using Wild type flies and the p-element mutant to look at maternal loading. For this complementation we were testing the hypothesis that we believed that the abnormal wing phenotype was due to maternal loading of mRNAs. If the wing phenotype *Sld5/+* presented itself in the *Sld5/+* F1 progeny then the *Sld5* wing phenotype could be due to inadequate maternal loading of *sld5*

$$\begin{array}{c}
 \underline{w} ; \underline{sld5^{A462}} \\
 w \quad \underline{Sb,ser}
 \end{array}
 \times
 \begin{array}{c}
 \underline{w} ; \underline{+} \\
 +
 \end{array}$$

$$\begin{array}{c}
 \boxed{\underline{sld5}} \\
 +
 \end{array}
 \begin{array}{c}
 \underline{+} \\
 sb
 \end{array}$$

Figure 4. Complimentation Cross to Examine Altered Wing Phenotype