Characterization of the GINS subunit psf1 in *Drosophila Melanogaster*

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Characterization of the GINS subunit psf1 in Drosophila Melanogaster

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Chapter 1: Project Summary

GINS (Go Ichi Ni San; 5,1,2,3 in Japanese) is a heterotetrameric protein complex known to be essential for the establishment of DNA replication forks and the progression of the replisome. The GINS complex is known to be integral in G1 to S phase transition. GINS may play a role in loading Cdc45 and DNA polymerases α and ε and has been suggested to function in the activation of the MCM helicase. The Psf1 subunit of the GINS complex is evolutionarily conserved and has been identified in all sequenced eukaryotic genomes. It has been inferred that Psf1 is required for an early step in DNA replication and the binding of Cdc45. What has been discovered of the Psf1 subunit would indicate an important role DNA replication and viability of the organism. Due to the pivotal role of GINS, we investigated the functional characteristics of Psf1 along with any protein interactions. Understanding Psf1 will give a better understanding of the GINS complex as a whole and possibly reveal its role in the initiation of DNA replication and other important cellular processes.

Our research has focused on a Drosophila psf1 hypomorphic allele. Our project has involved investigating the function of Psf1 using several approaches. First, we explored genetic interactions using a mutant allele of Psf1 in combination with mutant alleles for genes involved in DNA replication. Second, we determined the level of decrease in mRNA of the hypomorphic allele using reverse transcriptase PCR. Third, we used confocal microscopy to analyze the morphology and segregation of chromosomes in different tissues. Confocal microscopy was also used to assess any
cell cycle defects through the measurement of mitotic indices. Lastly, we created a true null psf1 mutant allele for further research.
Chapter 2: Introduction to the GINS complex

It has become increasingly clear that the appropriate regulation of the eukaryotic cell cycle is essential. Defects in this regulation have been linked to a variety of cancers (Blow & Gillespie, 2008). To prevent the occurrence of potentially cancer-causing alternations to the genome, it is crucial that chromosomal DNA is precisely duplicated during S phase of the cell cycle. It is equally important to ensure that no replication origin initiates more than once in each cell cycle, as this would lead to reduplication of the DNA (Blow & Gillespie, 2008). Therefore misregulation of the cell cycle may lead to DNA strand breaks or reduplication of DNA. The cell cycle can be thought of as consisting of two phases. The first phase comprises late mitosis and early G1, when CDK levels are low and licensing of DNA replication origins occurs. The second phase comprises late G1, S, G2 and early mitosis, when high CDK levels drive DNA replication and chromosome segregation (Blow & Tanaka, 2005).

Eukaryotic DNA is organized for replication as multiple replicons. DNA synthesis in each replicon is initiated at an origin of replication and proceeds bidirectionally until replication forks from adjacent replicons collide. In all eukaryotes there are several biochemical events that must take place before initiation of DNA replication can occur (Bryant, Moore, & Ayes, 2001). The eukaryotic initiator protein is a six-subunit protein complex, called the origin recognition complex, or ORC. This protein complex is required for replication in budding yeast, and it carries out its function by binding to
defined regions of the genome known as autonomously replicating sequences, or ARSs (Bell & Stillman, 1992). ORC has homologs in all eukaryotic organisms examined, and indeed most biochemical factors required for initiation of DNA replication are highly conserved (Bell & Dutta, 2002). ORC acts to recruit two more proteins, Cdc6, and Double Park (Dp), to the DNA. These proteins, in turn, are required for the chromatin loading of the MCM2–7 complex, which is believed to be the replicative DNA helicase (Labib & Diffley, 2001; Pacek & Walter, 2004; Shechter, Ying, & Gautier, 2004). ORC, Cdc6, and Dp act as a clamp loader that opens up the Mcm2–7 hexamer and closes it again around the DNA (Blow & Dutta, 2005). All together these proteins and protein complexes form a structure known as the pre-replication complex (Figure 1), or pre-RC (Diffley, Cocker, Dowell, Rowley, 1994).

The regulation of pre-RC formation is key for appropriately coordinating DNA replication with the cell cycle (Bell & Dutta, 2002). As the cell progresses from G1 to S phase, the pre-RC is made active by two proteins: cdc7/dbf4 and a cyclin dependent kinase (Cdk), whose activities rise in S phase. These kinases initiate a cascade of events that ultimately leads to origin unwinding, polymerase recruitment, and duplication of the cellular genome (Bell
Another protein, Cdc45, is involved in the conversion of pre-RCs to pre-initiation complexes (pre-ICs) (Figure 2). Cdc45 binds to chromatin after the formation of pre-RCs in a CDK- and DDK-dependent manner, and is required for the loading of polymerase alpha and epsilon onto chromatin (Takeda & Dutta, 2005). Initiation of DNA replication leads to disassembly of the pre-RCs (Diffley et al., 1994). Although all these replication factors are essential for initiation of chromosome DNA replication, precise mechanisms of assembly of individual factors or functions in DNA replication have yet to be sufficiently elucidated (Yabuuchi, Yamada, Uchida, Sunathvanichkul, Nakagawa, & Masukata, 2006).

A newly discovered complex known as GINS is required both during and after initiation of DNA replication for regulatory factors such as Cdc45 to interact with MCM in replisome progression complexes (RPC) (Gambus et al., 2006) (Figure 3). The main components of RPC are Mcm2–7, Cdc45 and GINS, in association with Mrc1, Tof1, Csm3 (involved in the stabilization of stalled forks), Ctf4 (required for sister chromatid cohesion), Spt16 and Pob3 (components of the histone chaperone FACT) (Fien & Hurwitz, 2006). This replisome progression complex migrates with the replication fork. Despite the essential role of GINS in DNA replication, how GINS interacts with MCM, Cdc45, and other protein factors at the replication fork remains unclear (Chang, Wang, Bermudez, Hurwitz, & Chen, 2007). GINS may serve as an accessory factor for eukaryotic DNA polymerases, including...
DNA polymerase Pol epsilon (Seki, Akit, Kamimura, Muramatsu, Araki, & Sugino, 2006) and the DNA Pol alpha-primase complex (De Falco, Ferrari, De Felice, Rossi, Hubscher, & Pisani, 2007). It has also been proposed that GINS binds to and enhances the activity of DNA polymerase a-primase (De Falco et al, 2007). Both Cdc45 and GINS appear to enhance the activity of the Mcm2–7 complex: they could do so by providing structural elements that improve its interaction with the DNA, translocation speed, or base pair separation activity (Kubota et al, 2003). Mcm2-7, GINS, and Cdc45 are required for initiation and the elongation stage of replication.

GINS (Figure 4) is a heterotetrameric complex consisting of Sld5 (synthetic lethal with dpb11 mutant-5), Psf1 (partner of Sld5-1), Psf2, and Psf3 and was first discovered by using a variety of genetic screens in *S. cerevisiae* (Takayama, Kamimura, Okawa, Muramatsu, Sugino, & Araki, 2003). The four GINS subunits are paralogs, among which the specific subunit pairs Psf1-Sld5 and Psf2-Psf3 are more closely related (Makarova, Wolf, Mekhedov, Mirkin, & Koonin, 2005). A subunit of GINS, Psf1 is required for cell proliferation in yeast and mice. Loss of Psf1 leads to early embryonic death in mice (Takayama et al, 2003). It is also known that Psf1 (Figure 5) is required for acute proliferation of hematopoietic stem cells in the bone marrow of mice (Ueno, Itoh, Sugihara, Asano, & Takakura 2008). Psf1 has a B domain that is functionally more important
than the stability of the core complex and may be a key contact point in replication initiation. It is possible that the peripheral surface with the Psf1 B domain is a functional interface for association with Cdc45, the MCM complex or both on chromatin (Katsuhiko, Yumiko, Toshiaki, Yosuke, & Fumio, 2007).

The study of the GINS complex may lead to a new understanding of how the replication fork is formed. Although extensively studied, complete mechanisms of the replisome and initial complexes are still unknown. Discovering the correct order in which proteins form these complexes can help us better understand their function and how they have evolved to what we see today.
Chapter 3: Specific Aims of Research

The main objective of this project was to begin to establish *Drosophila melanogaster* as a model organism for examining the function of the GINS complex. To accomplish this we used a variety of methods to characterize *psf1*, a subunit of the GINS complex. We began by verifying the correct positioning of a P-element that was reported by the Bloomington Stock Center to be inserted 10bp upstream of the translation start site of the *psf1* coding region. Further investigation revealed the P-element insertion resulted in a hypomorph of *psf1* and not a true null mutant. Transcription levels of *psf1* were quantified while genetic crosses investigated any genetic interactions. Informative phenotypes of the *psf1* hypomorph were investigated by imaging a variety of tissue samples using confocal microscopy. Also a true null mutant of *psf1* was produced by imprecise P-element excision that can be used for future research.

**Hypothesis:** *The GINS complex is necessary for proper DNA replication during the S phase of the cell cycle. Psf1 is integral to the formation of the GINS complex and the lack of sufficient Psf1 will result in improper DNA replication.*

**Specific Aim #1:** *Confirm correct P-element insertion.*

Ensure the proper location of the P-element insertion through PCR.
Specific Aim #2: **Quantify mRNA levels of psf1 in the P element insertion line.**

Perform reverse transcriptase PCR to compare mRNA levels with wild type flies. This will determine the level of psf1 expression in the mutant.

Specific Aim #3: **Create a true null mutant fly stock**

Create a true null mutant through multiple fly crosses for future research. A fly line with a knockout of psf1 can be compared to the psf1 mutant to reveal additional information about the function of the GINS complex.

Specific Aim #4: **Explore genetic interactions**

Perform fly crosses to discover any genetic interactions with other DNA replication proteins.

Specific Aim #5: **Investigate informative phenotypes using confocal microscopy**

Exploring phenotypes will be performed through the imaging of multiple tissue samples. First, salivary glands will be viewed for replication defects in polytene chromosomes. Secondly, brains will be viewed for both aneuploidy and mitotic indices. Third, early embryos will be viewed for asynchrony in nuclei or mitotic bridges indicating cell cycle defects. Fourth, ovaries will be viewed for condensation defects in the nurse cells.
Chapter 4: Methodology

Genomic DNA extraction

50 flies containing the \textit{psf1} P-element insertion and 50 wild type flies were anesthetized with \text{CO}_2 and collected into 1.5ml microcentrifuge tubes. Samples were homogenized using a disposable tissue grinder along with 200\mu l of Buffer A solution (100mM Tris-Cl (pH 7.5), 100mM EDTA, 100mM NaCl, 0.5\% SDS). Samples were incubated at 65°C for 30 minutes followed by the addition of 800\mu l of Buffer B solution (200ml of 5M potassium acetate, 500ml of 6M lithium chloride, stored at 4°C). Samples were mixed and incubated on ice for 10 minutes, then centrifuged at 12,000rpm for 15 minutes. Supernatant was transferred to a new microcentrifuge tube, careful to avoid floating precipitate, and had 600\mu l of isopropanol added to each tube and mixed. Samples were centrifuged at 12,000rpm for 15 minutes, supernatant discarded and pellet washed with 70\% ethanol and air-dried. Samples were then resuspended in 150\mu l of \text{dH}_2\text{O} and stored at -20°C.

PCR

Polymerase chain reaction was performed using Platinum Pfx DNA polymerase. Components added are shown on Table 1. PCR mixture was denatured for 2 min at 94°C and 35 cycles of PCR amplification were performed, shown in Table 2 on the
following page. Samples were stored at -20°C. Gel electrophoresis (70ml 1x TAE, 0.7% agarose, 0.5µl EtBr) was run at 120V for 30 minutes.

<table>
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<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
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</thead>
<tbody>
<tr>
<td>10x Pfx Amplification Buffer</td>
<td>5µl</td>
<td>1X</td>
</tr>
<tr>
<td>10mM dNTP mixture</td>
<td>1.5µl</td>
<td>0.3 mM each</td>
</tr>
<tr>
<td>50mM MgSO₄</td>
<td>1mM</td>
<td>1mM</td>
</tr>
<tr>
<td>Primer mix (10µM each)</td>
<td>1.5µl</td>
<td>0.3 mM each</td>
</tr>
<tr>
<td>Template DNA (10pg - 200ng)</td>
<td>≥1µl</td>
<td>As required</td>
</tr>
<tr>
<td>Platinum Pfx DNA Polymerase</td>
<td>0.4 - 1µl</td>
<td>1.0 - 2.5 units</td>
</tr>
<tr>
<td>Autoclaved, distilled water</td>
<td>to 50µl</td>
<td></td>
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</table>

Table 1. Components used for PCR.

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<th>Anneal: 55°C for 30 s</th>
<th>Extend: 68°C for 1 min</th>
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</thead>
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Table 2. PCR Temperature Cycle

<table>
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<tr>
<th>Primer Name</th>
<th>Reference Number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>psf1 start topo</td>
<td>38291796</td>
<td>5’- CAC CAT GAG CCG ACA AAC AAA AAT G -3’</td>
</tr>
<tr>
<td>psf1 with stop</td>
<td>38291797</td>
<td>5’- CTA GGC TAT GTG GTG AAG AAT GCC -3’</td>
</tr>
<tr>
<td>31a XP 3’ (20)</td>
<td>23170230</td>
<td>5’- CGA CAC TCA GAA TAC TAT TCC -3’</td>
</tr>
<tr>
<td>dorc2 fw promoter (37)</td>
<td>21378211</td>
<td>5’- CAC CTG CGC GAT TCA CAA CTC AC -3’</td>
</tr>
</tbody>
</table>

Table 3. Primer sequences used to verify P-element insertion.
RNA extraction

Approximately 1.0ml of psf1 mutant and wild type embryos were collected over a 3-day period. Stocks of psf1 mutant and wild type flies were placed in a collection bottle with a grape juice agar plate with a small amount of yeast paste used as the cover. Collection bottles were inverted so the agar plates were on the bottom and placed in the dark for 5 hours. Embryos were removed from the grape juice agar plate with a small paintbrush and transferred to a 1.5ml microcentrifuge tube and stored at -80°C in between collections. Embryos were homogenized using a disposable tissue grinder. RNA extraction was performed using a Totally RNA kit (Ambion) following the manufacturer’s instructions and stored at -80°C. The concentration of total RNA extracted from each sample was quantified using a NanoDrop Fluorospectrometer (Thermo Scientific).

RT-PCR

First-strand cDNA was synthesized from extracted RNA using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer’s instructions. PCR was performed at a range of cycles from 16-22 to ensure the cDNA was in the linear range of the amplification process. Gel electrophoresis was performed as described earlier.
Quantification of mRNA levels

Levels of mRNA were quantitatively calculated by comparing the density of the PCR bands for both psf1 mutant and wild type with rp49, a control gene. The gel was both imaged and analyzed using a FluorChem 8900 (Alpha Innotech) imager and analyzing software.

Creating fly line homozygous for psf1 mutation

To determine arrest points we crossed psf1 mutant flies with a fly line containing GFP. First generation flies were viewed under a fluorescent microscope and scored by their ability to glow. Glowing flies were self crossed and first generation offspring were viewed under a fluorescent microscope. Flies were scored for those that did not glow. These flies are homozygous for psf1 and were placed in a vial for future use.

Imprecise P-element excision

Fly line carrying psf1 mutant was crossed with a fly line carrying a transposase that mobilized the P-element insertion near the psf1 locus. Further crosses were performed (shown on following page) using dominant markers to identify the mobilized P-element and narrowed the flies to those that had an imprecise P-element excision. The transposase contained the dominant marker tubby that we could use to follow the P-element insertion. Tubby is easily recognized right before flies enclose, and are much shorter and wider than wild type pupae. The F1 cross produced a fly with both the mutant psf1 allele and the transposase. Collecting appropriate flies involved choosing
non-tubby pupae from the F1 vials. The F2 cross produced a fly that has a mobilized P-element insertion and were placed in separate, individual vials. These flies were backcrossed and the F3 generation flies were checked for wild type phenotypes. If the wild type phenotype existed in the next few generations we knew it was a precise P-element excision. If no wild type flies were generated then the excision was imprecise and may have resulted in a true null mutant. These flies will be kept and used in future research.

![Genetic Diagram]

- **V** indicates viability.
- **Sb** indicates a specific allele.
- **PsfI** indicates a restriction enzyme cut at the P-element boundary.
- **(-)** indicates a deletion or deletion varietal.

**Diagram Details:**

1. **First Cross:**
   - **Female** (V, PsfI/PsfI)
   - **Male** (Sb△2-3, Tb)
   - **Outcomes:**
     - Pick non-tubby Pupae

2. **Second Cross:**
   - **Female** (V, PsfI/Sb△2-3)
   - **Male** (Sb, Tb)
   - **Outcomes:**
     - Pick w-; non-tubby Pupae

3. **Third Cross:**
   - **Male** (Sb, Tb)
   - **Female** (Sb, Tb)
   - **Outcomes:**
     - Place in Individual Vials
Cross *psf1* fly line with fly lines containing DNA replication protein mutants

Virgin *psf1* mutant flies were collected over a period of 1 week, 130 virgin flies total. Five virgin female *psf1* mutant flies were placed in individual vials with five male flies from 13 DNA replication protein mutants. This was performed twice to ensure statistically significant figures. A list of the DNA replication protein mutants and their pathways can be found on Table 4 below.

Polytene chromosomes

Wandering third instar larvae were collected from the *psf1* and wild type fly lines. Larvae were placed in a solution containing 1XPBS pH 7.2 with 1% PEG 8000 and salivary glands were dissected out. Salivary glands were transferred to a fixative solution containing 100µl 37% formaldehyde and 900µl 55% acetic acid, 2.5% lactic acid and incubated for 2 minutes. Samples were then placed on a microscope slide with a siliconized cover slip placed on top. The cover slip was gently tapped repeatedly with the tip of a pencil as to spread out the polytene chromosomes. Another slide was placed against the sample with thin strips of paper in between to create a slide sandwich. The slide sandwich was then placed in a vice that was tightened to 15 N•m for 2 minutes. The slide was then placed in liquid nitrogen for 1 minute and the cover slip was removed with a razor blade. The slide was placed in 100% ETOH for 10 seconds and allowed to air dry. 7µl of Vectashield™ (Vector Laboratories) containing DAPI was added to the slide and a new cover slip was applied and sealed with clear nail polish. Finished slides were stored at -20°C.
Aneuploidy

Wandering third instar larvae were collected from the psf1 mutant and wild type fly lines. Larvae were placed in a solution containing 1XPBS pH 7.2 with 1% PEG 8000 and were dissected to remove the larval brains. Imaginal discs clinging to the brains were carefully removed. Samples were transferred to a hypotonic solution containing 0.5% sodium citrate and incubated for 10 minutes. The samples were then transferred to a fixative containing Acetic acid: Methanol: Water 11:11:2 and incubated for 20 seconds. Samples were then placed on a microscope slide with a siliconized cover slip placed on top. Another slide was placed against the sample with thin strips of paper in between to create a slide sandwich. The slide sandwich was then placed in a vice that was tightened to 15 Nm for 2 minutes. The slide was then placed in liquid nitrogen for 1 minute and the cover slip was removed with a razor blade. The slide was placed in 100% ETOH for 10 seconds and allowed to air dry. 7µl of Vectashield™ containing DAPI was added to the slide and a new cover slip was applied and sealed with clear nail polish. Finished slides were stored at -20°C. Slides were imaged at 100x magnification with the spinning disc confocal microscope in order to view individual chromosomes.

Mitotic indices

See Experiment 7a. Aneuploidy above to view slide preparation. 10 random fields of view were imaged per brain at 40x magnification using the spinning disc confocal microscope. 10 total brains were used for a total of 100 random images to score mitotic indices. Total nuclei were counted along with nuclei going through mitosis. Nuclei in
mitosis were divided by total number of nuclei to obtain the mitotic index for both wild type and psf1 flies.

**Early embryo imaging**

Stocks of psf1 mutant and wild type flies were placed in a collection bottle with a grape juice agar plate smeared with a small amount of yeast paste. Collection bottles were flipped so the agar plates were on the bottom and placed in the dark for 5 hours. Embryos were collected from the grape agar plates and placed on a fine mesh held by a modified screw cap and tube to create a basket and rinsed with dH$_2$O. Samples were then dechorionated by washing with a 50% bleach solution until the dorsal appendages had dissolved and then washed with 1X Embryo Wash Solution (7% NaCl, 0.5% Triton X-100, dH$_2$O), with a final wash with dH$_2$O. Samples were transferred from the mesh to a 5ml glass vial via an addition of 800µl of heptane followed by the addition of 800µl of methane. Vial was shaken vigorously for 15 seconds and allowed to stand at room temperature for 1 minute. Devitellinized embryos sink to the bottom and the remaining embryos are removed along with most of the solution. 5 minute washes were performed in the following series of methanol: PBS (NaCl 80g, KCl 2g, Na$_2$HPO$_4$ 14.4g, KH$_2$PO$_4$ 2.4g, dH$_2$O, adjusted to pH 7.4 with HCl) solutions: 80µl: 20µl, 60µl: 40µl, 20µl: 80µl. The remaining solution was removed and 1ml PBS was added for 1 minute, removed then 1ml PBTA (10X PBS 50ml, BSA 5g, Triton X-100 250µl, Sodium azide 0.1g, dH$_2$O) was added and incubated at room temperature for 30 minutes on a rotator. PBTA was removed and 495µl PBTA was added followed by 5µl 100X DAPI and incubated for 5
minutes on a rotator in the dark. Solution was removed and embryos were rinsed in 3x in PBTA solution followed by a 1 hour rinse in PBTA. Samples were transferred to a microscope slide and a plastic membrane was placed over the embryos before adding an aluminum cover slip mount on top secured to the slide by masking tape. Slides were stored at -20°C.

**Ovary imaging**

Females from *psft* and wild type fly lines were collected and anesthetized using CO$_2$. The abdomen of several females were removed and placed in PBS solution. Ovaries were dissected out of the abdomen, ovarioles teased apart, and placed in a PBT (10x PBS 10ml, 20% Triton X-100 2 ml, dH$_2$O 88ml) solution containing 4% formaldehyde for 20 minutes. Samples were then transferred by tweezers to a PBT solution containing DAPI (200µl 100x DAPI, 800µl PBT) for 5 minutes in the dark. Samples were then washed three times in PBT for 5 minutes followed by a 1-hour wash in PBT and three 10-minute washes with PBT. Samples were then transferred by tweezers onto a Lifter Slide™ with 7µl of Vectashield™ added. Slides were stored at -20°C.
Chapter 5: Results

Specific Aim #1: Confirm correct P-element insertion.

DNA extraction from the psf1 mutant

We received a mutant stock from the Drosophila stock center in Bloomington, IN that was reported to contain P-element insertion 10 base pairs upstream of the translation start site for psf1. P-element insertions are a common tool used in Drosophila melanogaster to generate tagged mutations. Primers used are shown in Table 3. We performed polymerase chain reaction (PCR) (Table 4) using various P-element forward primers (Figure 1a). Psf1 is 609bp long with a P-element inserted 10bp upstream of the locus (Flybase). Therefore we were looking for PCR product around the length of 620bp that would indicate a correct positioning of the P-element insertion.

Polymerase chain reaction (PCR) to detect P-element insertion

After extracting DNA from the psf1 mutant, the P-element insertion was confirmed using PCR (Figure 1b). Considering the size of the psf1 locus and location of the P-element, we were expecting a product of 620 base pairs (Figure 1a). Results confirmed the P-element insertion in the correct direction and position relative to the psf1 locus.
Specific Aim #2: Quantify mRNA levels of psf1 in the P element insertion line.

RNA extraction from psf1 mutant embryos

Embryos were collected from psf1 mutant and wild type fly lines over a 3 day period. This allowed us to obtain a sufficient amount of tissue for the Totally RNA kit (Ambion) to perform RT-PCR.
Reverse transcriptase polymerase chain reaction (RT-PCR) analyses of \textit{psf1} in embryos

It was essential to know the extent to which the P-element decreased mRNA levels of \textit{psf1} transcript. RT-PCR provides a method of quantifying mRNA levels. After extracting RNA from embryo tissue using a Totally RNA kit (Ambion), we performed RT-PCR to determine mRNA levels of \textit{psf1} with the P-element insertion compared to those levels in wild type embryos (Figure 2). Ribosomal protein 49 (rp49) mRNA was used as an internal loading control. The \textit{psf1} hypomorph was normalized to wild type \textit{psf1} mRNA levels. RT-PCR showed a 78% decrease in mRNA levels of \textit{psf1}.

Fig 2. RTPCR of Psf1 hypomorph. Rp49 mRNA was a control to quantify the density of the bands. The PSF1 hypomorph was normalized to wild type mRNA shown in the graph. The PSF1 hypomorph shows a 78% decrease in Psf1 expression compared to +/+. 
Specific Aim #3: Create a true null mutant fly stock

Creating fly line homozygous for psf1 mutation

Flies containing a balancer with GFP were crossed with the mutant fly line and subsequent generations were picked to allow for homozygous embryos to be produced. The homozygous embryos were placed in vials to verify an arrest point. The homozygous embryos did not arrest and become full adults able of reproduction.

Imprecise P-element excision

Observing phenotypes with decreased psf1 levels can be of further value if compared to a true null psf1 mutant. Therefore creating a null mutant would prove valuable to further understanding Psf1’s role. Through a series of crosses described in the methods section, a total of 15 possible null psf1 mutants were produced by imprecise P-element excision. These flies were observed to have mutant phenotypes indicating an imprecise excision and are being kept for future research.

Specific Aim #4: Explore genetic interactions

Cross psf1 fly line with fly lines containing DNA replication protein defects

We were curious to explore the possibility that Psf1 may genetically interact with other DNA replication proteins (Table 5). Therefore we preformed several crosses
between *psf1* mutant flies and other flies that have been previously found to contain mutations in DNA replication proteins. Unlinked non-complementation is a term describing two proteins that play a role in the same pathway and therefore produce a phenotype if both proteins are decreased. We looked for unlinked non-complementation by scoring F1 generations of flies. Fly crosses were scored for dominant markers and wild type phenotypes. If no wild type phenotypes were observed then the combination of the reduction in both functional proteins resulted in a new mutant phenotype and would indicate unlinked complementation. Fly crosses involving *psf1* and other DNA replication proteins revealed no unlinked non-complementation (Table 4).

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<tr>
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<th>Mcm2</th>
<th>Hp1</th>
<th>Geminin</th>
<th>Orc2</th>
<th>PCNA</th>
<th>Orc5</th>
<th>Dp</th>
<th>Mcm4</th>
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Table 4. Results of unlinked non-complementation experiment. F1 generation flies were scored for dominant markers over a week period and recorded above.
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Table 5. DNA replication mutants with specific pathways
Specific Aim #5: Investigate informative phenotypes using confocal microscopy

Salivary glands

Salivary glands consist of polytene chromosomes. Polytene chromosomes are of interest because they undergo repeated rounds of DNA replication without cell division, also known as endomitosis. These chromosomes have light and dark regions. The dark regions correspond to inactive chromatin called heterochromatin, while the light regions have a high level of transcriptional activity and are called euchromatin. Salivary glands were dissected from 3rd instar larvae to view polytene chromosomes. Chromosomes were either spread apart to view the arms or kept condensed to show any size differential. Chromosomes were stained with DAPI and viewed under the confocal microscope. Polytene chromosomes showed under-replication as compared to

Fig 3. Polytene Chromosomes of Psf1 and +/- . Salivary glands were dissected from 3rd instar larvae. Polytene chromosomes were both spread apart and left condensed. DAPI was used as a stain. Images were viewed using a spinning disc confocal microscope.
wild type glands. Glands appear much less full in both spread and non-spread chromosomes (Figure 3). This can be an indication that psf1 plays a role during S-phase of the cell cycle. Since the flies are viable the most likely explanation would be an S-phase delay that would slow down DNA replication but not cause lethality.

**Aneuploidy**

Aneuploidy is an abnormal number of chromosomes. In *Drosophila* you would expect to see a total of 8 chromosomes including two dot chromosomes. Aneuploidy present in psf1 mutants would suggest psf1 plays a roll in mitotic checkpoints or spindle formation. Brains were dissected from 3rd instar larvae and either sandwiched between two slides or kept whole. Sandwich brains were stained with DAPI while whole brains were left unstained, both types of samples were viewed under the confocal microscope. Images of chromosomes showed no aneuploidy in psf1 mutant flies (Figure 4). Whole brains

![Fig 4. Brain images of +/+ and Psf1 mutant. Brains were dissected from 3rd instar larvae. Brains were either sandwiched between slides or left whole to view. No aneuploidy was found in Psf1 mutant. Scale bar is equal to 10µm. Whole brains appear normal in psf1 mutant. DAPI was used as a stain. Images were viewed using a spinning disc confocal microscope.](image-url)
showed no difference in size. Based on this data \textit{psf1} does not have a pivotal role in either mitotic checkpoints or spindle formation.

**Mitotic Indices**

A mitotic index measures the cell cycle progression of cells by determining the amount of cells in M phase vs. Interphase phase. It is calculated by dividing the number of cells in mitosis by the total number of cells in the viewable area. Mitotic indices were scored in the brain as it goes through a complete normal cell cycle and can be easily dissected. Calculations were used to analyze if decreased \textit{psf1} levels disrupted the time periods of the cell cycle. Mitotic indices show a significant decrease of nuclei in mitosis in \textit{psf1} compared to wild type (Figure 5). A decrease in cells in mitosis would indicate a delay in S phase. We also looked at whole brains to in order to observe any size differences due to the level of mitotic indices. Whole brains showed no difference in size (Figure 4).

![Fig 5. Mitotic indices show a decrease of cells in mitosis in \textit{psf1} mutant compared to wild type. A whisker plot was produced from the data to graphically depict a statistical significant difference between the number of nuclei in M phase compared to I phase. The wt group \((M = 0.016, SD = 0.003)\) had significantly more cell growth during mitosis than the \textit{psf1} group \((M = .009, SD = .002)\), \(t(17) = 5.50, p = .0000, CI_{95} = .004, .009)\).](image-url)
Early embryo

The cell cycles during stages of embryogenesis have been extensively studied in *Drosophila melanogaster*. We will focus on the cell cycles shown in Figure 6.

These stages allow for the best view of the nuclei as they are at the surface of the embryo. During embryogenesis, nuclei go through a rapid cell cycle consisting of M & S phases. Wild type embryos show synchrony in the cell cycle during these stages of embryogenesis. Embryos with a mutant *psf1* allele may show asynchrony or metaphase bridges, both of which indicate a defect in either M or S phase of the cell cycle. Embryos were collected at the 5h stage, devitellinized, rehydrated and fixed. Embryos were stained with DAPI and viewed under a confocal microscope. Our results showed asynchrony in the nuclei in the *psf1* mutant embryo that is consistent with either an S or M phase delay (Figure 7).
Ovaries

Ovaries in *Drosophila melanogaster* go through several stages producing oocytes with multiple nuclei. Germline cells that are found in egg chambers in the oocyte are called nurse cells. Nurse cells are mitotic sisters of the oocyte and go through an observable condensation phase that can reveal phenotypes of mutants that play a role in chromatin condensation. The nurse cells change significantly through the different stages as shown in Figure 8 below. Nurse cells in stages 2-4 in the germarium appear polytene then form a blob-like appearance at stage 5-6 when they begin to dissociate. The blob-like appearance is caused by chromatin condensation and would show a visible phenotype if defects are present. Viewing the polyploid nurse cells in the ovaries of females containing decreased *psf1* level showed no phenotypes when compared to wild type (Figure 9).

Fig 7. Early embryo show asynchrony in Psf1 mutants compared to wild type. Embryos were collected at 5h, devitellinized and rehydrated before fixing. DAPI was used as a stain. Images were viewed using a spinning disc confocal microscope.
Fig 9. No condensation defects were observed in Psf1 mutants compared to wild type. Ovaries were dissected from female flies, tweezed apart and fixed. Stages 5 and 6 were imaged to look for condensation defects. DAPI was used as a stain. Images were viewed using a spinning disc confocal microscope.
Chapter 6: Discussion

Allowing DNA to only be replicated once per cell cycle is essential to the viability of the organism. The budding yeast GINS complex is present throughout the cell cycle, but is only recruited to origins of replication during S phase, around the time of initiation at each origin (Takayama et al, 2003). After initiation, budding yeast GINS moves away from origins with replication forks (Kanemaki & Labib, 2006). Moreover, GINS remains associated with stalled forks after inhibition of nucleotide synthesis (Takayama et al, 2003) and with forks that pause at protein–DNA barriers (Calzada et al, 2005). GINS only associates with chromatin during S phase in Xenopus (Kubota et al, 2003) and fission yeast (Yabuuchi et al, 2006) and it seems likely that the complex also functions at forks in these species. GINS is required for the unwinding step of DNA replication because ChIP experiments indicate that it is needed for the recruitment of the single-stranded DNA-binding protein RPA (Kanemaki & Labib, 2006). In the absence of GINS, MCM–Cdc45 does not move away from origins, and genomic footprinting indicates that the origin remains in the pre-replicative state (Kanemaki & Labib, 2006). Moreover, in xenopus, GINS is needed for Cdc45 to become stably associated with chromatin during the initiation of chromosome replication (Kubota et al, 2003). Much is known about the GINS complex in xenopus larval extracts, budding, and fission yeast. However in vivo experimentation with a metazoan has not been performed and is unique to my research.

A role in my research has been to use Drosophila melanogaster as a model organism for the function of the GINS complex, specifically the subunit Psf1. My studies
of Psf1 have included exploring genetic interaction as well as informative phenotypes using a hypomorph of the \textit{psf1} gene. We first obtained a strain of \textit{Drosophila} containing a P-element insertion 10bp upstream of the \textit{psf1} locus. Transposons are DNA sequences which contain at least one gene coding for a transposase and motives located on both ends of the transposons the role of which is to trigger integration (Houdebine, 2002). P-elements are class II transposons that can become mobile when bound by a transposase enzyme and are unique to \textit{Drosophila} melanogaster. A P-element insertion just before the coding region of a gene usually results in knocked out expression of any gene product. Therefore we were under the assumption that this specific fly line would have a complete null mutant for the \textit{psf1} gene. PCR of the genomic DNA verified the mutant fly line contained a P-element correctly inserted and in the right position (Fig 1).

We first investigated the arrest point of the mutant fly line. The GINS complex has a key role during both the establishment and the progression of DNA replication forks (Labib & Gambus, 2007). If \textit{psf1} was fully knocked out then you would not expect viability of the homozygous offspring. Flies containing a balancer with GFP were crossed with the \textit{psf1} mutant fly line and subsequent generations were picked to allow for homozygous embryos to be produced. Stocks of homozygous embryos were able to survive up to full adult. This indicated the \textit{psf1} mutant fly line was homozygous viable.

These unexpected results led us down a new path where we wanted to quantify the level of expression of the \textit{psf1} gene. Performing reverse transcriptase PCR gave us a quantification of the mRNA levels of the \textit{psf1} gene. This is of interest to us
since any phenotypes we find can be related back to the lower levels of psf1 expression. We could then compare the hypomorphic fly to a true null mutant in future research to see if there is any correlation. The RTPCR showed a 78% decrease in mRNA levels of psf1. This shows that only a 22% expression level of psf1 can still produce viable offspring. This can be explained either by an overabundance of Psf1 in wild type flies or that Psf1 is needed at only low levels for viability. Although the lower expression levels allowed for viable offspring, there can still be informative phenotypes we can observe. We know the GINS complex is important throughout the process of chromosome replication (Kanemaki et al, 2003). However a hypomorph may give us a clue to a more specific function of the GINS complex.

The first tissue we looked at was the salivary glands. Salivary glands in Drosophila contain polytene chromosomes that go through several rounds of endoreplication. Endoreplication is genome duplication without mitosis resulting in high cell volume and high gene expression levels. Figure 3 shows both spread and non-spread views of polytene chromosomes. There were noticeable size differences between the psf1 mutant chromosomes and the wild type chromosomes. The chromosomes of the psf1 mutant were less thick than wild type chromosomes indicating under replication. Under replication could be a result of a delay in S phase of the cell cycle. This would complement the findings in other species that the GINS complex is loaded on the replisome during the pre-initiation complex during the G1 phase and interacts specifically and stably during S phase with MCM and Cdc45 (Gambus et al, 2006).
The second tissue type we looked at were the brains of the *Drosophila*. The brains go through a normal cell cycle so we can look at tissue samples that do not go through endoreplication. We first looked for aneuploidy, which is an abnormal number of chromosomes. This occurs when cells do not properly separate during mitosis and would indicate the *psf1* may play a role during mitosis. We did not find any aneuploidy in the *psf1* mutant brain tissue (Fig 4).

The next step was to look at the mitotic index of the *psf1* mutant. Mitotic indices are the number of nuclei going through mitosis compared to nuclei going through interphase. The mitotic index would indicate if there is delay in the cell cycle. If the *psf1* mutant had a greater number of nuclei going through mitosis than you could assume there is an M phase delay since there is a greater number of nuclei in mitosis. Conversely, a decrease in nuclei in mitosis would indicate an S phase delay. As nuclei are slowed down in S phase you would expect to find fewer nuclei in M phase. We observed a significant decrease of nuclei going through mitosis indicating an S phase delay (Fig 5). The observed S phase delay in brain tissue strongly argues that the under replication of polytene chromosomes in salivary tissues is due to an S phase delay as well.

We then looked at the early embryo of the *psf1* mutant. *Drosophila* go through a well-studied sequence of events during the embryogenesis and we focused our observations on cell cycles 10-12 when the nuclei are on the outer edge of the embryo. During embryogenesis, nuclei quickly switch back and forth between M and S phase, bypassing G1 and G2. The synchrony of the changing M and S phases can be
viewed and any asynchrony would indicate a delay in either M or S phase. Asynchrony was viewed (Fig 7) in the psf1 mutant indicating a delay in either M or S phase. Although it cannot be singled out to a specific phase, the observations made in brain and salivary gland tissue suggest that this embryo phenotype is also due to an S phase delay in the psf1 mutant.

With several sources of evidence pointing an S phase delay in the psf1 mutant, we now wanted a true null mutant to corroborate our finding in the hypomorphic mutant. To create a null mutant we performed an imprecise P-element excision. A P-element construct can be easily mobilized using a separate source of Transposase, creating many lines with a single element inserted randomly in the genome (Ryder & Russell, 2003). Since the P-element is a class 2 transposon it can be moved to another area of the genome using a transposase enzyme. By crossing a fly line with a transposase we were able to lift out the P-element insertion from its location 10bp upstream of the psf1 coding region. Most excisions result in a precise excision of the P-element. However, occasionally, a few P-element excisions will be imprecise, deleting flanking DNA from the genome. Since the P-element is only 10bp upstream of the psf1 gene it would seem likely that most imprecise excisions would take at least a portion of the psf1 gene with it. This would create a true null mutant of psf1 that we could use for future research.

The original goal of my research project was to uncover informative phenotypes of a fly line containing a null psf1 allele. Preliminary data showed the correct placement of a P-element insertion required to produce a mutant psf1 gene. Further experimentation revealed homozygous viability of the psf1 mutant fly line that was
conflicting with our original hypothesis concerning the nature of the *psf1* mutant. This gave way to a change in my research plan as I began to focus my attention on the reasons behind the viability of the fly with two mutant copies of *psf1*. RTPCR results concluded a *psf1* expression level of only 22% in the mutant. This result proved that only a small percentage of Psf1 is needed for DNA replication to occur. This is most likely explained by a limited need of the GINS complex for viability of the organism. We did detect a delay in S phase through tissue samples of salivary glands, brains, and embryos that occurred with low levels of Psf1.

GINS associates with replication origins during S phase (Kanemaki et al, 2003). It has also been suggested that GINS promotes an interaction between the Sld3-Cdc45 complex and the Sld2-Dpb11-Pol epsilon complex and that this could facilitate initiation of DNA replication (Masumoto, Muramatsu, Kamimura, & Araki, 2002). Our results correlate well with the presently postulated role for Psf1 and the GINS complex in DNA replication. We saw a delay in S phase that may indicate with lower levels of Psf1 there are fewer GINS complexes formed to help with DNA replication. Therefore DNA replication occurs at a much slower rate as the limiting numbers of GINS complexes are not sufficient to perform replication of the genome at normal rates.

This is the first time that the GINS complex has been studied in a multi-cellular metazoan. Previous studies of the GINS complex have involved mouse tissue cultures, *in vitro* studies using xenopus larval extracts, and unicellular budding and fission yeast. *Drosophila* allows for in vivo experimentation at several different life stages. This gives new insight into the function of GINS without the potential artifact of *in vitro* and tissue
culture studies. *Drosophila* also provides the ability to study the function of the GINS complex at different stages of development from the embryo to the full adult allowing a more complete study of the complex throughout the life of the organism.

We have found it important for the GINS complex to be available for a normal cell cycle to occur. The lack of normal amounts of Psf1 and therefore GINS complex showed a much slower rate of DNA replication and therefore a slower cell cycle. To prevent the occurrence of potentially cancer-causing alterations to the genome, it is crucial that chromosomal DNA is precisely duplicated during S phase of the cell division cycle (Blow & Gillespie, 2008). Cell cycle regulation may become compromised when it becomes consistently delayed as seen in S phase with the hypomorphic *psf1* allele. Therefore the GINS complex is essential for an organism and a defect in its formation may disrupt cell cycle regulation.
Chapter 7: Appendix

Cloning psf1

Genomic DNA was isolated from the psf1 mutant and the psf1 allele was amplified with PCR as shown in Experiment 1. Genomic DNA was used instead cDNA because it lacked introns, therefore the entire sequence coded for the protein. The primers used were psf1 start topo and psf1 with stop (Table 2). Product was inserted into a pENTR/D-TOPO vector (Invitrogen) following manufacture’s instruction. The vector was then transformed into One Shot™ chemically competent E.coli cells were performed following manufacture’s instruction (Invitrogen). Samples were then spread across selective plates and incubated overnight at 37°C. Isolated colonies were picked from the selective plates and incubated overnight in LB at 37°C. Mini preps of the overnight samples were performed (Promega) following manufacture’s instruction and DNA was quantified using a NanoDrop Fluorospectrometer (Thermo Scientific). The quantified DNA was then sequenced to verify the mutant psf1 gene had been cloned into the E.coli cells and will be used in future research.
# Chapter 8: List of Abbreviations

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<tr>
<td>BSA</td>
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<tr>
<td>CDK</td>
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<td>DDK</td>
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<td>YPD</td>
<td>Yeast Peptone Dextrose</td>
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Chapter 9: Literature Cited


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