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THE ROLE OF PRE-REPLICATION COMPLEX PROTEINS IN DROSPHILA OVARIAN STEM CELL MAINTENANCE AND PROLIFERATION

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Abstract

Cancer stem cells (CSCs) may hold the key to advances in cancer research. Like adult tissue-specific stem cells, CSCs are able to self-renew and last a lifetime. These shared characteristics can thus be used to help learn more about CSCs and how they work. More specifically, understanding the molecular mechanisms that control adult tissue-specific stem cell proliferation can lead to better regenerative therapies and a better understanding of CSCs overall. The proliferation of *Drosophila* ovarian germline stem cells (GSCs), as well as their ability to create differentiated daughters, relies on proper DNA replication. By investigating the regulation of stem cell division, we can advance our knowledge of the fine line between regulated versus unregulated division, and the causes behind each.

One of the major components of replication control are the pre-initiation complex (PIC) proteins, which are essential for proper DNA replication. For example, Minichromosome maintenance protein 10 (*MCM10*) is recruited during the transition from G1 to S-phase, and aids in the recruitment of other PIC proteins to the replication fork. To investigate whether PIC proteins are essential for stem cell function, two comparisons were made. The average number of GSCs per germarium in control and mutant flies were compared. Next EdU staining was utilized to calculate the percentage of GSCs that were in S-phase. Once again, control and mutant percentages were compared to determine if *MCM10* mutants affect stem cell function. We demonstrate that the loss of *MCM10* results in a reduction in stem cell number and an increase S-phase length which is an indication of an altered cell cycle. Ongoing studies are investigating if stem cell loss in *MCM10* mutants is due to DNA damage and to observe the effects of other pre-replication initiation complex mutant proteins. Combined, our data suggests that PIC proteins are critical for stem cell function.

Introduction

Cancer is a sober topic that touches everyone. Numbers pulled from the National Cancer Institute state that nearly 40% of men and woman in the United States will be diagnosed with cancer. In 2016, nearly 1.5 million new cancer cases were diagnosed. Even though a rural county in Eastern North Carolina, Pitt County had 3,482 cancer cases with a little over 1,300 of the patients succumbing to their illnesses(Leung, 2017). Despite these staggering numbers, there are some bright spots to look at. There has been a general decrease in the diagnoses of cancer in men(1.8%), women(1.4%), and children(1.4%)(Leung, 2017). Also, the overall cancer death rate has decreased 13% from 2004-2013(Leung, 2017). By studying the smallest components of cancer, such as the mechanics behind what makes cancer cells so dangerous, a better understanding of how cancer cells work and how they develop can be gained

Stem cells have many unique properties that separate them from other cells in the body. For example, embryonic stem cells begin as totipotent, meaning they are able to differentiate into any type of cell((Bayat Mokhtari et al., 2017; Pasquier & Rafii, 2013; Wang, Zhu, & Pei, 2017). As the organism becomes more developed and organized, most stem cells lose their ability to become any type of cell; however, small populations of tissue-specific stem cells remain in tissues that have rapid cellular turnover(Bayat Mokhtari et al., 2017; Pasquier & Rafii, 2013; Wang et al., 2017). The cell cycle is kept under strict molecular control, to prevent tissue overgrowth or wasting(Bayat Mokhtari et al., 2017; Pasquier & Rafii, 2013; Wang et al., 2017).

Cancer cells do not adhere to a normal cell cycle. Recently, it has been hypothesized that the growth of tumors are fueled by limited number of cells that are capable of self-renewal, much like stem cells(Aharony, Michowiz, & Goldenberg-Cohen, 2017; Clevers, 2011; Hsu, Mohyeldin, Shah, Gokaslan, & Quinones-Hinojosa, 2012). Cancer stem cells(CSCs) can last a

lifetime(Aharony et al., 2017; Clevers, 2011; Hsu et al., 2012). They act as the parent of all other cells within the tumor. As their daughter/more specific cells die out, the cancer cell will keep dividing to either maintain or expand the tumor itself(Aharony et al., 2017; Clevers, 2011; Hsu et al., 2012). Given the similarities between tissue-specific stem cells and CSCs, a better understanding of stem cells or CSCs self-renewal processes could lead to more effective cancer drugs(Clevers, 2011; Hsu et al., 2012; Pasquier & Rafii, 2013). Finally, by looking into the division of stem cells and CSCs, we can further advance our knowledge of the fine line between checked versus unchecked division, and the causes behind each.

Control over DNA replication is central to a cell's ability to divide (Ruijtenberg & van den Heuvel, 2016; Shostak, 2017; Siefert, Clowdus, & Sansam, 2015; Takisawa, Mimura, & Kubota, 2000). One of the major components of replication control is the pre-initiation complex proteins which are essential in cell proliferation (Prasanth, Mendez, Prasanth, & Stillman, 2004; Sun & Kong, 2010; Takisawa et al., 2000). This group of proteins position DNA polymerase II over gene transcription start sites, denature the DNA, and position the DNA in the DNA polymerase active site for replication(Prasanth et al., 2004; Sun & Kong, 2010; Takisawa et al., 2000). Minichromosome maintenance protein 10 is a replication factor protein that is recruited during the transition from G1 to S-phase and aids in the recruitment of the pre-replication initiation complex(Chattopadhyay & Bielinsky, 2007; Christensen & Tye, 2003; Thu & Bielinsky, 2014).

MCM10 is a constitutively nuclear DNA binding protein that has the unique ability to bind to dsDNA or ssDNA(Chattopadhyay & Bielinsky, 2007; Christensen & Tye, 2003; Thu & Bielinsky, 2014; Vo et al., 2014). After *MCM10* incorporation occurs, *MCM10* facilitates the binding of Pol-a to DNA and acts as a replication fork factor that promotes DNA unwinding at origins along the sequence(Chattopadhyay & Bielinsky, 2007; Christensen & Tye, 2003; Thu &

Bielinsky, 2014; Vo et al., 2014). As *MCM10* is promoting unwinding, it activates helicases that split DNA apart and allow for replication to begin(Chattopadhyay & Bielinsky, 2007; Christensen & Tye, 2003; Thu & Bielinsky, 2014; Vo et al., 2014). In addition to the initiation of DNA replication, *MCM10* also aids in the elongation of DNA(Chattopadhyay & Bielinsky, 2007; Christensen & Tye, 2003; Thu & Bielinsky, 2014; Vo et al., 2014). Because of *MCM10*'s interactions with Pol-a, Ctf4, and RPA, *MCM10* is a key factor in lagging strand DNA synthesis(Chattopadhyay & Bielinsky, 2007; Christensen & Tye, 2003; Thu & Bielinsky, 2014).

MCM10 in humans is involved DNA replication and elongation(Chattopadhyay & Bielinsky, 2007). MCM0 binds directly to pol-a and plays a critical role in lagging strand synthesis(Chattopadhyay & Bielinsky, 2007). With this interaction, *MCM10* facilitates RNA-DNA primer synthesis and can possibly recruit PNCA or act as a primase itself(Chattopadhyay & Bielinsky, 2007). Recent studies demonstrate that *MCM10* deficiencies can lead to genetic diseases such as cancer(Thu & Bielinsky, 2014). Evidence behind this is supported by two genome wide screens that link *MCM10* to chromosome breakage suppression(Thu & Bielinsky, 2014). A link between replication stress(fork collapses, DSBs, and such) and chromosomal instability has been found as well(Thu & Bielinsky, 2014). The combination of these two can cause tumor heterogeneity(Thu & Bielinsky, 2014). Because deficiencies in *MCM10* cause replication stress which leads to chromosomal instability, misregulation of *MCM10* can lead to cancer development(Chattopadhyay & Bielinsky, 2007). Some studies show that *MCM10* expression levels either controls the aggressiveness of tumors or oncogenes seek out *MCM10* to control regulate the expression of the particular protein(Thu & Bielinsky, 2014). When *MCM10* or its human homologs is deficient, cancers such as gastric carcinoma, bladder cancer, esophageal cancer, and lung adenocarcinoma arise(Thu & Bielinsky, 2014).

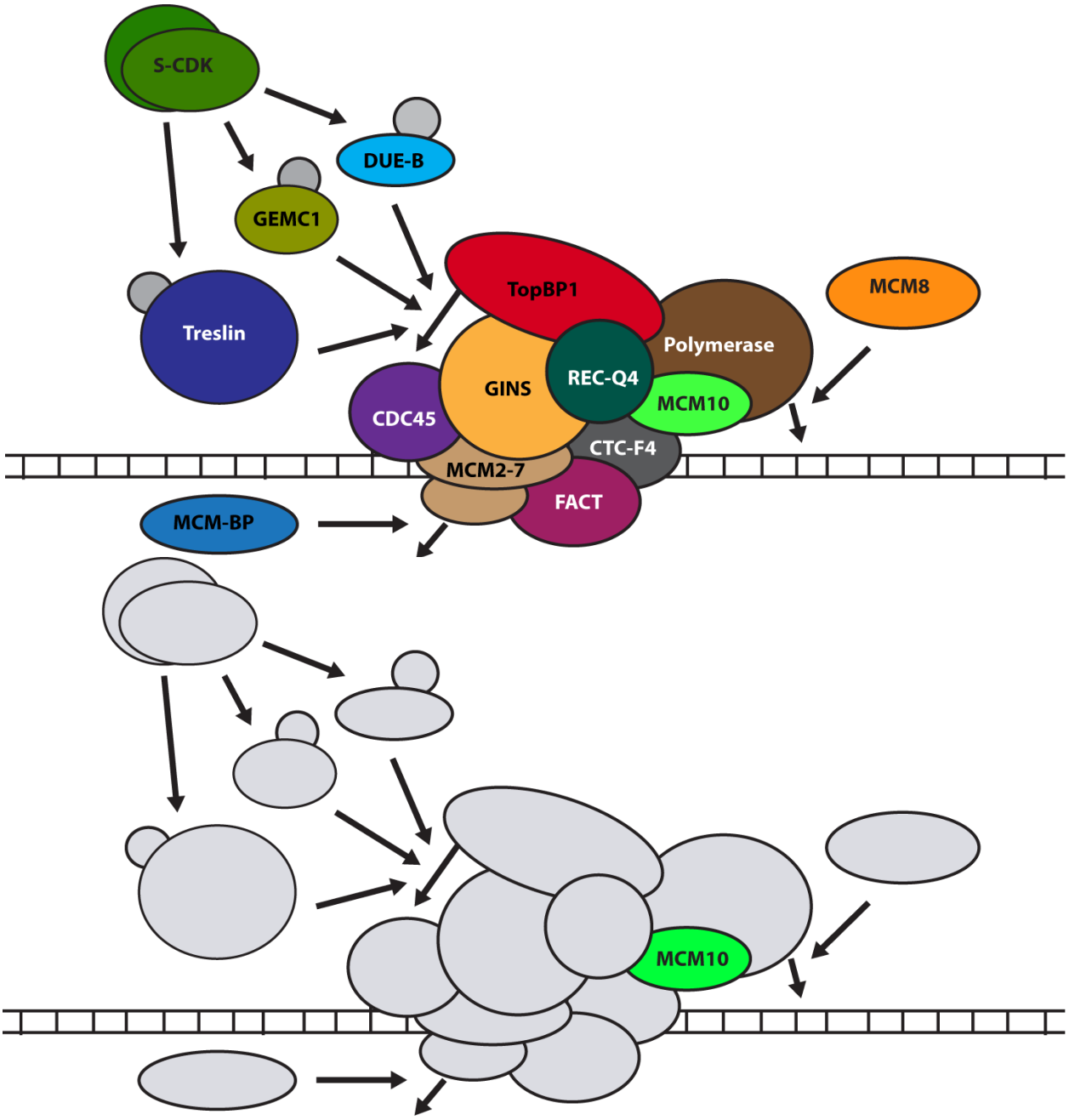
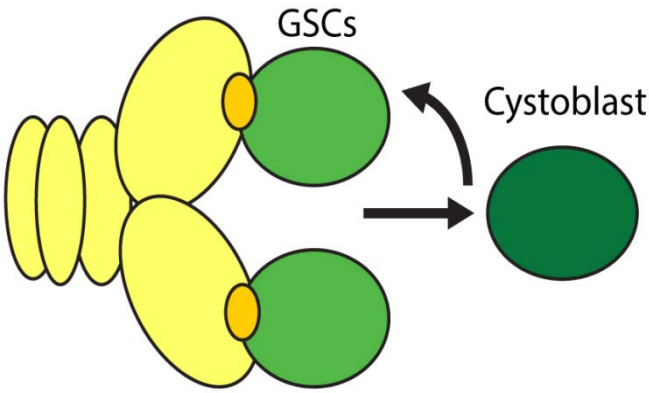


Figure 1 Pre-Initiation Complex for Replication is vital for the control of DNA replication. Diagram of the PIC and *MCM10* location.

Using model organisms, such as *Drosophila melanogaster*, allow researchers to use a variety of techniques to study their topics of interest(Lu, 2009; Martin & Krantz, 2014; Millburn, Crosby, Gramates, & Tweedie, 2016; Song, 2005). When it comes to categorizing the genes in stem cell renewal as well as observing the defects of stem cell renewal, *Drosophila* germline stem cells are a model system of a choice. GSCs are readily observable and can be immunostained with a multitude of protein markers (Dansereau & Lasko, 2008; de Cuevas & Matunis, 2011; Dunlop, Telfer, & Anderson, 2014; Sahai-Hernandez, Castanieto, & Nystul, 2012; Spradling, Fuller, Braun, & Yoshida, 2011) . On average, there are two to three germline stem cells at the anterior part of the germanium called the stem cell niche(Dansereau & Lasko, 2008; de Cuevas & Matunis, 2011; Dunlop et al., 2014; Sahai-Hernandez et al., 2012; Spradling et al., 2011). As a GSC divides, it does so asymmetrically(Dansereau & Lasko, 2008; de Cuevas & Matunis, 2011; Dunlop et al., 2014; Sahai-Hernandez et al., 2012; Spradling et al., 2011). This division produces another self-renewing GSC and a differentiated progenitor cell called a cystoblast(Dansereau & Lasko, 2008; de Cuevas & Matunis, 2011; Dunlop et al., 2014; Sahai-Hernandez et al., 2012; Spradling et al., 2011). The self-renewing GSC will stay in the niche while the cystoblast will go on and divide further(Dansereau & Lasko, 2008; de Cuevas & Matunis, 2011; Dunlop et al., 2014; Sahai-Hernandez et al., 2012; Spradling et al., 2011). After the cytoplast divides four times, it forms a 16 cell cyst. At this point, one of the sixteen cells becomes an oocyte while the others become nurse cells(Dansereau & Lasko, 2008; de Cuevas & Matunis, 2011; Dunlop et al., 2014; Sahai-Hernandez et al., 2012; Spradling et al., 2011).



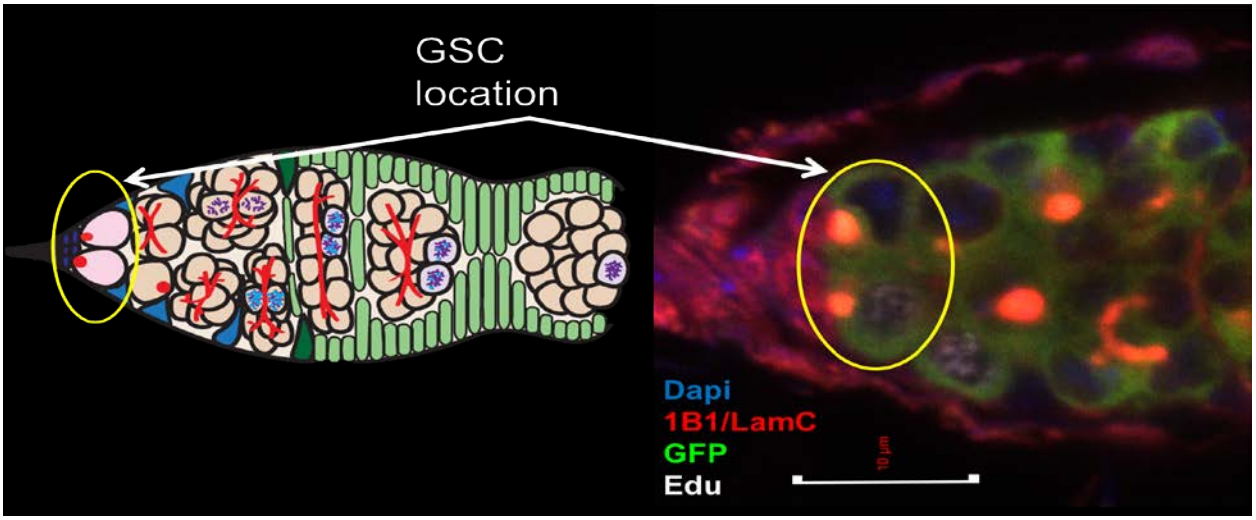


Figure 2 Germline Stem Cells used as model cell types. Top figure is a schematic of the GSCs. Bottom figure is a cartoon of what a germarium looks like compared to a germarium when immunostained. The germline stem cells are circled yellow.

A study used HeLa cells to determine the function of MCM10. In this experiment, MCM10 was found to be loaded on during G1/S transition in HeLa cells and would dissociate once S phase was completed(Chattopadhyay & Bielinsky, 2007). After deleting MCM10, the researchers discovered many defects that occur when MCM10 levels are depleted(Chattopadhyay & Bielinsky, 2007). Two major defects were that cells would accumulate large amounts of DNA damage and the cell cycle would arrest in late S/G2 phase(Chattopadhyay & Bielinsky, 2007). Therefore, it was determined that MCM10 is required for replication initiation and elongation because when absent, DNA damage and cell death are more prevalent, lagging strand synthesis is impeded, and replication fork arrest occurs(Chattopadhyay & Bielinsky, 2007). To further support these results, it was found that depletion of MCM10 causes degradation of p180, a subunit of pol-a, which is an essential protein that is involved in DNA replication(Chattopadhyay & Bielinsky, 2007). The arrest in S/G2 not only causes cell cycle arrest, but inhibits proliferation in cells as well(Chattopadhyay & Bielinsky, 2007). Inhibition of proliferation causes a severe reduction in cell number(Chattopadhyay & Bielinsky, 2007). Due to the reduction in number and arrest, morphological changes and unusual phenotypes are readily observed(Chattopadhyay & Bielinsky, 2007).

Results also indicated that there was an increase in accumulation of cells in G2/M phase(Chattopadhyay & Bielinsky, 2007). Fewer cells were entering S phase which leads to a decrease in DNA synthesis completion and leads to cells not entering mitosis due to incomplete DNA replication or DNA damage(Chattopadhyay & Bielinsky, 2007). These cells do not enter metaphase and there is an overall increase in apoptotic cells due to stalled forks and dsDNA breaks(Chattopadhyay & Bielinsky, 2007).

In *Drosophila, MCM10* has been linked to play an important role in DNA replication (Reubens et al., 2015). Decreased levels of *MCM10* were found to delay S-phase in the central nervous system and generate unique genomic lesions(Reubens et al., 2015). Alterations to the *MCM10* gene (in the form of a frame shift, a splice junction, and a premature stop codon) were introduced(Reubens et al., 2015). Despite the genetic effects of these three mutant alleles, *MCM10* was found to not be required for adult viability (Reubens et al., 2015). It was then deduced that *MCM10* mutations caused transcript instability (Reubens et al., 2015). To back this assertion, *MCM10* was later found to promote genomic stability during early embryonic divisions. The study found that MCM10 is required for female fertility. The ovaries were missing portions of certain cysts that are characteristic of the cell cycle (Reubens et al., 2015). This led the researchers to study *MCM10's* role in germline development. They found that *MCM10* likely functions in germline development or maintenance during oogenesis. The morphological changes due to the three mutant alleles seemed to affect the germline. This led me to ask what are the effects of the mutant alleles on the germline stem cells present in the ovaries *Drosophila*.

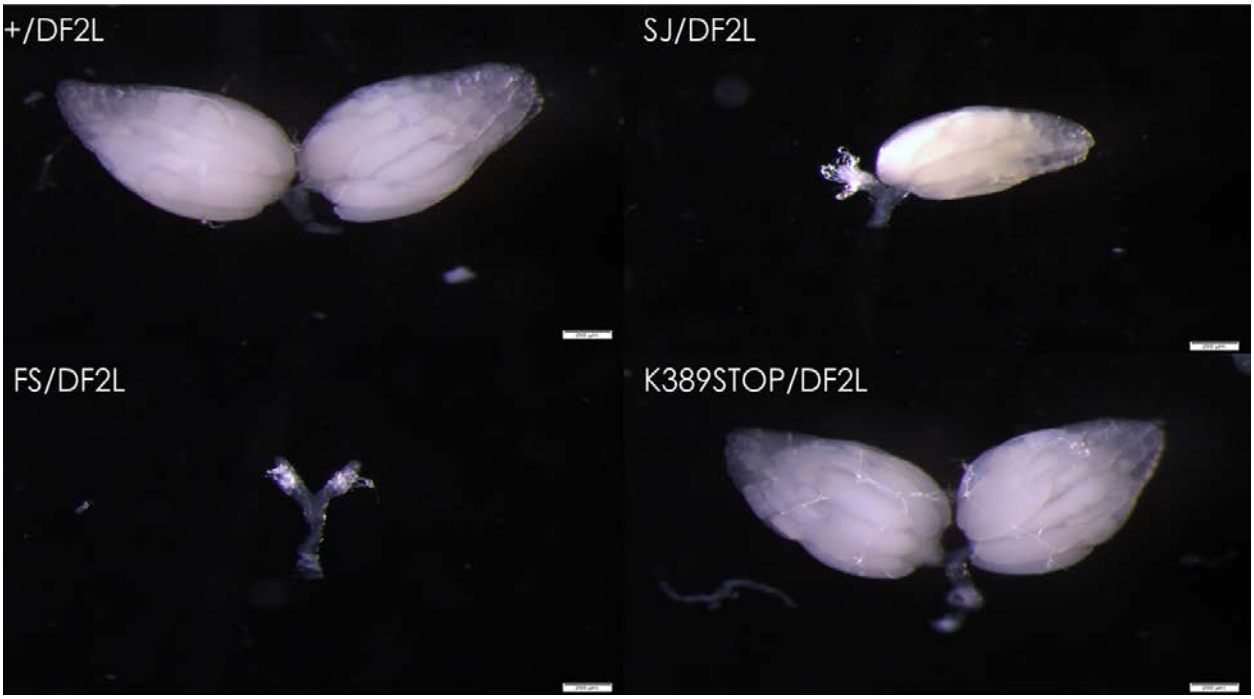


Figure 3 *MCM10* is required for *Drosophila* ovarial development
Wildtype germarium compared to three mutants.
B: Splice Junction
C: Frameshift
D: Premature stop

The purpose of this study is to increase the knowledge of stem cell proliferation and maintenance as a whole as well as draw attention to the importance of pre-initiation complex proteins like *MCM10* in stem cell proliferation and maintenance. Using the above *MCM10* deficient mutants, I attempted to answer two questions: What is the effect of *MCM10* on cell cycle progression in stem cells and what is *MCM10*'s role in stem cell maintenance?

Using immunostained germariums, I compared control versus mutant germariums at two time points, 4 DAE and 14 DAE. I first looked for EdU incorporation to determine which GSCs were in S-phase and which were not. I found that at 4 DAE, more mutant GSCs were in S-phase than their control counterparts. In addition, there was a visually significant difference between the number of GSCs present in mutants versus controls at both time points. This data indicates that *MCM10* plays a significant role in cell cycle progression and stem cell maintenance.

Methods(adapted from Reubens et al)

For all experiments presented, flies were cultivated at 25 °C on Drosophila dietmedia K12 (U.S. Biologicals D9600-07B), and w1118 was used as wild type. The following alleles were obtained from Dr. Tim Christensen: Df(2L)Exel6047,Mcm10 FS, Mcm10K389STOP, and Mcm10SJ. Mcm10mutants were analyzed in trans to Df(2L)Exel6047,which covers the entire Mcm10 locus(Reubens et al., 2015).

To assess ovarian morphology, newly eclosed females were collected for each genotype and mated to wildtype males. The females were fed on K12media and yeast paste for three days, placing the flies in a new vial with fresh yeast paste each day. On day four and fourteen days after eclosion, ovaries were dissected in fresh Grace's Insect Media (Lonza BioWhittaker).

For immunofluorescent analysis of ovarian morphology, ovaries were dissected, fixed, washed, and blocked as described (Ables and Drummond-Barbosa, 2010). The following primary antibodies were used overnight at 4 °C: mouse anti-Hts [1B1, Developmental Studies Hybridoma Bank (DSHB); 1:10] and mouse anti-Lamin C (LamC) (LC28.26, DSHB; 1:100). Following a two-hour incubation with Alexa Fluor 568-conjugated goat anti-mouse secondary antibodies (Life Technologies;1:200), ovaries were stainedwith0.5μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma). Ovaries were mounted in 90% glycerol containing 20 mg/ml n-propyl gallate (Sigma). Confocal Z-stacks (1 μm optical sections) were taken with a Zeiss LSM700 microscope using ZEN Black 2011 software. Images were analyzed and minimally and equally enhanced via histogram using Zeiss ZEN software.

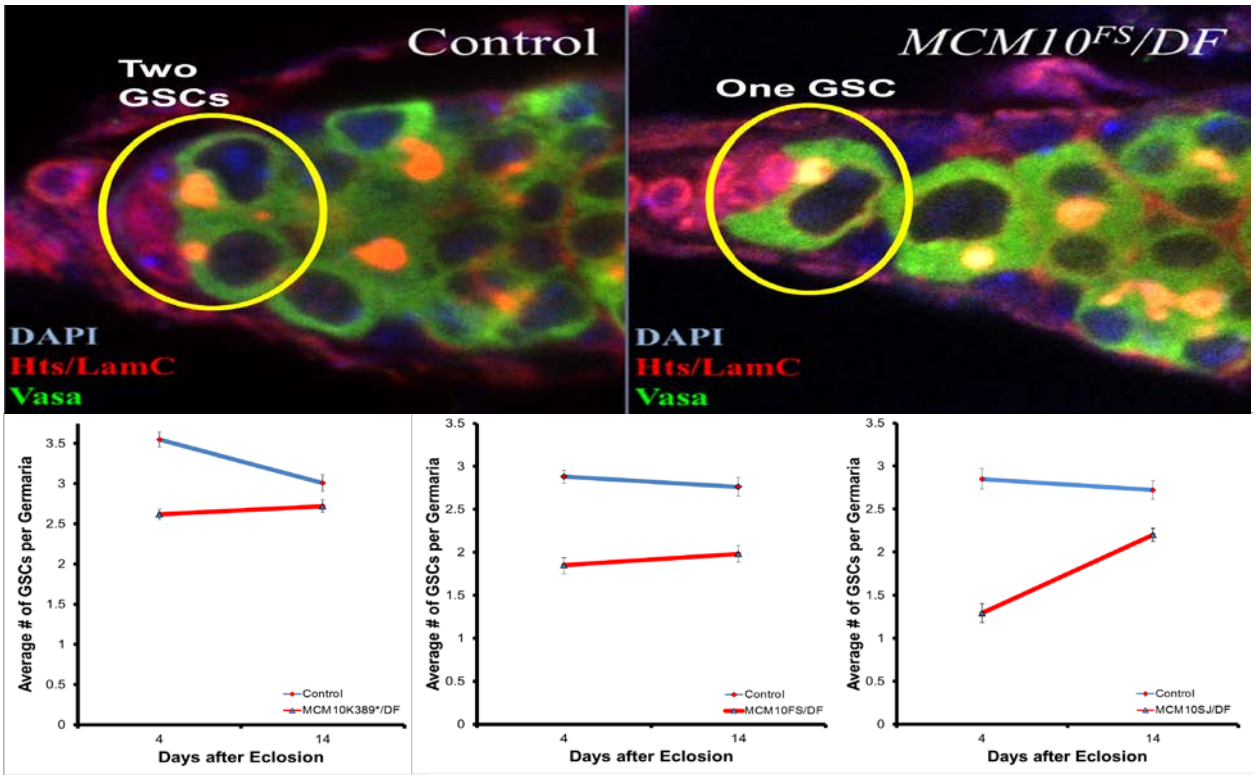
Results

MCM10 is required for adult GSC number

To determine if *MCM10* mutations altered stem cell number, the average number of GSCs per germarium was used to compare control germarium and mutant *MCM10* germarium. Germ Line Stem Cell numbers are determined by first identifying fusomes (colored in orange) that are attached to cap cells (colored in blue/purple), this signifies that the fusome is within the niche. Next, if a cell (outlined in green) if directly attached to a fusome which is located in the niche, then the cell is counted as a GSC. On average, a normal *Drosophila* would have 4 GSC located in the niche of the germarium. The same process was used for both control and mutant groups. To determine if the *MCM10* mutations have a greater effect over time on stem cell number, two time points were chosen. 4 d.a.e would be the first time point measured because the stem cells would have started the cell cycle. 14 d.a.e was chosen because the age of the cells would be a factor that could contribute to a decrease in stem cell numbers.

Results for 4 d.a.e show that there are fewer number of stem cells in *MCM10* mutants than in the control. Control averages were closer to 3 GSCs per germarium while mutant averages were below 3 and in some cases, below 2 GSCs. When comparing 4 d.a.e. Control *MCM10* vs. *MCM10* Frame Shift mutants, there was a statistically significant difference. The control average was 2.86 GSCs while the Frame Shift mutants averaged 1.85 GSCs. Control *MCM10* vs Splice Junction were also statistically significant different. Control lines averaged 2.85 GSCs while SJ lines averaged 1.84. Lastly, control lines were compared to the K389 mutants. Once again, the control lines averaged a higher GSC number, 3.55, while mutants averaged 2.62, and statistically significant different. These results show that *MCM10* plays a significant role in the early stages of stem cell placement.

14 d.a.e were looked at to determine stem cell loss over time. Usually, as cells age, they will become displaced from the niche. The older germaria showed a similar trend with fewer numbers of stem cells in *MCM10* mutants than in the control. Control averages were near 3 GSCs per germarium while mutant averages were around 2 GSCs. Comparing 14 d.a.e. Control *MCM10* vs. *MCM10* Frame Shift mutants showed a statistically significant difference. The control average was 2.76 GSCs while the Frame Shift mutants averaged 1.98 GSCs. Control *MCM10* vs Splice Junction was very similar with control lines averaging 2.72 GSCs while SJ lines averaged 2.20. This also was statistically significant. Lastly, control lines were compared to the K389 mutants. Once again, the control lines averaged a higher GSC number, 3.01, while mutants averaged 2.72, and were statistically significant.



*p < 0.0002

Figure 4. Comparing Control Germarium GSC numbers to Mutant Germarium GSC numbers. (A) is a sibling control with two GSC while (B) is a mutant sibling displaying one GSC. (C-E) display the average number of germline stem cells per germaria for each control and mutant line at 4 d.a.e and 14 d.a.e.

MCM10 is Necessary for GSC Cell Cycle Progression Through S-Phase

To determine the number of GSCs in S-phase, EdU was utilized because it is an S-phase marker. GSCs located in the niche that displayed EdU (colored in white) were counted and taken as a percent of all GSCs counted. The average number of EdU positive GSCs per germarium was used to compare control germarium and mutant *MCM10* germarium. We expected the 4 d.a.e controls and mutants to have a lower number of EdU positive cells compared to the 14 d.a.e controls and mutants since S-phase slows down with cell age.

I found that when *MCM10* is knocked down, there are a higher number of GSCs in S-phase. On average, results for 4 d.a.e show that there is a higher EdU incorporation in *MCM10* mutants than in the control. When comparing 4 d.a.e. Control *MCM10* vs. *MCM10* Frame Shift mutants, there was a statistic significant difference. The control average was 3.42 % of GSCs with EdU incorporation while the Frame Shift mutants averaged 10.73%. Control *MCM10* vs Splice Junction was statistically significant as well. Control lines averaged 4.82% incorporation while SJ lines averaged 13.68%. Lastly, control lines were compared to the K389 mutants. Once again, the control lines averaged a lower EdU percentage, 6.40%, while mutants averaged 11.76%, and were not statistically significant. This overall increase in cells that are in S-phase could indicate that the cell cycle has already slowed down in the mutants and that they have aged prematurely or have had their DNA replication machinery severely damaged.

14 d.a.e showed a leveling off effect for EdU incorporation. When comparing 14 d.a.e. Control *MCM10* vs. *MCM10* Frame Shift mutants, the control average was 10.55% while the Frame Shift mutants averaged 9.57%. This difference was not statistically significant. Control *MCM10* vs Splice Junction was very similar. Control lines averaged 9.30% while SJ lines averaged 13.66%. Lastly, control lines were compared to the K389 mutants. Once again, the

control lines averaged a higher GSC number, 12.93%, while mutants averaged 8.24%, and was not statistically significant. These numbers reflect previous research that has stated that S-phase increases with age. The controls finally caught up to the mutants with the number of GSCs in S-phase. This further supports that*MCM10* is required for progression through the cell cycle.

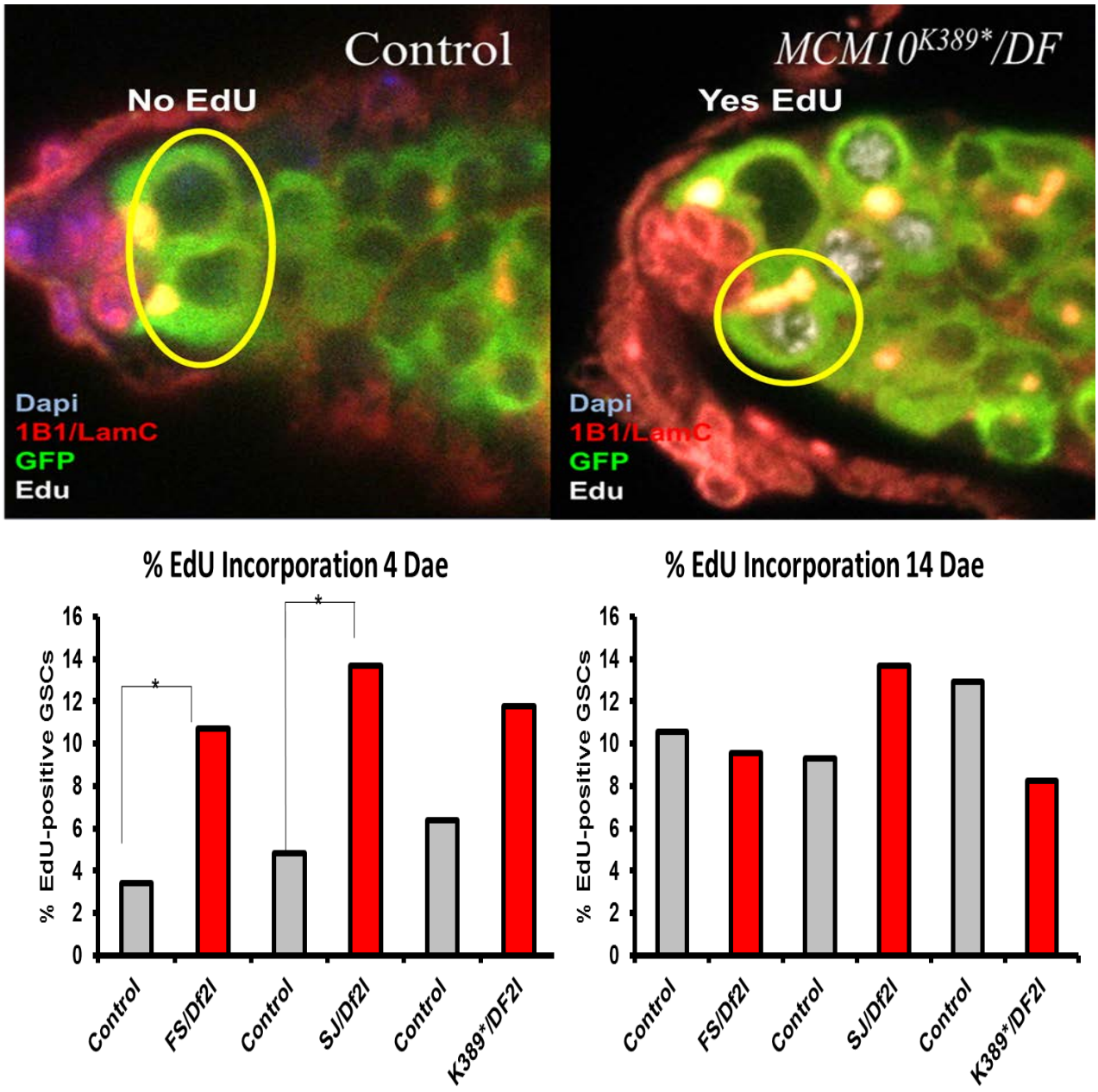


Figure 5 Comparing control sibling EdU incorporation to mutant sibling EdU incorporation.
(A) demonstrates what a germarium looks like when EdU is not incorporated. (B) does show what a germarium looks like with EdU incorporated. The white shows EdU in a stem cell and this signifies DNA is dividing. Panel C displays EdU incorporation in control and *MCM10* mutant fly lines. The difference in EdU incorporation (when compared to controls) for $MCM10^{FS}$ and $MCM10^{SJ}$ are statistically significant while $MCM10^{K389*}$ shows the same trend, just not statistically significant enough. Panel D is comparing controls to mutants at the 14 D.A.E mark. There are no statistically significant differences.

Discussion

I used *Drosophila* germline stem cells to categorize the effects when *MCM10* is deficient in stem cells. Results show that there is higher Edu incorporation in 4 Dae mutants, meaning there is a higher percentage of cells in S-phase. Germline Stem Cells located in the niche were counted. On average, results show that there are fewer number of stem cells in *MCM10* mutants than in the control.

I found that *MCM10* is necessary for GSC cell cycle progression through S-Phase. In the absence of *MCM10,* germline stem cells progress slowly through S-phase than wildtype GSCs. It took 10 days for the control cells to exhibit the same EdU incorporation levels. The higher incorporation is EdU in the 14 DAE control and mutants is not surprising since the cell cycle slows down as cells age. What is surprising was how high the EdU incorporation of the 4 d.a.e mutants where. The control cells showed normal levels of EdU while the mutants showed very high levels, showing that the 4 d.a.e mutants have early onset of DNA replication complications. These complications could show that the cells aged prematurely or age quicker than what they normally would have. Further testing should be done to determine if the longer cell cycle is due to DNA damage or other causes.

I found that *MCM10* is required for GSC maintenance between mutants and control. There was significant difference between all but one mutant vs control time point. This shows that when *MCM10* is knocked down, there are fewer GSCs per germarium. In general, more GSCs should become displaced as the cells age. Surprisingly, the controls actually lost more stem cells between the two time points than the mutants did. This could be due to the fact that the mutant germarium began with fewer stem cells so the difference was less when averaged out or just that the GSCs very rarely were displaced in the mutants.

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