Researching the Effects of iPLA2β on Fertility in Female *Drosophila melanogaster*

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Working Title

Null *iPLA2*β female *Drosophila* exhibit decreased fertility

Abstract

In the United States, 10–15% of couples report having trouble conceiving. Although two thirds of infertility cases have known causes, the remaining one third of cases have yet to be identified with specific physiological and biochemical processes. The goal of this thesis is to explore one potential cause of infertility in fruit flies and then extrapolate the results to shed light on causes of human infertility. Previous studies have indicated that the *iPLA2*β gene, which codes for a calcium-independent phospholipase, is implicated in infertility in mice and *Drosophila*. In the Steinhauer lab it was demonstrated that homozygous null *iPLA2*β female *Drosophila* exhibit decreased fertility when compared to their control counterparts. I performed egg laying assays with control and null *iPLA2*β female *Drosophila,* which showed reduced egg laying by *iPLA2β* females, confirming the link between iPLA2β and fertility. Then I dissected control and mutant *Drosophila* ovaries and utilized antibody staining techniques to determine the prevalence of apoptosis proteins and germline stem cells in both the control and mutant lines. The results of my research indicated that although null *iPLA2*β female *Drosophila* lay fewer eggs than the control females, they exhibit similar levels of apoptosis proteins and germline stem cells. Although these two proposed mechanisms do not explain reduced fertility in *Drosophila*, this research has paved the way for future research to explore other potential mechanisms and has enhanced our understanding of the link between *iPLA2*β and infertility.

Introduction

The cell membrane is largely composed of glycerophospholipids, macromolecules that contain a glycerol backbone, two fatty acid chains, and a phosphate group. Glycerophospholipids are remodeled in response to changing environmental conditions utilizing enzymes known as phospholipases. A wide range of phospholipids exists in nature due to the action of numerous phospholipase enzymes (Lands, 2000; van Meer et al., 2008; Shevchenko and Simons, 2010; Yamashita et al., 2014). These enzymes cleave phospholipids at various positions along its 3-carbon skeleton, producing phospholipids with varying head groups and acyl chain compositions (van Meer, 2005; Wenk, 2010). There are 4 superfamilies of phospholipase enzymes: A, B, C and D. Phospholipase A_2 is a specific type of acyl chain remodeling enzyme, involved in the Lands cycle, that cleaves the ester bond at the middle carbon (sn-2 position) of glycerophospholipids to yield lysophospholipids and fatty acids (Lands, 1965; Murakami et al., 2011; Zarringhalam et al., 2012; Yamashita et al., 2014; Renne et al., 2015). It was originally assumed that phospholipases A_2 were only involved with maintaining the phospholipid bilayers of the cell. However, more recently, studies have indicated that phospholipases A_2 are also involved with many other cellular activities, including mitochondrial function, cell death and nervous system function (Marszalek and Lodish, 2005; Schlame, 2013; Malhotra et al., 2009; Kinghorn et al., 2015; Iliadi et al., 2018).

Calcium-independent phospholipase A_2 is one of the 5 major classes of the superfamily of phospholipase A_2 (PLA2). iPLA2β is a calcium-independent phospholipase A_2 that has been found to be localized to mitochondria and is involved in phospholipid remodeling (Murakami et al. 2011). iPLA2β has been implicated in various neurodegenerative human diseases such as infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (atypical NAD), and dystonia-Parkinsonism (Gregory et al. 2017). The gene for iPLA2β exists in humans and has an ortholog in *Drosophila*, known as *CG6718*. However, its mechanism and function are not well understood and will be the focus of this study.

In addition to its other effects, iPLA2β has been implicated in fertility. Studies have demonstrated that *iPLA2*β knockout mice exhibit reduced male fertility due to impairment of spermatozoa motility (Bao et al., 2004). Additional studies demonstrated that iPLA2β is also implicated in male *Drosophila* fertility (Malhotra et al., 2009), which was consistent with the fact that iPLA2β was found to be highly expressed in the wild-type male germline (Steinhauer, unpublished data). However, the Steinhauer lab unexpectedly demonstrated that male *iPLA2*β null mutants undergo normal spermatogenesis, (Steinhauer, unpublished data). Strong expression of iPLA2β was also discovered in wild-type female adult germlines. Furthermore, unlike the mutant males, homozygous null *iPLA2*β females were found to be less fertile than their wild-type counterparts.

Female *Drosophila melanogaster* contain one pair of ovaries in their abdomen. On average, each ovary is composed of 16 ovarioles (Lin, 2004). Each ovariole contains egg chambers of successive stages, developing from anterior to posterior (**Figure 1**). Each egg chamber is surrounded by a layer of somatic follicle cells. The nurse cells within each egg chamber are the oocyte's sister germline cells that provide the maturing oocyte with vital nutrients. Ultimately, at the posterior of each ovariole, the nurse cells degenerate and the oocyte gives rise to a single mature egg. The follicle cells produce the eggshell (chorion). Since homozygous null *iPLA2*β females were found to be less fertile than their wild-type counterparts, it must be hypothesized that iPLA2β has an effect on this developmental process. The mechanism for decreased fertility in null homozygous *iPLA2*β female *Drosophila* will be examined in this study.

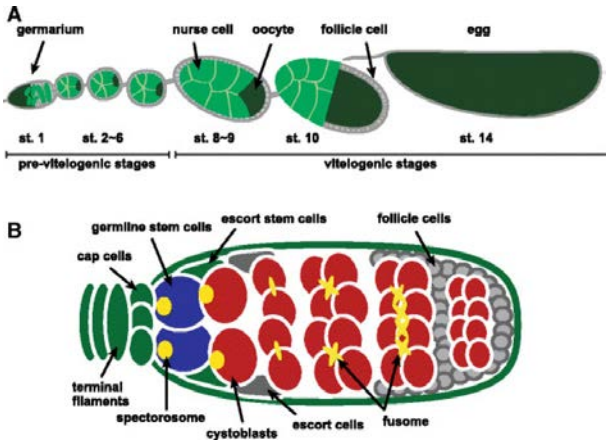


Figure 1. (A) The 14 developmental stages of an oocyte in an ovariole. The most anterior portion of the ovariole, pictured at stage 1, is known as the germarium and is pictured in **Figure 1B** with greater detail. (B) Germline stem cells (GSCs), the focus of this study, are pictured in blue. GSCs are surrounded by several somatic cell types, including terminal filament cells and cap cells. The cap cells are positioned just anterior to the GSCs and make special contact with them. The number of cap cells is correlated to the number of GSCs in a germarium. The cap cells are critical to formation, maintenance and regulation of the GSC niche (Ameku et al., 2016).

Hypothesis

Studying the mechanism of iPLA2β in *Drosophila* is an important endeavor, since it can provide us with insight for its role in human neurodegenerative diseases and human infertility. iPLA2β has been implicated in the apoptosis pathway in cultured human cells, albeit the mechanism by which this occurs is highly disputed (Ramanadham et al., 2004; Gadd et al., 2006; Bao et al., 2007; Seleznev et al., 2006; Yoda et al., 2010; Zhao et al., 2010; Song et al., 2014). It was therefore originally hypothesized in the Steinhauer lab that iPLA2β leads to infertility due to increased cell death via apoptosis in the germline. A similar mechanism may also operate in neurodegeneration.

Prior experiments performed in the lab indicated that there was a five-fold increase in degenerating egg chambers in *iPLA2*β null homozygous females compared to controls, seemingly confirming this hypothesis. However, when I performed the same experiment numerous times, no statistical difference between the number of dead egg chambers per ovariole in the wild-type and mutant flies existed. If apoptosis is not the driving force behind the decreased fertility in female flies, what is a potential mechanism? My research project was to investigate the mechanism that causes infertility. One possible mechanism that I will be exploring is reduced fertility due to iPLA2β stem cell activity in the germline.

Results

iPLA2β mutant females demonstrate reduced egg laying compared to controls

The Steinhauer lab found previously that *iPLA2*β mutant females produce fewer progeny than controls (**Figure 2A, B**). To confirm that *iPLA2*β mutant females produce fewer eggs, I performed egg laying assays with 1-week old and 2.5-week old mutant females alongside isogenic wild-type controls. The results demonstrated that *iPLA2*β mutant females lay fewer eggs than wild-type females, confirming the correlation between iPLA2β and infertility in female flies (**Figure 2C, D**).

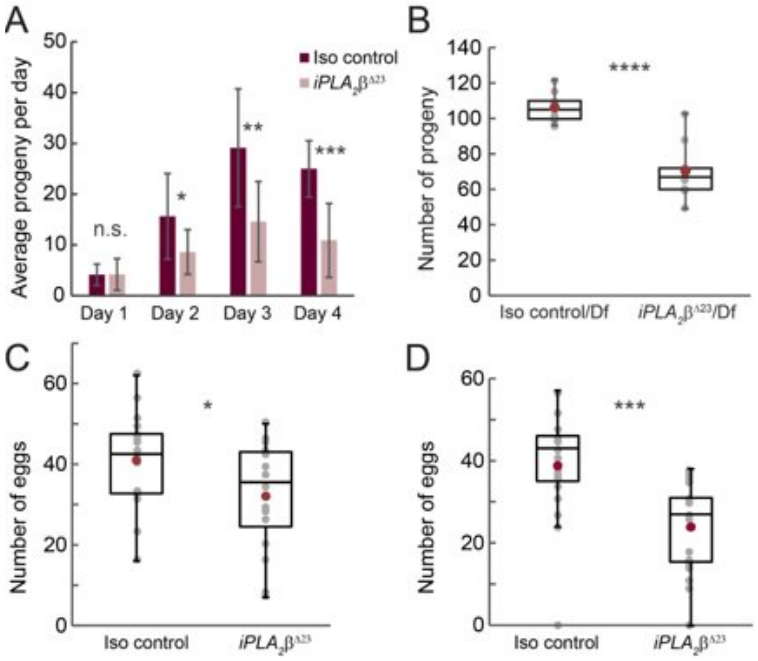


Figure 2. (A) Average number of progeny produced per day by young (<1 week) control female *Drosophila* ("iso control") displayed in maroon and null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$) displayed in salmon. Error bars are standard deviations. Both lines were mated to w^{1118} males, and females were passed to new vials daily for four days. Adult progeny per vial were counted. Differences were significant for days 2, 3, and 4. (B) Total progeny produced by hemizygous isogenic control and *iPLA2β* $^{\Delta 23}$ females over the course of 4 days. (C) Total number of eggs produced by 1-week old control female *Drosophila* and by 1-week old *iPLA2*β $^{\Delta 23}$ female *Drosophila* over the course of 4 days. Both lines were mated to w^{1118} males. (D) Total number of eggs produced by 2.5-week old control female *Drosophila* and by 2.5-week old *iPLA2β* $^{\Delta 23}$ female *Drosophila* over the course of 4 days. Both lines were mated to w^{1118} males. Plots in B-D show minimum, maximum, median, and first and third quartiles of progeny numbers. Averages are shown by the red dots. n.s. not significant, * p<0.1, ** p<0.02, *** p<0.001, **** p<0.0001 by unpaired t-test.

iPLA2β mutant females and control females demonstrate equal number of ovarioles

Because *Drosophila* eggs are produced in the ovarioles of the ovary, we asked whether *iPLA2*β mutant females have fewer ovarioles than controls. The number of ovarioles per ovary was obtained for 10-day old and 30-day old null *iPLA2*β female *Drosophila* and control female *Drosophila* (**Figure 3B).**

A **B**

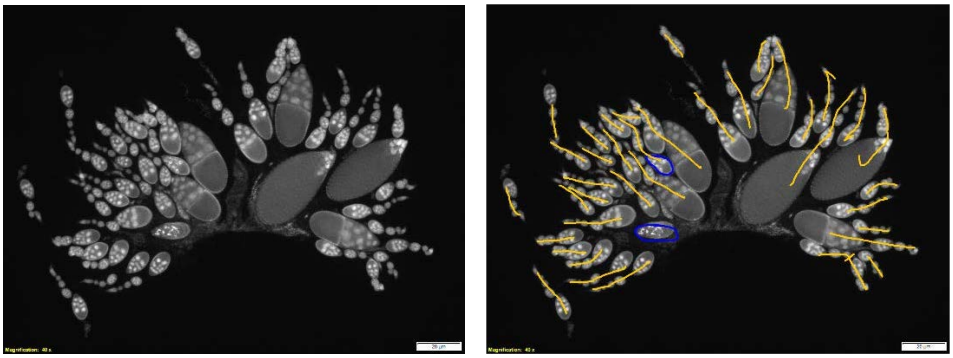


Figure 3. (A) Image of the ovarioles present in the ovaries of a female *Drosophila*. The literature states that the female *Drosophila* has two ovaries, each containing an average of 16-18 ovarioles (Lin, 2004). The stalk in the middle connects the two ovaries. Female *Drosophila* were mated on yeast paste, which enlarges the ovaries. Ovaries were then dissected and removed from the abdomen of the *Drosophila*. Slides were mounted with flouromount and then viewed with high-powered fluorescence microscope. (B) Yellow lines were drawn using Image J to mark each individual ovariole. In this fly, 40 ovarioles (marked with yellow lines) were counted. Each ovariole is composed of multiple egg chambers. In healthy nurse cells, the nuclei appear plush and circular. In egg chambers where cell death has occurred, the nuclei appear smaller, disfigured and brighter. Dead egg chambers were circled in blue. In this case, there were two dead egg chambers.

The results (**Figure 4)** demonstrated that there was no significant difference in the number of ovarioles per ovary between null *iPLA2*β female *Drosophila* and control female *Drosophila.* Therefore, another potential mechanism for the decreased number of eggs in null *iPLA2*β female *Drosophila* had to be proposed.

A **B**

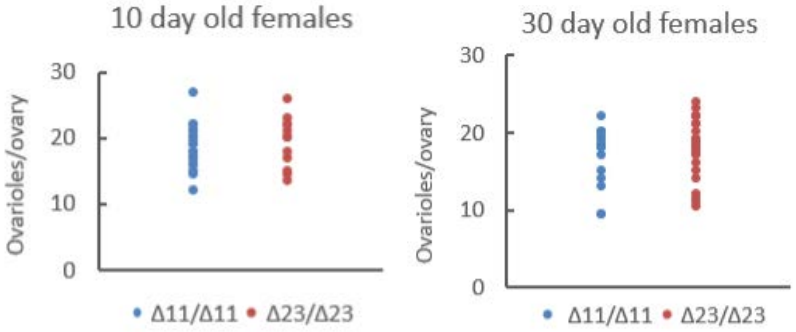


Figure 4. (A) Number of ovarioles per ovary in 10-day old control female *Drosophila* (blue) and 10-day old null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). A total of 29 ovaries and 531 ovarioles were counted in the 10-day old control female *Drosophila* (blue). The average number of ovarioles per ovary was 18.64±3.24 (standard deviation). A total of 16 ovaries and 304 ovarioles were counted in the 10-day old null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). The average number of ovarioles per ovary was 19.33±3.80. Difference between mutant and control was p=0.60 by unpaired t-test. (B) Number of ovarioles per ovary in 30-day old control female *Drosophila* (blue) and 30-day old null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). A total of 20 ovaries and 328 ovarioles were counted in the 30-day old control female *Drosophila* (blue). The average number of ovarioles per ovary was 16.94±3.73. A total of 32 ovaries and 549 ovarioles were counted in the 30-day old null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). The average number of ovarioles per ovary was 18.26±3.87. Difference between mutant and control was p=0.46 by unpaired t-test. Number of ovarioles was recorded using the method stated in **Figure 3B**.

Apoptosis is not a likely mechanism for decreased fertility in null iPLA2*β *female Drosophila

To investigate the hypothesis that *iPLA2*β mutants produce fewer mature eggs because their eggs die during oogenesis, I conducted antibody staining experiments on fly ovaries using an antibody that recognizes cleaved Caspase-3, an apoptosis factor. This demonstrated that apoptosis is not consistently elevated in mutant flies. This experiment was repeated, and the same results were found, indicating that another pathway was responsible for the decreased fertility in *iPLA2*β mutant females (**Tables 1** and **2**, **Figure 5**).

Young females (<1 week old)		
	Δ11	Δ23
Expt 1	9.9% (131)	17.4% (144)
Expt 2	4.8% (186)	2.4% (170)
Expt 3	5.37% (149)	8.11% (296)
Expt 4	0.86% (350)	4.43% (203)

Table 1. Percentage of dying egg chambers in young control female *Drosophila* (Δ11) and young null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$). Number of egg chambers for each experiment is shown in parentheses.

Aged females (20 d old)		
	Δ11	Δ23
Expt 1	4.4% (296)	8.1% (260)
Expt 2	7.37% (217)	2.81% (249)
Expt 3	6% (282)	3.125% (184)

Table 2. Percentage of dying egg chambers in 20-day old control female *Drosophila* (Δ11) and in 20-day old null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$). Number of egg chambers for each experiment is shown in parentheses.

A **B**

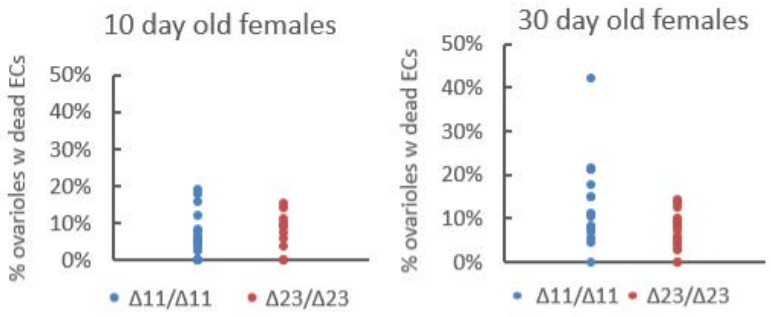


Figure 5. (A) Percentage of ovarioles per ovary that contain dead egg chambers in 10-day old control female *Drosophila* (blue) and 10-day old null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). 531 stage seven egg chambers and 28 dead egg chambers were counted in the 10-day old control female *Drosophila* (blue). The percentage of ovarioles with dead egg chambers was therefore 5.27%. 304 stage seven egg chambers and 24 dead egg chambers were counted in the 10-day null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). The percent ovarioles with dead egg chambers was therefore 7.89%. Difference between mutant and control was p=0.13 by two proportion z-test. The method of counting and identifying dead egg chambers is outlined in **Figure 3B**. (B) Percentage of ovarioles that contain dead egg chambers in 30-day old control female *Drosophila* (blue) and 30-day old null iPLA2β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). 328 stage seven egg chambers and 43 dead egg chambers were counted in the 30-day old control female *Drosophila* (blue). The percent ovarioles with dead egg chambers was therefore 13.11%. 549 stage seven egg chambers and 41 dead egg chambers were counted in the 30-day null *iPLA2*β female *Drosophila* (iPLA2β $^{\Delta 23}$, red). The percent ovarioles with dead egg chambers was therefore 7.47%. Difference between mutant and control was p=0.006 by two proportion z-test, indicating that the mutant females had statistically less apoptosis in their ovaries than controls.

*Exploring decreased germline stem cell production as a potential mechanism for decreased fertility in null iPLA2*β *female Drosophila*

The next step of the study entails researching an alternative mechanism for decreased fertility in *iPLA2*β mutant female flies. If the reduced fertility is not due to death of the egg chambers, another proposed mechanism for iPLA2β's role in infertility could be its effect on germline stem cell maintenance. We hypothesized that iPLA2β may be implicated in germline stem cell maintenance, leading to the production of eggs and ultimately progeny. Therefore, if *Drosophila* are homozygous null for iPLA2β, fewer germline stem cells will be produced and therefore fewer progeny will be observed.

Germline stem cells are located in the most anterior portion of each ovariole in the ovaries, called the germarium (**Figure 1A** and **B**). The literature indicates that normal ovarioles contain 2-3 germ line stem cells on average (Ameku et al., 2016), which are involved in producing the eggs throughout the life of the female fly. If fewer germline stem cells are present, then fewer eggs will be produced, and thus fewer progeny. Using antibody stains for germline stem cells, I gathered data on the number of germline stem cells in the control and iPLA2β mutant flies (**Figure 6**).

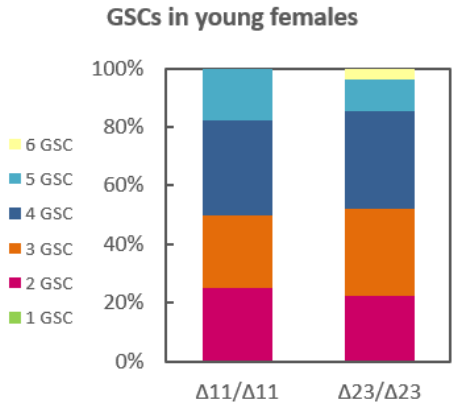
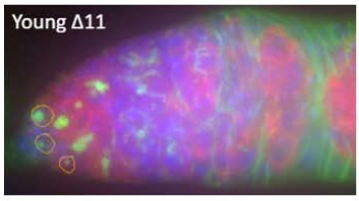


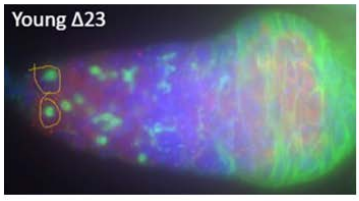
Figure 6. Percentage of germaria that contained 1 – 6 germline stem cells. Left column displays results for 6-day old control female *Drosophila* and the right column displays results for 6-day old null *iPLA2*β female *Drosophila*. 28 6-day old control female *Drosophila* germaria contained a total of 96 germline stem cells, yielding an average of 3.4 germline stem cells per germarium. 27 6-day old null *iPLA2*β female *Drosophila* germaria contained a total of 93 germline stem cells, yielding an average of 3.4 germline stem cells per germarium.

The results indicated that there was no difference between the number of germline stem cells per germarium in the mutant and control lines.

A



B



C

Young Females (20 d old)		
	Δ11	Δ23
Number of germaria	28	27
Total number of germline stem cells	96	93
Average number of germline stem cells per germarium	3.4	3.4

Figure 7. (A) Germarium of a 1-week old control female *Drosophila*. Image was taken with high-powered fluorescence microscope. Multiple Z-sections were captured and then stacked using Image J. Primary antibodies used to stain the germarium were: mouse anti-HTS (green), DAPI (cell nuclei, blue), and rabbi anti-Vasa (germline cells, red). The green dots are the spectrosomes of the germline stem cells and their daughters. Stem cell daughters have two different fates: germline stem cell renewal and cystoblasts, which also have round spectrosomes. To distinguish the differentiating cystoblasts from the renewing stem cells, the anteriormost cells, distinguished by their red Vasa staining from Vasa- adjacent cap cells, were circled with Image J (orange) and counted as germline stem cells. The more posterior green dots mark the differentiating cystoblasts. As cystoblasts undergo mitosis, the round spectrosomes branch into elongated fusomes, also visible with green anti-HTS staining in more posterior cells. (B) Germarium of a 1-week old null *iPLA2*β female *Drosophila* (C) Table indicating the average number of germline stem cells per germarium in 20-day old control and null *iPLA2*β female *Drosophila.* Results indicated that there was no difference.

Discussion

Null iPLA2β females demonstrate reduced fertility but exhibit normal production of ovarioles

We started with the observation that mutant females produce fewer progeny than their control counterparts. To determine whether reduced fertility was related to problems with oogenesis, I performed egg laying assays for 1-week old and 2.5-week old control and mutant flies. My data supported the conclusion that mutant females do not make eggs as efficiently as the controls (**Figure 2**). The old and young controls produced more eggs that the mutants throughout the four days following their mating with w^{1118} males.

The first hypothesis I tested was to see if there was an effect on the number of ovarioles present in the ovaries of the mutants. The results indicated that mutant females have the capacity to produce the normal number of ovarioles (**Figure 4)**. These findings pointed to the conclusion that there was some issue with the development of eggs, not the overall production of eggs.

*Exploring two potential oogenesis deficiencies in null iPLA2*β *female Drosophila*

Since the potential for egg production appeared to be equal in the control and mutant *Drosophila,* we turned our attention to exploring possible defects in the development of eggs in null *iPLA2*β female *Drosophila* during oogenesis. iPLA2β has been implicated in the apoptosis pathway in cultured human cells and therefore we hypothesized that iPLA2β may play a role in the apoptosis pathway, thereby preventing cell death in the germline (Ramanadham et al., 2004). Antibody staining experiments demonstrated that the percentage of ovarioles that contained dead egg chambers was comparable in both the control and mutant flies (**Figure 5)**. We concluded that another mechanism must be responsible for the decreased fertility in *iPLA2*β mutant females.

The second hypothesis that I explored was iPLA2β's effect on germline stem cell maintenance. We hypothesized that iPLA2β may be implicated in germline stem cell maintenance, which is crucial for the continuous production of eggs throughout the lifetime of the female fly. I discovered that on average, young control and null *iPLA2*β female *Drosophila* contain 3.4 germline stem cells per ovariole, indicating that there was no difference between the controls and mutants (**Figure 7C**). Therefore, it was concluded that iPLA2β has no major effect on the number of germline stem cells.

In conclusion, two potential mechanisms were tested to explore the observation that null *iPLA2*β female *Drosophila* produce less eggs than control females. The apoptosis pathway and germline stem cell production appeared to function at similar levels in the mutants and controls, leading us to assume that these two mechanisms are not major contributors to the decrease in production of eggs in null *iPLA2*β female *Drosophila.* Although the mechanism was

not determined, two key hypotheses were ruled out, paving the way for future research to explore other potential mechanisms. In the future, the Steinhauer lab will be exploring other potential mechanisms for decreased fertility in null *iPLA2*β female *Drosophila* and revisiting the proposed germline stem cell mechanism.

Future directions

In the future, they will investigate the role of germline stem cells in aged flies, as opposed to the young flies that I utilized for the research project, in the case that an issue with germline stem cell production may only become obvious in older *Drosophila*. The second phase of the research will involve exploring the size of the egg chambers in the both the mutants and controls to determine if there is an interference with normal growth in null *iPLA2*β female *Drosophila.* The third stage will be to investigate whether there are differences in mitochondrial distribution between *iPLA2*β mutants and controls as a result of decreased *iPLA2*β expression in the germline which in turn may be linked to decreased fertility.

Methods

Fly lines

In this study, two lines of flies were used: Δ11/ Δ11, the isogenic control stock and Δ23/ TM3, the mutant stock. Both Δ11 and Δ23 chromosomes were generated previously in the Steinhauer lab. The Δ11/ Δ11 stock contains two wild-type copies of the iPLA2β gene while the Δ23/ TM3 stock contains one normal copy of iPLA2β on the balancer (stubble) chromosome and one mutated copy of iPLA2β. When selecting mutant flies, all stubble flies are discarded since they contain one normal copy of iPLA2β on the balancer chromosome. Non-stubble flies are labeled as Δ23/ Δ23 which are null homozygous for iPLA2β and used for all subsequent experiments.

Fertility assays

To conduct the egg laying assays, I set up 20 vials of Δ11/ Δ11 flies with 1 female each and 20 vials of Δ23/ Δ23 flies with 1 female each. Subsequently, 2 w^{1118} males were added to the female in each vial, to allow for mating to occur. The following day all the males were removed, while females were transferred to fresh labeled vials. The same procedure was repeated for four days in a row. Each day the number of eggs in the vial were counted and recorded.

Fluorescent stainings

Ovary dissections were performed by mating 10 Δ11/ Δ11 females with 10 w^{1118} males and 10 Δ23/ Δ23 females with 10 w^{1118} females on live yeast paste. After several days, male flies were discarded, and females were dissected, and their ovaries were extracted and combed open in PBS. Ovaries were fixed in 4% formaldehyde, followed by 4 washes with PBX. The fix solution was removed and replaced with a block containing PBS + 1% Triton X-100 and 5% normal donkey serum (NDS). After 2 hours of rocking, the block was replaced with a primary antibody solution containing antibody, PBX, 10% NDS and 0.02% Azide. Ovaries were incubated overnight. After 3 washes with PBX, a secondary antibody and DAPI were added to the ovaries (covered) and left to rock. After 3 more washes with PBX, the slide was mounted with Flouromount. An Olympus IX-81 was utilized to image the samples. Image J software was used for analysis.

Primary antibodies used were mouse anti-HTS (1:20), rabbit anti-Caspase 3 (1:100), and rabbit anti-Vasa (1:5000). Secondary antibodies included 488 anti-mouse (1:1000) which labels green and cy3 anti-rabbit (1:200) which labels red.

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