INVESTIGATING THE ROLE OF *CG8093* IN *DROSOPHILA* FEMALE GERMLINE STEM CELLS

by

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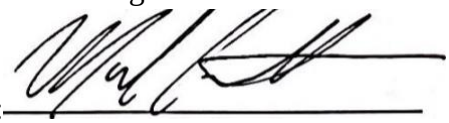
April 27th, 2023

I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed:  Date: 04/27/2023

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Investigating the Role of *CG8093* in the Germline Stem Cells

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ABSTRACT - Cell division and differentiation are imperative for the maintenance of living organisms. Germ cells, which serve as the progenitor cells necessary to create oocytes and sperm, are maintained in many organisms by the activity of a tissue-resident stem cell population called germline stem cells (GSC). To understand how germ cells are maintained, we used the well-characterized model reproductive tissue, the *Drosophila* ovary, which contains approximately 45 GSCs in young flies. Advances in whole genome sequencing have recently made possible the identification of the GSC transcriptome. In this data set, we identified an uncharacterized coding gene (*CG8093*) that is enriched specifically in GSCs during oogenesis. *CG8093* is a predicted acid lipase that is regulated by diet. When cells experience an abundance of dietary sugars, the downregulation of *CG8093* is observed, suggesting that *CG8093* serves as a cellular sensor of nutrient availability. However, the role of *CG8093* specifically in the GSCs for the division and differentiation of germ cells is not known. Here, I show that depletion of *CG8093* in germ cells results in a complete block to GSC establishment, manifesting as agametic ovaries. In contrast, depletion in differentiated germ cells does not alter oocyte differentiation. This data suggests that *CG8093* is a key player in the division and differentiation of GSCs in *Drosophila melanogaster*. Understanding the complexity of proteins needed for successful division and differentiation of germ cells is necessary for understanding how these cells are maintained to support organismal fertility.

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I would sincerely like to thank the people and organizations that made this study possible:

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Introduction

The regeneration of cells is paramount to the maintenance of life, as cells are continuously damaged by intrinsic and extrinsic factors. To combat this damage, organisms have evolved a variety of niches that house specialized cells called stem cells, which are pertinent to the restoration of specific cell populations [Voog and Jones, 2010]. An advantageous method for studying stem cell function is through their transcriptomics, as the transcription of genes into complementary RNA regulates activities within the cell. Historically, gene function was studied in *Drosophila melanogaster* using single-element insertion strains. These strains contain an inserted single P-element which causes a new mutation [Cooley et al. 1988]. This allowed for insertional mutagenesis, genic mapping, and chromosomal rearrangements [Cooley et al. 1988]. Insertional mutagenesis played a strong role, as many novel genes were characterized using this methodology. For example, *bag-of-marbles (BAM)* was first characterized by using P-element insertional mutagenesis to disrupt *Drosophila* gametogenesis [McKearin and Spradling, 1990]. However, as time progressed, so has the understanding of transcriptomics. New techniques today allow for efficient and cost-effective cell type transcriptional profiles through single-cell RNA sequencing.

RNA sequencing technology has the capability of providing an understanding of the transcriptome of an organism [Kukurba and Montgomery, 2015]. Generally, the sequencing is conducted by extracting a tissue sample, isolating the RNA, converting it to complimentary DNA, and then sequencing the sample via a Next-Generation Sequencing (NGS) platform [Kukurba and Montgomery, 2015]. Many single-cell and bulk RNA sequencing studies have been conducted with *Drosophila melanogaster* which has allowed for a variety of different methods to visualize gene expression. For example, the digital tool Oo-site is a culmination of

several single-cell and bulk RNA sequences that allow for the visualization of gene expression in the germarium [Martin et al. 2022]. Utilizing this novel technique, RNA sequencing in *Drosophila melanogaster* has led to the study of many uncharacterized genes which are gaining new importance in various cell types, including germline stem cells (GSCs) [Rust et al. 2021]. Characterization of genes in stem cells is paramount, as it will continue to enhance the understanding of how stem cells work in maintaining cell type populations.

The transcriptomics and metabolomics of stem cells play a dynamic, interconnected role that aid the stem cell's ability to properly maintain various cell type populations [Shyh-Chang and Ng 2017]. Interestingly, stem cells have been shown to undergo aerobic glycolysis, even in an oxygen rich environment [Abdel-Haleem et al. 2017]. This phenomenon was initially characterized in cancer cells, now known as the Warburg effect [Warburg 1956, Heiden et al. 2009]. *Drosophila melanogaster*, the fruit fly, has many stem cell niches that provide a useful foundation to study stem cell metabolism [Drummond-Barbosa and Tennessen, 2020]. Specifically, previous studies have indicated that *Drosophila* GSCs are affected by nutritional conditions, providing an excellent system to investigate the interconnected mechanisms regulating of stem cell metabolism and transcriptomics [Drummond-Barbosa and Spradling 2001].

Drosophila melanogaster is an elegant model organism to study cellular regeneration because of the linear arrangement of GSCs and their progeny (Figure 1). The female fly has one pair of ovaries, each containing 15-20 smaller segments called ovarioles [Hinnant et al. 2020]. The individual ovariole is then further segmented into three regions and 14 stages, where each stage represents progressively older egg chambers [Riparbelli et al. 2021]. In the most anterior portion of each ovariole, known as the germarium (Figure 1C), a combination of somatic and

gametic cell types make up the stem cell niche [Armstrong, 2020]. Terminal filament and cap cells are somatic cells that coalesce into the stem cell niche where two to three undifferentiated GSCs reside [Armstrong, 2020]. The fusome, a cytoplasmic organelle, can be used to identify the GSCs, as their fusomes are anteriorly localized in the cell directly adjacent to the neighboring cap cells [Riparbelli et al. 2021, Lin et al. 1994]. GSCs will divide asymmetrically into daughter cells known as cystoblasts [Ables and Drummond-Barbosa, 2013]. From here, the cystoblast will undergo incomplete mitotic divisions exactly four times, producing a 16-cell cyst, interconnected by the fusome [Hinnant et al. 2020]. The initial movement of the cysts posteriorly is facilitated by somatic cells known as the escort cells. These cells have protrusions that extend medially, which encapsulate and release the cysts [Ben-Zvi and Volk, 2019]. In the 8-cell cyst, there are two cells known as pre-oocyctes which are in the most posterior region of the cyst and indicated by 4 ring canals [Riparbelli et al. 2021]. Once the 16-cell cyst has formed, one of these pro-oocytes will be established as the oocyte and mature into the embryo. The remaining 15 are nurse cells and will aid in this process [Riparbelli et al. 2021]. Additionally, the movement of the 16-cell cyst is facilitated by another somatic cell type known as the follicle cells [Hinnant et al. 2020]. The linearization of such a complex process provides an excellent foundation for understanding the delicate balance of transcription that allows GSCs to proliferate, maintain a stem cell niche, and aid in the fertilization process.

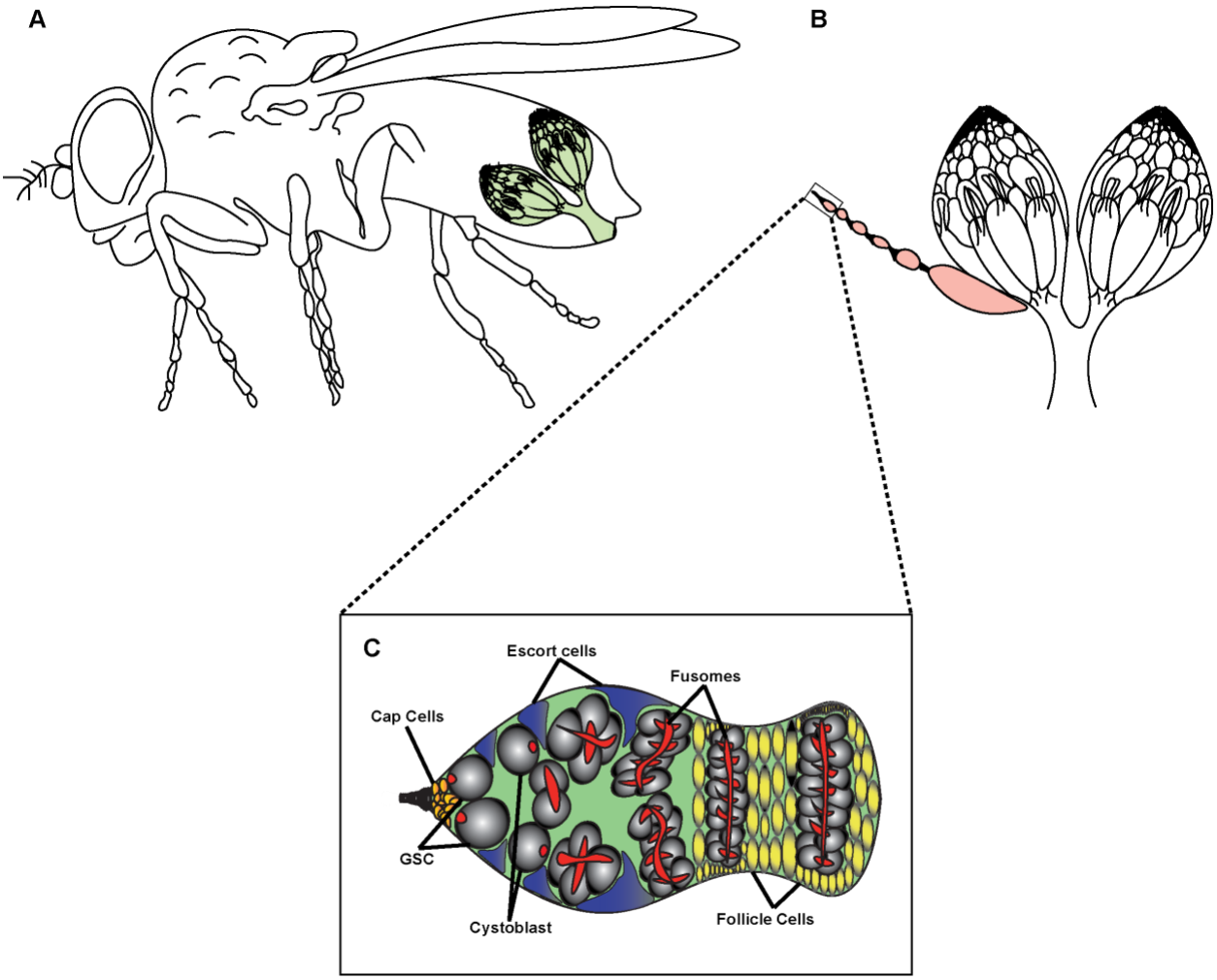

Figure 1: *Drosophila ovaries provide a clear segmental array of the development of an oocyte.* [A] The female fruit fly has one pair of ovaries (blue), which contain 15-20 ovarioles. [B] The structure of the fruit fly ovary, showing the ovariole (peach). [C] An enlarged view of the most anterior tip of the ovariole called the germarium. The beginning of oogenesis starts in the germarium, where germ cells divide into cysts and the movement of these cysts are facilitated by a variety of somatic cells. Cap cells (orange), GSC (anterior grey), escort cells (blue), germ cells (grey), follicle cells (yellow).

Single-cell RNA sequencing of the different cell types in the *Drosophila* ovary led to transcriptional profiles of each cell type, including the GSCs. Through manual curation of the GSC transcriptome, I identified *CG8093* as a novel uncharacterized gene whose mRNA was enriched specifically in GSCs, but not their progeny [Rust et al. 2021, Martin el al. 2022]. *CG8093* is a predicted triacylglycerol acid lipase, a specific type of acyl carrier protein [Mueller et al. 2004]. Studies in *Drosophila* larvae indicated that the transcription of *CG8093* is regulated by diet, where down regulation occurs upon an abundance of dietary sugars, as the hydrolysis of triacylglycerol is not needed to meet the energy demands of the organism [Zinke et al. 2002]. Yet, prior to eclosion, triacylglycerol is an imperative energy reserve that is used during the developmental stages to maintain energetic homeostasis [Heier and Kühnlein, 2018]. It has also been shown that in the latter half of oogenesis, anabolism of triacylglycerol from diacylglycerol is imperative to egg chamber development, where adequate synthesis of triacylglycerol is the key to pass through the mid-oogenesis checkpoint [Buszczak et al. 2002]. Considering GSCs are the facilitators of oogenesis and actively transcribe *CG8093*, the catabolism and anabolism of triacylglycerol could play a potential role in early oogenesis. Previous studies have suggested that GSCs are nutritionally-responsive and especially sensitive to dietary sugar [Laws and Drummond-Barbosa, 2017]. Therefore, understanding the function of *CG8093* in the GSCs will help elucidate the role of triacylglycerol metabolism in oogenesis. In this study, I used conditioned RNA interference to specifically deplete *CG8093* mRNA from GSCs and/or their progeny. I found that *CG8093* is necessary for GSC development as loss of *CG8093* from GSCs during development abolished GSC establishment. Further, I discovered that loss of *CG8093* from differentiating germ cells does not alter their differentiation or growth in nutrient-rich conditions. Taken together, this data suggests that further study of *CG8093* is imperative to

understanding the larger role of *CG8093* in the GSCs, and continue our understanding how triacylglycerol metabolism plays a role in oogenesis.

Materials and Methods

Drosophila Strains and Husbandry

Drosophila stocks were maintained at ambient temperature within a standard medium (cornmeal/molasses/yeast/agar). To assess *CG8093* significance in the ovary, the following fly lines were used:

Table 1: *Drosophila* Stocks used in Experimentation

Fly Line	**Supplier**
P{GAL4::VP16-nanos.UTR}	Van Doren et al. 1998
y[1] w[*]; P{w^{+mC}=GAL4-nos.NGT}40; P{w^{+mC}=GAL4-nos.NGT}A	BLM #64308 Tracey et al. 2000
Y[1]w[*]P{w^{+mC}=bam-GAL4:VP16}1	BLM #80579 Chen and McKearin, 2003
P{otu-GAL4::VP16.R}1, w1118	BLM #58424 Rorth, 1998
Y^{1} sc v^{1} sev^{21}; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMC04363}attP40*	BLM #56924 Ni et al,. 2010
UASp-LacZ	Gift of Drummond-Barbosa

For RNAi experiments, stocks were maintained at 20℃ temperature and crosses were set at 25℃. Crosses were tossed back every three days to minimize over-crowding of larvae. Adult

females were collected within one day of eclosion and kept in fresh food supplemented with wet yeast paste with an equivalent number of males until their dissection three days after eclosion.

Immunofluorescence and Microscopy

Drosophila ovary samples were prepared and analyzed following our published protocol [Blake et al. 2017]. Grace's insect medium was used to dissect and tease apart the ovaries. Following dissection, ovaries were placed in 3% Bovine Serum Albumin (BSA) coated 1.5 mL microcentrifuge tubes. Samples were fix with 5.3% formaldehyde, solvated in Grace's insect medium, for thirteen minutes on a rotating nutator. Samples were washed with 0.1% phosphate-buffered saline (PBS)-Triton X-100 and blocked at ambient temperature for three hours in blocking solution (5% Bovine Serum Albumin, 5% Normal Goat Serum, and 0.1% PBS-Triton X-100). Primary antibodies were diluted in blocking solution, added to the samples, and placed in a 4°C environment on a rotating nutator overnight. The primary antibodies used were mouse anti-Hts (1B1, 1:10)(DSHB)(incubated two nights); mouse anti-Lamin C (1:100)(DSHB) (incubated two nights); rat anti-Vasa (1:50)(DSHB)(incubated one night). The primary antibodies were removed, and the samples were washed with 0.1% PBS-Triton. Secondary antibodies were diluted in blocking solution and placed on the samples at ambient temperature for two hours. Goat species-specific secondary antibodies were conjugated with AlexaFlour 488 and 568. Secondary antibodies were removed, washed with 0.1% PBS-Triton, and stained with 0.5 µg/mL 4'-6-diamidino-2-phenylindole (DAPI), diluted in 0.1% PBS-Triton, for fifteen minutes. DAPI was removed from the samples, washed with 0.1% PBS-Triton, and stored in mounting media (90% glycerol, 20% n-propyl gallate) in 1.5 ml microcentrifuge tubes at 4°C. Imaging was conducted with two microscopes: the Olympus SZX7 light microscope (4x) and the Zeiss LSM700 laser scanning microscope (63x). The Olympus SZX7 was used at four times

magnification for all images and analyzed with the Olympus cellSens software. The Zeiss LSM700 used confocal z-stacks, 63x oil lens with one micrometer slices and analyzed with the ZEN Black 2012 analysis software. All images were minimally and equally enhanced using the Adobe Photoshop Creative Suite.

Analysis and Statistics

The number of germline stem cells were found based on the visualization of the round fusome that are adjacent to the cap cells using Hts/ LamC [de Cuevas and Spradling, 1998]. GSC counts between driver-only and experimental ovaries were compared by using Student's two-tailed T-test, with $p < 0.001$ considered statistically significant. Analysis was plotted using the GraphPad Prism software.

Results

Uncovering CG8093 in the GSCs

A variety of single-cell and bulk RNA sequencing studies have been conducted which has allowed for the identification of many uncharacterized genes in the *Drosophila* genome. I initially found *CG8093* as one of fifty unique statistically significant transcripts in the GSCs [Rust et al. 2021]. I also used the digital tool Oo-site, which congregates 4 separate sequencing data sets into a software to visualize gene expression in the germarium. Using Oo-site bulk RNA-sequencing, I found that *CG8093* is expressed solely in the GSCs as well, corroborating the initial single-cell RNA sequencing data (Figure 2a). However, using Oo-site polysome sequencing data, I found high translational activity of *CG8093* in the differentiating daughter cells (Figure 2b). Taken together, the loss-of-function approach was used to assess the significance of *CG8093* in the GSCs and differentiated daughter cells, where *CG8093* is

localized, adumbrated by the single-cell RNA sequencing, bulk RNA sequencing, and polysome sequencing.

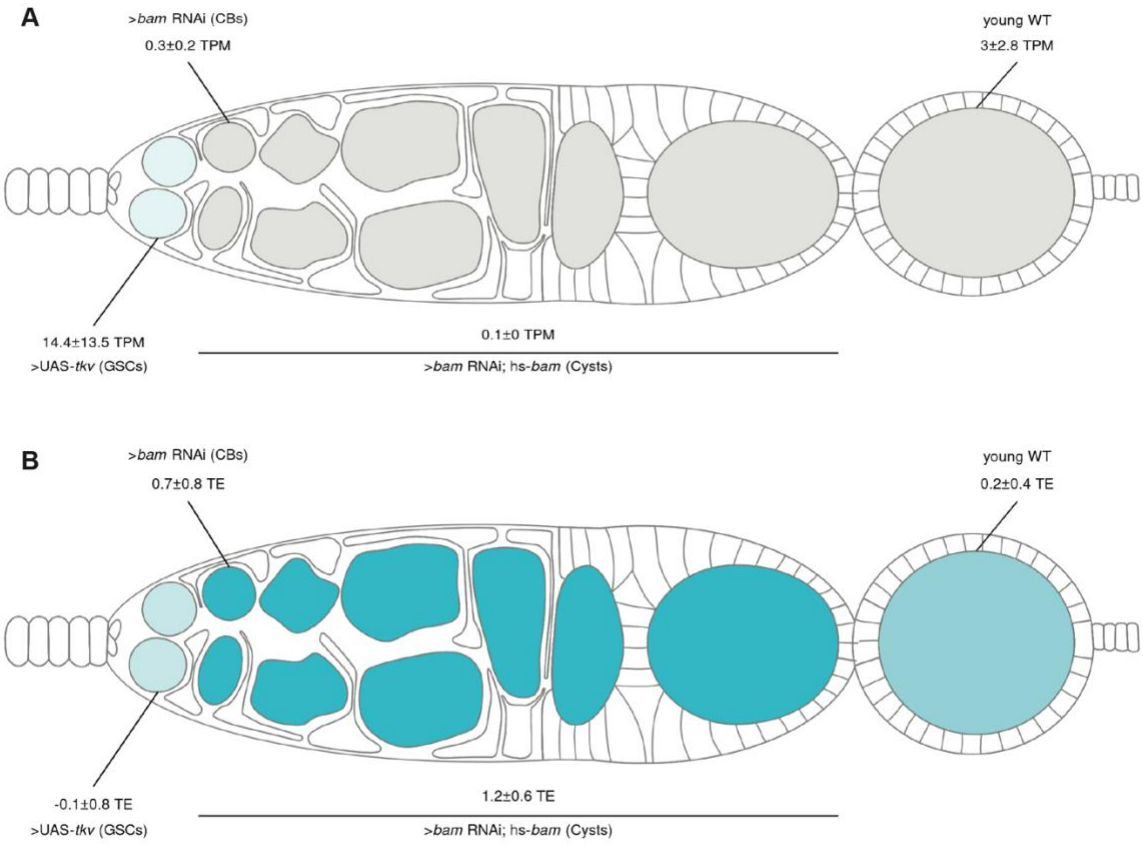

Figure 2: *CG8093 bulk RNA sequence and polysome sequence expression in the female Drosophila germarium.* [A] Germarium developed through Oo-site highlighting bulk RNA sequencing data showing the expression of *CG8093* solely in the GSCs, in concurrence with the single-cell RNA sequence [Martin el al. 2022, Rust et al. 2021]. [B] Germarium developed through Oo-site highlighting polysome translational sequencing data [Martin el al. 2022].

CG8093 is essential for the development of the GSCs

There is little understanding of how metabolic pathways influence stem cell lineages [Drummond-Barbosa and Tennessen, 2020]. Previous experiments indicate that rates of oogenesis change depending on the type of diet the fly consumes prior to conception [Matsuoka et. al, 2017]. Furthermore, it has also been shown that adipocytes sense amino acid levels and transmit this information to the GSC lineage, in turn modulating GSC numbers [Armstrong et al. 2014]. This previous evidence suggests the need for accompanying proteins, potentially *CG8093*, for the metabolic processes essential for stem cell development. To assess whether *CG8093*, a predicted acid lipase, may contribute to GSC maintenance, I first asked depletion of *CG8093* would affect GSC development. To carry out GSC loss-of-function of *CG8093*, I used the *Nanos-Gal4::VP16* driver that is expressed in the GSCs and subsequent germ cells. Flies carrying *Nanos-Gal4::VP16* were crossed with one germline-enhanced *CG8093* RNAi. Upon eclosion, adult females were fed every day with wet yeast paste for high nutrient environment and dissected three days after eclosion. The ovaries were stained with Hts to visualize the fusomes, LamC to visualize nuclear membranes, and Vasa to visualize gametic cells.

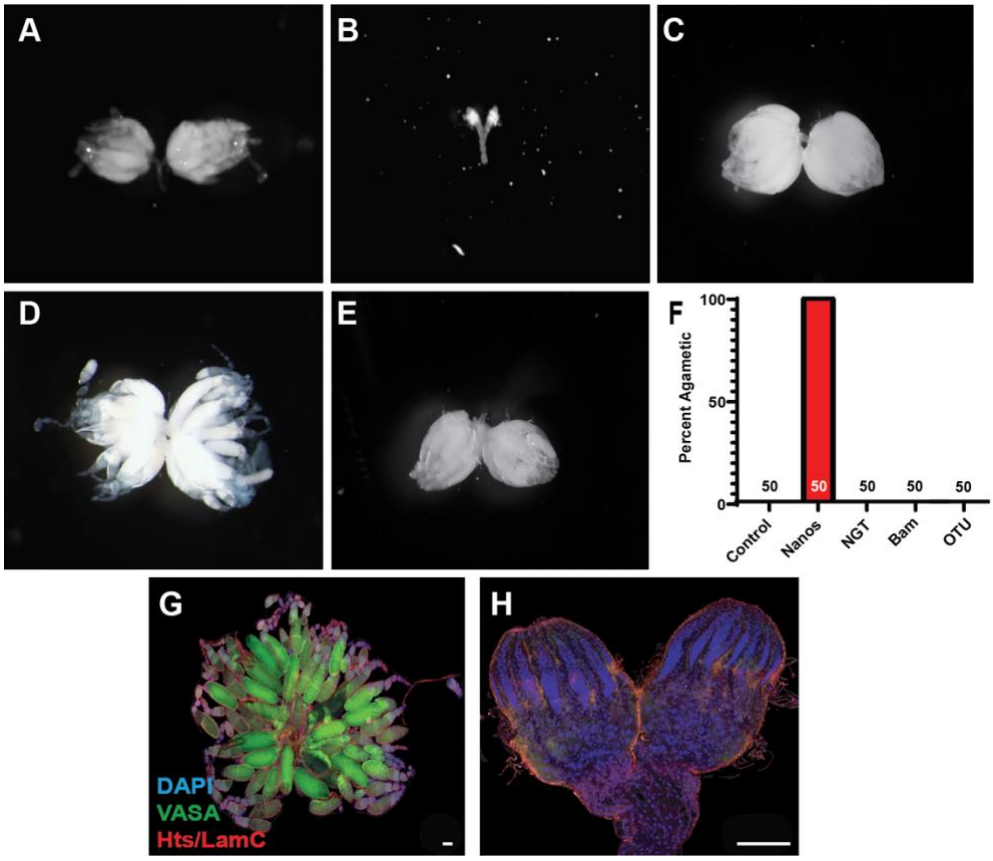

Figure 3: *CG8093 knockdown in the GSCs of adult female causes complete agametic ovaries.* [A-E] Full ovary light microscope images from females and induced knock down of [A] germline specific LacZ (control) or [B-E] *CG8093* using [B] *Nanos-Gal4::VP16*, [C] *NGT-Gal4*, [D] *Bam-Gal4*, [E] *Otu-Gal4*. [F] Percent agametic ovaries 3 days after eclosion (n=50). [G-H] Full ovary immunofluorescent images from female with induced knock down of [G] germline specific LacZ (control) or [H] *CG8093* using *Nanos-Gal4::VP16*. Labeled with Vasa (germ cells, green), DAPI (DNA, blue), and Hts/LamC (fusome and nuclear membrane, red). Scale bars represent 200 μm.

If *CG8093* aids in GSC development, I asked if the lost-of-function maintains putative GSC counts. Typically, two to three GSC's are found within the GSC niche [Laws and Drummond-Barbosa, 2017]. However, it was found that the loss-of-function with *Nanos-Gal4::VP16* resulted in agametic ovaries (Figure 3b, 3h). Therefore, I depleted *CG8093* in the GSCs (Figure 3b, 2c) and differentiate daughter cells (Figure 3d, 3e) to assess the prevalence of agametic ovaries in the female fly. Loss-of-function with *Nanos-Gal4::VP16* resulted in agametic ovaries 100% of the time (Figure 3f) and this phenotype was not observed in the differentiated daughter cells (Figure 3d, 3h). The *Nanos* promoter is expression in the developing ovary [Van Doren et al. 1998]. Taken together, there was a phenotypic result with depletion in the GSCs but not the differentiated daughter cells, which suggests *CG8093* is likely necessary for GSC establishment during pre-adult development.

CG8093 is not essential downstream for the development of GSCs

Dietary factors play a significant role in stem cell behavior. Typically, nutrient signaling occurs through intracellular pathways. However, there are other proposed indirect nutritional effects on stem cells [Ables et al. 2012]. To further investigate the drastic morphological disruption caused by the knockdown of *CG8093* with *Nanos-Gal4::VP16*, I next asked if the transcription of *CG8093* in subsequent cell divisions plays a role in GSC development. Previous evidence suggests there is a complex stem cell signaling network facilitated by nutrition that may require specific types of proteins [Ables et al. 2012]. For example, the rate of proliferation of GSCs and their progeny decrease on a poor diet, exhibiting the consequences of how nutrition plays a role with stem cell function [Drummond-Barbosa and Spradling, 2001].

To understand the role of *CG8093* in this signaling network, I conducted loss-of-function in the 4, 8, and 16 cell cysts to assess if *CG8093* plays a role in downstream nutritional signaling that affects the development of GSCs. Furthermore, I also conducted loss-of-function in the GSCs and subsequent germ cells with a weaker driver compared to *Nanos-Gal4::VP16* to further assess how *CG8093* plays a direct role in the development of GSCs. Specifically, I used *NGT-Gal4* to knock down *CG8093* in the GSC and subsequent germ cells, *Bam-Gal4* to knock down *CG8093* in the 4 and 8 cell cysts, and *OTU-Gal4* to knock down *CG8093* in the 16-cell cysts. These drivers were crossed with one UASp-CG8093 RNAi where the progeny adult females were fed every day and dissected 3 days after eclosion. The ovaries were stained with Hts/LamC to visualize the fusomes and Vasa to visualize gametic cells.

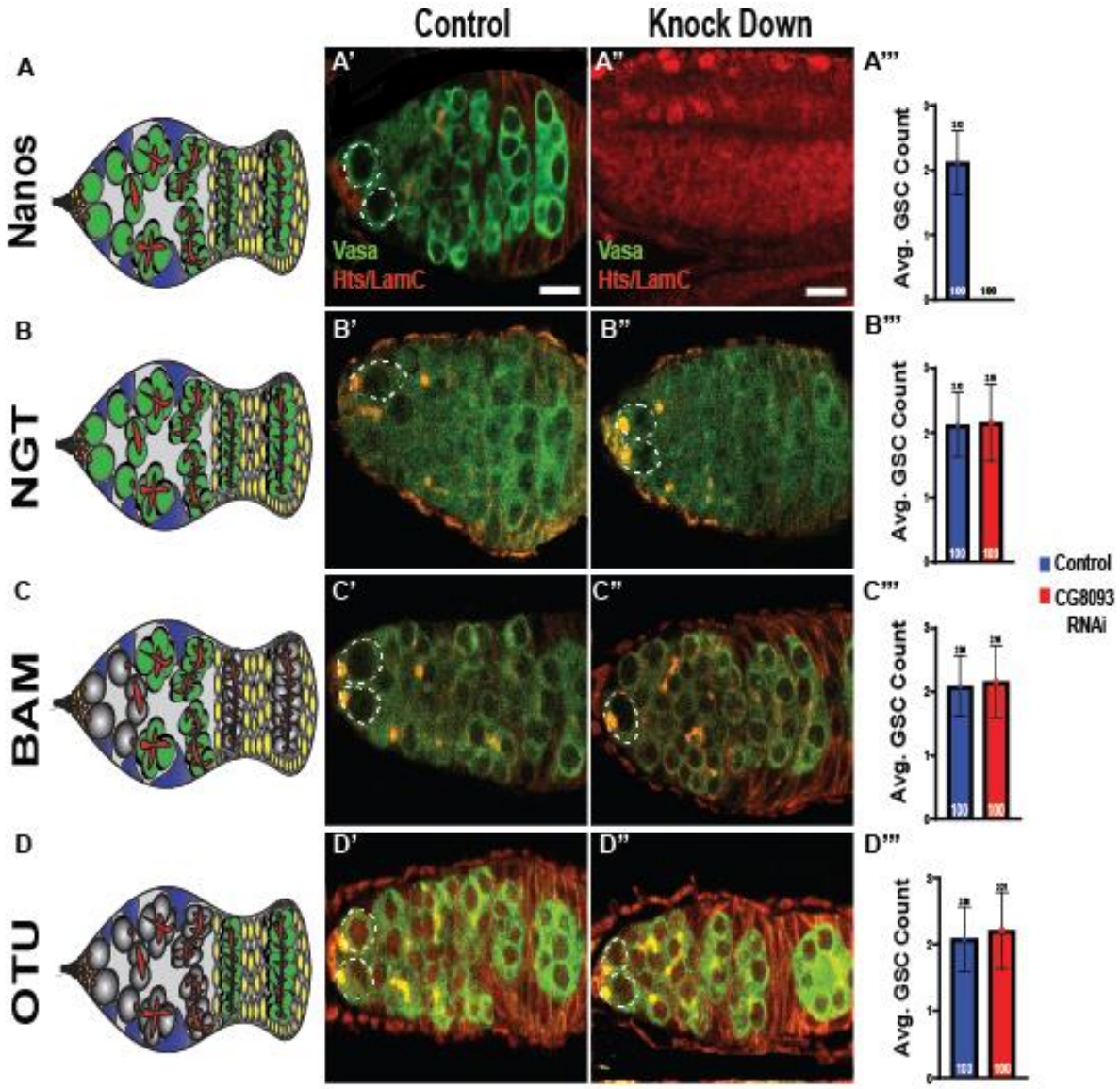

Figure 4: *CG8093 knockdown downstream of the GSCs in the adult female is not necessary for the development of GSCs.* [A-D] Germarium schematic highlighting where the *Nanos-Gal4::VP16, NGT-Gal4, BAM-Gal4, and OTU-Gal4* will initiate the knock down of *CG8093* (green). [A'-D'] Germarium immunofluorescent images 3 days after eclosion and induced knockdown of germline specific LacZ (control) or [A''-D''] *CG8093*. Labeled with Vasa (germ cells, green) and Hts/LamC (fusome and nuclear membrane, red). Scale bars represent 10 μm. GSCs circled in which dashed lines. [A'''-D'''] The average GSC count per germarium of *Nanos-Gal4::VP16* (control (n=100): 2.12, knockdown (n=100): 0), *NGT-Gal4* (control (n=100): 2.12, knockdown (n=100): 2.16), *BAM-Gal4* (control (n=100): 2.09, knockdown (n=100): 2.16), and *OTU-Gal4* (control (n=100): 2.08, knockdown (n=100): 2.21). Error bars represent standard deviation.

If *CG8093* plays a role in downstream nutritional signaling that affects the development of GSCs, I asked if the loss-of-function maintains putative GSC counts. Furthermore, since *CG8093* plays a role in direct GSC development, I asked if the lost-of-function with a weaker driver would maintain putative GSC counts. After quantification, I found no significant change in the number of GSCs between the wild type and RNAi germarium for all experiments. The putative GSC counts between wild type and RNAi germarium with all drivers suggest that *CG8093* does not play a role in downstream nutritional signaling and only a small quantity of *CG8093* resides in the GSCs.

Discussion

There have been no previous studies on the role of *CG8093* in the GSCs. However, previous data suggests the localization of *CG8093* in the GSCs, which may aid in our understanding of GSC metabolism due to its predicated role as an acid lipase [Rust et al 2021, Mueller et al. 2004]. Generally, proliferation of stem cells occurs rapidly, facilitated by the high rate of glucose consumption that will provide sufficient energy for this process [Shyh-Chang and Ng 2017]. Furthermore, stem cells also have been shown to prefer aerobic glycolysis as a consequence of this rapid proliferation, known as the Warburg effect [Heiden et al. 2009]. This suggests metabolism plays a crucial role in the ability for stems cells to proliferate properly. Therefore, I hypothesized that if *CG8093* was essential in the development of GSCs, then a decrease in the proliferation of GSCs would occur when *CG8093* is depleted in the GSCs. My research first investigated the role of *CG8093* in the GSCs using a loss-of-function approach with the *Nanos-Gal4::VP16* driver. I found that *CG8093* knockdown in the GSCs significantly affected the development of GSCs, as this knockdown exhibit agametic ovaries in all experimental ovaries. To better understand the role of *CG8093* GSCs, a different driver was used

to initiate the loss-of-function. I hypothesized that if *CG8093* was essential in the development of GSCs, then a decrease in the proliferation of GSCs would occur when *CG8093* is depleted in the GSCs with a weaker driver. This was facilitated using the *NGT-Gal4* driver. I found that depletion of *CG8093* in the GSCs using the *NGT-Gal4* did not affect GSC proliferation. These results together suggest that *CG8093* is localized in the GSCs, however not abundant, and essential for development of GSCs.

Nutritional signaling of stem cells has been shown in previous studies as a means of cellular communication [Ables et al. 2012]. To understand how *CG8093* may play a potential role in downstream nutritional signaling of the GSCs, I conducted a loss-of-function approach at various stages of germ cell development. I hypothesized that if *CG8093* was essential downstream for the development of GSCs, depletion of *CG8093* in the 4-, 8-, and 16-cell cysts would result in a decrease in proliferation of GSCs. This was explored by using the loss-of-function approach with a *Bam-Gal4* driver that initiated the knockdown of *CG8093* in the 4- and 8-cell cysts. I found that depletion of *CG8093* in the 4- and 8-cell cysts did not affect GSC proliferation. Furthermore, a *OTU-Gal4* driver was used to initiate the knockdown of *CG8093* in the 16-cell cysts. I found that depletion of *CG8093* in the 16-cell cysts did not affect GSC proliferation. These results indicate that *CG8093* does not play a role downstream for the regulation of GSCs.

In summary, my results identify an uncharacterized gene *CG8093* that plays an essential role in GSC development. Fabrication of new genetic tools and temporal knockdowns are two important directions to further study *CG8093* in the GSCs. Currently, there is only one available RNA interference line that can be used in gametic cells [Ni et al. 2010]. These findings highlight the significance of *CG8093* in the GSCs and more RNA interference lines will continue to

elucidate *CG8093*'s role in the GSCs. My results were produced by imposing a spatial knockdown of *CG8093* in specific germ cells within the germarium. There are other systems that allow for temporal control of the knockdown as well. The FLP-Out system can be used to conduct a mosaic analysis, allowing manipulated cells and wild-type cells to be directly juxtaposed throughout the germarium [Phipps et al. 2023, Caygill and Brand 2016]. Furthermore, the temperature sensitive Gal80 system can also be employed [Caygill and Brand 2016]. Both systems hinder the expression of the RNA interference until after eclosion of the fly, allowing for the GSCs to develop prior to depletion of *CG8093*. These systems, couple with a variety of RNA interference lines, will continue our understanding of *CG8093* in the GSCs. The effects of *CG8093* in the GSCs begins to elucidate the greater relationship between stem cell and their metabolism, highlighting the dynamic relationship between proliferation and the require metabolic needs for proper development.

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