# CHARACTERIZING THE ROLE OF THE *EARLY GENE AT 23* IN *DROSOPHILA MELANOGASTER* OOGENESIS

by

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by

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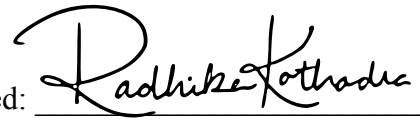
I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed:  Date: April 26, 2016

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This Senior Honors Thesis has been formatted according to the guidelines required for publication in the scientific journal *Development*.

## ABSTRACT

*Drosophila melanogaster* (fruit fly) females undergo oogenesis to create oocytes from undifferentiated stem cells in the ovary. Similar to estrogen in humans, the steroid hormone ecdysone in fruit flies has a known role in facilitating oogenesis. The ecdysone signaling pathway is highly studied as it can help us draw parallels between *Drosophila* and human reproductive processes. After ecdysone binds to an ecdysone receptor (EcR), EcR activates transcription of many genes, such as *E74*, *E75*, and *E78*. These early genes code for proteins that are essential for *Drosophila* tissue repair, development, and reproduction. One of the early genes, the *Early Gene at 23* (*E23*), is thought to be a target of ecdysone signaling, but little is known about its function.

Because *E23* is expressed in multiple cell types and highly expressed in *Drosophila* ovaries, we hypothesize that *E23* plays a role in *Drosophila* viability, reproduction, and oogenesis. To determine the role of *E23* in *Drosophila* viability and reproduction, flies harboring transposable element insertions in the *E23* locus were analyzed for potential mutant phenotypes. Our experiments using these lines suggested that *E23* is not needed for viability or fertility. We also tested whether *E23* affects oogenesis in more subtle ways, using high-resolution confocal microscopy, but results indicate that it does not affect early oogenesis or adult stem cell numbers. Although *E23* does not play a role in *Drosophila* oogenesis, it may have other important functions within the fly.

## ACKNOWLEDGMENTS

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## INTRODUCTION

Sexually reproducing organisms have the ability to form male and female gametes from undifferentiated germline cells (Matova and Cooley, 2001; Pepling et. al 1999; Sun and Spradling, 2013). These germ cells undergo differentiation to form eggs and sperm that unite to form a zygote, a cell capable of creating a new organism (Trounson and Roger, 2003). Metazoans undergo a process known as oogenesis to form the female gamete, an egg. Despite major physiological differences between sexually reproducing organisms, the machineries by which the germline is established and oogenesis occur are highly conserved (Trounson and Roger, 2003). Experimental evidence suggests that the molecular mechanisms by which *Caenorhabditis elegans* nematodes, *Drosophila melanogaster* fruit flies, *Xenopus laevis* frogs, chickens, mice, and even humans undergo oogenesis have common themes (Matova and Cooley, 2001).

In metazoans, oogenesis begins with primordial germ cells (PGCs) that make up the germline (Matova and Cooley, 2001). Localized molecules determine the fate of these PGCs and actively maintain their identity (Blackler, 1958). The germ cells of extragonadal origin migrate to the somatic gland, where germ cells unite with the somatic gonadal primordium and undergo differentiation (Matova and Cooley, 2001). After this union, germ cells begin proliferating to become gonial cells, or oogonia (Matova and Cooley, 2001). The oogonia undergo various mitotic divisions to form clusters of interconnected cells known as cysts (Matova and Cooley, 2001). From these cysts, one cell develops as an oocyte. The follicle develops and then the egg is released after fertilization. Although there are many conserved strategies, the process can vary by species. To study oogenesis in detail, biologists often use organisms that produce many eggs throughout their lifetime.

*Drosophila melanogaster*, commonly known as the fruit fly, is used as a model system to characterize oogenesis because it is easy to grow, upkeep, and study (Figure 1A). Female *Drosophila* lay anywhere between 750 and 1,500 eggs in their lifetime, making it easy for biologists to study oogenesis (Powell 1997). Fruit flies have a short generational time of 10-12 days, so experiments can be conducted quickly (Powell 1997). *Drosophila* are easy to grow and maintain due to their small size, making it possible to raise hundreds of flies at once. Furthermore, the fly is an easily manipulated genetic tool due to its fully sequenced genome of 165 million base pairs. Humans have 23 pairs of chromosomes whereas *Drosophila* only have 4 pairs, so genetic analysis is manageable. Most importantly, many genes in *Drosophila* are conserved in humans, thus allowing researchers to use the fly as a tool to study human genes, behavior, diseases, and developmental processes (Pandey and Nichols, 2011). In fact, approximately 75% of human genes have a functional homolog in the fruit fly (Pandey and Nichols, 2011).

*Drosophila* oogenesis occurs in a production line fashion beginning in the germarium and ending with a mature oocyte (Cox and Spradling, 2003). *Drosophila* ovaries come in pairs of two (Figure 1A), each consisting of 10-15 ovarioles (Figure 1B). Oogenesis begins at the anterior end of ovarioles in a structure known as the germarium. In the germarium (Figure 1D), germline stem cells (GSCs) undergo four mitotic divisions to produce a 16-cell cyst of interconnected germ cells (Matova and Cooley, 2001). Of these 16 cells, one cell develops as an oocyte and the other 15 cells become supporting nurse cells that provide nutrients to the oocyte (Sun and Spradling, 2013). Somatic follicle cells surround the cyst to create a follicle. The growing follicle undergoes 14 developmental stages to develop the oocyte that is ready for fertilization (Figure 1C).

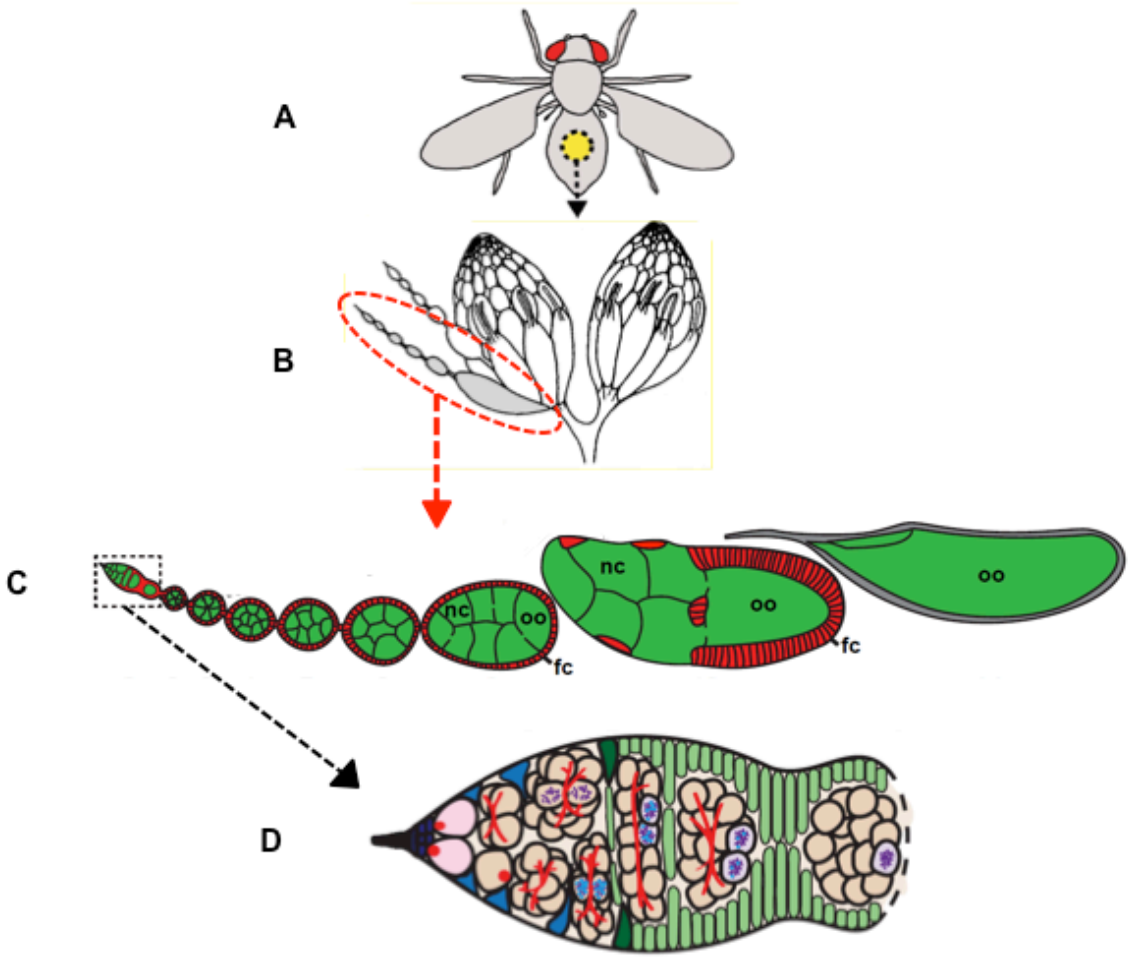


**Figure 1:** ***Drosophila melanogaster*** **Oogenesis**
**(A)** A pair of ovaries is located in the abdomen of *Drosophila* (yellow circle). **(B)** Each ovary consists of 10-15 ovarioles (red circle). **(C)** These ovarioles consist of a germarium and fourteen progressively older follicles. **(D)** The germarium (black square) that contains germline stem cells (GSCs: pink). GSCs divide to form daughter cells which undergo four divisions to form an interconnected 16-cell cyst (tan) composed of 15 nurse cells (nc) and one oocyte. Progressively older follicles bud off of the germarium, ultimately resulting in a mature egg that is ready to be fertilized (oo).

A variety of molecules, including steroid hormones are required to drive mammalian and *Drosophila* oogenesis (Pestka et. al, 2013). In mammals, steroid hormones like progesterone and estrogen have known roles in mammalian sexual organ development and reproduction. Both hormones promote development of female sex characteristics during puberty, facilitate ovulation, prepare females for birthing, and onset menopause (Manson, 2015). After puberty, females release an egg during the menstrual cycle. As the egg travels through the fallopian tube, progesterone is released to prepare the woman for pregnancy. If the egg is unfertilized, estrogen is released to prevent egg maturation. Progesterone and estrogen also induce uterine contractions and milk production in mothers (Manson, 2015)

Like mammals, *Drosophila* have well-conserved steroid hormones that are involved in reproduction (King-Jones and Thummel, 2005; Belles and Piulachs, 2015; Gancz and Gilboa, 2013). *Drosophila* therefore provides an excellent model for the study of how steroid hormones regulate developmental processes. The steroid hormone ecdysone, a member of the nuclear receptor superfamily, is arguably one of the most well-studied steroid hormones in *Drosophila* and is similar in structure and function to the mammalian hormone estrogen (Mangelsdorf et. al, 1995). For example, like estrogen, ovarian ecdysone promotes sex-specific feeding rates (Sieber and Spradling, 2015). Ecdysone also controls multiple aspects of *Drosophila* oogenesis, including stem cell functioning, germ cell growth and survival, and many more (Figure 2).

Ecdysone regulates gene expression by means of the ecdysone signaling pathway (Figure 2). The hormone binds to the ecdysone receptor, which is a nuclear receptor heterodimer of two proteins: Ecdysone Receptor protein (EcR) and Ultraspiracle (Usp) (Koelle et. al, 1991; Yao et. al, 1992; Yao et. al, 1993; Thomas, et. al, 1993; Riddiford et. al, 2000). Although EcR can bind to ecdysone alone, the addition of Usp optimizes activation of transcriptional gene targets (Grad

et. al, 2001; Grebe, Fauth, and Spindler-Barth, 2004). The ecdysone receptor complex then binds to ecdysone response elements, or promoter sequences, to induces or repress ecdysone-responsive genes (Koelle et. al, 1991). Nuclear hormone receptors all have common genetic and mechanistic roles in reproduction, thus indicating similarities between *Drosophila* and mammalian physiology and development (Mangelsdorf et. al, 1995). Therefore, studying ecdysone gives us insight into the role of receptors in mammalian sexual characteristics and reproduction.

Ecdysone regulates expression of numerous genes, many of which have yet to be studied. Ecdysone signaling induces and represses many genes that encode proteins with essential tissue specific effects in the germarium, cysts, and oocyte (Belles and Piulachs, 2015). Some early genes that have fundamental roles in *Drosophila melanogaster*, like *Ecdysone-induced protein 78C* (*E78*), *Ecdysone-induced protein 74EF* (*E74*), and *Ecdysone-induced protein 75B* (*E75*), are known to be downstream of ecdysone (Buszczak et. al, 1999; Carney and Bender, 2000; Ables and Drummond-Barbosa, 2010). For example, the *Ecdysone-induced protein 78C* (*E78*) is essential for establishment of the somatic germline stem cell niche, survival of follicles, and proper egg production (Ables et. al, 2015). Also, loss of the *Ecdysone-induced protein 74EF* (*E74*) results in failure for follicles to develop past early oogenesis (Buszczak et. al, 1999). Furthermore, removal of *Ecdysone-induced protein 75B* (*E75*) results in the degeneration of egg chambers during stages 8 and 9 of oogenesis, ultimately leading to female sterility (Buszczak et. al, 1999).

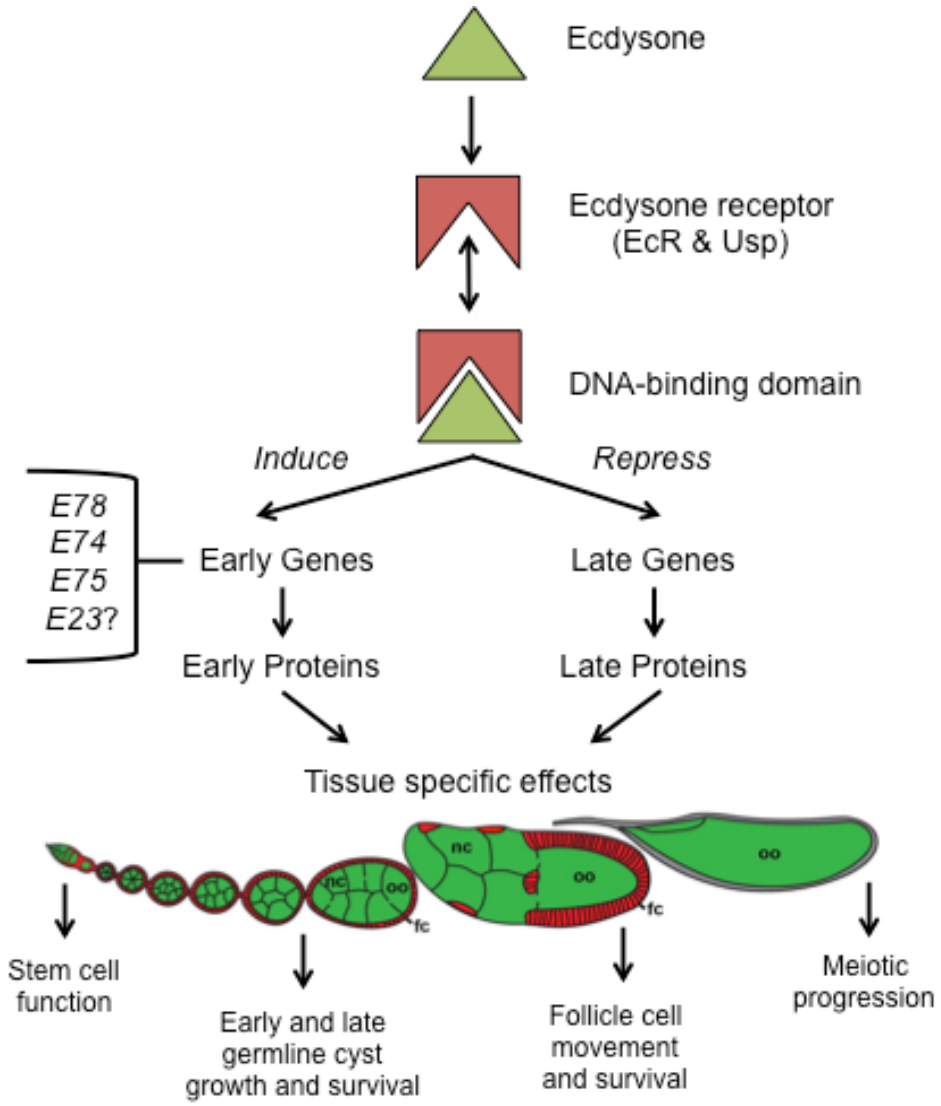


**Figure 2: Ecdysone Signaling Pathway**
The steroid hormone ecdysone binds to the ecdysone receptor, a nuclear heterodimer of Ecdysone Receptor (EcR) and Ultraspiracle (Usp) to induce early genes or repress late genes. These genes code for early and late proteins that have many tissue specific effects including germline stem cell proliferation and follicle cell movement. Early genes like *Ecdysone-induced protein 78C* (*E78*), *Ecdysone-induced protein 74EF* (*E74*), and *Ecdysone-induced protein 75B* (*E75*) are known downstream targets of ecdysone. The gene *Early Gene at 23* (*E23*) may be induced by ecdysone as well.

Genome-wide approaches have been used to identify putative ecdysone-response targets. Previous studies have found nearly 3000 genes that may be expressed downstream of ecdysone (Beckstead, Lam, and Thummel, 2005; Gauhar et. al, 2009; Shlyueva et. al, 2014). In one study, microarray technology and larval organ RNA culturing identified numerous genes that are regulated by ecdysone but not 20-hydroxyecdysone (*20-HE*; Beckstead, Lam, and Thummel, 2005*)*. Another study identified many direct gene targets in the Kc167 cell line by treating flies with *20-HE* and combining physical binding-site profiling with gene expression profiling (Gauhar et. al, 2009). More recently, Self-Transcribing Active Regulatory Region Sequencing (STARR-seq) was used to create a genome-wide quantitative map of enhancers that are induced or repressed by ecdysone in ovarian somatic cells (Shlyueva et. al, 2014). A comparison of the target genes identified in each of these studies revealed only 32 common ecdysone target genes are expressed in the three cell types, suggesting a common core of ecdysone targets.

One of the 32 putative ecdysone-response gene targets is *Early Gene at 23* (*E23*). *E23* is approximately 20 kbp long and is located on the left arm of chromosome II (Hock et. al, 2000; FlyBase). It contains eight coding exons, three non-coding exons, and five introns (FlyBase). There are three transcripts of *E23*, each with fairly similar structure (FlyBase).

The *E23* locus encodes an ATP-binding cassette transporter that is capable of repressing ecdysone-mediated gene activation independently of the Golgi apparatus and secretory pathway (ABC transporter; Hock et. al, 2000). The ABC transporter superfamily is a large protein family that is represented in all forms of life, including

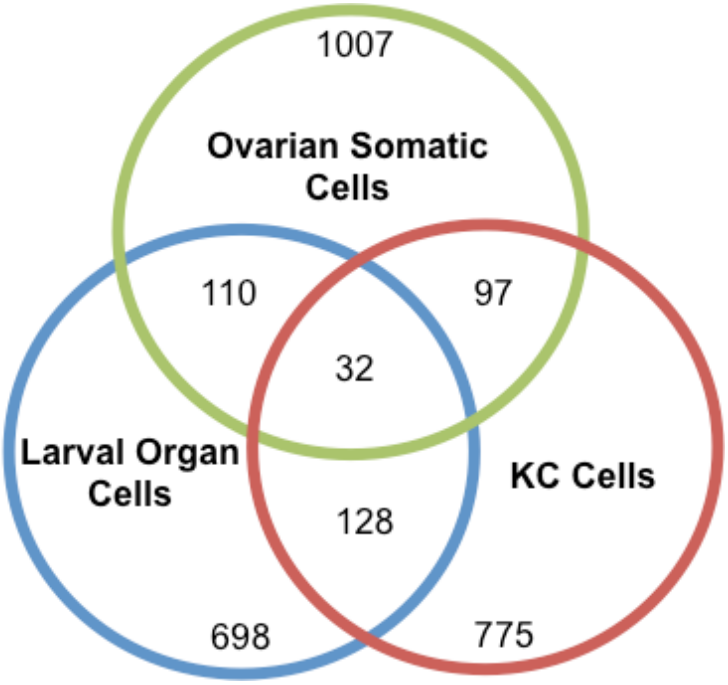


**Figure 3: Expression of *E23* in Multiple Cell Types**
Genome-wide datasets predict that transcriptional response to ecdysone is mostly non-overlapping in various cell types. Only 32 putative genes identified by various methods are expressed in ovarian somatic cells, larval organ cells, and KC cells. These 32 genes account for less than 5% of genes studied to date, suggesting that these genes may have important roles in *Drosophila melanogaster.*

mammals (Velamakanni et. al, 2007). *E23* is a part of the ABCG subfamily of ABC transporters that is associated with the efflux of cholesterol and other steroid hormones, like estrogen, in mammals (Imai et. al, 2003; Janvilisri et. al, 2003; Klucken et. al, 2000; Suzuki et. al, 2003; Wang et. al, 2004; Yu et. al, 2005; Yamanaka, Marques, and O'Connor, 2015).

*E23* has an established role as an ATP-binding cassette transporter that is capable of repressing ecdysone-mediated gene activation independently of the Golgi apparatus and secretory pathway (ABC transporter; Hock et. al, 2000). The ABC transporter superfamily is a large protein family that is represented in all forms of life, including mammals (Velamakanni et. al, 2007). *E23* is a part of the ABCG subfamily of ABC transporters that is associated with the efflux of cholesterol and other steroid hormones, like estrogen, in mammals (Imai et. al, 2003; Janvilisri et. al, 2003; Klucken et. al, 2000; Suzuki et. al, 2003; Wang et. al, 2004; Yu et. al, 2005; Yamanaka, Marques, and O'Connor, 2015). One isoform of ABCG transporters, ABCG2, has been identified as a multidrug transporter that is linked to breast cancer tumor resistance (Velamakanni et. al, 2007). Mutations in ABC transporter genes have been linked to cystic fibrosis, Tangier's diseases, Stangardt's disease, and many other inherited human disorders (Hock et. al, 2000). By characterizing *E23* as an ABC transporter in the ABCG subfamily, we can study its potential roles in steroid binding and relocation in relation to human disease, drug resistance, and drug therapies.

*E23* has also been characterized as a clock gene that regulates *Drosophila* circadian rhythm in developmental stages and in the adult fly (Itoh, Tanimura, and Matsumoto, 2011). *E23* is highly expressed in brain pacemaker neurons (Itoh, Tanimura, and Matsumoto, 2011). When *E23* levels are reduced, 50% of the flies became arrhythmic while the other 50% slept for abnormally long periods of time (Itoh, Tanimura, and Matsumoto, 2011). When *E23* was

overexpressed, 75% of the flies became arrhythmic (Itoh, Tanimura, and Matsumoto, 2011). These discoveries, if applied to humans, could help explain insomnia, sleep apnea, and other sleeping disorders.

Although the role of *E23* in developmental processes is fairly understudied, the role of its ecdysone responsive gene counterparts is well studied. Many of the 32 identified genes have key roles in *Drosophila* viability, fertility, and oogenesis (Tables 1 and 2). For example, flies with mutant *folded gastrulation* and *Jupiter* genes are partially lethal and often die during development (FlyBase). Also, flies lacking the genes *bip1* and *pointed* are sterile or semi-sterile (FlyBase). Furthermore, *broad* facilitates ovarian follicle cell development and *taiman* regulates border follicle cell migration and helps establish the germline stem cell niche (Bai et. al, 2000; Mathieu et. al, 2007; Jang et. al, 2009; Konig et. al, 2011; Jia et. al, 2015;).

Because *E23* is amongst the 32 genes expressed in multiple cell types, we hypothesize that it may have similar roles in *Drosophila* viability, fertility, and oogenesis to its counterparts. Furthermore, *E23* is highly expressed in adult ovaries. It is expressed nearly three times more in ovaries than any other adult tissues (Graveley et. al, 2011; See Figure 4). Because *E23* is expressed much more in the ovaries than other tissues, the gene may be maternally loaded. This high expression also suggests that *E23* may be involved in oogenesis. In order to test this hypothesis, I reduced the function of *E23* in *Drosophila* using transposable element insertions.

| Gene Name | Required for Viability? |
|---|---|
| *Chitinase 3* | ? |
| *broad* | Yes (some die during larval stage, increased mortality) |
| *Hr4* | Yes (increased mortality) |
| *Mnt* | Yes (increased mortality) |
| *raspberry* | Yes (increased mortality, partially lethal) |
| *folded gastrulation* | Yes (all die before end of embryonic stage) |
| *Early gene at 23* | ? |
| *rau* | Yes (partially lethal) |
| *taiman* | Yes (increased mortality) |
| *cactus* | Yes (all die before end of embryonic stage) |
| *Tetratricopeptide repeat protein 2* | No |
| *Misexpression suppressor of ras 3* | No |
| *brain tumor* | Yes (increased mortality) |
| *Hormone receptor-like in 39* | No |
| *Ecdysone receptor* | Yes (some die during pupal and pharate adult stage) |
| *lingerer* | Yes (some die during pupal stage) |
| *regular* | No |
| *Hormone receptor-like in 46* | Yes (increased mortality) |
| *CG9005* | No |
| *CG30089* | No |
| *Eps15 homology domain containing protein-binding protein 1* | Yes (increased mortality) |
| *hippo* | Yes (increased mortality) |
| *bip1* | No |
| *Heat shock protein 27* | No |
| *brummer* | Yes (all die before end of embryonic stage) |
| *Ecdysone-induced protein 74EF* | Yes (increased mortality) |
| *Ecdysone-induced protein 75B* | Yes (increased mortality) |
| *Ecdysone-induced protein 78C* | Yes (increased mortality) |
| *Tenascin major* | Yes (increased mortality) |
| *Jupiter* | Yes (partially lethal, majority die during pupal stage) |
| *aluminum tubes* | No |
| *pointed* | Yes (all die before end of embryonic stage) |

**Table 1: Putative Genes Involved in *Drosophila* Viability**

(Data from FlyBase) Because such a small percentage of genes are common to OSC, KC, and LO cells, it is suspected that these genes have vital functions. Of the 32 putative ecdysone early-response targets, 21 of these genes have a known role in *Drosophila* viability. Without these genes, many or all progeny die during development. The role of *E23* in viability is unknown.

| Gene Name | Required for Fertility? | Required for Oogenesis? |
|---|---|---|

| | | |
|---|---|---|
| *Chitinase 3* | ? | No |
| *broad* | Yes | Yes (somatic) |
| *Hr4* | ? | No |
| *Mnt* | ? | No |
| *raspberry* | ? | No |
| *folded gastrulation* | ? | No |
| *Early gene at 23* | ? | ? |
| *rau* | No | No |
| *taiman* | ? | Yes (somatic) |
| *cactus* | ? | No |
| *Tetratricopeptide repeat protein 2* | ? | Yes (germline & somatic) |
| *Misexpression suppressor of ras 3* | ? | Yes (germline & somatic) |
| *brain tumor* | ? | Yes (germline) |
| *Hormone receptor-like in 39* | Yes | Yes (ovary development) |
| *Ecdysone receptor* | Yes (females are sterile) | Yes (germline & somatic) |
| *lingerer* | No | No |
| *regular* | ? | ? |
| *Hormone receptor-like in 46* | ? | ? |
| *CG9005* | ? | ? |
| *CG30089* | ? | ? |
| *Eps15 homology domain containing protein-binding protein 1* | ? | ? |
| *hippo* | ? | ? |
| *bip1* | Yes (females are sterile) | No |
| *Heat shock protein 27* | No | No |
| *brummer* | ? | No |
| *Ecdysone-induced protein 74EF* | Yes | Yes (germline) |
| *Ecdysone-induced protein 75B* | Yes | Yes (germline & somatic) |
| *Ecdysone-induced protein 78C* | Yes | Yes (germline) |
| *Tenascin major* | ? | No |
| *Jupiter* | No | No |
| *aluminum tubes* | No | No |
| *pointed* | Yes (semi-sterile) | Yes (somatic) |

**Table 2: Putative Genes Involved in *Drosophila* Fertility and Oogenesis**
(Data from FlyBase) Because such a small percentage of genes are common to OSC, KC, and LO cells, it is suspected that these genes have vital functions. Of the 32 putative ecdysone early-response targets, 8 of these genes play a role in fertility and 11 genes have a role in facilitating oogenesis. However, the role of many of these genes in fertility and oogenesis has not yet been studied. *E23* may have similar roles in fertility and overall ovary development.

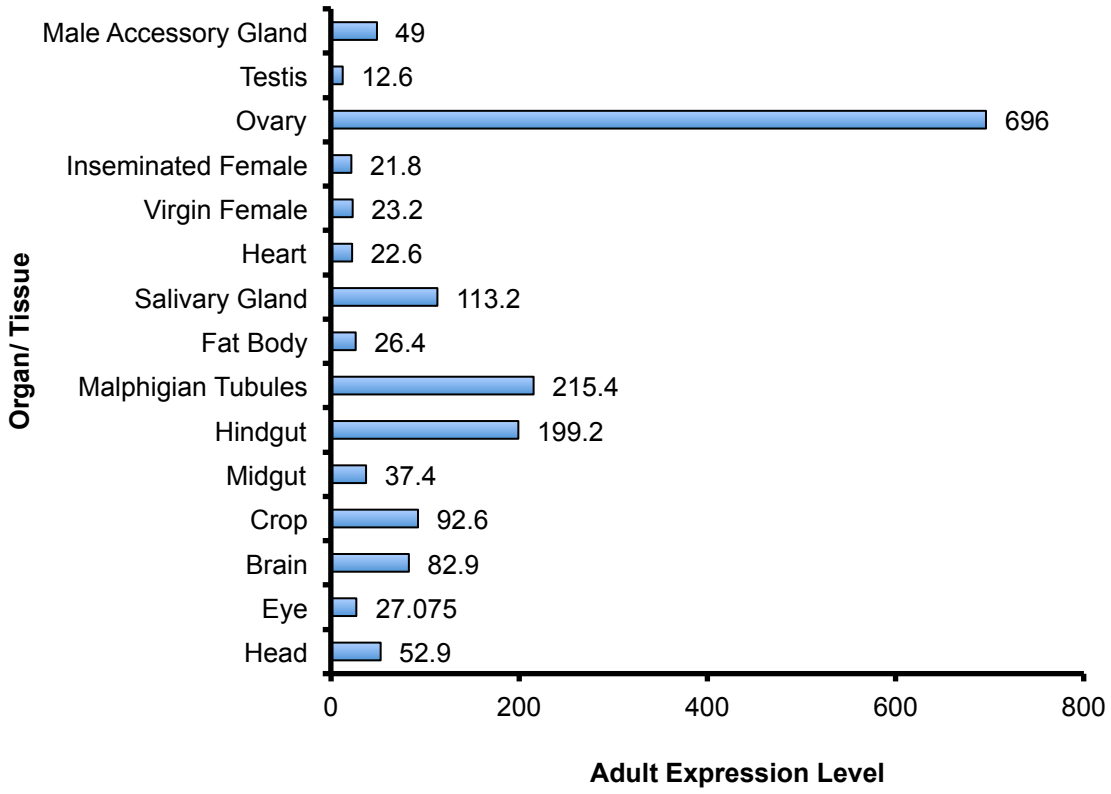


**Figure 4: Expression of *E23* in the Adult Ovary**
(Graveley et. al, 2011) RNA deep sequencing shows that *E23* is highly expressed in the adult ovary. It is expressed nearly 20 times higher in ovaries than other adult tissues. Most other tissues tested showed signs of low and moderate expression.

## METHODS

*Drosophila strains and culture conditions*

*Drosophila* stocks were maintained at 22-25$^{o}$ C in light medium (Nutri-Fly MF). For assessment of *E23* phenotypes, flies harboring transposable element insertions in the *E23* locus were analyzed for potential mutant phenotypes (Figure 5). In order to knock down the function of *E23* in *Drosophila*, two deficiency lines, *Df(2L)BSC163* (Blm #9598) and *Df(2L)Exel8008* (Blm #7786) were crossed with two *minos* insertion lines $E23^{MB07029}$ (Blm #25636) and $E23^{MI10587}$ (Blm #55506). Virgin female flies from each deficiency line were crossed with male flies from each *minos* insertion line in triplicates. The number of control and mutant progeny, distinguished by wing morphology, were counted to assess viability. Female and male reproductive capacity was measured by crossing progeny from the $F_1$ generation with siblings. The number of progeny per cross was counted to determine if the presence or absence of *E23* in flies affected reproductive capability.

*Immunofluorescence, staining, and microscopy*

Ovaries were stained, dissected, and viewed to determine if the lack of *E23* affects oogenesis. Female progeny were maintained on wet yeast at 23$^{o}$C for 5 days prior to ovary dissection. Ovaries were dissected in Grace's medium (BioWhittaker), teased apart, and then moved to a bovine serum albumin (BSA)-pre-coated tube on ice. Ovaries were then fixed in 5.3% formaldehyde (one part 16% formaldehyde to two parts Grace's medium; Ted Pella) for 13 minutes on a nutator. After removing the fix, ovaries were rinsed and washed in phosphate-buffered saline (PBS) with 0.1% PBS-Triton-X100 (Sigma) multiple times. Ovaries were left in 0.1% PBS-Triton-X100 for 4 days at 4$^{o}$ C and then incubated in blocking solution (5% BSA (BSA; Sigma), 5% normal goat serum (NGS; Jackson ImmunoResearch), and 0.1% Tween-20

(Sigma)) for 3 hours at room temperature. Ovaries were incubated in Rabbit anti-Vasa (1:1000 dilution in blocking solution; a gift from Paul Lasko) at 4$^{o}$C overnight and then washed in 0.1% PBS-Triton-X100 extensively. After removal of the primary antibody, ovaries were incubated in Mouse anti-Hts (1:10 in blocking solution dilution) and Mouse anti-LaminC (1:100 in blocking solution; 1B1; Developmental Studies Hybridoma Bank LC28.26) to stain the plasma membrane and nuclear lamina. After washing ovaries, they were incubated in Goat anti-rabbit-488 (1:200) and Goat anti-mouse-568 (1:200) for 2 hours in the dark. Ovaries were washed twice for 15 minutes, incubated in DAPI for 15 minutes, and washed again for 5 minutes in the dark. Ovaries were mounted in 90% glycerol with 20.0 ug/mL N-propyl gallate (Sigma) and stored at 4$^{o}$C for 12 days before viewing. Stained ovaries were imaged with a Zeiss LSM700 laser scanning confocal microscope and analyzed with Zen Blue 2012 Software.

*Statistical analysis*

For viability and fertility assays, standard error, indicated by error bars, was calculated in Microsoft Excel. The number of GSCs per germarium was counted to analyze if there is germline stem cell loss. For each cross, n=70. The average number of GSCs per control germaria was compared to the average number of GSCs per mutant germaria. Statistical analysis for GSC assays was performed using a students' two-tailed t-test in Microsoft Excel.

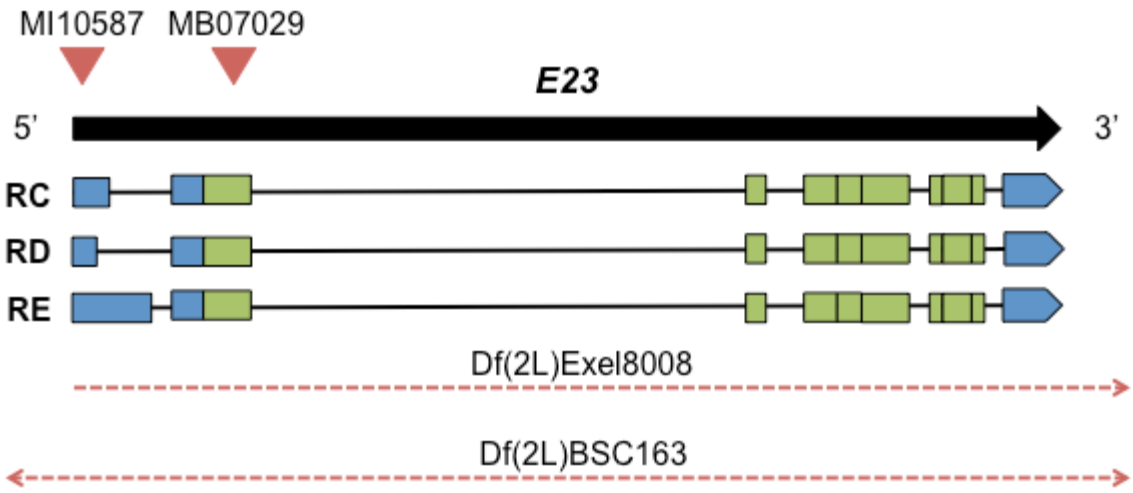


**Figure 5: Using Transposable Element Insertions to Reduce Function of *E23***
(Metaxakis et al., 2005; Bellen et al., 2011) *Minos* insertions are large pieces of DNA that can be used to separate a gene locus. Although there are many *minos* insertion lines located on the *E23* gene locus, we chose *MI10587* and *MB07029* because these insertions would separate the transcriptional start site and a large part of the first coding exon, thus creating a potential non-functional *E23* allele. The two deficiency lines were chosen to ensure that the entire gene is non-functional. *Df(2L)Exel8008* begins in the same location as *E23*, so it is not guaranteed that the gene will be affected. Therefore, the deficiency *Df(2L)BSC163* was also used as it spans the entire gene locus.

## RESULTS

*E23 is not required for viability.*

*E23* is amongst genes that are expressed downstream of ecdysone in larval organ cells, ovarian somatic cells, and KC cells (Figure 3). Many of these genes are required for *Drosophila* viability. Without these genes, some or all progeny die during development. Because *E23* is widely expressed, we hypothesized that it may have an important function in *Drosophila* viability. In order to test this hypothesis, transposable element insertions were utilized to knock down function of *E23* in flies. By crossing two deficiency lines with two *minos* insertion lines, four possible mutant lines were created (Figure 6A). The two deficiency lines are balanced with curly wings and the two *minos* insertion lines are balanced with *SM6A*. The expected progeny from each cross was 33.3% mutant flies indicated by straight wings and 66.6% control flies indicated by curly wings. One fourth of flies would be lethal because they contained both balancers. Twelve days after setting crosses, the number of progeny with straight wings and curly wings were quantified. Results indicate that for each cross, nearly 33.3% of progeny were mutant and 66.6% of progeny were control, as predicted by Mendelian ratios (Figure 6B). Because *E23* mutant progeny eclosed at the expected frequency, we conclude that *E23* is unlikely to affect viability.

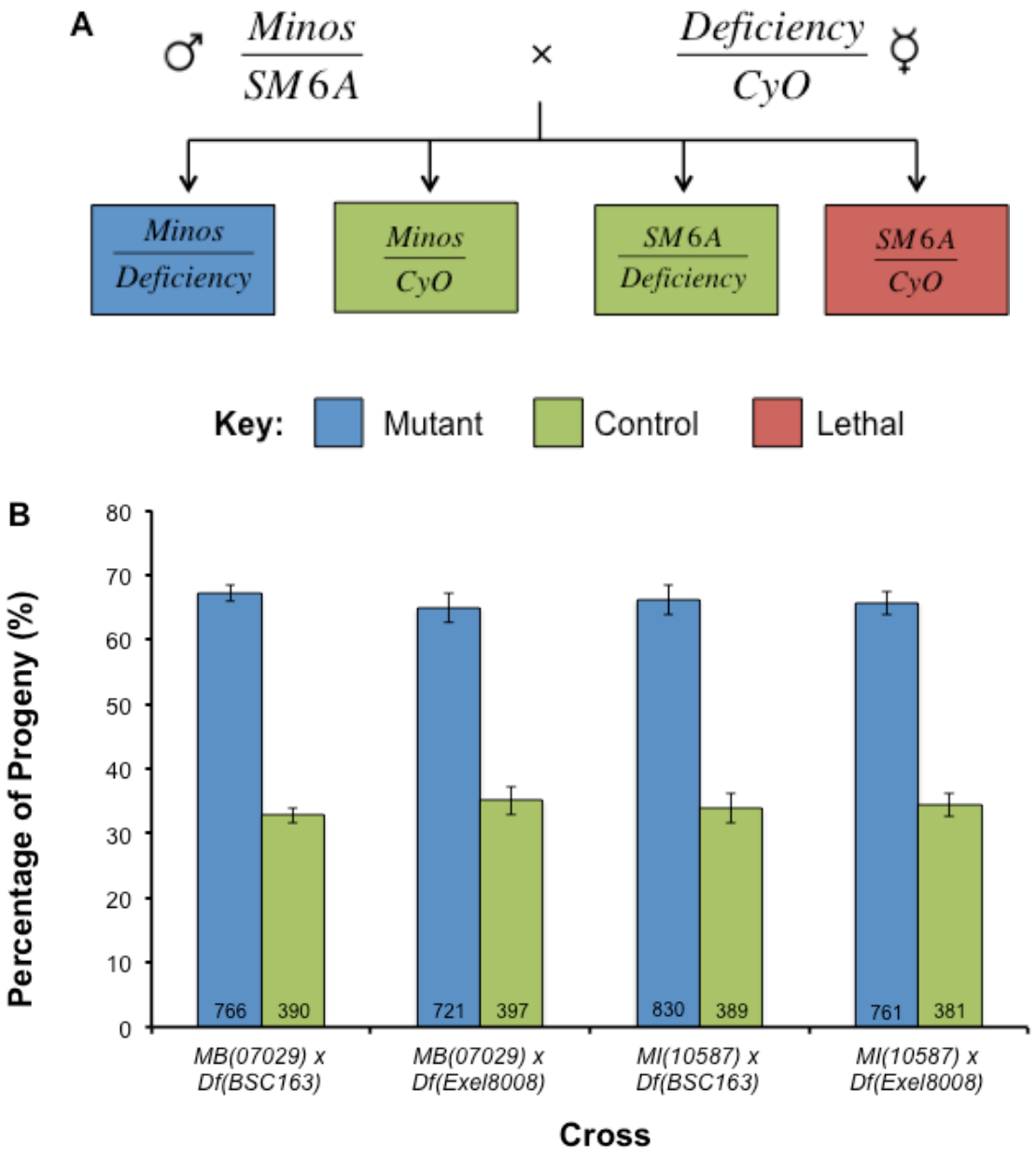


**Figure 6: Role of *E23* in Viability**
**(A)** When a *minos* insertion line is crossed with a deficiency line, one-fourth of the progeny are expected to be mutant (blue), one-half are expected to be control (green), and one-fourth are expected to be lethal (red). **(B)** The progeny of all four crosses, *MB(07029)* x *Df(BSC163)*, *MB(07029)* x *Df(Exel8008)*, *MI(10597)* x *Df(BSC163)*, and *MI(10587)* x *Df(Exel8008)*, came out in expected ratios with nearly 66.6% of flies being controls and 33.3% of flies being mutant. There is no significant difference in the number of progeny. Error bars represent standard error.

*E23 is not required for fertility.*

*E23* is highly expressed in adult ovaries, suggesting that the gene may be maternally loaded and play a role in fertility. To test this hypothesis, progeny from *MB(07029)* x *Df(BSC163)*, *MB(07029)* x *Df(Exel8008)*, *MI(10597)* x *Df(BSC163)*, and *MI(10587)* x *Df(Exel8008)* were crossed against each other (Figure 7A). A total of four crosses were set in triplicate using flies from the original four crosses. Mutant males and females were crossed together as a positive control. Control males and females were crossed together as a negative control. Mutant females were crossed with control males to assess female sterility. Control females were crossed with mutant males to assess male sterility. After twelve days, the total number of progeny per cross was counted and the mean per cross was calculated. There was no significant difference in the number of progeny per cross for any of the four crosses, therefore indicating that *E23* may not play a role in *Drosophila* fertility (Figure 7B).

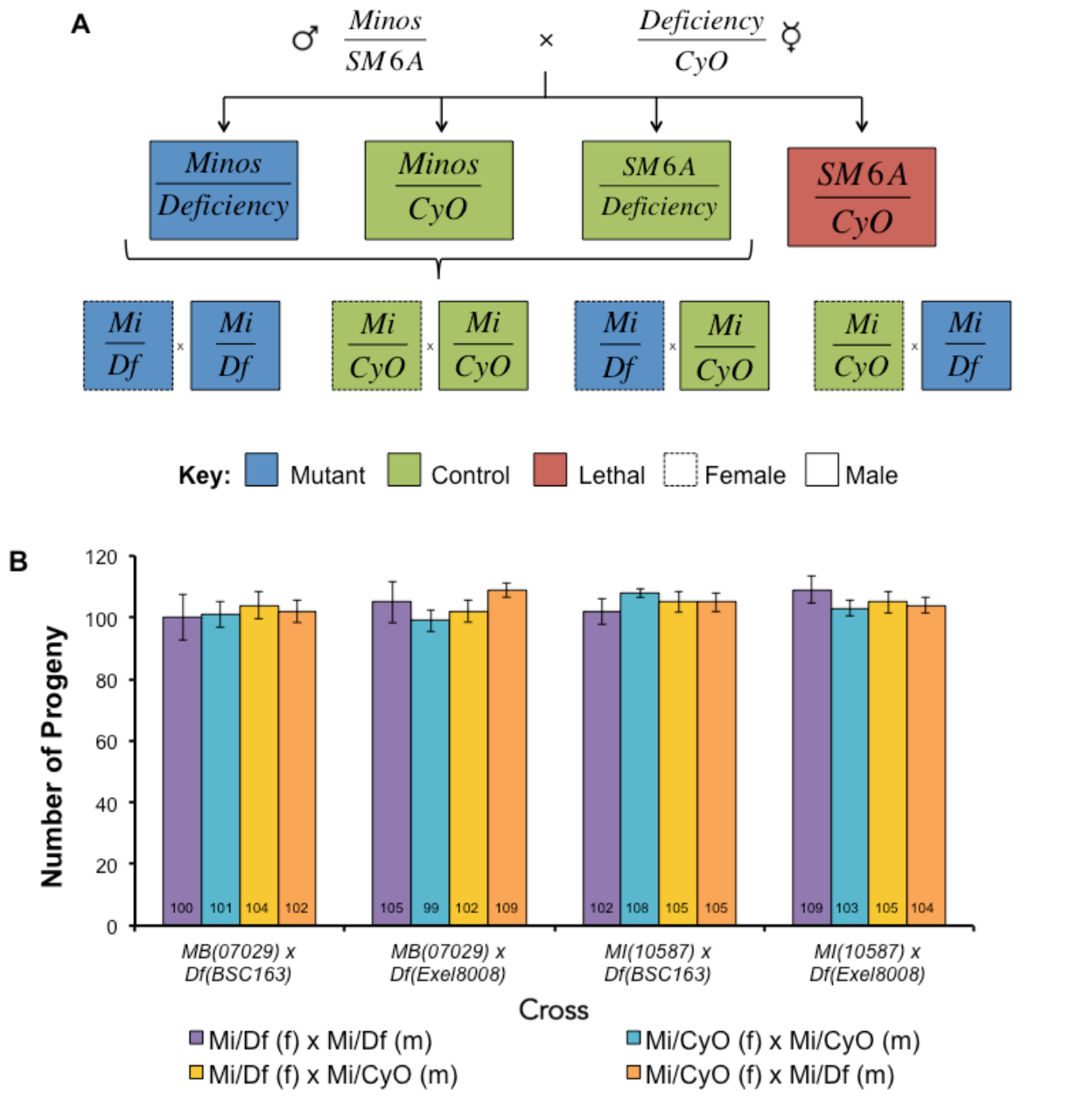


**Figure 7: Role of *E23* in Fertility**

**(A)** After back-crossing progeny from the $G_0$ generation, the number of progeny per cross was counted. The number of progeny for crosses including mutant flies were compared to the number of progeny to the control cross, *Mi/Df* (female; f) x *Mi/Df* (male; m). If there were significantly less progeny for the *Mi/CyO* (f) x *Mi/CyO* (m) cross, it would indicate that *E23* affects fertility. If there were significantly less progeny for the *Mi/Df* (f) x *Mi/CyO* (m), it would indicate that *E23*-deficient females affect fertility. If there were significantly less progeny for the *Mi/CyO* (f) x *Mi/Df* (m) it would indicate that *E23*-deficient males affect fertility. **(B)** However, there were no significant differences in the number of progeny per cross. Therefore, *E23* does not affect fertility rates. Error bars represent standard error.

*E23 is not required for early oogenesis or germline stem cell number.*

Genome-wide datasets suggest that transcriptional response to ecdysone does not typically overlap in multiple cell types. Of the approximately 3000 genes studied to date, only 32 genes are expressed in ovarian somatic cells, larval organ cells, and KC cells. Nearly half of these genes play a role in the germline or somatic cells to facilitate oogenesis. *E23* is one of the 32 putative gene targets, so we hypothesized that it may play a role in oogenesis like its counterparts. In order to test this hypothesis, transposable element insertions were utilized to knock down function of *E23* in flies. By crossing two deficiency lines with two *minos* insertion lines, four possible mutant lines were created. Ovaries from control and mutant progeny (indicated by wing morphology) were collected, dissected, stained, and viewed under a confocal microscope.

Although *E23* was not involved in viability or fertility, we looked for more subtle effects in the ovaries using confocal microscopy. The overall morphology of germaria did not differ between control and mutant ovaries (Figure 8). Ovaries appeared to be healthy, and ovarioles progressed normally in fourteen stages. In the germarium, there was clear indication of germline stem cells dividing into daughter cells and forming the 16-cell cyst. Therefore, we concluded that *E23* is not involved in early oogenesis.

Besides morphology, we studied if *E23* affects the number of adult stem cells in the germline. For each germarium, we counted the number of adult stem cells in 70 control and mutant germaria. Results demonstrated that there is no significant difference in the average number of germaria per control or per mutant for any of the four crosses (Figure 9). We also measured the distribution of how many germaria have 0, 1, 2, or 3+ stem cells. There were no significant differences between these distributions as well.

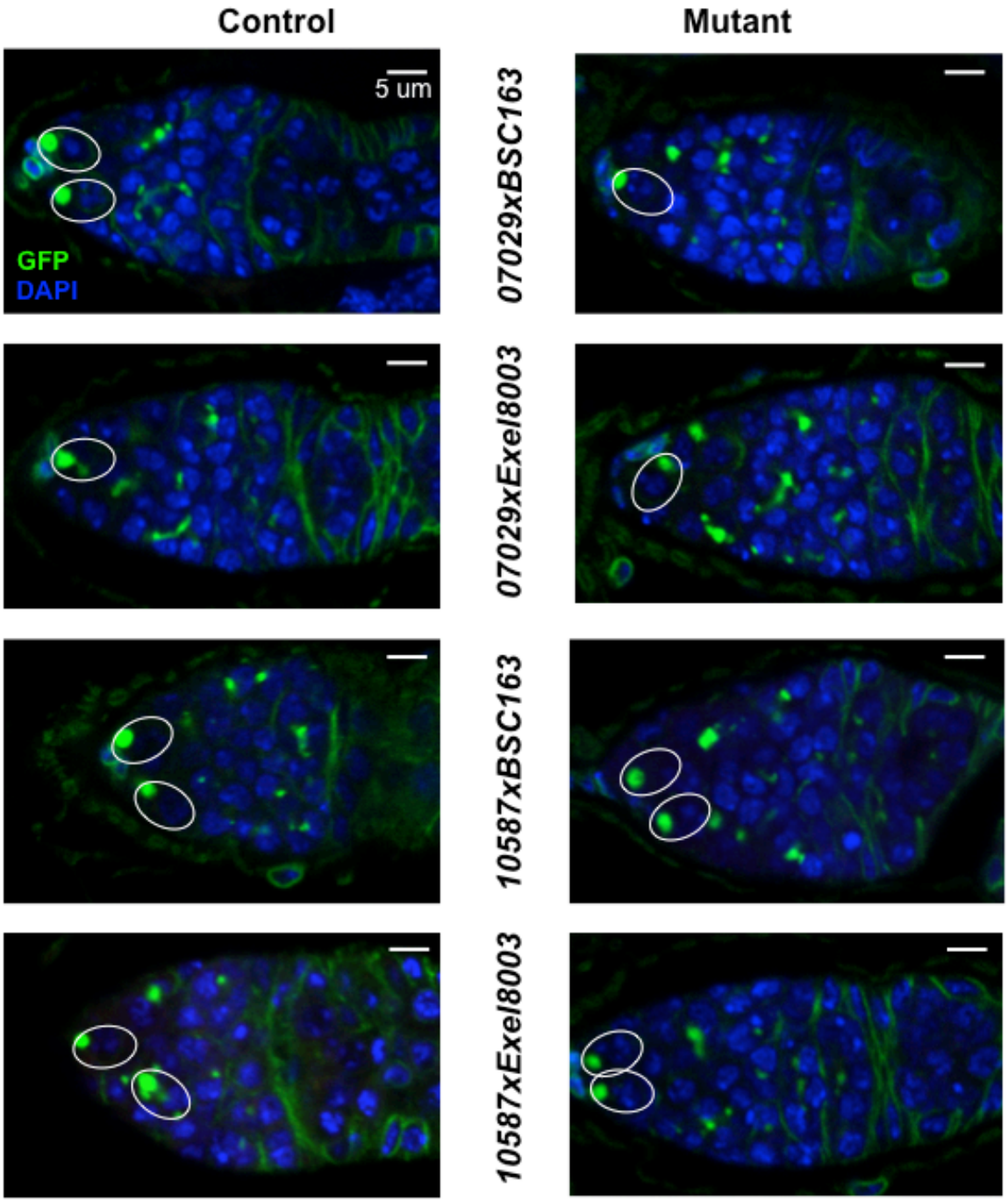


**Figure 8: Role of *E23* in Early Oogenesis**
*Drosophila* germaria have anywhere from zero to four adult stem cells. These adult stem cells divide to form daughter cells that undergo four divisions to form an interconnected 16-cell cyst. Both control and mutant germaria showed normal progression from adult stem cells (white circles), to daughter stem cells, and then the cyst. There were no obvious morphological differences between control and mutant germaria.

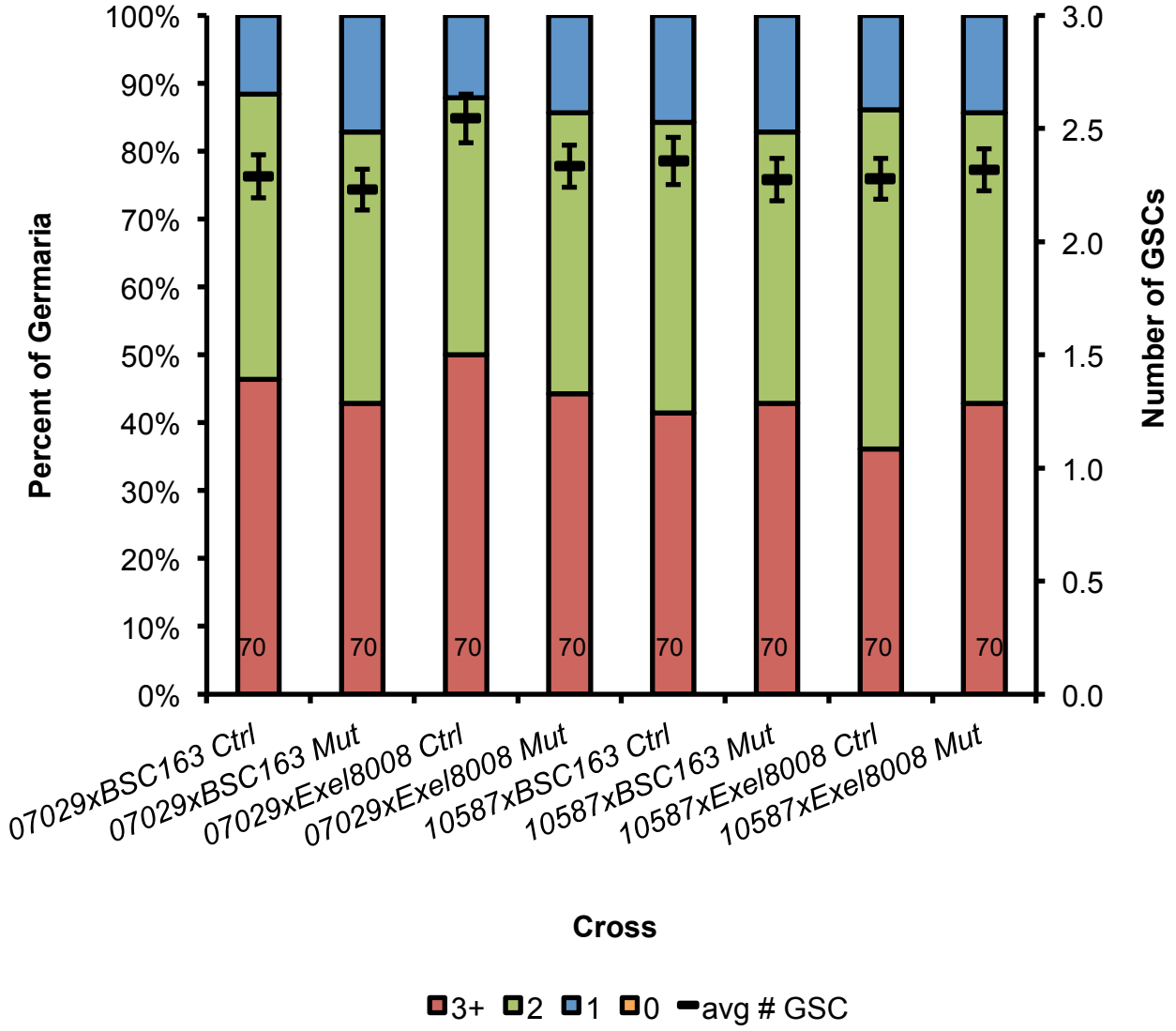


**Figure 9: Role of *E23* in Stem Cell Number**
Two *minos* insertion lines were crossed with two deficiency lines to knock down function of *E23*, resulting in a total of four crosses. Progeny from these crosses were collected, with curly wings representing control flies and straight wings representing mutant flies. Control and mutant ovaries for each cross were dissected, teased apart, stained, and viewed under a confocal microscope. The number of adult stem cells per germarium was counted. This number varied from 1-4 stem cells per germarium. There is no significant difference in the distribution of number of stem cells or the average number of stem cells for all crosses. Error bars represent standard error measurement.

## DISCUSSION

Although many early genes are known to have important roles in *Drosophila* developmental processes, our studies suggest that the *E23* may not share this role. The expression of *E23* in multiple cell types and high expression in the ovary suggested that the gene may be vital for development. It remains unclear how *E23* functions as a part of the ecdysone signaling pathway in the ovary, but literature suggests that many gene targets of this pathway are essential for *Drosophila* survivability, reproduction, gamete formation, and stem cell function. Our study found that *E23* does not play a role in *Drosophila* viability, fertility, early oogenesis, and germline stem cell number. Our work adds to a growing body of knowledge about the role of early genes in *Drosophila* development, and specifically what *E23* may be involved in.

In order to study the role of *E23,* we crossed flies harboring transposable elements with flies harboring large deletions in the *E23* genome. Although this method typically knocks down function of genes, the use of transposable insertions does not guarantee that there will be a non-functional *E23* allele. Indeed, we have not yet tested RNA or protein levels in the mutants used in this study; it is possible that some functional *E23* protein is made. In the future, we could use the FLP/FRT site-directed recombination technology or CRSPR/Cos9 system to engineer a more precise *E23* mutant. This mutant fly line could be used to replicate previously conducted viability, fertility, oogenesis, and germline stem cell experiments.

To create an *E23* null allele using FLP/FRT, a series of four crosses would have to be set (Figure 10). In the $G_0$ generation, *P{hsFLP}*$^{1}$*, y*$^{1}$ *w*$^{1118}$*; P{hs-hid}2, wg*$^{Sp\text{-}1}$*/CyO* (Blm #26901) virgin females would be crossed with *Pbac{WH}E23*$^{f04823}$ (Exelixis) male flies. Male progeny would be crossed with female virgin *Pbac{RB}E23*$^{e00859}$ (Exelixis) virgin females and heat shocked one time per day in a water bath set at 37$^{o}$C on days 3-8. In the $G_2$ generation, female

virgin progeny would be crossed with wildtype *Gla/CyO* wildtype flies (Blm #44227). In the $G_3$ cross, individuals with the deletion phenotype would be crossed with *Gla/CyO*. To test for the deletion, DNA would be extracted, amplified using PCR, and then analyzed using gel electrophoresis. These mutant flies could then be crossed against each other and progeny could be evaluated for viability, fertility, and oogenesis defects.

One reason why we studied the role of *E23* in fertility and oogenesis was because the gene is highly expressed in the adult ovary. However, this high expression may not be indicative of function in the ovary. According to FlyBase, many genes that have low expression in the ovary play key roles in oogenesis (Figure 11). Of the putative 32 gene targets expressed in ovarian somatic cells, KC cells, and larval organ cells, eleven genes are involved in oogenesis. Of these eleven genes, only *E23*, *brain tumor* (*brat*), and *Tetratricopeptide repeat protein 2* (*Tpr2*) are highly expressed in the adult ovary (Figure 12). The remaining eight genes show low expression or no expression at all. Although high expression of *E23* in the ovary was a valid reason for studying its role in oogenesis, the expression alone was not enough to determine if *E23* did indeed have a role in development.

Although our studies found that *E23* does not have vital developmental roles, it is possible that *E23* must pair with other genes to produce phenotypic defects. Many early genes repress late genes, which in turn result in tissue-specific effects. Furthermore, some genes interact with one another to produce larger effects in the ovaries. For example, *E23* acts with other genes to function as an ecdysone-inducible ABC transporter (Hock et. al, 2000). *E23* is a half-type transporter that must form a dimer in order to function as an ABC transporter (Yokomizo and Murakami, 2015). However, there is no evidence to suggest if *E23* must form a heterodimer with

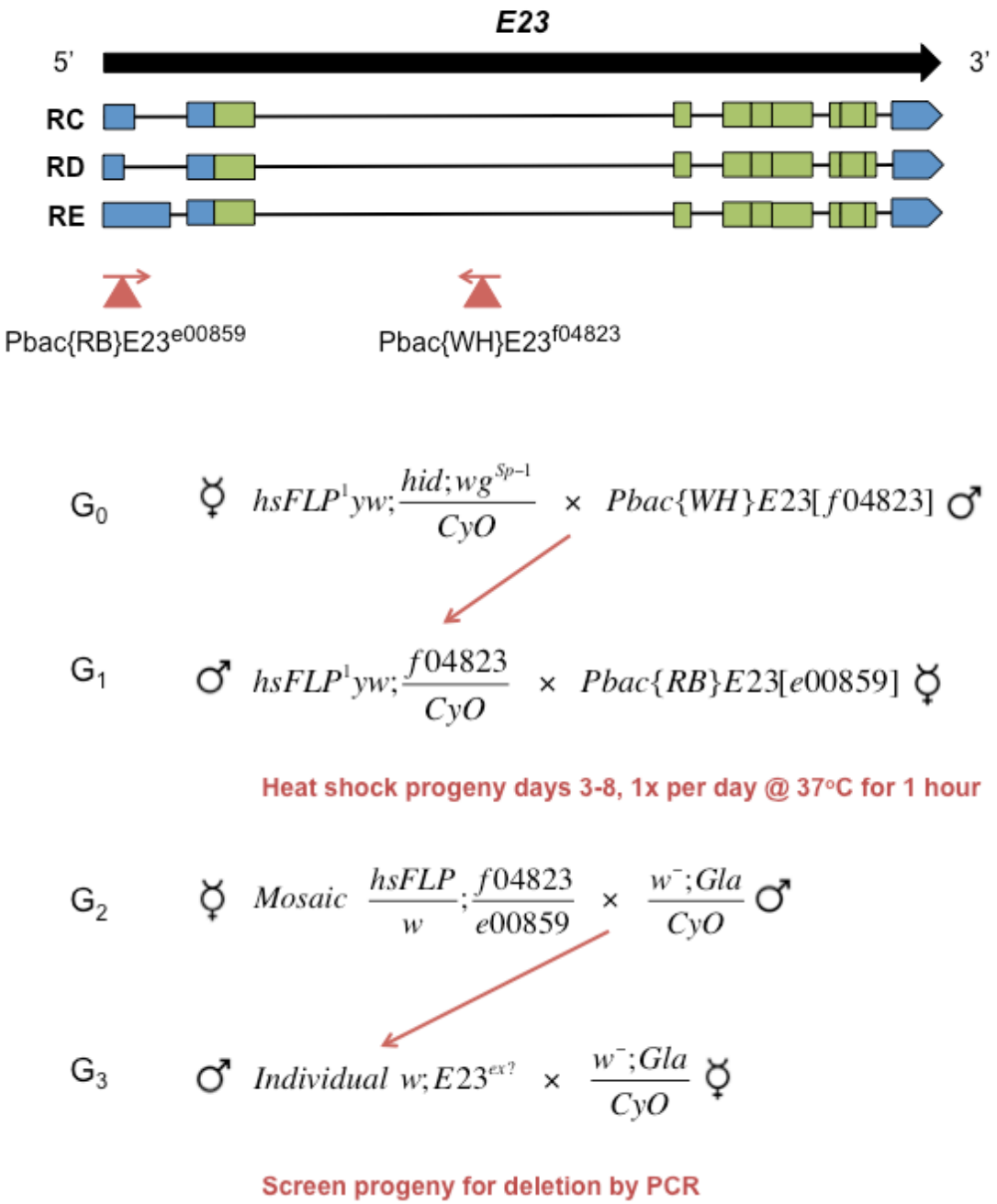


**Figure 10: Generating a Mutant with a Non-Functional *E23* Allele**
Piggy-back elements, or Pbac elements, are insertions used to remove large portions of DNA. The Pbac elements chosen (red triangles) remove DNA spanning the transcriptional start site, the majority of the first coding exon, and nearly half of the large intron. Removing a piece of DNA this large will likely create a non-functional *E23* allele. The Flp-FRT recombination system can be used to remove the DNA between the two Pbac elements. The $G_0$ and $G_1$ crosses added two FRT sites into the fly line. When the $G_1$ cross is heat shocked, the heat induces a recombination event. These flies are crossed with wildtype flies to make the mutation germline.

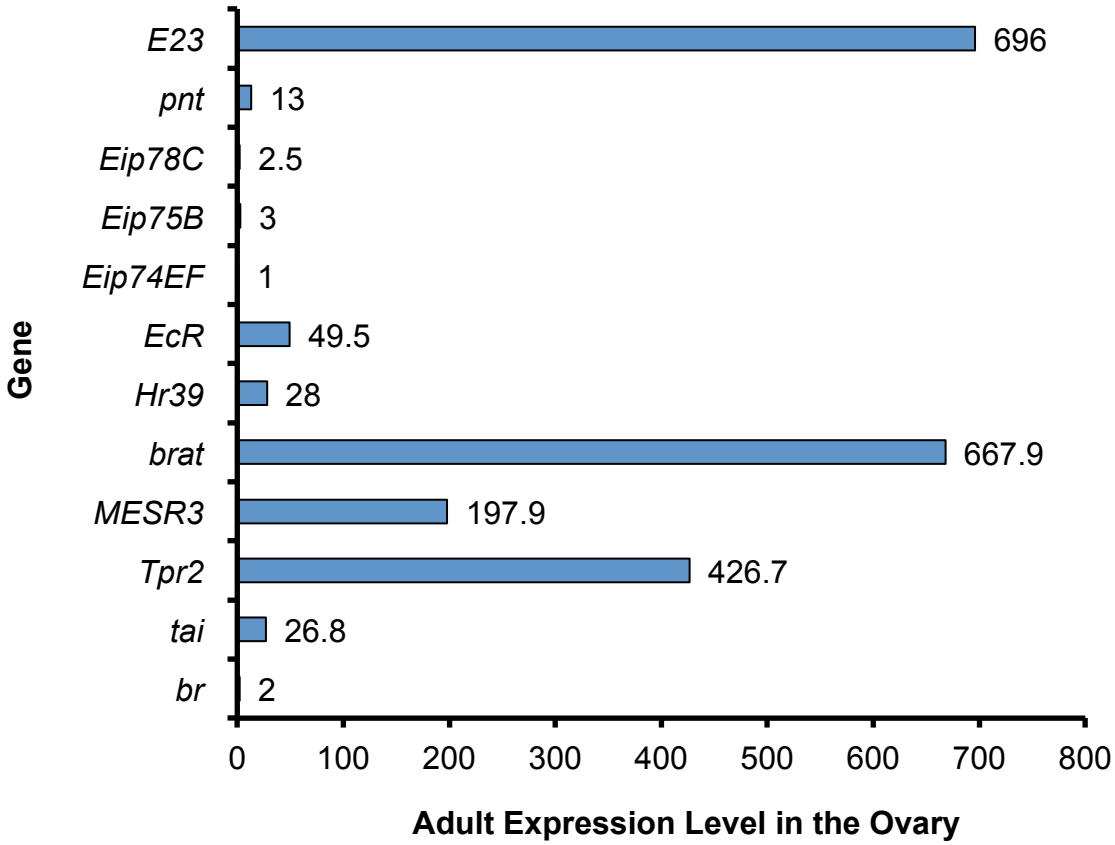


**Figure 11: Expression of Putative Gene Targets in the Adult Ovary**
(Graveley et. al, 2011) RNA deep sequencing shown above is for putative gene targets that have a known role in oogenesis in the germline or somatic cells. Of these genes, only two genes are highly expressed in the adult ovary. One gene is moderately expressed, while the remaining genes are lowly expressed or show no expression at all. Therefore, expression of a gene in the adult ovary does not necessarily correlate with a role in oogenesis.

another half-type transporter or a homodimer to carry out its function (Yokomizo and Murakami, 2015). In order to fully understand how *E23* fits into the ecdysone signaling pathway, it is necessary to understand the mechanism by which *E23* partners with other genes to act as a transporter (Yokomizo and Murakami, 2015). It is possible that the half-type transporter with which *E23* forms a heterodimer must also be knocked down in order to affect development.

*E23* also has a known role as a regulator of circadian rhythm by acting with the clock gene *vrille* (Itoh, Tanimura, and Matsumoto, 2011). When *E23* was knocked down in *Drosophila*, there was increased expression of *vrille (*Itoh, Tanimura, and Matsumoto, 2011). *E23* forms a feedback loop in the ecdysone signaling pathway to control circadian rhythm by ecdysone-mediated *vrille* expression (Itoh, Tanimura, and Matsumoto, 2011). Without the regulation of *vrille*, *E23* would not be able to alter the circadian rhythm of fruit flies. Although the knockdown of *E23* increased *vrille* expression, it decreased the expression of other clock genes including *per*, *tim*, *Clk*, and *Pdp1e* (Itoh, Tanimura, and Matsumoto, 2011). It is evident that there are many factors involved in controlling circadian rhythmicity, and that *E23* does not act alone.

Although our studies did not support our hypotheses, there are many modifications that can be made to these experiments to garner more accurate results. In the future, it will be helpful to create a guaranteed mutant and then study the function of *E23*. Even though using transposable element insertions is easier and shorter process for experimentation, creating a mutant will ensure that no trace amounts of *E23* will be expressed. If the replicated experiments present consistent results, it is possible that *E23* in fact does not play a role in any developmental processes or that *E23* potentially acts with other genes to affect development. Future studies will add to a set of data that either confirms or denies the role of *E23* in viability, fertility, and oogenesis.

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