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Investigating the Role of ATP-Binding Cassette Transporters in Drosophila melanogaster Testis Stem Cells

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INVESTIGATING THE ROLE OF ATP-BINDING CASSETTE TRANSPORTERS IN DROSOPHILA MELANOGASTER TESTIS STEM CELLS

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ABSTRACT

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Multidrug resistance is among the most pressing obstacles in cancer treatment today. Resistance is thought to arise from the ability of cancer stem cells to efflux therapeutic molecules using a collection of membrane proteins called ATP-binding cassette (ABC) transporters. There is strong interest in targeting ABC transporters to preserve and improve drug efficacy and reduce cancer recurrence. Many studies have been performed *in vitro* using cultured cell lines, but currently there is a lack of simple models in which to study ABC transporters *in vivo*. As a solution, I propose to use the fruit fly *Drosophila melanogaster* for the study of ABC transporters, and specifically the *D. melanogaster* testis stem cell niche—one of the best-characterized adult stem cell niches. Stem cells have several traits in common with cancer cells, including the ability to divide indefinitely, the ability to give rise to many different kinds of daughter cells, and chemoresistance. In invertebrates there is mounting evidence for a role ABC transporters play in insecticide resistance, but to date there is no peer-reviewed evidence for invertebrate *stem cell* drug resistance in the literature. Here, I present evidence of cytotoxic drug efflux in the germline stem cells of the *D. melanogaster* testis. This was accomplished by feeding the chemotherapy drug doxorubicin to wild type flies for two days and measuring fluorescence levels using confocal microscopy. Using enhancer trap lines, I also present ABC transporter expression evidence in both the germline and cyst lineages of the testis. Finally,

through RNAi knockdown of several ABC transporters, I present evidence of their contribution to germline stem cell drug efflux. I also report unexpected phenotypes in the male germline resulting from the knockdown of two ABC transporters, CG32901 and CG3164, which points to a role for these proteins in development of the normal testes niche.

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CHAPTER I

INTRODUCTION

ATP-Binding Cassette Transporters, Multidrug Resistance, and Cancer Stem Cells

One of the most pressing obstacles in cancer treatment is the resistance of cancer stem cells to conventional chemotherapy drugs [1-4]. In many instances, drugs may eradicate the majority of a tumor mass but leave behind the cancer stem cells, ultimately leading to cancer recurrence. Multidrug resistance is thought to arise from the ability of cancer stem cells to efflux therapeutic molecules using a collection of transmembrane proteins called ATP-binding cassette (ABC) transporters. This family of proteins has been implicated not only in cancer drug resistance [5], but also in cystic fibrosis [6, 7], and antibiotic resistance [8, 9]. Collectively, the ABC transporter family are capable of transporting an extremely wide variety of substrates—from small inorganic ions to both drugs and polypeptides. The family is divided into seven classes or subfamilies (ABCA - ABCG) based on amino acid sequence similarities, but so far only subfamilies A, B, C, and G have been implicated in cancer multidrug resistance [10]. The unifying feature of all ABC transporters is that they consist of two distinct protein domains: a *transmembrane domain* (TMD) embedded within the cell membrane, and a cytosolic *nucleotide binding domain* (NBD) which binds and hydrolyzes adenosine triphosphate (ATP) to fuel substrate transport. Structurally, ABC transporters exist as either *full transporters*, usually containing two TMD and two NBD each, or *half transporters* with only one TMD and NBD, which must form homo- or heterodimers to function [11]. While the ABC transporter family as a

whole is collectively capable of transporting a wide variety of substrates, most individual ABC transporters are specific for one or a few substrates. Such specificity is determined by the structure of their TMD. Many ABC transporters have overlapping substrate specificity [12], while the specific substrates and functional roles of many ABC transporters remain unknown.

Cancer multidrug resistance (MDR) is a phenomenon which occurs when cancer cells simultaneously become resistant to a host of structurally and functionally unrelated drugs. MDR has been observed in a number of different cancers, including acute myelogenous leukemia (AML), sarcomas, and breast, ovarian, and lung cancer [13-15]. There are a number of cellular mechanisms by which MDR can arise, such as blocking apoptotic pathways or activating DNA repair pathways (Figure 1.1), but the mechanism most often encountered is increased efflux by ABC transporters [13]. The contribution of ABC transporters to the development of MDR in cancer cells has been known for some time. ABCB1 (also known as P-glycoprotein or MDR1), first discovered in 1976, was shown to be expressed in drug-resistant mutants of the Chinese hamster ovary, or the CHO cell line [16]. Subsequently, numerous ABCB1 small-molecule inhibitors were developed in hopes of treating MDR, but not a single one of them became an enduring treatment option for patients [17]. Three generations of ABCB1 small-molecule inhibitors were tested in dozens of clinical studies throughout the 1980s and 1990s. While there were some apparent early successes (such as verapamil to treat lymphoma) [18], these inhibitors ultimately failed to prove safe and effective [15, 19]. These and subsequent disappointments led to outspoken pessimism regarding the pursuit of future ABC transporter inhibitors [20, 21], and ultimately the suppression of further research in this area for some decades [22]. In hindsight, it appears that the failure of those early clinical trials was due, at least in part, to their narrow focus on just one of the 48 distinct human ABC transporters, ABCB1, as a therapeutic target. This is

obviously problematic, because we now understand that many distinct ABC transporters may be expressed simultaneously within a single tumor. Today, at least 12 human ABC transporters from four subfamilies (ABC-A, ABC-B, ABC-C, and ABC-G) have been implicated in cancer MDR, and studies using cultured cancer cell lines have revealed that two proteins other than ABCB1 are predominantly responsible for MDR, ABCC1 and ABCG2, neither of which was a target in those early clinical trials [13]. Another issue was the lack of appropriate tools, such as methods for accurately detecting ABCB1 expression in human cancers (let alone other ABC transporters) [15], as well as robust tools for studying gene expression and mutation in general [14].

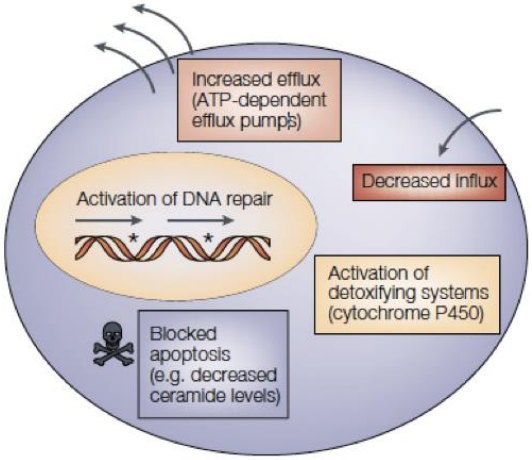


Figure 1.1: Cellular mechanisms of multidrug resistance, including increased efflux activity conferred by ABC transporters. Borrowed from Gottesman *et al*., 2002. [15]

Today there is renewed interest in developing ways to target ABC transporters, and a growing body of evidence suggests that such efforts could make both classical chemotherapy drugs and small-molecule inhibitors more effective and reduce the rate of cancer recurrence [3, 4, 22-25]. Currently, however, there is a lack of simple models in which to study ABC transporters *in vivo.* Many of the today's investigations into cancer stem cell drug resistance are

being performed *in vitro* using cultured cell lines [23, 26]. While these kinds of experiments have proven useful in identifying some resistance-thwarting drugs [27, 28], they are limited in their ability to account for the influence of a particular tissue's microenvironment. Not only are cancer cells exposed to various cytokines and growth factors from the surrounding tissue, but solid tumors often have areas which are hypoxic, nutrient-deprived, and highly acidified [5]. Indeed, hypoxia is even thought to be a biomarker for resistant cancer cells [29]. As such, *in vitro* models that do not take the physiology of these microenvironments into account are missing potentially valuable insights [30].

A solution to this problem may lie in the idea that chemoresistance is not exclusive to cancer cells but appears to be a general stem cell trait. Not all stem cells exhibit chemoresistance through elevated efflux activity. Those that do have come to be referred to as "side population" cells, first identified by their ability to exclude Hoechst 33342, a blue, fluorescent dye which binds to AT-rich regions of DNA [31]. Side population cells have since been identified in a variety of tissues (e.g. skin, heart, brain) [32]. Some cancer cells also exhibit this elevated efflux ability, and these cells are thought to be capable of both tumor initiation and repopulation [33]. In other words, these cells seem to function as cancer stem cells (CSCs). The theory of CSCs is now well established in the scientific community [1-4], and there is growing interest in targeting CSCs to potentially increase the efficacy of anti-tumor therapies (Figure 1.2). Furthermore, there is accumulating evidence that both ABC transporter expression and elevated efflux might be general stem cell properties, shared by both CSCs and normal stem cell populations alike [34]. (Also see Appendix A for evidence of dye exclusion in normal stem cells). This finding makes sense conceptually, as it would be important for stem cells to have a defense mechanism against harmful toxins in order to repopulate their respective tissues. Interestingly, it also suggests that

ABC transporter overexpression might be an evolved stem cell defense mechanism that has been hijacked for cancer chemoresistance. Given this, it is quite possible that well-established stem cell model systems could be used to shed new light on the workings of ABC transporters and their role in cancer MDR.

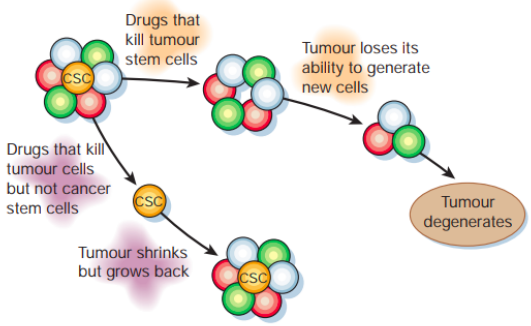


Figure 1.2: The theory of cancer stem cells posits that some cancer cells have stem-like properties enabling them of tumor initiation and repopulation. Targeting cancer stem cells could increase the efficacy of chemotherapies and reduce tumor recurrence. Borrowed from Reya *et al.*, 2001. [3]

ATP-Binding Cassette Transporter Expression: A General Stem Cell Trait

The finding that ABC transporters are expressed within some normal stem cell populations may present the opportunity to study proteins important to cancer MDR using well-established *in vivo* stem cell models. The fate of any stem cell daughter is either renewal of the stem cell state or differentiation into a cell type specific to the tissue in which the stem cell resides. That is, stem cells daughters either *self-renew* or *differentiate*. When germline stem cells divide, for example, their progeny either retain the stem cell state or begin to develop into mature germ cells, ultimately giving rise to gametes. To make this fateful decision, the stem cell depends on signals from its local microenvironment, or *niche* [35]. These signals might come from nearby somatic cells or other stem cells. There is evidence that these local signals suppress

differentiation, and that distance away from the niche and its signals allows for differentiation [36]. As local signals appear to be instrumental to maintaining the stem cell state, it makes sense to study stem cell properties such as efflux ability in the context of these signals, and not isolated from them. One of the obstacles to doing so, however, is the complexity of stem cell niches and the difficulty of genetic manipulations in mammals. Such complexity makes it difficult to identify and genetically manipulate specific cell populations [37], as well as difficult to elucidate the precise signaling interactions between cell populations in the niche [38, 39]. Such difficulties could be overcome by employing a simpler model organism, such as *Drosophila melanogaster*.

The benefits of using *D. melanogaster* are numerous. They include a short generation time, inexpensive maintenance, and a well-developed catalogue of robust genetic tools such as the Gal4-UAS system [40-42]. The breadth of both Gal4 lines and UAS reporter lines available make this a powerful system for manipulating and studying gene expression *in vivo*. Moreover, it is thought that ~ 65% of disease-causing genes in humans have fly homologs [43], giving them significant relevance to biomedical research. One area of research this organism is especially well-suited for is stem cell biology. In particular, the *D. melanogaster* testis stem cell niche (Figure 1.3), with its ease of manipulation and imaging and its well-characterized tissue architecture, is among the best models for studying adult stem cells *in vivo* [37, 44]. This niche supports two distinct populations of stem cells—germline stem cells and cyst stem cells—both of which cluster around a small group of somatic, nondividing cells called the hub. This clustering allows the stem cells to be identified by their position within the testis, as the stem cells are always found adjacent to the hub, and thus stem cell-specific stains are not necessary for identification. Furthermore, stem cells have several traits in common with cancer, including the ability to divide indefinitely, the ability to give rise to different kinds of daughter cells in some

cases, and chemoresistance, often through ABC transporter expression and elevated efflux activity. Given the similarities between stem cells and cancer, the *D. melanogaster* testis stem cell niche could, in theory, serve as a useful tool for the study of ABC transporters *in vivo.*

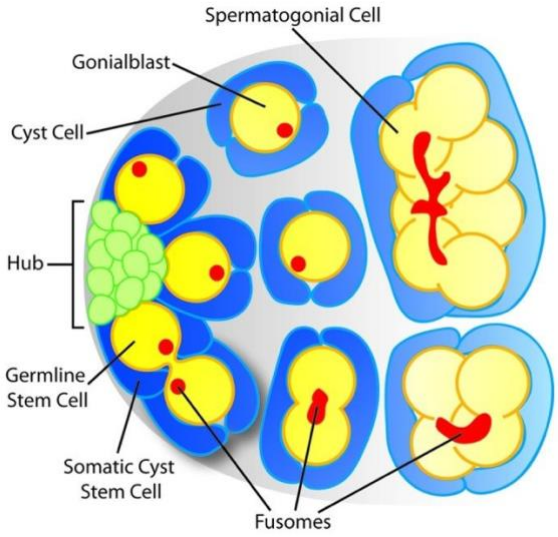


Figure 1.3: Conceptual model of the *Drosophila melanogaster* testis stem cell niche. Two distinct kinds of stem cells, germline and cyst, cluster around a small group of somatic cells called the hub. Distance away from the hub ultimately leads to differentiation. Borrowed from de Matunis *et al*., 2012. [44]

Which populations of regular stem cells express ABC transporters? Most of the evidence comes from vertebrate stem cell populations. The three ABC transporters most notorious for cancer multidrug resistance, ABCB1, ABCC1, and ABCG2 [45], are also expressed within some normal hematopoietic stem cell populations [46]. It is thought that these ABC transporters protect hematopoietic progenitor and stem cells from potentially harmful xenobiotics. ABCB1 is expressed in human $CD34^{+}$ stem cells [47]. ABCG2 is highly expressed in most (if not all) hematopoietic stem cells, including $34^{+}/38^{-}$ and $34^{+}/KDR^{+}$ populations, but expression is largely lost as these cells mature [48]. Likewise, ABCC1 is expressed at higher levels in hematopoietic

stem cells than in mature blood cells [49]. Other, less notorious ABC transporters are also expressed in human $34^{+}/38^{-}$ hematopoietic stem cells, including several members of subfamilies ABCA, ABCB, and ABCC [46]. Interestingly, *Abcg2* mRNA has been shown to be expressed within side population cells of both murine embryonic stem cells and skeletal muscle, as well as its ortholog in rhesus monkey bone marrow, indicating that ABC transporter expression may be a highly conserved feature of certain stem cell populations [50].

Even though the majority of the evidence for ABC transporter expression in regular stem cells is specific to hematopoietic stem cells, there is evidence of ABC transporter expression in non-hematopoietic stem cell populations as well. As mentioned above, ABCG2 is thought to be expressed in both murine embryonic stem cells and skeletal muscle. Moreover, Tang *et al*. characterized the expression profiles of several non-hematopoietic stem cell types, including adipose-derived human mesenchymal stem cells and human embryonic stem cells (HUES1, HES2, and HES3), and while they found the overall expression levels of ABC transporters to be lower in non-hematopoietic compared to hematopoietic stem cells, they did find similar expression profiles in these other stem cell types, as well as specific transporters unique to non-hematopoietic stem cells [51]. This suggests that stem cells depend not on any single ABC transporter, but on collections of transporters unique to their cellular identity, resulting in a kind of redundancy thought to both protect stem cell integrity and contribute to the difficulty of combating cancer multidrug resistance [51]. Unfortunately, it remains unclear whether many populations of tissue-specific adult stem cells express ABC transporters, and there have been few definitive studies on the matter. However, using lineage tracing experiments, Fatima *et al*. were able to show that ABCG2 is expressed not only within murine blood stem cells (as is already widely known), but also within stem cells of the murine small intestine and testis [52]. ABCG2

has also been shown to be highly expressed within human neural stem and progenitor cells [53]. Future studies will, in all likelihood, identify ABC transporters expressed in even more adult stem cell populations.

Comparatively less is known about the expression of ABC transporters within invertebrate stem cell populations. While it has yet to be shown in the literature whether ABC transporters are active within *D. melanogaster* testis stem cells, there are a number of reasons to believe it might be so. First, ABC transporters are found across all three domains of life, from bacteria to mammals, including a family of 56 proteins encoded in the *D. melanogaster* genome (Table 1). Second, there is a growing body of evidence that suggests ABC transporter expression is a general stem cell trait, shared by both cancer and normal stem cells alike [34, 35], and it would make evolutionary sense for stem cells of the germline to have a defensive mechanism such as ABC transporters to protect against cytotoxins. Third, previous research has demonstrated that *D. melanogaster* insecticide resistance has coincided with the upregulation of genes coding for several ABC transporters, and that knockdown of these genes was able to increase drug susceptibility [54-56]. Using the 91-R strain of *D. melanogaster*, which is highly resistant to the insecticide 4,4'-dichlorodiphenyltrichloroethane (DDT), Gellatly *et al*. showed that RNAi knockdown of three ABC transporters (Mdr50, Mdr65, and Mrp1) led to decreased resistance, reducing LT50 values by 13%, 12%, and 15% respectively [54]. Similarly, Kim *et al*. induced tolerance to the insecticide Ivermectin in Canton S (wild type) flies and showed that such tolerance both correlated with the transcriptional upregulation of three ABC transporters (Mrp1, CG1824, and CG3327) and was reduced following RNAi knockdown of these transporters. Finally, Denecke *et al*. used CRISPR-Cas9 knockouts to investigate the contribution of Mdr49, Mdr50, and Mdr65 in resistance to a variety of insecticides, with knockout mutants

showing increased susceptibility to 1, 3, and 5 insecticides, respectively [56]. Interestingly, they also found that treatment with verapamil (an ABC transporter-inhibitor) was able to inhibit resistance only in flies with at least one function copy of Mdr65, further implicating the transporter's role in causing resistance.

Table 1.1: A list of the 56 ABC Transporters encoded in the *Drosophila melanogaster* genome.

FAMILY	GENE	HUMAN ORTHOLOGUE*
ABC-A		
	ABCA	ABCA3
	CG1494	
	CG1801	
	CG6052	ABCA3
	CG8908	
	CG31213	
	CG34120	ABCA12
	CG42816	
	CG43672	
	Eato	
ABC-B		
	CG10226	
	Mdr49	ABCB4/ABCB11
	Mdr50	ABCB1
	Mdr65	ABCB4
	ABCB7	ABCB7
	CG1824	ABCB8
	CG3156	ABCB10
	Hmt-1	ABCB6
ABC-C		
	CG4562	ABCC4
	CG5789	
	CG7627	ABCC4
	CG7806	ABCC10
	CG9270	ABCC4
	CG10505	ABCC4
	CG11898	ABCC4
	CG31792	ABCC4
	CG31793	ABCC4
	l(2)03659	ABCC4
	MRP	ABCC3
	Mrp4	ABCC4
	rdog	ABCC4
	Sur	ABCC9

Table 1.1, (Continued)

FAMILY	GENE	HUMAN ORTHOLOGUE*
ABC-D		
	ABCD	ABCD2
	Pmp70	ABCD3
ABC-E		
	pix	ABCE1
ABC-F		
	CG1703	ABCF1
	CG9281	ABCF2
	CG9330	ABCF3
ABC-G		
	Atet	ABCG4
	bw	
	CG3164	
	CG4822	
	CG5853	ABCG1
	CG9663	
	CG9664	
	CG11069	ABCG5
	CG17646	
	CG31121	
	CG31689	
	CG32091	
	E23	
	st	
	w	ABCG2
ABC-H		
	CG11147	
	CG33970	
	snu	

Note. *Only top human orthologues with a FlyBase protein alignment score of 8 out of 15 or higher were included (corresponding to hits from at least 8 different tools or algorithms used by FlyBase to determine orthology). Multiple orthologues were listed if they tied for top FlyBase scores.

While evidence supporting the role ABC transporters play in insecticide resistance is mounting, the only direct evidence of invertebrate *stem cell* drug resistance to date (to the best of my knowledge) comes from Dayton *et al.* [57]. In their BioRxiv preprint, they provided the first evidence of ABC transporter expression within the intestinal stem cells (ISC) and enteroblast (EB) progenitors of the *D. melanogaster* midgut. By feeding flies two cytotoxic drugs (bortezomib and actinomycin D), dissecting their intestines, and evaluating cell response, they showed that ISC and EB exhibit increased drug tolerance relative to mature enterocytes. Then, by feeding flies fluorescent dyes and using RNAi to knockdown 55 of the 56 ABC transporters in the *D. melanogaster* genome, they showed that ISC and EB have elevated efflux ability conferred by seven ABC transporters (CG1494, CG1819, Mdr50, ABCB7, MRP, CG32091, and CG5853). Next, they used enhancer trap lines to characterize the expression patterns of ABC transporters in ISCs and EBs relative to mature enterocytes, and found that two of the seven previously identified ABC transporters, ABCB7 and CG32091, were specific to ISC and EB cell populations. Finally, they found that RNAi knockdown of these two ABC transporters was sufficient to restore drug susceptibility to ISCs and EBs.

All of this evidence taken together led to the question of whether ABC transporters play a role in drug efflux and resistance in *D. melanogaster* testis stem cells. Here, I present evidence of cytotoxic drug efflux in the germline stem cells of the *D. melanogaster* testis. This was accomplished by feeding doxorubicin to wild type flies for two days and measuring fluorescence levels using confocal microscopy. Then, using enhancer trap lines, I also present ABC transporter expression evidence in both the germline and cyst lineages of the testis. Finally, through RNAi knockdown of several ABC transporters, I present evidence of their contribution to germline stem cell drug efflux. I also report unexpected phenotypes in the male germline

resulting from the knockdown of two ABC transporters, CG32901 and CG3164. This work lays the foundation for future studies involving ABC transporters in the *D. melanogaster* testis stem cell niche.

CHAPTER II

GENERAL METHODS

Drosophila melanogaster Fly Strains

The Canton S (wild type), Nanos-Gal4, and UAS-GFP fly lines were obtained from Dr. Stephen DiNardo, and the c587-Gal4 line from Dr. Erica Matunis. All transgenic fly lines listed in Tables 2 and 3 were obtained from the Bloomington *Drosophila* Stock Centers (BDSC). All of the RNAi transgenic lines were created through the joint efforts of the Vienna Drosophila RNAi Center, the National Institute of Genetics, and the Drosophila Transgenic RNAi Project (TRiP) at Harvard Medical School [58]. The lines were validated by the RNAi Stock Validation and Phenotypes Project [58]. All stocks were grown at room temperature and fed on standard fly medium. For all fly crosses, resulting F1 progeny were incubated at 29° C for approximately one week in order to induce Gal4 expression prior to any further experimental treatment (drug-feeding, dissection and immunofluorescence staining, etc.).

Immunofluorescence Staining

Testis samples were dissected in *Drosophila* Ringer's solution, then incubated in 0.5 mL of fixative for 20 minutes at room temperature. Fixative solution is 4% formaldehyde and 0.02% Triton X-100 diluted in Buffer B (75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$, 16.7 mM KPO_4); 0.5 mL applied per sample. Following fixation, samples were rinsed twice with PBTx (1X PBS, 0.1% Triton X-100), washed with PBTx, then incubated overnight with 0.5 mL blocking solution (4% normal donkey serum in PBTx + 0.01% Na Azide) at 4°C. The next day, primary antibodies

diluted in blocking solution were applied to samples and incubated overnight at 4°C. Fluorescent antibodies were used to visualize the testis niche. Proteins that accumulate in distinct cell populations within the testis niche were bound by primary antibodies, which themselves were bound by secondary antibodies containing fluorophores (i.e. fluorescent compounds that can be visualized upon light excitation). After removing primary antibodies, samples were washed in PBTx overnight at 4°C. Secondary antibodies diluted in blocking solution were applied the next day and incubated overnight at 4°C. Following secondary antibodies, samples were rinsed in PBTx, incubated with Hoechst nuclear stain (if applicable), and washed overnight at 4°C. Finally, samples were soaked in a 50% Ringers/50% glycerol solution for at least 30 minutes, mounted on microscope slides, and imaged using a Zeiss 700 confocal microscope. A three-dimensional image of the testis niche was recreated by compiling Z-stacks of 0.5 μm intervals. All image analysis was done in ImageJ.

The hub, germline cells, and cyst lineage cells were stained using mouse anti-fasciclin 3 antibody (Developmental Studies Hybridoma Bank, 1:50), rabbit or goat anti-vasa antibody (Santa Cruz, 1:300), and guinea pig anti-traffic jam (TJ) antibody (1:10,000), respectively [59]. Rabbit anti-GFP antibody (Invitrogen, 1:1000) was used to enhance GFP detection in experiments involving the UAS-GFP reporter line, and rabbit anti-Cleaved Caspace-3 antibody (Asp175) (Cell Signaling Technology, 1:400) was used to detect apoptosis. All secondary antibodies were raised in donkey (Jackson ImmunoResearch, 1:400). *D. melanogaster* testis stem cells are identified by their proximity to the hub, and therefore do not require stem-cell specific markers. Hoechst 33342 (Invitrogen; 350 nm excitation maximum and 461 nm emission maximum), a fluorescent dye that binds to AT-rich regions of DNA, was used at 1 μg/mL.

Enhancer Traps

To investigate the expression patterns of select ABC transporters, the upstream activating sequence (UAS)-GFP reporter line was used. To verify its utility, UAS-GFP flies were first crossed to the driver lines Nanos-Gal4 and c587-Gal4, which drive Gal4 expression in the germline and cyst lineage, respectively. Following verification, UAS-GFP virgin females were crossed to males of each of the Gal4 enhancer trap lines from the BDSC (Table 2). Each of these enhancer trap lines have Gal4 insertions near the gene-of-interest, possibly bringing Gal4 expression under the control of the gene-of-interest's regulatory sequences. Thus, an ABC transporter-Gal4 enhancer trap x UAS-GFP cross may result in the expression of GFP within cells where Gal4 is expressed and able to bind UAS, and therefore recapitulate endogenous ABC transporter expression. The F1 progeny from each of these crosses were incubated at 29° C for approximately one week prior to dissection, immunofluorescence staining, and imaging.

Table 2.1: Transgenic *Drosophila melanogaster* lines used for enhancer trap crosses.

FAMILY	CG#	SYNONYM	STOCK #	LINE TYPE	VENDOR
ABC-B type	3879	Mdr49	24312	Gal 4 Enhancer Trap	BDSC
ABC-C type	6214	MRP	77697	Gal 4 Enhancer Trap	BDSC
ABC-C type	5772	Sur	25638	Gal 4 Enhancer Trap	BDSC
ABC-D type	2316	Abcd1	78384	Gal 4 Enhancer Trap	BDSC
ABC-G type	3164	N/A	76211	Gal 4 Enhancer Trap	BDSC
ABC-G type	4822	N/A	77564	Gal 4 Enhancer Trap	BDSC
ABC-G type	32091	N/A	29892	Gal 4 Enhancer Trap	BDSC
ABC-G type	3327	E23	25636	Gal 4 Enhancer Trap	BDSC
ABC-H type	11147	N/A	25548	Gal 4 Enhancer Trap	BDSC

Quantifying Efflux in *Drosophila melanogaster* Germline Stem Cells

To investigate potential drug efflux in the germline stem cells, Canton S flies were fed food containing the naturally fluorescent chemotherapeutic, doxorubicin (Thermo Fisher Scientific; 470 nm excitation maximum and 585 nm emission maximum). Doxorubicin is an intercalating agent that interferes with DNA replication by inhibition of topoisomerase II [60]. It

is also known to induce cell death via the generation of reactive oxygen species (ROS) [61, 62]. 100 μL of 1 mM doxorubicin were added to fresh vials of perforated fly food and allowed to soak overnight. Flies were added the following day and allowed to feed for two days. Flies fed on food containing dimethyl sulfoxide (DMSO) served as a negative control. Following feeding, testes were dissected in *Drosophila* Ringer's solution, fixed, and stained using immunofluorescence as previously described.

Image Analysis and Statistics

All image analysis was done in ImageJ. Differential efflux activity was measured by comparing the fluorescence pixel intensity of doxorubicin in stem cell populations vs. differentiating cell populations (i.e. germline stem cells vs. 4- and 8-cell spermatogonia). Rules for data analysis were as follows: (1) a minimum of three of each cell type was used per testis; if three of each cell type could not be identified, then that testis was not used. (2) All pixel intensity measurements were taken with a circular area 2.0 μm in diameter. (3) Pixel intensity measurements were taken from the brightest part of each cell (with respect to the x, y, and z planes) clearly within the bounds of the cell nucleus. Differences in mean fluorescence pixel intensity quantifications between germline stem cells and spermatogonia were tested for statistical significance using a two-tailed Paired t-Test in Excel. To do this, intensity measurements for both germline stem cells and spermatogonia were averaged within each testis, and sample size was determined by the number of testes analyzed. Thus, the Paired t-Test compared the mean germline stem cell intensity measurement with the mean spermatogonia intensity measurement within each testis. Statistical significance was further verified using a pairwise analysis involving nine independent Paired t-Tests in Excel, comparing the first, second, and third germline stem cell of each testis with the first, second, and third differentiating

spermatogonia within that same testis. Because the minimum number of testes for any of these doxorubicin efflux experiments was twenty (n = 20), this resulted in a minimum of 180 cell-to-cell pairwise comparisons.

Ribonucleic Acid Interference or Knockout of ATP-Binding Cassette Transporters

For germline RNAi experiments, the Nanos-Gal4 VP16 driver line was used [63]. This driver has expression of the Gal4 protein under control of the *nos* promoter, which is active in both the male and female *D. melanogaster* germline. Nanos-Gal4 virgin females were crossed with males of each of the upstream activating sequence (UAS)-RNAi lines from the BDSC (Table 2.2), with the exception of stock #58885, which has a loss-of-function genomic mutation in ABCA and thus required no cross. These RNAi lines were designed to express a double-stranded RNA hairpin loop when UAS binds Gal4, ultimately degrading the mRNA transcript of interest. Thus, a Nanos-Gal4 x UAS-RNAi cross results in the expression of dsRNA for RNAi only within Nanos-expressing cells (e.g. germline stem cells and progenitors). The F1 progeny from each of these Nanos-Gal4 x UAS-RNAi crosses were incubated at 29° C for approximately one week prior to doxorubicin feeding and analysis (as previously described). The c587-Gal4 driver line was used to investigate RNA interference of CG32091 and CG3164 in the cyst lineage. To investigate RNA interference of CG32091 and CG3164 in the female germline, ovaries from progeny of both Nanos-Gal4 x UAS-RNAi crosses were dissected, stained using immunofluorescence, and imaged in the same manner as the testes. Nanos-Gal4 male and female flies served as controls for both apoptosis and ovary RNAi experiments.

Table 2.2: Transgenic *Drosophila melanogaster* lines used for RNAi knockdown crosses.

FAMILY	CG#	SYNONYM	STOCK #	LINE TYPE	VENDOR
ABC-A type	1718	ABCA	58885	Mutant KO	BDSC
ABC-B type	3879	Mdr49	32405	UAS-RNAi	BDSC
ABC-C type	6214	MRP	38316	UAS-RNAi	BDSC
ABC-C type	5772	Sur	67246	UAS-RNAi	BDSC
ABC-G type	3164	N/A	57478	UAS-RNAi	BDSC
ABC-G type	4822	N/A	62475	UAS-RNAi	BDSC
ABC-G type	32091	N/A	57783	UAS-RNAi	BDSC
ABC-G type	3327	E23	57782	UAS-RNAi	BDSC
ABC-H type	11147	N/A	57741	UAS-RNAi	BDSC

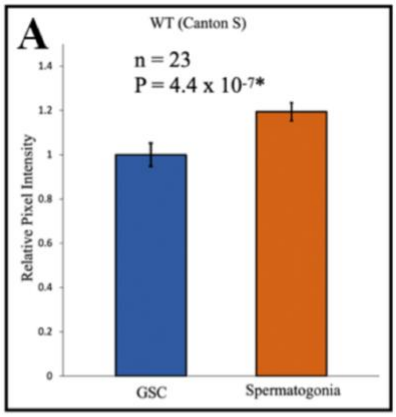
CHAPTER III

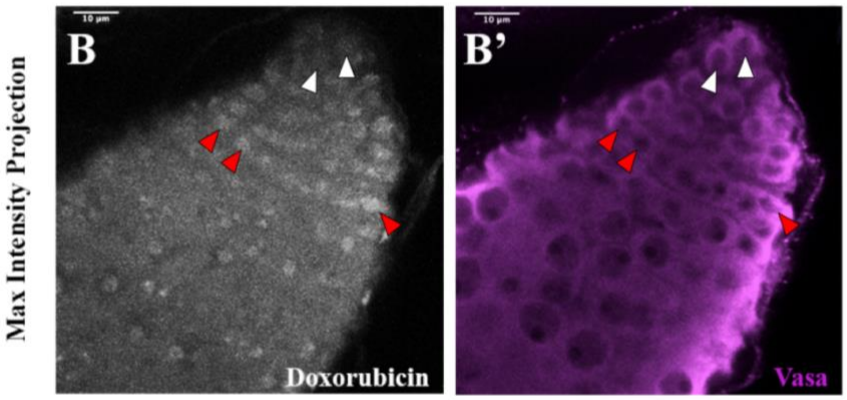
RESULTS

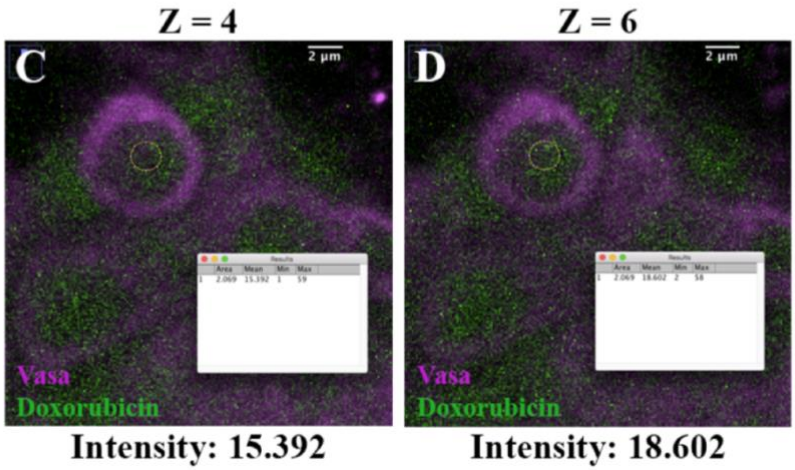
Investigating Drug Efflux in Germline Stem Cells

Drug efflux via ABC transporters is a hallmark of resistant cancer cells and stem cells alike. In order to investigate drug efflux in *D. melanogaster* testis germline stem cells (GSCs), the drug efflux ability of both GSCs and their differentiating daughter cells (spermatogonia) were compared. During spermatogenesis, GSCs undergo asymmetric cell division resulting in two daughter cells, one GSC that self-renews to maintain the stem cell state, and a gonialblast that exits the niche [37, 44, 64]. Differentiating gonialblasts are enclosed by somatic cyst cells and undergo four rounds of transit-amplifying spermatogonial divisions before ultimately giving rise to sperm-producing spermatocytes (see Figure 1.3). Because differentiating germline cells lose their stem cell characteristics as they move away from the niche, I hypothesized that any cytotoxic drug efflux ability of the GSCs would be reduced or lost during differentiation. To test this, wild type (Canton S) flies were fed for two days on food soaked in 1 mM doxorubicin. Doxorubicin is a common chemotherapeutic used to treat cancer. It is an intercalating agent that interferes with DNA replication by inhibition of topoisomerase II [60], and is also known to induce cell death via the generation of reactive oxygen species (ROS) [61, 62]. This drug was chosen for its natural fluorescence (470 nm excitation maximum, 585 nm emission maximum), as it can be readily detected using confocal microscopy. DMSO-fed flies served as a negative control (Figure 3.1 F). Following feeding, fly testes were dissected and imaged using immunofluorescence confocal microscopy. As seen in Figure 3.1 E, ingested doxorubicin clearly

reaches the testis. The pixel intensity of the doxorubicin stain was then measured using ImageJ in both GSCs and differentiating spermatogonia (both 4- and 8-cell spermatogonia were measured), and the mean GSC pixel intensity value was compared to the mean spermatogonia pixel intensity value within each testis. After analyzing twenty-three testes (n=23), GSCs were found to have, on average, lower doxorubicin pixel intensity measurements than differentiating spermatogonia ($P = 4.4 \times 10^{-7}$; Figure 3.1 A). The statistical significance of this finding was further verified using a pairwise analysis involving nine independent t-Tests (for each: $P < 0.05$; see methodology for details). This result supports the hypothesis that GSCs have increased cytotoxic efflux ability relative to their differentiating daughter cells. While this difference in doxorubicin accumulation seems to be modest, in some cases the intensity difference can be clearly visualized (Figure 3.1 B). To measure the doxorubicin stain in each cell, both the x- and y-planes were varied, as well as the z-plane (Figure 3.1 C-D), in an attempt to locate the brightest part of each cell (i.e., the area of highest doxorubicin accumulation). As an additional negative control, pixel intensity was also measured in the channel used to visualize the Vasa germline stain. As expected, this did not result in any significant difference between the GSCs and spermatogonia (Figure 3.1 G-H).


A
WT (Canton S)
n = 23
P = 4.4 x 10-7*
Relative Pixel Intensity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GSC
Spermatogonia


Max Intensity Projection
B
Doxorubicin
B'
Vasa


Z = 4
Z = 6
C
D
2 µm
Vasa
Doxorubicin
Intensity: 15.392
Intensity: 18.602

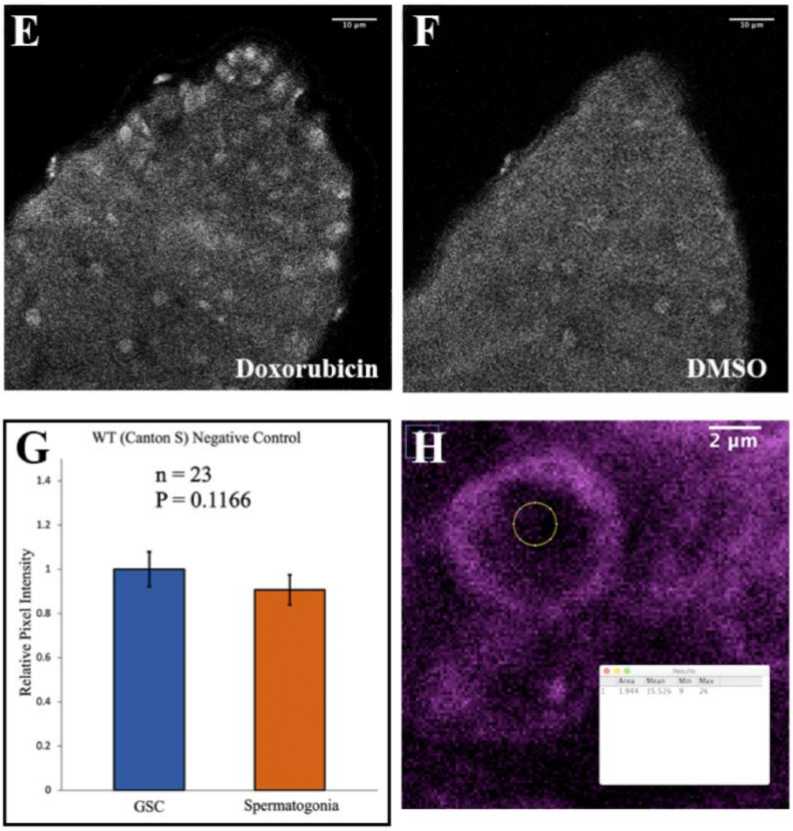


Figure 3.1: Quantifying cytotoxic drug efflux in GSCs and differentiating spermatogonia. (A) Bar graph representing the mean pixel intensity of the doxorubicin stain for both GSCs and spermatogonia. Three of each cell type was measured per testis, and sample size (n) was determined by the number of testes analyzed. Error bars represent standard error of the mean. Statistical significance was determined using a two-tailed Paired t-Test in Excel. (B) Maximum intensity projection showing reduced doxorubicin accumulation in GSCs compared to differentiating spermatogonia. White and red arrowheads point to GSCs and spermatogonia, respectively. (C-D) Pixel intensity of the doxorubicin stain was measured using a circular area 2.0 µm in diameter and by varying the x-, y-, and z-planes. Here the intensity measurements can be seen to increase between z=4 and z=6, representing a 1 µm movement. (E) Representative testis from a wild type (Canton S) fly fed 1mM Doxorubicin for two days. (F) Representative negative control testis from a wild type fly fed DMSO for two days showing background staining in the doxorubicin channel. (G) Bar graph representing the mean pixel intensity of the Vasa channel showing no significant difference between GSCs and spermatogonia. (H) Example of measuring pixel intensity in the Vasa channel.

Selection of ATP-Binding Cassette Transporter Gene Candidates

Following the observation that GSCs exhibit cytotoxic drug efflux, the next aim was to determine whether such efflux was due to ABC transporter activity. In order to identify potential gene candidates from the 56 ABC transporters encoded in the *D. melanogaster* genome, RNA-seq and microarray expression data from four separate studies were consulted [65-68]. Gan *et al.* analyzed the mRNA profiles of both wild type and *bag of marbles* (*bam*) mutant testes (which are enriched with undifferentiated stem-like cells) in order to investigate differential gene expression [65]. This data allows for the identification of genes with potential significance to the testis stem cell populations. Similarly, Terry *et al.* carried out microarray analyses in testes genetically manipulated to be enriched for both cyst stem cell and GSC populations [66]. In another, more recent approach, Shi *et al*. analyzed the gene expression profiles of the male Drosophila germline at several distinct stages of spermatogenesis, including GSCs, gonialblasts, the two-, four-, eight-, and sixteen-cell transit amplifying stages of spermatogonia, and early and late spermatocytes [67]. Finally, Vedelek *et al*. analyzed the transcriptomes of distinct regions of the testis, including the apical, middle, and basal regions, allowing for the identification of genes upregulated in the apical region where the stem cell niche is located [68]. By cross-referencing data from these studies with genes previously implicated in *D. melanogaster* drug resistance (Gellatly *et al.*, Kim *et al.*, and Dayton *et al.*, discussed above) [54-57]**,** 10 ABC transporter candidates were identified (Table 3.1).

Table 3.1: Selection of ABC transporter gene candidates. Genes selected through reported mRNA transcript evidence and drug resistance for enhancer trap, RNA interference, and/or knockout experiments.

FAMILY	CG#	SYNONYM	mRNA EXPRESSION	DRUG RESISTANCE
ABC-A type	1718	ABCA	[65, 67, 68]	
ABC-B type	3879	Mdr49	[65, 67]	[56]
ABC-C type	6214	MRP	[68]	[54, 55, 57]
ABC-C type	5772	Sur	[65, 67]	
ABC-D type	2316	Abcd1	[65, 68]	
ABC-G type	3164	N/A	[65, 67]	
ABC-G type	4822	N/A	[65, 66, 67]	
ABC-G type	32091	N/A	[65]	[57]
ABC-G type	3327	E23	[65, 67]	[55]
ABC-H type	11147	N/A	[65, 67]	

ATP-Binding Cassette Transporter Expression in Cyst and Germline Stem Cells

While a primary objective of this study was to determine whether or not ABC transporter activity was behind GSC cytotoxic drug efflux, an even more fundamental question was whether or not ABC transporters are expressed in either of the *D. melanogaster* testis stem cell populations. To the best of my knowledge, no one has investigated ABC transporter expression patterns in either cyst stem cells or GSCs. To do this, enhancer trap lines were acquired for 9 of the 10 ABC transporter gene candidates identified (see Table 2.1). Each of these lines have Gal4 insertions near the gene of interest and may have Gal4 expression under the control of ABC transporter regulator sequences. Thus, by crossing each of these enhancer trap lines to a UAS-GFP reporter line, it is possible to recapitulate endogenous ABC transporter expression. First, the integrity of the UAS-GFP reporter line was tested by crossing it to two separate Gal4 driver lines: Nanos-Gal4, which drives Gal4 expression in the germline, and c587-Gal4, which drives Gal4 expression in the cyst lineage (Figure 3.2). As expected, this resulted in GFP staining patterns consistent with cells positive for Vasa, a germline-specific marker (Figure 3.2 A), and cells positive for Tj, a marker of early cyst cells (Figure 3.2 B). Next, the UAS-GFP reporter line

was crossed to each of the ABC transporter enhancer trap lines, and the GFP expression patterns of the F1 testes were analyzed (Figure 3.3 A-I). The results of these enhancer trap experiments are summarized in Table 3.2. In general, GFP expression fell into four categories: cyst stem cells and somatic cyst cells only (ABCD); somatic cyst cells only (CG11147); cyst stem cells, somatic cyst cells, and spermatogonia, but not within GSCs (CG3164); and all four cell types, including cyst stem cells, somatic cyst cells, GSCs, and spermatogonia (CG4822, CG32091, E23, Mdr49, MRP, SUR).

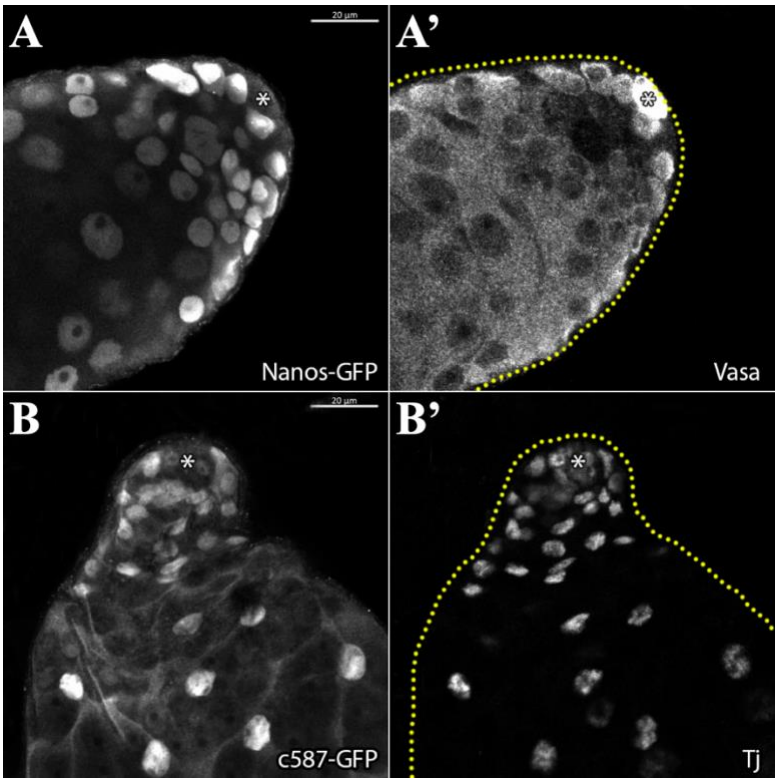
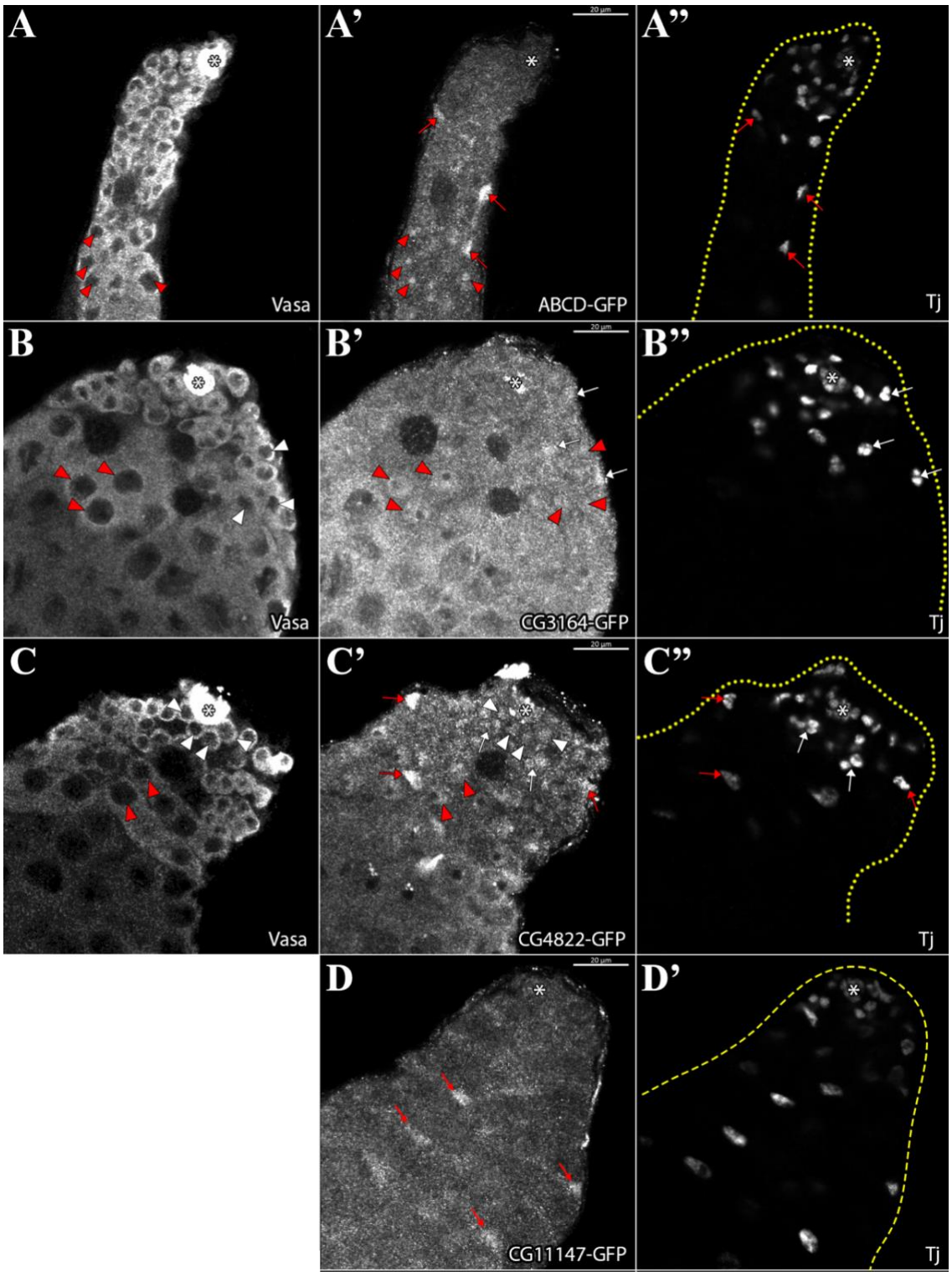
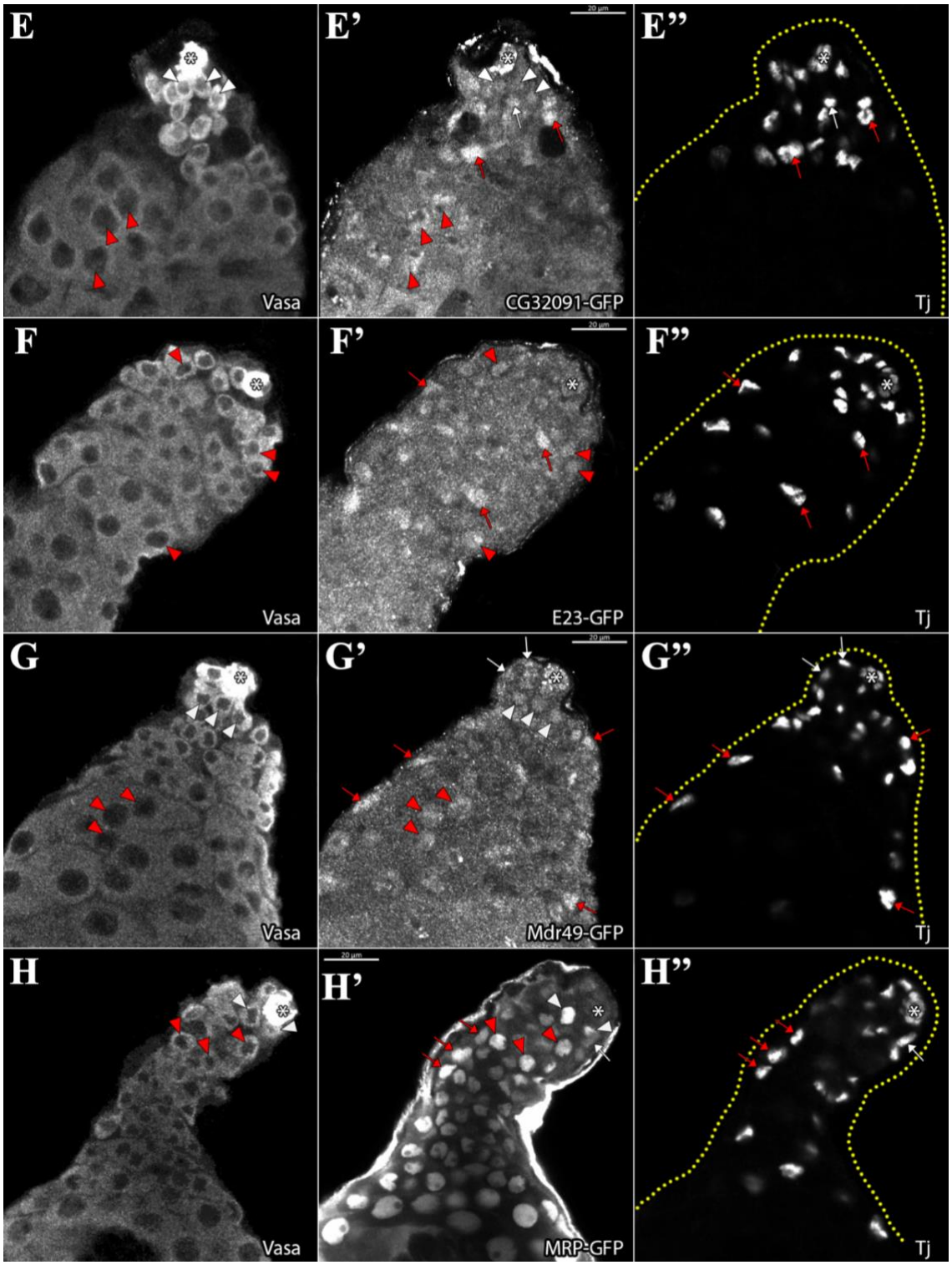


Figure 3.2: Positive controls for enhancer trap experiments. (A) Representative testis from Nanos-Gal4 x UAS-GFP cross showing GFP expression in a pattern consistent with the Vasa germline stain. (B) Representative testis from c587-Gal4 x UAS-GFP cross showing GFP expression in a pattern consistent with the Tj cyst lineage stain. All stains (Vasa, GFP, and Tj) shown in grey. Scale bars are 20 μm. Stem cell hubs marked by asterisks (*).


A
Vasa
A'
ABCD-GFP
A''
Tj
B
Vasa
B'
CG3164-GFP
B''
Tj
C
Vasa
C'
CG4822-GFP
C''
Tj
D
CG11147-GFP
D'
Tj


E
Vasa
E'
CG32091-GFP
E''
Tj
F
Vasa
F'
E23-GFP
F''
Tj
G
Vasa
G'
Mdr49-GFP
G''
Tj
H
Vasa
H'
MRP-GFP
H''
Tj

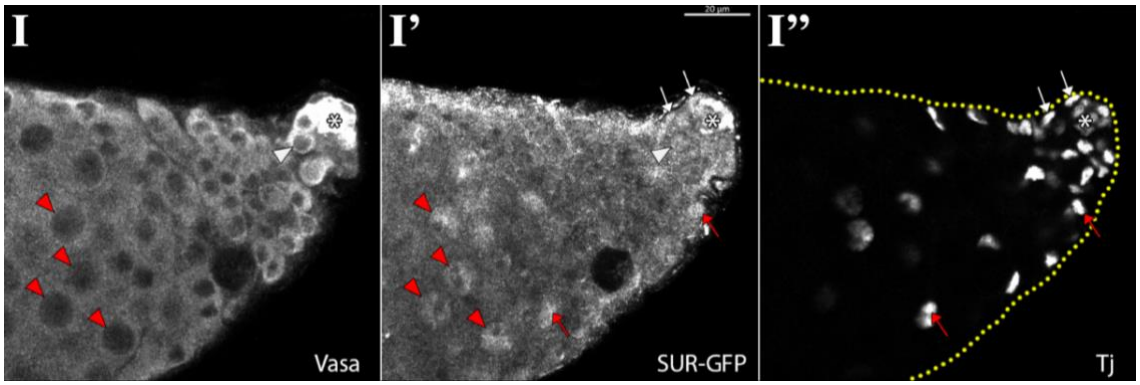


Figure 3.3: ABC transporter expression patterns in the *Drosophila melanogaster* testis niche. (A-I) Representative testes from each Gal4-ABC Transporter x UAS-GFP crosses. White and red arrowheads point to GSCs and spermatogonia or spermatocytes, respectively, while white and red arrows point to cyst stem cells and cyst cells, respectively. GSCs were identified by their adjacency to the hub, and cyst stem cells were identified as such if within two GSCs from the hub. All stains (Vasa, GFP, and Tj) shown in grey. Scale bars are 20 μm. Stem cell hubs marked by asterisks (*).

Table 3.2: Summary of gene expression patterns in the *Drosophila melanogaster* testis niche from enhancer trap experiments.

GENE	GSC	CySC	Germ	CYST
ABCD		X		X
CG3164		X	X	X
CG4822	X	X	X	X
CG11147				X
CG32091	X	X	X	X
E23	X	X	X	X
Mdr49	X	X	X	X
MRP	X	X	X	X
SUR	X	X	X	X

Note. *GSC and CySC stand for Germline Stem Cell and Cyst Stem Cell, respectively. Germ refers to both Spermatogonia and Spermatocytes. Cyst refers to Late-stage Cyst Cells.

These results seem to support the hypothesis that ABC transporters are expressed in *D. melanogaster* testis stem cells and their progenitors. However, a high degree of background staining may call into question the validity of these results. While negative controls involving only secondary antibodies did not reveal concerning levels of background staining in the stem cell niche (Figure 3.4 A-B), the results do seem to be confounded by the use of an anti-GFP

primary antibody (Figure 3.4 C-D). Anticipating that the native GFP expression driven by the Gal4-UAS system in these enhancer trap experiments might be difficult to detect, an additional anti-GFP antibody was used and tagged with a fluorescent secondary antibody, with the intention of enhancing the native GFP expression. This indicated that a portion of the experimental GFP staining appears to be due not to GFP driven by the Gal4-UAS system, but rather to the non-specific background staining of the anti-GFP antibody, especially in regions further away from the stem cell niche (i.e., the region of the spermatocytes and transit-amplifying spermatogonia). In order to differentiate between true experimental GFP staining and this unwanted background, repeat experiments should be performed without the anti-GFP antibody. However, there are aspects of the experimental GFP staining that cannot be explained by background alone. These include staining in the area of the stem cell niche, staining only in the cyst lineage, such as in the case of CG11147, and the strong, clean staining observed in the case of MRP, which looks the most like the Nanos-GFP and c587-GFP positive controls. Additionally, these results could be further validated using other methods such as *in situ* hybridization or RT-PCR.

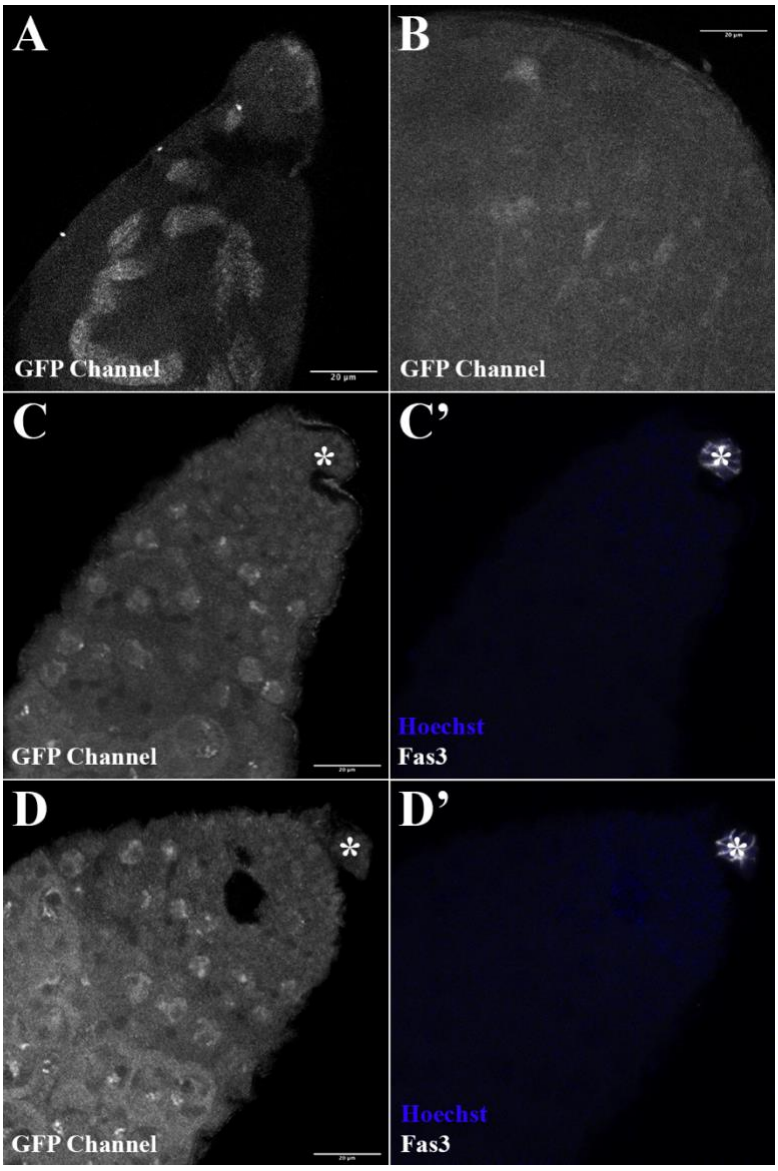


Figure 3.4: Negative controls for enhancer trap experiments. All testis samples from WT Canton S flies. (A-B) Representative testes treated with only secondary antibodies. (C-D) Representative testes treated with secondary antibodies, as well as Hoechst, anti-GFP, and anti-Fas3. Scale bars are 20 μm. Stem cell hubs (if identifiable) marked by asterisks (*).

Ribonucleic Acid Interference in Germline Stem Cells

In order to determine whether ABC transporter activity was behind GSC cytotoxic drug efflux, RNA interference (RNAi) lines were acquired for 8 of the 10 ABC transporter gene candidates identified, and a true mutant knockout (KO) line was obtained for ABCA (see Table

2.2). Each of these RNAi lines were crossed to a Nanos-Gal4 driver line, resulting in the expression of a double-stranded RNA hairpin loop and ultimately the degradation of the ABC transporter mRNA transcript within Nanos-expressing cells (i.e., GSCs and their progenitors). Surprisingly, germline RNAi knockdown (KD) of two of these ABC transporters resulted in extremely abnormal phenotypes in the testis stem cell niche (discussed later). To investigate whether the other ABC transporters are required in GSC cytotoxic drug efflux, F1 progeny from each of the RNAi crosses were fed doxorubicin before their testes were dissected, imaged, and analyzed as previously described. If an ABC transporter is required in GSC drug efflux, then loss of that transporter should result in an increased accumulation of doxorubicin in the GSCs, and potentially reduce the difference in doxorubicin accumulation between the GSCs and spermatogonia. The results of these KD or KO experiments implicated six ABC transporters (ABCA, CG4822, CG11147, Mdr49, MRP, and E23) in GSC drug efflux. The loss of each of these proteins resulted in no significant difference in the mean doxorubicin pixel intensity between GSCs and spermatogonia. The only genotype that was found to maintain a lower doxorubicin accumulation in the GSCs relative to spermatogonia was the SUR KD, suggesting that the SUR protein does not function in GSC drug efflux. These results suggest a high level both of redundancy in export function and overlap in efflux substrate specificity. Experiments involving other cytotoxic drugs, double or triple RNAi knockdowns, dominant negative mutants (which might be several members of the fly ABC-B subfamily), and treatment with ABC inhibitors such as verapamil could help to further illuminate the role these proteins are playing in GSC cytotoxic efflux.

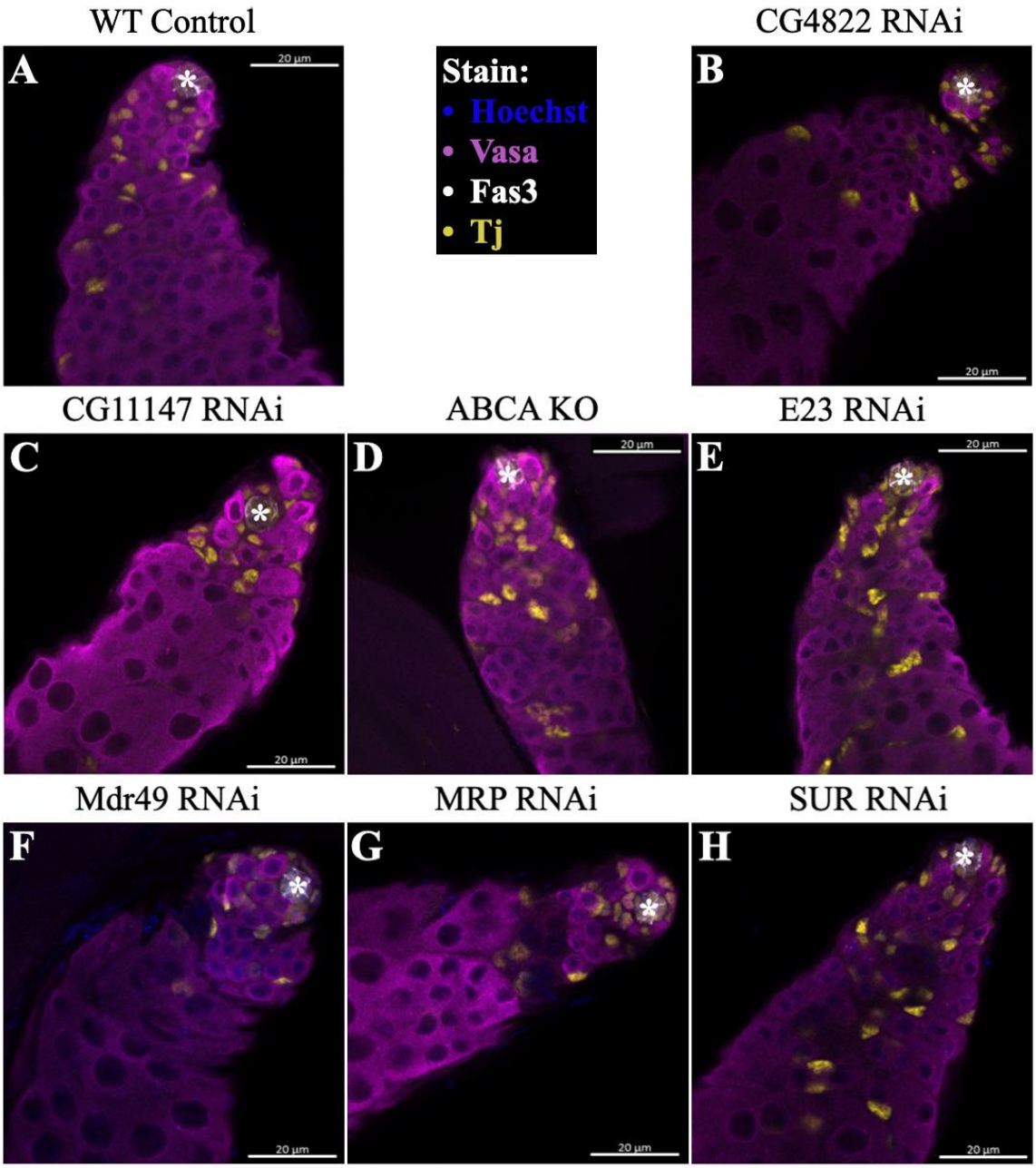


Figure 3.5: Germline RNAi KD or KO of select ABC transporters. (A) Representative testis from wildtype control fly (Nanos-Gal4). (B-H) Representative testes from seven germline RNAi knockdown crosses. Scale bars are 20 µm. Stem cell hubs marked by asterisks (*).

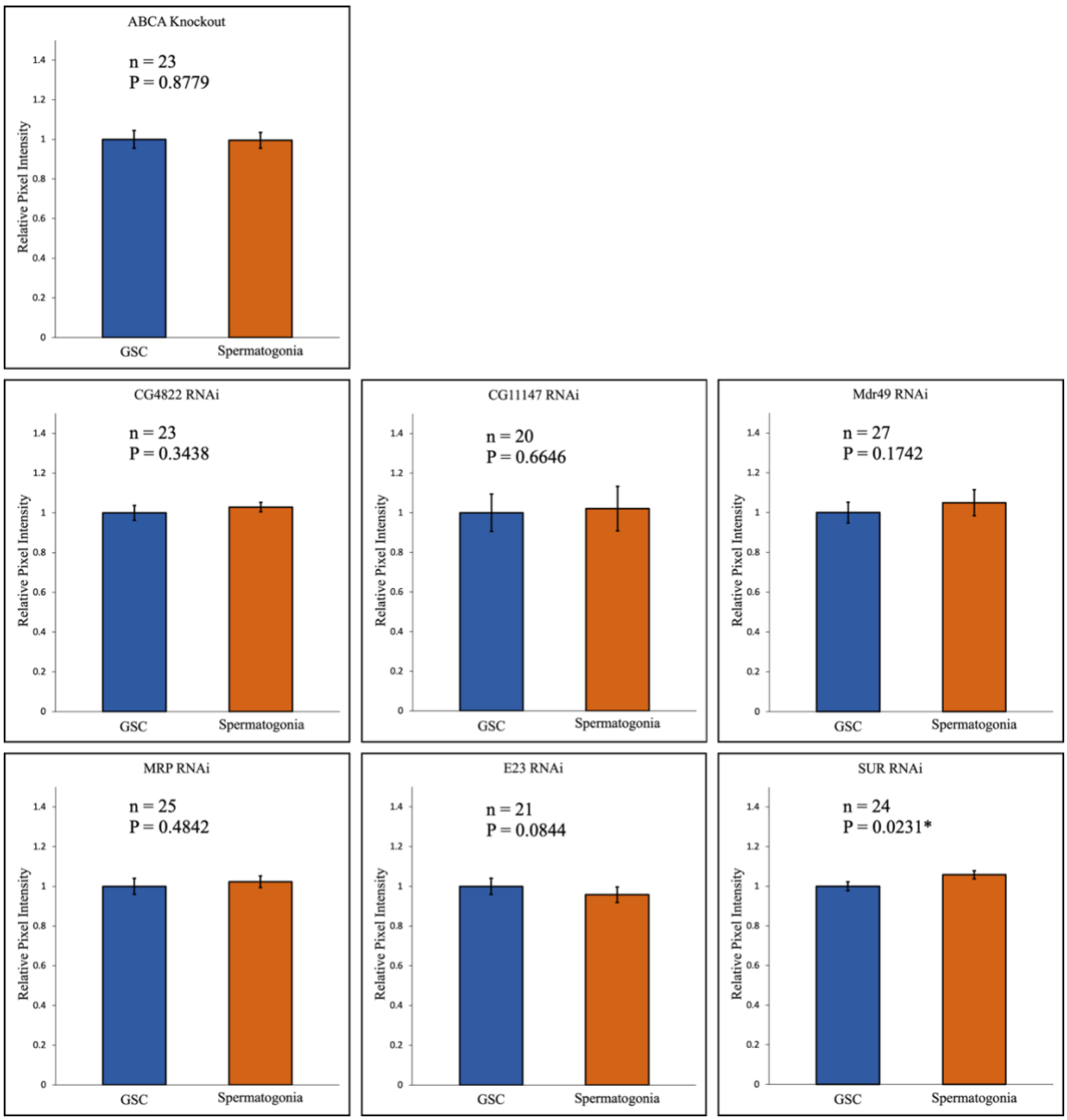
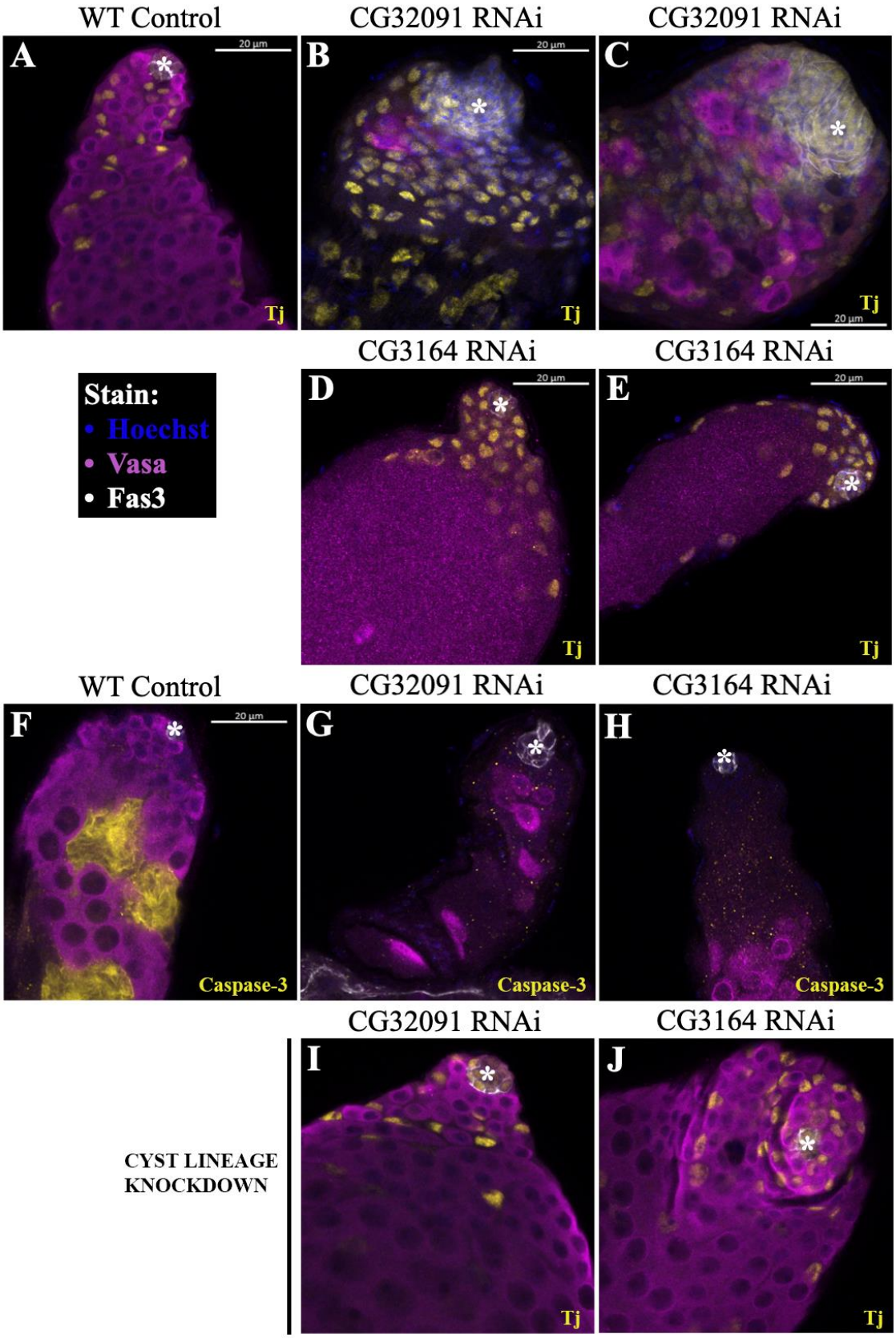


Figure 3.6: Effect of germline RNAi KD or KO on cytotoxic drug efflux. Sample size (n) was determined by the number of testes analyzed per genotype. Error bars represent standard error of the mean. Statistical significance was determined using a two-tailed Paired t-Test in Excel. The SUR knockdown (KD) was the only genotype found to have a significant difference between mean germline stem cell (GSC) pixel intensity and mean spermatogonia pixel intensity. However, pairwise analysis of the SUR KD resulted in a $P < 0.05$ for only 3 out of 9 independent t-Tests.

Surprisingly, RNAi KD of two ABC transporters, CG32091 and CG3164, resulted in total or near total loss of the germline cell lineage (Figure 3.7 A-E). Loss of CG32091 also resulted in dramatic expansion of both Tj-positive cyst cells and Fas3-positive hub cells (Figure 3.7 B-C). It is not clear why loss of these transporters should warrant such extreme phenotypes, and naturally such observations inspired a number of new lines of inquiry. First, it was not clear whether germline loss was due to increased levels of cell death or increased differentiation and movement away from the stem cell niche. To test the hypothesis that loss of CG32091 and CG3164 was inducing apoptosis in the germline, testes from both KD genotypes were treated with an anti-Cleaved Caspase-3 antibody. While three instances of cell death were observed in the Nanos-Gal4 wildtype control testes (n=12), there were no detectable signs of Cleaved Caspase-3 dependent apoptosis in either KD testes (Figure 3.7 F-H). This suggests that loss of these transporters does not induce Caspase-3 dependent apoptosis. Next, we wondered if this phenotype was specific to the germline or whether loss of these transporters might be generally cell lethal. To test this, both RNAi lines were crossed to the c587-Gal4 driver line to knockdown CG32091 and CG3164 in the cyst lineage. Interestingly, loss of these transporters in the cyst lineage resulted in no abnormal phenotype, suggesting a specificity to the germline cell lineage (Figure 3.7 I-J). Finally, we wondered if loss of these transporters might also impact the female germline. To investigate this, ovaries from CG32091 and CG3164 KD flies were dissected and stained using immunofluorescence in the same manner as the testes and imaged with confocal microscopy. As with the testes, ovaries from Nanos-Gal4 flies served as a control (Figure 3.7 K). Unlike in the testes, KD of these transporters did not result in any apparent loss or disruption of the female germline (Figure 3.7 L-M). To confirm that this phenotype is in fact due to loss of

transporters, future experiments might repeat these loss-of-function experiments using, for example, CRISPR-Cas9 knockouts instead of RNAi.


WT Control
CG32091 RNAi
CG32091 RNAi
A
B
C
20 µm
Tj
CG3164 RNAi
CG3164 RNAi
Stain:
• Hoechst
• Vasa
• Fas3
D
E
WT Control
CG32091 RNAi
CG3164 RNAi
F
G
H
Caspase-3
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CG3164 RNAi
CYST LINEAGE KNOCKDOWN
I
J

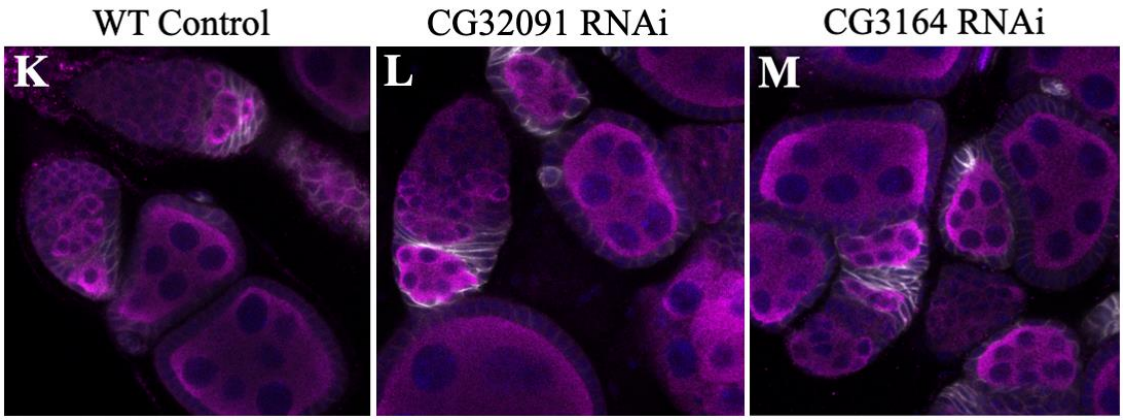

Figure 3.7: Germline RNAi KD of CG32091 and CG3164 results in male-specific germline loss. (A) Representative testis from wildtype control fly (Nanos-Gal4). (B-C) Germline RNAi of CG32091 results in loss or disruption of germline and expansion of both the hub and cyst lineages. (D-E) Germline RNAi of CG3164 results in loss of germline. (F) Wildtype (Nanos-Gal4) testis showing Caspase-3-dependent cell death. (G-H) Germline RNAi of CG32091 and CG3164 does not result in detectable Caspase-3-dependent cell death. (I-J) Cyst lineage RNAi knockdown of CG32091 and CG3164 does not result in any abnormal phenotype. (K) Representative wild type ovary showing germarium and ovarioles. (L-M) Germline RNAi of CG32091 and CG3164 does not result in loss or disruption of the female germline. Scale bars are 20 μm. Stem cell hubs marked by asterisks (*).

CHAPTER IV

DISCUSSION

The Utility of *Drosophila melanogaster* in Cancer Research

D. melanogaster has a rich history in biological research. Much of our modern understanding of genetics is heavily indebted to the work of fly geneticists of the 20st century through to the present. Not only has *Drosophila* research unveiled many of the fundamentals of genetics (including the chromosomal basis of inheritance), but it has also contributed heavily to biomedical research, including the discovery of many developmental signaling pathways [38, 69]. For example, both the Hedgehog pathway and the Notch pathways were first discovered in *D. melanogaster* and were named after their fly mutant phenotypes. Today it is understood that misregulations of both pathways are contributors to many diseases including cancer [70], and it is thought that abnormal activation of Sonic Hedgehog signaling (which plays a role in the regulation of adult stem cell populations) is responsible for the transformation and self-renewal of cancer stem cells [71]. Additionally, the Hippo signaling pathway was first discovered in *D. melanogaster* as a regulator of organ size, and later was recognized as an important tumor suppressor pathway in humans [72]. In general, the fact that many of the main human signaling pathways regulating cell growth are conserved in *D. melanogaster*, including both oncogenic and tumor suppressor pathways, make the fruit fly a powerful *in vivo* model for the study of cancer [42].

While the cancer stem cell hypothesis was first derived from studies involving mammals, the *D. melanogaster* stem cell model systems have the potential to shed new light on many aspects of cancer stem cells, not the least of which is the question of how cancer stem cells arise in the first place. Many models for the origin of cancer stem cells have been proposed. Some posit that cancer stem cells arise directly from normal stem cells, possibly by gaining niche-independence, while others suggest they arise from transit-amplifying progenitor cells [73]. While probably there will not be one single answer to this question, and the origin of cancer stem cells will likely vary between different types of cancer, researchers are beginning to answer such questions with the help of simple model organisms. Using *D. melanogaster*, Song *et al.* was able to show that cancer stem cells of the brain can arise from transit-amplifying progenitors dedifferentiating back into a stem-like state [74]. These *in vivo* stem cell model systems also allow researchers a valuable tool in the effort to find ways to eradicate cancer stem cells without harming the tissue-specific stem cell populations crucial to an organism's health and survival. This is another point well illustrated in the work of Song *et al*., who showed that cancer stem cells of the *D. melanogaster* brain and ovary, because of their dependence on eukaryotic translation initiation factor 4E (eIF4E), could be eliminated without harming the tissue-specific stem cell populations residing in those locations [74]. Additionally, many tumor models have been developed in *D. melanogaster,* including in areas such as the eye [75], the brain [76], and a variety of epithelial tumors which have been used to investigate molecular and cellular mechanisms governing tumorigenesis [77]. Moreover, several stem cell tumor models have also been developed in *D. melanogaster,* including an intestinal stem cell model developed by Markstein *et al*., used to screen for novel inhibitors of stem cell-derived tumors [78]., as well as several germline tumor models [65, 79]. The work of Gan *et al*. was briefly discussed earlier in

the context of selecting ABC transporter gene candidates, but one point not mentioned was that their *bam* mutant testes, which are enriched with undifferentiated stem-like cells, is a stem cell tumor model of the testis. Interestingly, when comparing the transcriptional profiles of *bam* and wild type testes, they reported upregulation of over half the ABC transporters encoded in the *D. melanogaster* genome (32/56). Given such a finding, future studies might investigate the role of ABC transporters in these *D. melanogaster* tumor models, and especially those derived from stem cells and/or progenitors.

Evidence of ATP-Binding Cassette Transporter Expression and Function in the *Drosophila melanogaster* Testis Stem Cell Niche

Regarding the current study, the results have potential implications for both cancer and developmental biology. Rather than being unique to cancer stem cells, there is mounting evidence that ABC transporter expression is a general stem cell trait, and the current study lends evidence in favor of that view. Given the significance of ABC transporter activity in both cancer multidrug resistance and antibiotic resistance (among other biomedical concerns), there is much interest in targeting ABC transporters therapeutically, as well as growing evidence that such targeting might prove effective [3, 4, 22-25]. As previously mentioned, *D. melanogaster* has already been established as a model organism for large-scale drug screens [78, 80], but the research proposed here could set the foundation for similar screens using the *D. melanogaster* testis niche to identify, for example, small molecule inhibitors for future combinatorial therapies to make conventional chemotherapies more effective. The establishment of the *D. melanogaster* testis niche as a tool for the study of ABC transporters would allow researchers to study these proteins *in vivo*, an ability that is especially important for stem cells which are heavily influenced by signaling from their local microenvironments. Since the *D. melanogaster* testis niche is one of

the best understood stem cell niches, it would also be an ideal setting in which to study ABC transporter gene expression, many aspects of which are not yet well understood, such as how gene expression is regulated between stem cells and their progeny and whether their expression is induced by chemotherapeutics (see Appendix B).

The current work has presented evidence not only of ABC transporter expression in the *D. melanogaster* testis stem cell niche, including stem cells and progenitors of both stem cell lineages, (Figure 3.3; Table 3.2) but also evidence of six ABC transporters functioning in GSC cytotoxic drug efflux, specifically when exposed to doxorubicin (Figure 3.1; Figure 3.6). While the natural fluorescence of doxorubicin makes useful for quantifying efflux, this drug did not cause any detectable disruption or stress to the testis architecture, even after longer periods of exposure at a ten-fold higher concentration (data not shown). Treatment with other drugs (perhaps Actinomycin D or Bortezomib) causing detectable disruption of the testis architecture might better illuminate the resistance properties of the germline or cyst stem cells. If such resistance were found relative to the surrounding tissue, then the resistance properties of ABC transporters could be further investigated. Expanding to other drugs would also make sense in the context of ABC transporter substrate specificity. While there is considerable overlap in efflux substrates, they are not identical. For instance, doxorubicin is a substrate shared by the three most notorious proteins involved in human multidrug resistance, ABCB1, ABCC1, and ABCG2, but the ABC inhibitor verapamil is a substrate only of ABCB1 [12]. Experiments with a diverse range of drugs could lead to insights regarding the substrate specificities of the *D. melanogaster* ABC transporter family.

This investigation has focused on just 10 of the ABC transporters encoded in the *D. melanogaster* genome, but there is ample evidence to broaden the scope of this research and

investigate the other 46 as well. And while most of the functional RNAi experiments reported here focused on the germline, there is no reason not to investigate the role ABC transporters might play in the cyst lineage as well. After all, results from the GFP enhancer trap experiments suggest that some ABC transporters, such as ABCD and CG11147, are exclusively expressed in the cyst lineage and not in the germline (Figure 3.3; Table 3.2). Although there does appear to be a discrepancy here, because RNAi KD of CG11147 also resulted in decreased GSC cytotoxic drug efflux (Figure 3.6), a result which should not be possible if CG11147 were only expressed in the cyst lineage. Further experiments will be needed to address this apparent discrepancy. In contrast, other results seem to complement each other nicely. RNAi KD of MRP also decreased GSC cytotoxic drug efflux (Figure 3.6), and this finding is consistent with the results of the enhancer trap experiments. Of the 10 ABC transporters investigated, MRP had the strongest, cleanest staining pattern, with compelling expression evidence in both the germline and cyst lineages (Figure 3.3 H). Given such strong expression evidence, it is possible that MRP is also playing some functional role in the cyst lineage as well.

An Unexpected Role for CG32091 and CG3164 in the Male Germline

A broader investigation of ABC transporters could also lead to interesting findings not only in the context of stem cell drug resistance and cancer, but also in development. Clearly, two of the ABC transporters investigated here, CG32091 and CG3164, are playing some previously undescribed role in the *D. melanogaster* testis stem cell niche. Why germline RNAi KD of these two transporters leads to total (or near total) loss of the germline is an open question. Since germline KD of CG32091 and CG3164 did not result in any detectable cleaved Caspase-3 (Figure 3.7 F-H), it would seem that loss of these transporters does not induce apoptosis. Perhaps a Caspase-3 independent cell death program is being activated. If not cell death, then perhaps the

GSC homeostasis is being biased toward differentiation instead of self-renewal, ultimately leading to loss of the lineage, or perhaps the self-renewal capabilities of the GSCs are being compromised altogether. It also seems plausible that loss of these genes is disrupting germline enclosure by the cyst cells. Further experiments will be needed to discriminate between these possibilities. In whatever case, it is clear that both these KD phenotypes are specific to the male germline, as neither cyst lineage KD nor female germline KD resulted in any abnormal phenotypes (Figure 3.7 I-M). There is a clear difference, however, between the CG32091 KD phenotype and the CG3164 KD phenotype. While germline RNAi KD of either gene results in germline loss, only RNAi of CG32091 results in dramatic expansion of both the hub and cyst lineages (Figure 3.7 B-E). This difference in phenotype might suggest a difference in function as well, indicating that in each case the germline is being lost for different reasons. Here again, further experimentation is needed.

Other instances of male germline loss have been reported in the literature. For example, Shields *et al.* illustrated that loss of the *chickadee* gene (which encodes profilin, a protein necessary for actin-polymerization) results in loss of GSCs, most likely due to defects at the GSC-hub interface [81]. Similarly, Chen *et al.* illustrated that *gilgamesh*, a homologue of casein kinase 1-γ, was necessary for GSC maintenance, and that loss of *gilgamesh* dramatically reduced the number of GSCs in the testis niche [82]. Interestingly, studies in yeast have also implicated *gilgamesh* in regulation of membrane transport, but precise details of this have yet to be worked out [83]. Other studies have illustrated that GSCs are susceptible to high levels of reactive oxygen species (ROS). ROS disrupt GSC homeostasis by favoring differentiation over self-renewal, ultimately decreasing GSC number, a phenomenon Tan *et al.* propose is due to changes in EGFR signaling [84]. What connection, if any, these studies may have to the extreme

phenotypes of CG32091 and CG3164 KD described here remains unclear. However, understanding both the intrinsic and extrinsic vulnerabilities of GSCs may amount to valuable clues in the effort to understand these new phenotypes.

In searching for possibilities, it may be fruitful to consider the known roles played by ABC transporters in other tissues, and perhaps especially those not directly relevant to cancer multidrug resistance. It may be that ABC transporters of the *D. melanogaster* testis niche function in hormone uptake. In 2018, Okamoto *et al.* showed that a membrane transporter, which they named Ecdysone Importer (Ecl), is responsible for cellular uptake of the steroid hormone ecdysone [85]. Ecdysone binds to a nuclear receptor to induce transcription of genes involved in insect molting and metamorphosis [86]. While Ecl is a member of the solute carrier organic ion (SLCO) superfamily of proteins, previous research demonstrated that the packaging of ecdysone into secretory vesicles is facilitated by the ABC transporter, Atet [87]. Enticingly, both Atet and the two ABC transporters identified in the current work, CG32091 and CG3164, are members of the ABC-G subfamily. This homology might suggest functional similarity as well. Another member of the ABC-G subfamily, E23, also functions in hormone regulation. Hock *et al.* illustrated that E23 can behave as a negative regulator of ecdysone, suppressing ecdysone-mediated gene transcription [88]. Taken together, these studies reveal not only that steroid hormones are clearly substrates of ABC transporters, but also that ABC transporters can play interesting and varied roles in regulating steroid hormones. Finally, Syed *et al*. recently found that ecdysone has a role in regulating gene expression in *D. melanogaster* neural stem cells [89]. Given the roles other members of the ABC-G subfamily have been found to play in hormone transport, this recent finding that ecdysone regulates gene expression in neural stem cells, and the detection of localized ecdysone in the testis [90], it would not be all that surprising to discover

similar roles related to hormone transport or regulation for CG32091 and CG3164 in the *D. melanogaster* testis stem cells.

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APPENDIX A

AN *EX VIVO* ASSAY TO INVESTIGATE DYE EXCLUSION

"Side population" cells were first discovered for their ability to exclude membrane-permeable dyes through increased efflux [31] , and have since been discovered in a variety of tissues including the skin, heart, and brain [32]. Cancer stem cells use this elevated efflux ability to attain chemoresistance, and the same has also been found to be true for many normal stem cell populations in vertebrates. In their BioRxiv preprint, Dayton *et al*. [57] have gone on to show that the phenomenon of dye exclusion also extends to invertebrate stem cells as well, specifically in the intestinal stem cells and enteroblast progenitors of the *D. melanogaster* gut. All of this taken together led to the question of whether such dye exclusion properties would be present in the stem cells of the *D. melanogaster* testis as well. To test this idea, *D. melanogaster* testes were exposed to a variety of membrane- permeable SYTO dyes. SYTO dyes 17 and 59-64 (Table A.1) are from the SYTO Red Fluorescent Nucleic Acid Sampler Kit (Thermo Fisher Scientific).

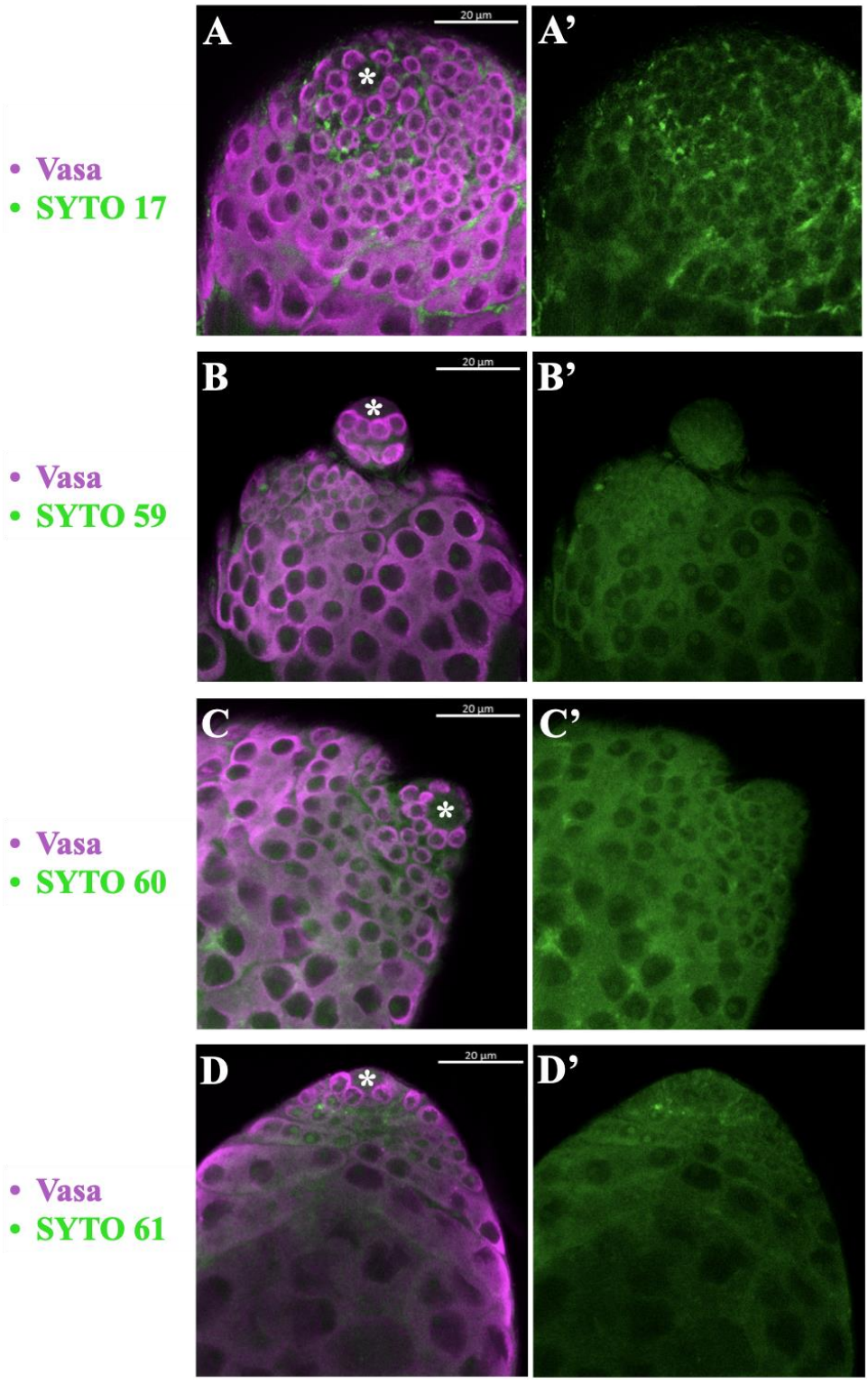
Table A.1: Spectral characteristics of SYTO 17 and SYTO 59–SYTO 64 (Thermo Fisher Scientific).

DYE	Excitation (nm)	Emission (nm)
SYTO 17	621	634
SYTO 59	622	645
SYTO 60	652	678
SYTO 61	628	645
SYTO 62	652	676
SYTO 63	657	673
SYTO 64	599	619

Because these SYTO dyes were not detectable following feeding, an *ex vivo* approach was used. Wild type (Canton S) testes were dissected in Ringer's and incubated for 2 hours in a 5 μM SYTO dye solution in Schneider's *Drosophila* Medium. 7 SYTO dyes were used in total

(SYTO 17, 59-64), and testes incubated only in Schneider's served as a negative control. Following incubation, testes were fixed and stained using immunofluorescence as previously described. In theory, ABC transporters would still be functional prior to sample fixation and capable of efflux.

The staining patterns of each of the SYTO dyes in the *D. melanogaster* testis niche can be observed in Figure A.1. Each of the SYTO dyes appear to be excluded, to somewhat varying degrees, from the nuclei of GSCs and their progenitors. In some cases, most dramatically illustrated by SYTO 64, it appears that encystment of the transit-amplifying spermatogonia is playing a role in excluding dye from the germline cells. Within germline nuclei, nucleolus staining was also present to varying extents, with SYTO 17 and 64 seeming to have the least, perhaps suggesting that these two dyes are the most susceptible to efflux. This experiment serves as a proof of concept, illustrating the possibility of studying dye exclusion using the *D. melanogaster* testis stem cell niche. This *ex vivo* approach could naturally be extended to future experiments with other dyes, drugs, and flies of varying genotypes. Future experiments with these SYTO dyes might also vary dye concentration and incubation time. A natural follow up to this experiment would be to investigate the effect of ABC transporter KD or KO on the phenomenon dye exclusion in the testis niche.


A
20 µm
A'
• Vasa
• SYTO 17
B
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B'
• Vasa
• SYTO 59
C
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C'
• Vasa
• SYTO 60
D
20 µm
D'
• Vasa
• SYTO 61

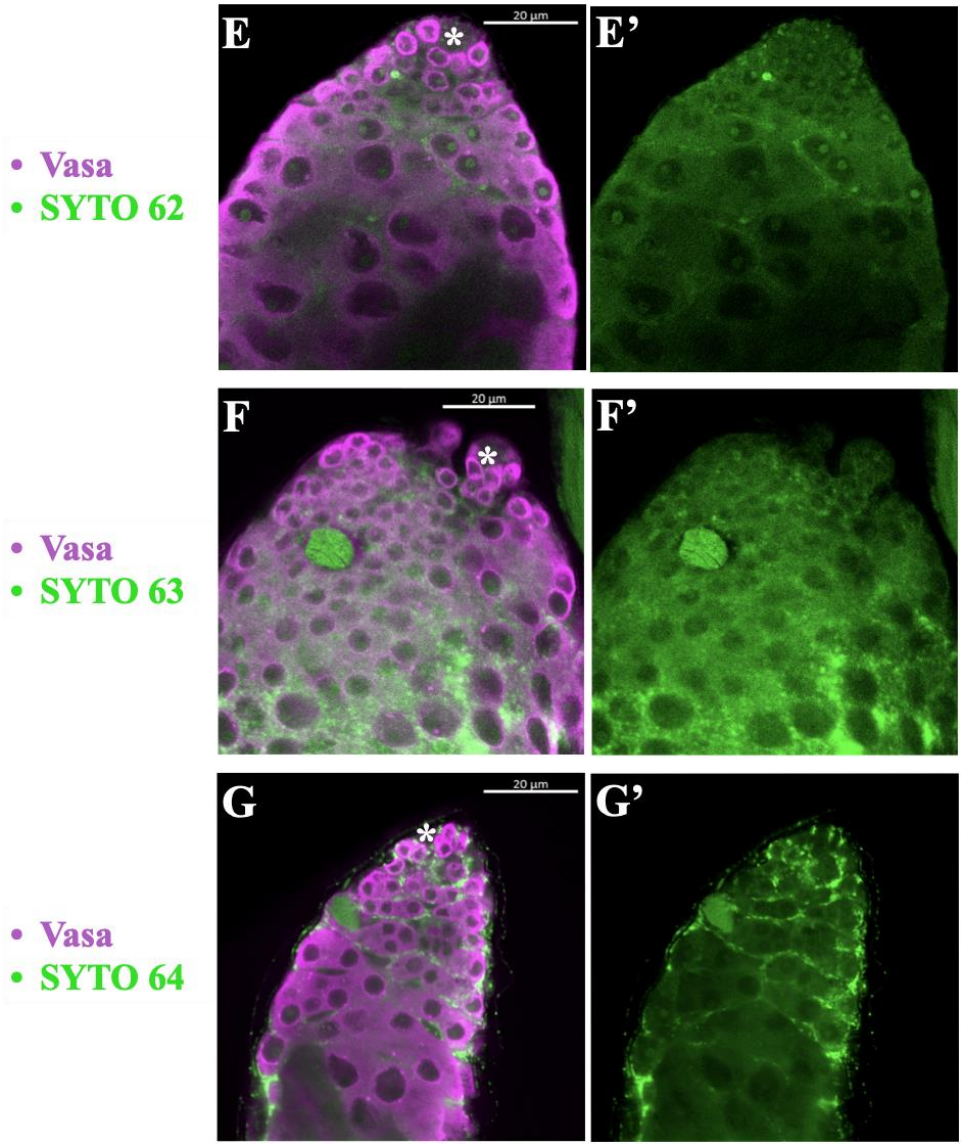


Figure A.1: Staining patterns of SYTO dyes 17 and 59-64 in the *Drosophila melanogaster* testis niche using an *ex vivo* approach. All testis samples came from wild type (Canton S) flies. Scale bars are 20 µm. Stem cell hubs marked by asterisks (*).

APPENDIX B

TRANSCRIPTIONAL UPREGULATION OF ATP-BINDING CASSETTE TRANSPORTERS FOLLOWING CYTOTOXIC DRUG EXPOSURE

Upregulation of ABC transporters upon cytotoxic exposure could be a protective mechanism that evolved in order to ensure successful gamete production. Understanding how these genes are activated and regulated within the stem cell niche could lead to important insights in how they function within the tumor microenvironments. Given that past studies in *D. melanogaster* have reported an upregulation of ABC transporter genes coinciding with cytotoxic drug resistance, we hypothesized that cytotoxic drug exposure will result in a transcriptional upregulation in the *D. melanogaster* testis niche.

To investigate differential RNA expression of four ABC transporters (MRP, E23, Mdr49, and CG32091) in the testis stem cell niche following *in vivo* drug exposure, wild-type flies (Canton S) were fed food containing the naturally- fluorescent chemotherapeutic, doxorubicin (Thermo Fisher Scientific). 100 μL of 1 mM doxorubicin were added to fresh vials of perforated fly food and allowed to soak overnight. Flies were added the following day and allowed to feed for three days. Flies fed on food containing dimethyl sulfoxide (DMSO) served as a negative control. Following feeding, 200 testes (100 experimental, 100 control) were harvested in *Drosophila* Ringer's solution, and testis tips (where the stem cell niche resides) were severed using sharpened forceps and immediately stored in TRIzol™. Total RNA was extracted using TRIzol™ Plus RNA Purification Kit (following Invitrogen user guide protocol for tissue samples). Testis samples were collected in 30-minute intervals before being transferred to 0.5 mL of TRIzol™ Reagent and stored at –20°C. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen 18080-044). For quantitative PCR, a PowerTrack SYBR Green (ThermoFisher) qPCR assay was utilized with a subsequent melt-curve analysis, following the manufacturer's protocols. The melt-curve analysis was used to validate the amplification curves and verify that the targets of interest were the only sequences being amplified. Samples were run

on a BioRad CFX384 Touch Real-Time PCR Detection System using the following parameters: 1 cycle at 95°C for 2 minutes followed by 40 cycles with 95°C for 5 seconds and 60°C for 30 seconds, and a final cycle of 95°C for 15 seconds prior to the melt curve. The melt curve ramped up from 60°C to 92°C in increments of 1.6°C with 1 minute at each temperature. As a positive control, a reference gene, *Zfh-1*, which encodes a zinc finger homeodomain protein known to be expressed in the *D. melanogaster* testis niche, was included [91].

Table B.1: Primers utilized for qPCR.

Name	Forward	Reverse	Product Length
MRP	CTTCTACTGGGCGTTCGTGA	GCTCACGTTCAGCTTGTTCC	88 bp
E23	CAGAGCGGTGATGTCGAGTT	CGCCCAGCAGTTACGAGATT	94 bp
MDR49	TGCATCGGAGTAGGACTTCAG	AGTGTCTGCTTTCGTCTACGG	139 bp
CG32091	GCGGGATGTATGTGGGTTTC	CCAAAGAACAGACCGCACAG	126 bp
ZFH1	TGCGGGGTTAATTCGGGAG	GGTCACACTGGTGTTAAAGGG	120 bp

An initial qPCR assay and melt curve analysis was performed using only the *ZFh1* positive control (Figure B.1), resulting in Cq values around 32 and melt peaks around 78°C for both control and experimental samples. A subsequent qPCR assay and melt curve analysis using all the genes of interest was performed. Results indicated that mRNA transcripts of only one gene of interest (*MRP*) were upregulated following drug exposure (Figure B.2), while the others remained unchanged (*Mdr49*) or appeared to be downregulated (*CG32091*, *E23*). Precise Cq values and melt peak temperatures for each gene are reported in Tables B.2 and B.3, respectively. The matching melt peak temperatures in both the experimental and control indicate that the same target gene was amplified in each sample.

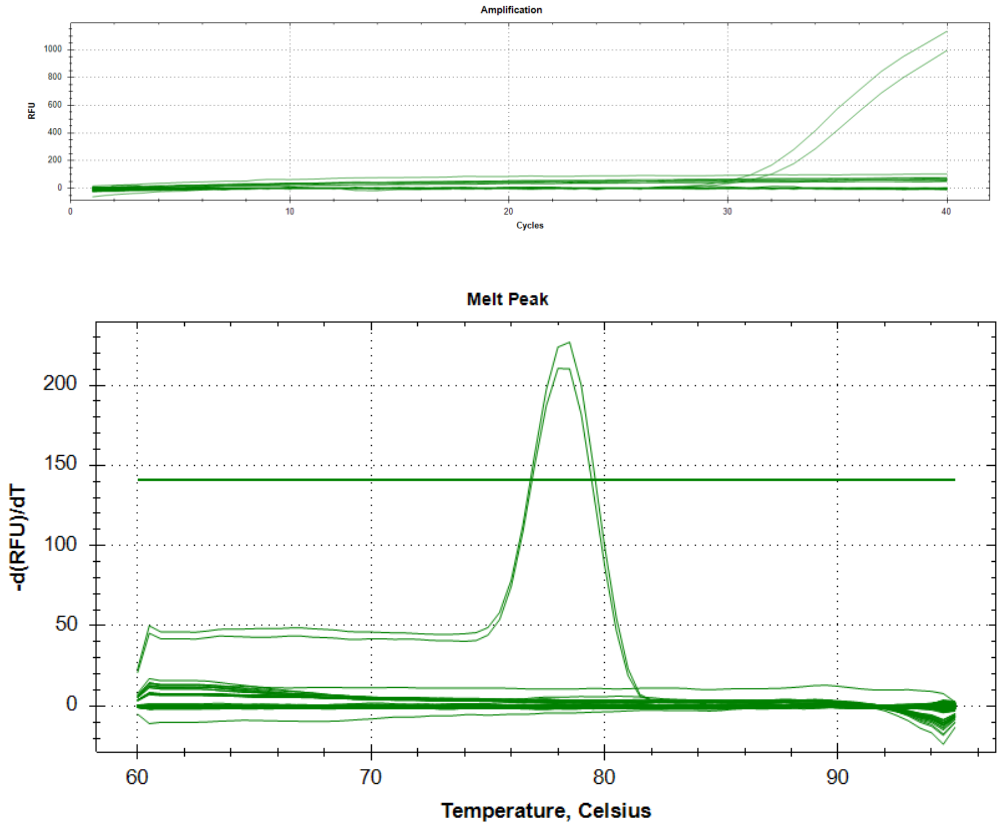
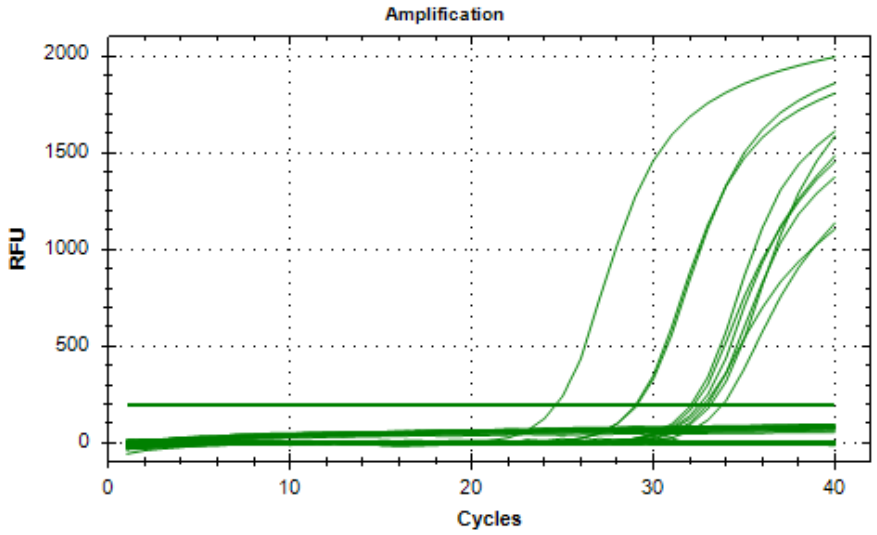


Figure B.1: Amplification cycles and melt curve peaks for the *Zfh1* control. Two melt peaks at 78°C confirm that the same gene target has been amplified in both the experimental and control.



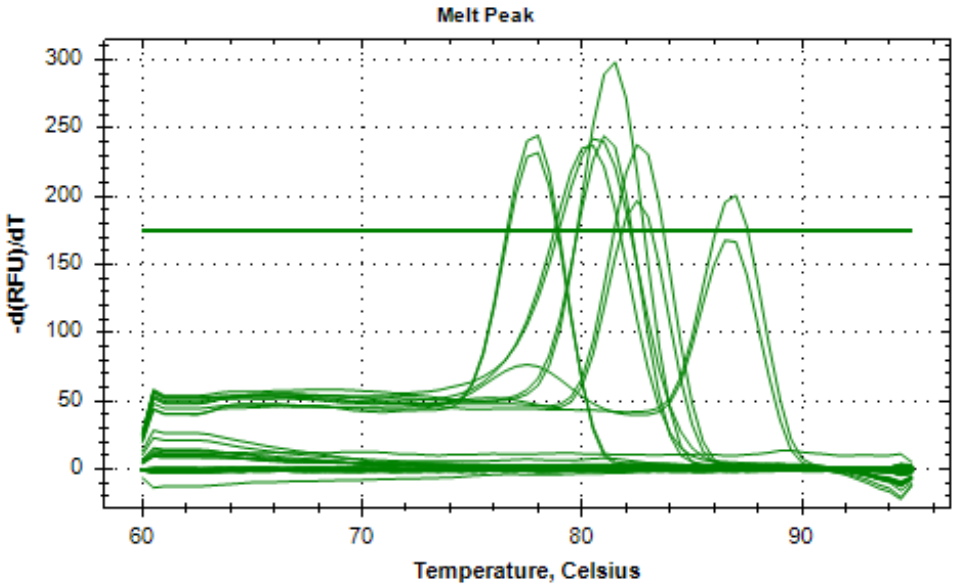


Figure B.2: Amplification cycles and melt curve peaks for all four genes of interest and *Zfh1* control. Two melt peaks at each distinct temperature confirm that the same gene targets have been amplified in both the experimental and control.

Table B.2: Cq Values

	Experimental	**Control**
MRP	**24.57**	**29.00**
E23	**33.11**	**29.06**
MDR49	**32.19**	**32.48**
CG32091	**32.92**	**32.04**
ZFH1	**33.74**	**32.70**

Note. Cq (quantitative cycle) values which correspond inversely to the amount of cDNA (and thus mRNA) present in sample.

Table B.3: Melt Curve Peaks

	Experimental	**Control**
MRP	**80.5**°C	**80.5**°C
E23	**81.0**°C	**81.5**°C
MDR49	**n.a. (approx. 87.0**°C**)**	**87.0**°C
CG32091	**82.5**°C	**82.5**°C
ZFH1	**78.0**°C	**78.0**°C

Note. Melt peaks from melt curve analysis of each qPCR sample. The matching melt peak temperatures in both the experimental and control indicate that the same target gene was amplified in each sample.

Of the genes investigated here, only *MRP* mRNA was upregulated following doxorubicin exposure. This suggests that MRP is the only ABC transporter of those studied whose expression levels in the *D. melanogaster* testis niche is sensitive to doxorubicin exposure. However, it is possible that the other three ABC transporters (E23, MDR49, and CG32091), which all appear to be transcriptionally expressed (Figure B.2), might be sensitive to cytotoxic compounds other than doxorubicin. This is entirely possible, as ABC transporters are known to vary widely in the substrates they transport. The results of this study also do not rule out the possibility that doxorubicin may be a transportable substrate of these three non-upregulated ABC transporters, as each appears to have a baseline level of expression already established. Although an absolute qPCR assay would be required to ascertain this level of expression for certain. Replicate experiments would be required to statistically validate the results of this study. In the future, the assay utilized here could serve to investigate the expression of other genes in the *D. melanogaster* testis niche. It would also be interesting to investigate the mechanism by which transcriptional regulation occurs (including the apparent doxorubicin-induced upregulation reported here). In cancer cells, expression of ABC transporters is regulated by a number of transcription factors, and upregulation can be influenced by components of malignant transformation such as P53, APC, and the MAPK/ERK pathway [92]. Comparatively less is known about the transcriptional regulation of ABC transporters in stem cells, and this could be the subject of interesting future studies.