Studies of cell migration and programmed cell death: characterization and analysis of scattershot, a mutation disrupting germ cell migration and programmed cell death in Drosophila melanogaster

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Studies of cell migration and programmed cell death: characterization and analysis of *scattershot*, a mutation disrupting germ cell migration and programmed cell death in *Drosophila melanogaster*

by

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TABLE OF CONTENTS

CHAPTER 1  GENERAL INTRODUCTION  1

CHAPTER 2  INVESTIGATION OF AMINO ACIDS CRITICAL FOR GPCR-MEDIATED GERM CELL MIGRATION IN DROSOPHILA  23

CHAPTER 3  IDENTIFICATION OF ADDITIONAL COMPONENTS OF DROSOPHILA GERM CELL MIGRATION AND PROGRAMMED CELL DEATH  49

CHAPTER 4  G PROTEIN-COUPLED RECEPTOR ROLES IN CELL MIGRATION AND CELL DEATH DECISIONS  70

CHAPTER 5  GENERAL CONCLUSIONS AND FUTURE DIRECTIONS  82

APPENDIX:  DATA  93

ACKNOWLEDGEMENTS  106
CHAPTER 1. GENERAL INTRODUCTION

Cell migration and programmed cell death (PCD) play crucial roles in development and homeostasis as well as in immune response. Often, cell migration and cell death are carefully coordinated in order for an appropriate number of cells to migrate successfully, while those superfluous or lost cells are efficiently eradicated. When cell migration and PCD are disrupted, disease conditions can result. Cancer is one of the most prevalent of these diseases. In fact, 1 in 3 Americans are predicted to develop some form of cancer over the course of their lifetime (Cancer, 2007). Metastatic cancer cells are of utmost concern. Over 90% of cancer deaths are due to the migration of cancerous cells away from the primary site and their invasion of a secondary location (Entschladen et al., 2005; Germanov et al., 2006; Gupta and Massague, 2006). A better understanding of the molecules and signaling networks acting to control cell migration and cell death may lead to new hypotheses as to the causes, implications, and potential treatments of these diseases.

Human benefit from research on cell migration and cell death is not limited to cancer prevention and treatment. In addition to cancer, many other diseases and developmental anomalies are impacted by the inability of an organism to effectively regulate cell migration and cell death decisions. These include diseases such as multiple sclerosis, atherosclerosis, autoimmune diseases and conditions leading to disease such as human immunodeficiency virus (HIV).

Using *Drosophila melanogaster* germ cell migration as a model, my research projects aim at elucidating currently unknown critical players in germ cell migration and programmed cell death in Drosophila. Additionally, further structural analysis of a known protein in
Drosophila germ cell migration, Tre1, will provide insight into the domains critical for proper function of this G protein-coupled receptor (GPCR). Drosophila germ cell development is a simple, genetically tractable system for the identification and characterization of signaling pathways involved in normal cell migration and cell death decisions. This research has the potential to impact human medicine as at least 60% of known human disease genes have direct orthologues in Drosophila (Chien et al., 2002; Fortini et al., 2000).

In this general introduction the following topics will be discussed: Drosophila germ cell migration, players in Drosophila germ cell death, GPCRs, and human disease states associated with defective cell migration, programmed cell death and GPCR signaling. These topics are of significance in my research projects as I will present my work on the characterization of the molecular defect in tre1, which encodes a GPCR involved in Drosophila germ cell migration and programmed cell death, discussed in Chapter 2. Further studies outlined in Chapter 3 will present a mutant screen designed to identify additional components of Drosophila germ cell migration and/or PCD. Comparison studies of germ cell migration in fruit fly, zebrafish, and mammals can be found in Chapter 4, a review published in the Annals of the New York Academy of Sciences.

**Drosophila germ cell migration**

Drosophila embryogenesis begins with a series of rapid-succestion nuclear divisions to form a single cell syncytium with nearly 6000 nuclei. Nuclei migrate to the posterior-most region of the syncytial blastoderm and are surrounded by a maternally supplied cytoplasm that contains germ cell determinants. This maternal cytoplasm, known as germ plasm, is
sequestered at the posterior pole during oogenesis. The nuclei that reside in the germ plasm are the first to be cellularized in the embryo and are destined to become germ cells (Sonnenblick, 1941). These primordial germ cells can undergo up to 2 cell divisions to produce a total germ cell population of 30-40 cells (Sonnenblick, 1941; Technau, 1986; Underwood et al., 1980; Williamson and Lehmann, 1996). Following these cell divisions the germ cells cease mitosis for the remainder of embryogenesis (Deshpande et al., 1999). Additionally, no RNA transcripts are produced in germ cells until around stage 9, a point just prior to the initiation of active cell migration (Kobayashi et al., 1996).

Drosophila germ cells, like germ cells of many other organisms, are formed at a site some distance from where they are ultimately required, the somatic gonad. The migratory steps of Drosophila germ cells have been the subject of careful genetic study; and it has been shown to be a multistep process (Campos-Ortega and Hartenstein, 1997; Santos and Lehmann, 2004a; Sonnenblick, 1941; Sonnenblick, 1950; Starz-Gaiano and Lehmann, 2001). The initial steps of migration for Drosophila germ cells are passive. The somatic cells of the presumptive posterior midgut are directly adjacent to where the germ cells initially form at the posterior pole. The germs cells adhere to the nearby tissue and are passively incorporated into the posterior midgut pocket during the extensive embryonic reorganization during gastrulation and germ band extension. The active part of the germ cells migration to the gonads begins at stage 10 with the transepithelial migration of the individual germ cells out of the posterior midgut pocket (Callaini et al., 1995; Jaglarz and Howard, 1995). Recently, critical signaling components involved in the transepithelial migration of germ cells were identified (Kunwar et al., 2008; Kunwar et al., 2003). The gene, *trapped in endoderm-1* (*tre1*), is required for germ cell polarity, individualization and successful migration out of the
midgut pocket (Kunwar et al., 2008; Kunwar et al., 2003). In a \textit{tre1} mutant allele lacking RNA expression in the germ cells, germ cells remain within the midgut pocket and are unable to complete their migration to the somatic gonad precursor cell populations (Kunwar et al., 2008; Kunwar et al., 2003). Germ cell transplantation experiments and tissue-specific expression of \textit{tre1} rescue experiments indicate that \textit{tre1} is required cell autonomously in germ cells (Kunwar et al., 2003). \textit{tre1} encodes a GPCR. Little else is known regarding \textit{tre1} signaling as its ligand remains elusive and the downstream signaling pathways that mediate transepithelial migration are poorly understood. Three additional proteins have been implicated downstream of \textit{tre1}. The G proteins, \textit{G}β\textsubscript{13f} and \textit{G}γ\textsubscript{1} are provided to the embryo maternally and loss of these proteins results in germ cells with defective migration out of the midgut pocket. (Kunwar et al., 2008; Kunwar et al., 2003). In germ cells expressing a dominant-negative form of the small GTPase, Rho1, a phenotype similar to the \textit{tre1} mutant results (Kunwar et al., 2003). In wild type embryos, expression of Rho1 and Gβ13f becomes localized to the lagging tail of the germ cells at stage 9 and helps to create polarization of the germ cell for successful transepithelial migration. However, in \textit{tre1}\textsuperscript{4EP5} mutants, this localization is disrupted and expression persists across the entire membrane of the germ cells (Kunwar et al., 2008). Not only must germ cells activate signaling cascades to successfully cross the epithelium but the somatic cells of the epithelial layer also must adjust to allow the passage of germ cells. About the time germ cells must migrate through the midgut epithelium, the normally tightly associated epithelial cells loosen intercellular junctions enabling germ cells to move between the cells (Callaini et al., 1995; Jaglarz and Howard, 1995).
As the germ cells leave the midgut, they migrate towards the lateral mesoderm. Both attractive and repulsive signals have been identified that guide these migrating germ cells to the somatic gonadal precursor cells to form the gonad. Following transepithelial migration, the germ cells orient themselves dorsally on the basal side of the midgut. Repulsion signals from the ventral region of the midgut assist in the dorsal positioning. The repulsive signal is generated by two related lipid phosphate phosphatases, Wunen (Wun) and Wunen2 (Wun2) (Starz-Gaiano et al., 2001; Zhang et al., 1997). The lipid phosphate phosphatases are hexahelical transmembrane proteins with their catalytic domains exposed to the extracellular space that act to dephosphorylate phospholipid substrates. Not only do Wun and Wun2 mediate the germ cell’s location on the midgut, subsequent expression in the central nervous system as well as in the ectoderm continues guiding the migratory path of the germ cells. This encourages the germ cells to bilaterally segregate to either side of the CNS yet prevents migration into the surrounding ectodermal tissues (Sano et al., 2005; Starz-Gaiano et al., 2001; Zhang et al., 1997). In this way, two germ cell populations are created in the lateral mesoderm that ultimately interact with the somatic gonad precursor cell populations in parasegments 10-12 (Boyle and DiNardo, 1995; Brookman et al., 1992). Interestingly, wun2 is also expressed in the migrating germ cells. Recently, it was proposed that the Wun2 expressed in the germ cells must compete with the Wun and Wun2 expressed in the somatic tissue to hydrolyze a currently unidentified phospholipid. In this way, germ cells avoid those areas of the mesoderm with the lowest concentrations of the desired phospholipid, which is directly along the CNS and chart a course on either side of the ventral midline (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005; Starz-Gaiano et al., 2001; Zhang et al., 1997). In wun/wun2 double mutant embryos, germ cells fail to orient
dorsally and do not bilaterally segregate but rather scatter throughout the mesoderm, suggesting that the phospholipid gradient has been destroyed (Sano et al., 2005; Starz-Gaiano et al., 2001; Zhang et al., 1997).

The migrating germ cells must not only be receptive to the repulsive cues acting on them, but also attractive signals guiding them to the somatic gonad precursor cells. The exact molecules that act as the attractive signal(s) still remains elusive, however, the synthesis pathway that creates this attractant continues to reveal the nature of this modified protein. It has been known for some time that the gene, columbia (clb), is required for attraction of germ cells. The fly homolog of 3-hydroxy-3-methylglutaryl coenzyme A reductase (hmgcr) is encoded by clb (Van Doren et al., 1998). Both loss of function and gain of function studies using clb have supported the notion that this reductase activity is required for production of an attractive signal. First, in clb mutant embryos, germ cells fail to laterally migrate into the mesoderm and those few germ cells that do manage to make it into the mesoderm fail to identify and interact with the somatic gonad precursor cells. Consistent with clb being necessary and sufficient for germ cell migration, clb RNA expression within the embryonic nervous system or ectoderm will attract germ cells to these locations (Van Doren et al., 1998). Hmgcr is involved in the synthesis of mevalonate. Mevalonate can be a precursor in the synthesis of cholesterol, ubiquinones, carotenoids, and isoprenoids (Santos and Lehmann, 2004b). Further investigation into the potential pathways downstream of mevalonate that could be generating the attractive signal reveals that the Drosophila genome lacks some of the key enzymes involved in the synthesis of cholesterol (Clayton, 1964; Santos and Lehmann, 2004a; Santos and Lehmann, 2004b). Therefore, components of the cholesterol synthesis pathway were ruled out as mediators of germ cell attraction. However,
blast searches of the Drosophila genome with human genes required for isoprenoid synthesis revealed fly homologs to all the necessary enzymes for isoprenoid synthesis (Santos and Lehmann, 2004b). Isoprenoids are lipids that are known to post-translationally modify proteins. Two enzymes involved in isoprenoid synthesis; *fpps* and *quemao*, have been shown to be involved in generation of a germ cell attractant signal. These encode farnesyldiphosphate synthase and geranylgeranyl-diphosphate synthase respectively. Mutants with severe reduction in expression of either of these genes result in a germ cell migration phenotype similar to that of *clb* mutants with germ cells unable to migrate into the mesoderm but instead remaining on the dorsal side of the midgut (Santos and Lehmann, 2004b). Additionally, misexpression studies on these mutants reveal they are sufficient to attract germ cells to ectopic locations (Santos and Lehmann, 2004b). A third enzyme not involved in the synthesis but rather the transfer of an isoprenoid product to target proteins has also been identified as having a role in germ cell migration. *Geranylgeranyl transferase type 1* encodes a protein that transfers geranylgeranylpyrophosphate to proteins as a post-translational modification. Again, loss of function studies reveal a germ cell migration phenotype when this protein is missing (Santos and Lehmann, 2004b). Taken together, these results indicate that an attractive signal generated by HMGCoA reductase also requires the downstream synthesis of isoprenoids and subsequent transfer of geranylgeranylpyrophosphate to a unknown target protein to elicit its attractive signal.

Yet another player implicated in germ cell attraction to the gonads is *hedgehog, hh*. The Hedgehog protein is expressed in the somatic gonad precursor cells and is involved in the specification of these precursor cells. Misexpression studies reveal a role in germ cell
migration. When *hh* is expressed in ectopic locations, this signal is sufficient to cause germ cell migration to those ectopic locations (Deshpande et al., 2001).

The relationship between HMGCoAr and Hedgehog attractant cues has been under investigation. Recent evidence suggests that the lipid produced in the HMGCoAr pathway acts to potentiate the Hedgehog signal that acts as a germ cell attractant. However, it is still possible that HMGCoAr’s role in germ cell migration may be directly related to a role in a completely independent germ cell signaling pathway. (Deshpande and Schedl, 2005; Deshpande et al., 2001).

Once the germ cells reach the two somatic gonadal precursor cell populations, they must form a tight ball of cells and coalesce to form the gonad. Once the germ cells have associated with the somatic gonadal precursor cells, they adopt a rounded, non migratory morphology indicating their completion of migration (Jaglarz and Howard, 1995). Two somatic cell transmembrane proteins, Shotgun (Shg) and Fear of intimacy (Foi) are required in gonad coalescence (Jenkins et al., 2003; Mathews et al., 2005; Van Doren et al., 2003). Foi is a zinc ion transmembrane transporter and *shg* encodes Drosophila E-cadherin. In wild type embryos, DE-cadherin levels are elevated in the somatic gonad precursor cells and this needed for compaction (Jenkins et al., 2003). In *foi* mutants, DE-cadherin protein and RNA levels are decreased, suggesting *foi* regulates its expression (Jenkins et al., 2003). Expression levels are DE-cadherin are proposed to regulate the compaction process. The germ cells complete migration to and compaction within the gonads by stage 14, which begins at approximately 10.5 hours of development.
**Drosophila germ cell programmed cell death**

The programmed cell death of Drosophila germ cells along their migratory route is largely uncharacterized. Recent germ cell counts have pinpointed the time frame at which the majority of germ cells undergo cell death. Cell death occurs between stages 10 and 12 (4.5-9.5 hours) of development (Yamada et al., 2008). At this time, the germ cells have left the posterior midgut pocket and are bilaterally segregating and navigating through the lateral mesoderm towards the presumptive somatic gonads. It is known that the lipid phosphate phosphatases, Wunen1 and Wunen2, that control the bilateral migration of germ cells, are also involved in mediating germ cell death. These proteins are expressed both in germ cells and somatic tissues of the central nervous system. It has been suggested that competition between germ cell and soma for substrate controls the directed bilateral migration and death of germ cells along the migratory route (Sano et al., 2005). Consistent with this hypotheses, both loss of expression of Wunens in the germ cells and overexpression of Wunens within the soma leads to germ cell death (Burnett and Howard, 2003; Hanyu-Nakamura et al., 2004; Renault et al., 2004; Starz-Gaiano et al., 2001).

Work from the Coffman lab, has found additional players in germ cell death. Other mutants isolated in our EMS mutagenesis screen affect a gene known as *outsiders*. The *outsiders* gene encodes a monocarboxylate transporter (Yamada, 2007; Yamada, 2008). In these mutants, a wild type number of germ cells reach the gonads. However, this defect causes germ cells ectopic to the gonads to persist when they should have undergone programmed cell death. Currently, it is unknown how a monocarboxylate transporter might fit into the mechanisms of cell death in germ cells (Yamada et al., 2008).
The transcription factor, p53, is also involved in the endogenous death of Drosophila germ cells (Yamada et al., 2008). Mutants in *p53* produce a germ cell phenotype similar to *outsiders*, successful germ cell migration, but an excessive number of germ cells persisting ectopic to the gonads. This transcription factor has been shown to be involved in two common modes of programmed cell death, apoptosis and autophagy.

While some players in Drosophila germ cell death have been identified, the specific cell death mechanisms remain to be elucidated. Three major forms of cell death are commonly discussed; apoptosis, autophagy, and necrosis. Apoptosis and autophagy have classically been implicated in programmed cell death. Autophagy was initially identified as a survival mechanism in times of low nutrient supply, while necrosis discussion tends to be restricted to cell damage-induced cell death. As this field of research grows, it is becoming apparent that specific cells do not have just one single mode of death; rather cell death can occur through various types, and combinations of these types of cell death programs depending on the molecules available to the cell. Molecules previously identified as controlling one form of programmed cell death have more recently been found to affect other forms of cell death as well (Crighton et al., 2006). Attempts to identify the form of cell death program working in Drosophila germ cells have been made. Analysis of the H99 deletion, that uncovers pro-apoptotic genes *reaper, hid,* and *grim,* reveals that endogenous germ cell death still ensues in the absence of zygotic expression of these proteins, suggesting caspase-mediated apoptotic cell death is not the cell death mechanism in Drosophila germ cells (Renault et al., 2004). The identification of the role of *p53* in germ cell death sheds little light on the potential modes of cell death, as *p53* has roles in multiple modes of cell death. Transcriptional activity of p53 has been shown to activate genes that control apoptosis.
(Laptenko and Prives, 2006), while p53 also has links to cell death via autophagy (Feng et al., 2005). Therefore, the mode or modes of germ cell death remain an outstanding question.

**G protein-coupled receptors**

GPCRs are found in many cell types and have the ability to regulate a myriad of cellular responses including cell growth and proliferation as well as cell migration and cell death decisions (Gether, 2000; Karnik et al., 2003). GPCRs are often targeted in the pharmaceutical industry. It is estimated that over 30% of all pharmaceuticals on the market today target GPCRs (Brink et al., 2004; Drews, 2000). The GPCR family comprises 1-5% of all proteins that are represented in the vertebrate and invertebrate genomes (Bockaert et al., 2002; Bockaert et al., 2003). While GPCRs are an expansive class of proteins, there are some conserved domains common to most GPCR classes. A shared factor among all GPCRs is its structure. GPCRs are seven-pass transmembrane proteins and are able to bind extracellular molecules that activate the receptor. The diversity of ligands for GPCRs is extremely broad. This family of receptors can respond to cues from ligands including: light, neurotransmitters, odorants, biogenic amines, lipids, proteins, amino acids, hormones, nucleotides, chemokines and many more (Kroeze et al., 2003). The ligand binding can occur through interactions with the extracellular N terminal tail, an extracellular loop, and the transmembrane domains. The receptor is held in an inactive state by intramolecular interactions that keep its structure constrained. In the β2-adrenergic receptor, one such intramolecular interaction is the ionic lock that links transmembrane domains 3 and 6 in the receptor. When the agonist binds, this connection is disrupted and changes shape of the receptor in such a way that opens up the intracellular loops for interactions with intracellular and transmembrane proteins (Ballesteros
et al., 2001). The strict requirement for proper orientation of transmembrane domains 3 and 6 in relation to each other has been shown to be important for maintaining the inactive state in a broad range of GPCRs (Ballesteros et al., 2001; Boucard et al., 2003; Gether, 2000; Miura et al., 2003; Sheikh et al., 1999; Sheikh et al., 1996). The conformational change that occurs when an agonist binds the receptor switches it from an inactive to an active state. This switch to an active state allows the GPCR to initiate signaling pathways within the cell.

The canonical pathway activated by GPCRs involves interaction with G proteins. Upon ligand activation, intracellular heterotrimeric G proteins can bind to the receptor. The association of the heterotrimeric G protein with the receptor allows the G protein subunits to replace bound GDP for GTP and to dissociate and activate downstream effector molecules. In this way, downstream signaling pathways are initiated to elicit a cellular response to external stimuli. Downstream effects of G proteins are dependent on the type of G protein bound, and their signal transduction includes: changes in cyclic AMP levels, activation or inactivation of adenylyl cyclase, activation of protein kinases, activation of phospholipase C, conversion of phosphotidylinositol-bisphosphate to diacylglycerol and inositol phosphate, release of calcium into the cytosol, and activation of small GTPases such as Rho to name a few (Radeff-Huang et al., 2004).

More recently, the paradigm of GPCR activation of only G protein-mediated signal transduction cascades has been revised. It is now known that GPCRs can activate signaling cascades previously assumed to be under the control of growth factor receptors (Daaka et al., 1998; Lefkowitz, 1998; Luttrell et al., 1999; Luttrell et al., 1996). The switch from G protein-dependent to noncanonical GPCR signaling is thought to involve phosphorylation of the receptor by G protein-coupled receptor kinases. This phosphorylation is known to be
involved in receptor desensitization by recruiting arrestins to the GPCR and targeting them to clathrin-coated pits. However, recent evidence suggests that not only does arrestin mediate receptor desensitization but it also acts as a scaffold to which proteins can bind to initiate alternate downstream signaling cascades (Lefkowitz and Shenoy, 2005). This flexibility of the GPCR in interacting with downstream binding partners creates a diverse and far-stretching ability to have a role in a significant number of different signaling cascades and cellular events including cell migration, chemotaxis and cell death (Lefkowitz and Shenoy, 2005).

**Human Disease States**

The research discussed in Chapter 2 connects a GPCR to roles in both the migration and programmed cell death of Drosophila germ cells. Cell migration to sites distant from the cells point of origin is a common theme in development, homeostasis, immune response and is defective in many diseased states (Franz et al., 2002; Horwitz and Webb, 2003; Ridley et al., 2003; Vicente-Manzanares et al., 2005). Commonly migrating cell types such as germ cells, immune system cells, leukocytes, and even metastatic cancer cells share many similarities in their migratory mechanisms. These cell types migrate individually using amoeboid-like movements towards the target location (Condeelis and Segall, 2003; Friedl and Wolf, 2003; Sahai, 2005; Wang et al., 2005). They adhere minimally to the substrate through which they migrate and have the ability to migrate across different tissues and through epithelial layers. Such similarities between germ cells and the migratory movements of cells typically involved in disease states make the study of Drosophila germ cell migration a wonderful model for the progression of disease. Cell death is often tightly linked to cell
migration as cells that fail to successfully migrate must be removed as they present the opportunity for tumor formation. Additionally, given the broad range of responses GPCRs can elicit upon activation, defective GPCR signaling leaves the host vulnerable to a plethora of disease states. In this section, I will discuss some disease states associated with cell migration/cell death decisions and/or GPCR signaling.

a) Secondary Site Cancer Formation

The study of cancer cell metastasis is critical to the prevention of death in cancer patients. Secondary sites of cancer formation can severely impact the quality of life and overall long-term survival of cancer patients (Chambers et al., 2002). Interestingly, secondary sites that are targeted for cancer colonization are not random. Chemokines and their receptors have been implicated in cancer cell metastasis (Koizumi et al., 2007). Recent studies have indicated the cause for nonrandom secondary site targets for breast cancer metastasis. The GPCR, CXCR4, is a chemokine receptor that has been identified to be active on breast cancer cells. Its chemokine ligand, SDF-1α, is highly expressed in those tissues that are common targets for metastasizing breast cancer cells. These include lymph nodes, bone marrow and lungs (Muller et al., 2001). CXCR4 and SDF-1 have been implicated in germ cell migration in other systems such as zebrafish, chick and mouse and therefore represents another GPCR, like tre1 that is involved in the successful incorporation of the germ cells into the gonad (Ara et al., 2003; Boldajipour et al., 2008; Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003).
b) Multiple Sclerosis

Multiple Sclerosis (MS) is a neurological disorder in which the body’s own immune system attacks the white matter of the central nervous system. Onset of this disease occurs in early adulthood and leads to both physical and cognitive degeneration. Inflammation of the brain and spinal cord are a result of hyper-migration of T-cells, B-cells and monocytes/macrophages to these regions (Cartier et al., 2005). Brain lesions in MS patients are a result of the invasion of these immune system cells. It has been discovered that regions of the CNS in MS patients express the chemokines MIP-1α and IP-10, which lead to the attraction of immune system cells expressing GPCRs specific to these ligands, CCR5 and CXCR3 (Balashov et al., 1999).

c) Human Immunodeficiency Virus (HIV)

HIV is the virus that leads to Acquired Immunodeficiency Syndrome (AIDS). This virus targets the host immune system, debilitating its response to infection. The virus specifically targets the CD4(+) T-cells for destruction by entering the cell. It was discovered that HIV entry into T-cells is accomplished with the help of the T-cells own CXCR4 and CCR4 G protein-coupled receptors on the cell surface. These GPCRs become binding sites for HIV particles and are hijacked to become part of the HIV entry complex, granting cell access to this devastating virus (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Feng et al., 1996).
d) Atherosclerosis

Atherosclerosis is a disease affecting the vessel walls of the heart. It can cause pathologies including myocardial infraction, stroke, and peripheral artery disease. A contributor to atherosclerosis is chronic inflammation due to attraction of leukocytes to the arterial wall. Chemokines and their chemoattractants have been implicated in the recruitment of these T cells and monocytes and as a result, inflammation (Liehn et al., 2006).

Overview of dissertation chapters

In these chapters, I present detailed analyses of germ cell migration in Drosophila and their significance in other systems.

Chapter 2. Research performed to identify the molecular defect in \textit{tre1^{sctt}}\textsuperscript{t}, a mutation that disrupts proper germ cell migration and programmed cell death. A single base pair change was identified that results in improper splicing and the loss of 8 amino acids from this GPCR. Further investigation revealed the necessity for the missing arginine of the highly conserved DRY motif in germ cell migration. (Antibody staining, germ cell counts and statistical analysis produced with the assistance of Margaret Pruitt, second author of a manuscript in preparation.)
Chapter 3. A reverse genetics approach performed to identify additional components of Drosophila germ cell migration signaling pathways. Genes of interest were selected based on readings that indicated a potential role for the gene in Drosophila germ cell migration, programmed cell death or roles in GPCR signaling and expression data suggesting presence at appropriate times and locations to be involved in these processes. Mutant alleles were assayed for germ cell migration phenotypes. I developed hypotheses based on my reading, designed the experiment, and ordered necessary stocks. Antibody staining and scoring was performed with the assistance of Deb Czarnecki, a summer undergraduate intern, and Margaret Pruitt, a current graduate student.

Chapter 4. A review published in the Annals of the New York Academy of Sciences that compares germ cell migratory movements in fruit flies, zebrafish and mammals. The roles of G protein-coupled receptors and their ligands in cell migration and cell death decisions are discussed.

Chapter 5. An overview of the findings of my research and the discussion of remaining questions and observations for investigation in future research.
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CHAPTER 2. INVESTIGATION OF AMINO ACIDS CRITICAL FOR GPCR-MEDIATED GERM CELL MIGRATION IN DROSOPHILA

Introduction

Cell migration mediated by G protein-coupled receptors (GPCRs) has emerged as a common theme that transcends many different cell types and organismal systems. The necessity for proper cell migration impacts an organism consistently throughout embryonic development and adult phases of life. Migrating cells in multiple systems share remarkable similarities in cell migration morphology and use of similar ligands for directional migration. For example, amoeboid-like migration morphologies are shared by metastatic cancer cells, cells of the immune system and germ cells of the reproductive system (Condeelis and Segall, 2003; Friedl and Wolf, 2003; Sahai, 2005; Wang et al., 2005). Additionally, chemokines and phospholipids have been identified as conserved attractants for cells of the reproductive system and in immune response as well as in the progression of diseased states such as cancer (Kunwar et al., 2006). These ligands activate receptors on the receiving cell’s surface that initiate a migratory response in the direction toward higher expression levels of the agonist. Both sensing of extracellular cues for directional migration and the cytoskeletal rearrangements of the cell necessary for movement are often perceived and regulated by GPCRs.

There is much interest in elucidating the molecular mechanisms and important players in cancer cell and immune cell migration as it relates to human health. Given the conserved nature of GPCR-mediated cell migration, it is critical to understand the common functional themes occurring at the GPCR level to mediate cell type-specific, yet evolutionarily conserved initiation of signal transduction. The migration of Drosophila
*melanogaster* germ cells provides a genetically tractable system for the study of GPCR-mediated cell migration, allowing identification of critical components of this global method of movement and providing the ability to study the function of these components in an intact organism.

The germ cell migration pathway in *Drosophila melanogaster* has been well characterized and involves a multi-step process (Campos-Ortega and Hartenstein, 1997; Santos and Lehmann, 2004; Sonnenblick, 1941; Sonnenblick, 1950; Starz-Gaiano and Lehmann, 2001). Drosophila germ cells are formed extraembryonically and must undergo a significant migratory journey through the developing embryo to reach the somatic gonad precursor cells. Upon cellularization, germ cells reside at the posterior-most region of the embryo and are directly adjacent to the somatic tissue that will eventually form the posterior midgut. The germ cells adhere to the midgut primordium and are carried along with this tissue during the extensive embryo reorganization that occurs during gastrulation, and they become incorporated into the posterior midgut as germ band extension proceeds. The first major active migratory movement taken by the germ cells is their crossing of the epithelial layer surrounding the midgut at stage 10 (Callaini et al., 1995; Jaglarz and Howard, 1995). The germ cells are directed in a specific path towards the somatic gonad precursor cells by both attractive and repulsive guidance cues and must sense and respond to these cues to reach their targets. Upon completion of transepithelial migration, the germ cells are oriented dorsally on the basal side of the midgut. At this time the germ cells bilaterally segregate into two populations on either side of the midline and migrate through the mesoderm towards the somatic gonad precursor cells in parasegments 10-12 of the embryo. In stages 12 and 13, the germ cells reach the somatic gonad precursor cells and coalesce to form the gonad. The
germ cell migration process is complete and germ cells have compacted with somatic gonad precursors cells to form the embryonic gonad by stage 14, approximately 10.5 hours into embryogenesis.

Drosophila germ cells share similarity in their migratory terrain with both metastatic cancer cells and cells of the immune system. All three must traverse multiple tissues and epithelial layers en route to their target. A Drosophila GPCR encoded by *trapped in endoderm* 1, *tre1*, is a maternally provided transcript that has been identified as having cell autonomous roles during germ cell dispersion within the midgut, presentation of germ cells in relation to the midgut and in the migration of germ cells through the epithelial layer surrounding the midgut (Kunwar et al., 2008; Kunwar et al., 2003). The *tre1*ΔEP5 allele is a deletion removing *tre1* sequence beginning just upstream of its start site, through exon 1 and a portion of intron 1. Transcripts of *tre1* were not detected in *tre1*ΔEP5 mutants prior to Stage 9 (Ueno et al., 2001). In *tre1*ΔEP5 mutants, germ cells are unable to exit the midgut and appear trapped within the posterior midgut pocket (Kunwar et al., 2008; Kunwar et al., 2003). This mutation is assumed to result in complete loss of function of the GPCR mediating germ cell migration.

An additional mutant, *scattershot* (*sctt*), was identified through an EMS mutagenesis screen targeting X-linked genes involved in Drosophila germ cell migration. In embryos from a cross between a female homozygous for the *sctt* mutation and a *sctt* male, proper germ cell migration to the gonads is severely disrupted (Coffman et al., 2002). In *sctt* mutants, many germ cells exit the posterior midgut normally and then scatter throughout the posterior half of the embryo, rather than the directed migration to the gonads observed in wild type embryos. Additionally, germ cells ectopic to the gonads persist. Genetic evidence
revealed that *sctt* failed to complement the *tre1<sup>AEPS</sup>* allele (Kunwar et al., 2003). When embryos from *tre1<sup>AEPS</sup>/*sctt females were assayed, an intermediate phenotype was produced. More germ cells remained clumped in the gut compared to the embryos derived from *sctt/*sctt mothers (Kunwar et al., 2003). While genetic experiments suggested that *sctt* was either an allele of *tre1* or a mutant representing a non-allelic non-complementing locus, the molecular defect causing the phenotype was unknown. We report the molecular lesion within *tre1* that definitively identifies *sctt* as an allele of *tre1* that severely alters the ability of this GPCR to function.

The *sctt* mutation provides a unique situation in which to study the critical domains of GPCRs in signal transduction leading to cell migration. The *tre1* transcripts are present at nearly wild-type levels in the *sctt* mutant (Camilla Burnett and Ken Howard; personal communication), yet a severe loss of function phenotype is produced that is phenotypically distinct from a complete loss of function mutation of the same gene. The molecular lesion causing the *sctt* phenotype is an 8 amino acid deletion due to alternative splicing. The loss of these 8 amino acids severely disrupts GPCR-mediated cell migration. Given the ability to genetically manipulate Drosophila using transgenics and analyze the outcome in the context of the whole organism, we have investigated the critical roles of a highly conserved GPCR motif in an intact organism down to single amino acid resolution. This research, to our knowledge, represents the first in-organism amino acid mutagenesis study that investigates the role of the amino acids at the transmembrane domain 3/intracellular loop 2 junction in a rhodopsin family GPCR.
Materials and Methods

Fly stocks

The sctt allele was generated in an EMS mutagenesis screen (Coffman et al., 2002). The T’G+ transgenic line was kindly provided by John Carlson (Dahanukar et al., 2001; Ueno et al., 2001). w^{1118}, P\{w+, fat facets-lacZ\}, the unmutagenized parental strain of sctt, was used as a wild-type control in these studies (Fischer-Vize et al., 1992).

Embryo collections

Embryos were collected on standard apple juice agar plates and aged 12-16 hours at 25 °C. Embryos were harvested and dechorionated using 50% bleach.

Whole mount antibody staining

Immunostaining was performed according to standard procedures (Johansen and Johansen, 2004). Embryos were fixed in 4% paraformaldehyde and devitellinized by shaking in heptane/methanol. The primary antibody used was chicken anti-Vasa, (a gift from Ken Howard, 1:10,000) and the secondary antibody used was biotinylated anti-chicken IgG (1:500) (Vector Laboratories). Antibody detection was performed using the ABC Elite Kit (Vector Laboratories) with 3,3’-diaminobenzidine tetrahydrochloride as a substrate.

X-Gal staining

Following embryo collection, the embryos were washed with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.3% Triton X-100, pH 7.2; (Sambrook, 1989)) and fixed for 10 minutes using a 2.5% glutaraldehyde-saturated heptane fixative.
(Holmes et al., 1998; Simon et al., 1985). Following fixation, embryos were washed with PBST for 4 hours. Staining was performed with a 0.08% X-Gal solution for 2 hours at 37 °C.

**Germ cell counts**

Embryonic germ cells were labeled either using X-Gal staining or anti-vasa antibody. Both methods detect similar numbers of primordial germ cells (Yamada, 2007). A differential interference contrast microscope was used to count the germ cells. Embryos were aged 12-16 hours and age was confirmed using embryo morphology as previously described (Campos-Ortega and Hartenstein, 1997). Criteria for scoring a germ cell as in the gonad included migration to abdominal segment 5 and presence within the correct bilateral region to be incorporated into the gonad. Gonadal sheath cells were used to delimit the gonad boundaries when possible.

**Sequencing**

Genomic template was extracted from a single adult fly using a buffer containing 10mM Tris-HCl, 1mM EDTA, 25mM NaCl, and 10 mg/ml Proteinase K (Gloor et al., 1993). Both sctt mutants and wild type flies were used as templates. The tre1 gene was PCR amplified using TripleMaster Taq DNA Polymerase (Eppendorf). The following primers were used to amplify the entire coding region of tre1: 5’- TCAAATAACCAAGCGGATGC-3’, 5’-CAAAAACGTTAGTTAGCGCC-3’, 5’-CACATCGTTGTGCTTGTTTCC-3’, 5’-GCGCAAAGATCTTTGATGAGC-3’, 5’-CCTGTGATCCTCTTTTG-3’, and 5’-GACAATGCGGACTAGCTTG-3’. A total of approximately 2000 base pairs were
sequenced on both strands. Seven sctt and five wild-type flies were used individually as genomic templates to confirm the critical single base pair change mutation identified between the two lines. Sequencing samples were run on an Applied Biosystems 3730xl DNA Analyzer (Iowa State University DNA Sequencing Facility).

**Reverse transcriptase PCR**

0-8 hour embryos were collected and dechorionated using 50% bleach. Embryos were homogenized in Trizol (Invitrogen) and total RNA was isolated. DNA was removed from the total RNA using Turbo DNase (Ambion). First strand cDNA synthesis was performed using RETROscript First Strand cDNA Synthesis Kit for RT-PCR (Ambion) according to the manufacturer’s instructions. PCR was performed on the cDNA template using Taq DNA polymerase (Eppendorf). Lack of genomic contamination in the PCR amplification was confirmed using primers that spanned multiple exons. The primers were as follows: 5’-TGCTCTTCTGCTCCTTCAGC-3’ and 5’-CCAGTGTCATTAACCCGATCA-3’. PCR products were sequenced and analyzed.

**Secondary structure protein predictions**

The Tre1 amino acid sequence of both wild-type and the sctt mutant were entered into the secondary structure prediction programs: SOSUI (Hirokawa et al., 1998), TopPred (von Heijne, 1992), and TMHMM (Krogh et al., 2001) to determine whether the 8 amino acid deletion from intracellular loop 2 was likely to affect overall topology.

**Engineering of the amino acid alteration cassette**
The T+G+ vector containing a 10 kb genomic fragment coding for both \textit{tre1} and \textit{gr5a} was used in the generation of the amino acid-substituted constructs (Dahanukar et al., 2001). A 1700 base pair fragment containing the target sequence for nucleotide replacement was excised by digesting with SphI and StuI restriction endonucleases (NEB) and cloned into a modified pSP72 vector containing an inserted StuI restriction endonuclease site and lacking a PstI site from the original polylinker. The resulting vector was further digested using PstI and Bpu10I restriction endonucleases (NEB) to excise a 160 base pair fragment of \textit{tre1} genomic DNA that housed the target sequence. Two sets of phosphorylated oligonucleotides were designed to replace the double-stranded 160 base pair fragment and insert an AloI restriction endonuclease recognition site directly into the target sequence region. A triple ligation was employed to ligate the digested pSP72 and the two sets of hybridized oligos to reconstitute the vector containing AloI. Vector sequencing confirmed the presence of the AloI restriction endonuclease sites. Two independent cassette vectors were designed with the AloI site engineered in different locations to allow the substitution of nucleotides encoding all 8 amino acids of interest. A schematic of the cassette construction is shown in the supplemental materials (Supplemental Figure 2.1).

**Creation of constructs with amino acid substitutions**

The pSP72 vector cassette containing the AloI restriction endonuclease site was digested with AloI (Fermentas). Due to the unusual double-cutting nature of the AloI enzyme, the AloI digest removed the AloI restriction site as well as 7 base pairs 5’ and 12-13 base pairs 3’ of the restriction site. A pair of complementary phosphorylated
oligonucleotides was designed to contain nucleotide changes to alter the amino acid sequence in the target region. The oligonucleotides were hybridized and ligated into the Alol-digested cassette. The Alol-containing pSP72 vectors were isolated via the Plasmid Midi Kit (Qiagen) and subsequent sequencing confirmed the presence of the desired nucleotide substitutions. The pSP72 vector was then digested with SphI and StuI to excise the original 1700 base pair fragment for insertion into the digested T+G+ vector to reconstitute the 10 kb genomic clone. Sequencing confirmed the presence of the nucleotide substitutions in the T+G+ vector. Supplemental Table 2.1 lists the phosphorylated oligonucleotides used in the creation of the amino acid substitution constructs.

**Generation of transgenic flies**

The modified T+G+ constructs were injected into a ready-for-injection w¹¹¹⁸ host strain using a modification of the standard transformation protocol (Spradling and Rubin, 1982), as outlined by Nicholas Gompel. The plasmid P{π25.7 Δ2-3 wc} was used as a transposase source (Karess and Rubin, 1984) and co-injected with the modified T+G+ constructs. Embryos were injected with the chorion intact. After injection, embryos were maintained on an apple juice/agar plate for 24 hours at 25 °C. Embryos were transferred to a vial containing standard media and allowed to develop to adults. Hatched adults were backcrossed to the w¹¹¹⁸ host strain. Transgenic flies were identified from this cross by screening for the presence of P[w+] eye color. Balanced stocks were established by crossing to the w¹¹¹⁸, wgSp-1/CyO; Sb/TM6b, Huf balancer stock. Precise transgenic insertion sites were determined by inverse PCR (Bellen et al., 2004).
Fly crosses

Transgenic chromosomes were crossed into the \textit{tre} \textit{l}^{sctt} mutant background. To test for rescue of germ cell migration, females homozygous for \textit{tre} \textit{l}^{sctt} and carrying one or two copies of the transgene were crossed to \textit{tre} \textit{l}^{sctt} males. The offspring were assayed for maternal rescue of germ cell migration.

Results

The \textit{sctt} mutation disrupts normal germ cell migration and programmed cell death

In \textit{sctt} mutants, the concerted movements of germ cells and the subsequent death of a subpopulation of the germ cells is disrupted (Coffman et al., 2002). In embryos produced from a cross between a homozygous \textit{sctt} female and a \textit{sctt} male, the germ cells fail to migrate to and coalesce with somatic gonad precursor cells. Instead, the germ cells scatter throughout the posterior half of the embryo. Few, if any of these germ cells reach the gonads. Consequently, approximately 70\% of the adults are sterile (Coffman et al., 2002). Thus, the migration of the germ cells is unable to deliver these cells to the gonad and additionally cell death of these ectopic germ cells is disrupted (Figure 2.1B). The \textit{sctt} allele shows a maternal effect, the mother must be homozygous for the mutation to observe a phenotype in the offspring. One maternal wild-type copy of the gene defective in \textit{sctt} in the mother is sufficient to completely rescue the germ cell migration process (Figure 2.1D). Additionally, in embryos from a homozygous \textit{sctt} mutant female, germ cell migration can be rescued through a paternally-supplied wild-type copy of the \textit{sctt} gene (Coffman et al., 2002). Embryos derived from a cross between \textit{sctt}/\textit{sctt} females and a \textit{sctt}^{+} male fall into one of two phenotypes depending upon whether they inherit the wild-type X chromosome or the Y from
the male. The *sctt* maternal/*zygotic*+ population are rescued for germ cell migration (Figure 2.1C) while those embryos with the *sctt* maternal/*zygotic* − background result in a severe germ cell migration phenotype (Figure 2.1B). Careful germ cell counts were performed to analyze the number of germ cells that reach the gonads in *sctt* mutant backgrounds. These germ cell counts establish a baseline for the upcoming transgenic rescue experiments. Germ cell counts at 12-15 hours reveal on average, 14.7 germ cells reach the gonads in wild type embryos (Table 2.1). These numbers are in agreement with previously published results from other genetic backgrounds (Hay et al., 1988; Sonnenblick, 1941; Sonnenblick, 1950; Underwood et al., 1980). Only a small number of germ cells, 0.5, persist ectopic to the gonads (Table 2.1). While the total number of germ cells in *sctt* maternal/*zygotic* − embryos is within a wild type range, on average 1.0 or less total germ cells per embryo reach the gonads. Rescue of germ cell migration by a paternally supplied copy of *tre1* is complete, an average of 13 germ cells was observed in the gonads of *sctt* maternal/*zygotic* + embryos from the cross of *sctt/*sctt* females to a *sctt*+ male (Table 2.1). The *sctt* maternal/*zygotic*− embryos from the same cross averaged 0.3 germ cells in the presumptive gonads, and a large number of germ cells remained ectopic to the gonads, 16.6, compared to 7.0 within their paternally rescued siblings (Table 2.1). This data supports and extends previous findings that germ cell migration to the gonad in *sctt* mutants is severely hindered but is successfully rescued when embryos zygotically express a wild-type copy of *tre1* (Coffman et al., 2002). The severe phenotype is only observed when embryos are derived from a mother lacking a wild-type chromosome.
**The sctt mutation is an allele of tre1**

The sctt maternal/zygotic mutation’s impact on proper germ cell migration and programmed cell death in embryonic development suggests that the molecular defect causing this phenotype represents a severe loss of function. However, the molecular lesion causing the sctt mutation was unknown. It was known that the sctt defect was an X-linked mutation, as the original EMS mutagenesis screen was designed to specifically isolate X chromosome mutants (Coffman et al., 2002). To narrow down the location of the sctt mutation, the sctt mutant chromosome was tested for complementation by crossing it to the Bloomington series of X chromosome deletion stocks that were available (Coffman et al., 2002). The sctt chromosome complemented all available deletion stocks. Recombination mapping of sctt using a labeled X chromosome placed sctt within 1 map unit of the crossveinless locus at 5A13 (Coffman et al., 2002). The translocation Dp(1;Y)dx^+5 carrying genomic sequence from 4C11-6D8 of the X chromosome on the Y rescues the sctt cell migration phenotype, indicating the sctt lesion falls within this range. However, the Dp(1;Y)dx^+1 translocation of genomic sequence from 5A8-6D8 failed to rescue the sctt defect. The culmination of the genetic experiments narrowed the sctt region to polytene chromosome bands between 5A3-4 and 5A8-9 as predicted by polytene chromosome squashes, between complementing deletions Df(1)JC70 and Df(1)C149.

Concurrent studies by Kunwar et al. revealed that the sctt chromosome failed to complement a known mutant allele of tre1, known as ΔEP5 (Dahanukar et al., 2001; Kunwar et al., 2003). However, it remained unclear whether this failure to complement indicated that the mutants were allelic or if non-allelic non-complementation was occurring. While the genetic mapping data suggested it was possible that sctt was an allele of tre1, genomic
sequencing by Kunwar et al. found no evidence of a molecular lesion in the \textit{tre1} gene in the \textit{sctt} mutant background (Kunwar et al., 2003). In addition, it was determined that \textit{tre1} mRNA levels were not significantly down-regulated in the \textit{sctt} background, arguing that the \textit{sctt} lesion was not resulting in a large change in \textit{tre1} transcription as might be expected for such a severe loss of function allele (personal communication, Camilla Burnett and Ken Howard). In an attempt to define the nature of the \textit{sctt} mutation, the entire coding region of \textit{tre1} was sequenced. A single adult fly was harvested and used as a genomic template. Both \textit{sctt} and wild type control flies were used. Primers directed at exons 2-7 of \textit{tre1} were used in PCR to amplify and subsequently sequence this region. Nearly 2000 base pairs were sequenced on both DNA strands and a single base pair substitution was observed between \textit{sctt} and the wild type control. An adenine within intron 4 was mutated to a thymine (Figure 2.2A).

\textbf{The \textit{sctt} defect is due to a mutated splice acceptor site in intron 4 of \textit{tre1}}

The single base pair change of an adenine to a thymine within intron 4 would not directly impact coding of the \textit{tre1} amino acid sequence. However, it was predicted that this single base pair change may interrupt the preferred splice acceptor site and impact proper splicing of the \textit{tre1} RNA product. The single base pair change altered the intron 4 splice acceptor site from AG to TG. Reverse transcriptase PCR and sequencing of the cDNA product was used to test this altered splicing hypothesis. To target a time frame of expression consistent with \textit{tre1}’s affect on germ cell migration within the embryo, a collection of 0-8 hour old \textit{sctt} and wild type embryos were used to isolate RNA templates. Reverse transcriptase PCR primers were designed to amplify the cDNA region surrounding the predicted altered splicing location. Splicing within the wild type control occurred as
predicted, all introns were properly spliced at the correct junctions (Figure 2.2A). With the 
scott template, intron/exon junctions through exon 4 were correctly spliced. However, directly 
following the intron 4 mutation within the splice acceptor site, the next 24 base pairs were 
missing from exon 5. The next suitable splice acceptor site, AG in the sequence is used. 
This change in splicing results in the deletion of 8 amino acids, RYILIACH, from the 
protein. The remainder of the protein proceeds in frame (Figure 2.2B). The deletion impacts 
the junction of the third transmembrane and the second intracellular loop of the GPCR’s 
heptahelical structure. Secondary structure analysis programs predict that while the deletion 
impacts the length of the second intracellular loop, the remainder of the protein structure 
remains intact (Hirokawa et al., 1998; Krogh et al., 2001; von Heijne, 1992). The removal of 
these 8 amino acids, including 2 residues of the highly conserved DRY motif of rhodopsin 
family GPCRs, from the total 392 amino acids constituting Tre1 has a significant impact on 
the function of this GPCR. This study definitively shows the scott is an allele of the tre1 gene 
and will now be referred to as tre1scott.

Identification of critical Tre1 amino acid residues in germ cell migration

Germ cell migration is severely affected in tre1scott mutants. While the molecular basis 
for this mutation was identified, little was known about the specific reason the deletion of 8 
amino acids had such a large effect on the ability of the Tre1 GPCR to function. Amino acid 
sequence comparisons of this 8 amino acid stretch revealed conserved residues among Tre1 
and human GPCRs (Figure 2.2C). To further elucidate the nature of the tre1scott defect and 
gain insight into the roles of these amino acids not only on tre1 function but in other GPCRs 
with high amino acid conservation to Tre1 in this region, structure/function analyses were 
undertaken where specific amino acid replacements with alanine were created. The T^G+
construct contains 10 kb of Drosophila genomic sequence including the sequence for *tre1* and an adjacent gene, *Gr5a* (Dahanukar et al., 2001). The T⁺G⁺ vector was previously shown to rescue the germ cell migration defect of a mutant allele of *tre1*, *tre1ΔEP5* (Kunwar et al., 2003). The T⁺G⁺ construct was modified to create a cassette containing an AloI restriction enzyme site within the genomic region encoding *tre1*. Upon digestion of the construct with AloI, oligonucleotides encoding variations within the RYILIACH region were inserted and tested for rescue of germ cell migration within the context of the developing embryo.

Multiple construct-bearing transgenic flies were created through microinjection of the various constructs into the standard w¹¹¹⁸ fly stock. Transgene insertion sites were identified using inverse PCR. Balanced transgenic stocks were generated for each of the P-element lines. Flies containing each transgene were crossed into the *tre1sctt* mutant background. Because it had been established that the *tre1sctt* is maternal effect and that one maternal wild type copy of *tre1* is sufficient to completely rescue germ cell migration (Figure 2.1D) (Coffman et al., 2002), females homozygous for *tre1sctt* bearing 1 or 2 copies of the transgene were crossed to *tre1sctt* males to assay the effectiveness of the transgene to rescue *tre1sctt* germ cell migration. As a positive control to confirm that addition of the 8 endogenous amino acids rescues germ cell migration, the original T⁺G⁺ vector was first assayed. In embryos from *tre1sctt/ tre1sctt*, T⁺G⁺ mothers, germ cells successfully reached the two somatic gonad precursor cell populations and coalesced to form the gonads (Figure 2.3). An average of 23 germ cells in the gonads were observed, compared to an average of 0.3 germ cell detected in the gonads of embryos from *tre1sctt/ tre1sctt* females lacking any transgene (Supplemental Table 2.2, Figure 2.4). These values are statistically different (P<0.0001, Student’s t-test) Reconstruction of the *tre1sctt* amino acid deletion was tested to confirm that a construct
lacking the 8 amino acids would be unable to rescue the \textit{tre1^{sctt}} defect. As predicted, the \textit{tre1^{sctt}} reconstruction allele lacking RYILIACH was unable to rescue the \textit{tre1^{sctt}} defect (Figure 2.3). As in \textit{tre1^{sctt}}/\textit{tre1^{sctt}} maternal/zygotic embryos, those embryos that also contained the \textit{tre1^{sctt}} reconstruction construct resulted in germ cells with no directed migration to gonads, germ cells were scattered throughout the posterior half of the embryo. A wild type number of germ cells were counted within the embryo. However, an average of only 1.4 germ cells reached the gonads, statistically similar to the no transgene control (P>0.05, Student’s t-test) (Supplemental Table 2.2, Figure 2.4).

The loss of RYILIACH from the Tre1 protein severely affects on the ability of germ cells to migrate. However, it was unclear whether this defect was due to the loss of a specific amino acid(s) or whether the deletion of these amino acids resulted in secondary structure defects elsewhere in the protein. To identify the critical amino acids within this deletion, constructs were designed with combinations of the original amino acids and alanine substitutions. If the Tre1 protein is dependent on any of these specific amino acids for proper germ cell migration, their replacement with alanine should fail to rescue the \textit{tre1^{sctt}} phenotype. All amino acids were systematically replaced with alanine to determine whether the amino acids were important or if the defect was caused by a spacing issue disrupting the structure of the protein. The RY AAA AAA construct was designed to test the role of the 6 amino acids, IILIACH, that are directly downstream of RY of the highly conserved DRY triplet.

Two independent RY AAA AAA transgenic insertion sites were tested to control for possible affects of transgene placement within the genome on the ability of the transgene to be expressed. The two RY AAA AAA constructs behaved in a similar manner, both rescuing the germ cell migration defect of \textit{tre1^{sctt}} mutants (Figure 2.3). An average of 20.9
and 20.4 germ cells were observed in the gonads of the two lines (Supplemental Table 2.2, Figure 2.4). Both lines were significantly different in their ability to rescue germ cell migration compared to the no transgene control (P values <0.0001, Student’s t-test). This suggests that RY of the conserved motif is sufficient to rescue germ cell migration and that the 6 amino acids immediately downstream of Arg-Tyr are not necessary for successful rescue of the germ cell migration phenotype.

A second construct, RY AAA ACH, which replaces the hydrophobic ILI with 3 alanines also confirms these 3 amino acids are not critical for germ cell migration (Figure 2.3). This construct was able to rescue germ cell migration with an average of 16.2 germ cells in the gonads (Supplemental Table 2.2, Figure 2.4). The RY AAA AAA constructs and to some extent the RY AAA ACH construct results, are suggestive of a critical role for the Arg and/or Tyr in the Tre1 GPCR function.

To test the specific role of both the Arg and Tyr of the conserved DRY motif of rhodopsin family GPCRs in germ cell migration, constructs were designed in which either the Arg or the Tyr was individually replaced by an alanine. Alanine replacement of the tyrosine of this motif was still able to rescue germ cell migration (Figure 2.3). Rescue was complete as the same number of germ cells reached the gonads as in the rescue construct, the positive control (Supplemental Table 2.2, Figure 2.4). When the Arg was replaced with an alanine, Tre1 function was not restored. An average of 0.4 and 1.1 germ cells in the gonads were documented (Supplemental Table 2.2, Figure 2.4). The phenotype appears similar to the tre1sctt severe mutants lacking any transgene and the sctt reconstruction construct that fails to rescue the defect (Figure 2.3). This result defines a role for the endogenous arginine in proper germ cell migration of Drosophila germ cells.
Cumulatively, this data demonstrates a clear role for the R of the highly conserved DRY motif in proper germ cell migration of Drosophila germ cells.

**Discussion**

The Tre1 GPCR has been previously linked to the migration of *Drosophila* germ cells through the posterior midgut epithelium (Kunwar et al., 2008; Kunwar et al., 2003). While the *tre1*Δ*EP5* mutation results in the majority of germ cells trapped within the epithelial layer surrounding the midgut, the *tre1*sc2 allele results in a greater number of germ cells able to cross through the midgut. However, germ cell migration is still severely defective (Coffman et al., 2002; Kunwar et al., 2003). We conclude that *tre1*sc2 represents a severe loss of function allele of *tre1*. This study shows that within *tre1*sc2 maternal/zygotic embryos, on average, ≤ 1 germ cells correctly locate and coalesce with the somatic gonad precursor cell population to form the gonad. This is compared to approximately 14.7 total germ cells that reach the gonads in the wild-type control.

Previous studies had revealed genetic evidence supporting the notion that the *tre1*sc2 mutation was a partial loss of function allele of *tre1* (Kunwar et al., 2003). Genomic sequencing results presented in this study revealed a single base pair change within the fourth intron that affects the splice acceptor site of that intron. This AG to TG mutation abolishes correct splicing and an alternate downstream splice acceptor site is used, resulting in the loss of the first 24 base pairs of exon 5. Further analysis indicates that in total, 8 amino acids, RYILIACH, are missing from the third transmembrane/second intracellular loop junction of the Tre1 GPCR while the rest of the protein proceeds in frame. Secondary structure
prediction programs calculate a shortening of the second intracellular loop but the remainder of the secondary structure is expected to be unaffected.

The identification of the small deletion causing the \( tre1^{sctt} \) phenotype prompted further investigation into this region of the GPCR. Through the design of a modular cassette vector containing the \( tre1 \) genomic sequence, the region encoding these 8 amino acids was able to be manipulated to insert amino acid substitutions in this region. Transgenic flies were created using these constructs and their ability to maternally rescue the \( tre1^{sctt} \) defect was assayed in the context of developing embryos. All of the amino acids deleted in the \( tre1^{sctt} \) mutation were systematically tested for functionality using an alanine scan approach. Through this replacement approach, it was discovered that the 6 amino acids following the RY were dispensable for function as the RYAAAAAAA construct restored germ cell migration back to wild type levels, indicating a rescue the \( tre1^{sctt} \) germ cell migration defect.

The latter two amino acids of a highly conserved amino acid triplet within this region, the DRY motif, are missing in \( sctt \) mutants. The tyrosine was found to be dispensible for germ cell migration. A construct containing an alanine replacement of this amino acid was still able to rescue the \( tre1^{sctt} \) germ cell migration defect when supplied maternally. Interestingly, it was found that the construct that replaced the first amino acid deleted in \( tre1^{sctt} \), arginine, with an alanine was unable to maternally rescue the \( tre1^{sctt} \) defect. In this cross, germ cells were unable to reach the somatic gonads and a \( tre1^{sctt} \)-like phenotype was observed. Therefore, the arginine appears to be necessary for proper function of Tre1 in the migration of germ cells. This arginine of the highly conserved DRY motif located at the cytoplasmic side of transmembrane 3 is considered the single most conserved amino acid residue in rhodopsin family GPCRs (Rosenkilde et al., 2005). Sequence alignments with a
variety of human rhodopsin family GPCRs involved in cell migration supports the highly
conserved nature of this residue (Figure 2.2C). Rhodopsin family GPCRs are a set of highly
diverse GPCRs both in their ligand binding ability and their elicited responses. In many
GPCRs, regardless of their ultimate function, the signal transduction has been shown to be
defective when the arginine of the DRY motif has been compromised (Scheer et al., 1996;
Zhu et al., 1994). Some studies have suggested that arginine can directly bind G proteins
(Acharya and Karnik, 1996). However, the vast majority of literature suggests that arginine
is involved in the stability of receptor conformation, particularly in holding the receptor in its
inactive state (Angelova et al., 2002; Ballesteros et al., 1998; Ballesteros et al., 2001;
Greasley et al., 2002; Shapiro et al., 2002; Zhang et al., 2005). Although some research
suggests the arginine could hold receptors in an active state as well (Flanagan, 2005). This
arginine is considered by some to be the most critical residue in signal transduction of
rhodopsin family GPCRs (Ballesteros et al., 1998; Oliveira et al., 1994; Scheer et al., 1996).
Cell culture experiments using nonconservative mutations of this residue have been found to
most commonly result in defective signal transduction from the receptor (Jones et al., 1995;
Scheer et al., 1996; Zhu et al., 1994). The tyrosine residue of the DRY motif is the least
conserved of the three within the motif with cysteine, histidine, and serine also commonly
found in this location (Rovati et al., 2007). Multiple cell culture studies have investigated the
role of this tyrosine in receptor function and have found that it has little or no role in receptor
function (Arora et al., 1997; Auger et al., 2002; Gaborik et al., 2003; Hawtin, 2005; Lu et al.,
1997; Ohyama et al., 2002; Rhee et al., 2000; Zhu et al., 1994). The results presented here
are consistent with these cell culture studies as replacement of the tyrosine with an alanine
did not inhibit the ability of the Tre1 GPCR to function in germ cell migration. While much
research has gone into the effects of amino acid alterations of the DRY motif in cell culture systems, their effects have not been studied within an intact organism. The arginine of this motif, while not completely disabling Tre1 function, severely impacts the ability of this GPCR to function in germ cell migration within the intact Drosophila embryo.

A common theme emerging in cell migration is the use of ligands such as chemokines and phospholipids as attractants for cells to specific locations. These ligands activate GPCRs on the receiving cell’s surface to initiate a migratory response in the direction toward higher expression levels of the agonist. GPCR function has been identified as having critical roles in the directed migration of a variety of cell types. The GPCR receptors S1P 1-4 recognize the phospholipid sphingosine-1-phosphate. Sphingosine-1-phosphate has been implicated in the process of lymphocyte recirculation and tissue homing critical in adaptive immunity response (Matloubian et al., 2004). Additionally, the SDF-1/CXCR4 ligand-GPCR pair has emerged as a conserved mechanism regulating a variety of cell migrations in cancer, immune response, and in development. In breast cancer, it has been found that secondary site tumor colonization due to cancer metastasis is not random in its selection of secondary sites but rather is due to direct migration of CXCR4-expressing cancer cells responding to the SDF-1 ligand at specific locations in the body. These locations include lungs and bone marrow, common secondary sites for breast cancer (Muller et al., 2001). This common ligand/receptor pair has also been found to play a role in lymphocyte trafficking of the immune system and leukocyte trafficking to sites of infection. The conserved molecular mechanism involving the CXCR4/SDF-1 receptor/ligand pair has been identified in mouse, chick, and zebrafish germ cell development. (Ara et al., 2003; Doitsidou et al., 2002; Dumstrei et al., 2004; Knaut et al., 2003; Molyneaux et al., 2003; Stebler et al., 2004). As in
Drosophila, the germ line stem cells in these vertebrates must navigate through multiple cell types and substrates to reach their ultimate destinations, the gonads (Molyneaux and Wylie, 2004; Raz, 2004; Santos and Lehmann, 2004).

Tre1 represents a member of a GPCR family with critical roles in the migration of cells (Figure 2.2C). Our understanding of the molecular details by which these GPCRs convert extracellular signals into intracellular signal transduction pathway(s) leading to both motility and directed migration to targets is incomplete. Use of the well-studied migratory movements of Drosophila germ cells provides an excellent system in which the study structure/function of GPCRs. This study provides conclusive evidence that the arginine of the highly conserved DRY motif at the start of intracellular loop 2 is critical for GPCR-mediated germ cell migration within the context of an intact, living organism. Given the highly conserved nature of this arginine residue in other GPCRs and its identification as a critical residue from cell culture studies, it is likely that it is critical to the function of many other GPCRs that transduce extracellular signals into migratory movements in a wide variety of cell types and organisms.

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CHAPTER 3. IDENTIFICATION OF ADDITIONAL COMPONENTS OF DROSOPHILA GERM CELL MIGRATION AND PROGRAMMED CELL DEATH

Research performed with the assistance of Margaret Pruitt and Deb Czarnecki

Introduction

Characteristic of many other organisms, the germ cells of Drosophila melanogaster are sequestered from the somatic cells of the embryo upon their formation (Boyle and DiNardo, 1995; Santos and Lehmann, 2004). In order to function, the germ cells must embark on a significant migration in order to reach their ultimate target, the somatic gonad precursor cells, to form the gonads of the organism. The migratory movements of Drosophila melanogaster germ cells have been extensively characterized (Campos-Ortega and Hartenstein, 1997; Santos and Lehmann, 2004; Sonnenblick, 1941; Sonnenblick, 1950; Starz-Gaiano et al., 2001). Initially, the germ cells are passively swept into the midgut through the movements of gastrulation and germ band extension. The active migratory movements of the germ cells are divided into phases of migration beginning with migration through the epithelial layer surrounding the midgut around stage 10 of development. This initial step is followed by: orienting dorsally on the basal side of the midgut, bilaterally segregating into two populations in the mesoderm, associating with the somatic gonad precursor cells, and finally gonad coalescence. Additional research has led to the identification of genes involved in these distinct processes and is reviewed in detail in Chapter 1. While the list of genes involved has provided much insight into the signaling
pathways through which germ cells are able to complete the multiple steps of migration, the story is far from complete.

For the transepithelial migration of germ cells, it is known that a G protein-coupled receptor encoded by *tre1* is required cell autonomously (Kunwar et al., 2008; Kunwar et al., 2003). Tre1 prepares germ cells for migration by controlling the relocalization of cell-cell adherens proteins from uniform distribution on the cell membrane to concentration at the lagging tail. In this way, germ cells become polarized, lose their tight association with each other, separate from the clump of germ cells and finally migrate through the epithelial layer surrounding the midgut (Kunwar et al., 2008). Additionally, it has been found that *rho1* acts downstream of *tre1* in this process (Kunwar et al., 2003). The G proteins Gβ13f and Gγ1 are also required for these processes. Mutants in these genes result in embryos with a *tre1* loss of function mutant phenotype, germ cells trapped within the midgut (Kunwar et al., 2008).

Two alleles of *tre1* mutants have been investigated. In *tre1ΔEP5* mutants no *tre1* transcript is detected in the germ cells until after wild-type germ cells are normally crossing the midgut epithelium (Kunwar et al., 2003). The germ cells are unable to pass through the epithelial layer and remain trapped within the midgut. In an additional allele, *tre1scct*, the germ cells are also unable to reach the gonads however, in *tre1scct* embryos, most germ cells exit the posterior midgut, but then scatter throughout the posterior half of the embryo (Coffman et al., 2002). While it is not uncommon for germ cells in wild type embryos to fail to reach their target, usually these ectopic germ cells are eradicated from the embryo by a currently uncharacterized form of programmed cell death. In *tre1scct*, not only do these germ cells mis-migrate but they also fail to carry out the cell death program and thus they persist in ectopic locations (Coffman et al., 2002). It is apparent that this GPCR has critical roles in the cell
autonomous signal transduction that must take place in order for the germ cells to successfully migrate and to commit cell death. However, very little is known regarding the molecular components downstream of this receptor. Additionally, the components of the remaining germ cell migration stages remain highly uncharacterized.

To elucidate the players downstream of the \textit{tre1} GPCR, a genetic screen of available P-element insertion stocks was performed. The pool of P-element insertion stocks tested was compiled through multiple rationales. First, P-elements were selected that could possibly impact function of genes that were involved in signaling downstream of GPCRs in other organisms and cellular systems. Additionally, targeted genes were selected based on their involvement in cell migration and/or cell death in other systems. And finally, gene expression profiles were investigated, if publically available, to see if the gene was expressed at a temporal and spatial time conducive for functioning in the germ cell migration process.

To investigate the mechanism through which Drosophila germ cell death occurs, a gene representing a common family of apoptotic proteins was tested. The gene \textit{dredd}, encodes a caspase. Caspases are cysteine-aspartic acid proteases that act to cleave proteins in apoptosis, one of the most common forms of cell death. This form of cell death is characterized by condensation of the nucleus and cytoplasm, fragmentation of cellular components, budding off of cellular membrane vesicles, and subsequent uptake by phagocytes to complete the cellular degradation process (Baehrecke, 2003). In apoptosis, caspases act to cleave proteins that lead to the death of the cell. Activation of cell death via caspases is a two step process. First, initiator caspases are processed into active forms that cleave effector caspases, thus activating them. Second, the effector caspases function to degrade the cell (Cashio et al., 2005). A stock containing a P-element insertion near the end
of the first exon of *dredd* was tested to determine whether *dredd* may be involved in the programmed cell death of germ cells.

An emerging theme of signal transduction downstream of GPCRs is signaling cascades initiated by the phosphorylation of the receptor. It has been established that intracellular and C-terminal tail phosphorylation is critical for GPCR sensitization but also has been implicated in the recruitment of additional proteins to the intracellular surface of the GPCR. First, recruitment of interactors will be discussed. Protein kinases and more specifically, GPCR kinases (GRKs) are able to phosphorylate receptors on specific tyrosine, serine and theonine residues (Gether, 2000). This phosphorylation can lead to G protein-independent signaling cascades downstream of GPCRs. One way in which this is done is through the SH2/SH3 family of adaptor proteins. Adaptor proteins carrying the SH2 binding domain are able to bind GPCRs via the docking site created by a phosphorylated tyrosine. The phosphorylation acts as a switch, SH2 domains are unlikely to bind proteins that lack the phosphotyrosine. Once bound to the receptor via its SH2 domain, the SH3 domain is able to recruit additional proteins to form a complex (Pawson et al., 2001). Four genes encoding SH2/SH3 adaptor proteins were targeted for investigation into potential roles in germ cell migration and/or programmed cell death. These included: *downstream of receptor kinase* (*drk*), *dreadlocks* (*dock*), *daughter of sevenless* (*dos*), and *crk*. P-element insertion stocks available from public stocks centers were tested for an embryonic germ cell phenotype.

The genes *src64b*, *c-terminal src kinase* (*csk*), and *GPRK2* encode Drosophila kinases. The Src family of kinases not only phosphorylate receptor tyrosines but also contain SH2 and SH3 binding domains for interaction with both the receptor and additional proteins. The *c-terminal src kinase* (*csk*) gene in Drosophila acts to negatively regulate src kinases
through phosphorylation of the c-terminal tail of Src protein while the Src protein can phosphorylate the receptor. P-element stocks with insertion sites near or within src64b and csk were tested for embryonic germ cell phenotypes. G protein receptor kinases also phosphorylate activated receptors. One of these, GPRK2 was also tested using two different P-element alleles.

In canonical GPCR signaling, the receptor conformation changes upon agonist binding. This conformational change allows the receptor to bind their respective G proteins. Activation of the G proteins involves the replacement of GDP bound to the inactive G protein with GTP. At that point, the GTP bound alpha subunit separates from the beta-gamma subunits. Signaling pathways are then activated (Karnik et al., 2003). The Rho GTPase family of G proteins has been shown to be involved in signaling cascades leading to a wide variety of functions including: actin and microtubule dynamics, gene expression, cell division, motility, cell polarity, adhesion, phagocytosis, and membrane transport (Buchsbbaum, 2007; Jaffe and Hall, 2005). It was suggested that Rho1 may be downstream of tre1 GPCR signaling as mutants of this gene display a phenotype similar to that of tre1^AEPS GPCR mutants (Kunwar et al., 2008; Kunwar et al., 2003). The transition of G proteins between GTP-bound and GDP-bound states is facilitated by two types of proteins; guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs promote the exchange of GTP for GDP, activating signaling, while GAPs facilitate GTP hydrolysis leading to GTPase inactivation. A Rho GTPase GEF, known as trio was targeted in the P-element insertion screen in order to determine whether it had a role in germ cell migration and/or programmed cell death.
A very critical component of GPCR signaling is desensitization of the receptor so as to prevent continued signaling through the activated receptor. The first step in this process involves phosphorylation of the receptor. This results in rapid desensitization of the receptor presumably by causing steric inhibition of GPCR interactions with its cognate G proteins. Additionally, this phosphorylation acts to recruit arrestins to the GPCR, which uncouples G proteins from the receptor (Benovic et al., 1987; Lohse et al., 1990; Pippig et al., 1993). Following arrestin binding, GPCRs are internalized via clathrin-coated pits (Ferguson et al., 1996; Ferguson et al., 1995). Once internalized, the GPCRs are either sent back to the membrane or are degraded by lysosomes (Anborgh et al., 2000; Krueger et al., 1997; Li et al., 2000; Pippig et al., 1995; Seachrist et al., 2002; Trejo and Coughlin, 1999). To investigate whether GPCR desensitization was critical for proper germ cell migration and/or programmed cell death, P-element alleles were tested for phenotypes. P-element insertions near or within three genes that were predicted to encode β-arrestin or proteins with arrestin-like N- and C-terminal domains were utilized. The kurtz gene encodes β-arrestin, while uncharacterized genes CG14696 and CG7047 have arrestin-like domains. A small Ras-like G protein, Rab5 is a critical component of the formation of the clathrin-coated pit. It resides in the membrane, in clathrin-coated vesicles as well as in the early endosomes and is involved in receptor endocytosis (Bucci et al., 1992). For this reason, rab5 P-element insertion alleles were examined for a role in germ cell migration and programmed cell death.

A recent paper from our lab identified Drosophila p53 as having a critical role in germ cell death (Yamada et al., 2008). Knowing that p53 was critical for germ cell death, I scanned available literature in an attempt to make a connection between the tre1 GPCR and p53. I identified a possible connection between arrestins and p53. GPCR signaling mediated
by arrestin and p53 attenuation mediated by the E3 ubiquitin ligase, Mdm2, are connected through binding of β-arrestin to Mdm2 (Wang et al., 2003). GPCRs may potentially regulate p53-mediated cell death through this interaction (Wang et al., 2003). In fact, overexpression of arrestin can increase levels of apoptosis by antagonizing, Mdm2, the negative regulator of p53 (Wang et al., 2003). Unfortunately, no homolog of mammalian Mdm2 has been identified in Drosophila. Therefore, the Drosophila genome was searched for other E3 ubiquitin ligases. Two of these were tested; neuralized and CG8184.

The ligand for the tre1 GPCR is currently unknown. One of the many ligands for the GPCR family is prostaglandin. A necessary step in the production of prostaglandin is peroxidation of arachidonic acid. In an attempt to identify critical components of ligand production for Tre1, Drosophila peroxidases were targeted. Two particular peroxidases displayed an RNA expression pattern very convincingly expressed in germ cells. This thioredoxin peroxidase, jafrac1, and the peroxidase, pxt, were added to the P-element screen list.

In this chapter, the results of a P-element allele screen for phenotypes defective in germ cell migration and programmed cell death are presented.

**Materials and Methods**

**Fly stocks**

Flies were maintained on standard media at 25 °C. Most stocks screened in this paper were obtained from the Bloomington Stock Center. The krz1 and krz2 alleles were a kind gift from G. Roman (Roman et al., 2000).
Embryo collections

Embryos were collected on standard apple juice plates at room temperature for 3 hours and aged 12-15 hours. Embryos were harvested and then dechorionated using a 50% bleach solution.

Antibody staining of germ cells

Immunostaining was performed as described in Johansen and Johansen (Johansen and Johansen, 2004). Embryos were fixed in 4% paraformaldehyde and devitellinized by shaking in heptane/methanol. The primary antibody used was Chicken anti-Vasa, (a gift from Ken Howard, 1:10,000) and the secondary antibody used was biotinylated anti-chicken IgG (1:500). Antibody detection was performed using the ABC Elite Kit (Vector Laboratories) and 3,3’-diaminobenzidine tetrahydrochloride as a substrate.

X-Gal staining

Following embryo collection, the embryos were washed with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.3% Triton X-100, pH 7.2; (Sambrook, 1989) and fixed for 10 minutes using a 2.5% glutaraldehyde-saturated heptane fixative (Holmes et al., 1998; Simon et al., 1985). Following fixation, embryos were washed with PBST for 4 hours. Staining was performed with a .08% X-Gal solution for 2 hours at 37 °C.

Mutant scoring

Embryos were observed under a dissecting microscope. The phenotype of each embryo was recorded. Embryos containing more than 3 germ cells ectopic to the gonads
were considered mutant for programmed cell death. Embryos in which germ cells failed to populate the gonads were scored as cell migration mutants.

**Balancer test crosses**

Some of the mutant stocks that produced a mutant phenotype were over a balancer chromosome as the mutant chromosome was homozygous lethal. Each chromosome was isolated in these balanced stocks to test which chromosome was producing the mutant phenotype. Males carrying one copy of the mutant chromosome and one copy of the balancer chromosome (mutant/balancer) were obtained. These males were crossed to homozygous wild type females carrying the \( P^{w^+}, \text{fat facets-lacZ} \) transgene that allowed visualization of germ cells through X-Gal staining (Fischer-Vize et al., 1992). Progeny from the resulting cross were used for two independent crosses. Flies carrying the mutant chromosome were selected based on the lack of balancer phenotype. Males carrying the mutant chromosome were crossed to females of the same genotype and their progeny were assayed. The second cross was set up between males and females both carrying the balancer chromosome and thus lacked the mutant chromosome. These progeny were assayed for a mutant phenotype.
Results and Discussion

Testing for additional components of germ cell migration and/or programmed cell death

To test for a gene’s involvement in Drosophila germ cell migration and/or programmed cell death, P-element insertions were selected in or near genes potentially mediating these processes. Embryos were collected from the stocks and antibody stained using an anti-vasa antibody to label germ cells. The percentage of mutant embryos was calculated. Of the 25 alleles tested, 10 produced significant germ cell phenotypes. Mutant heterozygous lines were considered significant if greater than 12% of its progeny were mutant, this would represent a 50% penetrant mutant phenotype as only ¼ of embryos from this cross would be homozygous mutant. For homozygous mutant lines, 30% of their offspring with a mutant phenotype was considered significant. The results are summarized (Table 3.1) and the resulting mutant phenotypes of some of the lines are shown (Figure 3.1).

The P-element allele inserted in the caspase dredd did not produce a mutant phenotype. This is in agreement with other studies that show caspase-dependent forms of cell death are not the predominant endogenous programmed death mechanism of Drosophila germ cells. It has been shown that Drosophila germ cell death does not appear to involve some of the key players in caspase-mediated cell death including pro-apoptotic genes grim, reaper, hid and the pro-survival genes diap1, diap2, and p35. Additionally, decreased expression of another initiator caspase, Nc/Dronc does not affect the death of germ cells (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005).
The majority of the P-element lines producing a mutant phenotype were selected based on potential disruption of genes with documented roles downstream of GPCRs. The SH2/SH3 protein family was tested to determine whether adaptor proteins might required for signal transduction downstream of Tre1. If adaptor proteins are required for cell migration/cell death or both, disruption of the gene may result in an impaired or loss of function mutant phenotype. Six stocks representing 4 different SH2/SH3 adaptor proteins were tested, but only one produced a mutant phenotype. When embryos from the P-element insert just 5’ of *crk* were assayed, a cell death phenotype was observed.

In order for SH2/SH3 adaptor proteins to bind, specific receptor tyrosines must be phosphorylated (Pawson et al., 2001). One group of proteins involved in phosphorylation of receptor tyrosines are the Src family of tyrosine kinases. The *src64h* gene encodes a Src kinase and a P-element insertion in intron 1 of this gene was tested for a germ cell migration and/or programmed cell death phenotype. If this Src kinase is responsible for the creation of phosphotyrosines on Tre1, we could expect that a defect in this gene might lead to a partial or complete loss of function phenotype. However, no phenotype was observed in embryos from this mutant stock. A second kinase associated with this family of kinases, *csk*, has a slightly different role. The regulation of Src kinases is controlled by Csks as they act to phosphorylate the C-terminal region of these kinases, inactivating them. The affects of loss of a negative regulator of kinases could completely inhibit signal transduction if phosphorylation blocks the signaling cascade or if proper switching between phosphorylated and unphosphorylated states are required to initiate distinct signaling cascades for germ cell migration and programmed cell death. This could potentially disrupt the phenotype in unknown ways. The P-element insertion near *csk* produced a mixed population of progeny.
The majority of the mutant embryos showed a programmed cell death defect while a significantly smaller subset of embryos had both a germ cell migration and programmed cell death defect indicating a potential role in the pathway leading to both processes. The two P-elements near the 5’ end of GPRK2 did not result in any observable mutant phenotype.

Rho family guanine nucleotide exchange factors (GEFs) facilitate the activation of G proteins by assisting in their exchange for GTP. The prediction would be that if a Rho GEF was mutated, then activation of G proteins that could be critical in signal transduction would either not be activated or not activated as efficiently. A P-element insert near the start of a Rho GEF known as trio was assayed for a mutant phenotype and was found to have a germ cell death phenotype. It has been previously shown that Rho1 is involved in Drosophila germ cell migration (Kunwar et al., 2008; Kunwar et al., 2003). It is surprising; however, that the defect observed in these studies was in programmed cell death, not germ cell migration. It is possible that the mutant Rho GEF could be specific to the signaling of a different Rho family G protein other than Rho1 that is integral to the cell death pathway rather than germ cell migration.

Arrestins have long been implicated in receptor desensitization and more recently have been shown to act as scaffolds for the recruitment of proteins to the bound receptor. One non-visual arrestin and two genes with arrestin-like domains were investigated for roles in germ cell migration and/or programmed cell death. If arrestin binding is critical for proper signal transduction, then the disruption of arrestin function could lead to the inability for germ cells to correctly migrate and perform cell death. The insertion of a P-element 5’ to the start of CG14696 produced embryos with an interesting phenotype. Germ cell migration to the gonads appeared normal, as did the programmed cell death of ectopic germ cells.
However, one gonad was abnormally localized. The mutant embryos displayed a “diagonal gonad” phenotype in which one gonad was located in the correct position while the second was displaced to the posterior. It is likely that this mutation affects the formation of the somatic gonad or patterning of the embryo as a whole. Three alleles of the β-arrestin, krz, were tested. One kurtz P-element insert allele, krz\(^{c01503}\), when mutated, resulted in a tre\(1^{scfr}\) phenotype as both germ cell migration and programmed cell death were consistently disrupted. The two other alleles, krz\(^{1}\) and krz\(^{2}\), initially produced germ cell death phenotypes however, these results were not able to be reproduced in subsequent staining of these stocks.

It is possible that the stocks were compromised in the time between the two stainings and had lost the mutant chromosome. These lines must be requested again and re-stained to confirm the initial mutant phenotype results.

A small G protein, Rab5, was also investigated to explore for a possible role in receptor desensitization. Three P-element insertion lines were tested but only one produced a potential germ cell death phenotype. Rab5 is localized to the plasma membrane and assists in the interaction of endocytic vesicles with the early endosome (Olkkonen and Stenmark, 1997). If receptor internalization is critical to the correct signaling of the GPCR, then disruption of this process would be expected to disrupt germ cell migration and/or programmed cell death. The one stock whose P-element insertion site is at the start of rab\(5\) coding produced a germ cell death defect.

Two P-element inserts near genes coding for E3 ubiquitin ligase activity, neur and CG8184, resulted in defective programmed cell death while germ cell migration appeared normal. The initial hypothesis was that E3 ubiquitin ligases could provide the link between arrestin-bound GPCRs and p53-mediated cell death. In this model, if the E3 ubiquitin ligase
is not present, then the link between GPCRs and cell death does not occur and cells survive. However, it is also possible that the ubiquitin-mediated degradation pathway is necessary for the act of cell killing through degradation of proteins. E3 ubiquitin ligases act to transfer ubiquitin to proteins targeted for degradation.

Prostaglandins are one of the many molecules that have been shown to be a ligand for GPCRs (Bos et al., 2004). One of the critical steps in the conversion of arachidonic acid to a prostaglandin intermediate is peroxidation (Tootle and Spradling, 2008). The ligand for Tre1 is currently unknown. To test the hypothesis that prostaglandins may be a ligand for Tre1, potential components of the prostaglandin synthesis pathway were investigated. The Drosophila genome was searched for peroxidases and two were discovered with a very striking germ cell expression pattern. A P-element insertion 5’ of the peroxidase, *pxt*, was chosen as well as an insert near the start of the thioredoxin peroxidase gene, *jafrac1*. The original hypothesis was the synthesis of prostaglandin was important for activation of Tre1 and the prediction was that removing a protein involved in synthesis of prostaglandin would inhibit activation of the receptor and thus both germ cell migration and programmed cell death would be disrupted. The *pxt* allele did not produce a germ cell phenotype. The P-element insert near *jafrac1* caused a very severe programmed cell death defective phenotype. The implication for a role of a thioredoxin peroxidase in germ cell death is currently unknown.

**Testing balancer chromosomes for mutant phenotypes**

Of the 10 mutant stocks producing phenotypes, 4 were from a homozygous stock suggesting the defect is due to the mutant chromosome. However, the 6 other lines were
heterozygous, making it necessary to test whether the balancer chromosome is causing the defect observed. To test this, crosses were set up between mutant males carrying one copy of the mutant chromosome and a copy of the balancer with a wild type female for three of the mutant lines (Figure 3.2A). The progeny were collected and those flies carrying the balancer chromosome were crossed to flies of the same genotype. In addition, those flies with the mutant chromosomes were also crossed. In this way, we were able to see if the mutant phenotype segregated with one of the above crosses. It was discovered that the mutant phenotype identified in the \textit{krz} mutant convincingly associated with the balancer chromosome, indicating that the mutant phenotype was most likely due to the balancer chromosome rather than the actual P-element insertion near \textit{krz} (Figure 3.2B). In the \textit{crk} mutant line, the phenotype also associated with the balancer chromosome, however, the percentage of mutant embryos in the balancer cross were still around wild-type levels (Figure 3.2B). The original calculation of the percent of mutant offspring in the P-element insert near \textit{csk} was abnormal. In a heterozygous stock, one would expect at most 25% of the offspring to be of a mutant phenotype, assuming the mutant allele is recessive. However, in the \textit{csk} mutants, nearly 63% of offspring had either a germ cell death or a germ cell migration and programmed cell death phenotype when only 25% was expected. Interestingly, the balancer and mutant crosses revealed that the germ cell death phenotype followed both chromosomes, suggesting that two separate phenotypes were possibly being produced, one from the balancer and the other from the \textit{csk} mutant chromosome (Figure 3.2B).
Controls required to validate preliminary observations

These preliminary results identify potential components of the Drosophila germ cell programmed cell death pathway. These include: *csk, trio, CG7047, rab5, CG8184, neur,* and *jafrac1.* Interestingly, the majority of P-element insertion stocks with a mutant phenotype produced a germ cell death defect while germ cell migration appeared normal. Additional research must be performed in order to validate these results. In total, 3 heterozygous mutant stocks were tested to determine which chromosome the defect was associated with, and it was discovered that balancer chromosomes can often produce abnormal germ cell phenotypes. Three other heterozygous stocks must be validated in this way. Those stocks over a balancer that are producing a mutant phenotype could be crossed into a different balancer background and again assayed for a germ cell defective phenotype to rule out a balancer effect.

For all stocks producing a mutant phenotype, outcrossing should be performed to ensure that the locus causing the defect is indeed located on the mutant chromosome and not elsewhere in the genome. Outcrossing would isolate the mutant chromosome and place it in a new genetic background with respect to the other 3 chromosomes in the Drosophila genome. These mutant stocks would be stained again to ensure that the phenotype was associated with the mutant chromosome.

Additionally, careful germ cell counts must be done in order to determine the severity of the programmed cell death defect, as well as to determine whether the number of germ cells reaching the gonads is consistent with wild type germ cell migration.

These mutants were selected in an attempt to find germ cell autonomous genes with roles in the germ cell migration and/or programmed cell death processes. However, we
currently cannot exclude the possibility that the defects are not germ cell specific but rather are caused by disruption of somatic cells involved in these processes. It seems likely that the defect in the \textit{CG14696} arrestin-like gene is caused by an overall embryo patterning defect as germ cells are able to reach the gonads, and programmed cell death efficiently eliminates ectopic germ cells. The defect appears to be in the proper placement of the somatic gonad. Germ cell transplants from mutant embryos into a wild type host could be done to ascertain whether each defect was germ cell specific or soma derived. Alternatively, gain of function experiments could be done to drive expression of wild type genes specifically in the germ line or in somatic tissue in the mutants to determine which location of wild-type expression can rescue the defect. This would suggest where the expression of this gene is needed.

All of the P-element insertions that produced a phenotype did not have defective germ cell migration, while programmed cell death was disrupted to varying degrees. While the reason is unclear, defects in germ cell death appear easier to identify than those in germ cell migration. One potential reason would be that severe germ cell migration mutants would be difficult to assay due to their severely decreased fertility. Perhaps these mutants are unable to be maintained. Also, if cell death is closely associated with cell migration in a way that causes cell death to all ectopic germ cells, then complete migration mutants could hypothetically have few or no germ cells. We have yet to find a mutant in which a significantly decreased number of germ cells are found.

The results in this chapter are very preliminary and much research still needs to be done to ascertain whether these genes are in fact active in the programmed cell death of germ cells. Other mutant alleles of these same genes could be tested to further confirm their roles. Additionally, those stocks that did not produce a mutant phenotype still do not rule out the
genes potential role in germ cell migration and/or programmed cell death. It is possible that the P-element insertion site in the tested stock is located in a region in or near the gene that does not affect protein function. Maternal expression of the genes tested could be essential for their roles in Drosophila germ cell migration and programmed cell death. The initiation of active migration and programmed cell death occurs only shortly after zygotic transcription begins in the germ cells. *tre1* maternal expression is important for proper germ cell migration and programmed cell death. The balanced P-element stocks would not be able to reveal maternal effect because the females in the stock one wild type copy of the gene. To test for a maternal requirement, homozygous mutants could be created using germline clones and their progeny could be assayed for a maternal effect phenotype. Also, genes encoding proteins of the same family were tested individually. It is possible that there is redundancy in function amongst these families. If one gene loses function then another of the same class is able to perform its role. Flies could be created that are mutant for multiple genes with redundant function and assayed to see if they have a germ cell migration and/or programmed cell death defect.

In conclusion, this research into genes that are involved in Drosophila germ cell migration and programmed cell death have provided some avenues that can be pursued in the attempt to further elucidate the signaling cascades controlling these processes.
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CHAPTER 4. G PROTEIN-COUPL ED RECEPTOR ROLES IN CELL
MIGRATION AND CELL DEATH DECISIONS

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Abstract

Recognition of external conditions and the elicitation of appropriate responses are critical to a cell’s ability to adjust to various developmental and environmental cues. G protein-coupled receptors (GPCRs) are a large class of receptors that act to relay external information into the cell by initiating signaling pathways that allow the cell to adapt to its present conditions. There are numerous ligands that activate GPCRs to initiate a multitude of intracellular signaling cascades involved in critical decisions including cell growth, differentiation, proliferation, migration, survival, and death. This article focuses on the signaling pathways involved in cell migration, survival and death decisions with an emphasis on germ cells from various organisms.

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Introduction

Migrating cells contact and interact with numerous cell types and substrates. The ability of these cells to react to an ever-changing environment is dependent on the recognition of external stimuli, followed by the activation of the appropriate responses. G protein-coupled receptors (GPCRs) are one of the mechanisms through which environmental cues are relayed into the cell in order to elicit a reaction. GPCRs are an extremely large class of proteins found in essentially all cell types. GPCRs recognize and respond to a wide variety of external signals including: organic molecules, ions, proteases, neurotransmitters, peptides, glycoproteins, hormones, nucleotides, lipids and light. (Gether, 2000; Karnik et al., 2003; Rader et al., 2004) The ligand can interact with extracellular regions of the GPCR, the transmembrane domains or both. A conformational change in the seven transmembrane helical structure results in the activation of the GPCR allowing it to interact with the appropriate heterotrimeric G protein within the cell. GPCRs relay signals into the cell utilizing a plethora of intracellular signal transduction pathways, and initiate signaling cascades that mediate diverse functions including cell growth, differentiation, proliferation, migration, survival, and death. (Radeff-Huang et al., 2004; Ye et al., 2002) In addition, kinases, proteases, adaptor proteins and ion channels often act within these pathways to modulate the actions of GPCRs. (Gether, 2000; Karnik et al., 2003; Rader et al., 2004)

This review will focus on GPCR-mediated cell migration and programmed cell death, emphasizing their roles in germ cell development. Current models of germ cell migration in mice, zebrafish, chick and Drosophila will be presented as well as the recently identified roles of GPCRs in the action of lysophospholipids in cell survival pathways. (Radeff-Huang et al., 2004) In addition, recent information will be discussed on the Drosophila GPCR, Tre1,
that has been shown to have a role in germ cell migration and programmed cell death during *Drosophila* development. (Coffman et al., 2002; Kunwar et al., 2003)

**GPCRs in Cell Migration**

GPCRs mediate the successful migration of numerous cell types in development, wound healing and immune responses. (Santos and Lehmann, 2004; Smith et al., 2004; Springer, 1995) In early development, the germ line stem cells must navigate through multiple cell types and substrates to reach their ultimate destinations, the gonads. (Molyneaux and Wylie, 2004; Raz, 2004; Santos and Lehmann, 2004) Population of the gonads with germ cells is essential to establish the next generation. Recently, a conserved molecular mechanism involving the CXCR4/SDF-1 receptor/ligand pair has been identified in mouse, chick, and zebrafish germ cell development. (Ara et al., 2003; Doitsidou et al., 2002; Dumstrei et al., 2004; Knaut et al., 2003; Molyneaux et al., 2003; Stebler et al., 2004) The chemoattractant SDF-1 and its GPCR counterpart, CXCR4, are known to function in many other developmental, homeostatic, and immune system response processes. Elucidation of the mechanisms of these signaling pathways will be a fruitful area of research in the near future as connections to other cell processes will no doubt be uncovered.

Murine germ cells originate during gastrulation. (Ginsburg et al., 1990) Time lapse analysis studies using immunofluorescence of the germ cells has provided wonderful detail of murine germ cell migration. (Anderson et al., 2000; Molyneaux et al., 2001) The migratory phase of these cells begins shortly after their formation as they move through the primitive streak on their way to the hindgut. While contained within the hindgut, the germ cells are highly motile, but no directed movement is observed until they cross the hindgut epithelium
and begin moving toward the genital ridges. The germ cells compact into a cluster as migration nears completion, and the germ cells interact and coalesce with somatic gonadal precursor cells to form the gonad. (Molyneaux et al., 2001)

Recently, it has been reported that the CXCR4/ SDF-1 ligand/receptor pair is involved in the final stages of murine germ cell migration, as the germ cells traverse the hindgut epithelium and migrate towards the genital ridges. (Molyneaux et al., 2003) SDF-1 appears to have no affect on germ cell movements prior to the crossing of the gut epithelium. The GPCR CXCR4 is expressed in the migrating germ cells and is able to respond to SDF-1 being expressed by the dorsal body wall and the genital ridges. The hypothesis that CXCR4 and SDF-1 are part of a germ cell chemoattractant mechanism in mice has been further supported by misexpression and knockdown experiments. When mice mutant in either the CXCR4 receptor or the SDF-1 ligand are generated, successful incorporation of germ cells into the gonads is severely impaired. (Ara et al., 2003; Molyneaux et al., 2003) Movement of germ cells lacking the CXCR4 receptor appears normal up to their incorporation into the hindgut. In the ensuing migratory steps, however, the germ cells are still observed on the migration path and few have reached the genital ridge at a time when wild-type germ cells have completed migration. In addition, misexpression of SDF-1 can attract some germ cells to ectopic locations and impede the directed migration of germ cells towards the endogenous ligand. (Molyneaux et al., 2003)

Germ cell migration in chick embryos is comparable to leukocyte migration in that germ cells utilize the vascular system for the initial passive migration steps on their way to somatic gonad tissue. (Stebler et al., 2004) The germ cells bind to the vasculature adjacent to the somatic gonad precursor cells. They must then pass through the blood vessel endothelium
in order to migrate to their target. As in mouse germ cell migration, SDF-1 appears to be the ligand that provides the attractive signals for the germ cells after crossing this epithelial layer. SDF-1 mRNA is expressed along the post-endothelial migration path of germ cells. Ectopic expression of SDF-1α causes aberrant germ cell migration and the accumulation of germ cells around the site of SDF-1α expression. (Stebler et al., 2004)

In zebrafish germ cells, SDF-1a appears to be required throughout germ cell migration. (Doitsidou et al., 2002; Dumstrei et al., 2004; Knaut et al., 2003) First, SDF-1a and the germ cell-expressed receptor, CXCR4b, are necessary for directed germ cell movements during the initial migrations of germ cells from random origination positions in the embryo. Second, this receptor-ligand pair aids in movement to, and coalescence with, the somatic gonad cells in the final stages of migration. (Raz, 2004) The somatic cells that act as intermediate targets of the germ cells express SDF-1a. Both loss-of-function and ectopic expression experiments support the conclusion that SDF-1a acts as the attractive signal for directed germ cell migration. Mutations that disrupt SDF-1a expression by altering somatic cell patterning affect the successful migration of zebrafish germ cells. (Doitsidou et al., 2002) Inhibiting translation of SDF-1a protein using morpholinos also disrupts germ cell migration. In these mutants, zebrafish germ cells are unable to consistently locate the gonads and scatter to ectopic locations. (Doitsidou et al., 2002) In additional experiments, it was found that ectopic SDF-1a was able to attract CXCR4b-expressing germ cells when endogenous SDF1a levels were reduced. (Doitsidou et al., 2002) In loss-of-function experiments where the gene encoding the CXCR4b receptor was mutated, germ cells scattered to ectopic locations rather than clustering at the gonadal anlage. (Knaut et al., 2003) Recent research in zebrafish has begun to elucidate the downstream components of the SDF-1a/CXCR4b interaction.
Inhibition of the G protein, Gi, results in a phenotype similar to loss of function sdf-1a or cxcr4b mutants. (Dumstrei et al., 2004)

**GPCRs in Regulation of Cell Death and Cell Survival Decisions**

Another crucial role for GPCRs in development is in the regulation of cell survival and cell death. The extensive networks of cellular signaling pathways connected to GPCRs allow fine control of cell survival and death in a context dependent manner. Recently, two lysophospholipids; sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), have been identified as GPCR ligands.(Radeff-Huang et al., 2004) The ligands signal through their respective receptor subtypes and the cellular response depends upon the cell type and/or the cellular context. In mammals, five S1P receptors (S1P1-5) and four LPA receptors (LPA1-4) have been identified.(Radeff-Huang et al., 2004; Ye et al., 2002) The S1P receptors are extremely selective in their ligand choice as they only recognize S1P and dihydroS1P.(Spiegel and Kolesnick, 2002) Both the LPA and S1P receptor subtypes have the ability to bind different types of G protein alpha subunits including G\(_{ai/o}\), G\(_{aq/11}\) and G\(_{a12/13}\). (Radeff-Huang et al., 2004; Ye et al., 2002) The difference in temporal and spatial expression patterns of the G\(_a\) subunits allows for control of multiple activities. However, regulation of cell survival appears to most often be regulated through the G\(_{ai}\) protein.

Apart from their roles in membrane structure, S1P and other sphingolipids are key players in cell growth, cell survival and cell death.(Spiegel and Kolesnick, 2002) (Kolesnick, 1987) S1P can act either as a ligand for S1P receptors or as a second messenger within the cell.(Radeff-Huang et al., 2004; Spiegel and Kolesnick, 2002) Extracellular S1P function is mediated through its GPCR and has been shown to promote cell survival in melanocytes,
neutrophils and leukemia cells. (Radeff-Huang et al., 2004) Interestingly, S1P can also protect the lives of cells without acting through its GPCR. Overexpression of S1P or sphingosine kinase, the enzyme that converts sphingosine into S1P, suppresses cell death, even in the absence of ligand-activated S1P function. (Edsall et al., 2001; Olivera et al., 2003; Van Brocklyn et al., 1998) For example, in mouse embryonic fibroblast cells that have lost S1P-receptor function, increased levels of S1P due to overexpression of sphingosine kinase protects these cells from death. (Olivera et al., 2003)

The S1P precursors ceramide and sphingosine are pro-apoptotic. It has been suggested that the balance between intracellular S1P and its precursors determines the fate of the same cells. (Spiegel and Milstien, 2003) Higher concentrations of S1P favor cell survival, while an abundance of ceramide and sphingosine leads to termination of the cell.

LPA has opposing roles in cell survival decisions depending on the cell type. LPA is anti-apoptotic in Schwann cells and ovarian cancer cells. (Ye et al., 2002) (Contos et al., 2000) Loss of LPA1 receptor function in these cells results in increased levels of apoptosis, indicating a requirement for LPA in the survival of these cells. However, in TF-1 hematopoietic cells or hippocampal neurons, LPA is pro-apoptotic. (Holtsberg et al., 1998; Lai et al., 2003) In some T lymphoblasts, LPA can elicit both apoptotic and pro-survival responses within the same cell type depending on the presence or absence of other regulatory molecules. (Ye et al., 2002)
GPCRs in Drosophila germ line development

Drosophila germ cell development also requires GPCR function. (Kunwar et al., 2003) Similar to mouse and chick germ cell development, Drosophila germ cells traverse an epithelial layer, the posterior midgut epithelium. (Campos-Ortega, 1997; Moore et al., 1998; Sonnenblick, 1941; Sonnenblick, 1950) This is followed by migration through mesodermal cell layers and coalescence with somatic gonadal precursor cells to form the gonads (Figure 4.1A). Drosophila germ cells require a GPCR encoded by the *tre1* gene. Maternally expressed *tre1* has several roles that include initiating the crossing of the midgut epithelium, path finding to the somatic gonad cells, and regulation of programmed cell death. (Coffman et al., 2002; Kunwar and Lehmann, 2003) In embryos lacking both maternal and zygotic *tre1* expression, the germ cells remain trapped within the primordial midgut. (Kunwar et al., 2003) Zygotic expression of *tre1* can partially rescue the germ cell migration phenotype of embryos from *tre1* mutant mothers. (Kunwar et al., 2003) Unlike mouse, chick and zebrafish germ cells where SDF-1 has been convincingly identified as a ligand of CXCR4, (Baggiolini et al., 1997; Bleul et al., 1996a; Bleul et al., 1996b; Doitsidou et al., 2002; Knaut et al., 2003; Ma et al., 1998; Stebler et al., 2004) the ligand for the GPCR located on the germ cell membrane of Drosophila remains elusive.

The *scattershot* (*sctt*) mutation, an EMS-induced partial loss-of-function allele of *tre1*, reveals roles for *tre1* in both germ cell migration and programmed cell death. (Coffman et al., 2002) In *sctt* mutants, the germ cells initiate migration by crossing the posterior midgut epithelium. However, directed migration to the gonads is disrupted and the germ cells scatter throughout the posterior half of the embryo (Figure 4.1B). Embryos from *sctt* mutant mothers also display a programmed cell death defect. In wild-type *Drosophila melanogaster*
development, approximately 50% of the germ cells that originate at the posterior pole fail to reach the gonads and are eradicated during migration. (Sonnenblick, 1941; Sonnenblick, 1950) While the signaling events that promote cell death and/or survival and the exact timing of programmed cell death in wild-type Drosophila embryos are unknown, few germ cells remain ectopic to the gonads in wild-type embryos after the germ cells and the somatic gonad cells have coalesced (Figure 4.1A). The germ cell migration and programmed cell death defects of sctt mutants can be genetically uncoupled by zygotic rescue. When a wild-type copy of trel is supplied paternally germ cell migration is restored but germ cells ectopic to the gonads still fail to complete the cell death program (Figure 4.1C). Elucidation of the upstream and downstream components of the trel-mediated signaling pathway will be crucial to our understanding of germ cell migration in Drosophila.

The multitudes of cellular processes mediated through the GPCR superfamily suggest an amazingly complex and well-regulated mechanism for the transduction of stimuli into various independent yet entangled cellular responses. The role of trel in Drosophila germ cell development is interesting as it demonstrates that multiple developmental functions are regulated through a single GPCR. One could imagine the usefulness of integrated pathways for cell death and cell migration. It would be beneficial to eliminate cells that are in excess, are misplaced, or fall behind during the path finding processes critical to so many developmental stages. The inability to efficiently rid the organism of ectopic cells is often detrimental. In humans, ectopic germ cells are etiologic in a variety of tumors including teratomas, endodermal sinus tumors, embryonal carcinomas, and choriocarcinomas, testicular and ovarian carcinomas. (Brandes et al., 2000; Fauci, 1998; Gatcombe et al., 2004; Ulbright, 2004) Further research on GPCR-mediated signaling in various model organisms will
undoubtedly deepen our understanding of the regulatory mechanisms controlling cell migration and programmed cell death and the common machinery that allows for crosstalk between these important cellular functions.

REFERENCES


CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The *scattershot* mutant is an allele of *tre1*

The *sctt* mutation was generated through an EMS mutagenesis screen designed to isolate X-linked genes involved in Drosophila germ cell migration (Coffman et al., 2002). Genetic map position on the X was determined through deletion complementation tests, recombination mapping with marked X chromosomes, and translocation rescue experiments. Mapping placed *sctt* within one map unit of the *crossveinless* locus (Coffman et al., 2002). In 2003, a manuscript from the Lehmann lab revealed that *sctt* failed to complement a mutant of the *tre1* (Kunwar et al., 2003). The *tre1* gene encodes a G protein-coupled receptor (GPCR) expressed in germ cells (Kunwar et al., 2003). While the complementation test suggested a link between *sctt* and *tre1* mutants, it did not rule out the possibility of non-allelic non-complementation. Investigation into the molecular lesion causing the *sctt* mutation revealed no disruptions of the coding region of the *tre1* gene in *sctt* mutants (Kunwar et al., 2003). Chapter 2 discusses my genomic sequencing of *tre1* in the *sctt* background and the identification of a single base pair change in intron 4. The location of the base pair change suggested that it could impact splicing of the *tre1* gene. Reverse transcriptase PCR was employed to target the mRNA sequence for sequencing and revealed the deletion of 24 base pairs from exon 5 of the mRNA. The data in Chapter 2 establishes for the first time, that *sctt* is in fact an allele of *tre1*. The *tre1*<sup>sctt</sup> allele is a severe loss of function allele, but it is not as severe as the *tre1*<sup>ΔEP5</sup> deletion which is a germ cell null mutation. In *tre1*<sup>ΔEP5</sup> mutants, germ cells are defective in transepithelial migration and thus remain trapped within the posterior midgut (Kunwar et al., 2003). Careful microscopic study of the
tissues in which \textit{tre1}^{scat} germ cells reside suggests that the majority of germ cells do exit the midgut but are unable to path find through the surrounding mesoderm to reach the gonads. Further assessment of the phenotypic differences of the \textit{tre1} alleles will require careful analyses of the \textit{tre1}^{\Delta EP5} phenotype and additional evidence supporting the observation that \textit{tre1}^{scat} germ cells can migrate the epithelium. Double-labeling using a germ cell specific antibody such as anti-vasa and a midgut marker could be done to score the percentage of \textit{tre1}^{scat} and \textit{tre1}^{\Delta EP5} germ cells that leave the midgut pocket. In addition, live imaging of germ cell migration in the \textit{tre1}^{scat} background would further elucidate the migratory defect in these mutants. Live imaging of germ cells in the \textit{tre1}^{\Delta EP5} mutant background shows an example of the inability of these germ cells to leave the midgut but does not detail the extent to which germ cells are able to exit the posterior midgut in the \textit{tre1}^{\Delta EP5} mutant embryos (Kunwar et al., 2008).

**The arginine of the DRY motif is necessary for maternal rescue of the \textit{tre1}^{scat} cell migration defect**

Maternal rescue experiments were performed using \textit{tre1}^{scat} homozygous females carrying at least one copy of a transgene. The transgenes contained amino acid replacements with alanine and were designed to identify the critical amino acid residues within the 8-amino acid deletion in \textit{tre1}^{scat}. The progeny resulting from a cross between the \textit{tre1}^{scat} / \textit{tre1}^{scat}; P[rescue] female and a \textit{tre1}^{scat} male were assayed for a germ cell migration phenotype. The arginine of the highly conserved DRY motif was identified as a critical residue for proper germ cell migration. When replaced with alanine, germ cell migration to the gonads is not restored. The other 7 amino acids in the \textit{tre1}^{scat} deletion are dispensable for rescue of germ cell migration. The arginine is considered the most conserved amino acid in all the
rhodopsin family GPCRs (Rosenkilde et al., 2005). Some studies have suggested that arginine can directly bind G proteins (Acharya and Karnik, 1996). However, the vast majority of literature suggests that arginine is involved in the stability of receptor conformation, particularly in holding the receptor in its inactive state (Angelova et al., 2002; Ballesteros et al., 1998; Ballesteros et al., 2001; Greasley et al., 2002; Shapiro et al., 2002; Zhang et al., 2005). Although some research suggests the arginine could hold receptors in an active state as well (Flanagan, 2005). This arginine is considered by some to be the most critical residue in signal transduction of rhodopsin family GPCRs (Ballesteros et al., 1998; Oliveira et al., 1994; Scheer et al., 1996). Nonconservative mutations of this residue have been found to most commonly result in defective signal transduction from the receptor (Jones et al., 1995; Scheer et al., 1996; Zhu et al., 1994). Therefore, my results within the context of an organism are consistent with numerous cell culture studies that previously identified arginine as a critical residue in GPCR signaling.

A transgenic construct designed to reconstruct the tre1scrit deletion has already been tested in maternal rescue experiments. As expected, germ cell migration is not restored in these flies. A second transgenic stock within an independent insertion site has been created and is also in the process of being crossed into the tre1scrit background for analysis.

The wild-type T+G+ rescue construct was able to completely rescue both germ cell migration and programmed cell death. Interestingly, only one other construct, the replacement of the endogenous tyrosine with an alanine, was able to completely rescue programmed cell death in addition to germ cell migration. The remaining constructs were unable to completely rescue this cell death defect. It is possible that programmed cell death of germ cells requires multiple amino acids in this deletion that were all present in the RY
ILI ACH and RA ILI ACH constructs but in none of the other constructs tested. For example, the RY AAA AAA construct is able to rescue cell migration but not cell death. It is possible that the wild type R is sufficient to rescue cell migration but perhaps additional amino acids downstream of RY that are replaced by alanine in this construct are required in combination to rescue cell death. Additional constructs could be designed to add back various combinations of amino acids in an attempt to find the critical amino acids for rescue of the germ cell death defect. Two other constructs tested were able to rescue cell migration but not cell death in a similar manner. These were the RY AAA AAA and RY AAA ACH constructs. Approximately 7-9 germ cells remained ectopic to the gonads in rescue of the tre1sctt mutation using these transgenic lines. Therefore, in both cases in which the three hydrophobic residues following the RY are missing, germ cell death is not restored. It would be interesting to design additional constructs that contain combinations of the arginine critical for proper germ cell migration, as well as the 3 hydrophobic residues following the tyrosine, RA ILI AAA, to see if both germ cell migration and programmed cell death is restored. Further research into these 4 amino acids may be able to define two separate amino acid requirements for germ cell migration and programmed cell death.

The maternal rescue experiments address whether maternal contribution of the transgene will rescue the defects. It has been established that the germ cell migration defects observed in embryos from homozygous mutant tre1sctt females can be rescued by supplying a wild type copy of tre1 paternally (Coffman et al., 2002). Drosophila germ cells are transcriptionally quiescent from their formation until Stages 8-9, about 3.5 hours after egg laying, just before active migration is initiated (Zalokar, 1976). Zygotic expression of tre1 at this time is sufficient to rescue cell migration but not cell death. Therefore, it seems unlikely
that any of the amino acid substitution constructs would be able to rescue germ cell death when supplied paternally. It would be interesting, however, to test whether those constructs that are able to maternally rescue germ cell migration would also be able to do so when zygotically expressed.

The *tre1* temporal requirement for proper programmed cell death of germ cells could be strictly necessary within the first 3.5 hours of development, before zygotic transcription is initiated in germ cells and thus paternal rescue of cell death is not observed. Alternatively, the programmed cell death mechanisms could be more sensitive to *tre1* transcript levels and require a higher level of those transcripts for cell death to occur. Perhaps the maternal deposition of these transcripts allows for higher amounts of this transcript compared to that which can be produced by zygotic transcription in the time frame prior to when germ cell death normally occurs. It has been shown that the majority of germ cell death occurs before stage 12, approximately 7.5 hours after egg laying (Yamada et al., 2008). Zygotic transcription in germ cells begins at about the time germ cells initiate migration, coincident with the onset of cell death. However, the death of germ cells is complete within 4 hours of the initiation of zygotic transcription and the embryo may not produce high enough levels of *tre1* transcript in that time frame. The *tre1* dose hypothesis for effective elimination of germ cells could be tested using the transgenic constructs designed for rescue of the *tre1<sup>sct</sup>* defects. A male fly could be designed that carried multiple copies of the rescue construct, either through recombination between two second chromosomes with independent insertion sites to obtain a chromosome with two insertion sites of the same construct or through generation of transgenic construct inserts on other chromosomes using a P-element hopping strategy (Cooley et al., 1988). The zygotic transcript dosage levels within the embryo from this male
would be increased due to multiple loci carrying the insertion. The rescue of germ cell death could be assayed in this embryo to determine whether higher levels of transcript will rescue the defect.

**tre1 roles beyond transepithelial migration**

Published analysis of tre1 has currently revealed only roles for tre1 at the time of and leading up to transepithelial migration. These results are based on the observed phenotype of tre1AEPS in which most germ cells never leave the midgut pocket (Kunwar et al., 2008; Kunwar et al., 2003). The tre1AEPS allele is considered to be an essentially null mutation. Roles of tre1 in subsequent steps would not be observed in this null mutant. The severe partial loss of function nature of the tre1sctt mutant argues additional roles for this protein beyond transepithelial migration. First, partial function of tre1 allows germ cells to exit the midgut. In tre1sctt mutants some of the germ cells are seen as far away from the midgut as being nearly in ectoderm (personal observation). Yet, even after successful migration through the midgut, germ cell migration to the gonads is still disrupted, suggesting roles for tre1 in mesodermal migration and pathfinding to the somatic gonad precursor cells. Also, the steps immediately following transepithelial migration are dorsally orienting on the basal side of the midgut and bilateral segregation in the mesoderm. These steps are controlled in part by the formation of a lipid phosphate gradient established through competition of lipid phosphate phosphatases, wunen and wunen2, expressed on the germ cells and in the ventral mesoderm and central nervous system (Burnett and Howard, 2003; Sano et al., 2005; Starz-Gaiano et al., 2001; Zhang et al., 1996; Zhang et al., 1997). In wild-type embryos germ cells will migrate towards regions of higher ligand concentration which are on either side of the
midline. Preliminary observations of \( tre1^{sct} \) mutant embryos at earlier time points suggest that the germ cells are unable to perceive this phosphogradient and some germ cells remain near the midline (personal observation). Germ cells in these mutants do not appear to bilaterally segregate upon migration through the mesoderm but rather migrate randomly towards the posterior pole of the embryo. Further study of \( tre1^{sct} \) germ cell migration using live imaging could be done to further confirm this observation. Yeast two hybrid experiments using the Tre1 protein as bait have identified \( wunen2 \) as being a high confidence interactor (Martin Schmidt and Margaret Pruitt-unpublished results). The \( wunen2 \) lipid phosphate phosphatase is expressed in the germ cells as well as in the soma (Hanyu-Nakamura et al., 2004). Interaction with \( wunen2 \) may indicate a role for \( tre1 \) in the migration of the germ cells upon sensing of the phospholipid gradient.

**Functional domains of \( tre1 \)**

How would \( tre1 \) be involved in so many different steps of germ cell migration yet allow defects in these specific steps to be uncoupled? For example, how is it that loss of 8 amino acids has little observable effect on transepithelial migration yet renders bilateral segregation incapacitated? One way this could occur would be the importance of different functional domains of \( tre1 \) for its different roles. The \( tre1^{AEPS} \) mutant causes complete disruption of protein function but because the protein’s first role is in the steps leading to and in transepithelial migration, the phenotype that results are germ cells trapped in the midgut. However, perhaps the \( tre1^{sct} \) mutant only affects a domain controlling bilateral segregation and programmed cell death but regions of the protein involved in transepithelial migration remain intact. Different signaling cascades could be downstream of these particular domains.
eliciting the various functions. To test the hypothesis that the 8 amino acid deletion in\( \text{tre}l^{sctt} \) disrupts an interaction with the \textit{wunen}2 lipid phosphate phosphatase protein involved in bilateral segregation, additional split-ubiquitin yeast two hybrid experiments are proposed. A modified \text{Tre}1 bait could be designed that lacks the 8 amino acids missing in \( \text{tre}l^{sctt} \) and could be used to test an interaction with the Wunen2 prey. If this region of \text{Tre}1 is involved in the interaction with Wunen2, the experiment will not detect an interaction. The wild type \text{Tre}1 bait would be used as the positive control in this experiment.

Additional functional domains could be assayed to determine their roles in \textit{tre}l through the creation of additional transgenics. Alternate regions of the protein could be targeted for amino acid substitution or even deletion in order to assess that particular region’s role in receptor function. Transgenics would need to be created and crossed into a null \textit{tre}l background. The resulting progeny would be analyzed for germ cell migration phenotypes. Of particular interest would be the C-terminal domain. The C-terminal tails of GPCRs have been identified as being able to interact with many GPCR interacting proteins. These interacting proteins connect the GPCR to critical functions such as targeting the receptor to cellular compartments, acting as scaffolds in which to assemble large complexes, trafficking of the receptor to and from the membrane, and in signaling (Bockaert et al., 2004). In T lymphocyte migration, the C-terminal tail of both chemokine receptors CCR5 and CXCR4 have been shown to interact with the Myosin heavy chain IIA to control actin-based motility (Rey et al., 2002).

My experiments indicated a potential critical role of the \text{Tre}1 C-terminal tail. In an attempt to label the transgenic constructs used in Chapter 2, an additional version of each construct was designed to contain a myc tag within its C-terminal tail. Transgenic flies were
created using some of these vectors and were crossed into the \textit{tre1}^{scrt} background. When wild-type rescue constructs containing all 8 amino acids were tagged and tested for their ability to rescue the \textit{tre1}^{scrt} mutation it was discovered that they were unable to rescue germ cell migration and programmed cell death. A \textit{tre1}^{scrt} phenotype resulted. However, it was known that a rescue construct without the myc tag was able to rescue both germ cell migration and programmed cell death. Therefore, it is possible that the insertion site of the myc tag is causing a disruption of the Tre1 protein. Given the C-terminal’s established role in interacting with proteins for localization, this could be due to improper trafficking of the receptor to the membrane (Bockaert et al., 2004). Also, it is possible that this region is involved in connecting Tre1 to downstream cascades controlling cell motility, as C-terminal domains in other GPCRs are linked to proteins involved in actin-based cell movements (Rey et al., 2002). This experiment raises the possibility that the C-terminal tail contains a critical functional domain of \textit{tre1}. C-terminal tail truncations could be created and assayed to see whether they were able to rescue different components of Tre1 function such as the transepithelial migration defect of the \textit{tre1}^{AEPS} mutation.

\textbf{REFERENCES}


Table 2.1. Germ cell distribution in \textit{sctt} mutants

<table>
<thead>
<tr>
<th>Maternal Genotype</th>
<th>Paternal Genotype</th>
<th>Zygotic Genotype</th>
<th>Germ Cells in Gonad Mean</th>
<th>S.E.M.</th>
<th>Germ Cells Ectopic Mean</th>
<th>S.E.M.</th>
<th>Total Number of Germ Cells Mean</th>
<th>S.E.M.</th>
<th>Range</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/ wt$^a$</td>
<td>wt/Y</td>
<td>(maternal$^+/zygotic^-$)</td>
<td>14.7</td>
<td>0.4</td>
<td>0.5</td>
<td>0.1</td>
<td>15.2</td>
<td>0.4</td>
<td>9-23</td>
<td>61</td>
</tr>
<tr>
<td>\textit{sctt}/sctt</td>
<td>\textit{sctt}/Y</td>
<td>(maternal$^-$/zygotic$^-$)</td>
<td>1.0</td>
<td>0.2</td>
<td>16.3</td>
<td>0.6</td>
<td>17.3</td>
<td>0.7</td>
<td>7-32</td>
<td>69</td>
</tr>
<tr>
<td>\textit{sctt}/sctt</td>
<td>wt/Y</td>
<td>(maternal$^-$/zygotic$^+$)$^b$</td>
<td>0.3</td>
<td>0.1</td>
<td>16.6</td>
<td>0.8</td>
<td>16.9</td>
<td>0.7</td>
<td>11-22</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(maternal$^-$/zygotic$^+$)$^b$</td>
<td>13.0</td>
<td>0.5</td>
<td>7.0</td>
<td>0.6</td>
<td>20.0</td>
<td>0.8</td>
<td>14-30</td>
<td>23</td>
</tr>
</tbody>
</table>

Germ cell counts performed on 12-15 hour old embryos

$^a$ wt denotes the non mutagenized \textit{w$^{118}$, P$^{w^+}$, fat facets-lacZ} parental strain

$^b$ two distinct phenotypic classes, presumed genotypes are in parentheses and based on genotyping experiments performed in Coffman et al. 2002.
Fig 2.1. The *brle*™ mutation severely disrupts germ cell migration and programmed cell death. (A-D) Dorsal views of 12-15 hour old embryos. Anterior is to the left. Germ cells are labeled brown with an anti-Vasa antibody. (A) A wild type embryo in which the germ cells have migrated to, and coalesced with, the somatic gonad precursor cells. No ectopic germ cells are observed. (B) A *brle*™ maternal/zygotic "embryo" resulting from a cross between a *brle*™/brle™ mother and a *brle*™/brle™ father. Germ cells scatter throughout the posterior half of the embryo and fail to align with the somatic gonad precursor cells. Germ cells ectopic to the gonads persist. Embryos from a *brle*™/brle™ female crossed to a wild type, +/Y, male fall into two phenotypic classes (C) A *brle*™ maternal/zygotic embryo that has a wild-type *brle* gene supplied paternally. Germ cell migration is restored as wild type numbers of germ cells reach the gonads. However, germ cells ectopic to the gonads remain. Male embryos from a *brle*™/brle™ female crossed to a wild type, +/Y, male are brle™/Y maternal/zygotic and display the phenotype shown in (B). (D) Maternal/zygotic *brle*™ embryos from a female that is heterozygous for *brle*™ crossed to a *brle*™/Y male. Germ cell migration is wild type.
Figure 2.2. The \textit{treI\textsuperscript{scct}} mutation results in an amino acid deletion of a conserved region of Rhodopsin class GPCRs.
Figure 2.2 continued. The \textit{tre}^{lsc} mutation results in an amino acid deletion of a conserved region of Rhodopsin class GPCRs.  (A) Reverse transcriptase PCR was performed on cDNA from 0-8 hour old \textit{tre}^{lsc} and wild-type embryos.  Wild type RT-PCR sequence reveals normal splicing at all intron/exon junctions.  The \textit{tre}^{lsc} template reveals a deletion of 24 base pairs of exon 5, immediately following the intronic A to T base pair change in \textit{tre}^{lsc} mutants.  The remainder of the sequence is unaffected.  Intron 4 is shown in gray.  The single base pair change from genomic sequencing is boxed in red.  The nucleotides missing in \textit{tre}^{lsc} mRNA are indicated by green highlight.  (B) Schematic of the predicted secondary structure of Tre1.  The 8 amino acid deletion in the \textit{tre}^{lsc} mutation results in an in frame loss of 8 amino acids from the third transmembrane domain/second intracellular loop junction.  The prediction programs SOSUI, TopPred, and TMHMM all suggest this deletion results in the shortening of the second intracellular loop.  Topology of the rest of the protein is not predicted to be affected by the deletion.  The missing amino acids, RYILIACH, are indicated.  (C) A sequence alignment comparing the \textit{tre}^{lsc} amino acid deletion to human GPCRs involved in cell migration.  Identical residues are in gray and similar residues are in blue.

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>R</th>
<th>Y</th>
<th>I</th>
<th>L</th>
<th>I</th>
<th>A</th>
<th>C</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tre1</td>
<td>R</td>
<td>Y</td>
<td>L</td>
<td>L</td>
<td>I</td>
<td>A</td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>Gpr84</td>
<td>R</td>
<td>Y</td>
<td>L</td>
<td>L</td>
<td>I</td>
<td>A</td>
<td>-</td>
<td>H</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>R</td>
<td>Y</td>
<td>L</td>
<td>A</td>
<td>I</td>
<td>V</td>
<td>-</td>
<td>H</td>
</tr>
<tr>
<td>Cxcr3</td>
<td>R</td>
<td>Y</td>
<td>L</td>
<td>N</td>
<td>I</td>
<td>V</td>
<td>-</td>
<td>H</td>
</tr>
<tr>
<td>Par-2</td>
<td>R</td>
<td>Y</td>
<td>W</td>
<td>V</td>
<td>I</td>
<td>V</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>S1PR3</td>
<td>R</td>
<td>H</td>
<td>L</td>
<td>T</td>
<td>M</td>
<td>I</td>
<td>K</td>
<td>M</td>
</tr>
<tr>
<td>S1PR4</td>
<td>R</td>
<td>P</td>
<td>V</td>
<td>A</td>
<td>E</td>
<td>S</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>Lpar3</td>
<td>R</td>
<td>H</td>
<td>M</td>
<td>S</td>
<td>I</td>
<td>M</td>
<td>R</td>
<td>M</td>
</tr>
</tbody>
</table>
Figure 2.3. Transgenic rescue identifies critical amino acid residues of Tre1 necessary for germ cell migration.

Dorsal views of embryos are shown. Anterior is to the left. Embryos aged 12-16 hours were stained with X-Gal to visualize the fat facets-lacZ transgene, a germ cell marker. Embryos were from tre1\textsuperscript{maternal} homzygous mothers containing at least one copy of the specified transgene. Replacement of the arginine with alanine results in a transgene that fails to rescue germ cell migration in tre1\textsuperscript{maternal} embryos. A (+) rescue of migration represents greater than 90% of embryos from the cross with the rescued germ cell migration phenotype. Lack of germ cell migration rescue (-), is less than 10% rescue of germ cell migration.

\[\begin{array}{|c|c|c|c|c|}
\hline
\text{Transgene} & \text{Rescue of Migration} & \text{Phenotype} & \text{Transgene} & \text{Rescue of Migration} & \text{Phenotype} \\
\hline
\text{RY ILI ACH} & + & & \text{No Transgene} & - & \\
\hline
\text{RY AAA AAA} & + & & \text{tre1\textsuperscript{maternal}} reconstruction & - & \\
\hline
\text{RY AAA ACH} & + & & \text{AY ILI ACH} & - & \\
\hline
\text{RA ILI ACH} & + & & & & \\
\hline
\end{array}\]

\[a\] The transgene is missing the 24 base pairs missing in tre1\textsuperscript{maternal} mutants.
Figure 2.4. Rescue of germ cell migration in *tre1^{scf}* mutants using transgenic constructs. The number of germ cells in the gonads of embryos in transgenic maternal rescue of the *tre1^{scf}* defect was analyzed. All test constructs assayed rescue germ cell migration with the exception of the arginine to alanine substitution, AYILIACH. The RYILIACH construct is the positive control and the no transgene and the *tre1^{scf}* reconstruction constructs are negative controls. Error bars represent the standard error of the mean (SEM). More data on these counts can be found in Supplemental Information Table 2.
Supplemental Figure 2.1. Design of Engineered Transgenic Cassette Construct.

(A) A 1700 base pair fragment of *tre1* genomic sequence was excised from the T+G+ vector using a SphI/StuI double digest and ligated into a modified pSP72 cloning vector. The ligated pSP72 construct was further digested with Bpu10I/PstI to excise a 160 base pair fragment containing the genomic region coding the second intracellular loop of Tre1. (B) Phosphorylated oligonucleotides were designed to replace the Bpu10I/PstI-excised sequence with the addition of an Alol restriction endonuclease recognition site. Phosphorylated oligonucleotides were hybridized to their complementary oligo. Two oligos were designed to cover the entire 160 base pair fragment on each strand of DNA. Triple ligation was done to reconstitute the pSP72 vector containing the addition of Alol to the target region (A). Two independent vectors were designed with the Alol site in different locations to target all 24 base pairs encoding the targeted RYILIACH. (C) Upon Alol digestion of the cassette, the nucleotides of the target sequence are removed due to the nature of the Alol enzyme cutting on each side of its recognition sequence and removing flanking genomic DNA. Two complementary phosphorylated oligonucleotides engineered with the nucleotide changes corresponding to the desired amino acid substitutions were hybridized and ligated back into the pSP72 vector. The pSP72 was then digested with SphI/StuI to excise the modified 1700 base pair fragment and insert it into the original T+G+ vector for use in the creation of transgenic flies. (D) Genetic cross designed to test the transgenic construct’s ability to maternally rescue the defect. Females homozygous for *tre1<sup>mut</sup>* and carrying one or two copies of the transgene were crossed to *tre1<sup>mut</sup>* males. The resulting offspring were phenotyped.
Supplemental Table 2.1. Description of transgenic constructs used to evaluate Tre1 function.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description of Amino Acid Change</th>
<th>Phosphorylated Oligos Used for Amino Acid Changes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYILIACH (T+G+ construct)</td>
<td>Rescue construct, Wild type amino acids</td>
<td></td>
</tr>
</tbody>
</table>
| trel<sup>mut</sup> reconstruction | The construct is missing the 24 base pairs missing from trel<sup>mut</sup> RNA | PGTAGCGGCT--------------------------GCCAGGGGATTA  
PCCCTGGCAG--------------------------CCGCTACTCGCA |
| RY AAA AAA         | ILI ACH replaced with 6 Alanines                  | PGTAGCGGCTGGCGGCGGCGGCGGCGGCGGATATCTGCAAGGGGATTA  
PCCCTGGCAGATATGCGCCCGCCCGCCCGCGCGCGAGCCGCTACTCGCA |
| RY AAA ACH         | ILI replaced with 3 Alanines                      | PGTAGCGGCTATTCGCAAGGGGATTA                      
PCCCTGGCAGATATGCCGCCGCCGCCGCCGCCACAGCCTACTCGCA |
| RA ILI ACH         | Tyrosine replaced with an Alanine                 | PGTAGCGGCTGTGGCAAGGGATGATATGCTGCAAGGGGATTA      
PCCCTGGCAGATATGCCGCCGCCGCCGCCGCCACAGCCTACTCGCA |
| ΔY ILI ACH         | Arginine replaced with an Alanine                | PGGCCTACGCGTTTACGGTATGCCACCACATGC              
PGTGGCCATCCACCTGAAAACGGTAGGGCCCAGT |

*Underlined sequence designates the nucleotide replacements used to create the amino acid substitutions
Supplemental Table 2.2. Germ cell distribution in tre1<sup>sex</sup> maternal/zygotic embryos containing modified tre1 transgenes

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Germ cells in gonad</th>
<th>S.E.M.</th>
<th>Germ cells ectopic to the gonads</th>
<th>S.E.M.</th>
<th>Total germ cells</th>
<th>S.E.M.</th>
<th>Range</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>RY ILI ACH *</td>
<td>23.0</td>
<td>0.9</td>
<td>1.2</td>
<td>0.4</td>
<td>24.2</td>
<td>0.7</td>
<td>11-39</td>
<td>69</td>
</tr>
<tr>
<td>RY AAA AAA</td>
<td>20.9</td>
<td>0.4</td>
<td>8.5</td>
<td>0.4</td>
<td>29.4</td>
<td>0.5</td>
<td>11-51</td>
<td>194</td>
</tr>
<tr>
<td>RY AAA ACH</td>
<td>16.2</td>
<td>0.3</td>
<td>7.3</td>
<td>0.8</td>
<td>23.5</td>
<td>0.3</td>
<td>17-53</td>
<td>64</td>
</tr>
<tr>
<td>RA ILI ACH</td>
<td>23.0</td>
<td>0.3</td>
<td>1.7</td>
<td>0.3</td>
<td>24.7</td>
<td>0.5</td>
<td>17-32</td>
<td>64</td>
</tr>
<tr>
<td>No transgene control</td>
<td>0.3</td>
<td>0.1</td>
<td>25.2</td>
<td>0.9</td>
<td>25.5</td>
<td>0.9</td>
<td>10-43</td>
<td>75</td>
</tr>
<tr>
<td>Tre1&lt;sup&gt;sex&lt;/sup&gt;reconstruction</td>
<td>1.4</td>
<td>0.5</td>
<td>23.3</td>
<td>0.7</td>
<td>24.6</td>
<td>0.7</td>
<td>12-48</td>
<td>103</td>
</tr>
<tr>
<td>ΔY ILI ACH</td>
<td>0.4</td>
<td>0.9</td>
<td>26.6</td>
<td>0.8</td>
<td>27.1</td>
<td>0.8</td>
<td>8-51</td>
<td>87</td>
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</table>

Embryos were collected and aged 12-16 hours

Germ cells were detected by staining for β-galactosidase activity using the P<sup>w+</sup>, fat facets-lacZ germ cell-specific marker

ND-Not determined. Germ cell counts have not yet been done on this line

*Wild type T<sup>°</sup>G<sup>+</sup> vector as described in Dahanukar et al. 2001
**Figure 3.1.** P-element insertion stocks producing germ cell migration and/or programmed cell death phenotypes.

Dorsal views of 12-16 hour old embryos. Germ cells are visualized with an anti-Vasa antibody. The name of the gene predicted to be impacted by P-element insertion is listed followed by the mutant allele tested. The predicted function of each gene is listed.

*Stock tested is over a balancer.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Mutant Allele</th>
<th>Predicted Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>crk</em> KG00336</td>
<td>SH2/SH3 adaptor*</td>
<td></td>
</tr>
<tr>
<td><em>csk</em> j1D8</td>
<td>C-terminal Src kinase*</td>
<td></td>
</tr>
<tr>
<td><em>trio</em> KG06642</td>
<td>Rho-GEF*</td>
<td></td>
</tr>
<tr>
<td><em>krz</em> KG015035</td>
<td>Arrestin*</td>
<td></td>
</tr>
<tr>
<td><em>CG7047-KG05089</em></td>
<td>Arrestin-like</td>
<td></td>
</tr>
<tr>
<td><em>rab5</em> KG08252</td>
<td>GTPase*</td>
<td></td>
</tr>
<tr>
<td><em>CG8184-KG02051</em></td>
<td>E3 ligase</td>
<td></td>
</tr>
<tr>
<td><em>neur</em> KG06174</td>
<td>E3 ligase*</td>
<td></td>
</tr>
<tr>
<td><em>jafrac1</em> KG05572</td>
<td>Thioredoxin peroxidase</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.1. Percentage of mutant embryos in tested alleles

<table>
<thead>
<tr>
<th>Gene Targeted</th>
<th>Allele</th>
<th>Gene Class</th>
<th>Expression</th>
<th>% Mutant&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>dredd</td>
<td>EY08404</td>
<td>caspase</td>
<td>Embryo</td>
<td>0.9 (100)</td>
<td>229</td>
<td>None</td>
</tr>
<tr>
<td>crk</td>
<td>KG00336</td>
<td>SH2/SH3</td>
<td>Embryo</td>
<td>17.3 (25) 20.3 (25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>324</td>
<td>400 Cell death defect**</td>
</tr>
<tr>
<td>drk</td>
<td>KG03077</td>
<td>SH2/SH3</td>
<td>Embryo</td>
<td>2.3 (100)</td>
<td>439</td>
<td>None</td>
</tr>
<tr>
<td>dock</td>
<td>04723</td>
<td>SH2/SH3</td>
<td>Embryo</td>
<td>6.5 (25)</td>
<td>123</td>
<td>None</td>
</tr>
<tr>
<td>dock</td>
<td>P{Epgy2}</td>
<td>SH2/SH3</td>
<td>Embryo</td>
<td>5.2 (100)</td>
<td>172</td>
<td>None</td>
</tr>
<tr>
<td>dock</td>
<td>k13421</td>
<td>SH2/SH3</td>
<td>Embryo</td>
<td>1.2 (25)</td>
<td>167</td>
<td>None</td>
</tr>
<tr>
<td>dos</td>
<td>EY04266</td>
<td>SH2/SH3</td>
<td>NA</td>
<td>4.0 (100)</td>
<td>206</td>
<td>None</td>
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<tr>
<td>gprk2</td>
<td>06936</td>
<td>Kinase</td>
<td>Embryo</td>
<td>9.5 (25)</td>
<td>200</td>
<td>None</td>
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<tr>
<td>gprk2</td>
<td>EY09213</td>
<td>Kinase</td>
<td>Embryo</td>
<td>10.7 (100)</td>
<td>121</td>
<td>None</td>
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<tr>
<td>src64b</td>
<td>KG00213</td>
<td>Src kinase</td>
<td>NA</td>
<td>5.0 (100)</td>
<td>140</td>
<td>None</td>
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<tr>
<td>csk</td>
<td>1D8</td>
<td>C-term. Src kinase</td>
<td>NA</td>
<td>62.9 (25)</td>
<td>278</td>
<td>Cell death Migration/ death*</td>
</tr>
<tr>
<td>trio</td>
<td>KG06642</td>
<td>Rho GEF</td>
<td>Embryo</td>
<td>46.0 (25) 51.6 (25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>137</td>
<td>155 Cell death defect*</td>
</tr>
<tr>
<td>kraz</td>
<td>c01503</td>
<td>Arrestin</td>
<td>Embryo</td>
<td>25.4 (25) 30.0 (25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>209</td>
<td>256 Cell death and cell migration defect**</td>
</tr>
<tr>
<td>kraz</td>
<td>kraz&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Arrestin</td>
<td>Embryo</td>
<td>15.2 (25) 0 (25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>Cell death defect None</td>
</tr>
<tr>
<td>kraz</td>
<td>kraz&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Arrestin</td>
<td>Embryo</td>
<td>20 (25) 0 (25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>Cell death defect None</td>
</tr>
<tr>
<td>CG14696</td>
<td>BG02595</td>
<td>Arrestin-like</td>
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<td>81.6 (100)</td>
<td>196</td>
<td>Gonad placement defect</td>
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<tr>
<td>CG7047</td>
<td>KG05089</td>
<td>Arrestin-like</td>
<td>Embryo</td>
<td>31.8 (100) 37.5 (100)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>201</td>
<td>200 Cell death defect</td>
</tr>
<tr>
<td>rab5</td>
<td>EY10619</td>
<td>GTPase</td>
<td>Embryo</td>
<td>9.4 (25)</td>
<td>283</td>
<td>None</td>
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<td>rab5</td>
<td>KG05684</td>
<td>GTPase</td>
<td>Embryo</td>
<td>5.0 (25)</td>
<td>126</td>
<td>None</td>
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<tr>
<td>pxt</td>
<td>EY03052</td>
<td>peroxidase</td>
<td>Germ cells</td>
<td>7.1 (100)</td>
<td>70</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>a</sup>The number in parentheses designates the highest percentage mutant expected based on the nature of the stock. Balanced stocks are only expected to produce 25% of embryos with mutant phenotypes while homozygous stocks could yield 100%.

<sup>b</sup>Second antibody staining results of the same stock

*Stock needs to be tested for a balancer effect

**Phenotype caused by balancer in stock

NA-Not available

Data collected with the help of Margaret Pruitt and Deb Czarnecki

Gray boxes indicate stocks that produced a percentage of mutants above threshold for study.
Cross 1:  \( \text{fafx/afa};+/+ \ x \ +/-Y; \text{P-element line/balancer} \)

Cross 2:  A (balancer chromosome)  \( \text{fafx}+/+; \text{bal}+/+ \ x \ \text{fafx}+/Y; \text{bal}+/+ \)

B (mutant chromosome)  \( \text{fafx}+/+; \text{P-line}+/+ \ x \ \text{fafx}+/+; \text{P-line}+/+ \)

### Table 1

<table>
<thead>
<tr>
<th>Stock</th>
<th>Isolated Chromosome</th>
<th>Wild Type Phenotype</th>
<th>( sctt ) Phenotype</th>
<th>Ectopic Germ Cells</th>
<th>Total Embryos</th>
<th>% Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>( krz^{01503} / \text{TM6b, Hu} )</td>
<td>( krz )</td>
<td>137</td>
<td>-</td>
<td>-</td>
<td>137</td>
<td>0.0</td>
</tr>
<tr>
<td>TM6b, Hu balancer</td>
<td>107</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>120</td>
<td>10.8</td>
</tr>
<tr>
<td>( \text{csk}^{13D8} / \text{TM3, Sb} )</td>
<td>( \text{csk} )</td>
<td>61</td>
<td>-</td>
<td>12</td>
<td>73</td>
<td>16.4</td>
</tr>
<tr>
<td>Sb balancer</td>
<td>68</td>
<td>-</td>
<td>11</td>
<td>79</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>( crk^{KG00356}/\text{ciD} )</td>
<td>( crk )</td>
<td>140</td>
<td>-</td>
<td>-</td>
<td>140</td>
<td>0.0</td>
</tr>
<tr>
<td>ciD balancer</td>
<td>88</td>
<td>1</td>
<td>4</td>
<td>93</td>
<td>5.4</td>
<td></td>
</tr>
</tbody>
</table>

### Figure 3.2

Identification of the chromosome causing the mutant phenotype.

(A) The crossing scheme to test which chromosome was causing the mutant phenotype. Males carrying one copy of the balancer and one copy of the P-element insertion chromosome were crossed to fafx/fafx (wild type) females. Progeny were collected from cross 1 and flies carrying the balancer were crossed to each other, cross 2A. Flies carrying the P-element lines were crossed together, cross 2B. (B) Embryos were collected from crosses 2A and 2B and aged 12-15 hours. Germ cells were visualized with X-Gal.
Figure 4.1. The scattershot (sctt) phenotypes. The sctt embryos have defects in both germ cell migration and programmed cell death. In all panels, dorsal views of 12-15 hour embryos are shown. Anterior is to the left. Germ cells are labeled using a fat facets-lacZ transgene. (A) A control embryo. At 12-15 hours of development, the germ cells have reached the somatic gonad precursor cells and have coalesced to form the gonads. (B) An embryo from a homozygous mutant mother that also lacks zygotic function of sctt. In these mutants, the germ cells lack directed migration and scatter throughout the posterior half of the embryo. In addition, the germ cells ectopic to the gonads fail to undergo programmed cell death. The somatic gonad forms normally. (C) An embryo from a homozygous mutant mother that has been crossed to a male harboring a wild-type copy of the tre1 gene. The germ cell migration defect is completely rescued. However, germ cells ectopic to the gonads remain.
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