

# **Investigations of potential roles of hypoxic response genes in *Drosophila* primordial germ cell development**

by

**Elizabeth Marie Asque**

A thesis submitted to the graduate faculty in partial fulfillment of the

requirements for the degree of

**MASTER OF SCIENCE**

Major: Genetics

Program of Study Committee:

Clark R. Coffman, Co-Major Professor

Jo Anne Powell-Coffman, Co-Major Professor

Erik Vollbrecht

Ning Fang

Iowa State University

Ames, Iowa

2011

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## CHAPTER 1: INTRODUCTION

Metastasis, the spread and colonization of cancer cells to distant tissues, of tumors is the leading cause of cancer patient mortality. The mechanisms controlling the initiation and progression of metastasis are diverse (Yilmaz and Christofori 2010). The tumor microenvironment largely influences the induction of certain biological pathways that are essential to tumor homeostasis and growth (Brizel *et al.* 1996; Dayan *et al.* 2008). Vasculature networks are unable to deliver sufficient oxygen and nutrients to rapidly proliferating tissues, such as solid tumors. Limited in the supply of oxygen, as the solid tumor grows it becomes increasingly at risk of having a hypoxic and even necrotic center (Semenza 2009). Hypoxic response networks are often invoked to cope with this stress. The elevated expression of the hypoxia-inducible factor-1 (HIF-1) hypoxic response network components is a notable characteristic of more aggressive cancers (Cichon *et al.* 2010; Coghlin and Murray 2010).

Investigation of cellular and developmental processes such as cell cycle progression, cellular differentiation, and programmed cell death have been central for understanding the diverse molecular pathways that direct multicellular development and pathologies such as cancer. Often, we can gain important insights to these processes in the contexts of model organisms. *Drosophila*, murine mammals, and zebra fish are model organisms that have been used as platforms for the investigation of the patterns and mechanisms of germ line development (Richardson and Lehmann 2010; Kunwar *et al.* 2006; Coffman 2003).

The mechanisms of primordial germ cell development, which are highly conserved across phyla, allow developmental biologists to address questions about the progression of

cellular development within the germ line. In many animals, including mammals, fruit flies, and zebra fish these cells are not formed in the location of the developing gonads (Sonnenblick 1994; Jaglarz *et al.* 1995; Knaut *et al.* 2003; Molyneaux *et al.* 2003; Santos and Lehmann 2004; Richardson and Lehmann 2010). Therefore during embryogenesis, the primordial germ cells actively migrate through developing epithelial and mesenchymal layers to reach their final destination, the gonads. This characteristic of the developing germ line allows us to address cellular mechanisms of migration in an *in vivo* context.

Utilizing *Drosophila* germ cell development as an *in vivo* model provides a platform for addressing outstanding questions about signaling networks that regulate cell migration, chemotaxis, survival during migration, and cell-cell interactions during migration in a dynamic environment, the developing embryo (Montell 2006). *Drosophila* primordial germ cells are a useful model because they are formed early in development, and the path of migration to the gonads can be altered, both genetically and by pharmaceuticals, for study (Santos and Lehmann 2004; Kunwar *et al.* 2006). These characteristics of *Drosophila* germ cells provide a means of investigating areas of cellular development such as migration, survival, stress response, cellular signaling, and differentiation. Here I have used the development of germ cells in *Drosophila* as a tool to study the effects of low oxygen stress, and to explore potential roles of hypoxic response genes in germ cell development. Utilizing low oxygen culture conditions and loss-of-function mutants I have observed that *Drosophila* embryogenesis is sensitive to oxygen tension and the zygotic loss of *Drosophila* HIF-1 $\alpha$  is not sufficient to induce primordial germ cell defects.

The following topics are essential to my research: *Drosophila* germ cell migration and programmed cell death, oxygen tension and the regulation of the Hypoxia-Inducible

transcription Factor-1 (HIF-1), the *Drosophila* regulatory mechanisms of HIF-1 signaling. These are discussed in detail in Chapter 2. The ideas and results of previous investigations shaped the central questions addressed in my research. The results and discussion of my work investigating potential roles of hypoxia and HIF-1 signaling network components are presented in Chapter 3. The results presented here provide the groundwork for further investigations of the role of *Drosophila* HIF-1 in germ cell programmed cell death. Discussion of these ideas can be found in Chapter 4.

Appendix A describes a collaboration with Anthony Stender, a graduate student in Ning Fang's group in the Department of Chemistry here at Iowa State University, where we investigate the potential of utilizing differential interference microscopy to image nanoparticles in a dynamic environment such as the developing *Drosophila* embryo.

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## **CHAPTER 2: LITERATURE REVIEW**

### **Drosophila Germ Cell Development**

Fertilized *Drosophila* embryos begin development with rapid rounds of karyokinesis without cytokinesis. The embryo is a syncytial blastoderm during initial phases of development. The nuclei that are most posteriorly located reside within a region of the embryo that contains germ cell determining factors, otherwise known as the region of “germ plasm” (Williamson and Lehmann 1996). The germ cells are the first to cellularize, and it is this event that distinguishes the primordial germ cells from the remainder of the developing somatic tissue (Sonnenblick 1994). The primordial germ cells have a distinct morphology from the soma; they are large and spherical in shape. This distinct morphology helps make the primordial germ cells a useful model system for tracking mechanisms of cellular development such as migration and death.

At the posterior end of the young embryo pole buds form, isolating the nuclei within this region. These isolated nuclei, before cellularization, divide twice. The germ cells are formed upon cellularization of the nuclei at the posterior end (Rabinowitz 1941; Sonnenblick 1994; Williamson and Lehmann 1996). These germ cells undergo up to 2 rounds of cellular division to increase their population to approximately 36-40 cells (Richardson and Lehmann 2010). Upon the last division the cells arrest in the G2 phase of the cell cycle and remain arrested at this phase until after the germ cells have been incorporated into the developing gonads (Sonnenblick 1994; Williamson and Lehmann 1996). After their division and arrest in G2, the germ cells employ RNA regulatory networks as well as transcriptional repression

mechanisms to prevent primordial germ cell differentiation into somatic tissues (Cinalli *et al.* 2008).

Despite cell cycle arrest after their initial divisions, the primordial germ cells remain translationally active (Zalokar 1976). Studies using radioactive uridine demonstrated that the primordial germ cells are not transcriptionally active until the invagination of the primordial midgut, approximately 4 hours post fertilization (Zalokar 1976; Sonnenblick 1994). Therefore, genetic assays of early embryonic development must consider the effects of maternally contributed transcripts, at least until zygotic transcription begins.

The migration of the germ cells to the gonads can be divided into three separate phases: (i) invagination of the germ cells in close association with the underlying primordial midgut epithelium to the interior of the embryo, (ii) initiation of active migration through the primordial midgut epithelium, and (iii) bilateral migration to the somatic gonadal precursor cells and coalescence of the germ cells and somatic gonad precursor cells to form a gonad (Montell 2006; Richardson and Lehmann 2010). Below I have elaborated on the progression of germ cell development for each of these milestones.

*Invagination of the germ cells to the interior of the embryo:*

At the start of gastrulation the germ band begins to elongate along the dorsal side of the embryo. This action initiates the formation of the primordial mesoderm and endoderm. Here the developing soma of the germ band rapidly undergo cellular divisions, this “pushes” the cluster of primordial germ cells towards the interior of the embryo. Along with the developing mesoderm this action creates the midgut pocket of the developing embryo. It is most notable here that the germ cells must avoid differentiation as the surrounding soma develops (Sonnenblick 1994). The completion of germ band extension at stage 10 and the



activation of zygotic transcription are indicators of the germ cells initiation of active migration through the primordial midgut epithelia (Campos-Ortega and Hartenstein 1997; Zalokar 1976; Jaglarz *et al.* 1995; Kunwar *et al.* 2003).

*Initiation of active migration through the primordial midgut epithelium:*

By the end of stage 10 (approximately 4 hours and 30 minutes post fertilization) the primordial germ cells have created cellular extensions and actively moved through the midgut epithelial layer (Campos-Ortega and Hartenstein 1997). This movement is proposed to be the result of several mechanisms that are both primordial germ cell autonomous as well as soma dependent (Jaglarz *et al.* 1995).

Recent studies demonstrated that a G-protein coupled receptor (GPCR) called Trapped-in-endoderm-1 (Tre1) is required for the initiation of germ cell migration through the midgut epithelium (Kunwar *et al.* 2003). Active Tre1 mediates germ cell polarization of the GTPase Rho1, G-protein subunit G $\beta$ 13f, and *Drosophila* E-cadherin (Shotgun) (Kunwar *et al.* 2008). In *tre1* mutants these proteins are uniformly distributed within the germ cells at stage 9-10, instead of being concentrated at the trailing ends of the germ cells. Therefore, at stage 9-10 the activation of Tre1 is necessary to disrupt the polarization of cell adhesion proteins within the germ cells to direct their exit through the primordial midgut (Raz 2004; Kunwar *et al.* 2008; Richardson and Lehmann 2010). However, further study is needed to identify what triggers the activation of germ cell migration.

*Bilateral migration to the somatic gonadal precursor cells and coalescence of the gonads:*

In wild-type embryos once the germ cells have traversed the midgut epithelium they split bilaterally into two independent migrating populations of cells. At stage 13 of embryogenesis the two clusters of germ cells make contact with the somatic gonadal

precursor cells. The germ cells and the somatic gonadal precursor cells then begin to coalesce, and complete coalescence of the gonads is observed by stage 15 (Jaglarz *et al.* 1995; Williamson and Lehmann 1996; Kunwar *et al.* 2006). The loss-of-function of several genes expressed by the germ cells and the somatic tissue demonstrated that the germ cells pathfind to the somatic gonadal precursor cells (Kunwar *et al.* 2006; Kamps *et al.* 2010). For example *tre1<sup>scd</sup>* mutants, a severe hypomorphic allele, exhibit primordial germ cells widely distributed across the posterior end of the embryo. Very few germ cells successfully migrate to the gonads (Kamps *et al.* 2010). Therefore, in addition to initiating polarization of adhesion proteins in the germ cells, germ cell expression of Tre1 also has roles in germ cell pathfinding.

Mutation of genes expressed by the somatic tissue, *wunen*, *wunen2*, *columbus*, and *mdr49*, display similar germ cell pathfinding defects. The resultant mutant phenotypes of these genes suggest that the two bilateral migrating clusters pathfind chemotactically to the somatic gonadal precursor cells (Kunwar *et al.* 2006; Richardson and Lehmann 2010). The expression of these genes by the somatic tissues is predicted to create a gradient, either attractive or repulsive, that “steers” the germ cells towards the gonads (Richardson and Lehmann 2010; Montell 2006; Raz 2004). Thus the migration and coalescence of the germ cells into the developing gonad is not entirely germ cell dependent. However, the nature of this gradient and the signals that either attract or repel the germ cells towards the somatic gonadal precursor cells are still unknown.

#### *Primordial germ cell death during development*

Approximately 12-13 hours post fertilization, the coalescence of the primordial germ cells and somatic gonadal precursor cells is complete, and the gonads are formed

(Sonnenblick 1994; Williamson and Lehmann 1996). The two gonads have roughly 10-15 germ cells each (this number is dependent upon genetic background) and are localized within the fifth abdominal segment on either side of the embryo upon completion of embryogenesis (Williamson and Lehmann 1996). Interestingly, between germ cell divisions after the initial cellularization of the germ cells until gonad coalescence, the number of germ cells decreases to approximately half of the starting population (Sonnenblick 1941; Coffman *et al.* 2002; Yamada *et al.* 2008; Richardson and Lehmann 2010).

It is still unclear as to which primordial germ cells are terminated during migration and what regulates programmed cell death in this population of cells. Multiple genes originally noted for their migration defects in germ cell development are also critical for germ cell survival or programmed germ cell death during development (Kamps and Coffman 2005; Coffman 2003). For example, mutations in genes *tre1*, *outsiders*, and *p53* confer a germ cell death defect (Coffman *et al.* 2002; Yamada *et al.* 2008; Kamps *et al.* 2010). These mutants have wild-type numbers of germ cells in the gonads. The mutant phenotype manifests as extra germ cells ectopic to the gonads. These ectopic cells still express germ cell markers, thus germ cell programmed cell death has been abrogated (Coffman *et al.* 2002; Richardson and Lehman 2010; Kamps *et al.* 2010)

Further research is needed to resolve the mechanisms controlling germ cell pathfinding and programmed cell death (Coffman *et al.* 2002; Coffman 2003; Coffman and Kamps 2005; Kamps *et al.* 2010). The transcription factor hypoxia-inducible factor-1 (HIF-1) has roles in both mediating cellular pathfinding and programmed cell death in mammals (Semenza 2009). The role of HIF-1 in these two processes is most notable in the progression of primary tumor cells to metastatic states (Chan and Giaccia 2007; Dayan *et al.* 2008;

Semenza 2009). HIF-1 function is conserved across diverse phyla (Rytönen *et al.* 2011). Thus using model systems to address the extent to which HIF-1 may be involved in cellular pathfinding and programmed cell death provides an excellent platform to further understand the roles of HIF-1 in pathogenesis.

Below I will discuss the conserved mechanism of HIF-1 regulation in vertebrates and invertebrates. I will then address how investigating what roles HIF-1 may have in cellular pathfinding and programmed cell death will advance our understanding of each of these dynamic biological processes.

### **Oxygen Tension Regulation of HIF-1 Dependent Hypoxic Response**

The hypoxia-inducible factor-1 (HIF-1) is a transcription factor complex that mediates response to low oxygen stress. It was first elucidated through studies of erythropoiesis (Semenza 1992). The complex is made up of two subunits and exhibits DNA binding activities (Wang and Semenza 1995). This activity of HIF-1 requires *de novo* protein synthesis, providing evidence that HIF-1 is a post-translationally regulated transcription factor (Wang 1993). Inducible by hypoxia in multiple cell and tissue types HIF-1 can be active both systemically and locally within an organism (Wang 1993; Semenza 1996). These initial observations provided the ground work for studying the activity of the transcription factor HIF-1.

Biochemical analysis, cloning techniques, and the utilization of sequence databases confirmed that the HIF-1 transcription factor is a heterodimer composed of two basic helix loop helix-Per ARNT Sim (bHLH-PAS) domain protein family members: HIF-1 $\alpha$  and HIF-1 $\beta$  (otherwise known as ARNT) (Semenza 1992; Wang 1993; Wang and Semenza 1995).

Hypoxia increased the stabilization of HIF and expression of HIF subunit RNAs. Elevated levels of HIF- $\alpha$  and HIF- $\beta$  within the nucleus are induced upon hypoxic exposure as well. This is the evidence that predicted these two proteins constitute the HIF-1 heterodimer (Wang *et al.* 1995).

The HIF-1 transcription factor complex is regulated by oxygen concentrations within the cell (Jiang *et al.* 1996). The oxygen sensing mechanism that regulates HIF-1 stabilization targets only the  $\alpha$ -subunit (Salceda and Caro 1997). Mammalian cells express more than one  $\alpha$ -subunit of HIF that, in conjunction with HIF- $\beta$ , is able to elicit response to hypoxic stress. Though response to hypoxia is mostly mediated by HIF-1 $\alpha$ , tissue specific expression has been observed for HIF-2 $\alpha$  and HIF-3 $\alpha$  (Wenger 2002; Huang and Bunn 2003). When the mammalian HIF-1 complex is mentioned from here onwards it is in reference to the subunits HIF-1 $\alpha$  and HIF-1 $\beta$ .

In normal oxygen conditions the hydroxylation of conserved proline residues 402 and 564 in HIF-1 $\alpha$  is mediated by a prolyl-4-hydroxylase (PHD) (Huang *et al.* 1998; Huang and Bunn 2003; Huang *et al.* 2002; Semenza 2004; Fong and Takeda 2008). Mammals have three PHDs (PHD1-3) that all exhibit prolyl hydroxylase enzymatic activity on HIF- $\alpha$  proteins (Epstein *et al.* 2001; Bruick and McKnight 2001; Lando *et al.* 2003). PHD2, however, has the highest specificity for hydroxylating HIF-1 $\alpha$ . Hypoxia slows down the degradation of HIF-1 $\alpha$ . PHDs utilize molecular oxygen, 2-oxoglutarate, iron, and ascorbate for substrate hydroxylation (Lando *et al.* 2003; Fong and Takeda 2008). Thus low oxygen conditions, 2-oxoglutarate analogs, and iron chelators are all inhibitors of PHD activity (Fong and Takeda 2008). As oxygen tension decreases the accumulation of stable HIF-1 $\alpha$  increases (Jiang *et al.* 1996; Semenza 2001; Semenza 2004). Interestingly, under hypoxia PHD2 is a target gene of

HIF-1 (Bruick and McKnight 2001; Huang *et al.* 2002; Berra *et al.* 2003; Fong and Takeda 2008). This negative feedback demonstrates that though HIF-1 is active in hypoxic conditions, the cellular environment is prepped for destabilizing HIF-1 $\alpha$  upon reoxygenation.

Once hydroxylated, degradation of HIF-1 $\alpha$  is mediated by the von Hippel Lindau (pVHL) tumor suppressor, a component of the E3 ubiquitin ligase complex (Salceda and Caro 1997; Ivan *et al.* 2001; Jaakola *et al.* 2001). The use of proteasome inhibitors and cell lines that lack pVHL, assayed independently and in conjunction, caused an accumulation of HIF-1 $\alpha$  in normal oxygen conditions. Thus demonstrating that HIF-1 $\alpha$  once hydroxylated requires pVHL and an ubiquitin proteasome for degradation (Wenger 2002; Huang and Bunn 2003; Ivan *et al.* 2001; Jaakola *et al.* 2001).

However, transcriptional activity of HIF-1 is not solely achieved by stabilization of HIF-1 $\alpha$ . To activate HIF-1 for transcription of hypoxic response genes, the subunits, HIF-1 $\alpha$  in particular, must undergo protein modification, nuclear translocation, heterodimerization, DNA binding, and recruit transcriptional co-activators (Wenger 2002; Bilton and Booker 2003; Lando *et al.* 2003; Metzen and Ratcliffe 2004; Semenza 1998; Semenza 2001; Semenza 2009). The interaction with CBP/p300 indicates that HIF-1 is not only stable, but active in transcription (Kallio *et al.* 1998; Ema *et al.* 1999; Wenger 2002). The hydroxylation modification of asparagine residue 803 of HIF-1 $\alpha$  inhibits CBP/p300 from interacting with the C-terminal activation domain of HIF-1 $\alpha$ . This hydroxylation event on asparagine 803, like proline 564 and 402, is catalyzed by a hydroxylase called factor-inhibiting HIF-1 (FIH) (Lando *et al.* 2002). FIH activity on asparagine 803, like PHD, is dependent on oxygen availability (Hewitson *et al.* 2002; Wenger 2002; Huang and Bunn, 2003). Regulation of HIF-1 $\alpha$  stability and activity by oxygen dependent hydroxylation of

proline 564 and 402, and asparagine 803 demonstrates the tightly controlled transcriptional activity of HIF-1. Once active, HIF-1 trans-activates genes that contain hypoxic response elements (HREs) within the promoter region (Wenger 2002). The consensus sequence of HREs is 5'-RCGTG-3' (Huang and Bunn 2003).

The hypoxic response network is conserved across diverse phyla. Invertebrates, such as *Drosophila* and *C. elegans*, unlike mammals, have only single genes encoding both HIF-1 subunits and regulators, PHD and VHL (Bacon *et al.* 1998; Powell-Coffman *et al.* 1998; Ma and Haddad 1999; Aso *et al.* 2000; Jiang *et al.* 2001; Bruick and McKnight 2001; Epstein *et al.* 2001; Rytönen *et al.* 2011). Recently, HIF-1 activation was demonstrated to prevent DNA damaged induced apoptosis of the *C. elegans* germ line (Sendoel *et al.* 2010). In mammals, HIF-1 over activation in tumors correlates with poor patient prognosis due to tumor resistance to anticancer therapies, such as ionizing radiation, to induce programmed cell death (Gray *et al.* 1953; Brizel *et al.* 1996; Chi *et al.* 2006; Chan and Giaccia 2007; Dayan *et al.* 2008; Semenza 2009). Results from Sendoel *et al.* 2010 also demonstrated that HIF activity in cancer cells, melanoma, abrogated programmed cell death by a mechanism homologous to that uncovered in *C. elegans* (Sendoel *et al.* 2010). Thus because of the high conservation of HIF across diverse phyla, the investigation of HIF in model systems can serve as a sound platform for further understanding the various mechanisms of HIF in mammals.

Yet to be investigated within the *Drosophila* model system, are the effects of hypoxia and the roles of HIF-1 in primordial germ cell development. *Drosophila* primordial germ cells provide a genetically tractable model system of active cellular migration and survival (refer to *Drosophila* Germ Cell Development section). The high conservation of the hypoxic

response network allows us to elucidate the roles of hypoxia and HIF-1 in *Drosophila* germ cell development.

Here I will describe investigations of hypoxic response within the *Drosophila* system and how the model platform of primordial germ cell development has been useful to address unanswered questions of cellular responses to low oxygen conditions.

## **Oxygen Tolerance in *Drosophila***

### *Discovery of a homologous HIF system: The *Drosophila* HIF components*

Many of the early experiments used to uncover the system of hypoxic response in *Drosophila* adopted approaches that were successful in resolving the human hypoxia-inducible factor (HIF-1) and its subunits. In 1996, Nagao *et al.* detected a hypoxia induced complex from *Drosophila* SL2 cells that bound to oligonucleotides consisting of known human HIF-1 DNA binding sites (Nagao *et al.* 1996). This complex and the human HIF complex exhibited characteristics such as induction in cells after 16 hours of exposure to 1% oxygen. The mammalian and *Drosophila* hypoxia inducible complexes were also unable to be eluted with oligonucleotides mutant for the HIF-1 DNA binding site (Nagao *et al.* 1996; Jiang *et al.* 1996).

In contrast to mammalian studies however, very little to no complex was pulled down from *Drosophila* cells treated with hypoxic mimetics (compounds that inhibit PHD activity), desferrioxamine and cobalt ions respectively (Nagao *et al.* 1996). These data revealed that *Drosophila* possesses a complex that is inducible under similar conditions to the mammalian hypoxia-inducible transcription factor, HIF-1. Further establishing this inducible transcription factor as the homolog to mammalian HIF was the elucidation of its subunits discussed below.



The search for the subunits of *Drosophila* HIF-1 began with examining proteins with bHLH-PAS domains. Proteins from the bHLH-PAS family have distinct motifs that place them within this class. The motifs that are conserved within this protein family are the bHLH region, a PAS domain (named after the first three members discovered of this family Per, ARNT and Sim), and C-terminal glutamine rich regions that typically function as transactivation domains (Zelzer *et al.* 1997). The *Drosophila* HIF-1 $\alpha$  homolog, *similar*, was uncovered using these criteria (Nambu *et al.* 1996; Zelzer *et al.* 1997; Bacon *et al.* 1998). A genomic screen of sequence from conserved regions of the bHLH-PAS domain from *single-minded*, a known member of the bHLH-PAS protein family, was conducted to identify a possible *Drosophila* HIF-1 $\alpha$  homolog (Nambu *et al.* 1996). From this screen one candidate clone expressed a protein with a conserved bHLH region. This gene was uncharacterized but had the highest identity to human HIF-1 $\alpha$  out of three known bHLH-PAS proteins, human HIF-1 $\alpha$ , and *Drosophila* genes *trachealess* (*trh*), and *single-minded* (*sim*). This novel gene had 63% identity with human HIF-1 $\alpha$ , 59% identity to *trh*, and 55% identity to *sim*; it was thus named *similar* (*sima*). Nambu *et al.* then analyzed the Similar peptide sequence in attempts to uncover possible conserved structural motifs (bHLH and PAS domain, and a C-terminal glutamine rich region) that are indicative of bHLH-PAS family members (Nambu *et al.* 1996; Zelzer *et al.* 1997). Similar does indeed have these conserved characteristics. The conserved bHLH-PAS family structural motifs and high identity to human HIF-1 $\alpha$  protein informs us that *similar* is a reasonable candidate *Drosophila* homolog to the mammalian HIF-1 $\alpha$ . Phylogenetic analyses strongly suggested that *Drosophila similar* and human HIF-1 $\alpha$  were homologs, and the next step was to test the hypothesis that they had analogous functions.

The functional *Drosophila* homolog to mammalian HIF- $\alpha$  was uncovered by DNA binding experiments with candidate proteins in hypoxic conditions. The bHLH-PAS domain of four candidate *Drosophila* HIF-1 $\alpha$  proteins (Sim, Sima, Trh, and Tgo) were fused to a GAL4 DNA binding domain and assayed for DNA binding activity under hypoxic and hypoxic mimetic conditions. Only two of the fusion proteins exhibited trans-activation activity under hypoxic conditions, Sima and Tgo. Of these two, the GAL4 fusion with the bHLH-PAS domain from Sima induced trans-activation in hypoxia, desferrioxamine, and cobalt ion exposure (these chemicals inhibit PHD function, refer to Oxygen tension regulation of the HIF-1 dependent hypoxic response network section). With the Tgo bHLH-PAS fusion, trans-activation was most detectable in hypoxic conditions, very little in desferrioxamine, and not detectable from exposure to cobalt ions. Trans-activation activity induced from GAL4 DNA binding domain fusions with Sima bHLH-PAS was compared to mammalian HIF-1 $\alpha$  trans-activation; both produced analogous results. *similar* is thus the gene in *Drosophila* that exhibits hypoxia inducible transcription factor activity and is the most likely homolog of mammalian HIF- $\alpha$  (Bacon *et al.* 1998). This conclusion provided evidence to support the hypothesis that *Drosophila* also has a transcription factor which functions in responding to hypoxic stress. However specific function of Tgo in eliciting hypoxic response was not yet examined.

The homologous *Drosophila* hypoxia-inducible transcription factor complex was confirmed by functional studies of the *Drosophila* HIF-1 $\beta$ -subunit, Tgo. Zelzer *et al.* investigated bHLH-PAS protein target gene specificity. Sequence alignments demonstrated that the *Drosophila* gene *aryl-hydrocarbon nuclear translocator* (ARNT) was 95% identical to the mammalian ARNT gene (Zelzer *et al.* 1997; Sonnenfeld *et al.* 1997). ARNT is the  $\beta$ -

subunit (HIF-1 $\beta$ ) in mammalian HIF-1, and the first member of the bHLH-PAS domain protein family identified (Wang and Semenza 1995; Zelzer *et al.* 1997; Ma and Haddad 1999). Like mammalian HIF-1 $\beta$ , the *Drosophila* homolog, Tango (Tgo), is a bHLH-PAS protein and functions as a common dimerization partner to confer transcription factor activities in numerous regulatory and signaling pathways (Sonnenfeld *et al.* 1997). Results from immuno-histochemical, yeast two-hybrid, and co-transfection assays all support the conclusion that Tgo is the *Drosophila* HIF-1 $\beta$  homolog (Jiang *et al.* 1996; Sonnenfeld *et al.* 1997; Ma and Haddad 1999).

#### *Regulation of Drosophila HIF*

A key characteristic of HIF-1 is its subunit regulation and activation. Of the two subunits only HIF-1 $\alpha$  (Sima in *Drosophila*) is regulated by oxygen tension within the cell (Wang *et al.* 1995; Jiang *et al.* 1996; Salceda and Caro 1997). Collectively results from previous studies provided an oxygen dependent model of the regulation of HIF-1 in *Drosophila* (Figure 1). In environments with sufficient oxygen a prolyl-4-hydroxylase post-transcriptionally modifies proline residue 564 of HIF-1 $\alpha$  by hydroxylation, VHL interacts with HIF-1 $\alpha$  once hydroxylated and poly-ubiquitinates it to be targeted for proteasomal degradation (Huang *et al.* 1998; Salceda and Caro 1997; Ivan *et al.* 2001; Jaakola *et al.* 2001). Homologs of these regulatory proteins are also found within the *Drosophila* genome.

The existence of PHDs, HIF- $\alpha$  prolyl-4-hydroxylases, in both mammals and *C. elegans* argues for the existence of a conserved prolyl-4-hydroxylase in *Drosophila* (Epstein *et al.* 2001; Bruick and McKnight 2001). To test the hypothesis that *Drosophila* HIF-1 prolyl hydroxylase (HPH) negatively regulated Sima-dependent transcription, the induction of an HRE reporter was monitored in *Drosophila* embryonic cell line KC167 transfected with HPH

RNAi. Results were analogous to mammalian HPHs, HRE reporter expression increased in cells transfected with HPH RNAi compared to controls. This indicated that Sima, like mammalian HIF-1 $\alpha$ , is post-transcriptionally modified by the hydroxylation of a proline residue catalyzed by a prolyl hydroxylase (Bruick and McKnight 2001).

The *Drosophila* von Hippel-Lindau (dVHL) protein was elucidated by interaction and co-immunoprecipitation assays. The candidate dVHL was first examined for its functional ability to interact with known E3 ubiquitin ligase components, Cul-2, Rbx-1, and Elongin B/C. Results from this assay revealed that dVHL was in fact able to associate with these proteins and form a complex that exhibited E3 ubiquitin ligase activity. Further evaluation of candidate dVHL examined its interaction with human HIF-1 $\alpha$  in comparison with human VHL (pVHL) (Aso *et al.* 2000).

Immuno precipitation assays revealed that dVHL and pVHL co-precipitated with mammalian HIF-1 $\alpha$ . This indicated that pVHL and dVHL are able to bind mammalian HIF-1 $\alpha$ . Incubation of pVHL and dVHL with E3 ubiquitin ligase components and HIF-1 $\alpha$ , demonstrated that both proteins formed complexes that were able to ubiquitinate HIF-1 $\alpha$ . pVHL is more proficient in the ubiquitination of HIF-1 $\alpha$  than dVHL (Aso *et al.* 2000).

Accordingly, these results demonstrated that the candidate dVHL is in fact a true homolog of mammalian VHL and has ubiquitin ligase activity. Genetic analyses of *Drosophila* tracheal development further supported the conclusion that the HIF regulatory pathway was evolutionarily conserved (see the HIF-1 Function in *Drosophila* section below) (Lavista-Llanos *et al.* 2002).

#### *HIF-1 Function in Drosophila*

Experiments substantiating the presence of a functional hypoxic response system in *Drosophila* began with analyzing mutant alleles of the candidate subunits of HIF-1, *similar* and *tango*, HIF- $\alpha$  and HIF- $\beta$  respectively. Mutations in either *similar* or *tango* produced no expression of a hypoxia inducible reporter. The expression of the hypoxia inducible reporter was still detected in *trachealess* and *single-minded* mutants, other *Drosophila* genes with sequence similarities to HIF subunits. These results argued that Sima and Tgo compose the *Drosophila* HIF-1 (Lavista-Llanos *et al.* 2002).

Like mammalian HIF- $\alpha$ , Sima also has an oxygen dependent degradation domain (ODDD) (Huang *et al.* 1998). When the amino acids 692-863 (the candidate ODDD) were removed from *sima*, the modified *sima* protein was detected in normoxia at elevated levels that were comparable to hypoxic exposure. Similar is likely regulated by post-transcriptional modification like HIF-1 $\alpha$ , i.e., subject to prolyl hydroxylation by a prolyl-4-hydroxylase. dsRNAi of *Drosophila Hph*, later named *fatiga* (*fga*) (Centanin *et al.* 2005), resulted in Sima localization to the nucleus and expression of a hypoxia inducible reporter (Lavista-Llanos *et al.* 2002). This result led to the analysis of *fga*, loss-of-function mutants which displayed analogous results to the dsRNAi knock down experiments. Thus *fga* is a functional prolyl-4-hydroxylase. Overall the data from these experiments support the model that the protein stability of Sima, like HIF-1 $\alpha$ , is regulated by oxygen concentration within the cell via Fga the prolyl-4-hydroxylase (Lavista-Llanos *et al.* 2002).

The aforementioned studies are supported by evidence that Sima is regulated by oxygen concentration within *Drosophila* SL2 cells. Gorr *et al.* in 2004 observed that *Drosophila* SL2 cells exposed to 1% oxygen exhibited an accumulation of Sima. This result was considered significant because it was comparable to accumulated levels of mammalian

HIF-1 $\alpha$  in cells exposed to analogous conditions. SL2 cells incubated in oxygen exposures other than 1% displayed lesser amounts of Sima bound to HRE oligonucleotides. When SL2 cells were co-transfected with *sima* and a hypoxia inducible reporter gene, expression of the reporter was ~8.5 fold greater when cells were exposed to 1% oxygen than under control conditions. Whereas cells co-transfected with *tgo* and the *sima* splice variant (missing the ODDD region and the glutamine rich repeat which is important for heterodimerization, exons 8-11) did not produce this result. Together these results support the hypothesis that *sima* is the *Drosophila* homolog of HIF-1 $\alpha$ , and that the ODDD region is important for protein stabilization and the glutamine rich region may be necessary for nuclear localization of Sima and dimerization with Tgo (Gorr *et al.* 2004).

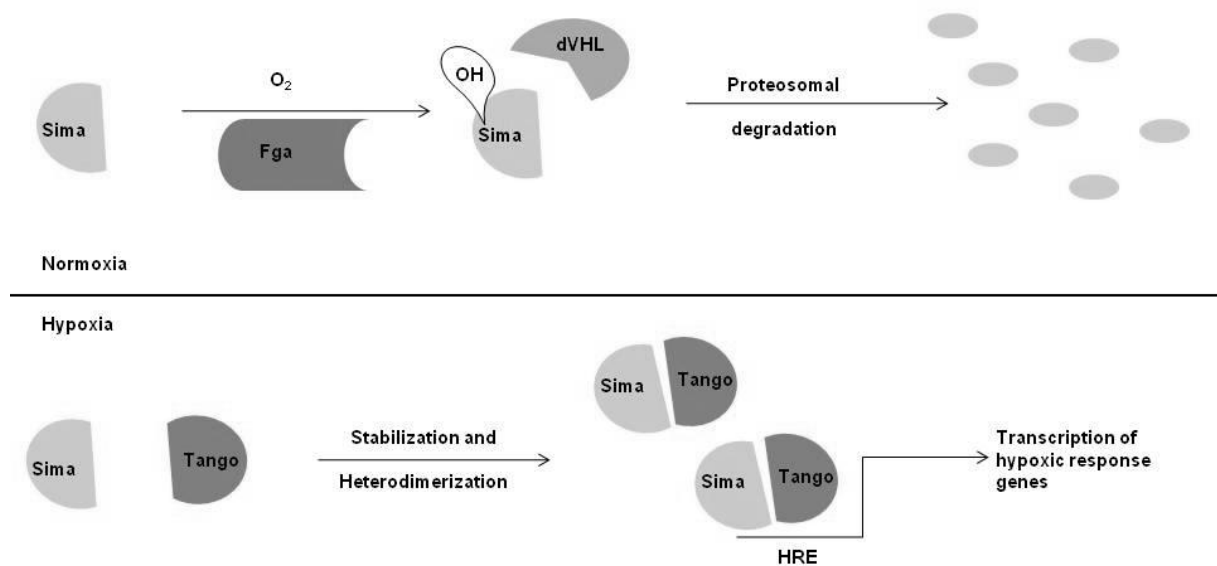
In addition to protein modification and degradation as mechanisms for regulating transcriptional activity of HIF in *Drosophila* and other organisms, oxygen concentrations also regulate the nuclear import and export of Sima. This mode of regulation is dependent on the nuclear localization signal in the C terminal activation domain, a nuclear export signal in the bHLH domain of Sima, and hydroxylation of the conserved proline residue 850. This conclusion is supported by the observation that Sima is mostly localized to the nucleus when proline 850 is mutated to alanine, the ODDD within the bHLH region is deleted, or when cells are incubated with Leptomycin B (LMB) a nuclear export receptor inhibitor (Gorr *et al.* 2006; Romero *et al.* 2008; Irisarri *et al.* 2009).

Other investigations of *Drosophila* Fga revealed that this prolyl hydroxylase is critical for functions outside of regulating hypoxic response. Using a deficiency screen to identify genes that may suppress a regulator of cellular growth, Cyclin D/Cdk4, one particular loss-of-function mutation produced a diminished growth phenotype. Flies with this deletion are

smaller than their wild type counterparts. Mosaic clones also exhibit homozygous mutant cells that are smaller in size compared to adjacent cells without the mutation. This suppressor mutation was an allele of *fga*. The diminished growth phenotype was rescued with *fga* cDNA injected into the mutant flies. *fga* is also needed for normal growth and cellularization. Homozygous mutants are lethal; indicating that *fga* is involved in developmental processes essential to organism development and homeostasis (Frei and Edgar 2004).

Tracheogenesis is not the only developmental event in *Drosophila* that involves the hypoxic response network. In adult females the ovary border cells migrate to the anterior end of the developing oocyte to create the micropyle. This directed migration requires HIF-1 activity for the transcription of the *Drosophila* homolog of C/EBP, *slow border cells (slbo)* (Doronkin *et al.* 2009). HIF-1 indirectly regulates E-Cadherin, Shotgun, expression in this system. In flies exposed to 1% oxygen or transgenic flies expressing *UAS-sima* in the border cells by a *slbo-GAL4* driver, border cell migration is severely delayed or halted. The hypothesis here is that Sima regulates *slbo* thus indirectly regulating the number of contacts between the border cells and the surrounding nurse cells of the ovary. Genetic *sima* loss-of-function mutants and *sima* mutants in mosaic clones do not display E-cadherin in the leading cell of the border cell cluster, only in wild-type cells is E-cadherin expressed at the leading edge. Within the ovary border cells the regulation of *slbo* and *shotgun* is dosage dependent of Sima. Thus Sima indirectly regulates the rate of border cell migration (Doronkin *et al.* 2009). Therefore *Drosophila* exemplifies roles of HIF-1 in cellular migration. This system can be analyzed further for investigating the rate of cell survival and response to cues within a dynamic environment, such as the developing embryo.

Sima, like HIF-1 $\alpha$ , is tightly regulated and is a part of the oxygen sensing mechanism in developing *Drosophila* embryos and adults (Figure 1). The information gained from the investigations of the HIF transcription factor to further understand oxygen sensing in development and disease is easily translatable because of its high conservation across phyla. However, many of the diverse mechanisms that regulate and are regulated by HIF are still heavily researched to develop better models of oxygen sensing within an organism.



**Figure 1 Oxygen dependent regulation of *Drosophila* Similar. (Top) Normoxic**

**conditions:** Similar (Sima) is hydroxylated at a conserved proline residue (P850) by the prolyl-hydroxylase Fatiga (Fga). Once hydroxylated Sima is targeted for degradation by dVHL, the *Drosophila* von Hippel Lindau protein. When dVHL has bound hydroxylated Sima the E3 ubiquitin ligase complex then ubiquitinates Sima. This ubiquitination, either poly- or mono-ubiquitination, targets Sima for proteosomal degradation. **(Bottom) Hypoxic**

**conditions:** In low oxygen Sima is stabilized. Fga is unable to use molecular oxygen as a substrate to hydroxylate Sima. Stable Sima is able to heterodimerize with Tango, the other subunit of the Hypoxia-Inducible transcription Factor (HIF-1). Sima and Tango dimerize via



their PAS domains. HIF recognizes Hypoxic Response Elements (HREs) located within the promoter region of genes that are transcriptionally regulated by hypoxia.

### Summary

HIF-1 activity increases in hypoxic tissues in both vertebrates and invertebrates, and diminishes in activity upon reoxygenation or upon becoming anoxic (Gorr *et al.* 2006).

Though the response to hypoxic stress by HIF in mammals and *Drosophila* is fairly consistent, response to hypoxia mimetics is somewhat reduced in *Drosophila* compared to mammals (Nagao 1996; Gorr 2004; Dekanty 2005; Gorr 2006).

Among diverse species, mammals to *Drosophila* or *C. elegans*, a target site of prolyl hydroxylation within HIF-1 $\alpha$  is conserved across these taxa; Pro 402 and 564 in mammals, Pro 850 in *Drosophila*, and Pro 621 in *C. elegans* (Huang *et al.* 1998; Epstein *et al.* 2001; Jiang *et al.* 2001). The regulatory mechanism of HIF-1 $\alpha$  is centered on this proline residue as a site of hydroxylation. Hydroxylation of the conserved proline in HIF-1 $\alpha$  by a prolyl-4-hydroxylase readies the protein for ubiquitination and subsequent proteasomal degradation (Rytönen *et al.* 2011).

Many of the essential proteins governing the hypoxic response network are conserved across various model organisms. Within mammals, *Drosophila* and *C. elegans* for example there are homologs to both  $\text{HIF-1}\alpha$  and  $\text{HIF-1}\beta$  subunits of the HIF complex, the prolyl-4-hydroxylase, and VHL, a component of the E3 ligase complex. The regulation of the hypoxic response system also appears to be conserved.

The high conservation of this response network supports its significance in development and homeostasis. In model systems, HIF-1 serves as a platform to study oxygen homeostasis, development, and oxygen-related pathogenesis. To further take advantage of

the conservation of HIF-1, we have investigated hypotheses of the potential roles of HIF-1 signaling in *Drosophila* germ cell migration and survival.

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## CHAPTER 3: INVESTIGATIONS OF POTENTIAL ROLES OF HYPOXIC RESPONSE GENES IN PRIMORDIAL GERM CELL DEVELOPMENT

### INTRODUCTION

Oxygen is essential to organisms that utilize metabolic pathways of cellular respiration to produce energy. Hypoxia can be defined as the condition of oxygen tension that is too low to sustain these normal physiological processes (Semenza 1996; Chi *et al.* 2006; Wenger 2002). A typical response to hypoxia is the alteration of metabolic gene expression, switching to a less energy intensive mode of metabolism is critical for avoiding programmed cell death (Wenger 2002; Liu *et al.* 2006; Weidman and Johnson 2008; Semenza 2009). The hypoxia inducible transcription factors (HIFs) are the chief transcription factors that mediate responses to hypoxic stress (Weidmann and Johnson 2008).

HIFs are heterodimeric transcription factors composed of two basic helix-loop-helix-Per Arnt Sim (bHLH-PAS) protein subunits, termed alpha and beta (Wang and Semenza 1995; Wang *et al.* 1995; Weidman and Johnson 2008). The alpha and beta subunits interact via the bHLH domain to form the HIF transcription factor. Mammals have multiple isoforms of the HIF $\alpha$  and HIF $\beta$  subunits (Wenger 2002). Invertebrates such as *Drosophila* and *C. elegans*, only have one isoform of HIF $\alpha$  and HIF $\beta$ ; *similar (sima)* and *tango (tgo)* in *Drosophila*, and *hif-1 $\alpha$*  and *aha-1* in *C. elegans* (Nambu *et al.* 1996; Zelzer *et al.* 1997; Sonnenfeld *et al.* 1997; Bacon *et al.* 1998; Powell-Coffman *et al.* 1998; Ma and Haddad 1999; Jiang *et al.* 2001; Rytkönen *et al.* 2011; Zhao and Haddad 2011).

In normal oxygen conditions Sima is hydroxylated on a conserved proline residue by prolyl hydroxylase Fatiga (Fga) (Huang *et al.* 2002; Epstein *et al.* 2001; Bruick and



McKnight 2001; Centanin *et al.* 2005). This inhibits Sima from dimerizing with Tango (Lavista-Llanos *et al.* 2002; Gorr *et al.* 2006; Sonnenfeld *et al.* 2005). Once hydroxylated, Sima is then ubiquitinated by a component of the E3 ubiquitin ligase, the von Hippel Lindau protein (pVHL) (Adryan *et al.* 2000; Jaakola *et al.* 2001; Ivan *et al.* 2001). Both *Drosophila* Fga and pVHL are homologous to the mammalian prolyl hydroxylases (PHDs) and pVHL respectively (Aso *et al.* 2000; Adryan *et al.* 2000; Bruick and McKnight 2001; Centanin *et al.* 2005; Arquier *et al.* 2006; Gorr *et al.* 2006; Rytkönen *et al.* 2011). Upon ubiquitination Sima is then targeted for degradation (see also Chapter 2 Figure 1 normoxia) (Salceda and Caro 1997; Huang *et al.* 1998). In contrast, insufficient levels of oxygen restrict the activity of Fga due to limited molecular oxygen availability (Centanin *et al.* 2005; Arquier *et al.* 2006; Fong and Takeda 2008; Centanin *et al.* 2008). Sima, unhydroxylated, is able to dimerize with Tgo via the bHLH domain to constitute the HIF heterodimeric transcription factor (see also Chapter 2 Figure 1 hypoxia) and activate transcription of hypoxia response genes. The primary mechanism of oxygen dependent regulation of HIF $\alpha$  is conserved across a wide range of phyla (Nagao 1996; Bacon *et al.* 1998; Lavista-Llanos *et al.* 2002; Gorr *et al.* 2004; Gorr *et al.* 2006; Rytkönen *et al.* 2011; Zhao and Haddad 2011).

HIFs are often upregulated within a hypoxic tumor microenvironment (Chi *et al.* 2006; Weidman and Johnson 2008; Yilmaz and Christofori 2010). Studies have shown that this upregulation correlates with the metastatic progression due to the survival (resistance to cancer therapies) of primary tumor cells (Maxwell *et al.* 2001; Dewan *et al.* 2006; Chan and Giaccia 2007; Rankin and Giaccia 2008). Thus, because HIF-1 has a role in the initiation of cancer cell migration and survival it serves as a valuable diagnostic marker (Gray *et al.* 1953; Brizel *et al.* 1996).

*Drosophila* ovary border cell migration is also sensitive to oxygen tension and was used as a model to further examine the role of HIF-1 in cell migration. Doronkin *et al.* found that ovary border cell migration is mediated by Sima in a dose-dependent manner (Doronkin *et al.* 2009). The accumulation of Sima, blocked border cell migration, whereas heightened overexpression of Sima partially rescued blocked border cell migration (Doronkin *et al.* 2009). This demonstrates that there are cellular mechanisms in *Drosophila* that provide a testable system for investigating the influence of HIF on cell migration and survival. This can give further insight to understanding the roles of HIFs in metastatic progression.

The development of *Drosophila* primordial germ cells is another useful system for investigating cell migration and programmed cell death. Approximately 12 hours post fertilization the germ cells have coalesced with the somatic gonadal precursor cells; each gonad contains roughly 10-15 germ cells and is localized within the fifth abdominal segment (Sonnenblick 1994; Williamson and Lahmann 1996). During migration the germ cells respond to external cues that mediate their survival and migration to the gonads (Coffman *et al.* 2002; Coffman 2003; Kunwar *et al.* 2003; Raz 2004; Kunwar *et al.* 2006; Yamada *et al.* 2008; Richardson and Lehmann 2010). The effects of hypoxia however, have not yet been investigated in *Drosophila* germ cell development. *Drosophila* germ cell development is a suitable platform for elucidating the effects of hypoxia on cell migration because of the high conservation of the hypoxic response network, the early formation of the germ cells, their survival and migration patterns during development, and more than 100 years of genetic work using *Drosophila* as a model organism (Gorr *et al.* 2004; Arquier *et al.* 2006; Montell 2006; Gorr *et al.* 2006; Cinalli *et al.* 2008; Yamada *et al.* 2008; Richardson and Lehmann 2010).

Here we have used environmental and genetic approaches to elucidate the effects of hypoxia on *Drosophila* germ cell development. First, we used a low oxygen environment, 3% O<sub>2</sub>, to examine effects of hypoxia on germ cell development. Exposing wild-type embryos to 3% oxygen resulted in an approximate 3 hour delay in development. This is similar to the effect observed in *Drosophila* ovary border cells; exposure to 1% oxygen blocked border cell migration (Doronkin *et al.* 2009). Germ cells were also observed ectopic to the gonads in stage 15 embryos after 3% oxygen exposure. This phenotype is classified as extra ectopic germ cells because there are wild-type numbers of germ cells within the gonads at this stage (Coffman *et al.* 2002; Yamada *et al.* 2008). However, this phenotype could have been caused by other environmental components we have yet to examine. Using these results and observations from prior studies (Doronkin *et al.* 2009) we tested the hypothesis that HIF signaling mediated this oxygen-sensitive phenotype and asked if the gain or loss-of-function of *sima* has a role in *Drosophila* germ cell development. Here we have found that the zygotic gain-of-function and the zygotic loss-of-function of *sima* are not sufficient to confer germ cell defects like that observed in embryos treated with 3% oxygen.

## RESULTS

### *Exposure to 3% oxygen results in increase numbers of germ cells ectopic to the gonads*

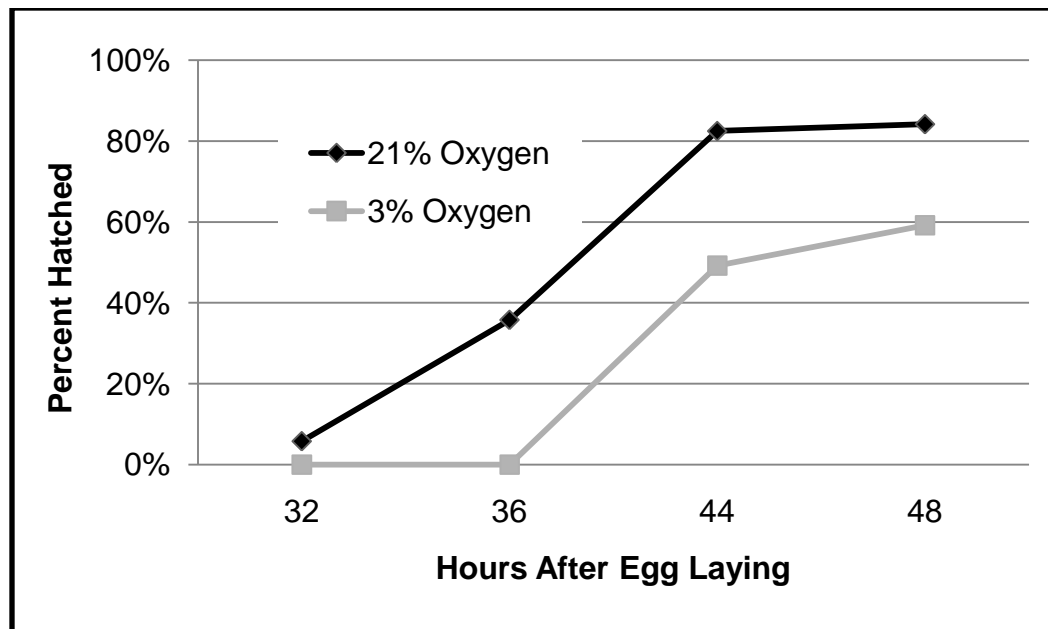
To test the hypothesis that primordial germ cell (PGC) migration and/or programmed death was sensitive to hypoxia, we examined PGC development in a range of oxygen concentrations. Prior studies had shown that wild-type flies cannot survive in or reproduce well in oxygen levels below 6% (Azad *et al.* 2009; Zhou *et al.* 2008). Reducing oxygen levels to 1% or 3% was sufficient to cause dramatic changes in tracheal development

(Adryan *et al.* 2000; Douglas *et al.* 2001; Lavista-Llanos *et al.* 2002; Centanin *et al.* 2005; Centanin *et al.* 2008). To examine the potential effects of hypoxia on PGC migration and death we first tested multiple oxygen levels, 0.5-5%, to find conditions in which embryogenesis could progress to stage 15 and beyond.

After 12 hours of development in standard conditions embryos have distinct morphological characteristics that define stage 15. Some of these are: dorsal closure, the gut forming a closed tube, and coalescence of the PGCs with the somatic gonadal precursor cells in abdominal segment 5 (Campos-Ortega and Hartenstein 1997). Initial results demonstrated that development in hypoxia resulted in a delay of approximately 3 hours. Compensating for this delay (see methods) we were then able to score developmental stage 15 embryos, a stage well after completion of germ cell migration to the gonads. Embryos developed in 3% oxygen survived to stage 15 of development, and 52.9% hatch if taken out of low oxygen conditions after 15 hours (Figure 1 and Table 1). 84.2% of control embryos cultured in room air hatched. Since the embryos hatched after incubation in 3% oxygen, albeit with a delay (Figure 1), we conducted subsequent studies in a 3% oxygen environment. Embryos cultured in 0.5% oxygen arrested early in development, and those cultured in 5% oxygen were developmentally indistinguishable from controls.

Table 1		
Oxygen Exposure	21% O <sub>2</sub>	3% O <sub>2</sub>
Percent Hatched	84.2	59.2

**Table 1. Wild-type embryos survive 15 hour incubations in 3% oxygen.** The majority of wild type embryos hatched after incubation in room air (21% oxygen) or 3% oxygen. 120 embryos were monitored in each condition.



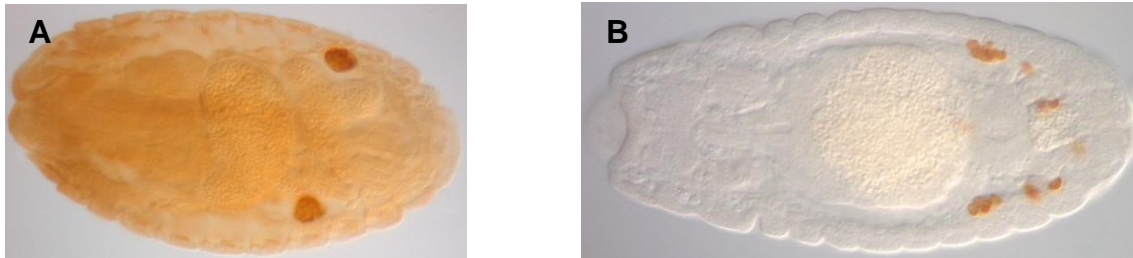
**Figure 1 Wild-type embryos incubated in 3% oxygen exhibit a delay in hatching.**

Embryos were monitored for a period 48 hours after egg laying, N=120 embryos in each condition.

Next, we examined PGC development in embryos cultured in 3% oxygen. In these experiments, embryos developed to stage 15 in 3% oxygen. Embryos were tested in each oxygen condition in parallel: a group of embryos in 3% oxygen for 15 hours and controls in room air for 12 hours in 21% oxygen (see methods). This provides enough time for the majority of the population in each oxygen condition to reach stage 15 of embryogenesis.

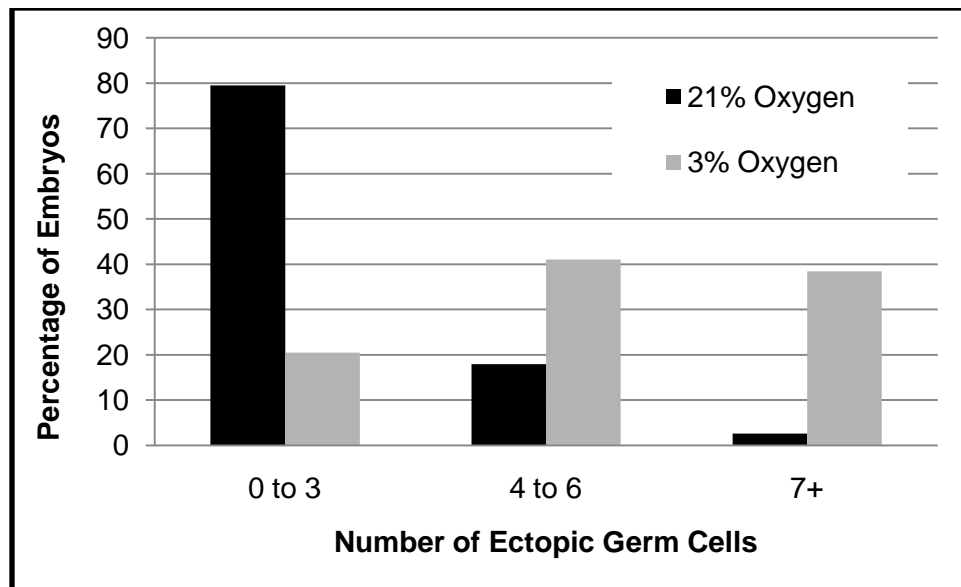
After developing in either 3% or 21% oxygen, embryos were fixed and the germ cells were stained for the presence of *fat facets-lacZ* transgene using X-Gal as a substrate or anti-Vasa staining using immunohistochemistry (see methods) (Fischer-Vize *et al.* 1992; Johansen and Johansen 2004; Burnett 2005). Wild-type stage 15 embryos incubated in control conditions (Figure 2A) exhibited PCGs tightly clustered bilaterally with the somatic

gonadal precursor cells. In sharp contrast, 79.5% embryos incubated in 3% oxygen exhibit PGCs ectopic to the gonads at stage 15 of development (Figure 2B and Figure 3).



**Figure 2. Wild type embryos exposed to 3% oxygen exhibit germ cell defects.** The embryos were fixed at developmental stage 15, and the PGCs were localized with a Vasa-specific antibody. Embryos are orientated with anterior to the left. A dorsal view is shown. **(A)** Wild type embryo raised in 21% oxygen (room air) (P[*fat-facets LacZ*]) with 0 PGCs ectopic to the gonads. **(B)** Wild type embryo raised in 3% oxygen with 8 PGCs ectopic to the gonads.

The PGCs of wild-type controls pathfind with great efficiency, and most embryos (80%) have fewer than 4 PGCs ectopic to the somatic gonad at stage 15 (Figure 3), with an average of 1.9 ectopic cells per embryo (Table 2). This is consistent with previous studies (Yamada *et al.* 2008). When embryos were incubated at 3% oxygen, 79% of the embryos exposed had  $\geq 4$  germ cells ectopic to the gonads (Figure 3), and the average number of ectopic PGCs was 6 (Table 2).



**Figure 3. 3% oxygen increases the percentage of embryos with more than four germ cells ectopic to the gonads.** Embryos were incubated in room air (black bars) or in 3% oxygen (grey bars) until they reached stage 15 of development.

Table 2		
	21% Oxygen	3% Oxygen
<b>Average Ectopic Germ Cells</b>	1.9	6.3
<b>N</b>	39	39
<b>SEM</b>	0.31	0.44
<b>*P-value</b>		1.25E-08

**Table 2. Embryos in 3% oxygen had an increase in the average number of germ cells ectopic to the gonads.** Embryos are grouped within one of the three following classes: 0 to 3, 4 to 6 or 7+ germ cells ectopic to the gonads. SEM is the Standard error of the mean.

\*Student's T-test.

To further understand the effects of hypoxia on germ cell development, we counted the total number of PGCs in each embryo (see Table 3). In control embryos before migration the PGCs begin as a population of approximately 33 cells, however when the PGCs coalesce with the somatic gonadal precursor cells this population has decreased by about half (Yamada *et al.* 2008). Therefore during PGC migration to the somatic gonadal precursor cells, there may be mechanisms of programmed cell death that occurs within PGCs.

We found that hypoxia did not cause a significant change in the number of cells in the somatic gonads (Yamada *et al.* 2008), but did increase the total number of germ cells by approximately 4 cells. This suggests that hypoxia may suppress the programmed cell death of some PGCs.

<b>Table 3</b>			
	<b>Average number of germ cells within the gonads</b>	<b>Average total number of germ cells</b>	<b>N</b>
<b>21% oxygen</b>	19.6	21.7	32
<b>3% oxygen</b>	19.0	25.4	34
<b>SEM</b>	0.86	0.87	
<b>P-value=</b>	*0.58	**0.005	

**Table 3. Embryos in 3% oxygen have an increased total number of germ cells.** \*P-value compares the difference between the mean number of germ cells within the gonads of embryos at stage 15 of development after 21% or 3% oxygen treatment. \*\*P-value compares the difference in the mean total number of germ cells between the populations of embryos developed to stage 15 in either 21% or 3% oxygen. SEM is the standard error of the mean. \* \*\*Student's T-test.



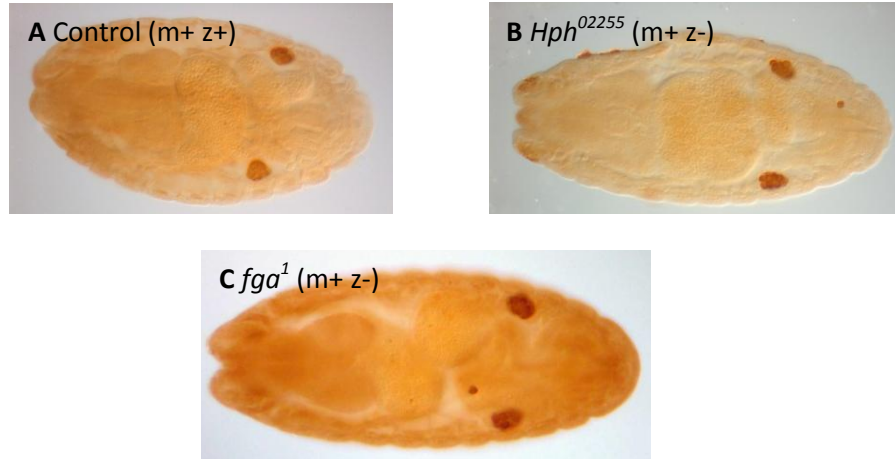
Embryos committed to embryogenesis until stage 15 in low oxygen conditions (3% O<sub>2</sub>) displayed an interesting germ cell defect phenotype. These initial results support our hypothesis that PGC development is sensitive to oxygen concentrations. We followed up with the hypothesis that the HIF-1 pathway components mediate this response to culture in low oxygen conditions by examining PGC development in HIF-1 pathway component loss-of-function mutants.

*Zygotic loss-of-function of fga does not cause germ cell defects*

Sima stabilization often occurs under hypoxic stress. We next considered the hypothesis that the effects of hypoxia on PGC number were due to over-expression of the Sima transcription factor. To test this, we used genetic strategies to increase Sima levels in normal oxygen culture conditions. Thus, embryos with the zygotic loss-of-function of *fatiga*, the prolyl hydroxylase of Similar, were scored for a germ cell defects. Alleles, *fga*<sup>l</sup> and *Hph*<sup>02255</sup>, of *fatiga* have strong loss-of-function mutant phenotypes in other studies, as shown by increased expression of a HIF-1 inducible reporter (Centanin *et al.* 2005; Acevedo *et al.* 2010). We chose these two alleles to analyze the gain-of-function of *sima* in normal oxygen conditions. These alleles are homozygous lethal. The genotype of the mothers are *fga* (*fga*<sup>l</sup> or *Hph*<sup>02255</sup>)/TM3, P[*ftz lacZ*] *Sb* and thus contain a wild-type copy of *fga* on the TM3 balancer. If *sima* mediates PGC development, then the gain-of-function of *sima* might mimic the PGC defects observed in 3% oxygen conditions.

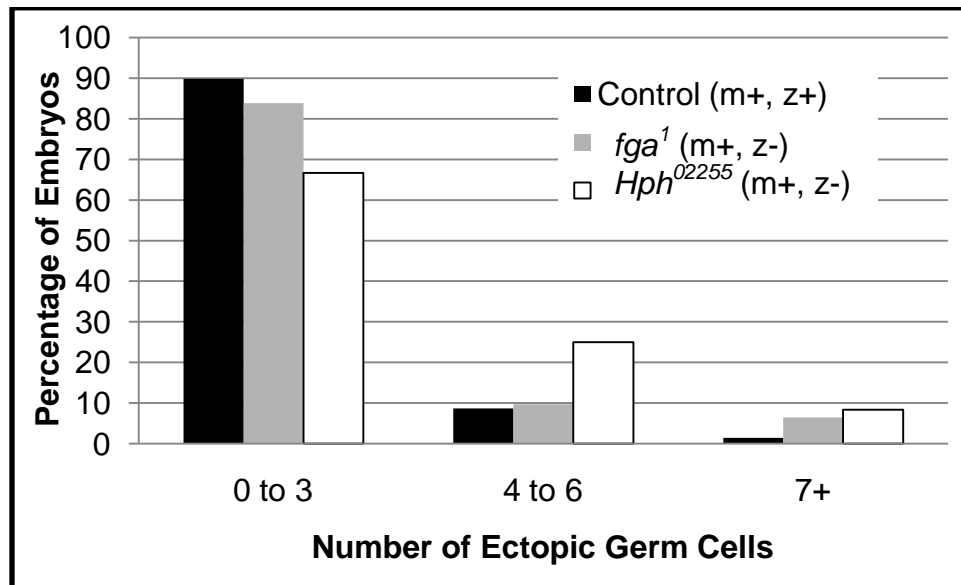
There was no obvious PGC defect phenotype in these embryos compared to control embryos developed in normal oxygen conditions (Figure 4). 89.8% of stage 15 control embryos had fewer than 3 PGCs ectopic to the gonads, N=69. The majority of homozygous

*fga* loss-of-function embryos also had fewer than 3 PGCs ectopic to the gonads; 83.8% and 66.7% with allele *fga*<sup>1</sup> (N=31) and *Hph*<sup>02255</sup> (N=48) respectively (Figure 5). In addition, control and homozygous loss-of-function *fga* embryos (irrespective of allele *fga*<sup>1</sup> or *hph*<sup>02255</sup>) have an average of fewer than 3 PGCs ectopic to the gonads (Table 4).



**Figure 4. *fatiga* zygotic loss-of-function mutants, *fga*<sup>1</sup> and *hph*<sup>02255</sup>, show no germ cell defects. (A-C)** Embryos are orientated as in Fig. 2. All embryos have a functional maternal (m+) copy of *fatiga*. **(B-C)** *fga*<sup>1</sup> and *Hph*<sup>02255</sup> embryos have a zygotic loss-of-function (z-) copy of *fatiga*. These embryos are double anti-body stained with anti-Vasa and anti-β Galactosidase to separate the homozygous loss-of-function mutants from the embryos carrying a TM3 balancer chromosome (See methods). Homozygous loss-of-function *fatiga* embryos do not show anti-β Galactosidase staining. **(A)** Control embryo (P[*fat facets-lacZ*]) with 0 PGCs ectopic to the gonads. **(B)** Homozygous loss-of-function *fatiga* embryo (*Hph*<sup>02255</sup>) with 1 ectopic PGC posterior to the gonads. **(C)** Homozygous loss-of-function *fatiga* embryo (*fga*<sup>1</sup>) with 1 ectopic PGC anterior to the gonads.

By Student's T-test, the means are significantly different between the *fga* loss-of-function lines and the P[*fat facets-lacZ*] control embryos (Table 4). However, when this mutant is compared to other control genetic backgrounds (Canton S) no significant difference is observed (Table 5). Thus, the stabilization of Sima does not cause PGC defects in stage 15 homozygous mutant *fga* embryos.



**Figure 5.** The zygotic loss-of-function of *fatiga* did not increase the percentage of embryos with  $\geq 4$  germ cells ectopic to the gonads. All embryos have a functional maternal (m+) copy of *fatiga*. *fga*<sup>1</sup> and *Hph*<sup>02255</sup> embryos are zygotic loss-of-function (z-) mutants of *fatiga*.

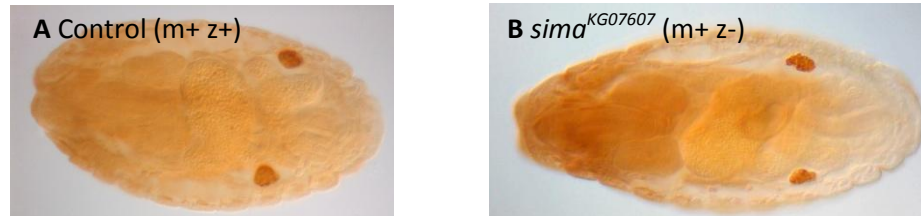
<b>Table 4</b>				
<b>Genotype</b>	Control	<i>fga</i> <sup>1</sup> (m+, z-)	Control <sup>2</sup>	<i>Hph</i> <sup>02255</sup> (m+, z-)
<b>Average Ectopic Germ Cells</b>	1.7	1.9	3.9	2.9
<b>N</b>	69	31	27	48
<b>SEM</b>	0.2	0.34	0.5	0.37
<b>P-value</b>		*0.49		**0.136

**Table 4. There is no difference in the average number of ectopic germ cells between control and *fatiga* zygotic loss-of-function embryos.** \*P-value compares the difference between the mean of the control and homozygous zygotic loss-of-function *fatiga*<sup>1</sup> mutant embryos, Student's T-test. \*\*P-value compares the difference between the mean of control<sup>2</sup> and homozygous zygotic loss-of-function *Hph*<sup>02255</sup> embryos, Student's T-test.

*Zygotic loss-of-function of similar does not cause germ cell defects*

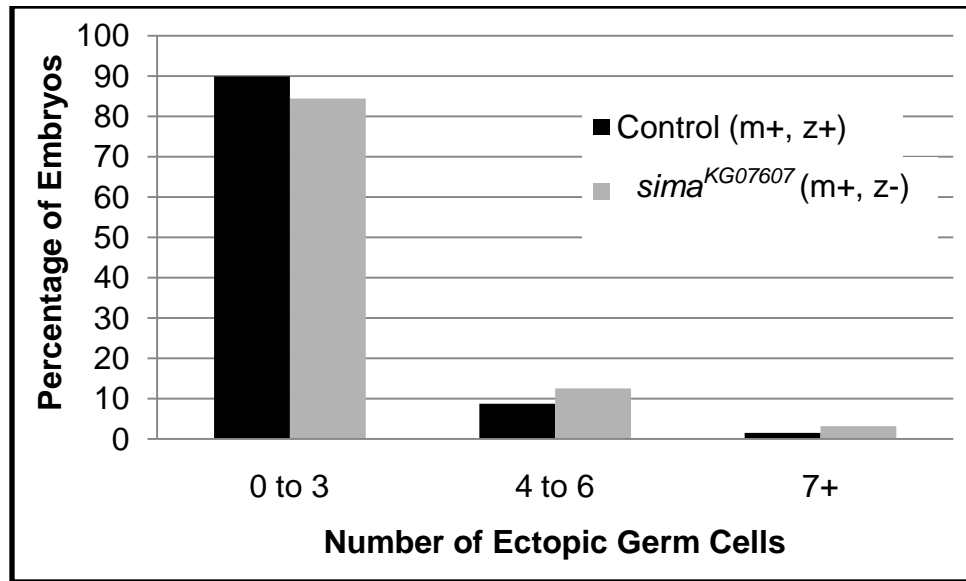
To further investigate the roles of Sima and its regulators in embryonic germ cell development, we examined the phenotypes of embryos homozygous for loss-of-function mutations in *sima*. Within *Drosophila* embryos the expression of HIF-1 $\alpha$  is required for survival in hypoxic environments and development of the trachea (Lavista-Llanos *et al.* 2002; Centanin *et al.* 2005; Centanin *et al.* 2008). We asked if the zygotic loss-of-function of *similar* in developing embryos would exhibit germ cell defects to address the hypothesis that *Drosophila* embryos require Sima for normal PGC development. Approaching this question we chose to analyze the strongest loss-of-function allele of *similar* (*sima*<sup>KG07607</sup>) in normal oxygen conditions. To examine the zygotic loss-of-function, this allele was placed over a marked balancer (TM3, P[ftz-lacC] *Sb*). We used the TM3, P[ftz-LacC] *Sb* balancer as above to identify homozygous zygotic loss-of-function embryos.

Zygotic loss-of-function embryos and control embryos were then collected, allowed to develop to stage 15 of embryogenesis, and fixed for staining (see methods). Homozygous *sima*<sup>KG07607</sup> zygotic loss-of-function embryos did not display germ cell defects when cultured in room air (Figure 6). As shown in Figure 6 the majority of embryos within both populations scored, control (P[*fat facets-lacZ*]) 89.9% (N=69) and *sima*<sup>KG07607</sup> zygotic loss-of-function mutants 84.4% (N=32), had 3 or fewer PGCS ectopic to the gonads (Figure 7). The numbers of PGCs ectopic to the somatic gonads are also not significantly different, when comparing embryos lacking zygotic *sima* to controls (Table 6).



**Figure 6. Zygotic loss-of-function of *similar* did not exhibit germ cell defects. (A-B)**

Embryos are oriented anterior (left) to posterior (right). The germ cells are antibody stained with anti-*vasa* and are clustered within the gonads. **(A)** Control embryos (P[*fat facets-lacZ*]) have both maternal and zygotic functional (m+, z+) copies of *similar*. This embryo has no germ cells ectopic to the gonads. **(B)** Zygotic loss-of-function (z-) *similar* mutant embryos (*sima*<sup>KG07607</sup>) only have a functional maternal (m+) copy of *similar*. This embryo has no germ cells ectopic to the gonads.



**Figure 7. Zygotic loss-of-function of *similar* did not increase the percentage of embryos with  $\geq 4$  germ cells ectopic to the gonads.** Control embryos (P[*fat facets-lacZ*]) have both maternal and zygotic functional (m+, z+) copies of *similar*. Zygotic loss-of-function (z-) mutant *sima*<sup>KG07607</sup> has a maternal functional (m+) copy of *similar*.

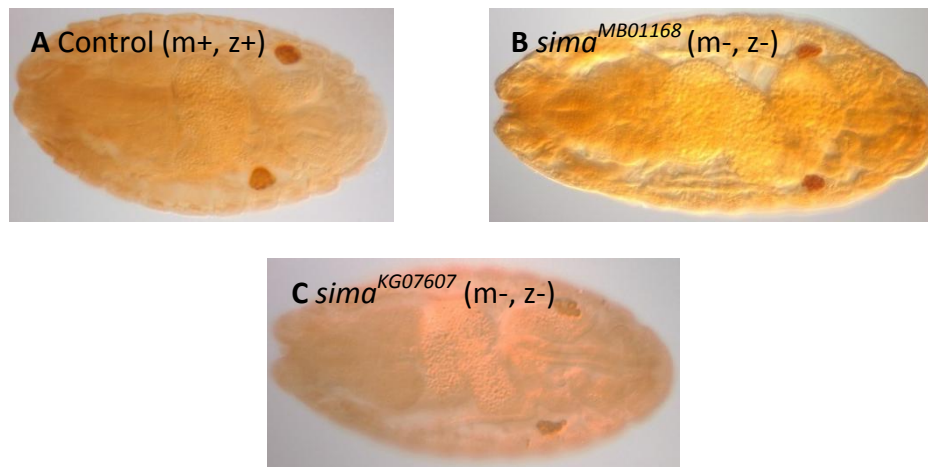
Table 6		
	Control (m+, z+)	<i>sima</i> <sup>KG07607</sup> (m+, z-)
<b>Average Ectopic Germ Cells</b>	1.7	1.7
<b>N</b>	69	32
<b>SEM</b>	0.20	0.39
	<b>*P-value</b>	0.95

**Table 6. *similar* zygotic loss-of-function did not increase the average number of germ cells ectopic to the gonads.** Table compares the average number of germ cells ectopic to the gonads between *similar* loss-of-function mutant and control embryos. SEM is the standard error of the mean. \*Student T-test.

*Maternal loss of function of similar does not cause germ cell defects*

Thus far we have examined the differences in the germ cell defects between wild type, *similar* and *fatiga* zygotic loss-of-function mutants. Embryonic germ cell development is directed by both maternal and embryonic gene products. We next investigated whether the maternal loss-of-function of *similar* can cause germ cell defects. *In situ* hybridization data confirm that *similar* and *fatiga* mRNAs are detectable in *Drosophila* embryos before most embryonic transcription begins (Nambu 1996; Lavista-Llanos *et al.* 2002). To examine embryos that did not contain any functional *Sima*, we generated a homozygous loss-of-function *sim*<sup>KG07607</sup> line, in addition to the *sim*<sup>MB01168</sup> homozygous line (see methods). Homozygous *sim*<sup>KG07607</sup> embryos do not produce *sim* transcript (Centanin *et al.* 2005), whereas the strength allele *sim*<sup>MB01168</sup> is unknown. Homozygous *similar* mutants are viable in normoxic conditions (Centanin *et al.* 2005). Wild-type and homozygous mutant *sim*<sup>KG07607</sup> and *sim*<sup>MB01168</sup> embryos were allowed to develop to stage 15 of development in room air conditions.

There was no discernable difference in appearance between wild-type stage 15 embryos and each *similar* maternal and zygotic loss-of-function mutant (*sim*<sup>KG07607</sup> and *sim*<sup>MB01168</sup>) (Figure 8). As seen in Figure 9, 89.9% (N=69) of wild-type embryos, 97.5% (N=40) of *sim*<sup>MB01168</sup> embryos, and 95% (N=40) of *sim*<sup>KG07607</sup> embryos, had fewer than 3 PGCs ectopic to the gonads. In Table 7, we also did not observe increased numbers of ectopic germ cells in embryos lacking maternal and zygotic *sim*. These data support the conclusion that *sim* is not required for normal germ cell development when oxygen is abundant.



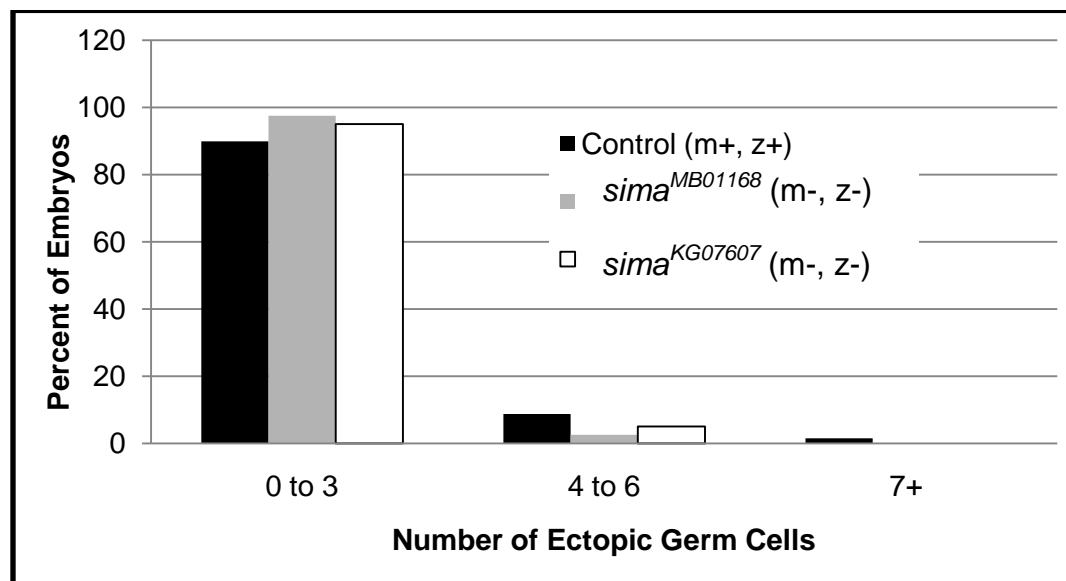
**Figure 8. *similar* maternal loss-of-function mutants did not exhibit germ cell defects. A-**

**C** All embryos are orientated anterior to the left. All embryos have 0 ectopic PGCs. **(A)**

Control embryos (P[*fat facets-lacZ*]) have both maternal and zygotic functional copies of

*similar* (m+, z+). **(B and C)** Maternal and zygotic loss-of-function *similar* embryos

*sima*<sup>KG07607</sup> do not have any functional copies of *similar* (m-, z-).



**Figure 9. Maternal and zygotic loss-of-function of *similar* did not increase the**

**percentage of embryos with  $\geq 4$  germ cells ectopic to the gonads.** Control embryos (P[*fat*

*facets-lacZ*]) have both maternal and zygotic (m+, z+) functional copies of *similar*.



*sima*<sup>MB01168</sup> and *sima*<sup>KG07607</sup> are both maternal and zygotic loss-of-function (m-, z-) mutants of *similar*.

<b>Table 7</b>			
	Control (m+, z+)	<i>sima</i> <sup>MB01168</sup> (m-, z-)	<i>sima</i> <sup>KG07607</sup> (m-, z-)
<b>Average Ectopic Germ Cells</b>	1.7	0.8	0.9
<b>N</b>	69	40	40
<b>SEM</b>	0.20	0.17	0.19
<b>*P-value</b>		0.008	0.002

**Table 7. *similar* maternal and zygotic loss-of-function does not increase the number of germ cells ectopic to the gonads.** SEM is the standard error of the mean. \* P-value compares the difference between the mean of the control and maternal loss-of-function *similar* mutants in each column, Student's T-test.

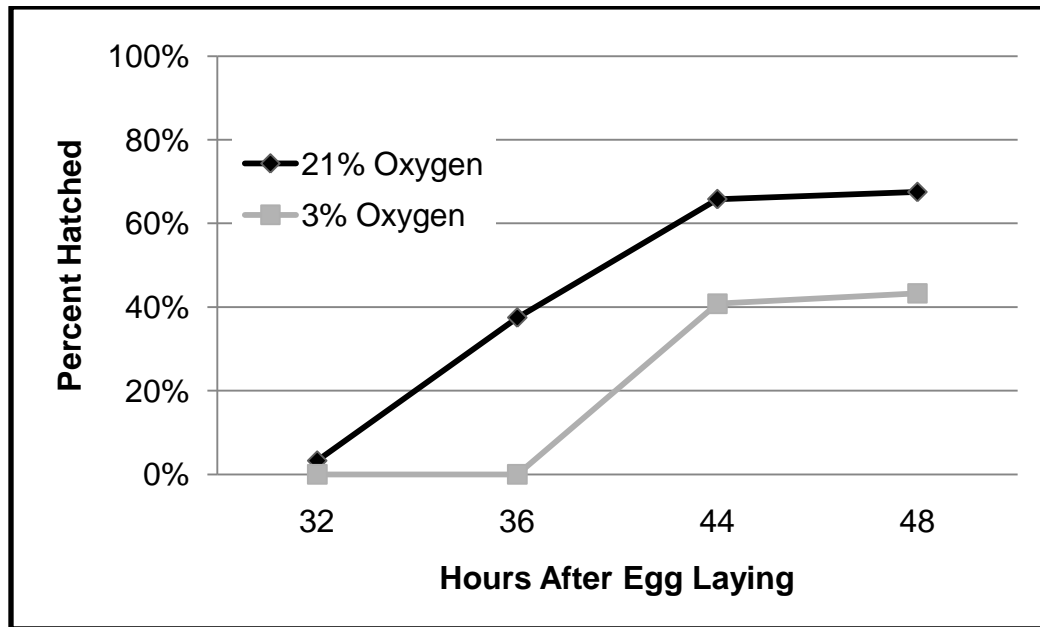
In contrast, we examined the effect of 3% oxygen exposure on primordial germ cell development in complete loss-of-function *sima* mutants. Results from previous studies have demonstrated that homozygous *sima*<sup>KG07607</sup> larvae were not able to continue development in a 5% oxygen environment (Centanin *et al.* 2005). Before investigating the potential effects of 3% oxygen on PGC development in *sima* maternal and zygotic loss-of-function mutants, we analyzed the viability of these mutants after exposure 3% oxygen. To measure the viability of these mutants the hatching rates of *sima*<sup>KG07607</sup> maternal and zygotic loss-of-function mutants and control embryos were monitored in room air after 3% oxygen exposure.

43.3% of homozygous maternal and zygotic loss-of-function *sima*<sup>KG07607</sup> embryos hatched after exposure to 3% oxygen (Table 8). These embryos in comparison to wild-type exhibited an increased delay in hatching time after exposure to 3% oxygen (refer to Figure 1

and 10). 3% oxygen exposure delayed wild-type embryos by approximately 3 hours whereas homozygous maternal and zygotic loss-of-function *sima*<sup>KG07607</sup> hatched approximately 9 hours slower.

Table 8		
Oxygen Exposure	21% O <sub>2</sub>	3% O <sub>2</sub>
Percent Hatched	67.5	43.3

**Table 8. *sima* maternal and zygotic loss-of-function embryos survive 3% oxygen incubation.** N= 120 in each oxygen condition.



**Figure 10. *sima* maternal and zygotic loss-of-function embryos (*sima*<sup>KG07607</sup>) exhibit a delay in hatching after incubation in 3% oxygen.** N= 120 in each oxygen condition.

Adjusting experimental conditions we then examined PGC development in homozygous maternal and zygotic *similar* mutants exposed to 3% oxygen. A group of *sima*<sup>KG07607</sup> embryos was exposed to 3% oxygen for 21 hours and compared to a group of *sima*<sup>KG07607</sup> embryos exposed to room air for 12 hours. This adjustment attempted to

compensate for the increased developmental delay. We were therefore allowed us to score stage 15 homozygous maternal and zygotic loss-of-function *sima*<sup>KG07607</sup> embryos from both oxygen conditions. We examined 47 homozygous loss-of-function maternal and zygotic *sima*<sup>KG07607</sup> embryos exposed to room air. Of these, 85.1% exhibited wild-type stage 15 characteristics and were scored for PGC defects. 14.9% of these embryos exhibited significant morphological defects and were not scored. In contrast, 144 homozygous loss-of-function maternal and zygotic *sima*<sup>KG07607</sup> embryos exposed to 3% oxygen that were examined. 72.2% of these embryos were not scored because they exhibited mild to severe morphological defects such as no dorsal closure, poor gut formation, and poor segmentation. 27.7% of these embryos exhibited stage 15 characteristics and were scored for PGC defects. Malformation of these characteristics suggests that 3% oxygen culture is detrimental to the development of homozygous maternal and zygotic loss-of-function *sima*<sup>KG07607</sup> embryos. Thus we cannot attribute the PGC defects observed in these embryos exposed to 3% oxygen solely to the lack of functional Sima.

## DISCUSSION

We have examined the affects of low oxygen stress and the loss-of-function alleles of hypoxic response constituents on germ cell development. Previous studies have identified a functional hypoxic response pathway in *Drosophila* that is homologous to other model organisms and humans (Zhao and Haddad 2011; Rytönen *et al.* 2011). The majority of these studies in *Drosophila* focused their efforts on tracheogenesis to develop models for further understanding vascular development, tumorigenesis, and ischemia (Adryan *et al.* 2000; Lavista-Llanos *et al.* 2002; Centanin *et al.* 2005; Centanin *et al.* 2008).

To date, outside of tracheal development and molecular characterization of HIF-1 subunits, the only reported roles of HIF-1 signaling in *Drosophila* development have been within ovary border cell migration. Ovary border cells are sensitive to oxygen tension and accumulated levels of Sima during oogenesis. Hypoxia and too much or too little Sima hindered the rate of border cell migration to the oocyte (Doronkin *et al.* 2009). The border cell migration phenotypes exhibited by the dose dependent expression of Sima in this system is analogous to tumor cell accumulation of HIF-1 $\alpha$ , of which has been demonstrated to be a key marker of metastasis progression (Dewan *et al.* 2006; Ali and Lazennec 2007; Chan and Giaccia 2007).

Here we have approached the involvement of HIF-1 in primordial germ cell development by testing the hypothesis that *Drosophila* embryos require sufficient levels of oxygen for normal germ cell development. Embryos cultured in 3% oxygen for 15 hours exhibit germ cell defects. The percentage of embryos with 4 or more germ cells ectopic to the gonads increased compared to embryos exposed to 21% oxygen. In addition to the germ cell defects observed, we also noted that hypoxic exposure (3% oxygen) confers approximately a 3 hour delay in development (refer to Figure 1).

The germ cell phenotype associated with culture in 3% oxygen led us to test the hypothesis that the HIF-1-dependent hypoxic response pathway was responsible for mediating the germ cell defects observed under hypoxic stress. We examined germ cell development in embryos with zygotic loss-of-function of *fga*. Fga is an oxygen-sensitive prolyl hydroxylase and regulator of Sima in normoxic conditions (Centanin *et al.* 2005; Irisarri *et al.* 2009; Avededo *et al.* 2010). *fga* mutants are unable to hydroxylate Sima in normoxic conditions, resulting in the accumulation of Sima and increased *Drosophila* HIF-1

transcriptional activity as observed by HIF-1 dependent reporter expression (Centanin *et al.* 2005). We analyzed the strongest alleles of *fga*, *fga*<sup>l</sup> and *Hph*<sup>02255</sup>. Our prediction was that the loss-of-function of *fga* would mimic the conditions observed in 3% oxygen, resulting in defects in germ cell development. We found that embryos with the zygotic loss-of-function of *fga*, by either allele, do not display germ cell defects when developed in normoxic conditions (refer to Figures 4 and 5, and Tables 4 and 5).

We then examined the zygotic and maternal loss-of-function of *Sima* in embryos exposed to 21% oxygen to determine if *Sima* was required for germ cell development. Previous investigations have determined that the loss of *similar* does not affect development or viability under standard conditions (Centanin *et al.* 2005). Two alleles of *similar* were examined, *sima*<sup>KG07607</sup> and *sima*<sup>MB01168</sup>. *sima*<sup>KG07607</sup> loss-of-function mutants are not able to exhibit a response to hypoxia. No expression of a HIF-1 dependent reporter was detected in *sima*<sup>KG07607</sup> loss-of-function embryos exposed to hypoxia (Centanin *et al.* 2005). The strength of the loss-of-function by *sima*<sup>MB01168</sup> is unknown. The zygotic and maternal loss-of-function of *sima* has no effect on germ cell development in embryos exposed to 21% oxygen conditions (refer to Figures 6-9 and Tables 6-7). We conclude that *similar* is not necessary for germ cell development under normoxic conditions.

We next addressed the requirement for *similar* in germ cell development when embryos are exposed to hypoxic conditions. Before interpreting possible PGC defects in maternal and zygotic *sima* loss-of-function embryos, we questioned whether these embryos can survive 3% oxygen culture. Like wild-type embryos *sima* maternal and zygotic loss-of-function embryos exhibit a delay in hatching, however, this delay is increased (refer to Figure 10). A low population of these embryos, 43.3%, hatched despite being cultured 3% oxygen

exposure (refer to Table 8). This initial result suggested that the homozygous maternal and zygotic loss-of-function of *sima* may have hindered development and viability of these embryos after exposure to 3% oxygen. In examination of the potential effects of 3% oxygen on PGC development in these embryos, we observed that 72.2% of the embryos exposed to 3% oxygen exhibited mild to severe morphological defects. Due to the low hatch rate and diminished population of stage 15 embryos that we could score after exposure to 3% oxygen we were not able to confidently score the observed PGC defects in these embryos.

Collectively, these studies provide evidence that *Sima* does not have a critical role in pathfinding of the germ cells to the somatic gonad when oxygen is abundant. However, when wild-type embryos are exposed to 3% oxygen a germ cell defect is observed. These embryos also exhibit an increased total number of germ cells, suggesting that hypoxic exposure hinders programmed cell death within the germ cells. Alternatively, the delay in embryogenesis from culture in 3% oxygen may have also contributed to the germ cell defects observed.

*Drosophila* embryos are sensitive to atmospheric oxygen concentration. Douglas *et al.* has shown that within the early *Drosophila* embryo there are two cell cycle arrest points induced by 5% oxygen culture: metaphase to anaphase and the transition to S phase. When cultured in 5% oxygen embryos exhibited cell cycle arrest before S phase, and upon reoxygenation resume the cell cycle (Douglas *et al.* 2001). Soon after the primordial germ cells are formed a few rounds of mitosis increase their population. However, over the course of migration half of the primordial germ cells are eliminated (Yamada *et al.* 2008). It is possible that culturing young embryos in 3% oxygen abrogates normal mechanisms of

programmed cell death of ectopic primordial germ cells, such as those observed in our investigations. HIF-1 activity has been shown to mediate programmed cell death in other organisms. *C. elegans* possesses a HIF-1-mediated hypoxic response system homologous to mammals and *Drosophila* (Epstein *et al.* 2001; Jiang *et al.* 2001; Gorr *et al.* 2006; Rytönen *et al.* 2011). A recent study demonstrated that activation of HIF-1 in ASJ sensory neurons upregulates TYR-2. This is a tyrosinase family member which prevents DNA damage induced apoptosis by antagonizing p53 (CEP-1 in *C. elegans*) function in the germline stem cells (Sendoel, *et al.* 2010). Investigating similar HIF-1 induced networks that may abrogate *Drosophila* primordial germ cell programmed cell death can address the observation of increased numbers of ectopic germ cells in wild-type stage 15 embryos after exposure to 3% oxygen.

Further research examining the amount of HIF-1 transcriptional activity within the germ cells can provide critical information as to whether the germ cells require functional HIF-1 activity for either timely development or programmed cell death. Yet to be examined is the complete loss-of-function of *fga*. Embryos that have a homozygous loss-of-function of *fga* do not develop (Frei and Edgar 2004; Centanin *et al.* 2005). Therefore it is difficult to examine the effects of the complete loss-of-function of *fga* on PGC development.

To address this, we have developed a strategy to examine the complete loss-of-function of *fga* by establishing *fga* germline clones (see methods). This strategy utilizes recombination and antibiotic resistance to select recombinant flies that may carry both the *fga* allele (*fga*<sup>1</sup>) and a flip recombination target (FRT) antibiotic resistant transgene (FRT(*neo82B*)) on the third chromosome. Through personal communication with Dr. Pablo

Wappner we have learned that the map distance between *fga* and FRT(*neo82B*) is less than 1:1000. Therefore the likelihood of retrieving a *fga*<sup>1</sup> FRT(*neo82B*) recombinant is very small. In addition to establishing loss-of-function *fga* germline clones, the knock down of *fga* with RNAi has not been examined in these investigations of PGC development. Thus, continued investigation of HIF-1 in *Drosophila* germ cell development, can provide insightful information for the study of HIF-1 in cellular migration and survival.

## METHODS

**Embryo collection in room air:** Adults of no more than 3-4 days of age were placed into collection chambers or tubes. These collection vessels are perforated with holes to allow gas exchange. Adults were fed with yeast paste on apple juice agar plates or caps (Parton *et al.* 2010; Figard and Sokac 2011). Adults are placed in collection vessels at the minimum 2 days prior to experimental embryo collection. Embryos are collected for 0 to 3 hours at 24°C in room air.

**Embryo collection for room air vs. hypoxia treatment:** Embryo collection for hypoxic versus room air experiments are performed the same as embryo collections in room air. These collections were done in room air at 21-22°C for 0-3 hours. Embryos to be treated with hypoxia were collected 3 hours prior to control embryos; this allowed correction for the 3 hour delay caused by hypoxic exposure.

**Hypoxia treatment:** Treatment of hypoxia for this study utilized several oxygen concentrations, 0.5, 3, 5, or 8% oxygen. These oxygen concentrations were stably achieved by allowing room air to mix with nitrogen in a regulated hypoxia chamber. Hypoxia exposure was conducted for 15 hours consecutively. Embryos exposed to hypoxia were



placed in the chamber at the end of the 0 to 3 hours collection period and kept in the chamber for 15 hours; the temperature inside the chamber was 22°C. Embryos exposed to room air are kept outside of the chamber at the end of the collection period and kept in this environment for 12 hours; the temperature outside the chamber was 22°C.

**Scoring Germ Cell Defect Phenotypes:** Embryos were scored following the techniques of Kamps *et al.* 2010. Germ cells and embryos were visualized using a differential interference contrast microscope. Embryos scored were staged by morphology to be in stage 15 of embryogenesis. Germ cells were counted within the gonads if present within the fifth abdominal segment and surrounded by the somatic gonadal precursor cells. Germ cells outside of this scoring guideline were counted as ectopic to the gonads. This method produced similar results for both immunohistochemical and X-Gal staining protocols. Statistical analysis of germ cell counts utilized the Student's T-test with two-tails and  $\alpha=0.05$ .

**Scoring Hatched Embryos:** Embryos were collected and treated with 21% and 3% oxygen conditions as stated previously. After the exposure period embryos were removed from their respective conditions. Embryos were then washed off of the agar plates with ddH<sub>2</sub>O and into a mesh capped vial. Using a stereoscope, embryos were plated (120 embryos/plate) on an apple juice agar plate with yeast paste only around the brim to attract hatched larvae. Plates were kept in a box with a damp paper towel to provide humidity and prevent drying out. Plates were monitored every 4 hours by counting the number of egg shells. 48 hours after the eggs were laid served as the cutoff point to end scoring.

**G418 Medium preparation:** Preparation of G-418 Sulfate fly food media followed the technique utilized within Xu, T and Rubin, G 1993. 0.025g of fine powder G-418 Sulfate (US Biologicals G-418 Geneticin ® C<sub>20</sub>H<sub>40</sub>N<sub>4</sub>O<sub>10</sub>•2H<sub>2</sub>SO<sub>4</sub> Catalog # G1000) was mixed with

1mL ddH<sub>2</sub>O. Larger quantities of this stock can be made proportionally, aliquoted and frozen for later use. Vials with 7mL of corn meal agar fly food were perforated using a dissection needle, approximately 10-12 holes equally spaced per vial. 175µL of G-418 Sulfate 0.025g/mL stock was placed onto the perforated fly media and allowed to dry for at least 4 hours prior to use; to achieve a final concentration of 0.625mg/mL of G-418 Sulfate. Lethal dose is 200-300µL of 0.025g/mL of G-418 Sulfate for every 10mL of fly food, reported in Xu and Rubin 1993. G-418 Sulfate fly media was prepared fresh for the day of use.

**Drosophila Culture-Maintaining Stocks:** All stocks were kept in standard conditions: fresh bottles or vials maintained at the minimum of every two weeks. Stocks are kept in a temperature and humidity regulated diurnal Percival© incubator. Day period is set to 12 hours of light at a temperature of 23°C. Night period is set to 12 hours of no light at a temperature of 23°C.

**X-Gal staining:** Immediately after room air or experimental oxygen concentration exposure, the chorion was removed from embryos by wash with 50% bleach. Embryos were then fixed with 2.5% glutaraldehyde in phosphate buffer saline 4% triton (PBST) for 10 minutes.

Embryos are then rinsed with PBST for a minimum of 4 hours. Embryos are then incubated in 37°C with an X-Gal staining solution with a 0.08% X-Gal solution for 2 hours. Technique adopted from (Kamps *et al.* 2010). See table of reagents Table 1.1

**Immunohistochemistry:** Immunohistochemical techniques were adopted from Yamada *et al.* 2008 and Kamps *et al.* 2010. Immunohistochemical reagents and protocol was followed from Johansen and Johansen 2004. Single antibody staining utilized the primary antibody chicken anti-Vasa (1:10,000) and secondary antibody biotinylated anti-chicken IgG (1:500) vector labs. Primary antibody is a gift from Ken Howard (Burnett 2005), secondary antibody

is from Vector Laboratories. Double antibody staining utilized primary antibody mouse anti- $\beta$ -Galactosidase and secondary antibody biotinylated goat anti mouse and goat anti chicken in addition to single antibody staining materials. In double antibody staining assays both primary antibodies and both secondary antibodies were added to the reaction tubes simultaneously at the appropriate step in the procedure. Visualized antibody the reaction within the germ cells using Vectastain ABC Kit ® (Elite PK-6100 Standard) and brief treatment with 3, 3'-diaminobenzidine tetrahydrochloride (10mg/mL).

### **Crosses to establish hypoxic response alleles over a marked balancer**

Alleles *sim*<sup>KG07607</sup>/TM3, *Sb Ser*, *fga*<sup>1</sup>/TM3, *Ser*, and *Hph*<sup>02255</sup>/TM3, *Sb Ser* crosses were done with both male and female of each genotype. Alleles were crossed to a double 3<sup>rd</sup> chromosome balancer TM2, *Ubx e*/TM6, *Sb e*. The F<sub>1</sub> selected from cross #1 were: allele/TM2, *Ubx e*. These offspring were then crossed to *sim*<sup>2</sup> *kar*<sup>1</sup>/TM3, P[*ftz-lacC*] *Sb*, the marked 3<sup>rd</sup> chromosome balancer line. The F<sub>1</sub> selected from cross #2 were: allele/TM3, P[*ftz-lacC*] *Sb*; these lines were expanded as the zygotic loss-of-function lines of *similar* and *fatiga* alleles (Figures 11-16). *sim*<sup>KG07607</sup> homozygous individuals were selected from sibling crosses of *sim*<sup>KG07607</sup>/TM3, P[*ftz-lacC*] *Sb* (Figure17).

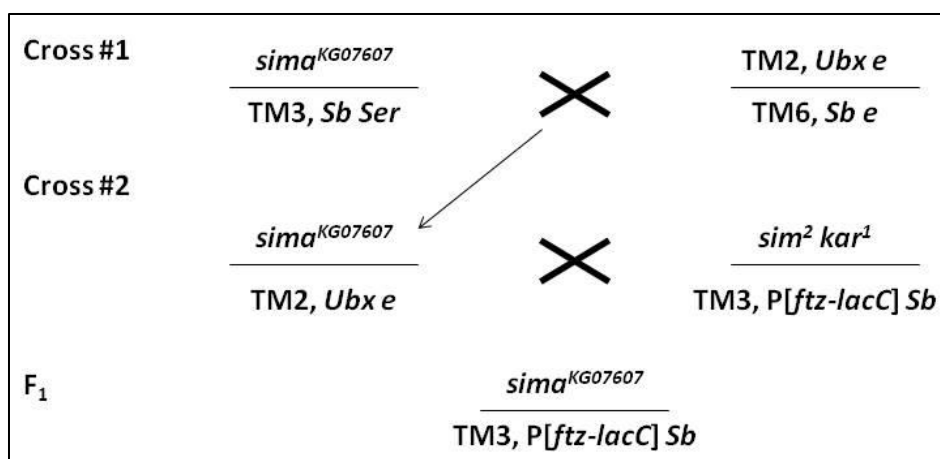


Figure 11. Crossing  $sim^a^{KG07607}$  to a marked third chromosome balancer.

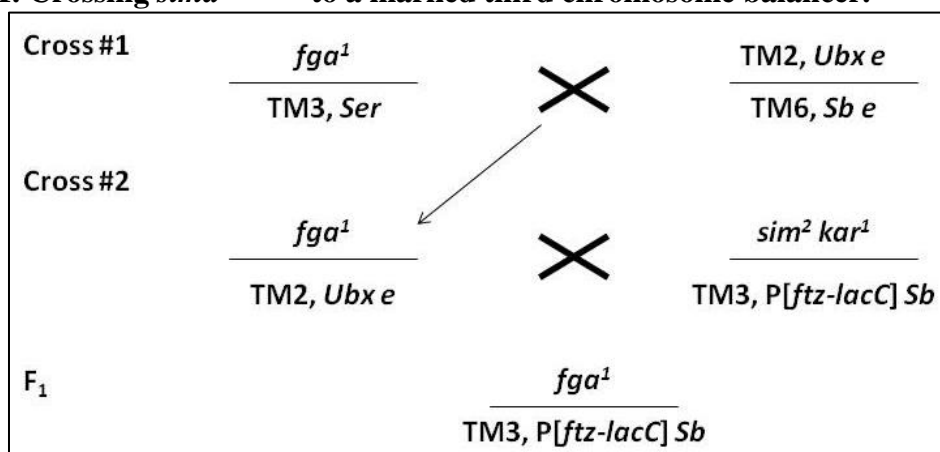


Figure 12. Crossing  $fga^1$  to a marked third chromosome balancer.

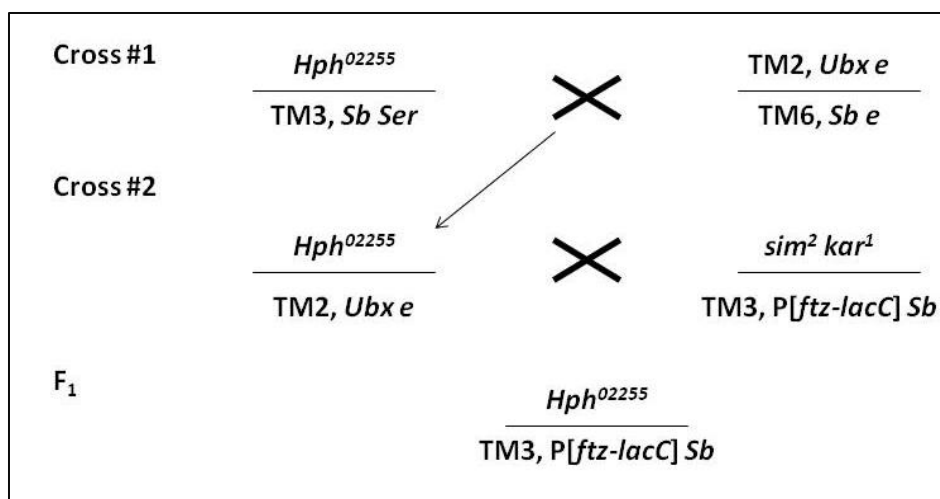


Figure 13. Crossing  $Hph^{02255}$  to a marked third chromosome balancer.

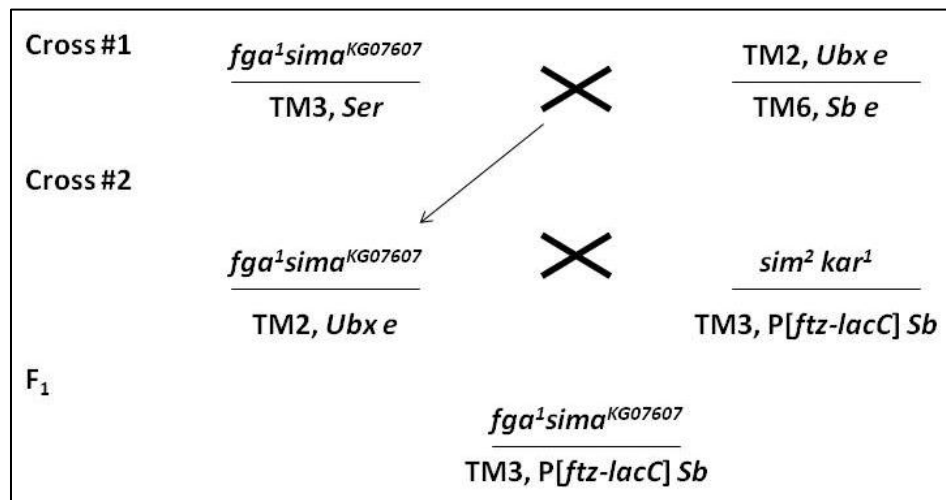


Figure 14. Crossing  $fga^1 sima^{KG07607}$  to a marked third chromosome balancer.

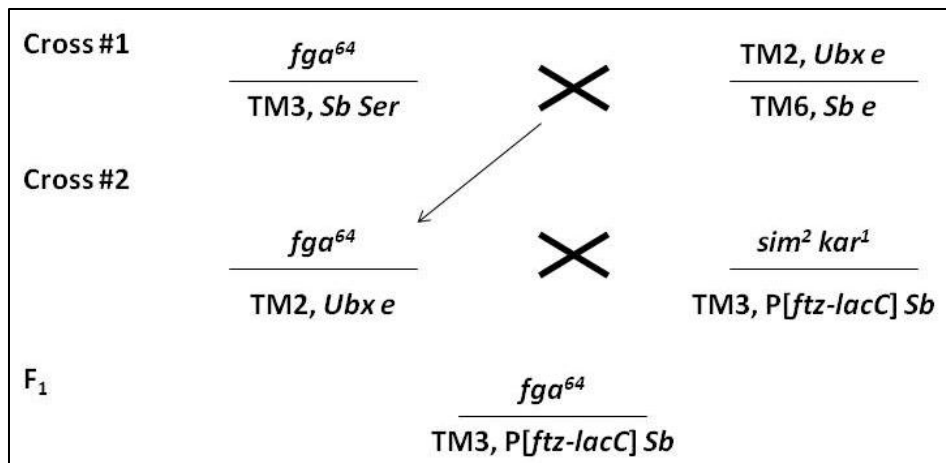


Figure 15. Crossing  $fga^{64}$  to a marked third chromosome balancer.

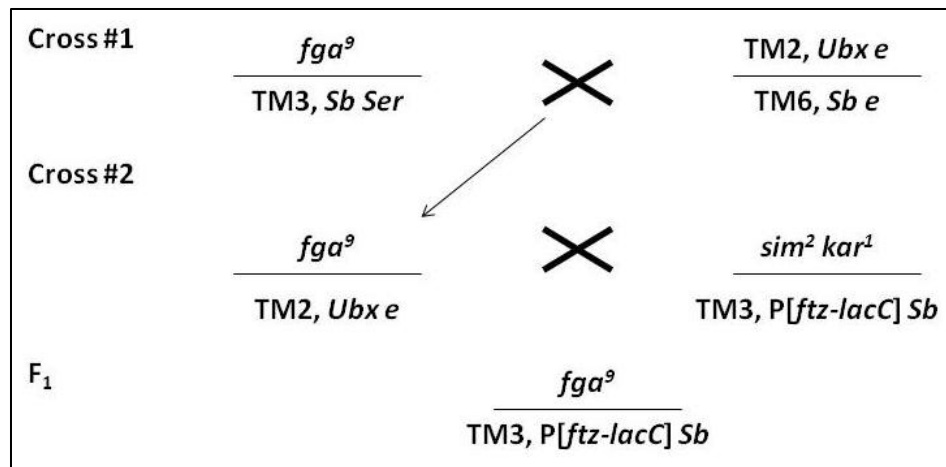


Figure 16. Crossing  $fga^9$  to a marked third chromosome balancer.

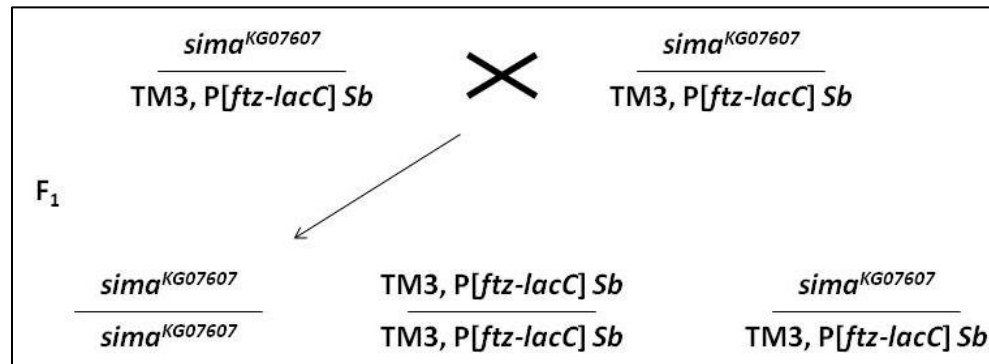
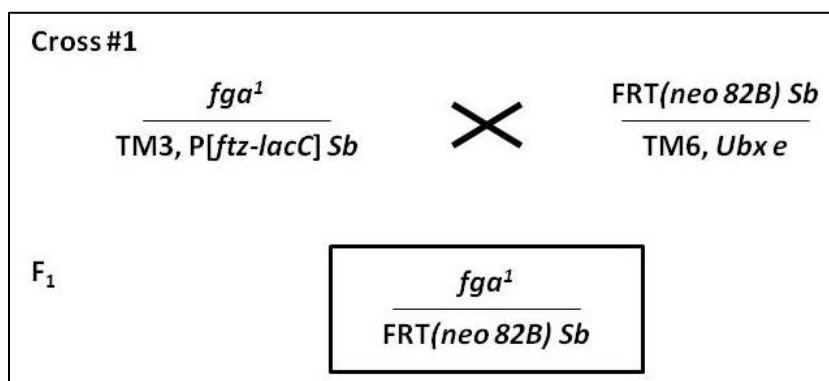


Figure 17. Selecting for  $sima^{KG07607}$  homozygotes for maternal-zygotic experiments.

#### Crosses to establish recombinants for maternal germ line clones:

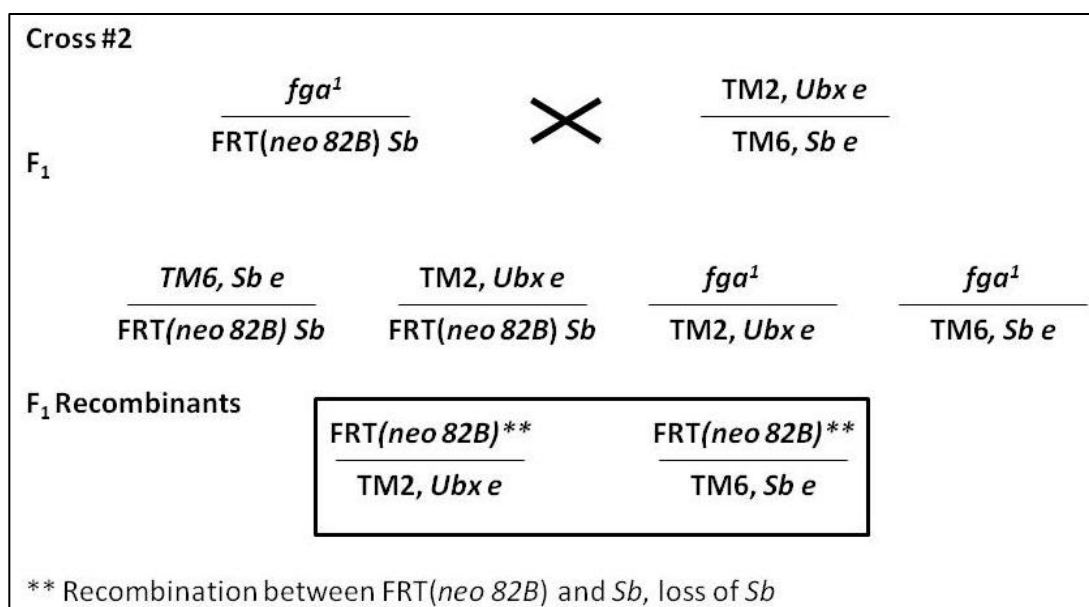
$fga^1$  is the strongest known loss-of-function allele of the *fatiga* gene (Centanin *et al.* 2005; Aveledo *et al.* 2010). This characteristic drove use to use the  $fga^1/TM3, P[ftz-lacC] Sb$  line to perform crosses to achieve *fga* germ line clones. To produce maternal and zygotic loss-of-function *fatiga* mutant embryos our strategy relies on the recombination of the third chromosome to establish a stock of FRT(*neo82B*)  $fga^1$ /Balancer (TM2, *Ubx e* OR TM6, *Sb e*). Recombination between the *fatiga* locus and the insertion site of the FRT(*neo 82B*) is very low,  $<<1:1000$  (personal communication with Dr. Pablo Wappner). To map possible recombination we chose to follow the loss of *Stubble* from the insert FRT(*neo 82B*) *Sb* on the

right arm of the third chromosome. Stubble is 10 map units distal to the FRT insert. Loss of Stubble would indicate a recombination event between the FRT insert and Stubble. The first cross proceeds as follows (Figure 18):



**Figure 18. Cross number 1 to give females for recombination.**

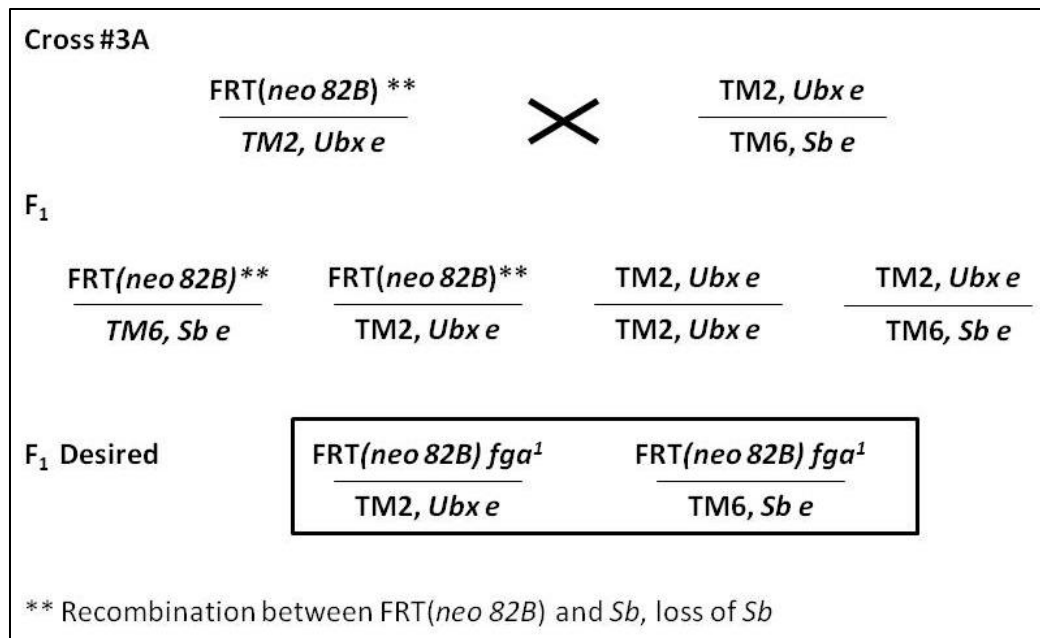
The genotype on the third chromosome  $fga^1/FRT(neo\ 82B)\ Sb$  drove the selection of virgin adults for the next cross. Cross number two was made with these virgin adults to a double balancer to select for possible recombination between the  $FRT(neo\ 82B)$  and *Stubble* on the 3R with the  $FRT(neo82B)\ Sb$  insert (Figure 19). This cross was performed on G-418 Sulfate medium, only offspring with the  $FRT(neo\ 82B)$  chromosome will survive to adulthood when raised on G-418 Sulfate. This cross proceeds as follows:



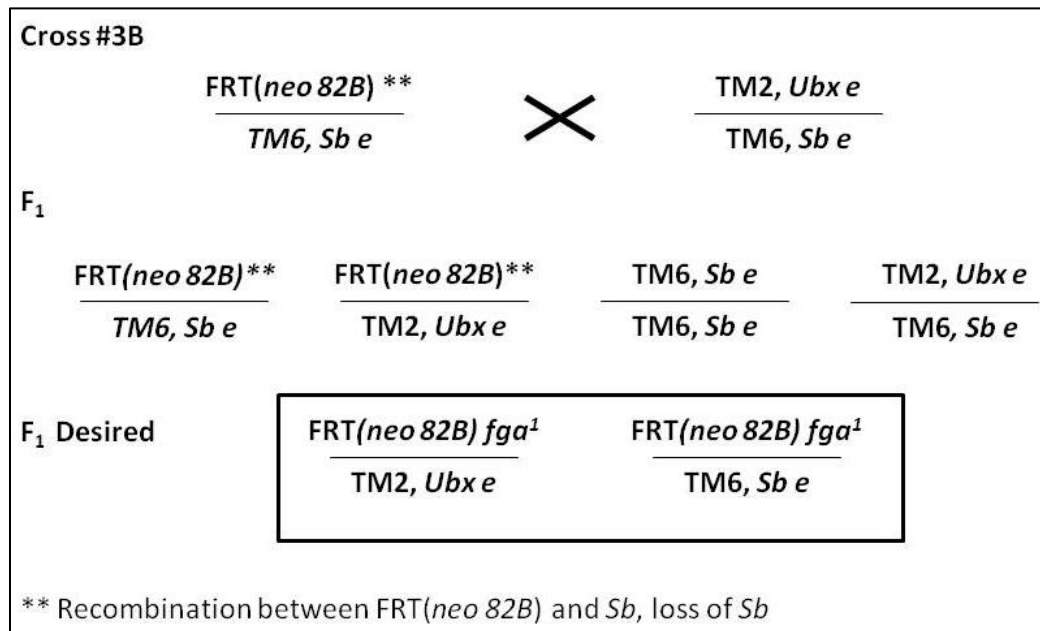
**Figure 19. Cross number 2.**

Only the offspring from cross two that survive G-418 Sulfate treatment and have lost *Stubble* from the *FRT(neo 82B) Sb* insert on 3R are selected for cross number 3. These offspring can either have a *TM2, Ubx e* or *Tm6, Sb e* third chromosome balancer. Cross 3A (Figure 20) and 3B (Figure 21) are performed to ensure that *Stubble* recombined away from the *FRT(neo 82B) Sb* chromosome. Cross 3A proceeds as follows:





**Figure 20. Cross Number 3A.**



**Figure 21. Cross Number 3B.**

To continue with establishing a stock to perform maternal germ line clones using the *fga*<sup>1</sup> loss-of-function allele we sacrificed the selected progeny from cross 3A and 3B, and screened molecularly with PCR to detect the presence of the P-element insert. Using primers

to screen for the presence of the 5' and/or the 3' end of the P-element *Hph*<sup>02255</sup> will display whether the *fga*<sup>1</sup> allele has recombined onto the third chromosome with the FRT(*neo82B*) *Sb* insert.

<b>Drosophila Fly Stocks</b>
<b>Bloomington Drosophila Stock Center at Indiana University</b>
y[1] w[*]; P{w[+mC]=lacW} sima[j11B7]/TM3, Sb[1]
y[1]; ry[506] P{y[+mDint2] w[BR.E.BR]=SUPor-P} sima[KG07607]/TM3, Sb[1] Ser[1]
y[1] w[67c23]; Mi{ET1} sima[MB01168]
P{ry[+7.2]=PZ} Hph[02255] ry[506]/TM3, ry[RK] Sb[1] Ser[1]
y[1] w[67c23]; P{y[+7.7] w[+mC]=wHy} Hph[DG03101]
w[1118]; PBac{w[+mC]=WH} Hph[f03923] mRpL13[f04195]
y[1] w[67c23]; P{w[+mC] y[+mDint2]=EPgy2} Hph[EY02317]
y[1] w[*]; P{ry[+7.2]=neoFRT} 82B Sb[1]/TM6
sim[2] kar[1]/TM3, P{ry[+7.2]=ftz/lacC} SC1, Sb[1] ry[RK]
<b>Gift Stocks</b>
P{w[+mW.hs]=faf-lacZ.F} JF1 (Dr. Janice Fischer University of Texas at Austin)
Canton S (Dr. Johansen ISU)
w[-]; TM2, Ubx e/TM6, Sb e (Dr. Jack Girton ISU)
fga[1]/TM3 (Dr. Pablo Wappner University of Buenos Aires)
fga[9]/TM3 (Dr. Pablo Wappner University of Buenos Aires)
fga[64]/TM3 (Dr. Pablo Wappner University of Buenos Aires)
fga[1] sima[KG07607]/TM3 (Dr. Pablo Wappner University of Buenos Aires)
<b>Generated Stocks</b>
sima[KG07607]/TM3, ftz LacC Sb
fga[1]/TM3, ftz lacC Sb
fga[9]/TM3, ftz lacC Sb
fga[64]/TM3, ftz lacC Sb
fga[1] sima[KG07607]/TM3, ftz lacC Sb
sima[KG07607]/sima[KG07607]

<b>Table 1.1</b>	<b>X-GAL Staining Reagents</b>
<u>Molarity</u>	<u>Reagent</u>
0.2 M	Na <sub>2</sub> HPO <sub>4</sub>
5 M	NaCl
1 M	NaH <sub>2</sub> PO <sub>4</sub>
50%	Clorox® Bleach
100mM	K <sub>4</sub> Fe(CN) <sub>6</sub> •3H <sub>2</sub> O
100mM	K <sub>3</sub> Fe(CN) <sub>6</sub>
10%	X-Gal (100mg/1mL DMSO)
See recipe list 1.1	PBST Triton X100
See recipe list 1.2	PBS
See recipe list 1.3	Heptane Fix

Recipe List 1.1	Phosphate Buffer Saline Triton (Triton X100)**
<u>Amount</u>	<u>Reagent</u>
160g	NaCl
4g	KCl
28.8g	Na <sub>2</sub> HPO <sub>4</sub> Anhydrous
4.8g	KH <sub>2</sub> PO <sub>4</sub> Anhydrous
20L	dH <sub>2</sub> O
60mL	Triton 100x

Recipe list 1.2	Heptane Fix
<u>Amount</u>	<u>Reagent</u>
190mL	PBS*
190mL	PBST**
20mL	50% Glutaraldehyde solution
~500mL	Heptane

Recipe List 1.3	Phosphate Buffer Saline* (PBS)
<u>Amount</u>	<u>Reagent</u>
8g	NaCl
0.2g	KCl
1.44g	Na <sub>2</sub> HPO <sub>4</sub>
0.24g	KH <sub>2</sub> PO <sub>4</sub>
1L	dH <sub>2</sub> O

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## CHAPTER 4: FUTURE DIRECTIONS

### *Further Studies of HIF in Drosophila melanogaster Germ Cell Development*

After many years of studying the regulation and activity of *Drosophila* HIF-1, the molecular and biochemical functions of *Drosophila* HIF-1 components are now under intense investigation (Gorr *et al.* 2004; Romero *et al.* 2008; Irisarri *et al.* 2009; Acevedo *et al.* 2010). Despite the insightful information gained through investigations of tracheogenesis and ovary border cell migration, and now germ cell development, much is still unknown about the functions of HIF-1 in *Drosophila* (Lavista-Llanos *et al.* 2002; Centanin *et al.* 2005; Centanin *et al.* 2008; Doronkin *et al.* 2009). *sima*, *fga*, and *dVhl* are maternally provided transcripts demonstrated by *in situ* hybridization data (Nambu *et al.* 1996; Sonnenfeld *et al.* 1997; Bacon *et al.* 1998). However, how much of those mRNAs are localized within the germ cells and at what point during development they are translated is still unknown. It is possible that maternally provided transcripts of HIF subunits and regulators are sufficient to allow normal effect germ cell development at early stages of embryogenesis.

Zygotic transcription in the germ cells of *Drosophila* begins approximately at stage 10 of development, 4 hours after fertilization and just before the onset of primordial germ cell migration (Richardson and Lehmann 2010). Quantification of the expression levels of *Sima*, *Fga*, and *dVHL* proteins in the germ cells before and during migration may provide useful information as to how essential the hypoxic response pathway is in mediating germ cell development. It may also be necessary to generate germline clones to deplete maternally provided components of HIF-1 signaling to discover the roles of these genes, if any, in early primordial germ cell development

*Drosophila* germ cell movement through the midgut is mediated in part by cellular adhesion protein dE-cadherin, Shotgun. In ovary border cell migration HIF-1 mediates dE-cadherin expression and affects the progression of border cell migration (Doronkin *et al.* 2009). It is possible that *Drosophila* HIF is active within the germ cells and regulates similar mechanisms of cellular adhesion. Thus germ cell development may be influenced by mechanisms that might include mediating cellular adhesion to the midgut primordium.

This hypothesis can be addressed by searching the promoter region of *shotgun* for HRE sites may determine if it is a potential target of HIF-1. Given this information we can then ask if dE-cadherin is regulated by HIF-1 directly. Overexpression of *sima* or loss-of-function of *fga* induces higher levels of expression of hypoxia inducible reporters (Centanin *et al.* 2005). Therefore, one might predict that if HIF-1 regulated dE-cadherin, then hypoxic exposure or stabilization of Sima would also induce the accumulation of dE-cadherin. Thus, *Drosophila* germ cell exit from the midgut may also be dependent upon the expression level of Similar, as it is observed in ovary border cell migration (Doronkin *et al.* 2009). In this study, ovary border cell migration is regulated by Sima in a dose-dependent manner.

Results from this current study revealed that when embryos are subject to development in a hypoxic environment (3% oxygen) that the total number of germ cells within the embryo by stage 15 of embryogenesis is elevated. This may be due to the induction of networks that prevent/inhibit programmed cell death within germ cells. For example, studies show that embryos exposed to 5% oxygen undergo cell cycle arrest, but are capable of normally resuming the cell cycle after reoxygenation (Douglas *et al.* 2001). Therefore, if an embryo is cultured in a hypoxic environment, then processes such as cell cycle progression and cell death may be too energy intensive to continue and are thus

avoided during development. Addressing the role of HIF-1 in *Drosophila* primordial germ cell death may give insightful clues as to what pathways are essential for primordial germ cell survival or termination during an energy intensive process such as migration.

Examining the maternal and zygotic loss-of-function of *fga* is challenging because most homozygous loss-of-function *fga* flies do not survive past embryogenesis (Frei and Edgar 2004). However because we are investigating germ cell development, we have developed two possible approaches that may allow us to examine the effects of the complete loss-of-function of *fga* on germ cell development. The first approach is to utilize RNAi to knock down *fga* expression in the germ cells. However the efficacy of this approach is difficult to obtain.

The second approach utilizes a series of genetic crosses to establish *fga* germline clones. *fga* germline clones will allow us to examine embryos that are homozygous for the loss-of-function of *fga* thus bypassing. This is important because what roles maternally provided *fga* may have in germ cell development are currently unknown.

#### *Challenges of studying germ cell development in Drosophila*

Fixing biological samples allows the study individual cells in a set environment while also enabling the identification and characterization of cellular structures and positions, however, dynamic biological processes cannot be reliably analyzed in this manner. To overcome this shortcoming, fluorescence microscopy has come to be the dominant practice for live imaging of cells (Aldaz, *et al.* 2010; Wessels *et al.* 2010; Schmolze *et al.* 2011).

Fluorescence microscopy utilizes fluorescent probes to tag and illuminate specific cellular features such as proteins or organelles. Transgenes created by fusing genes coding

fluorescent proteins, such as Green Fluorescent Protein, to a gene of choice allows scientists to study the localization, half-life, and interaction of gene products within a cellular environment (Wessels 2010). Fluorescence microscopy can also utilize more than one type of fluorescent probe simultaneously; thus allowing scientists to visualize multiple event or multiple levels of one event within the cell.

One major drawback of fluorescent microscopy is the photo-instability of the fluorescent probes used in practice. Fluorescent molecules suffer from photobleaching: the intensity of the fluorescent signal diminishes with time when the specimen is exposed to light, and the half-life of the fluorophore is typically a few seconds to a few minutes (Bernas *et al.* 2005; Schmolze *et al.* 2011). Thus, specimen examined using fluorescent microscopy can only be observed for short periods of time.

Recent developments in microscopy have produced a workable method for live cell imaging that overcomes the obstacle of photo-instability. Nanoparticles can be used for imaging of live and fixed cells, and nanoparticles are not susceptible to photo-instability. These particles can be manufactured from diverse materials such as metals, polymers, and silica (Wang *et al.* 2010b). Nanoparticles can also be visualized in either fixed or live samples with multiple forms of microscopy (Dark-field, Transmission Electron Microscopy, and Differential Interference Contrast) (Stender *et al.* 2010).

Nanoparticles have the advantage of being optical probes that are able to provide distinguishable evidence of their presence, location, and orientation within a cell (Wang *et al.* 2010b). Nanoparticles made of noble metals are only visible within a small spectral band, and outside of this region they become invisible. This feature, called the Plasmon resonance wavelength, distinguishes nanoparticles from other cellular material. Thus it is feasible to

confirm the presence of a gold or silver nanoparticle within a dynamic cellular environment (Wang *et al.* 2010a; Wang *et al.* 2010b). Gold nanoparticles do not affect cellular viability, whereas silver particles have been shown to be toxic (Chithrani *et al.* 2006; Lee *et al.* 2007).

Another dimension is added to the resolution of the nanoparticles when the “spinning” of the particle is taken into account. Differential interference contrast (DIC) microscopy relies on two orthogonal wave fronts (bright and dark) for image formation. Rod shaped nanoparticles, nanorods, are an ideal probe for imaging with this system. When a spinning nanorod comes into alignment with the bright wavefront its appearance is white, and when it is aligned with the dark wavefront its appearance is black. Intermediate angles appear to cast a shadow or grey area on each nanorod (Stender *et al.* 2010). Therefore to visualize live tissue samples nanorods and DIC microscopy have proven to be proficient in producing high resolution images and even video capture data (Stender *et al.* 2010; Wang *et al.* 2010a; Wang *et al.* 2010b).

In *Drosophila* embryos, the yolk within the middle of the developing embryo has auto-fluorescence characteristics that overlap the excitation wavelengths of GFP (Davis *et al.* 1995; Rudolph *et al.* 1999). The source of this auto-fluorescence is the composition of the yolk mass, mainly composed of proteins called vitellogenins (Tufail and Takeda 2008). To overcome this obstacle multiple filter sets are used to eliminate background illumination from the yolk in order to visualize the fluorescently probed structures within the embryo. This is yet another obstacle aside from photobleaching that hinders the amount of data that can be collected from a live sample within one given experiment. Alternatively, developing fluorescent probes other than GFP, such as Red Fluorescent Protein, can provide another method to visualizing cellular structures within the *Drosophila* embryo.

Due to the challenges of imaging live dynamic events in *Drosophila*, such as germ cell development, with utilizing fluorescent microscopy, we have chosen to use nanoparticles to design new techniques for imaging within a live embryo. These experiments have utilized injection techniques, nanoparticles (gold and silver of various sizes and shapes), and DIC microscopy to develop such a protocol for live whole organism imaging with nanoparticles. Thus this protocol will develop a technique that can be used as an alternative or complimentary to the current live imaging techniques that are used in developmental biology (see Appendix A for results and methods).

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## APPENDIX

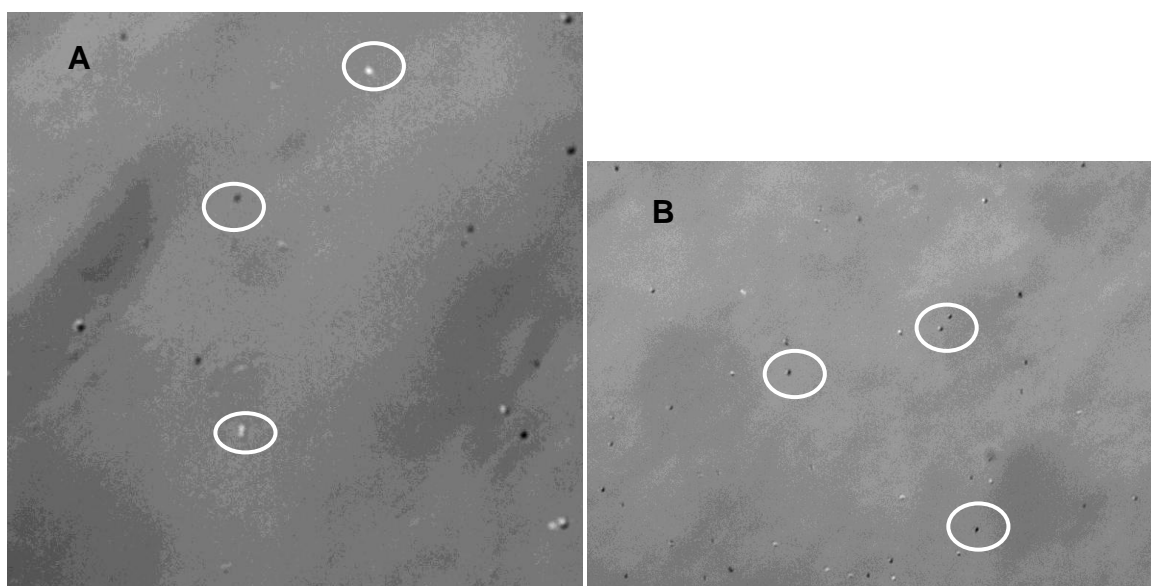
### INTRODUCTION

Currently two sets of microscopy techniques are used to visually study and image microscopic events within *Drosophila* embryos. The first relies on fixation of cellular tissue, such as conventional immunohistochemical staining techniques. The alternative is to image living tissues with fluorescence (Aldaz *et al.* 2010; Wessels *et al.* 2010; Seo *et al.* 2011). Though these techniques have provided scientists with useful tools for collecting data, each of these protocols has its limitations.

Nanoparticle imaging can be utilized independently of or in conjunction with fluorescent imaging to visualize dynamic biological processes in real time (Stender, *et al.* 2010; Wang *et al.* 2010b). The Coffman and Fang group have collaborated to develop a technique for live whole *Drosophila* embryo imaging, using nanoparticle probes injected into young embryos. Here the two laboratories contributed equally to the design and execution of this developing technique. Below I adopted methods for injecting nanoparticles into young embryos, and Anthony Stender has developed techniques for collecting and analyzing real time data from living injected embryos.

### RESULTS

Injection of nanoparticles into ~1hour old embryos has produced intriguing results. First examining the characteristics of immobilized nanoparticles was necessary to determine the signal strength of the different nanoparticles that were used in each experiment. Figure 22 depicts nanoparticles of different composition, size, and shape.

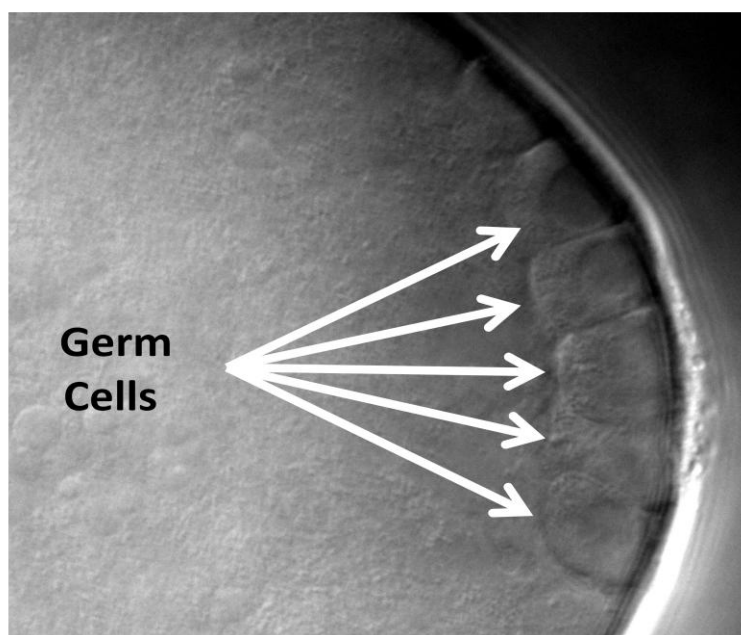


**Figure 22. Nanoparticles fixed to a coverslip.** (A and B) Three white circles demark nanoparticles. (A) 25x73nm gold nanorods were fixed to a coverslip and imaged with a 660nm filter at 140x. (B) 60nm silver nanospheres were fixed to a coverslip and imaged with a 540nm filter at 160x.

Common filters were used to stimulate gold and silver nanoparticle plasmon resonance for detection on a surface. 540nm and 720nm filters were used to detect the presence of both nanoparticles. It was found that the silver nanospheres were brighter in signal than the gold nanorods. Figure 22B displays fixed 60nm silver spheres though the majority of experiments performed used 80nm silver spheres that provided an even brighter signal (not shown).

Post injection, embryos were covered in halocarbon oil 700 and a coverslip (see methods). Embryos were then examined for signs of life, to assess viability, as well as the presence of nanoparticles using differential interference contrast microscopy. This was observed by noting stages of development (Campos-Ortega and Hartenstein 1997) and

detecting rapid movement of cellular material. Embryos that survived injection typically displayed cellularization of the primordial germ cells (approximately 30 minutes to 1 hour post injection) at the posterior end of the embryo (Figure 23).

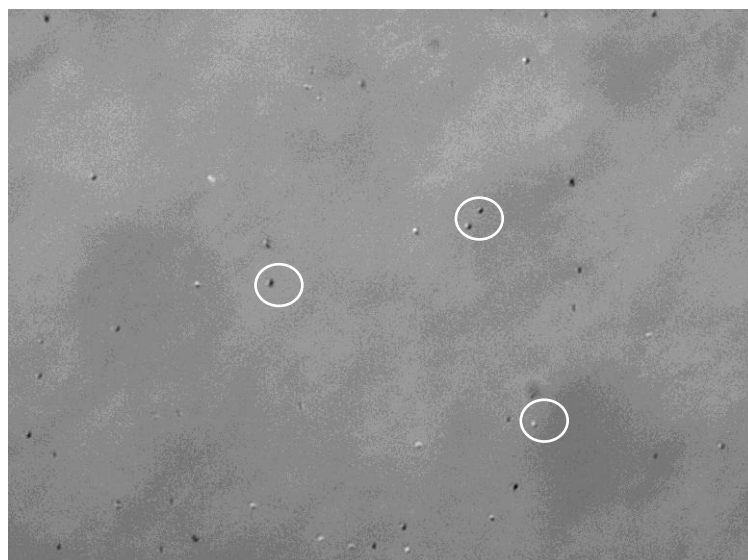


**Figure 23. The posterior end of an embryo ~2 hours old.** Arrows point to several germ cells that have visibly formed. Imaged at 80x.

Subsequent developmental events such as germ cell migration along the dorsal plate to muscle contraction have also been observed (not shown). However, embryo mortality post injection remained high. One hypothesis is that when embryos are covered in halocarbon oil underneath a coverslip they become hypoxic. Meaning, there is not enough oxygen to support dynamic developmental processes such as the cell cycle (Douglas *et al.* 2001). To overcome this obstacle we have utilized liquids with varying oxygen permeability (such as 70% Glycerol 30% PBST and halocarbon oil 95). Experimental trials with these liquids were

not ideal; both either evaporated or wicked underneath the double-stick tape used to mount the coverslip. Other reasons for this high mortality rate have still are still being investigated.

Figure 24 depicts 60nm silver spheres above an embryo. This was useful to determine the size ratio between the nanoparticles and the embryo. Thus scanning the embryo for nanoparticles was then more definitive.



**Figure 24. 60nm silver spheres above an embryo.** Imaged with a 540nm filter at 160x. The three white circles demark nanoparticles.

## CONCLUSION

We have yet to see nanoparticles within the embryos. Nanoparticles have not previously been imaged within such a dynamic, thick, and complex environment. Therefore, it is possible that the components (such as rapid cell division, movement, and vitellogenin proteins) of the developing embryo skew the signal of the nanoparticles in such a way that a typical filter set used to image the nanoparticles is no longer applicable to the system (Tufail

and Takeda 2008; Campos-Ortega and Hartenstein 1997). However, the potential of nanoparticle imaging within a developing *Drosophila* embryo is high.

This experimental technique has illuminated numerous variables that must be controlled to obtain ideal experimental parameters for live nanoparticle imaging within a young embryo. Such variables include concentration and date of preparation of nanoparticle solution, time-of-injection into young *Drosophila* embryos, methods of dechoriation, and length of post injection recovery time without a coverslip. All of these variables have been adjusted in attempts to improve experimental outcomes.

Preparing fresh or sonicating nanoparticle solution on the day of injection, we observed fewer aggregated particles. Allowing embryos to rehydrate and rest approximately 5 minutes post injection increased the number of embryos that could be examined. Despite the positive results these adjustments have made, overcoming hypoxia still remains a challenge.

Further research and experimentation utilizing various concentrations, sizes, and shapes of nanoparticles and filter sets may be necessary to continue the development of nanoparticle imaging within a whole organism system.

## METHODS

### Injecting Nanoparticles in *Drosophila* Embryos

**Fly Maintenance:** 2-5 day old adult flies were kept in embryo collection chambers at 24°C and relative humidity ~30%. These chambers were set up at least 2 days prior to each experiment. Yeasted plates were changed at least three times daily, to prevent females from holding eggs. On the day of injection apple juice plates with a thin layer of yeast paste were used (Parton *et al.* 2010; Figard and Sokac 2011).

**Collecting Embryos for Injection:** Apple juice agar plates were changed at least 2-3 hours prior to collecting embryos for injection. Embryos were collected for a maximum of 1 hour. Embryo collection must not exceed 1 hour in order to collect embryos that have not cellularized. At the end of collection adults that may have gotten stuck to the yeast during the collection period were removed from the agar plate. The plated was flooded with ddH<sub>2</sub>O. A paint brush was used to gently detach the embryos from the apple juice agar plate. Detached embryos were rinsed into an open ended mesh capped vial. The nylon mesh was then removed and placed on a wetted Kim-wipe to keep the embryos hydrated. A stereoscope and thin paint brush were used to mount 50-80 embryos onto a microscope slide for injection.

**Injecting Embryos:** Embryos were allowed to dehydrate for 10 minutes prior to injection. The rate of dehydration is dependent upon the humidity. Needles for injection were made prior to injection. Needles were opened manually by gently nudging the tip into the side of a microscope slide using a triple axis micromanipulator. Needles are loaded with 1  $\mu$ L of nanoparticle solution. Embryos were injected when appropriately dehydrated.

**Dechorionating the embryos:** Embryos were dechorionated with double stick tape. Injected embryos were gently touched with double stick tape on the end of a microscope slide. Lifting the double stick tape microscope slide from the embryos breaks the chorion around the embryos.

**Mounting embryos for Imaging:** Post-injection embryos are very fragile. A clean fine tipped paint brush was used to carefully transfer the injected embryos from either the injection slide or the double stick tape. Embryos are transferred to a new slide for imaging. 4-5 embryos are sufficient for data collection post injection. Once embryos were transferred they were covered with a small drop of Halo Carbon oil 700.

## Nanoparticle Imaging in Drosophila Embryos

**Preparing Nanoparticles for Injection:** An aliquot (50, 75, or 100  $\mu\text{L}$ ) of nanoparticle solution was pipetted into centrifuge tube and centrifuged for 10 minutes at 5500 rpm. The majority of liquid layer was removed. As necessary, the solution was re-centrifuged. To attain better concentrations less than 5  $\mu\text{L}$  of solution was retained with the nanoparticles. The nanoparticles were then suspended in Rhodamine B solution (suspended in DMSO). Nanoparticle solutions were sonicated in a water bath for 20 – 30 minutes prior to each injection.

**Nanoparticles:** Mesoporous silica nanoparticles, 60 nm gold nanospheres, 60 and 80 nm silver nanospheres, 25x60 nm gold nanorods, 25x73 nm gold nanorods were used over the course of these experiments.

**Imaging Slide Preparation Post Injection:** Two parallel strips of double-sided tape were placed on either side of the mounted embryos to prevent damage to the embryos and to hold a coverslip in place. A coverslip was placed above the mounted embryos after injection.

**Imaging:** All imaging was done with a Nikon Eclipse 80i microscope in differential interference contrast (DIC) mode. To locate and center the embryos, a 20x air objective was used. For high resolution imaging, a 100x objective was utilized. The magnification was fine-tuned with a zoom knob on the microscope. With the 100x objective, the actual magnification was able to be adjusted from 80x to 200x. Most images were collected at 80x or 100x. Exposure times were typically held under 0.1 seconds. To identify a nanoparticle, it was necessary to image the particle (or the region that contains the particle) at two wavelengths. Depending on the microscope setup, it was possible to either: a) swap filters or



b) use a dual-view component that allows simultaneous viewing of a region at two wavelengths.

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