CHAPTER SEVEN

Cellular and Developmental Adaptations to Hypoxia: A *Drosophila* Perspective

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Abstract

The fruit fly *Drosophila melanogaster,* a widely utilized genetic model, is highly resistant to oxygen starvation and is beginning to be used for studying physiological, developmental, and cellular adaptations to hypoxia. The *Drosophila* respiratory (tracheal) system has features in common with the mammalian circulatory system so that an angiogenesis-like response occurs upon exposure of *Drosophila* larvae to hypoxia. A hypoxia-responsive system homologous to

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mammalian hypoxia-inducible factor (HIF) has been described in the fruit fly, where Fatiga is a *Drosophila* oxygen-dependent HIF prolyl hydroxylase, and the basic helix-loop-helix Per/ARNT/Sim (bHLH-PAS) proteins Sima and Tango are, respectively, the *Drosophila* homologues of mammalian HIF-alpha (α) and HIF-beta (β). Tango is constitutively expressed regardless of oxygen tension and, like in mammalian cells, Sima is controlled at the level of protein degradation and subcellular localization. Sima is critically required for development in hypoxia, but, unlike mammalian model systems, it is dispensable for development in normoxia. In contrast, *fatiga* mutant alleles are all lethal; however, strikingly, viability to adulthood is restored in *fatiga sima* double mutants, although these double mutants are not entirely normal, suggesting that Fatiga has Sima-independent functions in fly development. Studies in cell culture and *in vivo* have revealed that Sima is activated by the insulin receptor (InR) and target-of-rapamycin (TOR) pathways. Paradoxically, Sima is a negative regulator of growth. This suggests that Sima is engaged in a negative feedback loop that limits growth upon stimulation of InR/TOR pathways.

1. Introduction

This chapter intends to provide an overview of the recent progress attained in the field of hypoxia and HIF biology in the fruit fly *D. melanogaster*. We will begin with a brief description of the model, its life cycle, its advantages as a genetic system, and a summary of some of the useful genetic methods available in this species. We will continue with a description of the *Drosophila* respiratory system and its oxygen-dependent plasticity, which shares many features with angiogenesis of vertebrate organisms. We will then summarize the current knowledge on the *Drosophila* HIF system, including the cellular mechanisms of oxygen-dependent regulation as well as a brief description of the target genes that mediate adaptation to oxygen starvation. We will finish by discussing the regulation that the InR and TOR pathways exert on the *Drosophila* HIF system and the current knowledge about the role of the hypoxia-responsive machinery in growth control and cell size determination.

2. *Drosophila melanogaster* as a Model System to Study Physiological Responses to Hypoxia

The fruit fly *D. melanogaster* is a cosmopolitan species with striking capacity to colonize a wide array of different habitats and environmental conditions (Berrigan and Partridge, 1997; Dillon and Frazier, 2006; Gibert *et al.*, 2001). It belongs to the so-called group of holometabolous insects in which the general body plan undergoes a dramatic reorganization in the larva-to-pupa transition. The duration of the entire life cycle is about 12 days at 25°

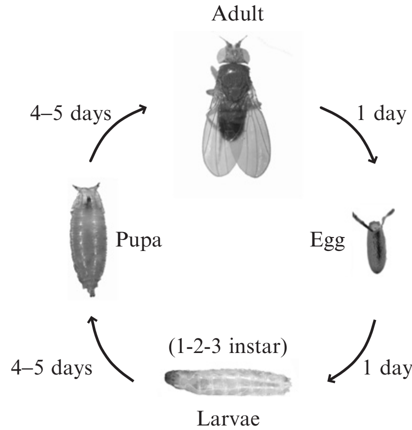

Figure 7.1 The life cycle of *Drosophila melanogaster*. The duration of the entire *Drosophila* life cycle is about 12 days at 25°. Adult females lay eggs; embryogenesis lasts 24 h and takes place within the eggshell. The three larvae instars take 4 to 5 days; the third instar larva gives rise to pupae that undergo metamorphosis to become an adult that attains sexual maturity within 24 h.

(Fig. 7.1) so that generations can be reared within a short time period (Ashburner *et al*., 2004). Females lay eggs into fermenting fruits, and, after completion of embryonic development, a first instar larva hatches from the eggshell. The resulting larva feeds very actively and increases its weight by several folds, while it molts twice to a second and a third larval instar. After attaining a critical weight, third instar larvae stop feeding, become immobile, and encapsulate in the pupal case, where they undergo a 4-day metamorphosis. During this period, the larval tissues are degraded to their basic components, and the entire body is rebuilt to give rise to a pupa that undergoes one additional cuticle molt to become a pharate adult. Finally, a fully formed adult emerges from the pupal case and attains sexual maturity within 24 h.

In nature, *Drosophila* larvae live mostly in fermenting fruits and feed with fruit pulp and yeast that usually grows therein. Thus, in its normal habitat, the larvae compete with microorganisms for limited amounts of oxygen. Therefore, *Drosophila* larvae are permanently exposed to a hypoxic micro-environment, anticipating a well-developed cellular machinery that responds to oxygen starvation (Gorr *et al*., 2006).

3. Experimental Advantages of the Model System

Genetic studies in *D. melanogaster* started about 100 years ago with the pioneering work of Thomas Hunt Morgan in 1909. Since then, genetic work in the fruit fly has become increasingly intense, and thousands of

mutations have been isolated, targeting a large portion of the genes of the fruit fly. This ample repertoire of mutations has been gradually enriched with sophisticated genetic tools that facilitated manipulations (Greenspan, 1997). The availability of molecular biology techniques in the early 1980s provoked a true revolution in the *Drosophila* field. *Drosophila* genetic mobile elements were isolated, molecularly characterized, and converted into gene-delivering vectors (Cooley *et al.*, 1988; Rubin and Spradling, 1982; Spradling and Rubin, 1982). In just a few years, transformation of *Drosophila* embryos and generation of transgenic fly lines became a routine practice in every *Drosophila* laboratory.

Progress in *Drosophila* gene technology has accelerated dramatically over the last decade and, nowadays, the *Drosophila* genome is fully sequenced (Adams *et al.*, 2000), mutations targeting most of the genes are available, genes can be readily overexpressed in almost any desired pattern (limited just by the availability of known promoters) (Brand and Perrimon, 1993), and gene expression can be conditionally silenced in spatially and temporally restricted patterns by delivering RNA interference (RNAi) (Carthew, 2001), by inducing loss-of-function mitotic clones (Chou and Perrimon, 1992), or by overexpressing dominant-negative constructs *in vivo* (Wilk *et al.*, 1996).

4. The *Drosophila* Respiratory System

The circulatory system of insects is primitive and basically composed of a single dorsal vessel that plays the role of a primitive heart. Insect blood (hemolymph) does not circulate through veins and arteries but, rather, fills the entire body cavity, delivering nutrients to organs and tissues throughout the body. Gas transport (i.e., oxygen delivery and carbon dioxide release) does not depend on such a primitive circulatory system. Instead, gases are delivered directly to organs and tissues of the organism through the respiratory system named *tracheal system* (Ghabrial *et al.*, 2003). The air enters the insect body through orifices called spiracles, which are directly connected to a complex network of ramified epithelial-like tubes, the *tracheae* (Samakovlis *et al.*, 1996a), which provide oxygen to every cell or tissue in the organism (Fig. 7.2). As we will discuss later, the *Drosophila* tracheal network shares many cellular features with the mammalian circulatory system. Development of the *Drosophila* tracheal system begins at mid-embryogenesis when 10 clusters of approximately 80 cells at each side of the embryo begin to express the transcription factors Trachealess (Isaac and Andrew, 1996; Wilk *et al.*, 1996) and Drifter/Ventral veinless (Anderson *et al.*, 1995; Llimargas and Casanova, 1997), which promote differentiation of ectodermal cells into a tracheal cell fate. Following differentiation, these cell clusters, called tracheal placodes, invaginate to form tracheal pits (Llimargas and Casanova, 1999),

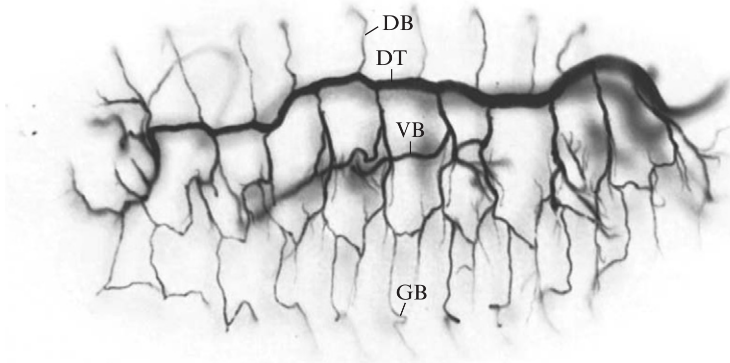

Figure 7.2 The *Drosophila* respiratory (tracheal) system. A stage 17 *Drosophila* embryo is stained with a 2A12 monoclonal antibody to visualize the tracheal tree, which is a continuous network of ramified tubes that conduces air to all tissues and cells in the body. Some of the main branches of the tracheal system are marked: DB, dorsal branch; DT, dorsal trunk; GB, ganglionic branch; VB, visceral branch.

and, immediately afterwards, discrete groups of cells in every cluster start migrating in highly stereotyped directions to give rise to the various tracheal branches (Llimargas, 2000; Wappner *et al*., 1997). As embryogenesis proceeds, tracheal branches from adjacent or contralateral clusters get in close proximity and fuse (Jiang and Crews, 2003; Samakovlis *et al*., 1996b; Tanaka-Matakatsu *et al*., 1996), generating a continuous tubular network—the tracheal tree (see Fig. 7.2). By the end of embryogenesis, one single cell at the tip of each branch (Llimargas, 1999), the terminal cell, emits subcellular processes that will provide air to all cells in target tissues (Guillemin *et al*., 1996). Noteworthy, from the moment tracheal pits have been formed, the entire process of tracheal development depends exclusively on tracheal cell migration in the complete absence of cell divisions. Thus, tracheal development begins with 20 tracheal placodes of about 80 cells each (total: ~1600 cells) and ends up with the same number of cells, forming a complex tubular network that supplies air to all the cells and tissues in the body.

This sophisticated process of guided cell migration depends mainly on the chemotactic activity of the fly fibroblast growth factor (FGF) homologue Branchless (Bnl) (Sutherland *et al*., 1996), which is expressed in target tissues outside the tracheae, attracting the extension of tracheal branches. Bnl binds the FGF receptor homologue Breathless (Btl) (Dossenbach *et al*., 2001; Klambt *et al*., 1992; Reichman-Fried and Shilo, 1995; Reichman-Fried *et al*., 1994), which is expressed in tracheal cells and relays the signal intracellularly, provoking modifications in the cytoskeleton that induce changes of cell shape that result in guided cell migration. Thus, the expression pattern of Bnl in target tissues predicts the direction of tracheal cell

migration and consequent branch extension. This expression pattern is very dynamic during tracheogenesis; once a leading cell of a given branch has reached a Bnl-positive cluster in the target tissue, *bnl* expression is switched-off, and the gene is turned on again a little further on the track of the growing branch. Thus, throughout the process of tracheal development, *bnl* expression is turned on and off many times along the path of migrating tracheal cells (Ribeiro *et al*., 2002, 2003; Sutherland *et al*., 1996). This tubulogenic process has features in common with mammalian vasculogenesis, where migration of blood vessel primordia is guided by the expression of different isoforms of the vascular endothelial growth factor (VEGF) that target receptors on the plasma membrane of epithelial cells (Metzger and Krasnow, 1999; Wappner and Ratcliffe, 2001).

By the end of embryogenesis, the stereotypic phase of tracheal development has been completed, and later, in larval stages, terminal tracheal branches are plastic and have the capacity to sprout out projections towards oxygen-starved areas in the surrounding tissues, very much like angiogenesis in mammals (Jarecki *et al*., 1999). This hypoxia-dependent response of tracheal terminal branches is also mediated by Bnl, which is induced in oxygen-starved target tissues, and its receptor, Btl, expressed in tracheal cells. This hypoxia-dependent behavior of the *Drosophila* tracheal system is remarkably similar to mammalian angiogenesis (Metzger and Krasnow, 1999; Wappner and Ratcliffe, 2001), where VEGF is induced in oxygen-starved cells, promoting the outgrowth of new blood capillaries that provide additional oxygenation to hypoxic target tissues.

5. Occurrence of a *Drosophila* System Homologous to Mammalian HIF

The occurrence in *Drosophila* of a system homologous to mammalian HIF was first inferred by electromobility shift assays (EMSA), in which nuclear extracts prepared from *Drosophila* S2 cells were incubated with oligonucleotides derived from enhancers of mammalian genes that are induced in hypoxia. In these conditions, hypoxia-inducible complexes were formed, suggesting the occurrence of an endogenous *Drosophila* nuclear protein that can bind HIF consensus motifs on the DNA (Nagao *et al*., 1996). Almost simultaneously, a ubiquitously expressed *Drosophila* gene encoding a 1505 amino acid basic helix-loop-helix Per/ARNT/Sim (bHLH-PAS) transcription factor, closely related to mammalian HIF-α, was cloned and named *similar* (sima) (Fig. 7.3) (Nambu *et al*., 1996). Sima is remarkably bigger than all mammalian HIF-α proteins described so far, exhibiting a molecular weight of approximately 180 kDa; it displays a 45% amino acid identity with human HIF-1α in the PAS domain

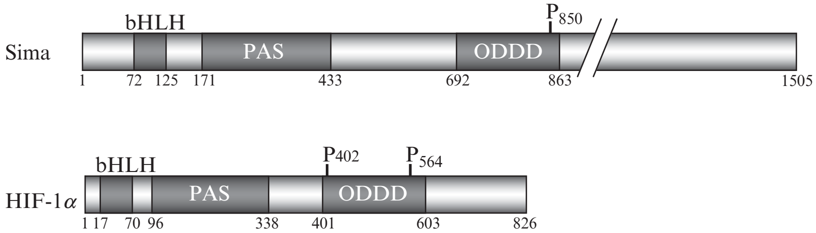

Figure 7.3 Schematic representation of the *Drosophila* hypoxia-inducible factor (HIF) homologue Sima and mammalian HIF-1α proteins. The basic-helix-loop-helix (bHLH), Per/ARNT/Sim (PAS), and oxygen-dependent degradation (ODDD) domains are shown. Note that the Sima prolyl 850 residue is the substrate of the *Drosophila* HIF prolyl hydroxylase, Fatiga.

(the highest among *Drosophila* bHLH-PAS proteins) and 63% in the bHLH domain. The first experimental evidence that Sima could be a *Drosophila* HIF-α functional homologue was reported soon afterwards. Sima protein is expressed in *Drosophila* S2 cells at low levels, but when cell cultures are exposed to severe hypoxia, Sima protein levels rise dramatically (Bacon *et al.*, 1998). Paralleling the regulation of mammalian HIF-α proteins (Huang *et al.*, 1998; Jiang *et al.*, 1997; Maxwell *et al.*, 1999; Pugh *et al.*, 1997), Sima regulation occurs at the level of protein stability, and a transferable central domain of the protein is responsible for oxygen-dependent proteasomal degradation (Bacon *et al.*, 1998).

The *Drosophila* homologue of HIF-β/ARNT was identified at approximately the same time and, in addition to forming a heterodimer with Sima, was found to be a common partner for several different *Drosophila* bHLH-PAS proteins, as occurs with HIF-β/ARNT in mammalian cells (Ma and Haddad, 1999; Ohshiro and Saigo, 1997; Sonnenfeld *et al.*, 1997; Zelzer *et al.*, 1997). As expected, Sima and Tango interact physically through their HLH motifs and PAS domains (Sonnenfeld *et al.*, 1997), and functional studies in cell culture and in developing embryos confirmed that Sima and Tango are absolutely required for inducing a transcriptional response to hypoxia (Bruick and McKnight, 2001; Centanin *et al.*, 2005; Dekanty *et al.*, 2005; Gorr *et al.*, 2004; Lavista-Llanos *et al.*, 2002). Expression of Tango protein is ubiquitous in all tissues of the fruit fly throughout development. Interestingly, Tango is primarily localized in the cytoplasm of all cells in the embryo, unless an α-subunit partner, such as Trachealess (involved in tracheal development) (Wilk *et al.*, 1996) or Single minded (involved in glial cell differentiation in the embryonic nervous system) (Nambu *et al.*, 1991), is coexpressed in the same cell. When α- and β-subunits are expressed in the same cell, they localize in the nucleus and can readily induce transcription of target genes (Ward *et al.*, 1998). A possible role of Tango in subcellular localization of Sima has not been studied so far. Given that subcellular localization of Sima seems itself to

depend on a complex cellular machinery controlled by oxygen tension (discussed later), the participation of Tango in this regulation might be more complex than in the subcellular localization of other bHLH-PAS protein partners. As with other aspects of HIF cell biology, *Drosophila* genetics might help to understand the role of Tango/HIF-β in the regulation of Sima/HIF-α subcellular localization.

Unlike Tango, Sima protein levels are far too low to be detectable in normoxic embryos by immunofluorescence. Only upon exposure to severe hypoxia can the Sima protein be observed in the nuclei of cells of the tracheal system (Lavista-Llanos *et al.*, 2002). Transgenic fly lines bearing transcriptional *lacZ* or green fluorescent protein (GFP) reporters that are specifically induced in hypoxia have been developed and, consistent with the expression pattern of Sima protein, are induced with maximal sensitivity in cells of the tracheal system (Fig. 7.4). This high sensitivity to hypoxia displayed by the tracheal cells is maintained throughout the life cycle of the fruit fly. Remarkably, all the rest of the tissues in the organism are also able to accumulate Sima protein and induce hypoxia-responsive reporters, though induction occurs at stronger hypoxic conditions (Lavista-Llanos *et al.*, 2002). The existence of enhanced responses to hypoxia in tracheal cells is of interest and raises the question as to the identity of tracheal endogenous inducible genes. These observations pose an interesting paradox as, in the currently accepted model of tracheal adaptation to hypoxia, Bnl upregulation in non-tracheal cells is the key determinant of oxygen-dependent tracheal plasticity (Jarecki *et al.*, 1999). Therefore, the physiological significance of the observation that Sima-dependent gene induction

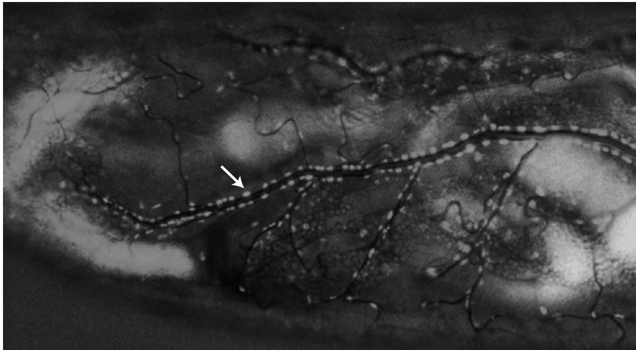

Figure 7.4 The transcriptional response to hypoxia is highly sensitive in cells of the tracheal system. *Drosophila* transgenic larvae bearing a hypoxia-inducible reporter exposed to 5% O_2 express the reporter mainly in the cells of the respiratory (tracheal) system, so that all nuclei of tracheal cells are decorated with green fluorescent protein (GFP; arrow) (for details, see Lavista-Llanos *et al.* [2002]). Reproduced with permission from Gorr *et al.* (2006).

is particularly sensitive in tracheal cells is unclear. The *Drosophila* system offers an opportunity to apply genetic tools to investigate this point, which might also contribute to better understanding the molecular basis of angiogenesis.

6. Regulation of Sima by Oxygen Levels

As in mammalian cells, the *Drosophila* HIF-α protein Sima is primarily regulated by oxygen at the level of protein degradation (Gorr *et al.*, 2004). An oxygen-dependent degradation domain (ODDD) encompassing amino acids 692 to 863 has been identified (Lavista-Llanos *et al.*, 2002), and, remarkably, this domain contains a prolyl residue (P850) (Arquier *et al.*, 2006; Jaakkola *et al.*, 2001) (see Fig. 7.3), which appears to be the substrate of a *Drosophila* HIF prolyl hydroxylase that operates as an oxygen sensor. Consistent with this, an open-reading frame encoding a *Drosophila* gene highly homologous to mammalian prolyl hydroxylase domains (PHDs) was discovered (Bruick and McKnight, 2001; Epstein *et al.*, 2001; Lavista-Llanos *et al.*, 2002) and named *fatiga* (Centanin *et al.*, 2005). RNAi-mediated silencing of this gene provokes constitutive accumulation of Sima protein both in normoxic cell cultures (Bruick and McKnight, 2001) and in *Drosophila* embryos (Centanin *et al.*, 2005), and, as expected, constitutive accumulation of Sima led to upregulation of genes that are typically induced in hypoxia. These results could be mimicked in various *fatiga* loss-of-function alleles; they all display higher-than-normal Sima protein levels accompanied by normoxic induction of hypoxia-responsive transgenic reporters (Centanin *et al.*, 2005).

In addition to being regulated by the prolyl hydroxylase Fatiga at the level of protein stability, experiments carried out in embryos revealed that Sima subcellular localization depends on oxygen tension as well. Studies of Sima subcellular localization have been carried out by overexpressing Sima in transgenic embryos, thereby overriding the rate of protein degradation. In this experimental setting, Sima is primarily cytoplasmic in normoxia and accumulates in the nuclear compartment upon exposure to hypoxia (Lavista-Llanos *et al.*, 2002). However, this is not an all-or-none response. Detailed studies in normoxia and graded hypoxia revealed that regulation of Sima subcellular localization is dose-dependent and modulated by developmental parameters (Dekanty *et al.*, 2005) (Fig. 7.5). Whereas in normoxic early embryos, Sima is localized exclusively in the cytoplasm, by the end of embryogenesis, a significant proportion of normoxic embryos show an even distribution of Sima within the cell, and a lower proportion of individuals exhibit Sima localized in the nuclear compartment. When challenged with increasingly stronger hypoxic stimuli, developing embryos have a higher proportion of Sima protein localized in the nucleus, becoming totally

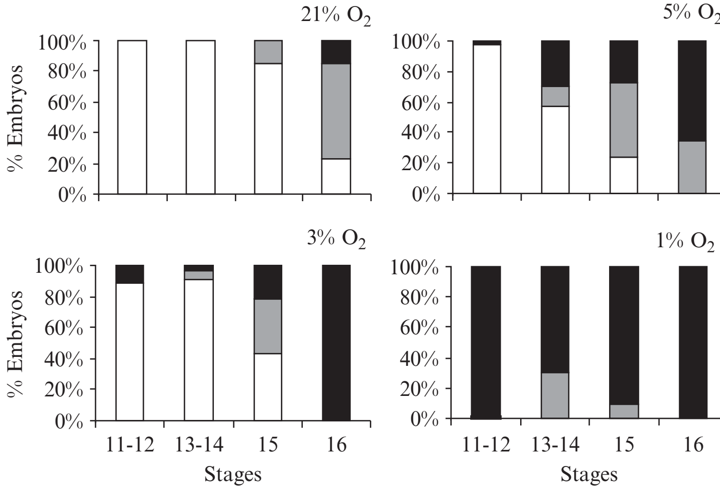

Figure 7.5 **Sima subcellular localization depends on oxygen concentrations in a dose-dependent manner and is modulated by developmental parameters. White: cytoplasmic location; grey: ubiquitous; black: nuclear. Numbers refer to embryonic stages. Reproduced from Dekanty *et al.* (2005).**

nuclear in embryos exposed to 1% O_2. Thus, Sima becomes increasingly nuclear as hypoxia becomes more severe and predominantly cytoplasmic in conditions of abundant oxygen supply (see Fig. 7.5). Contrary to some initial predictions, nuclear localization seems to be the "default state" of Sima subcellular localization, since deletion of the ODDD renders Sima constitutively nuclear, regardless of oxygen tension (Lavista-Llanos *et al.*, 2002). In mammalian cells, similar regulation of HIF-α subcellular localization seems to occur; as in von Hippel-Lindau (VHL)-lacking cells, HIF-α is constitutively localized in the nucleus, suggesting that the ODDD is involved in regulation of subcellular localization (Groulx and Lee, 2002). The molecular mechanism by which the ODDD mediates this regulation is unclear; two models in principle could account for the observations: the ODDD is necessary for cytoplasmic retention in normoxia or the ODDD is required for active nuclear export. Genetic experiments in *Drosophila* might help to understand this unresolved issue of HIF regulation.

7. Role of Sima and Fatiga in *Drosophila* Development

Analyses of "knockout" mouse strains have revealed that mammalian HIF proteins have essential functions in embryonic and postembryonic development. They have been shown to participate in the formation of the

embryonic heart, vasculature, brain, cartilages, and the placenta in adult females (Adelman *et al*., 2000; Covello and Simon, 2004; Giaccia *et al*., 2004; Iyer *et al*., 1998; Pfander *et al*., 2004; Tomita *et al*., 2003), suggesting that local oxygen tension might play a role in these developmental processes. Unexpectedly, the *Drosophila* homozygous Sima loss-of-function mutants are fully viable in normoxia, but fail to develop in mild hypoxic conditions (Centanin *et al*., 2005), indicating that Sima is necessary for development in hypoxia but not in normoxia. In contrast, *fatiga* mutant alleles provoke lethality at different stages of the life cycle in normoxia (none of them can attain the adult stage), implying that Fatiga is critically required for normal development. Strikingly, *fatiga sima* double mutants are viable, attaining the adult stage in normoxia (not in hypoxia) (Centanin *et al*., 2005) (Fig. 7.6), suggesting that the most fundamental functions of Fatiga/PHD in *Drosophila* development involve downregulation of Sima protein levels.

Noteworthy, *fatiga sima* double mutants are not entirely normal, as they show defects in ovary and wing development. This observation suggests that Fatiga is apparently involved in patterning these organs in a Sima-independent fashion. This conclusion is clearly of interest, since alternative target molecules for HIF prolyl hydroxylases have not been identified as yet. Forthcoming studies in the field of *Drosophila* developmental genetics might help in identifying these elusive target molecules of HIF prolyl hydroxylases.

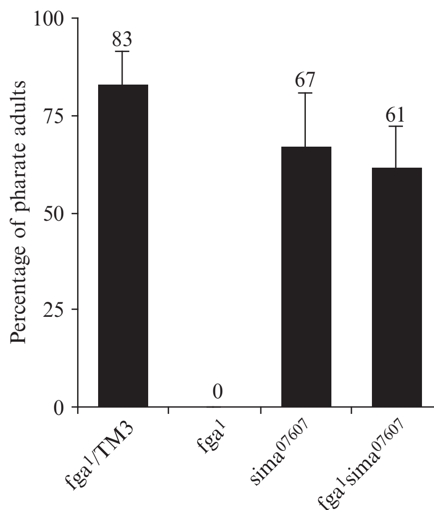

Figure 7.6 A mutation in *sima* gene reverts lethality of *fatiga* mutants. *fatiga* heterozygous individuals (*fga*^{1}/TM3) are viable to adulthood, but *fatiga* homozygotes (*fga*^{1}) are lethal; lethality is fully reverted in *fatiga sima* double mutants (*fga*^{1} *sima*^{07607}). Reproduced from Centanin *et al*. (2005).

8. Hypoxia-Inducible Genes and the Adaptation of *Drosophila* to Oxygen Starvation

Studies of oxygen-regulated genes have been carried out in *Drosophila*, and genetic screens based on behavioral responses to hypoxia have been successful in identifying loci that are relevant for adaptation to low oxygen conditions (Haddad, 1998; Haddad *et al*., 1997). *Drosophila* is extremely tolerant of oxygen starvation and can survive in hypoxia for long periods of time (Haddad, 2006; Vigne and Frelin, 2006). For instance, flies that are challenged with 0.5% O_2 for more than 6 h do not die, although they enter a state of stupor in which they do not move or respond to stimuli (Liu *et al*., 2006). A few minutes after reoxygenation, flies wake-up, recover their usual locomotor activity, flying capacity and normal behavior, and fertility is not significantly affected. Screens for genes that participate in the adaptation of *Drosophila* to such extreme hypoxic conditions have led to the isolation of loci that provoke a lengthened or shortened waking period after hypoxia-induced stupor. For instance, a mutation affecting the trehalose phosphate synthase gene was shown to increase the post-stupor recovering period; it was proposed that the disaccharide trehalose prevents protein denaturation in the central nervous system, thereby improving the outcome upon oxygen starvation (Chen *et al*., 2002).

More recently, a genome-wide expression screen was performed in *Drosophila* adults by comparing mRNA expression levels of each of the genes of the transcriptome in different conditions of oxygen deprivation (Liu *et al*., 2006). Different sets of genes could be defined according to their expression profile in different hypoxic conditions. Some of the transcripts are induced in mild hypoxia; others in stronger hypoxia; a third subgroup of transcripts is induced after an acute, but not a chronic exposure to hypoxia, and a set of transcripts is induced only if exposure to hypoxia has a certain minimal duration. The whole set of genes induced in hypoxic conditions is functionally diverse, reflecting the plethora of molecular and cellular changes that occur in an oxygen-starved organism. Interestingly, transcription factors that mediate responses to various types of (non-hypoxic) stresses are upregulated in low oxygen, suggesting that the physiological changes that occur in hypoxia activate multiple stress-responsive pathways simultaneously (Liu *et al*., 2006).

9. Regulation of Sima by the PI3K and TOR Pathways

In addition to oxygen-dependent mechanisms that control the abundance or activity of HIF proteins, non-hypoxic stimuli, such as nitric oxide, growth factors, hormones, and cytokines, also play a role in mammalian

HIF regulation (Conrad *et al.*, 1999; Feldser *et al.*, 1999; Fukuda *et al.*, 2002; Kasuno *et al.*, 2004; Richard *et al.*, 1999, 2000; Zhong *et al.*, 2000). For instance, insulin or insulin growth factors (IGFs) can increase HIF-α protein levels, triggering the induction of hypoxia-responsive genes (Kietzmann *et al.*, 2003; Zelzer *et al.*, 1998). The effect of these growth factors seems to depend mostly on the phosphoinositide-3-kinase (PI3K) signaling pathway (Roth *et al.*, 2004; Treins *et al.*, 2002; Zundel *et al.*, 2000), although the mitogen-activated protein kinase (MAPK) pathway seems to contribute to HIF activation as well. *Drosophila* insulin-like peptides (DILPs) displaying high-sequence identity with mammalian insulin are secreted by discrete groups of neurosecretory cells in the brain and target a unique *Drosophila* InR homologue that activates a conserved downstream kinase cascade that includes PI3K and the protein kinase B (PKB), also called AKT (Fernandez *et al.*, 1995; Ikeya *et al.*, 2002; Rulifson *et al.*, 2002). Like in mammalian cells, the activity of PI3K is antagonized by the phosphatase protein and tensin homolog (PTEN) (Goberdhan *et al.*, 1999; Huang *et al.*, 1999), and activation of the InR pathway brings about the activation of the TOR and phosphorylation of downstream effectors (Miron *et al.*, 2003; Oldham and Hafen, 2003; Oldham *et al.*, 2000). It has been recently demonstrated that InR and TOR pathways respond to the nutrition state of the organism, regulating larval growth (Hafen, 2004; Kim and Rulifson, 2004; Zhang *et al.*, 2000). When nutrients are abundant, InR and TOR pathways are fully active and promote growth, and, conversely, in conditions of nutrient deprivation, the activity of these pathways is reduced, leading to growth inhibition. The effect of TOR on cell growth depends at least in part on the activation of S6K, a kinase that phosphorylates the ribosomal protein S6, promoting an increase of protein translation and the inactivation of eIF4E-binding protein (4E-BP), a translation initiation inhibitor (Neufeld, 2004; Rintelen *et al.*, 2001).

Regulation of HIF by the InR and TOR pathways seems to be another conserved feature of the *Drosophila* hypoxia-responsive pathway (Dekanty *et al.*, 2005). Insulin can trigger the expression of a Sima-Tango–dependent luciferase reporter in *Drosophila* S2 cells to levels that are comparable with those observed upon exposure to extreme hypoxia. Induction of the luciferase reporter is paralleled by upregulation of Sima-endogenous target genes, such as *lactate dehydrogenase-A* (*ldh-A*), confirming the physiological relevance of this insulin-induced response. Pharmacological and RNAi-mediated silencing experiments revealed that Sima-dependent transcription upon induction with insulin depends on the PI3K and TOR pathways (Dekanty *et al.*, 2005). Genetically-induced over-activation of these pathways in living embryos also provokes the upregulation of Sima-dependent transcription, as revealed by the expression of a lacZ hypoxia-inducible reporter in transgenic embryos. Detailed studies carried out in cell culture and *in vivo* showed that an accumulation of Sima protein and an increase of

its nuclear localization account for Sima-dependent gene induction upon activation of PI3K and TOR pathways (Dekanty *et al.*, 2005).

10. Role of the HIF System in Growth Control and Cell Size Determination

From nematodes to humans, the PI3K and TOR pathways play a cardinal role in growth control and cell size determination (Oldham and Hafen, 2003). Do Sima and Fatiga participate in this regulation? It has long been appreciated that insects exposed to hypoxia grow at reduced rates and remain smaller than controls kept in normoxia (Frazier *et al.*, 2001; Peck and Maddrell, 2005), but the cellular basis of this phenomenon remains largely unresolved. We have recently observed that *fatiga* mutant pupae are strikingly smaller and their growth rate is reduced compared with that of their wild-type siblings (Centanin *et al.*, 2005) (Fig. 7.7A). Consistent with this, a recent study showed that cells in *fatiga* loss-of-function mitotic clones in the larval fat body, an organ analogous to the mammalian liver, are clearly smaller than wild-type cells of the same organ (Frei and Edgar, 2004). The same study showed that, conversely, overexpression of *fatiga* in wing imaginal discs (the primordia of adult wings) was sufficient to increase cell size. Thus, Fatiga seems to be required for normal growth and cell size determination. Given that Fatiga is a negative regulator of Sima/HIF-α, it was relevant to answer whether or not over-accumulation of Sima can account for cell size reduction in *fatiga* mutant cells. This appears indeed to be the case, since *sima fatiga* double mutant pupae have a normal size (see Fig. 7.7A), and normal growth rate is also restored in these double mutants (Centanin *et al.*, 2005). Consistent with this, experiments involving overexpression of Sima in cells of the fat body, an organ composed of endoreplicative cells, were strikingly smaller than wild-type control cells (see Fig. 7.7B), suggesting that Sima is a cell-autonomous negative regulator of growth (Centanin *et al.*, 2005).

These results pose an apparent paradox, since: (1) activation of InR and TOR pathways induce growth; (2) activation of InR and TOR pathways induce Sima-dependent transcription; and (3) Sima is a negative regulator of growth. A model accounting for these data might involve a negative feedback loop, where InR/TOR pathways promote growth but also activate Sima, which in turn downregulates InR/TOR signaling, thereby limiting growth. A recent genetic screen aimed to identify suppressors of the InR/TOR network has led to the discovery of a novel *Drosophila* gene, *Scylla*, a negative regulator of these pathways (Reiling and Hafen, 2004). *Scylla* is evolutionarily conserved—the mammalian orthologue is called REDD1 (Brugarolas *et al.*, 2004)—and induced by Sima/HIF upon exposure to

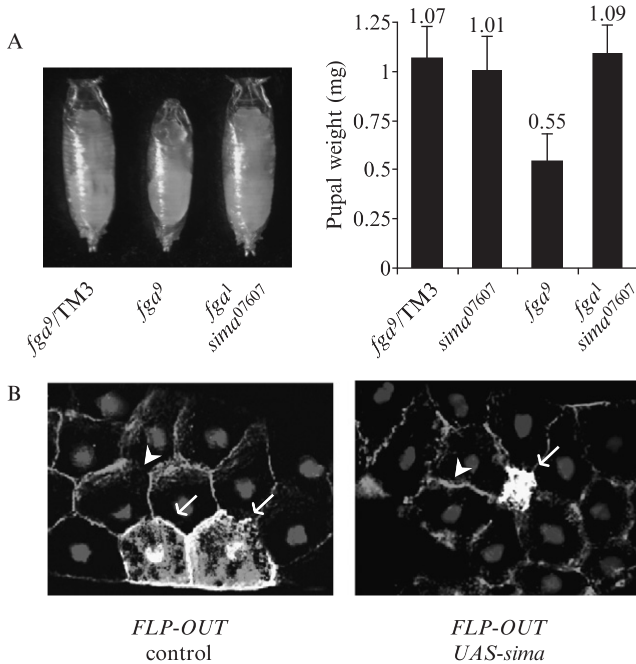

Figure 7.7 Fatiga and Sima are involved in growth control and cell size determination. (A) *fatiga* homozygous mutants (*fga*^{9}) are remarkably smaller than their heterozygous siblings (*fga*^{9}/TM3), but normal size is restored in *fatiga sima* double mutants (*fga*^{1} *sima*^{07607}). (B) Random expression of Sima in cells of the larval fat body provokes striking reduction of cell size. (Left panel) Control larvae in which green fluorescent protein (GFP) has been expressed in random cells of the fat body (arrow); these cells have the same size as the cells that do not express GFP (arrowhead). (Right panel) Sima was expressed in random cells of the fat body that also express GFP as a marker (arrow); these cells are remarkably smaller than control neighboring cells that do not express Sima protein (arrowhead). Reproduced from Centanin *et al.* (2005).

hypoxia. Thus, it seems likely that a negative feedback loop involving *Scylla*/REDD1, and perhaps other Sima/HIF-inducible negative effectors of InR/TOR signaling, accounts for size and growth-rate reduction in hypoxia (Fig. 7.8).

Yet, it cannot be ruled-out that Fatiga plays a Sima-independent role in *Drosophila* growth regulation. In a genetic screen for modifiers of CycD/Cdk4-induced overgrowth in eye imaginal discs, a mutation in the *fatiga* gene emerged as a dominant suppressor (Frei and Edgar, 2004). As this effect is not suppressed in *tango* mutant clones in the eye, it seems unlikely that the suppression of growth mediated by the *fatiga* mutant in this tissue might involve upregulation of Sima/Tango. Thus, a likely scenario is that growth

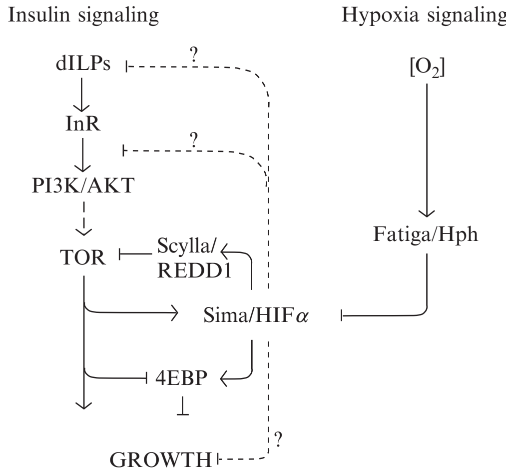

Figure 7.8 Model for insulin receptor (InR)/target-of-rapamycin (TOR) signaling through Sima and the role of Sima in growth control. *Drosophila* insulin-like peptides (DILPs) bind InR, activating the PI3K/AKT and TOR pathways, which in turn promote growth. TOR activates Sima-dependent transcription that in turn induces *Scylla*/REDD1, a negative regulator of TOR signaling, eIF4E-BP, whose induction inhibits growth and, possibly, other hypothetical genes that mediate downregulation of InR/TOR (dashed lines with question marks). Through such a mechanism, Sima would be engaged in a negative feedback loop that limits overgrowth induced by InR/TOR signaling. Modified from Dekanty *et al.* (2005).

impairment in *fatiga* mutant endoreplicative cells is due to over-accumulation of Sima, while growth impairment in *fatiga* mutant cells in the eye (non-endoreplicative) involves regulation of a putative HIF-independent pathway (Frei and Edgar, 2004).

11. Concluding Remarks

A system homologous to mammalian HIF is largely conserved in *Drosophila melanogaster*, a genetically tractable organism with advantages as an *in vivo* model system for cell and developmental biology studies. Initial analysis of the regulation of Sima/HIF-α has confirmed that all the basic features of mammalian HIF biology are largely maintained in *Drosophila*, suggesting that any progress in understanding hypoxic responses in this species can probably be extrapolated to human HIF. The availability of a wide array of mutants as well as the simplicity of the methods used for gene silencing, transgenesis, and overexpression studies provide an ideal framework for tracking HIF regulation *in vivo* and for investigating the cellular

basis of poorly understood aspects of HIF biology (e.g., oxygen-dependent control of subcellular localization). Mammalian HIF proteins are regulated by several different signaling transduction pathways, but the biochemical and molecular mechanisms involved in this regulation remain largely unclear. The genetic tools available in *Drosophila* might help to shed light on these poorly understood processes and contribute in better defining at what level and by which mechanisms the "hard wired" genetic networks controlling development interface with oxygen-sensing cellular machineries.

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