Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Insect Physiology 52 (2006) 349–364
Journal of Insect Physiology

Review

Sensing and responding to hypoxia via HIF in model invertebrates

Thomas A. Gorr[a,*], Max Gassmann[a], Pablo Wappner[b]

[a]*Institute of Veterinary Physiology, Vetsuisse Faculty and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 260, CH-8057, Zurich, Switzerland*
[b]*Instituto Leloir, CONICET, Facultad de Ciencias Exactas y Naturales, UBA, Patricias Argentinas 435, Buenos Aires 1405, Argentina*

Received 18 October 2005; received in revised form 4 January 2006; accepted 5 January 2006

Abstract

This past decade has brought considerable progress towards elucidating the molecular mechanisms of oxygen sensing pathways by which mammalian cells are able to detect and adjust, or succumb, to hypoxia. In contrast, far less is known about the protein and DNA constituents that endow many invertebrate species to withstand and recover from even more severe and prolonged O_2 limitations. In spite of these differences in hypoxia tolerance, inadequacy in oxygen supply is, from mammals to insects to nematodes, signaled onto the DNA level predominantly by hypoxia-inducible factors (HIFs). Across the animal kingdom, HIF accumulates in hypoxic, but not normoxic, cells and functions in a remarkably conserved pathway. Using crustacean (*Daphnia magna*) and insect (*Drosophila melanogaster*) models, work by us and others has implicated HIF in restoring O_2 delivery via stimulated hemoglobin synthesis (*Daphnia*) or tracheal remodeling (*Drosophila*). HIF is essential for these arthropods to adapt and survive during moderate O_2 limitations. A similar life-preserving role for HIF-signaling in hypoxic, but not anoxic, environments had previously been established for another stress-tolerant invertebrate model, the nematode *Caenorhabditis elegans*. Exploring regulations of oxygen-dependent *Daphnia* and *Drosophila* genes in cell culture and in vivo have furthermore aided in uncovering novel HIF-targeting mechanisms that might operate to fine-tune the activity of this transcription factor under steadily hypoxic, rather than changing, oxygen tensions. We conclude our review with yet another addition to the growing list of HIF's many functions: the control of cellular growth during fly development.

Keywords: *Daphnia*; *Drosophila*; Hypoxia-inducible factor; Hypometabolism; Tracheogenesis

Contents

1. HIF signaling: a brief history . . . 350
2. Sensing oxygen via HIF: mammalian lessons . . . 351
3. From oxygen to oxy-genes: a very conserved pathway . . . 351
4. Hydroxylation-independent control of HIF . . . 354
5. Invertebrate HIF: different framework, different functions? . . . 354
6. Strata of tolerance: hypoxic responses in *Daphnia* and *Drosophila* . . . 356
7. Fly HIF: a driver of tracheal remodeling? . . . 357
8. HIF and prolyl hydroxylases regulate cellular growth . . . 358
9. Conclusions and outlook . . . 359
Acknowledgements . . . 360
References . . . 360

*Corresponding author. Tel.: +41 44 635 8814; fax: +41 44 635 8932.
E-mail address: tgorr@access.unizh.ch (T.A. Gorr).

0022-1910/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2006.01.002

1. HIF signaling: a brief history

Transducing minutes to hours of a marked shortage in oxygen supply onto the level of DNA is, throughout the animal kingdom, mainly mediated by hypoxia-inducible factors, aka HIFs.[1] HIFs are a highly conserved family of basic-helix-loop-helix (bHLH)/PAS[2] transcription factors that confer a multifaceted adaptive response to hypoxia (see Bunn and Poyton, 1996; Taylor and Zhulin, 1999, for review). In mammals, teleosts, the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, HIF is a heterodimer of α- and β-subunits that specifically binds target gene sequences, so-called hypoxia-response elements (HREs), in response to low oxygen partial pressure (pO_2) (see Crews, 1998; Huang and Bunn, 2003; Shen and Powell-Coffman, 2003; Nikinmaa and Rees, 2005, for review). One of the most instructive HIF targets was, in retrospect, the peptide hormone known to promote the production of red blood cells: ERYTHROPOIETIN (EPO). Transcription of the human *epo* gene is robustly induced by hypoxia. The tripartite 3′ enhancer of the human *epo* gene contains an induction-contributing HRE that was identified as a $^{5'}$TACGTGCT$^{3'}$ octanucleotide (Blanchard et al., 1992; Semenza and Wang, 1992). This hypoxia-driven EPO synthesis was soon realized to represent an 'iceberg's tip' of a far more comprehensive and widespread oxygen sensing mechanism (Maxwell et al., 1993; Wang and Semenza, 1993). In 1995, the key signal transducer of this oxygen sensing cascade was eventually identified when Wang and Semenza, by utilizing an immobilized version of the *epo* enhancer HRE, purified the binding protein complement from human hepatoma cells as the HIF-1 protein complex (Wang and Semenza, 1995). HIF-1 was subsequently shown to consist of 120 kDa α- and 91–94 kDa β-subunits (Wang et al., 1995). In the wake of these early observations, numerous additional HRE-flanked HIF target genes (Firth et al., 1995; Wenger and Gassmann, 1997; Ebert and Bunn, 1999; Semenza, 1999) implicated this transcription factor in orchestrating essential cellular and systemic defenses to falling pO_2. In mammals, these HIF targets typically fall into two effector categories whose main functions aim to restore energy and oxygen homeostasis by:

(a) increasing anaerobic carbohydrate consumption and energy production via enhanced glycolysis;
(b) increasing tissue oxygenation through stimulated angiogenesis, vasodilation and erythropoiesis.

Approximately 2%–5% of all human genes might be transcriptionally controlled by HIF in response to hypoxia (Manalo et al., 2005). While not anywhere close to the expected list of some 500+ effector genes, to date experimental evidence for HIF-dependent expression has been provided, through HRE binding assays (i.e. electrophoretic mobility shift assay, EMSA), functional transactivation of reporter genes or loss of expression in HIF-α null cells, for a total of 70 hypoxia-regulated 'oxy-genes' (see Wenger et al., 2005, for recent compilation of HIF targets). Hence, HIF is viewed as global and ubiquitous regulator in transducing reduced oxygen availability into changes in gene activity (e.g. Guillemin and Krasnow, 1997; Semenza, 1999; Harris, 2002; Vogelstein and Kinzler, 2004; Cummins and Taylor, 2005; Wenger et al., 2005). It is, however, by no means the only pathway linking pO_2 with DNA. Depending on the degree, site and combinatorial nature of stimuli, hypoxic gene expression can alternatively be mediated, or modified, by different oxygen- and redox-sensitive transcription factors such as:

- nuclear factor-κB (NF-κB),
- tumor suppressor protein p53,
- c-Fos, c-Jun monomer subunits in activating protein-1 (AP-1),
- members of Sp-factor family (i.e. Sp-1, Sp-3),
- early growth response protein-1 (Egr-1),
- cyclic AMP-response-element-binding protein (CREB),
- metal-transcription factor-1 (MTF-1),
- nuclear factor for interleukin 6 (NF-IL6 aka C/EBPβ),

HIF-alternate hypoxia-induced transcription responses have been extensively reviewed (e.g. Acker and Acker, 2004; Murphy, 2004; Cummins and Taylor, 2005). Yet, little is known in regard to the activating mechanisms of these factors or their potential cross-talk with the HIF pathway (Cummins and Taylor, 2005). In contrast, these last few years uncovered a wealth of novel insights particularly in regard to the oxygen-mediated regulation of the HIF cascade in mammalian cells and tissues (reviewed in Lando et al., 2003; Acker and Acker, 2004; Schofield and Ratcliffe, 2004). For these reasons, this review will revisit adaptive responses mediated specifically by HIF with a fresh look across taxa. We will start our comparative survey with a description of the up-to-date basics of HIF structure, function and regulation as they have been unraveled in regard to the standard mode of oxygen sensing and hydroxylase-driven HIF control in mammalian cells (Sections 1, 2). Subsequently, we will elaborate on the role of invertebrate models in uncovering the remarkable 'worm-to-man' conservation in the O_2 sensing HIF pathway (Section 3). Crustacean and insect models, however, have also aided in discovering novel, hydroxylation-independent ways of controlling the activity of this transcription factor (Section 4). In later sections, we will apply the mammalian 'framework' of the HIF pathway onto invertebrate physiology and stress tolerance (Sections 5, 6) and, finally, onto invertebrate development (Sections 7, 8). Throughout this review, we will strive to

[1]For better distinction, gene and cDNA names will be given as italicized/lowercase, while protein names will be in uppercase.
[2]PAS: acronym for PER, ARNT, SIM, the first proteins discovered to contain this domain (see Glossary).

point to commonalities and differences in HIF signaling between vertebrate and invertebrate systems.

2. Sensing oxygen via HIF: mammalian lessons

Vertebrate and invertebrate cells alike are able to signal changes in pO_2 onto DNA through oxidative modifications of the α-subunits of HIF. Multiple isoforms of these regulatory HIF proteins exist in fish, amphibians, birds and mammals (Nikinmaa and Rees, 2005). Three HIF-α-subunits have been reported in human and rodent cells: HIF-1α of 826 amino acids, HIF-2α (870 aa) and HIF-3α (668 aa) (lengths refer to human proteins, see Fig. 1). All three factors share, aside from sequence homology within the N-terminal bHLH and PAS domains, the ability to heterodimerize with the ubiquitously expressed HIF-1β-subunit, aka aryl hydrocarbon receptor nuclear translocator or ARNT, thus producing HIF-1, -2 and -3. In hypoxic cell culture, HIF activity is induced at the protein level by the regulation of both the abundance and transcriptional activity of the 1α- and 2α-subunits. In contrast, steady-state levels of the ARNT protein, and of all transcripts, are unaffected by changes in oxygen tension (Huang et al., 1996). The mammalian HIF oxygen sensing protein that controls the stabilization, and thus, abundance of HIF-α proteins in the cell exists in three isoforms, named PHD1–3, for the characteristic prolyl hydroxylase domains contained within the reading frame. These PHDs are novel members of the 2-oxoglutarate-dependent dioxygenase superfamily with prolyl-4 hydroxylase activity (Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). PHDs catalyze the Fe(II)- and O_2-dependent hydroxylation of two prolyl residues (Pro^{402} and Pro^{564} in human HIF-1α) (Masson et al., 2001; Hirsilä et al., 2003; Acker and Acker, 2004) within the oxygen-dependent degradation domain (ODD) of HIF-1α (Huang et al., 1998) and HIF-2α (see Fig. 1). Once hydroxylated, HIF-1α/-2α rapidly bind to the von Hippel–Lindau (VHL) protein that is the recognition component of an E3 ubiquitin ligase complex, thereby tying prolyl hydroxylation to ubiquitination and proteasomal degradation of α-subunits' under high or rising pO_2 (e.g. Salceda and Caro, 1997; Maxwell et al., 1999). The efficacy of this protein-level mode of control is mirrored by the <5 min half-life of HIF-1α upon reoxygenation (Huang et al., 1996) and, conversely, the instantaneous accumulation of the transcription factor during declining oxygen tensions (Jewell et al., 2001).

A second O_2-requiring hydroxylation reaction, that of a single asparagine within the C-terminal activation domain (CAD) of HIF-1α and -2α, governs the transcriptional activation of HIF-1 and -2 (see highlighted prolyl (P) and asparaginyl (N) hydroxylation sites in human HIF-1α, (Fig. 1)). Hydroxylation of this asparagine (Asn^{803} in human HIF-1α) by another Fe(II)/2-oxoglutarate-dependent dioxygenase, an asparaginyl hydroxylase dubbed FIH-1 (factor inhibiting HIF-1), leads to steric hindrance of the interaction between α-subunits and the coactivator proteins p300/CBP, which, in turn, prohibits the transactivation of target genes under high pO_2 (Lando et al., 2002a, b). The absolute requirement of PHD- and FIH-1 activities for oxygen and Fe(II) (Lando et al., 2003; Acker and Acker, 2004; Schofield and Ratcliffe, 2004) has made these hydroxylation reactions susceptible to inhibition not only by declining pO_2 but also by hypoxia-mimicking agents such as transition metals (e.g. Co^{2+}) and iron chelators (e.g. desferrioxamine, DFO) (Goldberg et al., 1988). Upon exposure to hypoxia, cobalt- or DFO (Epstein et al., 2001), hydroxylation of prolyl and asparaginyl residues is therefore abolished. This inhibition enables the α-subunit to escape proteolytic degradation, to translocate into the nucleus and dimerize with ARNT, where the αβ heterodimer associates with the transcriptional coactivators p300/CBP to promote target gene transcription via HRE binding sites. The discovery of the hydroxylase-ubiquitin-proteasome (named HUP hereafter) cascade clearly was of great significance in unraveling the regulation between active (hypoxia) and inactive (normoxia) states of HIF signaling.

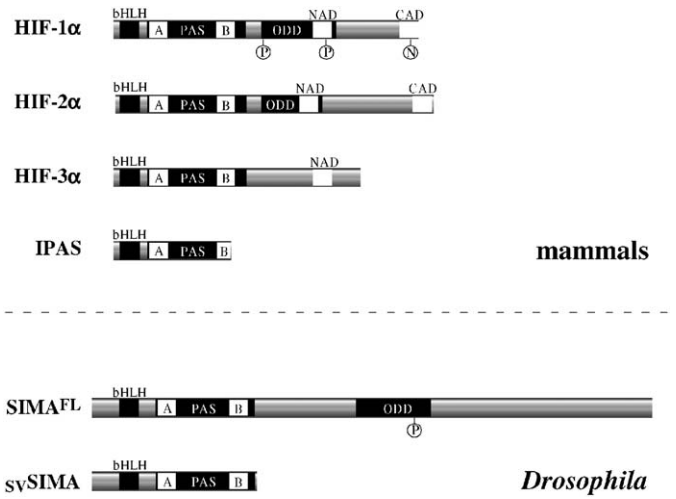

Fig. 1. *Structure and function of HIF-α proteins.* Top: mammalian HIF-1α, -2α, -3α proteins, and -3α splice variant (IPAS); bottom: *Drosophila* HIF-α homolog SIMA, as full-length ($SIMA^{FL}$) and splice variant (svSIMA) isoform. HIF-α functional domains (with function given in parenthesis): bHLH = basic region (HRE binding) with helix-loop-helix domain (HIF-α:HIF-β interaction); PAS = PER/ARNT/SIM domain (HIF-α:HIF-β interaction) with repeats A and B; ODD = oxygen-dependent degradation domain (HIF-α stability); NAD/CAD = N-terminal/C-terminal activation domain (HIF-α stability and transcriptional competence) with prolyl (P)/asparaginyl (N)-hydroxylation targets being highlighted (see text for details).

3. From oxygen to oxy-genes: a very conserved pathway

The HIF signaling pathway, and its HUP-based regulation, shows striking conservation throughout the animal kingdom, from nematodes to fish to humans (Bruick and McKnight, 2001; Epstein et al., 2001; Nikinmaa et al., 2004). In fact, the discovery that mammalian PHD1–3 prolyl hydroxylases served as HIF oxygen sensors emerged from a study in *C. elegans*. In 2001, Peter Ratcliffe and

associates (Epstein et al., 2001) identified EGL-9 as a 2-oxoglutarate-dependent dioxygenase that down-regulated *C. elegans* HIF-α protein levels via VHL-dependent ubiquitination and subsequent proteasomal degradation in an oxygen-dependent manner (e.g. Salceda and Caro, 1997; Maxwell et al., 1999). Rat and human EGL-9 homologs (i.e. PHD1–3) were subsequently identified to operate as HIF-α degrading oxygen sensors in mammalian systems (Epstein et al., 2001). At the same time, Bruick and McKnight (2001) identified all three human PHDs (termed HPHs or HIF prolyl hydroxylases) and provided in vitro and cell culture evidence of their activity in targeting human HIF-α proteins. Interestingly, this group extended their studies to a *Drosophila* system, by showing in a KC167 fly cell line that silencing of the fly PHD homolog in normoxia, provoked up-regulation of HIF-controlled, hypoxia-inducible transcripts (Bruick and McKnight, 2001).

The existence of homologous oxygen sensing cascades in various animal phyla includes, of course, HIFs per se. Among invertebrates, homologous α and *β* HIF subunits have been reported in *C. elegans* (Powell-Coffman et al., 1998; Jiang et al., 2001), *Drosophila* (Nambu et al., 1996; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997; Ma and Haddad, 1999) and the branchiopod crustacean *Daphnia magna*.[3] Not surprisingly, all examined *hif* transcripts were found to be, unaffected by changes in pO_2, ubiquitously expressed throughout embryogenesis and in nearly all tissues (Nambu et al., 1996; Sonnenfeld et al., 1997; Jiang et al., 2001). In contrast, functional HIF protein is confined to within the lower hypoxic pO_2 range through the action of HUP and possibly non-HUP control modes (see "*Hydroxylation-independent control of HIF*"). In nematodes (*C. elegans*), waterfleas (*Daphnia*) *and* fruitflies (*Drosophila*), HIF activity peaks, as it does in mammals (Jiang et al., 1996), in hypoxic tissue, while it sharply declines towards both normoxic and anoxic pO_2 (e.g. *Drosophila* HIF: maximal HRE-binding activity at ~3%–4% O_2 in fly embryos (Lavista-Llanos et al., 2002) and at ~1% O_2 in cell culture, (Gorr et al., 2004b). Along similar lines, the *hif-1α* gene product of *C. elegans* is essential to ensure growth and survival in hypoxic conditions (0.5%–1% O_2), whereas it is fully dispensable for the reversibly arrested growth and development (i.e. suspended animation) that is induced by a complete lack of oxygen (Jiang et al., 2001; Padilla et al., 2002; Shen and Powell-Coffman, 2003; Shen et al., 2005). From 'worm' to man, *anoxic* defenses, along with some, albeit less well characterized, hypoxia signaling pathways (see above), are known to occur in HIF-independent fashion (e.g. An et al., 1998; Wenger et al., 1998; Padilla et al., 2002; Shen and Powell-Coffman, 2003; Ameri et al., 2004).

Our own studies on oxygen sensing in fly SL2 cells (Schneider, 1972) revealed that the activity of *Drosophila* HIF (dHIF, Fig. 2, see "EMSA") and the abundance of the HIF-α-subunit homolog SIMA (Fig. 2, "Western blot") are both inversely correlated to oxygen levels. These findings underscored for dipteran cells the importance of HIF-α-subunit accumulation in determining HIF activity, thereby stressing once again the conservation of fly and human O_2 sensing machineries. Upon closer inspection, however, one realizes that the properties of insect and mammalian systems are similar, yet not identical (Gorr et al., 2004b). In cultured mammalian cells, iron chelation (e.g. by DFO) or exposure to transition metals (e.g. Co^{2+}) are able to mimic hypoxia by inhibiting both prolyl and asparaginyl hydroxylations of α-subunits (Epstein et al., 2001; see Maxwell and Salnikow, 2004, for discussion), which induces HIF activity to a similar degree in response to all three experimental conditions (Goldberg et al., 1988). dHIF, on the other hand, when assessed through target gene regulation or in vitro HRE-oligonucleotide binding studies, is predominantly activated by hypoxia, weakly so by DFO treatment, and refractory to Co^{2+} ions (Fig. 2, "EMSA") (Nagao et al., 1996; Gorr et al., 2004b; Dekanty et al., 2005). The potency of various stimuli in inducing *Drosophila* HIF activity, therefore, scales in the following relative order: $hypoxia\ (H) > DFO(D) \gg normoxia(N) \simeq cobalt(C)$. Interestingly, this scaling of stimuli appears to be directly linked to the relative abundance of SIMA protein (see SIMA-immunodetection in Fig. 2, "Western blot"), where hypoxia and DFO, but not cobalt, yielded measurable SIMA accumulation (Gorr et al., 2004b).

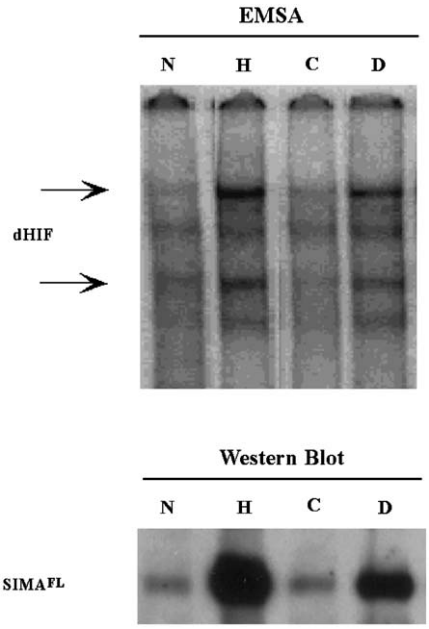

Fig. 2. *Fly HIF activity and HIF-α abundance. Drosophila* cell HIF (dHIF) activity (measured by electrophoretic mobility shift assay (EMSA)), and abundance of $SIMA^{FL}$ protein (Western Blot) as function of hypoxic (H: 1% O_2, 16 h), $CoCl_2$ (C: 100 μM, 16 h) or desferrioxamine (D: 100 μM, 16 h) exposure, relative to normoxic (N: 21% O_2, 16 h) controls (see text for details).

[3]Cloning of *Daphnia magna* HIF-α and -*β* cDNAs is currently underway; H. Yamagata, pers. communication; Tokyo University.

Further support for a tight conservation of the O_2/HUP-mediated control of HIF signaling was provided by the occurrence of homologous amino acid motifs around the proline^{850} candidate hydroxylation site within SIMAs ODD (highlighted in Fig. 1) (Ivan et al., 2001), thereby confirming earlier studies on the narrowed oxy-regulatory peptide regions within the fly HIF-α factor (Bacon et al., 1998; Srinivas et al., 1999). Eventually, proline^{850} was substantiated to confer at least part of SIMAs normoxic instability through the documented interaction of *Drosophila*'s PHD and VHL homologs with the hydoxylproline in this position (Arquier et al., 2006). Evidence for a physiological role of *Drosophila*'s PHD homolog was provided in 2002, when we demonstrated that this oxygen sensor also controls abundance of SIMA in living embryos. RNAi-mediated silencing or genetic inactivation of the *phd* gene caused accumulation of SIMA in normoxia, which, in turn, led to the constitutive induction of a hypoxia-responsive transgenic reporter gene (Lavista-Llanos et al., 2002). In addition, *Drosophila*'s *phd* gene is robustly induced by hypoxia in a HIF-dependent manner (see Lavista-Llanos et al., 2002 and Gorr et al., unpublished data), similar to equivalent inhibitor inductions that operate on mammalian (*phd*2 and *phd*3; but not *phd*1) or nematode (*egl*-9) HIF prolyl hydroxylase genes (Berra et al., 2003; del Peso et al., 2003; Marxsen et al., 2004; Shen et al., 2005). In all cases, this hypoxia-induced negative feedback loop apparently evolved to limit HIF-α accumulation even under moderate O_2 deprivation. The *Drosophila* PHD homolog, that we named "FATIGA" after its lack-of-oxygen phenotype, was later shown to be required for developmental progression, regardless of oxygen tension, and was also implicated in the cell autonomous regulation of growth (Frei, 2004; Frei and Edgar, 2004; Centanin et al., 2005); (see below: "*HIF and prolyl hydroxylases regulate cellular growth*").

However, cross-taxa conservation of the HIF pathway involves, with HIF-1α/SIMA not only α-subunits. *Drosophila's* protein TANGO mirrors its common ancestry with the mammalian ARNT factor, by being able to form tissue- and stage-specific heterodimers with multiple (at least five) partner proteins. Contrary to this functional promiscuity, only the SIMA:TANGO (i.e. dHIF) heterodimer was found to be absolutely required to regulate the transcription of HRE-reporter constructs in hypoxic flies (Lavista-Llanos et al., 2002) (see Fig. 3, "hypoxic nucleus"). Other α-like bHLH/PAS partners of TANGO (see Crews, 1998, for review), necessary for the tissue-specific nuclear translocation of TANGO under normoxic pO_2 (Ward et al., 1998), were unable to elicit this hypoxic response (Lavista-Llanos et al., 2002) (see Fig. 3, "normoxic nucleus"). These TANGO partners include the neurogenic factor SINGLE-MINDED (SIM) (Nambu et al., 1991); TRACHEALESS (TRH), the master regulator of tracheal development (Isaac and Andrew, 1996; Wilk et al., 1996); SPINELESS (SS), that is involved in antenna, leg and bristle development, and DYSFUSION (DYS), another bHLH/PAS factor with roles in tracheal development (Emmons et al., 1999; Jiang and Crews, 2003). However, this growing list of TANGO heterodimers with close, or

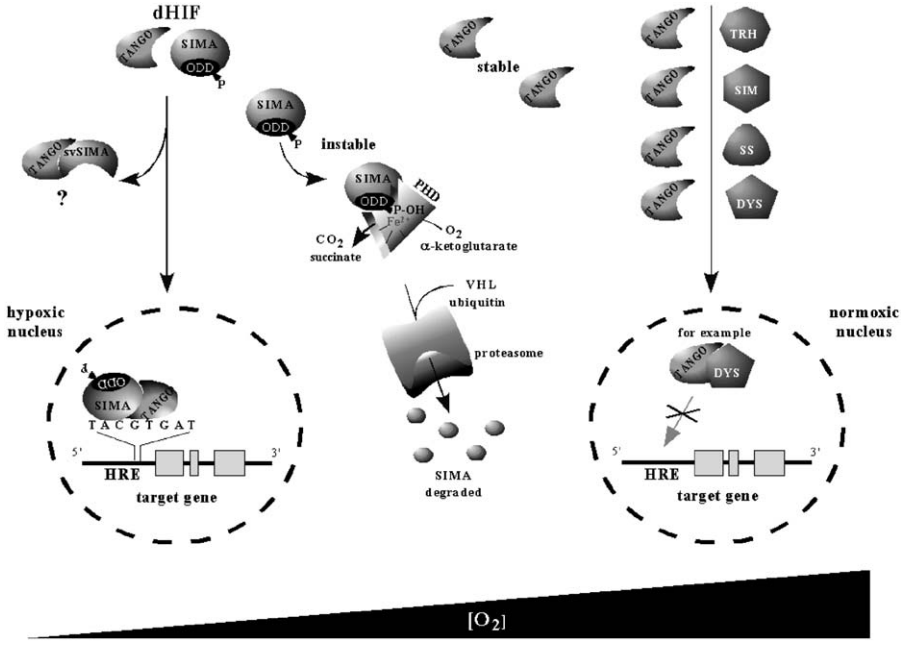

Fig. 3. *Fly HIF signaling*. Model of *Drosophila* HIF (dHIF) signaling in hypoxia (see [O_2] gradient, "hypoxic nucleus") with SIMA (SIMA^{FL}, HIF-α), TANGO (ARNT), svSIMA and HRE ($^{5'}$TACGTGAT$^{3'}$) constituents. Indicated are: SIMA inhibition under reoxygenation and normoxic-moderately hypoxic oxygen tensions, in response to proline hydroxylation (P-OH) by PHD/FATIGA and VHL-assisted ubiquitination plus proteolytic (proteasome) degradation. Also shown: alternate TANGO pathways under high oxygen (see [O_2] gradient, "normoxic nucleus") with various, tissue- or stage-specific dimerization partners: TRH, SIM, SS and DYS (see text for details).

even overlapping, DNA specificities also begs the question as to how the signaling pathways mediated by each α-like bHLH/PAS protein are maintained as distinct cascades when several co-expressed TANGO partners might compete for TANGO or binding sites (Zelzer et al., 1997).

4. Hydroxylation-independent control of HIF

General similarities in oxygen sensing cascades across taxa seem to extent even beyond the HUP-system onto additional HIF checkpoints. The requirement for further controls, which might operate even under steady and severely hypoxic pO_2, can best be inferred from the significance of *physiological* levels of HIF signaling for controlled cell growth and function. In many human malignancies, for example, a HIF-hyperactive (i.e. HIF-1α/-2α over-expressing) pathway is associated with tumorigenesis, increasing metastatic potential, resistance to radio- and chemo-therapy and an overall worsening of clinical outcomes (see Harris, 2002; Semenza, 2002, 2003; Höpfl et al., 2004; Vaupel, 2004, for recent reviews). Yet, several studies on cancer cells noted a *transient* induction of HIF-1α protein levels under hypoxic or even anoxic conditions with peak abundance after 4–8 h of exposure, followed by progressively declining levels during prolonged treatment (Wiesener et al., 1998; Graven et al., 2003; Ameri et al., 2004). This decline of HIF-1α levels by prolonged anoxia is noteworthy, since it occurs under conditions known to abolish (Epstein et al., 2001; D'Angelo et al., 2003; Stolze et al., 2004; Arquier et al., 2006) the O_2-dependent activity of PHD/FIH-1 enzymes (Hirsilä et al., 2003; Koivunen et al., 2004) and suggests the action of additional, and hydroxylation-independent HIF-targeting mechanisms.

A point in case was recently made with the discovery of hypoxia-induced variant HIF-α peptides. In both mammals and *Drosophila*, it was reported that HIF signaling can be blocked through the expression of truncated HIF-α forms that exert a dominant negative effect over the full-length HIF-α-subunit. In mammals, an endogenous HIF antagonist was found in 2001 that consists of the inhibitory PAS domain protein, aka IPAS (Makino et al., 2001). IPAS, a hypoxia-induced splice variant of the mouse *hif-3α* gene, encodes a 307 amino acid (aa) HIF-α truncated product. This HIF-α variant lacks the ODD (i.e. ΔODD) and C-terminal transactivation regions and only retains the N-terminal bHLH and PAS domains (Makino et al., 2001, 2002). Intense IPAS expression was predominantly seen within the epithelial layer of the cornea. Interestingly, IPAS formed abortive complexes with HIF-1α and was able to suppress expression of pro-angiogenic cytokines (e.g. *vascular endothelial growth factor* (*vegf*)). Thus, IPAS has been implicated in the maintenance of the avascular phenotype of the corneal epithelium by exerting a dominant negative effect over full-length HIF (Makino et al., 2001, 2002).

Similar to the link between *hif-3α* and IPAS, we have recently described the agonist/antagonist relationship among expressed products of the *Drosophila sima* gene (Gorr et al., 2004b). In cultured SL2 fly cells, the *sima* gene is expressed into at least two mRNAs: one constitutive transcript, encoding the 1510 aa full-length peptide (see Fig. 1: $SIMA^{FL}$), and one hypoxia-induced shorter isoform. This shorter transcript is a *sima* splice variant which lacks four exons close to the C-terminus and, in consequence, encodes a 426 aa ΔODD 'mini-HIF' that was termed svSIMA (see Fig. 1). SIMA full length ($SIMA^{FL}$) and svSIMA proteins are identical in sequence throughout the entire N-terminal portion (419 amino acids), including all of the bHLH and PAS domains. In hypoxia, the transcriptionally competent $SIMA^{FL}$ accumulates in the nucleus (see Fig. 3), where it either induces or suppresses the expression of target genes (Gorr et al., 2004b). In contrast, over-expressed svSIMA is constitutively cytosolic and fails to stimulate HRE-dependent transcription, yet is capable to inhibit $SIMA^{FL}$:TANGO-mediated reporter transactivation in a dose-dependent manner (Gorr et al., 2004b). We speculate, therefore, that the biological role of this ODD-deleted HIF-α truncation might serve to down-regulate HIF activity in *hypoxic* fly cells by creating a cytosolic 'sink' for TANGO (see Fig. 3). Elucidating the in vivo role of svSIMA will be of particular interest to understand its physiological function. These findings suggest that, like murine *hif-3α*, the *Drosophila sima* gene can generate, specifically under low pO_2, $SIMA^{FL}$ and svSIMA products to fine-tune hypoxic signaling. Cross-taxa explorations of hypoxia-sensitive splicing mechanisms of *hif-α* transcripts will surely be launched by these studies. These findings make also clear that hydroxylation-independent mechanisms are at work, even under conditions of extreme O_2 deprivation, to maintain HIF signaling within physiological limits, perhaps as a necessary requirement for the prevention of derailments in cellular growth and function that are usually associated with hyperactivity of this pathway. Enhancing our knowledge of these additional, PHD/FIH-independent HIF checkpoints is potentially of considerable biomedical relevance for the development of future HIF targeting therapies.

5. Invertebrate HIF: different framework, different functions?

As outlined above, HIF-mediated transcriptional regulation of oxy-genes and -genomes operates in the hypoxia-tolerant toxicological and genetic models *Daphnia*, *Drosophila* and *C. elegans*. Further, cloned or predicted, HIF-α cDNAs and expressed sequence tags have been reported from the honeybee *Apis mellifera* (Genbank accession XM 392382), the goldenrod gall fly *Eurosta solidaginis* (Genbank accession AY 845427; Morin et al., 2005), the silk moth *Lonomia obliqua* (Genbank accession CX 817129) and the grass shrimp *Palaemonetes pugio* (Genbank

accession AY 655698). Based on this preliminary sampling, one can tentatively deduce that the HIF pathway is common amongst arthropod (e.g. *Daphnia*, *Palaemonetes*, *Drosophila*, *Eurosta*, *Apis*, *Lonomia*) and bilaterian (e.g. *C. elegans*) invertebrates. Of course, future work needs to resolve more finely the representation of HIF signaling as functional environment/DNA interface by looking at other major metazoan groups (e.g. arachnids, myriapods, annelids, mollusks, deuterostomes, etc.).

While hypoxia tolerance (see Hochachka, 1986a,b, for concept) is widespread amongst invertebrates (Wegener, 1988, 1993), including some crustaceans and especially insects (Hoback and Stanley, 2001; Schmitz and Harrison, 2004), such ability to survive and recover from hours to days or weeks of exposure to little or no oxygen is rarely found in endothermic vertebrates, i.e. birds and mammals (with possible exceptions amongst neonatal developmental or hibernating physiological stages; see Singer, 1999; Drew et al., 2004; Heldmaier et al., 2004; Mortola, 2004, for review). For the most part, adult endotherms are oxyregulators and succumb to even slight degrees of oxygen deprivation lasting only minutes (e.g. Duffy et al., 1972). Notably in stress-sensitive mammalian organs (e.g. brain, kidney, heart; Hansen, 1985; Jaeschke et al., 1988; Buck et al., 1993; Erecinska and Silver, 2001; Rosenberger et al., 2005), HIF has acquired a critical role in promoting cell survival during hypoxic challenges. Such adaptation utilizes two main homeostatic strategies: (a) a systemic strategy in mild hypoxia (Ebbesen et al., 2004) based on enhancing oxygen delivery to the hypoxic tissue via induced angiogenesis, vasodilation, and erythropoiesis (restoring O_2 homeostasis); and (b) a cell autonomous strategy during more severe O_2 deprivation (Ebbesen et al., 2004) based on the enhanced uptake and breakdown of glucose through anaerobic glycolysis (restoring of energy homeostasis). The inverse relationship between glycolytic flux and oxygen availability is known as Pasteur effect (Storey, 1985; Schmidt and Kamp, 1996) and marks the switch from aerobic (mitochondrial) to anaerobic (fermentative) metabolism and energy production. In primary and transformed mammalian cells exposed to 0%–2% O_2, this switch is regulated by HIF (Seagroves et al., 2001) and manifests itself in the enhanced transcription of genes encoding specific glucose transporters and most glycolytic enzymes (Webster, 1987, 2003; Iyer et al., 1998). However, prominent stimulation of fermentative carbohydrate consumption by low pO_2 is a typical short-term attempt of hypoxia-sensitive and oxyregulating tissues to compensate for continuing ATP utilization demands (Suarez et al., 1989; Buck et al., 1993). Anaerobic glycolysis soon amasses toxic levels of end products (e.g. lactic acid), while it still fails to meet the ATP demands for ionic and osmotic equilibrium, thus producing an ultimately fatal ATP imbalance in the cell (Hochachka et al., 1996; Boutilier and St-Pierre, 2000; Hochachka and Somero, 2002) (see Fig. 4, hatched line).

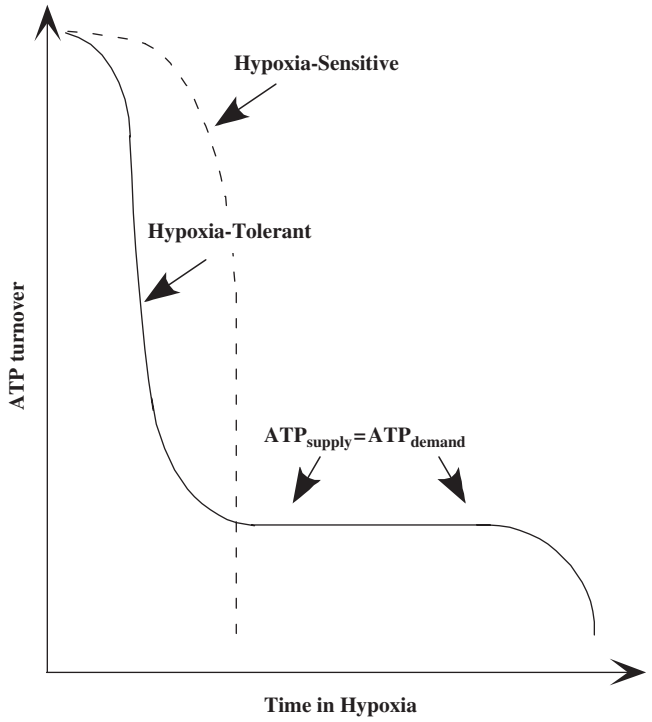

Fig. 4. *ATP turnover*. Simplified comparison of ATP-turnover schemes between hypoxia-sensitive ("HS", e.g. endothermic vertebrates) and hypoxia-tolerant ("HT", e.g. facultative anaerobe invertebrates) organisms during hypoxic challenges (adopted from Hochachka et al., 1996; Boutilier and St-Pierre, 2000; Hochachka and Somero, 2002; reprinted here with permission from Elsevier). In HS animals/tissues, loss of oxidative ATP production during O_2 limitation meets continually high ATP demands. The resulting energetic deficit ultimately manifests in a lethal drop of [ATP] (hatched line), despite various compensatory defenses orchestrated by HIF. In HT animals/tissues, a drastic, yet controlled, metabolic depression yields a new steady-state between ATP_{supply} and ATP_{demand} processes (continuous line) as premier defense against hypoxia (see text for details).

In hypoxia-tolerant animals or stages, markedly different survival strategies are employed. These are primarily based upon energy *conservation* rather than on energy *compensation*. Here, the ability to reversibly enter a state of a regular metabolic depression, characterized by a drastically reduced, yet balanced, steady-state between ATP supply and ATP consumption, prevents lethal falls in cellular ATP levels. This is the single most protective and unifying feature of hypoxia-tolerant organisms (Hochachka et al., 1996; Boutilier and St-Pierre, 2000; Hochachka and Somero, 2002) (see Fig. 4, continuous line). The extent of this metabolic depression (aka hypometabolism) is inversely related to the period of hypoxia tolerance (see Guppy and Withers, 1999, for review). Since ~80% of mitochondrial O_2-consumption is coupled to ATP synthesis (Rolfe and Brown, 1997), metabolic depression, monitored as a drastically reduced oxygen consumption rate, occurs immediately in these animals when exposed to hypoxia. At the molecular level, sustained hypometabolism involves the coordinated suppression of every major ATP-utilizing function in the cell (i.e. protein synthesis (~30%

of normoxic ATP turnover rate); protein degradation (~20%); ion-motive ATPases, notably Na^+-K^+-ATPase (~25%)) to match the concomitant decline in ATP production (Land et al., 1993; Land and Hochachka, 1994; Hochachka et al., 1996; Rolfe and Brown, 1997; Hochachka and Somero, 2002). During hypometabolic states, glycolytic flux only needs to be elevated and provide for ATP as much as the residual energy expenditures require. It should come as no surprise then, that particularly hypoxia-tolerant aquatic or intertidal invertebrates (e.g. *Sipunculus* peanut worms, *Mytilus* mussels, *Cardium* cockles, *Chironomus* midge larvae) display only weak or absent Pasteur effects (see Storey, 1985; Grieshaber et al., 1994; Schmidt and Kamp, 1996; Scholz and Zerbst-Boroffka, 1998, and papers cited therein). Indeed, bivalve muscles and insect brains (Wegener, 1993; Grieshaber et al., 1994) develop even a glycolytic rate inhibition during episodes of oxygen deprivation (i.e. reverse Pasteur effect), despite the occasional up-regulation of genes encoding glycolytic enzymes in hypoxic flies or fly cells (Gorr et al., 2004b; Dekanty et al., 2005).

6. Strata of tolerance: hypoxic responses in *Daphnia* and *Drosophila*

Daphnia and *Drosophila* exhibit a remarkable resilience to oxygen deprivation, as the adult stages are able to remain fully viable throughout a 4 h (*Drosophila*, Krishnan et al., 1997) or 24 h (*Daphnia*, Paul et al., 1998) anoxic challenge. As expected, late-stage embryos and larvae of *Drosophila* withstand even day-long exposures of lack of oxygen (Wingrove and O'Farrell, 1999). Despite this tolerance, adult daphnids (Kobayashi and Hoshi, 1982; Paul et al., 1997) and drosophilids (Csik, 1939; Chadwick and Gilmour, 1940) display oxyregulation in O_2-uptake or behavior as long as ambient pO_2 levels are non-limiting to sustain oxidative metabolism. Commonplace occurrence of oxygen independent respiratory rates amongst crustaceans (Childress, 1975; McMahon, 2001) or insects (Keister and Buck, 1974; Loudon, 1988; Hoback and Stanley, 2001) is, thus, faithfully reflected by waterflea and fruitfly models. However, once oxygen concentrations have sufficiently dropped to levels near or beyond the critical pO_2 (pC) of the species (~1.6%–3% O_2 for adult *Drosophila melanogaster*, Csik, 1939; Chadwick and Gilmour, 1940), both models express a multifaceted repertoire of defenses in correspondence to the severity of the stress signal. These defenses, listed below, include both systemic and cellular responses that range from epigenetic to behavioral changes ("fly" = *Drosophila melanogaster*):

- sensing and avoidance of hypoxia via a nitric oxide/cyclicGMP signaling pathway in fly larvae (Wingrove and O'Farrell, 1999);
- stupor, ranging from loss of coordination to complete immobility—used to define pC in adult drosophilids (Csik, 1939; Chadwick and Gilmour, 1940);
- stimulated synthesis of extracellular hemoglobin in daphnids (Pirow et al., 2001; Gorr et al., 2004a);
- increased heart rate and hemolymph perfusion rates in daphnids (Paul et al., 1998);
- remodeling of the tracheal respiratory system during development in fly larvae (Jarecki et al., 1999);
- induced expression of hypoxia/HIF-responsive genes in fly cells and embryos (Bruick and McKnight, 2001; Lavista-Llanos et al., 2002; Gorr et al., 2003, 2004b; and references cited therein);
- glycogen-fueled anaerobiosis with lactate as arthropod-typical endproduct in daphnids and flies (Wegener, 1993; Grieshaber et al., 1994);
- reduced O_2-consumption rates to ~20%–30% of normoxic controls in adult flies (Krishnan et al., 1997; Ma et al., 1999);
- cell cycle arrest in fly embryos (Foe and Alberts, 1985; Wingrove and O'Farrell, 1999; DiGregorio et al., 2001; Douglas et al., 2001);
- general and reversible chromatin condensation in fly embryos (Foe and Alberts, 1985).

In contrast to the recent transcriptome analyses of HIF-dependent and -independent hypoxic responses in *C. elegans* (Bishop et al., 2004; Shen et al., 2005), the impact of HIF on most of the above defenses in *Daphnia* or *Drosophila* has not been studied. A notable exception is a short list of HIF/SIMA-dependent targets in flies (see above). In addition, HIF was recently implicated in the hypoxic induction of *Daphnia magna globin* genes. From a systemic point of view, the transcriptional induction of hemoglobin synthesis in daphnids mirrors mammalian vascular and erythropoietic responses, since it has been shown to serve as 'first line of defense' of multiple vitality parameters by extending aerobic metabolism deeper into hypoxic pO_2 ranges (Pirow et al., 2001). Owing to the extracellular nature of this hemoglobin, the induction was, however, expected to operate on the level of the *globin* genes per se. Indeed, as we were able to show, elevated transcription of *hb*2, one of the four existing *Daphnia magna globin* genes, in response to low oxygen was entirely dependent upon three motifs within the promoter of this gene when monitored in heterologous transfection assays (Gorr et al., 2004a). While two of these cis-elements were shown to be standard HIF binding sites (i.e. HREs), the third motif, a $^{5'}$CACGTG$^{3'}$ E-box palindrome, acted as a docking sequence for a non-HIF, constitutive complex. Interaction between the CACGTG-sequence and this constitutive factor was found to restrain binding of HIF to nearby HREs. Thus, at least one *Daphnia magna* globin gene promoter appears to attract both inducing and inhibitory complexes via HREs and E-box palindromes, respectively (Gorr et al., 2004a). It remains to be determined, if the interplay between these complexes evolved to allow for a graded, rather than the stereotypical ON/OFF, activation of gene expression in response to declining pO_2.

Collectively, HIFs key role in hypoxia-sensitive organisms during standard homeostatic responses of improved oxygen supply (i.e. activated angiogenesis, erythropoiesis) and metabolic switches (i.e. activated anaerobic glycolysis) is beyond doubt. In contrast, our understanding of HIFs contribution to stress-resilience within tolerant systems is still very sketchy. We do not know, if, and to what extent, HIF controls, for example, the down-regulation of ATP-costly processes such as cell proliferation, apoptosis or macromolecular turnover in hypometabolic states of hypoxia-tolerant/invertebrate cells.

7. Fly HIF: a driver of tracheal remodeling?

Mammalian and teleost HIF proteins have been implicated in many developmental processes, including the formation of the vascular system, and development of the heart, brain, cartilages and placenta (Iyer et al., 1998; Adelman et al., 2000; Tomita et al., 2003; Covello and Simon, 2004; Pfander et al., 2004; Vuori et al., 2004). The underlying mechanisms are not well understood and whether local oxygen availability is relevant for these processes is not entirely clear. Nevertheless, the role of hypoxia in angiogenesis (i.e. formation of vascular capillaries from pre-existing blood vessels) has long been appreciated to involve the activity of oxygen-dependent angiogenic cytokines (Pugh and Ratcliffe, 2003). Steady-state levels of VEGF, a potent inducer of angiogenesis, are increased up to 10–15 fold in hypoxic cells (Shweiki et al., 1992, 1995; Levy et al., 1995). Part of this induction is due to increased mRNA stability and part due to transcriptional induction; the latter clearly depending on HIF activity. Therefore, in hypoxic tissues, inhibition of the HUP-cascade discussed above yields HIF activation, and consequently, the up-regulation of angiogenic growth factors like VEGF. VEGF over-production, in turn, attracts the outgrowth of blood capillaries towards hypoxic areas and improves oxygenation of the tissue.

Owing to the widespread absence of respiratory hemolymph pigments amongst insects, it is widely held for the majority of these animals that their circulatory system does not play a role in transporting oxygen to organs and tissues. Instead, oxygen is thought to be directly delivered to every cell in the organism through a complex, ramified network of epithelial tubes termed the tracheal system (Ghabrial et al., 2003). In *Drosophila*, development of the tracheal system begins at mid-embryogenesis when epithelial primordial, under the control of TRACHEALESS (see above), differentiate into a tracheal cell fate (Isaac and Andrew, 1996; Wilk et al., 1996). Thereafter, the whole tubular network is generated by a process relying exclusively on guided cell migration since no cell divisions occur throughout the entire tracheal development (Samakovlis et al., 1996). In *Drosophila*, cell migration is guided by the fibroblast growth factor (FGF) homolog BRANCHLESS (BNL), which is dynamically expressed in clusters of epidermal cells surrounding the tracheal primordia (Sutherland et al., 1996). At the molecular level, it has been shown that BNL binds to the FGF receptor homolog BREATHLESS (BTL), which is expressed in the plasma membrane of migrating tracheal cells (Sutherland et al., 1996; Ribeiro et al., 2003). Consistent with a guidance function of the FGF homolog, ectopic expression of BNL in an unusual ectodermal location causes tracheal cell migration and branch outgrowth towards the new location of expression (Sutherland et al., 1996). Thus, the shape of *Drosophila*'s tracheal tree is set up by the BNL expression pattern in target tissues (outside the tracheae) and the activation of its receptor BTL on the membrane of tracheal cells. By the end of embryogenesis, this hardwired migratory process has been completed. In larval stages, terminal tracheal branches are plastic and have the capacity to sprout-out projections towards oxygen-starved areas in the surrounding tissues (Jarecki et al., 1999), in a process which appears to resemble capillary outgrowth during mammalian angiogenesis (Metzger and Krasnow, 1999; Wappner and Ratcliffe, 2001).

The ability of insect tracheal cells to undergo remodeling in response to oxygen needs throughout the organism has been discovered more than five decades ago (Wigglesworth, 1954; Locke, 1958). In a classical experiment performed by V.B. Wigglesworth on the blood sucking bug *Rodnius prolixus*, a major abdominal tracheal branch was surgically severed, giving rise to an extensive hypoxic area in one of the abdominal segments (Wigglesworth, 1954, 1983). A few days later, a dramatic compensatory response was observed, with branches from neighboring segments extending terminal projections into the hypoxic zone (Wigglesworth, 1954; Locke, 1958). Much more recently, by taking advantage of the genetic tools available in *Drosophila*, it was shown that the embryonic guidance cue BNL is also responsible for driving the extension of oxygen-dependent tracheal terminal branches during larval stages, and consistent with this observation, BNL protein accumulates in hypoxia (Jarecki et al., 1999). These lines of evidence suggest for *Drosophila* larvae that tracheal plasticity is induced via BNL by the SIMA:TANGO dimer in hypoxic tissues (outside the tracheae), thus promoting tracheal terminal sprouting (see Fig. 5B). Although, this model is attractive and agrees with well-established mechanisms of VEGF-regulated mammalian angiogenesis, further experiments are required to confirm whether the *branchless* gene is indeed activated by HIF in response to hypoxia outside of tracheal cells (Fig. 5B). In fact there is evidence suggesting a somewhat more complex picture. Contrary to the prediction, expression of SIMA-dependent reporters in *Drosophila* larvae is induced by mild hypoxic exposure in tracheal cells, rather than in the surrounding tissues (Lavista-Llanos et al., 2002) (Fig. 5A). Non-tracheal expression of the transgenic reporters was weak and only noted when the larvae were exposed to a more severe oxygen deprivation. At the same time, the pattern of accumulation of endogenous SIMA protein paralleled perfectly the expression of these hypoxia-inducible reporters

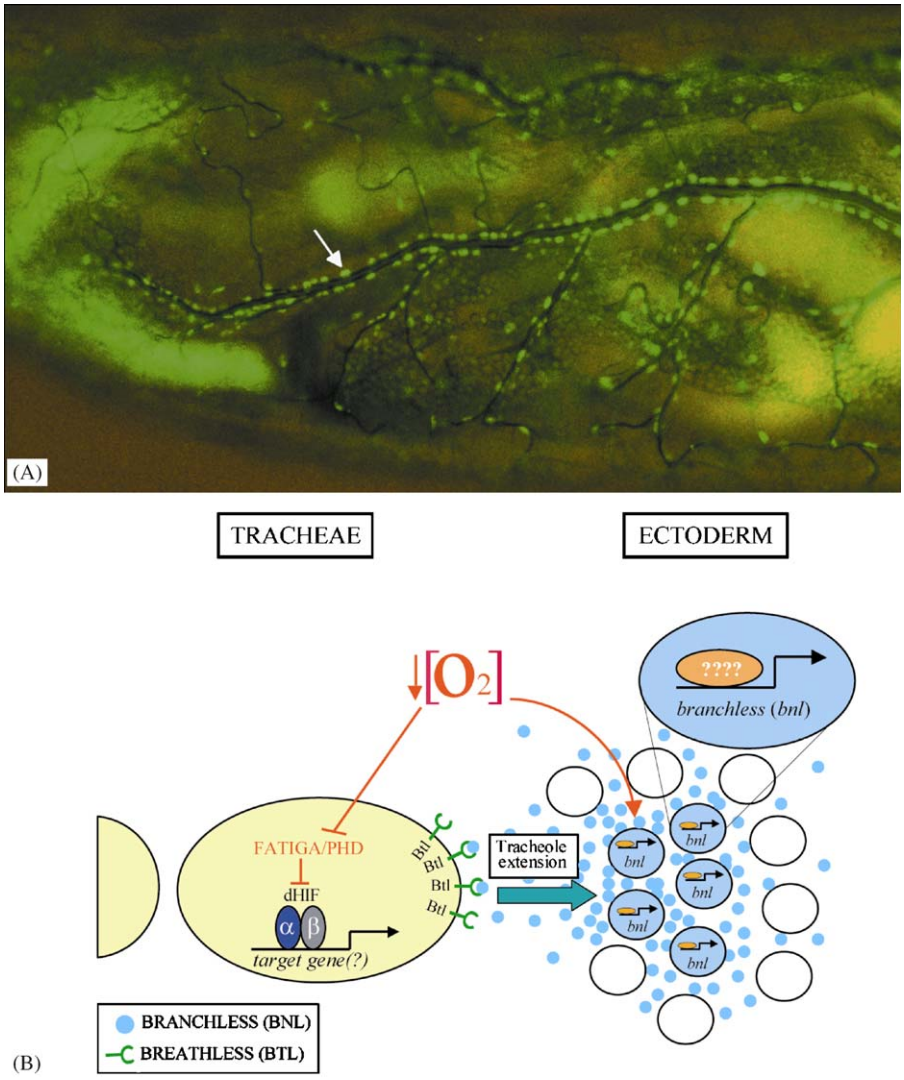

Fig. 5. *The SIMA:TANGO dimer functions as fly HIF (dHIF) specifically in hypoxic tracheae*. (A) A *Drosophila* first instar larva, that is transgenic for a SIMA:TANGO-inducible green fluorescent protein (GFP) reporter, has been exposed to a 5% oxygen atmosphere. The GFP reporter, that bears a nuclear localization signal, is expressed in the nuclei of the tracheal system (the arrow marks one nucleus), while no expression of the reporter is observed outside the tracheal system. (B) Model for oxygen-dependent plasticity of the *Drosophila* larval tracheal system: under low pO_2, the chemo-tactic ligand BRANCHLESS (BNL)/FGF is expressed in non-tracheal tissues, thereby binding to the BREATHLESS (BTL)/FGF receptor and promoting the extension of thin tracheal projections (tracheoles) towards the hypoxic tissue; the molecular mechanism that mediates oxygen-dependent BNL induction is unknown. In hypoxic tracheal cells, the *Drosophila* HIF (dHIF) α-subunit, SIMA accumulates, thereby inducing the transcription of tracheal-specific unknown genes that might contribute to O_2-dependent tracheal remodeling.

(Lavista-Llanos et al., 2002) (Fig. 5A). Based on these findings, tracheal cells seem to respond with a heightened sensitivity to declining pO_2 as compared to other cell types (Lavista-Llanos et al., 2002; Arquier et al., 2006), albeit neither the reason(s) nor the mechanism(s) for such tissue-specific thresholds of oxygen sensing and the HIF-signaling pathway are currently understood. Tackling the molecular bases of oxygen-dependent tracheal plasticity awaits novel conceptual approaches and experimental strategies.

8. HIF and prolyl hydroxylases regulate cellular growth

Hypoxic insects, including drosophilids, generally grow at reduced rates and remain smaller relative to normoxic control animals (Frazier et al., 2001). Until recently, however, very few insights had been uncovered in regard to the molecular interface between hypoxic responses and growth control of cells and organisms. Now, studies in the field of cell growth regulation have arrived at the unexpected conclusion that the *Drosophila* PHD homolog, a protein we named FATIGA (FGA) (Centanin et al., 2005), is involved in the determination of cell size. While looking for genes that might mediate the established growth- and proliferation stimulation by hyperactive CYCLIN D/CDK4 cyclin-dependent protein kinase complexes (CYCD/CDK4), Frei and Edgar discovered that partial loss of function of *fga* dominantly suppressed the growth, but not the proliferation, phenotype provoked by

the ectopic expression of *cycD* (Frei, 2004; Frei and Edgar, 2004). Moreover, mitotic clones of *fga* homozygous mutant cells in the fat body exhibited clear size reduction, revealing that FGA/PHD is likely to function as a cell autonomous positive regulator of cell growth. Consistent with this hypothesis, the same study also showed that ectopic expression of FGA/PHD is sufficient to increase cell size in wing imaginal discs, suggesting that not only decreasing but also increasing FGA/PHD levels can affect cell size (Frei, 2004; Frei and Edgar, 2004).

Extensive work over the last seven years by Hafen's group and other labs has characterized the role of the insulin-triggered phosphoinositol-3-kinase (PI3K)/target of rapamycin (TOR) pathway in *Drosophila* cell and body size regulation (Bohni et al., 1999; Oldham and Hafen, 2003). Increased activity of this pathway enhances growth, while diminished PI3K/TOR signal transduction leads to cell and body size reduction. It was therefore suggested that the main function of this pathway is to coordinate the extent of cellular and organismal growth in accordance with environmental conditions and the nutritional status of the organism (Britton et al., 2002). Since hypoxic flies remain small in size (Frazier et al., 2001), potential cross-talks between the PI3K/TOR and the hypoxia/HIF pathways in *Drosophila* are currently under active investigation. A genetic screen has recently implicated a hypoxia-inducible gene named *scylla* as a negative regulator of TOR signaling, thereby providing a mechanistic explanation for the well-known link between oxygen scarcity and body size reduction (Reiling and Hafen, 2004). Interestingly, this regulation is evolutionary conserved as the mammalian homolog of SCYLLA, a hypoxia-inducible product named REDD1/RTP801, has exactly the same effect on TOR signaling and growth control (Brugarolas et al., 2004). Taken together, these recent results suggested that *fatiga* loss-of-function causes SIMA protein up-regulation, which in turn could be a negative regulator of cellular growth. A relevant question in this regard is as to whether over-accumulation of SIMA can account by itself for the cell size reduction of *fga* mutant clones. Our own results revealed that this is indeed the case. Over-expression of SIMA in random clones throughout different larval polytenic tissues provoked a dramatic cell autonomous reduction of cell size, revealing that SIMA does indeed convey the *fga* cell growth phenotype (Centanin et al., 2005). In support of this conclusion, homozygous pupae for a hypomorphic *fga* allele were much smaller than their wild-type siblings, whereas *fga* sima double mutant pupae had reverted to a normal size (Centanin et al., 2005).

Nevertheless, the cross-talk between the HIF/SIMA and insulin/PI3K/TOR pathways appears to be far more complicated. We have recently shown, both in *Drosophila* SL2 cells and in living embryos, that insulin is a potent activator of SIMA-dependent transcription. The effect is mediated by the PI3K/TOR pathway and involves accumulation of SIMA protein as well as its enhanced nuclear localization (Dekanty et al., 2005). Thus, in vitro and in vivo, SIMA can inhibit cell growth by down-regulating the TOR pathway, while paradoxically, SIMA signaling is activated by the same pathway. What is the physiological meaning of this apparent negative feedback loop from hypoxia via SIMA to the insulin/PI3K/TOR cascade? Is the SIMA-dependent inhibition of cellular (and organismal) growth conferred entirely by SCYLLA, or do other SIMA targets contribute to this effect as well? These, and other, important issues related to the novel and exciting role of HIF/SIMA in growth regulation remain unresolved for now.

9. Conclusions and outlook

Invertebrate species will provide for exciting new directions and paradigms in future studies of HIF-mediated oxygen sensing. As outlined in this review, a homologous HIF pathway operates, with a remarkable degree of conservation from nematodes to flies to fish to humans, by governing essential aspects of physiological and developmental adaptations to oxygen limitation. Parallel and different responses alike are mediated via HIF when comparing our standard, hypoxia-sensitive human or rodent models with hypoxia-tolerant invertebrates. As overarching theme during the onset of hypoxic challenges, HIF signaling aids in maintaining systemic O_2 homeostasis and aerobic metabolism across a wide range of taxa by improving oxygen carrying capacities through hemoglobin- (*Daphnia*), tracheal remodeling- (*Drosophila*) or angiogenesis-/erythropoiesis-centered (vertebrates) strategies. Deeper into hypoxia, however, HIF-driven regulation of target genes might be instrumental in switching cellular metabolism to either energy compensation- (mammals) or energy conservation-based (many invertebrates) defenses. Exploring invertebrate HIF, therefore, might allow us to understand its significance in inducing and maintaining physiological adaptations such as hypometabolism and cellular quiescence that underlie the phenotypic tolerance towards reduced oxygen. These insights will subsequently enable us to compare the molecular basis of stress resilience between physiological (i.e. in these lower metazoans) and pathological states (e.g. quiescent regions in solid human tumors) and to interfere with it. Furthermore, the hypoxia-induced presence of an endogenous 'anti-HIF' molecule in fly cells (e.g. ODD-deleted HIF-α splice variant) suggests the need for fine-tuning, or even terminating, the activity of this transcription factor under *all* pO_2, including prolonged and severe O_2 deprivation. Understanding the physiological role of these α-subunit derivatives will certainly benefit us in generating novel and clinically relevant HIF-targeting mechanisms. Finally, HIFs control of invertebrate development is clearly seen in *Drosophila*, where it was shown to act as a growth inhibitor in response to hypoxia, presumably by antagonizing the growth-promoting function of the insulin/PI3K/TOR pathway. Thus, invertebrate models have yielded the first details of the molecular crosstalk between

HIF and insulin pathways to coordinate growth of cells and organisms in accordance with ambient oxygen tension.

Acknowledgements

TAG wishes to acknowledge Profs. H.F. Bunn (Brigham and Women's Hospital, Harvard Medical School) for years of fruitful mentorship and financial support of the *Daphnia*- and *Drosophila*-related projects (NIH Grant RO1 DK041234 rewarded to H.F. Bunn) and G.A. LeBlanc (North Carolina State University) for proof-reading this manuscript. TAG and MG are supported by the Swiss National Science Foundation and the EU's Sixth Framework Programme project "EUROXY". PW thanks members of his lab for fruitful discussions (Wellcome Trust Grant 070161/Z/03/Z).

References

Acker, T., Acker, H., 2004. Cellular oxygen sensing need in Cns function: physiological and pathological implications. Journal of Experimental Biology 207, 3171–3188.

Adelman, D.M., Gertsenstein, M., Nagy, A., Simon, M.C., Maltepe, E., 2000. Placental cell fates are regulated in vivo by Hif-mediated hypoxia responses. Genes & Development 14, 3191–3203.

Ameri, K., Lewis, C.E., Raida, M., Sowter, H., Hai, T., Harris, A.L., 2004. Anoxic induction of Atf-4 through Hif-1-independent pathways of protein stabilization in human cancer cells. Blood 103, 1876–1882.

An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., Neckers, L.M., 1998. Stabilization of wild-type P53 by hypoxia-inducible factor 1alpha. Nature 392, 405–408.

Arquier, N., Vigne, P., Duplan, E., Hsu, T., Therond, P.P., Frelin, C., D'Angelo, G., 2006. Analysis of the hypoxia-sensing pathway in *Drosophila melanogaster*. Biochemical Journal 393, 471–480.

Bacon, N., Wappner, P., O'Rourke, J., Bartlett, S., Shilo, B., Pugh, C., Ratcliffe, P., 1998. Regulation of the *Drosophila* bHLH-PAS protein Sima by hypoxia: functional evidence for homology with mammalian Hif-1α. Biochemical and Biophysical Research Communications 249, 811–816.

Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., Pouyssegur, J., 2003. Hif prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of Hif-1alpha in normoxia. EMBO Journal 22, 4082–4090.

Bishop, T., Lau, K.W., Epstein, A.C.R., Kim, S.K., Jiang, M., O'Rourke, D., Pugh, C.W., Gleadle, J.M., Taylor, M.S., Hodgkin, J., Ratcliffe, P.J., 2004. Genetic analysis of pathways regulated by the von Hippel–Lindau tumor suppressor in *Caenorhabditis elegans* PLoS Biology 2, e289, 1549–1560.

Blanchard, K.L., Acquaviva, A.M., Galson, D.L., Bunn, H.F., 1992. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Molecular and Cellular Biology 12, 5373–5385.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., Hafen, E., 1999. Autonomous control of cell and organ size by chico, a *Drosophila* homolog of vertebrate irs1–4. Cell 97, 865–875.

Boutilier, R., St-Pierre, J., 2000. Surviving hypoxia without really dying. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 126, 481–490.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., Edgar, B.A., 2002. *Drosophila's* insulin/Pi3-kinase pathway coordinates cellular metabolism with nutritional conditions. Developmental Cell 2, 239–249.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W., Kaelin Jr., W.G., 2004. Regulation of mTOR function in response to hypoxia by Redd1 and the Tsc1/Tsc2 tumor suppressor complex. Genes & Development 18, 2893–2904.

Bruick, R., McKnight, S., 2001. A conserved family of prolyl-4-hydroxylases that modify Hif. Science 294, 1337–1340.

Buck, L., Land, S., Hochachka, P., 1993. Anoxia-tolerant hepatocytes: model system for study of reversible metabolic suppression. American Journal of Physiology—Regulatory, Integrative and Comparative Physiology 265, R49–R56.

Bunn, H., Poyton, R., 1996. Oxygen sensing and molecular adaptation to hypoxia. Physiological Reviews 76, 839–885.

Centanin, L., Ratcliffe, P.J., Wappner, P., 2005. Reversion of lethality and growth defects in fatiga oxygen-sensor mutant flies by loss of hypoxia-inducible factor-alpha/Sima. EMBO Reports 6, 1070–1075.

Chadwick, L., Gilmour, D., 1940. Respiration during flight in *Drosophila Repleta* Wollaston: the oxygen consumption considered in relation to the wing-rate. Physiological Zoology 13, 398–410.

Childress, J.J., 1975. The respiratory rates of midwater crustaceans as a function of depth of occurrence and relation to the oxygen minimum layer off Southern California. Comparative Biochemistry and Physiology Part A: Physiology 50, 787–799.

Covello, K.L., Simon, M.C., 2004. Hifs, hypoxia, and vascular development. Current Topics in Developmental Biology 62, 37–54.

Crews, S., 1998. Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes & Development 12, 607–620.

Csik, L., 1939. The susceptibility to oxygen want of different *Drosophila* species. Zeitschrift für vergleichende Physiologie 27, 304–310.

Cummins, E.P., Taylor, C.T., 2005. Hypoxia-responsive transcription factors. Pflügers Archiv European Journal of Physiology 450, 363–371.

D'Angelo, G., Duplan, E., Boyer, N., Vigne, P., Frelin, C., 2003. Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits Hif-1 responses during reoxygenation. Journal of Biological Chemistry 278, 38183–38187.

Dekanty, A., Lavista-Llanos, S., Irisarri, M., Oldham, S., Wappner, P., 2005. The Insulin/Pi3k/Tor pathway induces a Hif-dependent transcriptional response in *Drosophila* by promoting nuclear localization of Hif-1alpha/Sima. Journal of Cell Science 118, 5431–5441.

del Peso, L., Castellanos, M.C., Temes, E., Martin-Puig, S., Cuevas, Y., Olmos, G., Landazuri, M.O., 2003. The von Hippel Lindau/hypoxia-inducible factor (Hif) pathway regulates the transcription of the Hif-proline hydroxylase genes in response to low oxygen. Journal of Biological Chemistry 278, 48690–48695.

DiGregorio, P.J., Ubersax, J.A., O'Farrell, P.H., 2001. Hypoxia and nitric oxide induce a rapid, reversible cell cycle arrest of the *Drosophila* syncytial divisions. Journal of Biological Chemistry 276, 1930–1937.

Douglas, R., Xu, T., Haddad, G., 2001. Cell cycle progression and cell division are sensitive to hypoxia in *Drosophila melanogaster* embryos. American Journal of Physiology—Regulatory Integrative & Comparative Physiology 280, R1555–R1563.

Drew, K.L., Harris, M.B., LaManna, J.C., Smith, M.A., Zhu, X.W., Ma, Y.L., 2004. Hypoxia tolerance in mammalian heterotherms. Journal of Experimental Biology 207, 3155–3162.

Duffy, T.E., Nelson, S.R., Lowry, O.H., 1972. Cerebral carbohydrate metabolism during acute hypoxia and recovery. Journal of Neurochemistry 19, 959–977.

Ebbesen, P., Eckardt, K.U., Ciampor, F., Pettersen, E.O., 2004. Linking measured intercellular oxygen concentration to human cell functions. Acta Oncologica 43, 598–600.

Ebert, B., Bunn, H., 1999. Regulation of the erythropoietin gene. Blood 94, 1864–1877.

Emmons, R., Duncan, D., Estes, P., Kiefel, P., Mosher, J., Sonnenfeld, K., Ward, M., Duncan, I., Crews, S., 1999. The spinless-aristapedia and Tango bHLH-PAS proteins interact to control antennal and tarsal development in *Drosophila*. Development 126, 3937–3945.

Epstein, A., Gleadle, J., McNeill, L., Hewiston, K., O'Rourke, J., Mole, D., Mukherji, M., Metzen, E., Wilson, M., Dhanda, A., Tian, Y., Masson, N., Hamilton, D., Jaakola, P., Barstead, R., Hodgkin, J., Maxwell, P., Pugh, C., Schofield, C., Ratcliffe, P., 2001. *C. elegans* Egl-9 and mammalian homologs define a family of dioxygenases that regulate Hif by prolyl hydroxylation. Cell 107, 43–54.

Erecinska, M., Silver, I.A., 2001. Tissue oxygen tension and brain sensitivity to hypoxia. Respiration Physiology 128, 263–276.

Firth, J.D., Ebert, B.L., Ratcliffe, P.J., 1995. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and camp response elements. Journal of Biological Chemistry 270, 21021–21027.

Foe, V., Alberts, B., 1985. Reversible chromosome condensation induced in *Drosophila* embryos by anoxia: visualization of interphase nuclear organization. The Journal of Cell Biology 100, 1623–1636.

Frazier, M.R., Woods, H.A., Harrison, J.F., 2001. Interactive effects of rearing temperature and oxygen on the development of *Drosophila melanogaster*. Physiological and Biochemical Zoology 74, 641–650.

Frei, C., 2004. Cyclin D/Cdk4: new insights from *Drosophila*. Cell Cycle 3, 558–560.

Frei, C., Edgar, B.A., 2004. *Drosophila* Cyclin D/Cdk4 requires Hif-1 prolyl hydroxylase to drive cell growth. Developmental Cell 6, 241–251.

Ghabrial, A., Luschnig, S., Metzstein, M.M., Krasnow, M.A., 2003. Branching morphogenesis of the *Drosophila* tracheal system. Annual Review of Cell and Developmental Biology 19, 623–647.

Goldberg, M.A., Dunning, S.P., Bunn, H.F., 1988. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science 242, 1412–1415.

Gorr, T., Cahn, J., Hradecky, P., Bunn, H.F., 2003. Genome-wide computational screen for candidate Hif target genes in *Drosophila melanogaster* and *Caenorhabditis elegans*. In: Lahiri, S., Semenza, G., Prabhakar, N. (Eds.), Oxygen Sensing—Responses and Adaptation to Hypoxia, vol. 175. Marcel Dekker, New York, pp. 175–199.

Gorr, T., Cahn, J., Yamagata, H., Bunn, H., 2004a. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. Journal of Biological Chemistry 279, 36038–36047.

Gorr, T., Tomita, T., Wappner, P., Bunn, H., 2004b. Regulation of *Drosophila* hypoxia-inducible factor (Hif) activity in Sl2 cells—identification of a hypoxia-induced variant isoform of the Hifα homolog gene similar. Journal of Biological Chemistry 279, 36048–36058.

Graven, K.K., Bellur, D., Klahn, B.D., Lowrey, S.L., Amberger, E., 2003. Hif-2alpha regulates glyceraldehyde-3-phosphate dehydrogenase expression in endothelial cells. Biochimica et Biophysica Acta (BBA)—Gene Structure and Expression 1626, 10–18.

Grieshaber, M., Hardewig, I., Kreutzer, U., Pörtner, H.-O., 1994. Physiological and metabolic responses to hypoxia in invertebrates. Reviews of Physiology, Biochemistry and Pharmacology 125, 43–147.

Guillemin, K., Krasnow, M., 1997. The hypoxic response: huffing and hifing. Cell 89, 9–12.

Guppy, M., Withers, P., 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews 74, 1–40.

Hansen, A.J., 1985. Effect of anoxia on ion distribution in the brain. Physiological Reviews 65, 101–148.

Harris, A.L., 2002. Hypoxia–a key regulatory factor in tumour growth. Nature Reviews Cancer 2, 38–47.

Heldmaier, G., Ortmann, S., Elvert, R., 2004. Natural hypometabolism during hibernation and daily torpor in mammals. Respiratory Physiology & Neurobiology 141, 317–329.

Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K.I., Myllyharju, J., 2003. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. Journal of Biological Chemistry 278, 30772–30780.

Hoback, W.W., Stanley, D.W., 2001. Insects in hypoxia. Journal of Insect Physiology 47, 533–542.

Hochachka, P., 1986a. Metabolic arrest. Intensive Care Medicine 12, 127–133.

Hochachka, P.W., 1986b. Defense strategies against hypoxia and hypothermia. Science 231, 234–241.

Hochachka, P., Buck, L., Doll, C., Land, S., 1996. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proceedings of the National Academy of Sciences of the United States of America 93, 9493–9498.

Hochachka, P.W., Somero, G.N., 2002. Biochemical adaptation—Mechanism and process in physiological evolution. Oxford University Press, New York.

Höpfl, G., Ogunshola, O., Gassmann, M., 2004. Hifs and tumors—causes and consequences. American Journal of Physiology—Regulatory, Integrative and Comparative Physiology 286, R608–R623.

Huang, L., Arany, Z., Livingston, D., Bunn, H., 1996. Activation of hypoxia-inducible transcription factor depends primarily upon redox sensitive stabilization of its α subunit. Journal of Biological Chemistry 271, 32253–32259.

Huang, L., Gu, J., Schau, M., Bunn, H., 1998. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proceedings of the National Academy of Sciences of the United States of America 95, 7987–7992.

Huang, L.E., Bunn, H.F., 2003. Hypoxia-inducible factor and its biomedical relevance. Journal of Biological Chemistry 278, 19575–19578.

Isaac, D.D., Andrew, D.J., 1996. Tubulogenesis in *Drosophila*: a requirement for the trachealess gene product. Genes & Development 10, 103–117.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., Kaelin Jr., W.G., 2001. Hifalpha targeted for Vhl-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 292, 464–468.

Iyer, N., Kotch, L., Agani, F., Leung, S., Laughner, E., Wenger, R., Gassmann, M., Gearhart, J., Lawler, A., Yu, A., Semenza, G., 1998. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. Genes & Development 12, 149–162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., 2001. Targeting of Hif-alpha to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292, 468–472.

Jaeschke, H., Smith, C.V., Mitchell, J.R., 1988. Hypoxic damage generates reactive oxygen species in isolated perfused rat liver. Biochemical and Biophysical Research Communications 150, 568–574.

Jarecki, J., Johnson, E., Krasnow, M., 1999. Oxygen regulation of airway branching in *Drosophila* is mediated by branchless Fgf. Cell 99, 211–220.

Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H., Gassmann, M., 2001. Induction of Hif-1alpha in response to hypoxia is instantaneous. FASEB Journal 15, 1312–1314.

Jiang, B.H., Semenza, G.L., Bauer, C., Marti, H.H., 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. American Journal of Physiology—Cell Physiology 271, C1172–C1180.

Jiang, H., Guo, R., Powell-Coffman, J., 2001. The *Caenorhabditis elegans* Hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proceedings of the National Academy of Sciences of the United States of America 14, 7916–7921.

Jiang, L., Crews, S.T., 2003. The *Drosophila* dysfusion basic helix-loop-helix (bHLH)-PAS gene controls tracheal function and levels of the trachealess bHLH-PAS protein. Molecular and Cellular Biology 23, 5625–5637.

Keister, M., Buck, J., 1974. Respiration: some exogenous and endogenous effects on rate of respiration. In: Rockstein, M. (Ed.), The Physiology of Insecta, vol. 6. Academic Press, New York, pp. 469–509.

Kobayashi, M., Hoshi, T., 1982. Relationship between the haemoglobin concentration of *Daphnia magna* and the ambient oxygen

concentration. Comparative Biochemistry and Physiology Part A: Physiology 72, 247–249.

Koivunen, P., Hirsilä, M., Günzler, V., Kivirikko, K.I., Myllyharju, J., 2004. Catalytic properties of the asparaginyl hydroxylase (Fih) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. Journal of Biological Chemistry 279, 9899–9904.

Krishnan, S., Sun, Y.-A., Mohsenin, A., Wyman, R., Haddad, G., 1997. Behavioral and electrophysiologic responses of *Drosophila melanogaster* to prolonged periods of anoxia. Journal of Insect Physiology 43, 203–210.

Land, S., Buck, L., Hochachka, P., 1993. Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. American Journal of Physiology—Regulatory, Integrative and Comparative Physiology 265, R41–R48.

Land, S., Hochachka, P., 1994. Protein turnover during metabolic arrest in turtle hepatocytes: role and energy dependence of proteolysis. American Journal of Physiology—Cell Physiology 266, C1028–C1036.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., Bruick, R.K., 2002a. Fih-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes & Development 16, 1466–1471.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., Whitelaw, M.L., 2002b. Asparagine hydroxylation of the Hif transactivation domain a hypoxic switch. Science 295, 858–861.

Lando, D., Gorman, J.J., Whitelaw, M.L., Peet, D.J., 2003. Oxygen-dependent regulation of hypoxia-inducible factors by prolyl and asparaginyl hydroxylation. European Journal of Biochemistry 270, 781–790.

Lavista-Llanos, S., Centanin, L., Irisarri, M., Russo, D.M., Gleadle, J.M., Bocca, S.N., Muzzopappa, M., Ratcliffe, P.J., Wappner, P., 2002. Control of the hypoxic response in *Drosophila melanogaster* by the basic helix-loop-helix PAS protein similar. Molecular & Cellular Biology 22, 6842–6853.

Levy, A.P., Levy, N.S., Wegner, S., Goldberg, M.A., 1995. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. Journal of Biological Chemistry 270, 13333–13340.

Locke, M., 1958. The co-ordination of growth in the tracheal system of insects. Quarterly Journal of Microscopic Science 99, 373–391.

Loudon, C., 1988. Development of *Tenebrio molitor* in low oxygen levels. Journal of Insect Physiology 34, 97–103.

Ma, E., Haddad, G., 1999. Isolation and Characterization of the hypoxia-inducible factor 1β in *Drosophila melanogaster*. Molecular Brain Research 73, 11–16.

Ma, E., Xu, T., Haddad, G., 1999. Gene regulation by O_2 deprivation: an anoxia-regulated novel gene in *Drosophila melanogaster*. Molecular Brain Research 63, 217–224.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., Poellinger, L., 2001. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 414, 550–554.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H., Poellinger, L., 2002. Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor 3 alpha locus. Journal of Biological Chemistry 272, 32405–32408.

Manalo, D.J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B.D., Ye, S.Q., Garcia, J.G., Semenza, G.L., 2005. Transcriptional regulation of vascular endothelial cell responses to hypoxia by Hif-1. Blood 105, 659–669.

Marxsen, J.H., Stengel, P., Doege, K., Heikkinen, P., Jokilehto, T., Wagner, T., Jelkmann, W., Jaakkola, P., Metzen, E., 2004. Hypoxia-inducible factor-1 (Hif-1) promotes its degradation by induction of Hif-alpha-prolyl-4-hydroxylases. Biochemical Journal 381, 761–767.

Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., 2001. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO Journal 20, 5197–5206.

Maxwell, P., Salnikow, K., 2004. Hif-1: an oxygen and metal responsive transcription factor. Cancer Biology & Therapy 3, 29–35.

Maxwell, P., Pugh, C., Ratcliffe, P., 1993. Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. Proceedings of the National Academy of Sciences of the United States of America 90, 2423–2427.

Maxwell, P., Wiesener, M., Chang, G., Clifford, S., Vaux, E., Cockman, M., Wykoff, C., Pugh, C., Maher, E., Ratcliffe, P., 1999. The tumour suppressor protein Vhl targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399, 271–275.

McMahon, B.R., 2001. Respiratory and circulatory compensation to hypoxia in crustaceans. Respiration Physiology 128, 349–364.

Metzger, R., Krasnow, M., 1999. Genetic control of branching morphogenesis. Science 284, 1635–1639.

Morin Jr., P., McMullen, D.C., Storey, K.B., 2005. Hif-1alpha involvement in low temperature and anoxia survival by a freeze tolerant Insect. Molecular and Cellular Biochemistry 280, 99–106.

Mortola, J.P., 2004. Implications of hypoxic hypometabolism during mammalian ontogenesis. Respiratory Physiology & Neurobiology 141, 345–356.

Murphy, B.J., 2004. Regulation of malignant progression by the hypoxia-sensitive transcription factors Hif-1alpha and Mtf-1. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 139, 495–507.

Nagao, M., Ebert, B., Ratcliffe, P., Pugh, C., 1996. *Drosophila melanogaster* Sl2 cells contain a hypoxically inducible DNA binding complex which recognises mammalian Hif-1 binding sites. FEBS Letters 387, 161–166.

Nambu, J.R., Lewis, J.O., Wharton Jr., K.A., Crews, S.T., 1991. The *Drosophila* single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of Cns midline development. Cell 67, 1157–1167.

Nambu, J., Chen, W., Hu, S., Crews, S., 1996. The *Drosophila melanogaster* similar bHLH-PAS gene encodes a protein related to human hypoxia-inducible factor 1α and *Drosophila* single-minded. Gene 172, 249–254.

Nikinmaa, M., Rees, B.B., 2005. Oxygen-dependent gene expression in fishes. American Journal of Physiology—Regulatory, Integrative and Comparative Physiology 288, R1079–R1090.

Nikinmaa, M., Pursiheimo, S., Soitamo, A.J., 2004. Redox state regulates Hif-1alpha and its DNA binding and phosphorylation in salmonid cells. Journal of Cell Science 117, 3201–3206.

Ohshiro, T., Saigo, K., 1997. Transcriptional regulation of breathless Fgf receptor gene by binding of trachealess/darnt heterodimers to three central midline elements in *Drosophila* developing trachea. Development 124, 3975–3986.

Oldham, S., Hafen, E., 2003. Insulin/Igf and target of rapamycin signaling: a Tor de force in growth control. Trends in Cell Biology 13, 79–85.

Padilla, P.A., Nystul, T.G., Zager, R.A., Johnson, A.C., Roth, M.B., 2002. Dephosphorylation of cell cycle-regulated proteins correlates with anoxia-induced suspended animation in *Caenorhabditis elegans*. Molecular Biology of the Cell 13, 1473–1483.

Paul, R.J., Colmorgen, M., Hüller, S., Tyroller, F., Zinkler, D., 1997. Circulation and respiratory control in millimetre-sized animals (*Daphnia magna*, *Folsomia candida*) studied by optical methods. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 167, 399–408.

Paul, R., Colmorgen, M., Pirow, R., Chen, Y.-H., Tsai, M.-C., 1998. Systemic and metabolic responses in *Daphnia magna* to anoxia. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 120, 519–530.

Pfander, D., Kobayashi, T., Knight, M.C., Zelzer, E., Chan, D.A., Olsen, B.R., Giaccia, A.J., Johnson, R.S., Haase, V.H., Schipani, E., 2004. Deletion of Vhlh in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. Development 131, 2497–2508.

Pirow, R., Bäumer, C., Paul, R.J., 2001. Benefits of heamoglobin in the Cladoceran custacean *Daphnia magna*. Journal of Experimental Biology 204, 3425–3441.

Powell-Coffman, J., Bradfield, C., Wood, W., 1998. *Caenorhabditis elegans* orthologs of the aryl hydrocarbon receptor and its heterodimers partner the aryl hydrocarbon receptor nuclear translocator. Proceedings of the National Academy of Sciences of the United States of America 95, 2844–2849.

Pugh, C.W., Ratcliffe, P.J., 2003. Regulation of angiogenesis by hypoxia: role of the Hif system. Nature Medicine 9, 677–684.

Reiling, J.H., Hafen, E., 2004. The hypoxia-induced paralogs scylla and charybdis inhibit growth by down-regulating S6k activity upstream of Tsc in *Drosophila*. Genes & Development 18, 2879–2892.

Ribeiro, C., Petit, V., Affolter, M., 2003. Signaling systems, guided cell migration, and organogenesis: insights from genetic studies in *Drosophila*. Developmental Biology 260, 1–8.

Rolfe, D., Brown, G., 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiological Reviews 77, 731–758.

Rosenberger, C., Heyman, S.N., Rosen, S., Shina, A., Goldfarb, M., Griethe, W., Frei, U., Reinke, P., Bachmann, S., Eckardt, K.U., 2005. Up-regulation of Hif in experimental acute renal failure: evidence for a protective transcriptional response to hypoxia. Kidney International 67, 531–542.

Salceda, S., Caro, J., 1997. Hypoxia-inducible factor 1α (Hif-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Journal of Biological Chemistry 272, 22642–22647.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D.C., Guillemin, K., Krasnow, M.A., 1996. Development of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. Development 122, 1395–1407.

Schmidt, H., Kamp, G., 1996. The Pasteur effect in facultative anaerobic metazoa. Experientia 52, 440–448.

Schmitz, A., Harrison, J.F., 2004. Hypoxic tolerance in air-breathing invertebrates. Respiratory Physiology & Neurobiology 141, 229–242.

Schneider, I., 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. Journal of Embryology and Experimental Morphology 27, 353–365.

Schofield, C.J., Ratcliffe, P.J., 2004. Oxygen sensing by Hif hydroxylases. Nature Reviews Molecular Cell Biology 5, 343–354.

Scholz, F., Zerbst-Boroffka, I., 1998. Environmental hypoxia affects osmotic and ionic regulation in freshwater midge-larvae. Journal of Insect Physiology 44, 427–436.

Seagroves, T., Ryan, H., Lu, H., Wouters, B., Knapp, M., Thibault, P., Laderoute, K., Johnson, R., 2001. Transcription factor Hif-1 is a necessary mediator of the Pasteur effect in mammalian cells. Molecular and Cellular Biology 21, 3436–3444.

Semenza, G., 1999. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annual Review of Cell and Developmental Biology 15, 551–578.

Semenza, G.L., 2002. Hif-1 and tumor progression: pathophysiology and therapeutics. Trends in Molecular Medicine 8, S62–S67.

Semenza, G.L., 2003. Targeting Hif-1 for cancer therapy. Nature Reviews Cancer 3, 721–732.

Semenza, G.L., Wang, G.L., 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Molecular and Cellular Biology 12, 5447–5454.

Shen, C., Powell-Coffman, J.A., 2003. Genetic analysis of hypoxia signaling and response in *C. elegans*. Annals of the New York Academy of Sciences 995, 191–199.

Shen, C., Nettleton, D., Jiang, M., Kim, S.K., Powell-Coffman, J.A., 2005. Roles of the Hif-1 hypoxia-inducible factor during hypoxia response in *Caenorhabditis elegans*. Journal of Biological Chemistry 280, 20580–20588.

Shweiki, D., Itin, A., Soffer, D., Keshet, E., 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843–845.

Shweiki, D., Neeman, M., Itin, A., Keshet, E., 1995. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proceedings of the National Academy of Sciences of the United States of America 92, 768–772.

Singer, D., 1999. Neonatal tolerance to hypoxia: a comparative-physiological approach. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 123, 221–234.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., Crews, S., 1997. The *Drosophila* Tange gene encodes a bHLH-PAS protein that is orthologous to mammalian arnt and controls Cns midline and tracheal development. Development 124, 4571–4582.

Srinivas, V., Zhang, L., Zhu, X., Caro, J., 1999. Characterization of an oxygen/redox-dependent degradation domain of hypoxia-inducible factor α (Hif-α) proteins. Biochemical and Biophysical Research Communications 260, 557–561.

Stolze, I.P., Tian, Y.M., Appelhoff, R.J., Turley, H., Wykoff, C.C., Gleadle, J.M., Ratcliffe, P.J., 2004. Genetic analysis of the role of the asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (Hif) in regulating Hif transcriptional target genes. Journal of Biological Chemistry 279, 42719–42725.

Storey, K., 1985. A re-evaluation of the pasteur effect: new mechanisms in anaerobic metabolism. Molecular Physiology 8, 439–461.

Suarez, R., Doll, C., Buie, A., West, T., Funk, G., Hochachka, P., 1989. Turtles and rats: a biochemical comparison of anoxia-tolerant and anoxia-sensitive brains. American Journal of Physiology—Regulatory, Integrative and Comparative Physiology 257, R1083–R1088.

Sutherland, S., Samakovlis, Krasnow, M., 1996. Branchless encodes a *Drosophila* Fgf homolog that controls tracheal cell migration and the pattern of branching. Cell 87, 1091–1101.

Taylor, B., Zhulin, I., 1999. PAS domains: internal sensors of oxygen, redox potential, and light. Microbiology and Molecular Biology Reviews 63, 479–506.

Tomita, S., Ueno, M., Sakamoto, M., Kitahama, Y., Ueki, M., Maekawa, N., Sakamoto, H., Gassmann, M., Kageyama, R., Ueda, N., Gonzalez, F.J., Takahama, Y., 2003. Defective brain development in mice lacking the Hif-1alpha gene in neural cells. Molecular and Cellular Biology 23, 6739–6749.

Vaupel, P., 2004. The role of hypoxia-induced factors in tumor progression. Oncologist 9 (Suppl 5), 10–17.

Vogelstein, B., Kinzler, K.W., 2004. Cancer genes and the pathways they control. Nature Medicine 10, 789–799.

Vuori, K.A., Soitamo, A., Vuorinen, P.J., Nikinmaa, M., 2004. Baltic Salmon (Salmo Salar) Yolk–Sac Fry mortality is associated with disturbances in the function of hypoxia-inducible transcription factor (Hif-1alpha) and consecutive gene expression. Aquatic Toxicology 68, 301–313.

Wang, G.L., Semenza, G.L., 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proceedings of the National Academy of Sciences of the United States of America 90, 4304–4308.

Wang, G., Semenza, G., 1995. Purification and characterization of hypoxia-inducible factor 1. Journal of Biological Chemistry 270, 1230–1237.

Wang, G., Jiang, B., Rue, E., Semenza, G., 1995. Hypoxia-inducible factor 1 is a basic helix-loop-helix-Pas heterodimer regulated by cellular O_2 tension. Proceedings of the National Academy of Sciences of the United States of America 92, 5510–5514.

Wappner, P., Ratcliffe, P., 2001. Development of branched structures and the cellular response to hypoxia—an evolutionary perspective. In: Haddad, G., Xu, T. (Eds.), Genetic Models in Cardiorespiratory Biology. Marcel Dekker Inc., New York, pp. 91–138.

Ward, M., Mosher, J., Crews, S., 1998. Regulation of bHLH-PAS protein subcellular localization during *Drosophila* embryogenesis. Development 125, 1599–1608.

Webster, K., 2003. Evolution of the coordinate regulation of glycolytic enzyme genes by hypoxia. Journal of Experimental Biology 206, 2911–2922.

Webster, K.A., 1987. Regulation of glycolytic enzyme Rna transcriptional rates by oxygen availability in skeletal muscle cells. Molecular and Cellular Biochemistry 77, 19–28.

Wegener, G., 1988. Oxygen availability, energy metabolism and metabolic rate in invertebrates and vertebrates. In: Acker, H. (Ed.), Oxygen Sensing in Tissues. Springer, Berlin, pp. 13–35.

Wegener, G., 1993. Hypoxia and posthypoxic recovery in insects: physiological and metabolic aspects. In: Hochachka, P., Lutz, P., Sick, T., Rosenthal, M., Thillart, G.V.D. (Eds.), Surviving Hypoxia: Mechanisms of Control and Adaptation. CRC Press, Boca Raton, FL, pp. 417–434.

Wenger, R., Gassmann, M., 1997. Oxygen(es) and the hypoxia-inducible factor-1. Biological Chemistry 378, 1–8.

Wenger, R.H., Camenisch, G., Desbaillets, I., Chilov, D., Gassmann, M., 1998. Up-regulation of hypoxia-inducible factor-1alpha is not sufficient for hypoxic/anoxic P53 induction. Cancer Research 58, 5678–5680.

Wenger, R.H., Stiehl, D.P., Camenisch, G., 2005. Integration of oxygen signaling at the consensus HRE. Science STKE 2005 vol. 306 p. re12.

Wiesener, M.S., Turley, H., Allen, W.E., Willam, C., Eckardt, K.U., Talks, K.L., Wood, S.M., Gatter, K.C., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., Maxwell, P.H., 1998. Induction of endothelial Pas domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood 92, 2260–2268.

Wigglesworth, V., 1954. Growth and regeneration in the tracheal system of an insect, *Rhodnius Prolixus* (Hemiptera). Quarterly Journal of Microscopical Science 95, 115–137.

Wigglesworth, V., 1983. The physiology of tracheoles. Advances in Insect Physiology 17, 86–148.

Wilk, R., Weizman, I., Shilo, B., 1996. Trachealess encodes bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. Genes & Development 10, 93–102.

Wingrove, J., O'Farrell, P., 1999. Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila*. Cell 98, 105–114.

Zelzer, E., Wappner, P., Shilo, B., 1997. The PAS domain confers target gene specificity of *Drososphila* bHLH-PAS proteins. Genes & Development 11, 2079–2089.

Glossary

Angiogenesis: growth and proliferation of capillaries from pre-existing vasculature

Anoxia: lack of oxygen; decline of [O_2] down to trace amounts (i.e. [O_2] $\ll 0.1\%$)

ARNT: aryl hydrocarbon receptor nuclear translocator, constitutive HIF-β subunit and common partner protein of various other bHLH/PAS proteins

bHLH: basic helix-loop-helix region; function of basic region: DNA (HRE) binding; function of HLH region: protein:protein (HIF-α:HIF-β) interaction

BNL: BRANCHLESS, *Drosophila*'s fibroblast growth factor (FGF) homolog, ligand to BTL and guidance cue during tracheal outgrowth, expressed in epidermal cells adjacent to tracheal primordia

BTL: BREATHLESS, *Drosophila*'s FGF-receptor homolog, expressed by tracheal cells

CBP: CREB binding protein, CBP and its paralog p300 are protein/histone acetyltransferases and coactivators of HIF and numerous other transcription factors

Co^{2+}: cobalt salt, transition metals Co^{2+}, Ni^{2+}, Mn^{2+} can inhibit PHD and FIH enzymes and stabilize HIF-α, mimetics of mammalian hypoxia response

CYCLIN D/CDK4: CYCLIN-DEPENDENT KINASE 4, activated by CYCLIN D; cell growth and proliferation promoting complex

DFO: desferrioxamine, iron chelator; inhibits PHD and FIH enzymes and stabilizes HIF-α; iron chelating agents are general mimetics of hypoxia responses

E-box: family of short cis-elements of general $^{5'}$CANNTG$^{3'}$ denotation; HREs are E-box members

EMSA: electrophoretic mobility shift assay, in vitro assay for *trans*-factor binding to a specific DNA sequence (e.g. detection of HIF/HRE interaction)

Erythropoiesis: formation of red blood cells

FGA: FATIGA, *Drosophila*'s PHD (HIF prolyl hydroxylase) homolog

FIH-1: factor-inhibiting HIF; dioxygenase with asparaginyl hydroxylase activity, HIF oxygen sensor

HIF: hypoxia-inducible factor, heterodimer of α- and β-subunits, recognizes specific cis-elements (HREs)

HIF-α: oxy-sensitive, regulatory HIF subunit

HRE: hypoxia-response element, HIF binding site; current consensus: $^{5'}$VNVB(A/G)CGTG(C/GTA)N$^{3'}$, with B = all bases except A; V = all bases except T; N = any base; core, essential for HIF binding: underlined

HUP: hydroxylase-ubiquitin-proteasome cascade, role, e.g. in HIF-α regulation

Hypoxia: marked decline of [O_2] below normoxic levels of 20.95% O_2; (e.g, 0.1%–5% O_2 is typically used as HIF-activating hypoxia for in vitro or in vivo studies)

IPAS: inhibitory PAS domain protein, hypoxia-induced ODD-deleted splice variant of murine HIF-3α

NAD/CAD: N-terminal (NAD) and C-terminal (CAD) transcriptional activation domain of HIF-α-subunits; CAD contains asparaginyl residue, which, upon hydroxylation, blocks interaction between HIF and coactivator proteins in normoxia, thereby terminating HIF-mediated gene transcription

ODD: oxygen-dependent degradation domain in HIF-α-subunits; contains NAD and prolyl residue(s), which, upon hydroxylation, confer normoxic instability of α-subunit via ubiquitination and proteasomal degradation

Oxyconformity: O_2/energy demands of metabolism decrease in accordance with falling pO_2; this respiratory regulation can occur even with fully oxygenated mitochondria (i.e. is independent from oxidative limitations)

Oxyregulation: O_2/energy demands of metabolism are high and unaffected by changing pO_2 values until oxygen tensions have declined to, or beyond a, critical threshold (pC)

Palindrome: DNA sequence that reads the same on opposite strands (in opposite directions), e.g. $^{5'}$CACGTG$^{3'}$ / $^{3'}$GTGCAC$^{5'}$

PAS: PER-ARNT-SIM domain (see footnote #2); contains A and B repeats, function of PAS domain: protein:protein (e.g. HIF-α: HIF-β) interaction

PHD: prolyl hydroxylase domain containing dioxygenase, HIF oxygen sensor

PI3K: phosphoinositol-3-kinase, insulin pathway component involved in growth regulation

pC: critical pO_2, marks the physiological transition from oxyregulatory to oxyconforming O_2/energy demands, and the biochemical transition from an oxidative (mitochondrial) to anaerobic (glycolytic) metabolism

pO_2: oxygen partial pressure

SIMA: bHLH/PAS factor named "SIMILAR" (abbrev. SIMA) in *Drosophila*, HIF-α homolog

SL2: schneider line 2, macrophage-like cell line of late stage *Drosophila* embryos

svSIMA: hypoxia-induced ODD-deleted splice variant of *Drosophila*'s full-length ($SIMA^{FL}$) HIF-α homolog

TANGO: bHLH/PAS factor in *Drosophila*, homologous to mammalian ARNT proteins

TOR: target-of-rapamycin, kinase with reported roles in growth control

VHL: von-Hippel–Lindau protein; tumor suppressor and E3 ubiquitin ligase component