Integrative and Comparative Biology Advance Access published August 30, 2007

From critters to cancers: bridging comparative and clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia

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Synopsis The objective of this symposium at the First International Congress of Respiratory Biology (ICRB) was to enhance communication between comparative biologists and cancer researchers working on O_2 sensing via the HIF pathway. Representatives from both camps came together on August 13–16, 2006, in Bonn, Germany, to discuss molecular adaptations that occur after cells have been challenged by a reduced (hypoxia) or completely absent (anoxia) supply of oxygen. This brief ''critters-to-cancer'' survey discusses current projects and new directions aimed at improving understanding of hypoxic signaling and developing therapeutic interventions.

Introduction

Relaying minutes to hours of reduced oxygen supply onto the level of DNA is, throughout the animal kingdom, chiefly mediated by hypoxia-inducible factors (HIFs). Discovered in 1995 by Wang and Semenza as the inducing activity of erythropoietin, the hormone that controls red blood cell production (Wang and Semenza 1995), HIFs are now recognized as a highly conserved family of basic-helix-loop-helix (bHLH)/PAS (acronym for PER, ARNT, SIM, the first proteins discovered to contain this domain) transcription factors that confer a multifaceted adaptive response to hypoxia via changes in gene expression. In mammals, teleosts, the fruitfly *Drosophila melanogaster*, the crustacean *Daphnia magna*, and the nematode *Caenorhabditis elegans*, HIF is a heterodimer of α-subunits and β-subunits that specifically binds target gene sequences, the so-called hypoxia-response elements (HREs), in response to low partial pressure of oxygen (pO_2). Across all these species, declining pO_2 stabilizes and activates the regulatory α- subunit of HIF, while physiological oxygen tensions quickly lead to its destruction. Thus, HIF activity is conservedly controlled at the level of its α-subunits. To date, three HIF-α proteins, HIF-1α/-2α/-3α, have been reported in human and rodent cells, with the function and regulation of the first two being far better understood than is true for HIF-3α. All three α-subunits, however, share the ability to heterodimerize with the constitutively present HIF-1β

This paper summarizes one of the 22 symposia that constituted the First International Congress of Respiratory Biology held August 14–16, 2006, in Bonn, Germany.

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Integrative and Comparative Biology, pp. 1–26
doi:10.1093/icb/icm072

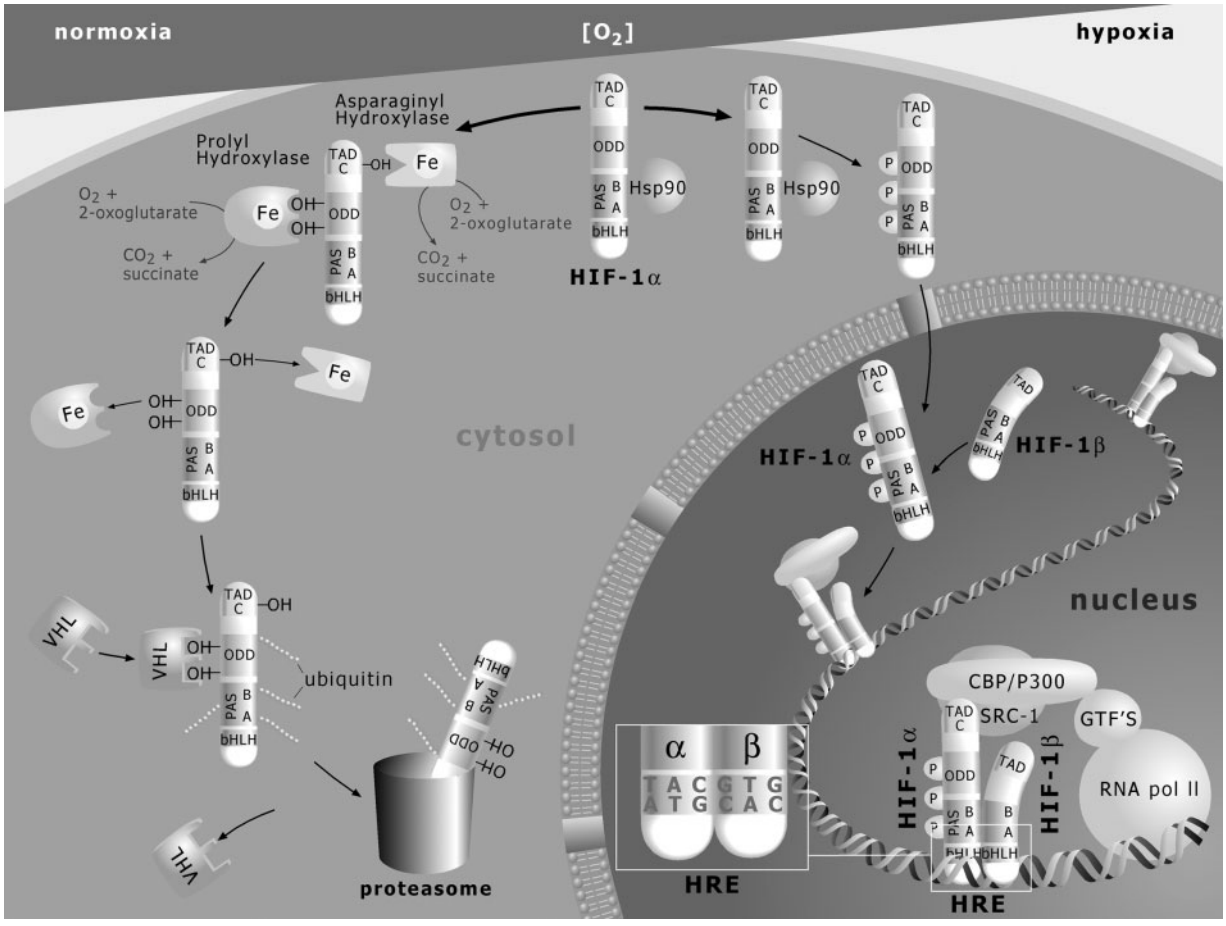

Fig. 1 HIF-α mediated pathway of cellular O_2 sensing. Molecules are not drawn to scale. Prolyl hydroxylase in the figure represents PHD1-3. GTF's = general transcription factors of the basal machinery. For further details, see text. Figure has been previously published. Reprinted here from Fandrey et al. 2006, with permission from the European Society of Cardiology.

subunit (Huang et al. 1996), originally known as ARNT (aryl hydrocarbon receptor nuclear translocator), thus producing complexes HIF-1, HIF-2, and HIF-3. As shown in Fig. 1, the HIF oxygen sensor that controls the abundance of the α-subunits as a function of pO_2, is a family of novel prolyl hydroxylases named PHD1-3 (prolyl hydroxylase domain containing 1-3; Epstein et al. 2001). As members of the 2-oxoglutarate-dependent dioxygenase superfamily, the PHDs catalyze the Fe(II)- and O_2-dependent hydroxylation of two specific prolyl residues within the oxygen-dependent degradation domain (ODD) of the HIF-1α/-2α factors (Pro^{402} and Pro^{564} in human HIF-1α). Once hydroxylated, HIF-1α/-2α rapidly bind to the von Hippel–Lindau (VHL) tumor suppressor protein that acts as 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 (Maxwell et al. 1999). The efficacy of this protein-level mode of control is mirrored in cell culture 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, that of a single asparagine within the C-terminal transactivation domain of HIF-1α or HIF-2α (Fig. 1: TAD-C; Asn^{803} in human HIF-1α) by 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. This, in turn, prohibits the transactivation of target genes under high pO_2 (Lando et al. 2002). The absolute requirement of PHD1–3 and FIH-1 activities for oxygen and Fe(II) has made these hydroxylation reactions susceptible to inhibition by severely hypoxic and anoxic pO_2. That is why, during sufficiently low pO_2, the α-subunit is free to escape proteolytic degradation and, assisted by accessory factors (e.g., HSP90) and/or

stimulatory kinase signals, to efficiently translocate into the nucleus. Here HIF-1α/-2α dimerizes with ARNT and the resulting αβ-heterodimer associates with transcriptional cofactors (e.g., p300/CBP), thereby activating or suppressing target genes at HIF binding sites (Fig. 1). Studies in hypoxia tolerant invertebrate model systems have immensely contributed to this state of knowledge of HIF-mediated hypoxic signaling. For example, the discovery of mammalian PHD1-3 proteins functioning as HIF O_2 sensors was made possible through the identification of a *C. elegans* ortholog (EGL-9, egg-laying abnormal 9) and the fact that this dioxygenase regulates HIF function by prolyl hydroxylation in the nematode in a well conserved HIF-VHL-prolyl hydroxylase pathway (Epstein et al. 2001). Since hypoxia and activation of HIF-1/-2 correlate with malignant progression and poor prognosis in many cancers, and since tumor hypoxia forms a source of resistance to many of the current surgical, chemical, and radiation therapies, efforts to use model organisms for further analysis of the HIF signaling pathway continue to grow. This 12-speaker symposium summarized our contributions and new insights from model organisms and human pathologies to further elicit, integrate, and exploit incoming signals and outgoing effects of the HIF pathway across taxonomies and physiologies.

To set the stage, the first article by *Thomas Gorr* introduced tumor hypoxia both as a concept and a menace in today's cancer treatments. He then compared typical hypoxic defenses in tumors with those of hypoxia sensitive and hypoxia tolerant, healthy animal tissues, respectively. Next, *Keith Webster*'s contribution portrayed the overall molecular evolution of various hypoxia response pathways that evolved in prokaryotes, yeasts, as well as in multicellular plants and animals. As HIF signaling is, thus far, exclusive for animals, this article concluded with a brief summary of HIF's most widespread and, probably, primordial function: the up-regulated expression of glycolytic enzyme genes for an elevated substrate flux and ATP production by anaerobe glycolysis (i.e., the Pasteur effect). The phylogenetic section then began with two articles dedicated to the hypoxia tolerant nematode *C. elegans*. In the first of these articles, *Jo Anne Powell-Coffman* presented her group's latest findings in support of a 2-fold regulation of *hif-1*, the HIF-α homolog in the nematode, by EGL-9: (1) via the classical VHL-dependent pathway that regulates the oxygen-dependent degradation of the *hif*-1 protein (see above and Fig. 1) and (2) via a novel VHL-independent pathway in which EGL-9 inhibits *hif*-1 activity, as assayed by expression of *hif*-1 target genes. Data by *David Hoogewijs*, on the other hand, suggested induction of multiple *C. elegans* globin-like genes under anoxia by *hif-1*-dependent regulation. If confirmed, this result will, even for ''lowly'' nematodes that survive without any angiogenic or erythropoietic components to their defensive arsenal, dramatically underscore the importance of inducible tissue globins (and other respiratory pigments?) in the recovery of oxygen homeostasis after a hypoxia assault. Moving on to crustaceans, *Shinichi Tokishita* and *Hideo Yamagata* recently cloned the cDNAs of the HIF-α and -β (ARNT) subunit homologs of the cladoceran *Daphnia magna*. By making use of conserved protein–protein interactions, the *D. magna* HIF-α cDNA was actually isolated after a yeast-two-hybrid screen identified its expressed product as interactor with a *D. magna* ARNT bait. The current article presents *in situ* evidence for tissue specific expression patterns of *Daphnia* HIF-α/-β subunits, including the presumed O_2 respiring epipodites of adult waterfleas. In contrast to *Daphnia*, higher crustaceans such as decapods express not globins but the blue, copper-containing hemocyanins as extracellular respiratory pigments. *Nora Terwilliger*'s laboratory added the complete cDNA of the HIF-α homologue of the Dungeness crab *Cancer magister* to the growing body of invertebrate HIFs. Multiple candidate HREs in the upstream regions of four of the six hemocyanin genes in *C. magister* imply hypoxic, and possibly even nonhypoxic, sensitivity of hemocyanin production in decapods. The invertebrate section closed with *Thomas Hankeln*'s and *Thorsten Burmester*'s surprising discoveries of tissue–globin expression in insects, a taxon that, due to the exquisite O_2 supply from trachea, was supposed not to need any diffusive aid. Hankeln and Burmester also showed that *Drosophila* expresses at least three globins, of which the dominant *glob1* is found, as yet another surprise, to be hypoxia-suppressed in embryonic cells or specimens. In contrast, larvae slightly elevate *glob1* transcription after intermittent hypoxia. Now, we only need to know what these globins are actually doing in insect tissues.

Since a given volume of water contains only 1/30th of the amount of oxygen that is in the same volume of air at the same partial pressure, and since the rate of diffusion of oxygen in water is 10,000 times slower than it is in air, oxygen availabilities are, as expected, far more variable and act far more selectively during the evolution of aquatic organisms than is the case for air breathers. No wonder then that fishes have come forward as

the vertebrate champions in displaying not only extremely diverse, but also protective, adaptations to varying degrees of hypoxia. This topic was squarely addressed by the following two presentations. First, *Kalle Rytkönen* and *Mikko Nikinmaa* reported on their recent comparison of variation in HIF-1α sequences amongst several fish species, which took into account a total of eight newly determined cDNAs. They further attempted to correlate this variation, particularly the distribution of redox-sensitive cysteines, with the development of tolerance to hypoxia in these teleosts. Next, *Doris Abele*, *Katja Heise*, and *Magnus Lucassen* examined HIF activation in temperate versus cold-adapted teleosts. Their previous work had already noted the ''early onset'' (at higher pO_2 levels) stabilization of fish HIF-1α-proteins, relative to mammalian factors, which had been attributed to cysteine-enriched sequences within the ODD and TAD-C domains. Now, they support a heightened redox-regulation of fish HIF by contrasting increased DNA binding under the more reducing conditions that follow acute cold shock (temperate species) with signs of oxidative stress and comparatively lower constitutive levels or activities of HIF-1α (polar species). The section on mammalian HIF signaling was launched by the contribution by *Joachim Fandrey* on live imaging of the activation and assembly of the HIF-1 complex in hypoxic nuclei. State-of-the-art 2-photon-laser-microscopy and fluorescence resonance energy transfer (FRET) techniques afforded a very high degree of resolution and the unambiguous demonstration that, after photobleaching, HIF-1 subunits return independently, yet somehow coordinatedly, to the same specific loci within the nucleus, presumed to be HRE motifs within the chromosomes. The final two reports gave examples of the pathophysiological site of deranged HIF signaling in cancer. Interestingly, both reports indicated HIF-2α, rather than HIF-1α, to be the tumor progressing culprit. Evidence from *Patrick Maxwell*'s recent work on Ewing's sarcoma indicated that hypoxia not only activates angiogenesis, it also promotes vascular mimicry by the tumor cells as well, which results in tumor-lined channels that are linked to the circulation and may be important in providing a ''venous'' drainage system. The clearest case in support of HIF's role in cancer, however, emerged from the impressive genetic evidence that links loss-of-function of the VHL tumor suppressor with the commonest form of kidney cancer: clear-cell renal-cell carcinoma. Particularly informative is VHL disease caused by an inherited mutation in the VHL gene. This carries a very high risk of clear-cell kidney cancer. Following somatic ''second-hit'' inactivation of both VHL alleles, HIF-α subunits are constitutively stabilized in large numbers of pre-malignant foci in the kidney. As a result of HIF activation, these foci show a markedly downregulated expression of the intercellular adhesion molecule, E-cadherin, which might suggest a potential mechanism by which loss of VHL function could contribute to the evolution from precancerous lesions to renal carcinoma. An added requirement for frank tumors to emerge in the kidney is a shift in expression from HIF-1α to HIF-2α. Another HIF-2α driven cancer is the childhood tumor neuroblastoma. As presented by *Sven Påhlman*, hypoxic neuroblastoma cells are known to dedifferentiate by reducing expression of neuronal markers and developing into a less mature, more aggressive phenotype. In support of nonredundant functions in this cancer, the HIF-1α subunit was found to be transiently induced by 1% O_2, while HIF-2α gradually accumulated in mildly deprived (5% O_2) neuroblastoma cells as well as in tumor regions adjacent to capillaries. To better comprehend such disparate induction profiles and oxygen sensitivities of the different HIF-α isoforms, future studies need to correlate PHD and FIH specificities and activities more closely to either subunit, and/or to reveal additional mechanisms in control of these transcription factors.

This concluded the cross-taxa/cross-physiology, and most importantly, cross-boundary journey on oxygen sensing and HIF signaling. May this ''true meeting of disciplines'' at the ICRB 2006 inspire and initiate fruitful exchanges and collaborations to further enhance our understanding of hypoxic signaling, and through it, the fight against cancer.

Trio vitale: hypoxia responses in sensitive, tolerant, and tumorigenic tissues (T.A. Gorr)

In contrast to the highly oxygen-dependent and homeostatic organs and tissues of adult mammals (Krogh 1919), most solid tumors develop regional radiobiological hypoxia as they accumulate mass (Thomlinson and Gray 1955). Since vascular beds in neoplasms are generally comprised of immature, disarrayed, and poorly functional capillaries, the tumor will ultimately outgrow vessel-to-cell oxygen diffusion distances that can range from ~50 to 230 μm (Tannock 1968, 1972). The resulting breach of O_2 supplies (low) and demands (high) triggers a severely limited and extremely heterogeneous oxygenation of the tumor tissue with oxygen partial

pressures (pO_2) frequently <5 mmHg ($\approx 0.7\%$ O_2) [reviewed by Vaupel et al. (1998) and Brown and Wilson (2004)]. Several groups recently elucidated metabolic shifts in O_2-deprived cancer cells that go a long way towards understanding the multifaceted resistance by which human tumors can cope with these erratic and, at times, completely lacking supplies of oxygen, glucose, and other blood-born nutrients [reviewed by Höckel and Vaupel (2001), Wouters et al. (2004), and Feldman et al. (2005)]. Curiously enough, these tumor adaptations appear to recapitulate main paradigms that previously had emerged as the quintessential defense of hypoxia *tolerant* invertebrates, ectotherms, and neonatal or hibernating endotherms. Inspired by these conceptually novel contributions by noted physiologists to tumor biology (Hochachka et al. 2002), this article will continue down their ''critter-to-cancer'' linking path through a brief review of tumor hypoxia and its correlation with the prevailing safeguards against low pO_2's in three existing frameworks: (1) hypoxia sensitive, healthy (=nontransformed) tissues; (2) hypoxia tolerant, healthy tissues, and (3) hypoxia tolerant, transformed tissues.

Tumor hypoxia manifests itself on many levels. It renders transformed cells more radio-resistant, promotes their growth through angiogenesis and increases the odds for local recurrences, metastasis or genetic lesions. It also is known to select for the expansion of apoptosis-defying cell variants [reviewed by Semenza (2003), Vaupel (2004), Maxwell (2005), and Zhou et al. (2006)]. In short: a low oxygen microenvironment drives malignant progression towards a more invasive, therapy-resistant phenotype. Many, if not most, of these adverse changes during human tumorigenesis are conferred by altered gene expression profiles that are mediated through the oxygen sensing response pathway of the hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) [reviewed by Semenza (2003), Vaupel (2004), Maxwell (2005), and Zhou et al. (2006)]. Due to scarcity in oxygen supply, and/or hyperactive oncogenic upstream signals, HIF's regulatory subunits (HIF-1α, HIF-2α) are frequently overexpressed in multiple malignancies as opposed to undetectable levels in healthy, oxygenated tissues. Consequentially, HIF-1α/-2α protein abundance can serve for many, but not all, cancers as independent prognostic marker for disease progression and poor clinical outcome. Thanks to global research efforts of the past decade, we are now able to unquestionably link tumor hypoxia and the subsequent activation of HIF-1/-2 signaling to the specific reprogramming of cellular fates and physiology, whereby, on balance, the tumor's survival and growth is promoted. Cellular transformation is, however, not the only way to acquire stress resilience. Many invertebrate, and some lower vertebrate, species are similarly endowed to withstand and recover from otherwise lethal limitations in oxygen availability. Of note in this context, the pioneering work of Hochachka, Boutilier, and many others, which laid down the unifying principles of stress defenses in tolerant animal models, (Hochachka 1986; Hochachka et al. 1996; Boutilier 2001), also had a tremendous impact on studies regarding the role of hypoxia in the genetic underpinnings of tumor development (Murphy 2004).

Oxygen tensions in normal tissue (e.g., skeletal muscle), unlike those in tumors, are, even under the most extreme work loads, in a near-perfect supply/demand homeostasis (reviewed by Hochachka 1999). This exquisite equilibrium between metabolite delivery and metabolic output works well across a wide range of pO_2. As long as ambient oxygen tensions exceed a critical threshold (pC), usually occurring at a tension of $\sim$1.8–18 mmHg ($\sim$0.2–2% O_2) (Rumsey et al. 1990; Arthur et al. 1999; Gnaiger 2003), the mitochondrial electron flux is substrate saturated and proceeds at or near its maximal velocity. Thus, O_2 uptake and the ensuing oxidative metabolism function more or less independently from supplies (Gnaiger 2003). Upon chronic (minutes–hours) declines of tissue pO_2 to near-pC levels, however, the typical array of *physiological* responses during fetal and adult stages of mammalian development include hypoxia-induced:

(a) *erythropoiesis* (production of red blood cells);
(b) *angiogenesis* (development of new blood vessels from pre-existing ones);
(c) *vasodilation* (lumen widening due to relaxation of smooth muscles in vessel wall);
(d) *high-flux glycolytic carbohydrate consumption* (the Pasteur effect);

and, upon further deprivation of oxygen:

(e) *initiation of apoptotic* (programmed) *or necrotic* (accidental) *pathways of cell death.*

With currently several hundred potential (Manalo et al. 2005) and 70 validated hypoxia-responsive gene targets (Wenger et al. 2005), including those for critical erythropoietic or angiogenic growth factors and nearly all glycolytic enzymes, oxygen sensing via HIF-1/-2 signaling is clearly the key in controlling each one of the above defense categories in *healthy* tissues (reviewed by Schofield and Ratcliffe 2004; Semenza 2004; Fandrey et al. 2006). As hallmarks of hypoxia sensitive mammalian organs (e.g., brain,

kidney, and liver) (Duffy et al. 1972; Suarez et al. 1989), the earlier-listed *energy compensating strategies* function to either keep vascular O_2-carrying capacities steady (*a–c*), or, at least for brief periods of time (seconds–minutes), to maintain preexisting rates of ATP demand during periods of dwindling oxidative ATP synthesis (*d*). Upon HIF-mediated induction of the genes that launch the Pasteur effect [see Webster 1987; Iyer et al. 1998; Seagroves et al. 2001; Stubbs et al. 2003; Webster 2003 and Webster (subsequently)], however, this high-flux glycolysis quickly depletes finite stores of fermentable substrate (e.g., glycogen) and amasses toxic levels of end products (e.g., H^+ per ATP hydrolysis) in sensitive, hypoxic cells. In the absence of energy expending reductions, anaerobe fermentations fail to meet ATP maintenance demands of ionic and osmotic equilibrium, and are thus unable to prevent an ultimately fatal ATP imbalance in central neurons, renal tubular cells, or hepatocytes (Duffy et al. 1972; Buck and Hochachka 1993; Hochachka et al. 1996; Krumschnabel et al. 2000; Boutilier 2001).

Hypoxia tolerant animal models, on the other hand, predominantly rely on *energy conserving strategies* that center around a stress-induced and stress-maintained, yet fully reversible, suppression of the metabolic rate (hypometabolism) (reviewed by Gorr et al. 2006a). This metabolic suppression down to a newly balanced ATP supply = ATP demand steady-state, prevents lethal falls in cellular ATP levels and is the single most protective and unifying feature of nontransformed hypoxia tolerant tissues (Hochachka et al. 1996; Boutilier 2001). The lower the ATP turnover can be suppressed, the longer the cells remain protected during hypoxic or anoxic challenges (Guppy and Withers 1999; Krumschnabel et al. 2000). To match the synchronously declining ATP production in O_2-depleted cells, hypometabolism requires the immediate and coordinated down-regulation of every major ATP-utilizing function in the cell, including (1) protein synthesis and degradation; (2) ion-motive ATPases, notably Na^+/K^+-ATPase; and (3) gluconeogenesis (Rolfe and Brown 1997). Therefore, the large-scale drop in protein turnover (Land et al. 1993; Land and Hochachka 1994) and ion-motive pump activities maintain, as a tolerance hallmark, electrochemical gradients across membranes at reduced permeabilities (*channel arrest*) (Buck and Hochachka 1993; Krumschnabel et al. 2000). As further advantage of hypometabolic states, glycolytic fluxes only need to be elevated and provide for ATP as much as the residual energy expenditures require (i.e., weak-absent Pasteur effect) (Storey 1985; Grieshaber et al. 1994; Schmidt and Kamp 1996). Hence, entry into a hypometabolic state spares fermentable fuel, reduces metabolic waste and extends survival time. Along with hypometabolism, cells also enter a state of quiescence due to a slowing down, or complete blockade, of cell cycle proliferation from defined hypoxia-sensitive checkpoints. Finally, since ~80% of mitochondrial O_2-consumption is coupled to ATP synthesis (Rolfe and Brown 1997), transitions into anaerobiosis and metabolic depressions are indicated by proportionally declining oxygen consumption rates. Particularly tolerant systems show extended oxygen-dependent respiration even under mild hypoxia (Pörtner and Grieshaber 1993; Gnaiger 2003). This so called oxyconformance is characterized by O_2 uptake rates that decline proportionally with ambient pO_2. It contrasts with the regulated respiration of most cells in culture, where the rate of O_2 uptake remains constant and independent of pO_2 across a wide range of oxygen tension. Mechanistically, the switch from oxyregulated to oxyconforming respiration is, in mammalian cells at least, also orchestrated via HIF-1. During $pO_2 \leq pC$, the HIF-1-induced pyruvate dehydrogenase kinase 1 (Pdk1) inhibits pyruvate dehydrogenase from using pyruvate to fuel the mitochondrial tricarboxylic acid (TCA) cycle. This TCA block was recently shown to actively suppress respiration, redirect both O_2 and glucose utilization towards cytosolic sinks, and rescue cells from hypoxia-induced apoptosis (Kim et al. 2006; Papandreou et al. 2006; Simon 2006).

Tumors typically survive conditions of "little" or "no" oxygen through a mixed repertoire of energy-compensating and energy-conserving defenses, respectively. As established by Folkman and colleagues, tumors depend on the process of angiogenesis for growth beyond a few millimeters in size and for subsequent spreading to other sites (reviewed by Hanahan and Folkman 1996; Carmeliet and Jain 2000). However, angiogenetic capacities and competence vary greatly among types of tumors. Pronounced angiogenetic switches are known to occur in rapidly proliferating, metabolically active cancers of the brain (e.g., glioblastomas) and kidney (e.g., renal cell carcinomas), less so in mammary carcinomas, and least in lung and prostate carcinomas (Eberhard et al. 2000). Moreover, oxyregulated respiration of cancer cells is commonplace and reveals critical thresholds ranging from ~0.15% O_2 for Ehrlich ascites cells (Froese 1962) to ~1.5% O_2 for neuroblastoma cells (Robiolio et al. 1989). Respiratory and metabolic switches, it seems, occur with corresponding oxygen sensitivities in normal

and neoplastic tissues. Along similar lines, cultured aerobic cancer cells are not inherently more glycolytic than are normal ones, as transformed and nontransformed cells alike partition their total normoxic ATP production into ~80% oxidative and ~20% glycolytic contributions (Guppy et al. 2002; Zu and Guppy 2004). Rather than being causally linked with tumorigenesis, these data reinforce that prominent glycolysis only reflects active proliferation (Brand and Hermfisse 1997) and/or exposure to low pO_2 (above) of any kind of cells (discussed by Stubbs et al. 2003). However, near-anoxic cancer cells (Ebbesen et al. 2004) and perinecrotic layers in spheroids or experimental tumors can also adapt to diffusion-limited nutrient supplies via energy-conserving strategies of metabolic depression and quiescence. "Critter-like," these cancer cells *halt* growth (reviewed by Giaccia 1996), reduce O_2 consumption rates and, thus, efficiently alleviate local gradients of hypoxia. They lower ATP consumption by (1) inhibiting DNA replication, (2) inhibiting the majority of mRNA and protein syntheses, and (3) arresting cell cycle proliferation at a single G1/S (hypoxic pO_2) transition point or multiple (anoxic pO_2) transition points (reviewed by Höckel and Vaupel 2001). "Critter-Unlike," our information to date is wholly inadequate in regard to relative changes of activities and ATP demands on ion pumps (e.g., Na^+/K^+-ATPase) to generally assess if, and how, whole-cell membrane currents and ion transport are maintained in O_2-deprived neoplasms (but see, Wodopia et al. 2000; Karle et al. 2004). In contrast, several groups have recently made strides towards discerning the molecular mechanisms by which hypoxic/anoxic cancer cells downregulate O_2 consumption (i.e., HIF-1/Pdk1 switch, mentioned earlier) and mRNA translation (Wouters et al. 2003, 2004; Feldman et al. 2005).

The greatly simplified classification of hypoxia responses in Fig. 2 shows for transformed cells and solid tumors an overall mixed pattern of adaptations, whose individual aspects will vary with the type or stage of tumor, the host microenvironment and the severity of hypoxia. One way for gaining tolerance that seems unique to tumors (Fig. 2) is the hypoxia-mediated selection of apoptosis-resistant cell variants that acquired mutations in apoptosis-triggering tumor suppressor genes such as p53 (reviewed by Wouters et al. 2004; Zhou et al. 2006). Indicated through the intersection with hypoxia sensitive physiologies, the energy compensating hypoxia responses in tumors are, at least in part, inherited from the hypoxia-sensitive precursor tissue, yet can certainly be modified and exaggerated. This "first

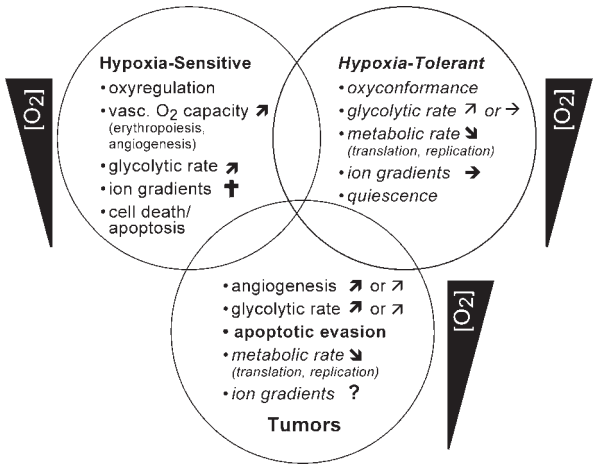

Fig. 2 Venn diagram of different hypoxia responses. Tumor responses in general consist of a mixed pattern with some defensive components derived from the hypoxia sensitive precursor tissue (regular text), while others emerged as newly developed defenses that gain the tumor a measurable degree of hypoxia tolerance (italic text). Evading apoptosis seems to be a defense unique to progressing oncogenesis (boldface type). Intersecting areas in the diagram symbolize great variability in the overlaps between three response categories. Controlled regulations: process induced: arrow tilted up; process maintained: horizontal arrow; process suppressed: arrow tilted down; uncontrolled insult of process: cross; bold arrows: strong response; thin arrows: weak response.

line of defense" is induced by more or less moderate hypoxia (i.e., $pO_2 \sim 0.15$–1.5% $O_2 \cong$ pC) and includes the HIF-mediated activation of angiogenesis along with a variably strong Pasteur effect. During severe O_2 limitation (i.e., $pO_2 \ll$ pC), transformed and malignant cells defend themselves by an immediate switch into O_2-conforming respiration (Fig. 2) and entrance into energy-conserving hypometabolic and quiescent stages (intersection to hypoxia tolerant responses). Intervening with these tolerance-conferring defenses which, physiologically, set the tumor apart from its hypoxia-sensitive host organ, holds great potential for more selective cancer diagnostics and therapies (Wouters et al. 2003, 2004; Feldman et al. 2005). In contrast, intervening with anaerobe glycolysis will, as a more or less ubiquitous response, affect transformed and healthy hypoxic tissues alike. Unfortunately, relative to angiogenetic or glycolytic switches, the existence of hypometabolic, quiescent states in hypoxic cancer cells and tumors has yet to emerge as mainstream tenet within the oncological community. It is here where resilient "critters" with sequenced genomes such as *C. elegans*, *Drosophila*, zebrafish, and others to

follow (e.g., *Daphnia*) can help pave the way for characterizing, and eventually blocking, key players and pathways that underlie tolerance to hypoxia in human pathologies.

Molecular evolution of the regulation of glycolytic enzyme genes by hypoxia (K.A. Webster)

Life evolved on earth for about 2 billion years under anaerobic conditions, close to one half of the total time period of biological evolution (Barnabas et al. 1982; Papagiannis 1984). Therefore, the fundamental features of biology and genetics, including DNA synthesis, transcription, translation, and their regulation were established under anaerobic conditions (Segerer et al. 1985). Perhaps because of this, certain proteins, pathways, and regulatory processes function preferentially under hypoxia. This is particularly true for glycolysis, where oxygen regulates both enzyme activity and gene expression (Webster 1987; Webster et al. 1990). The regulation involves contributions from at least four separate molecular pathways, some of which may have been conserved through 4 billion years of evolution dating back to the anaerobic origin of life. There are numerous molecular modulators of glycolytic flux, the most famous of which was reported by Louis Pasteur (1861). He showed that oxygen inhibited fermentation and that glucose consumption was inversely proportional to oxygen availability, i.e., that glycolysis was positively regulated by hypoxia (Pasteur effect) (see Gorr earlier). Over a century later, in 1987, our group reported that the expression of multiple glycolytic enzyme genes was also coordinatedly regulated by oxygen tension, and that there was a reciprocal effect on the transcription of some mitochondrial genes (Webster 1987; Webster et al. 1990). Whereas the Pasteur effect was probably a rather early acquisition in the regulation of glycolysis by oxygen tension, the control of gene expression by hypoxia appears to have evolved gradually and possibly in a series of stages. Figure 3 illustrates some of the DNA sequence elements that may have been involved in the evolution of this regulation.

Oxygen-regulated gene switching in bacteria and archaea includes the activation and/or repression of genes and operons of key metabolic enzymes (Bunn and Poyton 1996). This includes positive and negative factors regulated by oxygen tension or redox potential and involves contributions of at least three major regulatory pathways, including the ARC, FNR, and CSRA–CSRB systems.

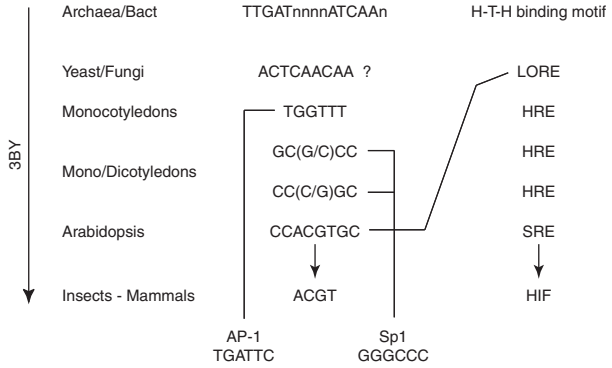

Fig. 3 Evolution of hypoxia response elements of glycolytic enzyme genes. The earliest oxygen response element is found in archaebacteria and bacteria; the sequence resembles a classical helix-turn-helix (H-T-H) DNA binding motif and binds the factor FNR. The low oxygen response element (LORE) of yeast binds a protein that is activated by hypoxia and transition metals reminiscent of the later HIF-1 pathway. Hypoxia response elements (HREs) and stress response elements (SREs) of plants have sequences that resemble AP-1, Sp1, and HIF-1 sites, each of which can function as hypoxia responsive elements in insects, fish, and mammals.

Features of the ARC and FNR systems suggest that they may be the predecessors of the hypoxia regulation of eukaryotic glycolytic enzyme genes. The ARC system is involved in the repression of aerobic functions under anaerobic conditions, whereas the FNR system is involved in the anaerobic induction of metabolic enzyme genes (Bunn and Poyton 1996). FNR, like HIF-1α, is a transcription factor with redox-regulated cysteine residues that determine conformation and DNA binding (see Abele et al. subsequently). The target sequence for activated FNR includes the consensus sequence nTTGATnnnnATCAAn, which is a typical palindromic binding site for helix-turn-helix motifs common to many mammalian transcription factors, including HIF-1. This is perhaps the earliest example of a redox-regulated helix-turn-helix transcription factor involved in the regulation of bioenergetic genes. Yet, there are even closer parallels between these regulatory pathways and those that regulate glycolytic enzyme genes in higher mammals. In the yeast, *Saccharomyces cerevisiae*, the OLE1 gene encodes a delta9 fatty acid desaturase that is essential for the synthesis of unsaturated fatty acids. OLE1 is induced by hypoxia, transition metals, and iron chelators. An element in the OLE1 gene promoter with the sequence ACTCAACAA is responsible for the response to hypoxia. This element named LORE (low oxygen responsive element) can confer hypoxia inducibility to a heterologous promoter and binds a

specific hypoxia-inducible protein. Additional LORE elements have been identified in the promoters of other hypoxia-inducible genes, suggesting a mechanism for a global synchronized response to hypoxia in *S. cerevisiae*. This may be the earliest evidence of a regulatory system capable of mediating a global response of multiple unlinked genes to changes in oxygen tension. Although the sequence of the LORE does not resemble any identified mammalian LORE (Fig. 3), the common responses of *S. cerevisiae* LOREs and mammalian HREs to hypoxia, transition metals and iron chelators suggest related pathways (Vasconcelles et al. 2001; Jiang et al. 2001b).

Exposure of maize root cells to hypoxia results in the induction of approximately 20 proteins, deemed anaerobic polypeptides (Dennis et al. 1988; Olive et al. 1991; Dolferus et al. 1994). Two anaerobic response elements (ARE) were identified in the proximal promoters of the aldolase and ADH1 (alcohol dehydrogenase1) genes. The first site contained the consensus sequence TGGTTT and the second site contained the consensus GC(G/C)CC (Olive et al. 1991). Mutation of these elements resulted in the loss of response to hypoxia. Further studies revealed the specific binding of a protein to the GC-rich element and this protein was designated GCBP-1 (GC-rich binding protein-1). This protein has not been fully characterized; its abundance is not changed by hypoxia, it requires accessory proteins and/or posttranslational modifications to mediate transcriptional activation by hypoxia, and its binding to the GC site is in competition with members of the SP1 family of zinc finger transcription factors. There are strong parallels between this regulatory pathway and that described recently for the regulation of mammalian muscle-specific pyruvate kinase (PKM) and β-enolase genes (Discher et al. 1998). These elements represent the earliest examples of hypoxia response elements directly controlling individual glycolytic enzyme genes. Plants also provide a clue as to how hypoxia response elements were selected from other stress response pathways of regulation, including temperature and osmolarity, both of which featured significantly as evolutionary selective pressures. Hypoxia, dehydration, and hypothermia induce the ADH gene in the roots of the dicotyledon, *Arabidopsis thaliana*. The promoter contains a single GT/GC motif, which has a similar sequence to the *Zea mays* GC site, except that the GT motif is in reverse orientation (Dolferus et al. 1994). The *Arabidopsis* ADH promoter contains a second motif with the sequence CCACGTGC. The core sequence of this motif, ACGTG, is the binding site for HIF-1, the major hypoxia regulatory binding protein in mammalian cells (see above and Fig. 1). Interestingly, this motif appears to be required for ADH gene induction by hypothermia, dehydration, and UV light, but not hypoxia, whereas the hypoxia response is determined by the GT/GC sequence.

The regulation of glycolytic enzyme genes by hypoxia in nematodes, insects, crustaceans, fish, reptiles, birds, and mammals and possibly all mobile multicellular species is multifactorial, with clear origins in the prokaryotic and fungal regulatory systems (Webster and Murphy 1988; Hochachka and Lutz 2001). Animal glycolytic enzyme genes are regulated both coordinatedly and individually by hypoxia-responsive transcription factors including HIF-1, SP-1 family factors, AP-1, and possibly metal response elements (Murphy et al. 1999). HIF-1 is probably the main component and is largely responsible for coordinating the induction (Webster et al. 2000; Webster 2003). The core consensus sequence for HIF-1 binding is ACGT, and active HIF-1 binding sites have been reported in at least eight glycolytic-enzyme genes, usually in the proximal promoter regions (reviewed by Wenger et al. 2005).

In conclusion, HIF functions in nematodes, insects, crustaceans, and vertebrates but not in plants or fungi, and it is possible that the pathway developed in the Silurian period about 500 million years ago, when highly mobile metazoans were evolving. The sequence ACGTC is essential, although not sufficient, for gene activation by HIF-1. The same sequence is required for the hypothermia, dehydration, and the UV response of *Arabidopsis* genes involved in carbohydrate metabolism. It seems likely that this recognition sequence, and the protein that binds it, are related in plants and animals, and thus may provide the link between gene regulation in hypoxic root tips and the HIF-1 pathway seen from "worm to man" (Webster et al. 2000).

Genetic analysis of hypoxia signaling and response in *C. elegans* (J. A. Powell-Coffman)

Regulation of HIF-1 protein levels by the EGL-9 enzyme and the VHL tumor suppressor is evolutionarily conserved. The *C. elegans* homologs of the HIF-1α and HIF-1β subunits are *hif-1* and *aha-1*, respectively (Powell-Coffman et al. 1998; Epstein et al. 2001; Jiang et al. 2001a). The majority of the transcriptional responses to hypoxia require *hif-1* function, and *hif-1* loss-of-function mutants fail to adapt to hypoxia, yet thrive in normoxia and are able to survive anoxia-induced arrest (Jiang et al. 2001a;

Padilla et al. 2002; Shen and Powell-Coffman 2003; Shen et al. 2005). *Caenorhabditis elegans* is amenable to both forward and reverse genetic approaches, and it is an immensely powerful model system for gene discovery and characterization of genetic networks (Jorgensen and Mango 2002; Gunsalus and Piano 2005). The *egl-9* encodes a member of the 2-oxoglutarate-dependent oxygenase superfamily (Darby et al. 1999; Aravind and Koonin 2001). When oxygen levels are sufficiently high, EGL-9 hydroxylates HIF-1. The *C. elegans* ortholog of the von Hippel–Lindau tumor suppressor, VHL-1, then targets HIF-1 for degradation (Epstein et al. 2001) (see above and Fig. 1). There are three mammalian homologs of EGL-9, and they are termed PHD, HPH, or EGLN genes (Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2002).

The *C. elegans egl-9* clearly has functions independent of *vhl-1*. The *C. elegans egl-9* gene was originally isolated as a gene required for normal egg-laying (Trent et al. 1983). More recently, *egl-9* mutants have also been shown to have decreased sensitivity to cyanide (Gallagher and Manoil 2001). The *vhl-1*$^{-/-}$ mutants have not been shown to have either of these defects. Bishop et al. (2004) showed that while *egl-9* RNAi increased expression of a HIF-1 target gene in a *vhl-1* mutant, the dioxygenase inhibitor 2,2′-dipyridyl (DIP) did not. This suggested that some *egl-9* functions may not require prolyl hydroxylation of HIF-1. The Powell-Coffman group compared the *vhl-1*$^{-/-}$ and *egl-9*$^{-/-}$ phenotypes more extensively, and determined that, while HIF-1 target genes are expressed at much higher levels in *egl-9* mutants, HIF-1 protein is expressed at similar levels in *vhl-1*- and *egl-9*-defective animals (Shen et al. 2006). Collectively, these data are consistent with a model in which EGL-9 acts via two pathways to regulate HIF-1 function: (1) EGL-9 hydroxylates HIF-1 and targets it for VHL-1-dependent degradation; (2) EGL-9 acts via a second VHL-independent pathway to inhibit HIF-1 expression or activation, and this pathway may not require EGL-9 hydroxylase activity (Fig. 4) (Shen et al. 2006).

The Powell-Coffman group has also developed genetic screens to identify and characterize HIF-1 regulatory networks in *C. elegans*, and they have isolated several ''rhy'' mutations (regulators of the hypoxia-inducible factor). Mutations in the *rhy-1* gene cause over-expression of HIF-1 target genes and morphological defects that are similar to those exhibited by *egl-9* mutants. These defects are suppressed by a loss-of-function mutation in *hif-1*. Expression of HIF-1 target genes is higher in *rhy-1*(*ok1402*); *vhl-1*(*ok161*) double mutants than in single mutant and this suggests that RHY-1 and VHL-1 act via two independent pathways in limiting expression of these HIF-1 targets. The *rhy-1*(*ok1402*) mutation causes a slight increase in *hif-1* mRNA (1.8-fold) and HIF-1 protein (2.2-fold) levels. This increase in HIF-1 protein levels is smaller than that caused by loss-of-function mutations in *vhl-1* or *egl-9* (which over-express HIF-1 protein 4.8-fold and 4.5-fold, respectively). These data are consistent with a model in which RHY-1 has a minor effect on HIF-1 protein expression and functions primarily in a VHL-1-independent pathway to limit HIF-1 function (Fig. 4) (Shen et al. 2006). Important questions for future studies include: How does RHY-1, an integral membrane protein, inhibit expression of HIF-1 target genes? What proteins or pathways interact with RHY-1 to regulate HIF-1 function? Do similar proteins regulate mammalian HIF?

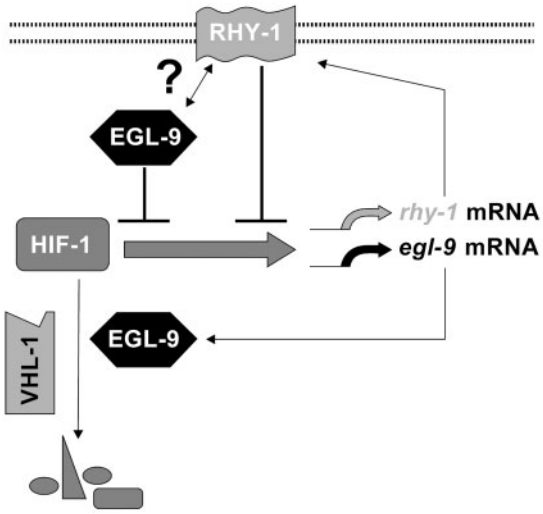

Fig. 4 Model for regulation of *C. elegans* HIF-1 by multiple negative feedback loops. When oxygen levels are high, the EGL-9 enzyme hydroxylates HIF-1. This covalent modification allows VHL-1 to target HIF-1 for degradation. Genetic data in *C. elegans* show that EGL-9 also inhibits HIF-1 activity via a separate, VHL-1-independent pathway. Additionally, *C. elegans* RHY-1 functions in a negative feedback loop to inhibit HIF-1 function.

Quantitative expression analysis of *C. elegans* globins under anoxic conditions (D. Hoogewijs)

The genome of *C. elegans* encodes at least 33 putative globins (Hoogewijs et al. 2004; Vinogradov et al. 2006). Their sequences all align with the classical globin fold composed of eight helices (A–H), and, consistently possess the invariant proximal histidine

at position F8 (i.e., the eighth residue in the F-helix). All putative *C. elegans* globins have orthologs in the sibling species *C. briggsae* and *C. remanei* and some display significant similarity in sequences with BLASTP Expect (E) values ranging from e^{-02} to e^{-12} to vertebrate myoglobin, neuroglobin, and cytoglobin (the *E*-value describes the number of expected hits that occur by chance for similarly scoring matches between the query and a database entry). Comparison of all globin-like sequences revealed major differences in genome organization, gene structure, and expression profiles (Hoogewijs et al., submitted for publication). Phylogenetic analysis has shown that the majority of these globins are highly divergent from each other, indicative of long and independent evolutionary histories within the gene family (Hoogewijs et al. 2004). Preliminary analysis of spatial expression suggests that many of the *C. elegans* globins are expressed in tissue-specific patterns. Evaluation of their relative abundance using real-time quantitative PCR (qPCR) experiments shows, moreover, that all globin genes are transcribed at low level in wild-type adults, except T22C1.2 and ZK637.13 which are expressed more prominently.

To investigate which globins might be regulated in response to oxygen deprivation, synchronized young adult worms (1–2 days of adult age) were exposed to anoxic conditions (<0.001 kPa O_2) for 12 h and the changes in levels of gene expression relative to normoxia were analyzed by qPCR. Results demonstrate a robust ~2–5-fold upregulation for several globin genes under anoxic conditions (Table 1). To verify whether the elevated transcription of these globin-like genes is regulated by HIF, qPCR experiments were performed in age-synchronized adult *hif-1* mutants. In support of a widespread HIF-1 control of globin synthesis, eight out of 10 putative globins were no longer induced by anoxia in *hif-1* mutants (Table 1). Additional support for a HIF-1/globin link in *C. elegans*, as also seen in *Daphnia* (see Tokishita and Yamagata subsequently) or *Drosophila* (see Hankeln and Burmester subsequently), came from a computational analysis of globin genomic regions that detected the presence of putative HREs in all anoxia-induced globin genes. These globins are also induced under normoxia in *hif-1* mutants (data not shown), however, suggesting more complex, and possibly HIF-independent regulations that might compensate for the loss of HIF-1 function. More questions emerged upon realizing that anoxic worms arrest development along with cell-cycle progression and enter a reversible state of suspended animation for which HIF-1 function is not required (Padilla et al. 2002; Shen and Powell-Coffman 2003). It thus appears that hypoxia responses via HIF-1 are activated as the pO_2 drops below a threshold, and may extend, for at least some genes, even into anoxia. Further, experimental evidence is needed to discern hypoxic from anoxic inductions of *C. elegans* globins and to understand why HIF-1, albeit being dispensable for anoxic survival, still regulates target genes during extreme O_2 deprivation.

Table 1 Differential globin expression in wild-type and *hif-1* mutant worm under normoxia and anoxia

Globin gene	Wild-type A/N	*hif-1* A/N
F21A3.6	2.248*	1.074
Y17G7B.6	2.118*	1.121
C26C6.7	5.574*	0.919
R13A1.8	2.024*	1.056
W01C9.5	1.958*	1.007
C18C4.9	2.538	1.192
Y75B7AL.1	1.897*	1.144
C36E8.2	2.279*	0.929
T22C1.2	2.247	1.116
C18C4.1	2.572*	0.957

The figures represent fold differences in expression of at least three independent biological repeats (Hoogewijis et al., Submitted for publication). A = anoxia, N = normoxia. *$P < 0.05$.

Globin gene expression was additionally evaluated in mutants of the *daf-2* insulin/insulin-like growth factor receptor homolog of *C. elegans*. *daf-2* mutants are known to be hypoxia resistant (Scott et al. 2002). The *daf-2* pathway regulates development and life-span in nematodes, whereby a reduction in signaling is transduced onto downstream targets through activation of the DAF-16 forkhead transcription factor that produces an extended life-span and elevated stress resistance. Results of qPCR amplifications have shown that, following anoxia, nearly all globin genes are expressed at lower levels in *daf-2* mutants compared to wild-type worms. In contrast, ZK637.13, the first detected globin species in *C. elegans* (Mansell et al. 1993), is significantly induced (4-fold), possibly indicating an insulin-like pathway dependent regulation (Hoogewijs et al., submitted for publication). Interestingly, expression of ZK637.13 was demonstrated only in a subset of epidermal cells (Lynch et al. 1995). RNAi experiments are ongoing to ascertain whether ZK637.13 is required for resistance to oxygen deprivation of *daf-2* worms.

Expression of HIF and hypoxic induction of hemoglobin in the crustacean *Daphnia magna* (S. Tokishita and H. Yamagata)

We previously found four genes for hemoglobin (Hb) chains constituting a compact cluster on the chromosome of *D. magna* (Kimura et al. 1999). Our recent analysis showed that at least six Hb genes (*dhb1–dhb6*) constitute that cluster. All genes consist of seven exons and six introns; i.e., they all encode a di-heme domain, ~31 kDa globin subunit. All intergenic regions within the cluster contain numerous candidate HREs that are homologous to the binding sites of mammalian HIF-1 (see above and Fig. 1). Gorr et al. (2004a) showed that HREs located upstream of the *dhb2* gene interact *in vivo* and *in vitro* with human and *Drosophila* HIF complexes, and with the presumed *Daphnia* HIF complex, in response to hypoxia. To elucidate whether the structure of the HIF complex and overall features of HIF signaling pathway are conserved between mammals and *Daphnia*, we cloned *D. magna* cDNAs that encode the HIF-α and HIF-β (ARNT) subunits.

By screening a *D. magna* cDNA library with DNA probes that were amplified from conserved regions in ARNT genes of various organisms, cDNAs encoding a *D. magna* homolog of ARNT were isolated and the structural and functional features, as well as the expression pattern of their product, DmagARNT, were analyzed (Tokishita et al. 2006). Among known bHLH-PAS proteins, the deduced amino-acid sequence of DmagARNT showed the highest degree of identity to that of *Drosophila* ARNT (aka TANGO). Expression of DmagARNT in ARNT-lacking mouse Hepa-c4 cells fully recovered the loss of hypoxia response, suggesting the formation of a functional heterodimer with mouse HIF-1α and the subsequent transactivation of the downstream luciferase reporter.

DmagARNT was produced in an *Escherichia coli* expression system and then used to immunize rats. Whole-mount immunostaining of *D. magna* embryos was performed with the aid of an antibody thus raised against DmagARNT. Expression of DmagARNT was evident at the middle to late stages of embryonic development (about 25–48 h after ovulation) in several tissues, including a pair of the 1st antenna, 2nd antenna, 2nd maxilla, five pairs of the thoracic limbs, the central nerve system, anus, dorsal organ, maxillary gland, and carapace. As observed in other species, the DmagARNT is likely to function broadly as expressed dimerization partner in various developmental and sensory processes. In contrast, expression of ARNT in adult *D. magna* was limited to the epipodites of thoracic limbs, suggesting that ARNT plays a role solely in the hypoxia response in adult *Daphnia*.

A traditional cDNA library screening, as used for the cloning of *D. magna* ARNT, failed to identify a cDNA for *D. magna* HIF-α. Therefore, a yeast two-hybrid system was employed using an upstream half of a cDNA for *D. magna* ARNT fused with GAL4-DNA binding domain as bait. A *D. magna* cDNA library fused with GAL4-activation domain was used as prey. A cDNA for *D. magna* HIF-α was found among the GAL4-positive clones. Alignment of the amino acid sequence encoded by the cloned *D. magna* HIF-α cDNA with those of other organisms showed a high degree of sequence conservation of bHLH and PAS domains. Two proline residues within the oxygen-dependent degradation domain, hydroxylation of which leads to proteosomal degradation of the protein (see above and Fig. 1), are conserved in *D. magna* HIF-α. On the other hand, an asparagine residue in the C-terminal transactivation domain, hydroxylation of which leads to hindrance of interaction with the coactivater p300/CBP (see above, and Fig. 1), is not conserved in *D. magna* HIF-α. This TAD-C asparagine residue, therefore, is present in HIF-α's of decapodan crustaceans, amphibians, fish, and other vertebrates, while it is missing in those of *Daphnia*, *Drosophila*, and *C. elegans*. The mechanisms that activate the HIF complex may thus be different between the former and latter, and one might speculate that prolyl-hydroxylation and α-subunit degradation phylogenetically preceded the transcriptional inactivation under high pO_2 via asparagine hydroxylation. Phylogenetic analysis of the conserved bHLH and PAS domains, however, showed the expected close relationships of cladoceran, decapodan, and *Drosophila* HIF-α polypeptides. Whole-mount immunostaining was carried out on *D. magna* embryos using an antibody prepared against *D. magna* HIF-α. Hypoxia-induced expression of HIF-α was observed in several tissues including epipodites, a presumed respiratory organ (Fig. 5).

HIF-1 regulation in crustaceans (N.B. Terwilliger)

Hypoxic conditions in coastal waters around the world, resulting primarily from pollution runoff, are a global problem that threatens the wellbeing of numerous marine organisms (Diaz 2001).

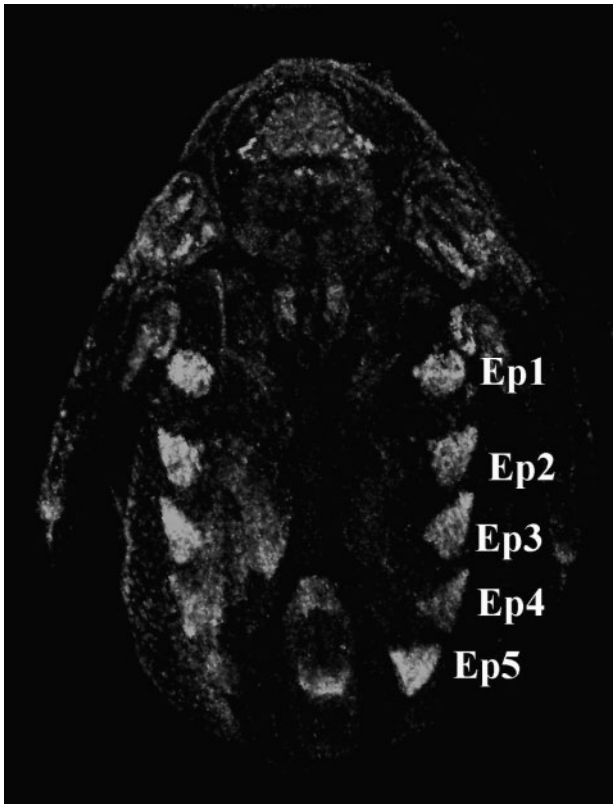

Fig. 5 Tissue specific expression of *D. magna* HIF-α at the late stage of embryonic development following exposure to hypoxia (3% O_2, 20h). Whole mount embryos were subjected to fluorescent immunostaining with a rat antibody raised against *D. magna* HIF-α. Fluorescent signals were visualized by a confocal laser scanning microscope (Leica TCS SP2). Hypoxia-induced expression of *D. magna* HIF-α was observed in epipodites of the thoracic limbs at the late stage of embryonic development. Ep1–Ep 5, epipodites on the five thoracic limbs.

Oceanic waters in the Northeastern Pacific are normally oxygen rich due to a combination of extensive upwelling of cold, nutrient-rich waters, and strong northwest winds that blow surface waters away from the coast in a counterclockwise direction. For the past five summers, however, a large region of oxygen-poor water has formed off Oregon's coast, probably due to shifting wind patterns, and large numbers of dead crabs and fish have been reported. In August 2006, a 30-mile ''dead zone'' containing as little as 0.1 ml oxygen per liter seawater wreaked major destruction on marine organisms in Oregon oceanic waters. Thus, not only coastal but oceanic organisms are subject to the effects of hypoxia. In addition to these severe episodes, the challenge of responding to normal tidal fluctuations in oxygen levels extends to marine organisms living in rocky intertidal pools and estuaries. It is, therefore, of major interest to understand what role HIF might play in the response of marine crustaceans to environmental stressors, including hypoxia.

In the phylum Arthropoda, HIF has been described in one group of Crustacea, the Branchiopoda, and in the Insecta. Branchiopod crustaceans, including *Daphnia*, *Artemia*, and *Triops*, increase hemoglobin (Hb) synthesis after hypoxic exposure (Fox 1948; Kobayashi and Tanaka 1991; Terwilliger 1992). HREs have been identified in the upstream regions of all globin genes (i.e., *dhb1–dhb6*) of *Daphnia magna* (see Tokishita and Yamagata earlier). Some of the motifs located in the 5′ flank of the *dhb2* gene were found to be essential in conferring HIF-mediated transactivation during hypoxia onto a heterologous reporter (Kimura et al. 1999; Gorr et al. 2004a). The sequences of both HIF cDNA's have recently been described in *D. magna* (see Tokishita and Yamagata earlier). Interactions between HIF and globin synthesis have also been described in insects (Hankeln et al. 2002; Gorr et al. 2004b; Hankeln and Burmester subsequently). Prompted by these earlier reports, we started to examine the role of HIF in regulating gene expression in the blue-blooded Malacostraca, the large group of crustaceans including crabs and shrimp that typically express copper-containing hemocyanin rather than Hb (Terwilliger 1998).

To this end, we determined the complete cDNA sequence of a HIF-α homolog in *Cancer magister*, the Dungeness crab (Ryan, Terwilliger, and Head, Genbank #DQ535030). The sequence aligns well with alpha subunit homologs from other arthropods, the grass shrimp *Palaemonetes pugio*, the fly *Drosophila melanogaster*, and the honeybee *Apis mellifera*. The sequence from *C. magister* also shows good alignment with those of chordate species, including the fish *Oncorhyncus mykiss*, the amphibian *Xenopus laevis*, and human *Homo sapiens*. The sequence from *C. magister* includes the bHLH region, the PAS A and B domains, the ODD, and the C-terminal activation domain, regions conserved in HIF sequences from other phyla (Fig. 1). The total length of the HIF-α proteins shows distinct phylogenetic differences, however. Among the Arthropoda, insect HIF-α's are longer (*D. melanogaster: HIF-a homolog SIMA*, 1505aa; *A. mellifera*, 1576aa) than those of crustaceans (*C. magister*, 1047aa; *P. pugio* 1057aa). The HIF-1α sequence in Chordata is even shorter (*X. laevis* 802aa, *H. sapiens* 826aa, and *O. mykiss* 766aa). These differences in length reflect major insertions and/or deletions in concert with conservation of key functional domains.

Increases in concentration and oxygen affinity of hemocyanin have been reported in response

to hypoxia and/or hyposalinity in several crustacean species, including *Carcinus maenas*, *Nephrops norvegicus*, and *Callinectes sapidus* (Boone and Schoffeniels 1979; Baden et al. 1990; Mangum 1997). These changes suggest that synthesis of hemocyanin, like that of the extracellular Hbs of branchiopod crustaceans, might be regulated by hypoxic, nonhypoxic, and possibly even developmental stimuli (Rider et al. 2005; Gorr et al. 2006b). The genes coding for six hemocyanin subunits of *C. magister* have been sequenced (Ryan and Terwilliger, manuscript in preparation) and the upstream regulatory regions have been examined for putative HIF-binding sites. Multiple consensus (RCGTG) and core (CGTG) sequences in the upstream regions of four of the six genes and in the intergenic region between linked hemocyanin genes 1 and 2 are present, consistent with a role for HIF-1 in the regulation of hemocyanin synthesis. Three of these genes code for constitutive hemocyanin subunits 1, 2, and 3, whereas the gene for hemocyanin subunit 6 is developmentally regulated (Terwilliger et al. 2006).

The ODD region of a HIF-α homolog in the blue crab *Callinectes sapidus* has recently been amplified using primers based on the HIF sequence of *C. magister*. These results illustrate the conserved nature and broad expression of HIF in brachyuran crustaceans.

Globins in *Drosophila* and other insects: respiratory proteins, oxygen scavengers, or else? (T. Hankeln and T. Burmester)

Respiratory proteins in insects have long been considered a specialty of taxa that regularly encounter hypoxic conditions in their habitats (reviewed by Burmester and Hankeln 2007). Notable examples are the aquatic mud-dwelling larvae of nonbiting midges (chironomids). Their extracellular globins, encoded by an unusually large gene family of more than 40 genes (Hankeln et al. 1998), account for more than 90% of the larval hemolymph proteins. Thus, their function is the storage and transport of oxygen, facilitating survival even under almost anoxic conditions. Larvae of the horse botfly (*Gasterophilus intestinalis*) also survive in the hostile environment of the horse alimentary tract by extracting oxygen via intracellular globins, mainly expressed in tracheal cells and the fat body (Dewilde et al. 1998). Backswimmers (Insecta: Notonectidae), diving predators that breathe under water with the aid of an abdominal air bubble, have intracellular globins at high concentration in a specialized ''tracheal organ.'' The oxygen bound by the globins is used to replenish the air bubble at later stages of the dive, thus maintaining neutral buoyancy (Matthews and Seymour 2006).

Globins have been totally unexpected in those insects which have almost unlimited access to oxygen via their tracheal system. Our survey of available sequence databases, however, revealed the presence of globin genes in various *Diptera*, *Hymenoptera*, *Lepidoptera*, *Hemiptera*, and *Coleoptera*. Thus, globins are a natural and ubiquitous constituent of the insect gene repertoire. While being phylogenetically more diverse than anticipated, all these insect globins feature the sequence hallmarks necessary for reversible oxygen-binding and probably localize to the cytoplasm. On the tissue level, a common theme is their expression in cells of the tracheal system, as demonstrated experimentally in *Drosophila*, the honeybee, and mosquitoes (Hankeln et al. 2002, 2006; Burmester et al. 2007). Other prominent globin expression sites like the fat body, visceral muscles, testes, or malpighian tubules were also observed, depending on the species and the developmental stage.

In *Drosophila*, three globin genes have been identified (Burmester and Hankeln 1999; Burmester et al. 2006). *glob1* is the most prominently expressed globin, found in the tracheal system and fat body in embryos, larvae and adults. Potential HREs are conserved in *glob1* from various *Drosophila* species, suggesting potential upregulation of this gene after hypoxia. However, *glob1* mRNA levels did not change in adult *Drosophila melanogaster* after experimental hypoxia, and were even diminished by about 50% in embryos and larvae. This is in agreement with *in vitro* cell culture data, suggesting a transcriptional downregulation of *glob1* via the HIF system (Gorr et al. 2004b). Slightly increased *glob1* expression (~1.5-fold) in larvae was observed only after hypoxia/normoxia cycles and following hyperoxia (95% O_2). These data can be unified to suggest that *glob1* preferentially fulfils the role of a regional O_2 buffer in tracheal cells, either supplying oxygen at peaks of most intensive oxidative metabolism, or binding excessive O_2 during periods of rest, in order to prevent the accumulation of toxic reactive oxygen species.

Evolution of HIF-1α in fishes (K.T Rytkönen and M. Nikinmaa)

Oxygen has been a major force in evolution of aquatic organisms, including fishes (Val 1995; Farmer 1999; Janis and Farmer 1999; Chapman and

Hulen 2001; Nikinmaa 2002; Powell 2003; Nikinmaa and Rees 2005). Oxygen availability is a more critical environmental factor for aquatic animals than for terrestrial ones, since at the same partial pressure, water contains only 1/30th of the oxygen contained in the same volume of air. Moreover, the rate of diffusion of oxygen in water is only 1/10,000 of that in air (Dejours 1975). As there are both phylogenetically closely related fishes with differing oxygen requirements and distantly related species with similar oxygen requirements, fishes are the primary choice among vertebrates for comparative evolutionary studies of oxygen-dependent systems.

We investigated whether sequence variation in the HIF-1α gene associates with oxygen demand among several fish species, including novel HIF-1α sequences from protacanthopterygian, cypriniform, and perciform lineages. Our results indicate that the HIF-1α genes of teleost fishes are somewhat shorter than are those of tetrapods (see Terwilliger earlier). We inspected the variation in specific amino acid residues and concentrated especially on redox-sensitive cysteines that are involved in signaling by reactive oxygen species (ROS) (Barford 2004), and consequently also by oxygen levels. Initially, it was found that human HIF-2α, but not HIF-1α, function is modulated by redox reagents (Lando et al. 2000). Partially, this effect is due to a serine–cysteine substitution in the DNA binding basic region of the bHLH feature. Human HIF-2α has a cysteine at position 25, which aligns with a serine at position 28, in mammalian HIF-1α. Site-directed mutagenesis of Ser28 of mammalian HIF-1α to Cys28 conferred redox sensitivity to DNA binding (Lando et al. 2000). Studies in salmonid cells revealed that DNA binding of some fish HIF-1α is also regulated by redox state (Nikinmaa et al. 2004). Cloning of the rainbow trout HIF-1α sequence showed indeed the presence of a cysteine at position 28 (Soitamo et al. 2001), while in the hypoxia tolerant cyprinids, HIF-1α position 28 is occupied by serine. In correspondence with HIF sensitivities, ROS appear, at least in some cases, to be involved in mediating oxygen sensitivity (Bogdanova and Nikinmaa 2001). For these reasons, we set out to correlate variation in HIF-1α sequence with hypoxia defense strategies in fishes: i.e., does HIF-1α of hypoxia tolerant species generally have Ser28 and is this residue a cysteine in hypoxia sensitive species. Careful sequence analysis demonstrated, however, that position 28 is occupied by cysteine in the hypoxia tolerant *Protacanthopterygii* (pike), as is the case in the hypoxia sensitive salmonids, and by serine in the hypoxia tolerant cyprinids, whereas the hypoxia sensitive asp possesses an asparagine in this location. Thus, the presence or absence of cysteine in HIF-1α position 28 does not appear to correlate with species specific hypoxia defense strategies but rather may reflect phylogeny.

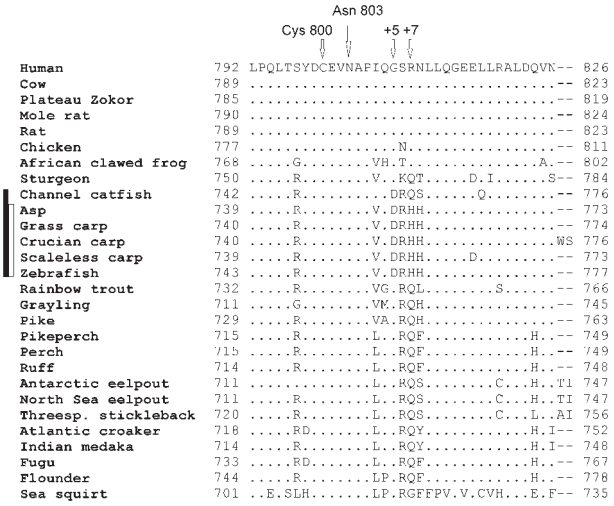

Fig. 6 Alignment of the C-terminus of the HIF-1α amino acid sequence of available fish species and selected other species. Cysteine 800, Asparagine 803 (human HIF-1α nomenclature), and positions +5 and +7 upstream from Asparagine 803 are marked with arrows. In these positions, *Ostariophysi* (black line) and *Cypriniformes* (white line) have lineage-specific amino acid residues. *Ostariophysi* have aspartic acid (D) in position +5, whereas other vertebrates have glycine (G) instead; in the +7 position *Cypriniformes* have histidine (H), whereas other vertebrates have arginine (R) or glutamine (Q). The figure is similar to the original one published in Rytkönen et al., (2007). Reproduced here with the permission from Elsevier.

On the other hand, the most notable group-specific variation was found in the vicinity of Asn-803 (human HIF-1α nomenclature). As previously shown, 15 amino-acid residues before, and 20 after, Asn-803 in human HIF-1α appear to be important for FIH activity (Koivunen et al. 2004). The alignment of our data set (Fig. 6) shows that positions −11 to +20 (with regard to Asn-803) are well conserved in all vertebrates, with some group-specific exceptions. The most notable of these are the *Ostariophysi*/*Cypriniformes*-specific amino-acid residues in the +5 and +7 positions after Asn-803. *Ostariophysi* have aspartic acid (D) in position +5, whereas other vertebrates have glycine (G) instead. In position +7 *Cypriniformes* have histidine (H), whereas other vertebrates have arginine (R) or glutamine (Q). Substituting glycine (G) for aspartic acid (D) in position +5 introduces an extra negative charge, and the presence of histidine makes the charge variable within the physiological pH range.

It remains to be investigated whether these group-specific amino acid changes are correlated with the generally good hypoxia resistance of *Ostariophysi/Cypriniformes*.

To summarize, no clear HIF-1α signatures could be associated with hypoxia tolerant versus sensitive phenotypes. If species-specific differences in oxygen dependence of HIF-1α function have evolved, they probably occur at other levels of the HIF-1α pathway.

HIF and cold adaptation in fish (D. Abele, K. Heise and M. Lucassen)

Hypoxia tolerant champions like the crucian carp, which survive long winter months under a fully closed ice cover, exhibit constitutive HIF-α protein levels in various central organs and gill tissue. Short term hypoxic exposure further elevates HIF-1α protein levels in the crucian carp, especially at low water temperature, which presumably supports hypoxic survival during winter cold (Rissanen et al. 2006). Recently, constitutive HIF-1α expression was also confirmed in less tolerant, and even hypoxia sensitive, fish like the North Sea eelpout, *Zoarces viviparus* (Heise et al. 2006b) and its polar confamilial *Pachycara brachycephalum* from Antarctic deep waters, where environmental hypoxia is unlikely to occur (Heise et al., 2007, in press).

To specify changes in HIF-1α gene structure and HIF pathway functioning that may relate to cold adaptation in marine fish, we compared partial cDNA sequences of different cold-adapted species, covering the C-terminal half of HIF-1α from North Sea and Antarctic eelpout, as well as from two Antarctic nototheniids and the icefish *Chionodraco myersi*. Stabilization of HIF-1α at much higher pO_2 in fish cells (i.e., 5–10% $O_2 \cong$ 38–76 torr) than in mammalian cells has been attributed to the exchange of up to four amino acids of the human sequence for redox-sensitive cysteines in the ODD of rainbow trout (Soitamo et al. 2001) and both zoarcid (position of *Zv*HIF-1α: 442, 496, 512, 578) ODD regions. All Antarctic fish investigated, thus far, carry three cysteine-yielding exchanges in the ODD and two additional cysteines in, or next to, the C-terminal transactivation domain (Fig. 1), which could be important in conferring an increasingly redox sensitive HIF transactivation mechanism (Nikinmaa et al. 2004). Cysteine exchanges in HIF-1α sequences from marine and fresh water fish reported to date support the idea that HIF-dependent gene control might be very sensitive to redox regulation within the normoxic (i.e., noncritical) pO_2 range, hence illustrating a clear difference between the pathway controls of fish and mammals.

The cellular redox environment is strongly determined by the glutathione redox ratio GSH/GSSG (i.e., reduced/oxidized glutathione) that rapidly destabilizes once environmental or physiological stressors trigger an unbalanced formation of reactive oxygen species (ROS). To examine the direct impact of the redox potential (E_{mV}) on HIF function, we heat-shocked (12→18, 22, 26°C) and cold-shocked (12→5, 1°C) North Sea eelpout for 2 h and measured HIF binding to the human erythropoietin enhancer in gel shift assays of liver extract. Figure 7A shows the direct correlation between a reduced redox potential with lowered ambient temperatures for eelpout liver (heat shock −245 mV, control −260 mV, cold shock −275 mV), whereas Fig. 7B indicates a significant linear relationship between an increase of HIF-DNA binding and a reducing redox potential in *Zoarces viviparus* liver (Heise et al. 2006a, 2006b).

Comparing fish from different latitudes (Antarctic eelpout versus North Sea eelpout) at their natural, low, water temperatures (0°C for the polar and 6°C for the temperate eelpout), we found different levels of oxidative stress indicators and different glutathione contents. Antarctic eelpout maintain oxidative stress indicators in liver, specifically the lipid peroxidation marker TBARS (thiobarbituric acid reactive substances) and chemiluminescence rates, indicative of singlet oxygen formation and oxidative stress in general (Gonzalez Flecha et al. 1991), at significantly lower levels than do North Sea eelpout. Cellular glutathione content was significantly increased (3-fold) in the polar eelpout over North Sea eelpout and, moreover, oxidized to GSSG by over 50%. Although E_{mV} did not differ significantly ($P = 0.13$) between these two species, HIF–DNA binding in gel shift assay was significantly lower in the polar than in the temperate fish. Furthermore, antibodies newly produced against the eelpout HIF-1α detected lower constitutive HIF-1α protein levels in the polar species (Heise et al., 2007, in press). Taken together, both findings are in line with a lower hypoxia tolerance of the cold-adapted Antarctic species. Similar to the geographic cold adaptation in the Antarctic fish, seasonal cold acclimatization of temperate eelpout (summer T: 12°C and winter T: 6°C) was associated with an oxidation ($P < 0.01$) of cellular E_{mV}. North Sea eelpout, however, accumulated higher levels of oxidative damage indicators (protein carbonyls and TBARS) in liver tissue during the winter season. Yet, as in acutely cold shocked

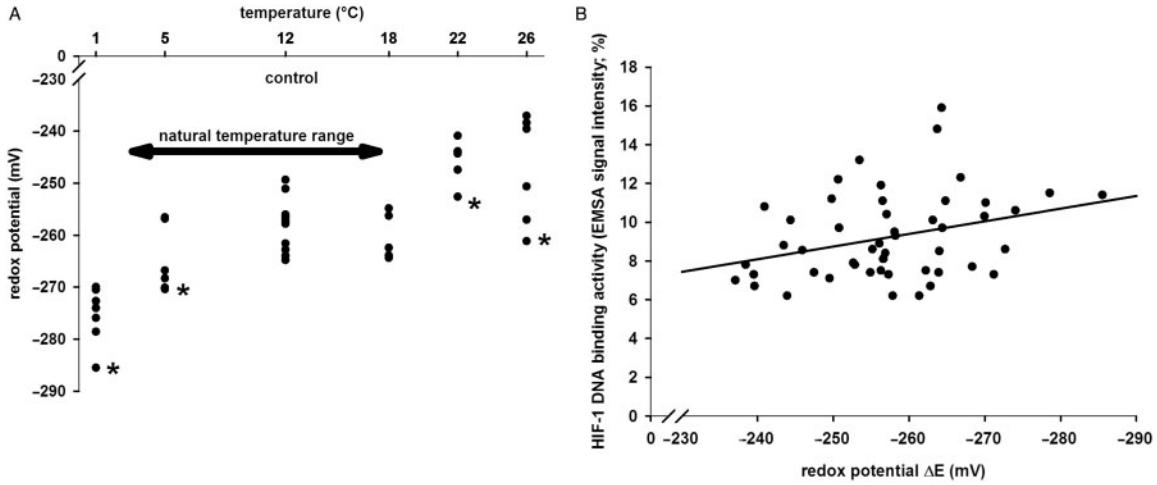

Fig. 7 (**A**) Cellular redox potential as a function of temperature. The cellular redox potential was calculated on the basis of the GSH/GSSG ratio at *in situ* temperature and *in situ* intracellular pH (Schafer and Buettner 2001) in liver samples of *Zoarces viviparus* after 2 h cold exposure to 1 and 5°C (unstressed fish at control temperature 12°C), as well as after 2 h heat exposure to 18, 22, and 26°C. Each dot represents an individual fish sample, * = significantly different from the unstressed group at 12°C, $P < 0.05$. (**B**) HIF-1 activity as a function of cellular redox potential. Linear regression demonstrating increased HIF-1 DNA binding at a more reduced redox environment ($R^2 = 0.3$, $n = 50$, $P < 0.05$, Statview 5.0).

eelpouts (Fig. 7B), HIF–DNA binding increased significantly in liver extracts of winter *Z. viviparus*, in spite of the more oxidized redox conditions. Thus, in these eurythermal fish, either other signaling components overwrite the oxidizing cellular milieu in favor of an elevated HIF-1/DNA binding during the winter cold, or the transcription factor response to the cellular redox signal is somehow changed or desensitized in response to winter acclimatization. Another possible explanation for this conundrum is that protein turnover and removal of peroxidized lipids is slowed in the cold, resulting in higher tissue concentrations of oxidative damage markers, without necessarily requiring higher rates of ROS production.

We conclude that seasonal, as well as evolutionary, cold adaptation leads to more oxidized redox conditions, and only maintenance of high antioxidant defence levels seems to offset oxidative damage in liver tissue of cold-adapted fish. HIF-1 DNA-binding is lowered, possibly by reactive oxygen species production during heat shock. Whereas HIF seems to be involved in seasonal cold acclimatization in eurythermal eelpout, lower HIF protein levels and DNA-binding capacity of the transcription factor in the stenothermal Antarctic species may, in part, explain the comparably lower tolerance to hypoxia and generally higher stress susceptibility of the Antarctic fish. Future studies need to scrutinize a possible role of HIF during the cold adaptation of fish when there is no temperature-induced tissue hypoxia.

Imaging the activation and assembly of the mammalian HIF-1 complex (J. Fandrey)

Imaging the components of the oxygen sensing mechanisms is difficult. We have previously localized the nuclear distribution of HIF-1α and of the O_2-sensors PHD1, PHD2, PHD3, and FIH-1 (Metzen et al. 2003; Berchner-Pfannschmidt et al. 2004). While the localization of HIF-1α and HIF-1β in hypoxic nuclei has been unambiguously shown by many groups, it is not clear when dimerization of the two partners actually occurs. Models so far are based on co-immuno-precipitation studies but not on imaging within living cells. Moreover, crystallization of full length HIF-α's and HIF-1β, or even the entire HIF-1 complex, to obtain information about potential structural changes within the complex upon transition from normoxia to hypoxia, and vice versa, has not been successful thus far. Partial crystal structures of the PAS domain B, which is present in HIF-α's and HIF-1β (Fig. 1), were recently unraveled (Card et al. 2005). It was proposed that, within the HIF-1 complex, the PAS B domains of HIF-1α and HIF-1β are oriented in an antiparallel manner to form the HIF-1 dimer. This is unusual with respect to other members of the PAS family (Yildiz et al. 2005), and

Card et al. (2005) pointed out that it needed to be shown how the active HIF-1 complex is formed in the nucleus of living cells.

To measure the mobility of HIF-1 subunits by fluorescence recovery after photobleaching (FRAP), we constructed HIF-1α-ECFP (enhanced cyan fluorescence protein) and HIF-1β-EYFP (enhanced yellow fluorescence protein) fusion proteins and determined their nuclear distribution in the human osteosarcoma tumor cell line U2OS by 2-photon-laser-microscopy. Subnuclear structures of heterogeneously distributed HIF-1 subunits were reliably detected. Interestingly, return of fluorescence after photobleaching led to the recovery of exactly the same subnuclear structures, indicating that HIF-1 subunits were directed to specific loci within the nucleus, i.e., putative sites of HRE binding. Moreover, we found different velocities whereby HIF-1α and HIF-1β returned into the bleached areas to recover fluorescence. This was unexpected because, until now, it was assumed that HIF-1α, upon entering the nucleus, immediately forms the dimer with HIF-1β. Our data clearly indicate that both subunits can move independently of each other and thus do not immediately associate inside the nucleus. To obtain even more detailed information about the geometry of the HIF-1 complex in living cells, we established FRET with different HIF-1α-ECFP fusion proteins as donor molecules and HIF-1β–EYFP as acceptors. We were able to unambiguously show close interaction of HIF-1α and HIF-1β under hypoxic conditions in living U2OS cells, using HIF-1α/β fusion proteins labeled at their N-terminal ends. The distance between the two HIF-1 subunits of only 6.6 nm was derived from calculations from FRET efficiencies determined over a range of acceptor/donor ratios. HIF-1α and HIF-1β labeled at their N-termini were found to be fully transcriptionally active which disproved the concern that the N-terminal ECFP/EYFP labels might impede DNA binding. When both HIF-subunits were labeled at their C-terminus, the distance between the HIF-1 subunits was 7.2 nm, which is fully compatible with the notion that co-activator proteins like p300/CBP are bound to the C-terminus (Fig. 1). Unexpectedly, using a HIF-1α–ECFP C-terminal deletion mutant that lacks the TAD-C of HIF-1α, HIF-1α, and HIF-1β, distances were only moderately closer.

Surprisingly, the distance between the C-terminally labeled HIF-1α and N-terminally labeled HIF-1β determined by FRET was only 7.9 nm, indicating that the C-terminus of the HIF-1 complex is much closer to the DNA binding domains than was previously thought (Wotzlaw et al. 2007). This could facilitate interaction with other tissue-specific transcription factors (Fig. 8). Our data are fully compatible with partial X-ray structure analyses of the PAS B domains of HIF-α's, suggesting an antiparallel orientation of the PAS B domains and thus a more compact assembly of the HIF complex (Card et al. 2005).

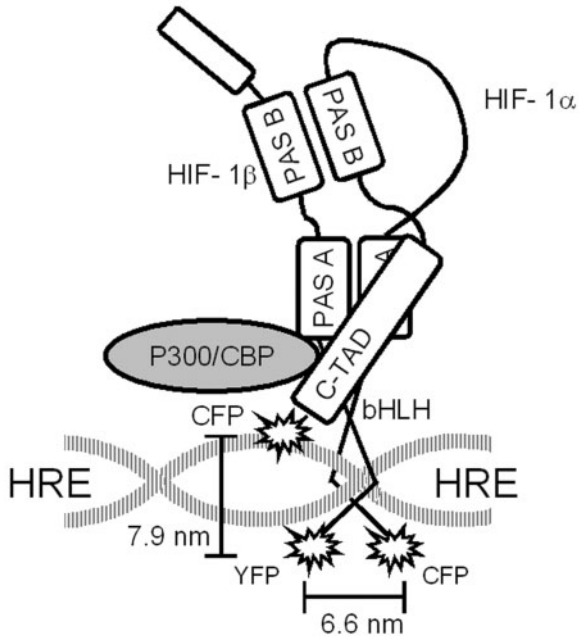

Fig. 8 Putative conformation of the HIF-1 dimer based on distance calculations derived from FRET measurements. The HIF-1 dimer is schematically drawn (not to scale!) with its bHLH domains bound to the DNA at the HRE (see Fig. 1 for orientation). Labeling HIF-1α with ECFP and HIF-1β with EYFP at their N-terminal ends results in a distance of 6.6 nm between the two fluorophores. PAS A and PAS B domains are shown in an anti-parallel orientation of the PAS B domain which results in a rather close proximity, i.e., 7.9 nm, of the C-terminal transactivation domain (TAD-C) of HIF-1α, labeled with ECFP, to the EYFP-labeled N-terminus of HIF-1β. The coactivator p300/CBP may thus come close to the DNA-bound other transcription factors.

Hypoxia-inducible factor and cancer (P.H. Maxwell)

There is extensive documentation of hypoxia in cancers compared to normal tissues. This is due to inefficient delivery of oxygen via a less robust vascular network, and also because proliferating tumor cells are metabolically active. Hypoxia is an important prognostic indicator. Not only do hypoxic tumors respond less well to chemotherapy and radiotherapy, but outcomes are also worse if they are managed surgically. Since HIF-1 is activated by hypoxia this suggested that HIF could play a role in cancer biology and might be important in aspects of

the tumor phenotype such as angiogenic signaling and enhanced glycolysis (see Gorr earlier).

Insight into the potential role of the HIF system in tumor biology has come from examining the behavior of model tumors grown from cells with genetic alterations in the HIF pathway. For example, we showed some years ago that xenografts of mouse hepatoma cells lacking HIF-1β do not show focal expression of the HIF target genes VEGF (vascular endothelial growth factor) and GLUT1 (glucose transporter 1), are less well vascularized and grow more slowly than do isogenic HIF-competent tumors (Maxwell et al. 1997). As expected for a pathway with extensive downstream targets, the net results on tumor growth vary from one cell background to another. At least in part, this is because HIF also activates expression of proapoptotic genes such as BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3). This illustrates that natural selection in a population of tumor cells could result in increased activity of a pathway with positive and negative consequences for cellular proliferation and survival—as long as the overall balance was positive. If increased HIF activation results in increased survival through metabolic changes, a number of neutral or deleterious effects of HIF could be co-selected. Particular interest has focused on the role of HIF activation in angiogenic signaling by tumors. An interesting new twist is that, with Dr Griffioen, we have shown that hypoxia results in vascular mimicry in Ewing's sarcoma (van der Schaft et al. 2005). As a result, blood channels are formed that are directly lined by tumor cells having many features normally associated with endothelial cells. Intriguingly, these channels appear to provide a route for deoxygenated blood to leave the tumor. Although much is known about how tumors increase blood inflow, an obvious consequence is that more drainage will be required. It is interesting to speculate that HIF activation will have a general role in coordinating this venous drainage.

Strong support for the importance of HIF in cancer biology comes from studies showing that HIF-1α subunits are commonly expressed in human tumors, whereas they are not generally detectable in normal tissue (Harris 2002). Increased HIF activation generally correlates with a poor prognosis. In some tumor series, however, the reverse has been seen, which is consistent with HIF defective tumors growing more rapidly in some experimental studies.

More direct evidence for a role of HIF in driving the initiation and promotion of tumors is that cancer-associated mutations in tumor suppressor genes and oncogenes commonly increase HIF activation. The clearest example to date is VHL. This is a classic two-hit tumor suppressor gene originally isolated from studies of kindreds with the multitumor autosomal dominant syndrome characterized by von Hippel and Lindau about 100 years ago. VHL acts as the recognition component of an ubiquitin E3 ligase enzyme that captures HIF-1α/2α subunits in the presence of oxygen, leading to their ubiquitination and destruction (Fig. 1). In the absence of VHL, HIF α-subunits are stabilized and are constitutively active. Complexity is added by the fact that VHL inactivation almost certainly has other consequences that are independent of HIF activation. We are particularly interested in two linked questions. First, how does inactivating VHL result in the initiation of tumors? Second, what are the consequences of genetic activation of HIF in normal human tissues? One route we have pursued was to examine tissues from humans with VHL disease due to a germline mutation in the VHL gene. Labeling the cell-surface enzyme carbonic anhydrase IX (CAIX), another strongly responsive HIF target, provides a robust way of identifying groups of cells that have a somatic "second hit" so that both VHL alleles are inactivated (Mandriota et al. 2002). Several interesting findings have emerged. In kidneys from VHL patients these foci of HIF activation are common, but show minimal evidence of increased proliferation compared to adjacent cells. Nevertheless, they show many hallmark features of clear cell renal cell carcinoma, including expression of the intermediate filament vimentin, increased angiogenesis, and a clear cell appearance. Recently, we have used these foci to show that HIF activation results in another hallmark feature of cancer—a striking decrease in expression of the intercellular adhesion molecule, E-cadherin. Furthermore, re-expression of VHL or antagonizing HIF in renal cancer cells with defects in VHL rescues E-cadherin expression (Fig. 9) (Esteban et al. 2006). Interestingly, the earliest lesions of VHL inactivation identified on the basis of CAIX only express HIF-1α. Progression to cysts, and to frank tumor, is associated with expression of HIF-2α in addition to HIF-1α, suggesting that additional genetic events that produce a shift to dominant expression of HIF-2α are important in tumor development in the kidney. Consistent with this, expressing HIF-1α into 786-O renal cancer cells (which usually only express HIF-2α) reduces their growth as xenografts. In renal cancer cells, and in the premalignant lesions, there are distinct consequences of HIF-1 and HIF-2 activation. For example, cyclin D1 is increased by HIF-2 exclusively, whereas CAIX and BNIP3 are only

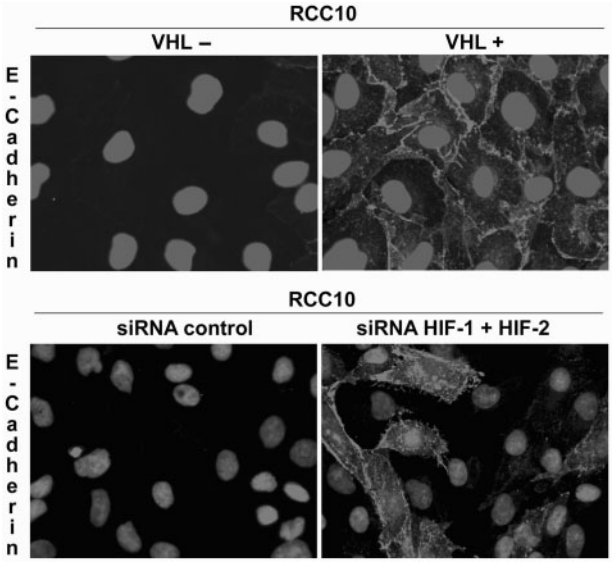

Fig. 9 The VHL-HIF pathway regulates expression of the cell adhesion molecule E-cadherin in renal cells. Immunofluorescence images for E-cadherin showing that the VHL defective renal cell cancer line, RCC10, expresses little or no E-cadherin (top left). DAPI-stained nuclei are visible as distinct ovals throughout the figure. Stable expression of a VHL gene rescues E-cadherin expression (top right). The siRNA for HIF-1α and HIF-2α also rescues E-cadherin expression (bottom right). Figure courtesy of Dr M. Esteban and Ms S. Harten.

increased by HIF-1 (Raval et al. 2005). Interestingly, the situation is different in spinal cord, where our studies with Dr Vortmeyer (NIH) have shown that the earliest foci of VHL inactivation express only HIF-2 (Vortmeyer et al. 2006). While CAIX is an exclusive HIF-1 target in the kidney, in spinal cord lesions expression of HIF-2 in isolation appears sufficient to activate CAIX. This illustrates that the isoform selectivity of HIF activation varies in different cell types, and that the downstream linkages also show cell-type specificity.

Effect of hypoxia on neuroblastoma phenotype and behavior. Molecular mechanisms and clinical consequences (S. Påhlman)

The childhood tumor neuroblastoma is derived from immature or precursor cells of the sympathetic neuronal lineage (Hoehner et al. 1996, 1998; De Preter et al. 2006). The clinical behavior of neuroblastomas is notoriously heterogeneous and ranges from benign (ganglioneuroma) to highly aggressive (neuroblastomas with amplification of the oncogene MYCN that encodes N-Myc) lesions, and in general, less mature neuroblastomas are more aggressive than are more differentiated forms. Hypoxia has been shown to be one cause of phenotypic heterogeneity, and recently we observed that hypoxic neuroblastoma cells reduce their expression of neuronal differentiation marker genes and become stem cell-like (Jogi et al. 2002). A similar loss of differentiation characteristics was also seen in hypoxic breast cancer cells (Helczynska et al. 2003) and we conclude that loss of differentiation adds to the general pattern of hypoxic tumor cells being more aggressive than are more well-oxygenated ones.

Cellular adaptation to hypoxia is to a large extent mediated by stabilization of the hypoxia-inducible transcription factors HIF-1α and HIF-2α. These factors are also important during normal development in general. More specifically, HIF-2α is implicated in the development of the sympathetic nervous system (SNS) (Tian et al. 1998). Consistently, HIF-2α is specifically expressed in mouse embryonic and human fetal SNS structures (Tian et al. 1998; Jogi et al. 2002; Nilsson et al. 2005) and high HIF-2α expression, observed in seemingly well-vascularized neuroblastomas, might be explained by their SNS origin (Holmquist-Mengelbier et al. 2006). HIF-1α could not be detected in these HIF-2α positive neuroblastoma cells, and *in vitro* growth of neuroblastoma cells at 21%, 5%, or 1% oxygen revealed that HIF-2α, but not HIF-1α, becomes stabilized and is active at 5% oxygen. We could further show that VEGF, as one of several classical hypoxia-driven genes, is already expressed at 5% oxygen via a HIF-2α-dependent process, which prompted us to conclude that HIF-2α acts as an oncogene at least in neuroblastoma. To address that issue, both HIF-α isoforms were transiently knocked-down in neuroblastoma cells and injected subcutaneously in nude mice. The resulting tumors grew more slowly when HIF-2α expression was reduced, while HIF-1α level reduction had no impact on tumor growth (Holmquist-Mengelbier et al. 2006). In a large clinical study on neuroblastoma material, we could demonstrate a correlation between HIF-2α protein levels and VEGF expression and even more importantly, a correlation between high HIF-2α protein expression and poor patient outcome. We conclude that HIF-2α in neuroblastoma cells is active at near-physiological oxygen concentrations and transcribes genes linked to a *bona fide* hypoxic phenotype, which could explain the association between high HIF-2α protein expression and aggressive neuroblastoma behavior.

Conclusion

The ''worm-to-man'' survey on oxygen sensing via HIF presented here spans a wide variety of taxa and highly divergent physiological frameworks (i.e., hypoxia sensitive, hypoxia tolerant, healthy, and neoplastically transformed). Studying HIF in model invertebrates such as *C. elegans*, *Drosophila*, and *Daphnia* has the advantage that many of the key players in this pathway (e.g., HIF-α, PHD, and VHL) are each encoded by single-copy genes. As the genetic redundancy is diminished, the *in vivo* functional analysis of these pathway members via the generation of knockdown or mutant phenotypes is greatly facilitated and simplified. Furthermore, HIF signaling is remarkably conserved across the animal kingdom, which is commonly being exploited for the design of experimental protocols to clone and characterize novel HIFs and downstream targets from related species (see above). Possibly the biggest asset of characterizing hypoxia responses in these model invertebrate and lower vertebrate ''critters,'' however, is their unparalleled tolerance towards even the most severe limitations in oxygen. Due to that, defenses in a nematode, a fly or a carp and those of a solid human tumor, resemble one another in fundamental aspects (see above). The fact that *C. elegans* or *Drosophila* are genetically tractable to the (1) analysis of, and (2) interference with, hypoxia tolerance on a genome-wide scale has already begun, and will certainly continue, to have a great impact on tumor biology through the improved understanding of the molecular mechanisms underlying hypoxia tolerance, and the development of new diagnostic markers and therapeutic targets of the HIF pathway.

Acknowledgments

The authors wish to acknowledge Steven Perry, Anke Schmitz, Stefan Hetz, Steve Morris, and Thomas Breuer for their invaluable contributions in having organized the ICRB 2006 meeting. The following funding agencies supported the work presented here: US National Science Foundation (N.B.T, J.A.P-C); US National Institutes of Health (K.A.W); American Heart Association Established Investigator Award (J.A.P-C); Ministry of Education, Science, Sports and Culture of Japan (S.T, H.Y); German Research Foundation (T.H, T.B, J.F); Academy of Finland and University of Turku (K.T.R, M.N); Alfred-Wegener-Institute for Polar and Marine Research (D.A, K.H, M.L); Cancer Research UK (P.H.M); Swedish Cancer Society and the Children's Cancer Foundation of Sweden (S.P); Swiss National Science Foundation (T.A.G) and EU's 6th Framework Programme EUROXY (P.H.M, T.A.G).

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