Hypoxia Tolerance in Animals: Biology and Application*

T. A. Gorr[1,†]
D. Wichmann[1]
J. Hu[1]
M. Hermes-Lima[2]
A. F. Welker[2]
N. Terwilliger[3]
J. F. Wren[4]
M. Viney[4]
S. Morris[4]
G. E. Nilsson[5]
A. Deten[6]
J. Soliz[1]
M. Gassmann[1]

[1]Institute of Veterinary Physiology, Vetsuisse Faculty and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland; [2]Departamento de Biologia Celular, Universidade de Brasília, Brasília, Brazil; [3]Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon; [4]Department of Biological Sciences, University of Bristol, Bristol, United Kingdom; [5]Department of Molecular Biosciences, University of Oslo, Oslo, Norway; [6]Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany

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ABSTRACT

Many invertebrates and ectothermic vertebrates successfully cope with a fluctuating supply of ambient oxygen—and consequently, a highly variable tissue oxygenation—through increasing their antioxidant barriers. During chronic deprivation of oxygen, however, the hypometabolic defense mode of the fruit fly *Drosophila*, the hypoxia-induced behavioral hypothermia of the crayfish *Pacifastacus leniusculus*, and the production of ethanol during anoxia by the crucian carp *Carassius carassius* all indicate that these animals are also capable of utilizing a suite of genetic and physiological defenses to survive otherwise lethal reductions in tissue oxygenation. Normally, much of an organism's gene response to hypoxia is orchestrated via the hypoxia-inducible transcription factor HIF. Recent developments expand our view of HIF function even further by highlighting regulatory roles for HIF in the hypometabolism of insects, in the molting and the normoxic immune response of crustaceans, and in the control—via the downstream effector gene erythropoietin—of the hypoxic ventilatory response and pulmonary hypertension in mammals. These and related topics were collectively presented by the authors in a symposium of the 2008 ICA-CBP conference at Mara National Reserve, Kenya, Africa. This synthesis article communicates the essence of the symposium presentations to the wider community.

General Background and Symposium Rationale

Inadequate oxygen supply followed by uncontrolled signaling represents a major aspect of the progression of cardiovascular disease, stroke, and cancer. One significant obstacle that limits the response of many cancer patients to conventional therapies is the reduced oxygenation (hypoxia) seen in most solid tumors. Tumor hypoxia, in turn, correlates with inherent tumor aggressiveness, incidence for spreading, and resistance to all commonly used medical treatments. Cancer cells respond to hypoxia by upregulating physiological mechanisms normally involved in maintaining the local oxygen supply while also switching their metabolism to a more prominent utilization of anaerobic fermentative rather than oxidative pathways. Importantly, key aspects of the hypoxia survival strategies—including multiple functional and regulatory details surrounding the main O_2-sensing signal transducer, a transcription factor called HIF (hypoxia-inducible factor)—are conserved across phyla, from humans to nematodes. To survive periods of diminished and/or erratic oxygen supply, organisms have evolved to tolerate such changes in their microenvironment. Similarly, the cancer cells in a tumor evolve as it develops, by acquiring mutations that help them survive in the local environment. Consequently, one major outcome of existing selection pressures under low oxygen that is common between many invertebrate or lower vertebrate species and human tumors is the gain of hypoxia tolerance. Tumor angiogenesis, glycolysis, and apoptotic evasion are regarded by many as the main adaptations that aid the tumor in acquiring tolerance and have all been well studied. Compared with these responses, the reduction of ATP-consumptive processes during hypoxic or anoxic challenges as physiological means to establish a lifesaving new hypometabolic-energy steady state and HIF's regulatory role of this metabolic slowdown are poorly understood with respect to most animal or neoplastic cells.

* We dedicate this article to Steve Morris (deceased 2009) in honor of his legacy as an inspiring teacher and scientist, a dedicated lover of wildlife biology, and a great guy. This article was prepared as an overview of a symposium at "Molecules to Migration: Pressures of Life," the Fourth International Conference in Africa for Comparative Physiology and Biochemistry, Maasai Mara National Reserve, Kenya, 2008.

† Corresponding author; e-mail: tgorr@access.uzh.ch.

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In addressing this knowledge gap, the symposium reported here (organized and chaired by Max Gassmann, University of Zurich) aimed to survey the mechanistic responses and adaptations facilitating hypoxia tolerance in a wide range of animal models. The synthesis reflects the breadth of animal adaptability by focusing on selected examples of models and their survival modes during hypoxic or anoxic challenges. Thus, the presented topics include (*a*) up-to-date reviews on the coping mechanisms of anoxia-tolerant organisms, including the challenge of reactive oxygen species; (*b*) normoxic, hypoxic, and cold-induced HIF responses in crustaceans; (*c*) interference with hypoxia tolerance effecting genes in fruit flies and cancer cells; and (*d*) the erythropoietin-mediated antihypertensive protection of hypoxic alveolar airways as well as the enhancement of the hypoxic ventilatory response with a particular augmentation of carotid body function in mice and humans.

The O_2-Sensing HIF Pathway

Transducing minutes to hours of a diminished oxygen partial pressure (Po_2) onto the level of DNA is mainly, though not exclusively, mediated by HIFs throughout the animal kingdom. Generally speaking, these responses to hypoxia via HIF or other cascades begin to occur at the cells' specific critical oxygen threshold (P_c), which separates the aerobe-oxyregulated ($Po_2 > P_c$) from the anaerobe-oxyconforming ($Po_2 < P_c$) physiological state. For mammalian cells subjected to a $Po_2 < P_c$, several hundred potential (Manalo et al. 2005) and more than 70 validated hypoxia-responsive gene targets of HIF have been identified to date (Wenger et al. 2005). Considerable progress has also been made in understanding the molecular basis of HIF and its regulation by oxygen (Höpfl et al. 2004; Schofield and Ratcliffe 2004; Maxwell 2005; Fandrey et al. 2006). A decade after the discovery of HIF, it is quite clear that, from mammals to teleosts, and in *Drosophila melanogaster*, the crustacean *Daphnia magna*, and the nematode *Caenorhabditis elegans*, the HIF complex is composed as a heterodimer of homologous α subunits and β subunits (Gorr et al. 2006; Hoogewijs et al. 2007). Both classes of HIF subunits are members of the family of basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS; Per-Arnt-Sim first reported PAS domain–containing proteins) transcription factors. During hypoxia, the functional heterodimer binds specifically to target gene sequences embedded within hypoxia-response elements (HREs). To date, three HIF-α proteins—HIF-1α, -2α, and -3α—have been reported in mammals to heterodimerize with the constitutively present HIF-1β subunit, originally known as aryl hydrocarbon receptor nuclear translocator (ARNT), thereby yielding complexes HIF-1, -2, and -3.

Regulation of HIF by oxygen, in cultured cells at least, occurs primarily at the protein level through the enzymatic hydroxylation of specific α-subunit prolyl and asparaginyl residues. In tissues of humans and mice, on the other hand, hypoxia additionally seems to operate by inducing the mRNA of HIF-1α, as reported by some (Wiener et al. 1996; Vogt et al. 2001; A. Deten, unpublished data) but not by others (Wenger et al. 1996). The mammalian family of HIF prolyl hydroxylases, PHD-1–3, have been established to function as the long-sought-after HIF oxygen sensors and thus are responsible for the protein-level control of HIF activity (Epstein et al. 2001; Ivan et al. 2001; Jaakkola 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 mammalian HIF-1α and -2α factors. This modification enables capture of HIF-α by the von Hippel–Lindau (VHL) tumor suppressor protein, which acts as recognition component of an E3 ubiquitin ligase complex. Therefore, prolyl hydroxylation is the oxygen-regulated step governing ubiquitination and proteasomal degradation of HIF-1α and -2α. A second O_2-requiring hydroxylation modifies an asparagine within the C-terminal transactivation domain of HIF-1α or -2α. The responsible asparaginyl hydroxylase is called FIH-1 (factor inhibiting HIF-1). This modification prevents recruitment of the coactivator proteins p300 and CBP, which in turn prohibits the transactivation of target genes under high Po_2 (Lando et al. 2002*a*, 2002*b*). The absolute requirement of PHD- and FIH-1 activities for O_2 and Fe(II) makes these hydroxylations susceptible to inhibition by hypoxia/anoxia, iron chelators (e.g., desferrioxamine), transition metals (e.g., Co^{2+}), and 2-oxoglutarate analogs (e.g., dimethyloxalylglycine). As a result, from cultured mammalian cells to fly cells, HIF signaling shows a well-preserved maximal activity between 0.5% and 2.0% O_2 (Jiang et al. 1996; Ebbesen et al. 2004; Gorr et al. 2004*b*).

It appears, therefore, that HIF signaling has evolved to operate precisely within the hypoxic niche of oxygen partial pressures (i.e., $Po_2 \leq P_c$). Although the P_c is a cell-specific variable, under standard culture conditions this critical oxygen threshold usually occurs for primary or transformed mammalian cells within the ~0.15%–1.5% O_2 range (Froese 1962; Robiolio et al. 1989). Thus, the P_c range overlaps well with the range of HIF activation, suggesting that HIF's sentinel and perhaps original function might have been safeguarding the cells' transition from aerobic to anaerobic ATP synthesis (e.g., Webster 1987; Iyer et al. 1998; Seagroves et al. 2001). Beyond these autonomous functions, active HIF is systemically required—for example, in nematodes and fruit flies—for the adaptation of the organism and its development during hypoxic, but not anoxic, stresses (Shen and Powell-Coffman 2003; Centanin et al. 2005).

HIF's maximal signal during low Po_2 and declining activity toward normoxic and anoxic conditions is the combined result of strikingly low oxygen affinities of the PHD and FIH-1 sensor proteins together with a pronounced hypoxia-driven transcriptional induction of some of the sensor genes (i.e., PHD-2, PHD-3). The in vitro and apparent K_M values for the hydroxylation of short synthetic HIF-1α peptide substrates by oxygen lie at approximately 64 mm Hg (FIH-1) and 178 mm Hg (average for PHD-1–3). These data imply that HIF hydroxylases operate in vivo at Po_2 levels far below their K_M values (Hirsilä et al. 2003; Acker and Acker 2004). Thus, the oxygen affinity of the PHD enzymes actually lies above the concentration of dissolved

oxygen in the air, which means that the availability of oxygen is predicted to be limiting for activity of HIF hydroxylases over the entire physiological range. These K_M data are therefore consistent with the enzymes' function as bona fide sensors of graded levels of oxygen. Moreover, the HIF-mediated hypoxic upregulation of PHD-2 and PHD-3 gene expression (Marxsen et al. 2004) prepares for the rapid destruction of HIF-α subunits following reoxygenation (D'Angelo et al. 2003) and enables PHD enzymes, because of their increased protein levels, to stay operative even under conditions of low P_{O_2} (Stiehl et al. 2006). This negative feedback loop not only limits the HIF response but also resets the hypoxia threshold by downshifting the HIF set point toward further O_2 depletion (Stiehl et al. 2006). Conversely, cells grown in high O_2 concentrations reset their HIF set point upward through the downregulation of PHD genes (Khanna et al. 2006). Activation of the HIF system can thus adapt to hypoxia because of its flexible and perhaps tissue-specific O_2 thresholds. These thresholds are adjusted according to the previous cell exposure to low or high O_2 concentrations and in turn are responsible for holding steady the pathway that is responsive to varying tissue oxygenation.

Under extreme oxygen deprivation, however, transcription factors that are distinct from HIF take over the regulation of cell survival. These HIF-independent pathways often center on the tumor suppressor protein p53, the known gatekeeper for cell cycle arrest or apoptosis in DNA-damaged, deoxygenated, and nucleoside triphosphate–depleted cells (Graeber et al. 1996; An et al. 1998; Wenger et al. 1998). Loss-of-function mutations of the p53 gene, which occur in more than 50% of human cancers (Levine 1997; Ryan et al. 2001), can lead to unchecked cellular proliferation and/or the selection for hypoxia-resistant, apoptosis-defying p53-mutant clones. In addition to p53, the translational induction of the basic leucine zipper–activating transcription factor 4 (ATF-4), indirectly mediated by the endoplasmic reticulum (ER) resident kinase PERK (Blais et al. 2004; Ye and Koumenis 2009), stimulates another truly VHL-independent cascade following the accumulation of un/misfolded proteins in the ER lumen of O_2-deprived cells (i.e., ER stress). ATF-4 activation has also been demonstrated in vivo near necrotic regions in invasive breast carcinomas (Ameri et al. 2004). Remarkably, PHDs (i.e., PHD-3) can also interact with ATF-4 via prolyl residues embedded in the factors' noncanonical ODD (Köditz et al. 2007). Thus, PHD-dependent sensing of changing oxygen levels recruits both the HIF and the ATF-4 systems to guide the cell along its trajectory from hypoxic adaptive to anoxic ER stress responses.

Oxidative Stress in Hypoxic/Anoxic-Tolerant Species

Of the oxygen consumed by animals, 0.1%–0.2% is routinely converted into reactive oxygen species (ROS), which are involved in several cellular signaling pathways, including the cell cycle, stress responses, and energy metabolism (e.g., Kowaltowski et al. 2009). When in excess, ROS can damage most biomolecules, and consequently they are implicated in the pathogenesis of many human diseases. A well-studied model for oxidative stress is ischemia and reperfusion. It was demonstrated in the late 1980s that tissue failure caused by ischemia/reperfusion stress is associated with the generation of excess ROS during the postischemic reperfusion, with subsequent oxidative stress. However, numerous animal species can survive up to many weeks with very little or no oxygen (Hermes-Lima and Zenteno-Savin 2002). How do they cope with the potential dangers of reoxygenation stress after hypoxia/anoxia exposure?

That hypoxic/anoxic-tolerant animals would have high levels of antioxidant defenses was first noticed by Reischl (1986) when he proposed that sulfhydryl (SH)-rich (i.e., cysteine-rich) hemoglobins in hypoxic turtles act as antioxidant defenses against a possible surge of ROS generation following tissue reoxygenation. Later, Rice and Cammack (1991) observed that ascorbate content in the brain of an anoxic-tolerant turtle remains high regardless of oxygenation state, whereas guinea pig brain slices presented a large decline in ascorbate levels. Rice et al. (1995) also reported that brain ascorbate concentrations are two- to threefold higher in anoxic-tolerant vertebrate species when compared with anoxia-sensitive species. Similarly, anoxia-exposed garter snakes presented increased superoxide dismutase (SOD) activities (in muscle and liver) and reduced glutathione (GSH) levels (in muscle) when compared with nonexposed animals (Hermes-Lima and Storey 1993). Moreover, exposure of garter snakes to 5 h of freezing caused an organ-specific increase in catalase and selenium glutathione peroxidase (Se-GPX) activities. Alterations in antioxidant defenses to hypoxia are now generally established. For example, anoxic goldfish and leopard frogs *Rana pipiens* both showed increases in Se-GPX and catalase activities (Hermes-Lima and Zenteno-Savin 2002). Turtles *Trachemys scripta elegans* exhibited high constitutive activities of most antioxidant enzymes in various organs (Willmore and Storey 1997*a*, 1997*b*), and although submergence caused decreased enzyme activities, these were considered to be sufficient to protect against generation of ROS on reoxygenation. Turtle hatchlings showed no changes in antioxidant capacity of plasma, brain, and liver following hypoxia or in lipid peroxidation and protein carbonyl (Baker et al. 2007). In contrast, five of seven species of hatchling turtles increased hepatic catalase activity following anoxic exposure (16 d; Dinkelacker et al. 2005). Among invertebrates, land snails *Helix aspersa* showed a twofold rise in foot Se-GPX activity induced by anoxia (A. F. Welker, unpublished data). Anoxia-induced increases in endogenous antioxidant defenses (enzymes or GSH) were also reported for the molluscs *Biomphalaria tenagophila* and *Littorina littorea* (Pannunzio and Storey 1998; Ferreira et al. 2003), the polychaete *Heteromastus filiformis* (Abele et al. 1998), and the crab *Chasmagnathus granulata* (de Oliveira et al. 2005).

Exposure to hypoxia also elicited increases in antioxidant enzymes in fish, for example, catalase and Se-GPX activities in the brain of carp (Lushchak et al. 2005*b*) and the liver of tilapia (Cardoso 2005). In the estuarine fish *Leiostomus xanthurus*, organ-specific increases in SOD activity were also observed under hypoxia (Cooper et al. 2002). In addition, hypoxia exposure in Pacific oysters prompted increased GPX expression in the

gills and mantle (David et al. 2005). This ability to elevate antioxidant enzyme activity seems to correlate with the capacity of an animal to tolerate anoxia/hypoxia. Thus, the Atlantic cod *Gadus morhua*, a hypoxic-sensitive species, showed no changes in catalase expression and a diminution in the expressions of GPX and SOD in response to hypoxia (Olsvik et al. 2006). Constitutively high activities of antioxidant enzymes are also relevant for posthypoxic stress in fish. Goldfish, for example, present levels of hepatic Se-GPX activity (0.3–0.5 U/mg protein) that are comparable with rodents. In *Piaractus mesopotamicus*, a fish that lives in seasonally oxygen-poor waters, high constitutive levels of the GPX protein were observed in liver (Bastos et al. 2007).

In addition, freezing (in freeze-tolerant vertebrates) harbors the danger of generating excess ROS during thawing and requires some control over free-radical metabolism. Freeze exposure for 24 h in the wood frog *Rana sylvatica* prompted significant increases in Se-GPX and total-GPX activities in different tissues (Joanisse and Storey 1996). Increased expression of thioredoxin, glutathione S-transferase (GST), and glucose-6-phosphate dehydrogenase (G6PDH) in heart and γ-glutamyltranspeptidase (γ-GT) activity in liver were also observed for wood frogs under exposure to freezing (Hemmings and Storey 1996; Storey 2004). In addition, a variety of reptiles show increased expression of antioxidant enzymes or enzyme activity during freezing (Hemmings and Storey 2000; Storey 2006; Voituron et al. 2006) reminiscent of those responding to anoxia (Storey 2007).

Air exposure is another situation that severely diminishes oxygen availability to water breathers. Mussels *Perna perna* increased GST activity in the hepatopancreas during a 4-h period of air exposure (de Almeida et al. 2005). In the subantarctic crab *Paralomis granulosa*, air exposure induced tissue-specific increases in SOD, catalase, and GST activities (Romero et al. 2007). A rise in muscle Se-GPX and catalase activity was also reported for *Callinectes ornatus*, a crab from intertidal zones, following 3 h of aerial exposure (Togni 2007).

Evidence for postanoxic oxidative stress eventually came from goldfish (Lushchak et al. 2001), where lipid peroxidation increased by twofold in liver after 1 h of reoxygenation (following 8 h of anoxia). Hypothetically, goldfish would have even higher levels of lipid peroxidation products during reoxygenation if antioxidant defenses were not activated on anoxic exposure. Indeed, hyperoxia exposure in goldfish induced significant augmentation in markers of oxidative stress (Lushchak et al. 2005*a*). Clear-cut evidence that ROS production is increased following postanoxic stress was recently obtained by Milton et al. (2007), who determined that hydroxyl radical ($OH\cdot$) generation in the brain of turtles *T. scripta* became very low during anoxia but was restored on reoxygenation. A reduction in ROS production was also reported in turtles *Chrysemys picta* when their cortical sheets were exposed to progressive hypoxia and anoxia (Pamenter et al. 2007). ROS generation returned to control levels on reoxygenation. The absence of a large increase in oxidative damage during reoxygenation has been attributed to the preactivation of endogenous antioxidant enzymes and/or the presence of well-developed antioxidant defense systems. The variation in ROS concentration could act on signaling pathways to control antioxidant enzyme synthesis in hypoxic-tolerant species (Lushchak and Bagnyukova 2006). Possible candidates to participate in these signaling cascades include H_2O_2 itself (Kowaltowski et al. 2009) and the Akt (protein kinase B) pathway (Loss et al. 2009). This increase in the endogenous antioxidant capacity under low oxygen concentrations has been termed "preparation for oxidative stress" (Hermes-Lima and Zenteno-Savin 2002) and is present in animal groups that share very distant evolutionary ancestors (Fig. 1). Moreover, the buildup of enzymatic antioxidant defenses under conditions of low metabolic rates (anoxia, hypoxia, freezing) is energetically costly, emphasizing the importance of ROS management for the control of oxidative stress on reoxygenation.

Activation of Se-GPX (activity or gene expression) occurs in various animals enduring hypoxia or stress related to changes in oxygen availability, for example, in hibernating bats and ground squirrels (Storey 2003), in supercooling lizards (Voituron et al. 2006), and in estivating gastropods (Ramos-Vasconcelos et al. 2005; Nowakowska et al. 2009). Therefore, it is tempting to propose that Se-GPX plays a crucial role in regulating the effects of ROS (as inducing oxidative stress or as a cellular second messenger) during cycling oxygenation and metabolic rate.

While increased antioxidant defenses during anoxia/hypoxia maintains intracellular levels of ROS at manageable levels on reoxygenation, the role of each specific antioxidant defense has not been investigated until very recently. When Cardoso (2005) injected the Nile tilapia with the catalase inhibitor aminotriazole (ATZ; Bagnyukova et al. 2005), it promoted a depletion in hepatic catalase activity but did not cause a relevant reorganization of other antioxidant defenses and markers of oxidative stress during normoxia. However, hypoxia exposure tended to increase hepatic Se-GPX activity, and after 30 min of reoxygenation the GSSG : GSH ratio was increased by 1.3-fold in ATZ-injected fish in comparison with saline-injected fish. Importantly, other markers of oxidative stress showed little change in either hypoxic or reoxygenated fish (Cardoso 2005). These results are consistent with a minor induction of posthypoxic oxidative stress in tilapia liver with suppressed catalase activity and, thus, with a minor role for catalase in the protection against reoxygenation stress in tilapia. However, we must bear in mind that activation of liver Se-GPX during hypoxia (Cardoso 2005) is consistent with Se-GPX playing a role in managing posthypoxic oxidative stress. Because there is no specific Se-GPX inhibitor, future RNA interference (RNAi) work must elicit this postanoxic stress paradigm.

HIF and Hypoxia Responses in Crustaceans

Tidal-zone crustaceans, similar to the turtles and freshwater fish described above, are also exposed to particularly variable environments. Many decapod crustaceans—including crabs, shrimps, and lobsters—experience frequent short-term changes

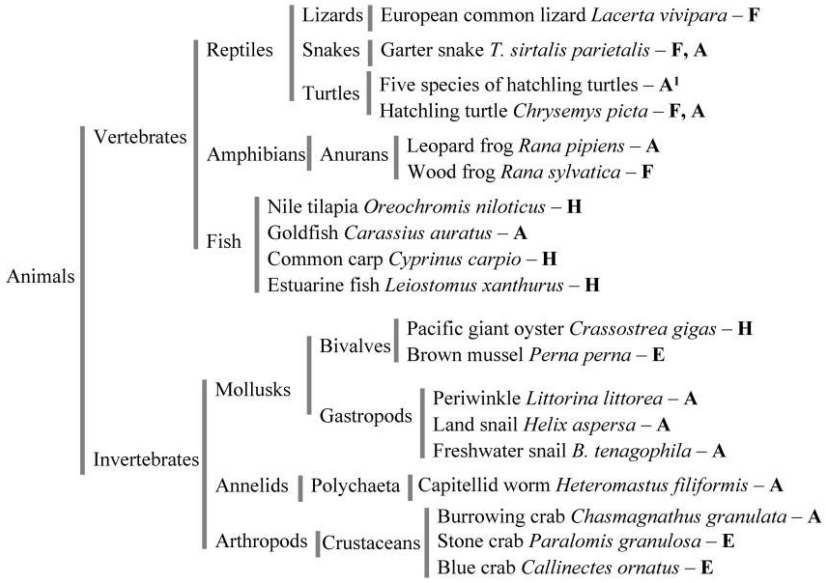

Figure 1. Animal groups for which preparation for oxidative stress is an adaptation strategy for enduring freezing (*F*), hypoxia (*H*), anoxia (*A*), and air exposure (*E*). Other stress situations where preparation for oxidative stress may be involved—such as hibernation, estivation, diapause, supercooling, and dehydration—are not listed. Hatchling turtles with increased liver catalase activity are *Chelydra serpentine*, *Trachemys scripta*, *Emydoidea blandingii*, *Chrysemys picta*, and *Malaclemys terrapin*.

in the abiotic factors oxygen, salinity, and temperature in their marine or estuarine habitats. The ability of a marine species to adjust to varying levels of these parameters as the tide rises and falls, for example, is a major factor in determining whether the animal is restricted to the subtidal zone or can penetrate the upper reaches of the estuary or indeed the supratidal zones. As the next two sections demonstrate, both marine and freshwater decapods are well endowed with behavioral and molecular responses when challenged by a nonconstant habitat, and thus they represent important new models with which to investigate both acute and chronic adaptations to stress.

One strategy for surviving in a changing world is to be relatively insensitive to oxic, ionic, or thermal fluctuations and to simply conform to the current conditions. This approach can potentially offer considerable energy savings. Another strategy is to maintain a heterogeneous array of responders that can regulate function across the spectrum of environmental changes that the organism might experience. An example is the oxygen-binding and -transporting protein hemocyanin (Hc) found in the circulating hemolymph of most decapod crustaceans. The protein is composed of multiple subunits that self-assemble into hexameric arrays; in the Dungeness crab *Cancer magister*, each Hc subunit is encoded by a separate gene (Terwilliger et al. 2006). The high degrees of plasticity and diversity at both the structural and functional levels afforded by the molecular heterogeneity of crustacean Hc is thought to provide these animals with flexible adaptation, both developmentally and evolutionarily (Markl and Decker 1992; Giomi and Beltramini 2007). Working in concert with this strategy of functionally heterogeneous oxygen transporters is the ability to rapidly mobilize a suite of defenses, both behavioral and molecular, against a variable thermal regime or fluctuating oxygen and salinity concentrations in the environment through closely coordinated pathways of gene regulation. A key regulator in tolerating hypoxia and possibly other stresses is the HIF transcription factor, as introduced above.

In a phylum such as Arthropoda, species diversity is so immense that comparative studies even within a class can yield a panoply of divergent results that makes the search for unifying patterns to understand mechanisms of stress response more challenging. Nonetheless, both the comparative approach and detailed examinations of single species are providing significant glimpses of the range of molecular adaptations to environmental stresses among crustacean species and how conserved these mechanisms are in comparison with those in other phyla. Gene-expression profiling studies coupled with real-time quantitative PCR (qPCR) analyses have identified novel stress-specific genes as well as shared multiple-stress-responsive genes in several species, including shrimp *Palaemonetes pugio* and *Penaeus monodon* (Brouwer et al. 2007; de la Vega et al. 2008) and crabs *Portunus pelagicus*, *C. magister*, and *Carcinus maenas* (Terwilliger et al. 2006; Kuballa et al. 2007; Towle and Terwilliger 2008). Within the decapods, however, it is likely that the detailed response to an environmental challenge or to stress has diverged widely across species, although the upstream control mechanisms have been conserved. In addition to its well-documented role in mobilizing the transcription of selected genes associated with hypoxia defense, HIF upregulation has been demonstrated during normoxia in both hypoxia-tolerant vertebrates such as the crucian carp (Rissanen et al. 2006) and specific cases in hypoxia-sensitive vertebrates (Blouin et al. 2004; Dery et al. 2005). Processes in which normoxic upreg-

ulation of HIF-1α is involved—including controlled cell proliferation, apoptosis, vascularization, tissue reconstruction, and a strong immune response—are also processes that are integral to the molt cycle and to the innate immune response of crustaceans. These parallels have led us to hypothesize that HIF-α is involved in recruiting certain genes during molting and infection in crustaceans (Terwilliger 2008).

Molting requires a periodic shutdown of extraneous physiological activities during which the animal focuses on simultaneously detaching itself from the old exoskeleton while synthesizing the new one. The epithelial surface to be shed and rebuilt includes the lining of the foregut and hindgut, explaining in part why the adult crab ceases to feed for several days preceding and immediately after ecdysis. Furthermore, just before ecdysis, oxygen uptake may be affected because of an increased diffusion distance across the gills resulting from multiple layers of the new flexible exoskeleton, the loosened extracellular space, and the old exoskeleton. The ligamentous supports between the heart and the dorsal carapace must be temporarily disconnected, further contributing to a decrease in effective oxygen uptake and delivery via the circulation. Thus, the potentials for transient metabolic depression, localized tissue hypoxia, and a role for HIF regulation are high when a crustacean molts, even though environmental oxygen is plentiful. Preliminary studies of HIF expression during molting in juvenile *C. magister* in normoxic conditions indicate cyclical upregulation of HIF-α as the molt cycle progresses (W. Miller, unpublished observations). These results call for further investigations into HIF-regulated pathways of gene expression in molting.

The upregulation of HIF-α proteins in normoxic vertebrate macrophages occurs in response to stimulation by lipopolysaccharide (LPS), an outer-wall component of Gram-negative bacteria (Blouin et al. 2004). Crustacean hemocytes are also mobilized by LPS, releasing antimicrobial peptides, clotting proteins, and prophenoloxidase that function in the crustacean innate immune response (Söderhäll and Cerenius 1998; Bachere 2000; Terwilliger and Ryan 2006). Hemolymph obtained from freshly caught crabs and shrimp often contains low levels of culturable bacteria (L. Burnett, unpublished observations), which suggests that crustaceans may naturally function in a state of chronic immunostress. In acute-infection studies, healthy adult blue crabs *Callinectes sapidus* were infected with the marine pathogenic bacterium *Vibrio campbellii* (N. Terwilliger, K. Burnett, L. Burnett, and D. Towle, unpublished manuscript), resulting in increased expression of HIF-α mRNA in *Vibrio*-injected crabs, consistent with the role of HIF-α in a normoxic immune response. In a separate study, juvenile *C. sapidus* were injected either with a specific HIF-α double-stranded RNA (dsRNA) or with the nonspecific poly-C/G dsRNA, and gene expression was measured by qPCR. As a result of both dsRNA injections, HIF-α expression was significantly higher than it was in noninjected crabs (Terwilliger et al. 2008). We suggested that the dsRNA activated a nonspecific immune response, similar to that observed in shrimp (Robalino et al. 2004). The increase in HIF transcript in *C. sapidus* supports the hypothesis that nonspecific immunostress in crabs triggers an upregulation of HIF. Changes in expression of downstream portions of the immune response would then be initiated. Together, solid evidence exists to support the notion that HIF in decapod crustaceans is key in coordinating responses in tissues that undergo developmental and ecdysial reprogramming as well as in oxygenated yet microbially challenged cells.

Physiological Response of Crayfish to Environmental Hypoxia and Temperature Change

The variability in the abiotic environment of intertidal crustaceans (above) can be even more extreme in freshwater bodies, especially isolated shallow ponds. Aquatic invertebrates in such habitats are regularly exposed to fluctuations in ambient oxygen concentration and temperature. These habitats can frequently become hypoxic, and as a result these animals exhibit a suite of defenses to cope with O_2 stress that can increase the efficiency of O_2 transport systems. A nice example of such adaptations is the hypoxia-inducible, HIF-driven synthesis of different globin genes in the planktonic crustacean *Daphnia magna* (Gorr et al. 2004*a*). As an alternative to upregulated O_2 delivery to maintain an aerobic, oxyregulated physiology while facing mild hypoxia, a lessening of energy demands through the reduction of metabolic activities together with a shifted balance from oxidative toward anaerobic (i.e., glycolytic) ATP production might also be invoked to protect cellular energy levels from becoming fatally depleted (Hochachka et al. 1996). However, an exaggerated glycolytic substrate flux is not sustainable in the long term, and mechanisms to reduce energy demand must be exploited. Metabolic rate depression is understood to facilitate this reduction in ATP demand in many invertebrate species (e.g., Storey and Storey 1990; Grieshaber et al. 1994). Endothermic species may also achieve metabolic rate depression by lowering the thermoregulatory set point (anapyrexia), slowing cellular processes and ultimately decreasing energy demand. Some ectotherms—for example, lizards (Hicks and Wood 1985), frogs (Wood and Malvin 1991), fish (Rausch and Crawshaw 1990), and crayfish (Dupre and Wood 1988)—actively seek out colder environments to reduce their body temperature, thereby reducing metabolic rate and their requirement for O_2, a behavior known as hypoxia-induced behavioral hypothermia (HIBH; Wood 1991; Morris 2004).

Ambient temperature (T_a) is a particularly important variable for ectotherms because it drives metabolism in these organisms and can have large effects on metabolism (Q_{10} effect). As such, the American signal crayfish *Pacifastacus leniusculus* is a valuable model in which to examine the interplay between temperature and physiological and molecular responses to hypoxia, an interaction that only rarely occurs in mammalian and avian models (e.g., Morris 2004). To study aspects of HIBH in *P. leniusculus*, crayfish were acclimated to 5°, 13°, or 20°C for a minimum of 2 wk and subsequently exposed to progressively decreasing oxygen partial pressures (Po_2, ~21.3–1.0 kPa). The critical point (P_c) of the oxygen consumption rate (Mo_2) below which crayfish recruit anaerobiosis and accumulate lactate was

elucidated using closed respirometry (Morris and Callaghan 1998; Morris et al. 2005) while hemolymph was sampled for L-lactate analysis.

Increasing the acclimation temperature of *P. leniusculus* from 13° to 20°C elevated the Mo_2 in normoxic crayfish by 14.2%, whereas a decrease in acclimation temperature from 20° to 5°C depressed Mo_2 by 7.5% (Fig. 2). However, these differences were largely apparent only for crayfish in normoxic or near-normoxic conditions, for example, comparing results at 20°C (Po_2 = 15 and 10 kPa) with those at 13° and 5°C. Regardless of the temperature employed, hypoxia eventually elicited an oxyconforming response with significant decreases in Mo_2 below the P_c. For example, at 20°C, $P_c = Po_2$ of 5 kPa, and importantly at 5°C, the P_c corresponded to a Po_2 of just 2.5 kPa (Fig. 2).

In normoxic crayfish, the hemolymph concentration of L-lactate remains universally low, even at 20°C (0.04–0.23 mmol L^{-1}). In general, hemolymph lactate concentrations increase when the water Po_2 falls below 2.5 kPa; thus, this value is used as the critical Po_2 for *P. leniusculus*, consistent with work on other crayfish (Morris et al. 2005). This P_c for anaerobiosis is largely unaffected by T_a, except that at the lowest Po_2 (~1 kPa), the extent of the anaerobiosis is greater at the highest temperature (20°C). Thus, predictably, crayfish held at lower temperatures resort less to anaerobiosis (less L-lactate), consistent with the suggestion that animals at lower temperatures are better able to maintain energetic status, probably because of Q_{10} effects lowering energy requirements. Thus, HIBH would provide some clear benefits during severe environmental hypoxia.

These findings are reflected in data gained using a crayfish cell line (OLGA-PH-J/92, obtained from American Type Culture Collection [ATCC]) from *Orconectes limosus*. These cells show considerable tolerance to severe and prolonged hypoxia (96 h, Po_2, ~0.2 kPa to ~1 kPa) with reduced temperatures (13°–5°C) and maintain ATP concentration in a steady state comparable to normoxic values (Wren et al. 2008). In general, these findings support the concept that cooler temperatures offer protection to ectotherms in hypoxic environments. Our investigation of the in vitro onset-decay profile of the HIF indicates that HIF binding to DNA in hypoxic OLGA cells is activated by hypoxia and is further potentiated by reduced temperatures (23°, 13°, 5°C comparisons; Wren et al. 2008), which is highly reminiscent of conclusions reached for the crucian carp (Rissanen et al. 2006). Importantly, it may thus also be the case that crayfish seek out colder temperatures in order to aid HIF induction (Morris 2004).

The identity of the crustacean OLGA cell line has recently come into question (Lee et al. 2009), and consequently, the commercial supply of OLGA cells (ATCC CRL-2576) has been discontinued. The preliminary report (Lee et al. 2009) regarding ATCC OLGA cell batches as containing clones of a potentially fungal cell contaminant encouraged us to characterize our batch of independently supplied cells (gift of Dr. T. Neumann; Neumann et al. 2000) by cloning and sequencing a fragment of the arginine kinase (Ak) gene. Among crustaceans, Ak is a widely identified and highly conserved gene, and the complementary

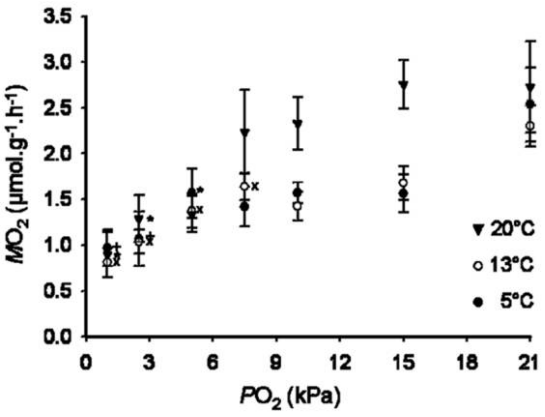

Figure 2. Oxygen consumption rate Mo_2 (μmol g^{-1} h^{-1}) of *Pacifastacus leniusculus* acclimated to either 20°C (*filled triangles*), 13°C (*open circles*), or 5°C (*filled circles*) for 14 d before exposure to progressive hypoxia. Error bars represent standard errors of the mean. Significantly lower values compared with normoxic rates within temperature groups are denoted by an asterisk (20°C), a plus sign (13°C), and a multiplication cross (5°C). Analyses employed a one-factor ANOVA, and where significant, post hoc testing used Tukey's test ($P < 0.05$).

DNA (cDNA) is partially known from the *P. leniusculus* crayfish (J. F. Wren, unpublished manuscript). Thus, assuming that our OLGA cells stem from the crayfish *O. limosus* (Neumann et al. 2000), we were able to include as a positive control the cloning of the homologous Ak fragment from the close relative *P. leniusculus*.

Using tissue from either *P. leniusculus* muscle or OLGA cells, we were able to TOPO-clone a 300-bp Ak PCR amplicon into the pCRII plasmid for subsequent sequence confirmation of the enclosed insert (Invitrogen, TOPO cloning kit with pCRII vector). BlastN searches against a nonredundant DNA database, and ClustalW-based sequence alignments revealed that (*a*) 99 out of 100 top-scoring matches between the OLGA or *P. leniusculus* Ak queries were obtained with arthropod Ak database entries, including numerous crustacean sequences with identity ranging from 80% to 84%; and (*b*) the short Ak fragment showed a 100% sequence identity between the OLGA and *P. leniusculus* sources. These Ak sequence data thus strongly encourage us to conclude that our OLGA cells are truly of crustacean origin and are not a fungal derivative. We are unable to make the same conclusion for other, discontinued OLGA supplies, and we urge caution because OLGA may comprise a heterogeneous strain in which some batches may include fungal contaminants. Potential users of OLGA cells should similarly consider specifically characterizing their cell batch for crustacean or noncrustacean sequences.

There have been few attempts to integrate the biochemical and molecular concepts of the hypoxia response with that of anapyrexia (or HIBH; Morris 2004 for review). However, it is important to consider that while acute lowering of temperature may afford some protection against severe or prolonged hypoxia, ectotherms ultimately show thermal acclimation in which metabolic rate partially recovers and Mo_2 reapproaches the

original rate shown at higher temperatures (hence the reduction in Mo_2 of only 7.5% when comparing 20°-acclimated to 5°C-acclimated crayfish). In these circumstances, HIBH is of short- to medium-term benefit, and in view of our findings, the acute effect of temperature change on the response of these animals to hypoxia must now be established.

Targeting Hypoxia-Tolerance Mechanisms: From *Drosophila* to Cancer Cells

As emphasized above, tolerance of severe hypoxia ($Po_2 \ll P_c$) or anoxia commonly rests on energy conservation as a long-term survival strategy. This hypometabolic state is defined by Po_2-conforming oxygen uptake rates, disengaged protein and DNA synthesis, and a noncycling, quiescent phenotype. Adoption and maintenance of hypometabolism underlies the enormous resistance in many invertebrate and some ectothermic vertebrate species to a large variety of stresses including hypoxia, ischemia, dehydration, and the hypothermia experienced in small mammalian hibernators (reviewed in Hochachka et al. 1996; Boutilier and St-Pierre 2000; Gorr et al. 2006). For a tumor, the degree of hypoxia tolerance may be an important determinant of the level of hypoxia that can be sustained within the mass. Therefore, hypoxia tolerance can be viewed as a protumor property that provides a molecular resistance toward effective therapy. Similar to that observed in invertebrate and ectothermic vertebrate animal models, hypometabolic defenses of severely hypoxic (0%–0.5% O_2) cancer cells integrate adaptations at all three levels of this metabolic slow down, that is, in the O_2 uptake rate, the macromolecular synthesis machinery, and within the progression of the cell cycle. Regarding the switch into the oxyconforming uptake mode of cancer cells (Froese 1962; Freyer 1994), a molecular explanation has recently been put forward in form of the partly HIF-mediated induction of the pyruvate dehydrogenase (PDH) inhibitor PDK1. Activation of PDK1 in turn suppresses respiration rates because the inhibition of the PDH reaction starves the tricarboxylic acid (TCA) cycle of pyruvate substrate (Kim et al. 2006; Papandreou et al. 2006). The general silencing of all ATP-costly macromolecular syntheses—including the rapid inhibition of replicon initiation in early S-phase of the cell cycle ("replicon arrest": Pettersen and Lindmo 1983; Probst et al. 1988; Åmellem and Pettersen 1991) and the marked reduction in the overall rate of protein synthesis ("translational arrest": Pettersen et al. 1986; Kraggerud et al. 1995; Guppy et al. 2005)—has also been reported from O_2-deprived cancer cells. Finally, cell cycle proliferation is arrested, often at the G1/S-phase transition and independent of p53 function (Graeber et al. 1994), to block or slow the growth of cancer cells when they become deoxygenated (Giaccia 1996; Höckel and Vaupel 2001). This ultimate defense, cellular quiescence, correlates with an extreme resistance to radiotherapy (Masunaga et al. 2002, 2006).

To introduce and assess the targeting of hypoxia-tolerance pathways as a conceptually novel strategy in oncology, we recently established a fly-to-cancer translational approach for the discovery of and interference with novel hypometabolic functions. When *Drosophila* is exposed to mild hypoxia, HIF functions in vivo in an angiogenesis-like fashion, mainly within the tracheal system (Lavista-Llanos et al. 2002), to convert the hypoxia signal into the activated expression of the fibroblast growth factor (FGF) receptor homologue. In this way, HIF primes the tracheal endings to sprout out toward hypoxic target tissues that are secreting increasing doses of FGF ligand (Centanin et al. 2008). However, when flies or fly embryos are exposed to a more severe shortage of O_2, they will switch from a tracheal (angiogenic) strategy to a hypometabolic one that at first glance seems to mimic the behavior of cancer cells, both in vitro and in vivo, during progressive deoxygenation. *Drosophila*'s remarkable ability to survive severe oxygen deprivation is reflected by the complete recovery after a 4-h exposure (adult flies) or one of several days (late embryos) to a N_2 atmosphere (Haddad et al. 1997; Wingrove and O'Farrell 1999). For the initial discovery of genes whose hypoxia-responsive products might play a role in governing entry and maintenance of a hypometabolic state (i.e., hypometabolic factors [HMFs]) and to that end, participate in conferring hypoxia tolerance, we recorded transcriptome profiles of S2 cells (Schneider cell, line 2) in response to graded hypoxia through microarray and Northern blot RNA-level surveys. S2 cells were cultured from the particularly hypoxia-tolerant late-stage embryos (Schneider 1972) and abundantly accumulated the functional HIF complex during O_2 deprivation (Gorr et al. 2004*b*). More than 300 S2-expressed genes responded with a greater than twofold induction or suppression during the most severe period of hypoxia (not shown). Because of the general paucity of commercial *Drosophila* antibodies, this initial survey recorded only transcriptional responses. However, during the subsequent validation and fly/cancer translation stage exemplified for the homologous *Thor/4E-BP1* (fly/human) HMF candidate below, we assessed the gene's functions at the RNA and protein levels.

Our survey (J. M. Gómez, T. Tomita, N. Romero, K. Clough-Gorr, M. Gassmann, H. F. Bunn, A. Deten, P. Wappner, and T. A. Gorr, unpublished manuscript) revealed the gene *Thor* as one of the most strongly hypoxia-induced hypometabolic factor candidate genes. *Thor* is *Drosophila*'s single-copy homologue of the mammalian translation repressors 4E-binding proteins 1–3 (4E-BP1–3). Active and hypophosphorylated 4E-BPs act by sequestering the rate-limiting, cap-binding protein eIF4E from the key translation initiation complex eIF4F (see Fig. 3). Thus, induction of *Thor* in hypoxic fly cells (see Fig. 3, thick "induction" arrow from hypoxia label to 4E-BP1) might contribute to inhibiting this branch of the ATP-costly protein synthesis. Indeed, fly genetics in collaboration with Pablo Wappner (Leloir Institute, Buenos Aires) were used to expand our S2 results to the in vivo level by showing (*a*) the pronounced induction of *Thor* mRNA in hypoxic flies, (*b*) the strict requirement of HIF for in vivo *Thor* expression, and (*c*) the requirement of *Thor* function for *Drosophila* to adapt to and recover from hypoxia. Observation (*b*) implicates HIF for the first time in the control of a candidate hypometabolism-mediating factor and thus in the downregulation of ATP-costly protein synthesis activities in hypoxia (J. M. Gómez, T. Tomita,

N. Romero, K. Clough-Gorr, M. Gassmann, H. F. Bunn, A. Deten, P. Wappner, and T. A. Gorr, unpublished manuscript).

Do cancer cells also acquire hypoxia tolerance and therapeutic resistance in part via the 4E-BP-mediated translational slowdown? A rather weak and heterogeneous transcriptional control of 4E-BP genes in different human cancer cells (i.e., hepatoma cells [Hep3B], breast carcinoma cells [MCF7], cervical carcinoma cells [HeLa]) during 16-h episodes of hypoxia or anoxia (not shown) prompted us to study regulation of 4E-BP1 proteins in these cell lines. In order to act as translational suppressor, 4E-BP1 must be hypophosphorylated to be able to compete for eIF4E binding with the protranslational factor 4G (see Fig. 3). Decrease in the phosphorylation status of 4E-BPs is usually accomplished through the tuberous sclerosis complex (TSC) 1/2-REDD1/RTP801-mediated inhibition and the Bcl2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3)- or promyelocytic leukemia tumor suppressor (PML)-mediated inhibition of the upstream mammalian target of rapamaycin (mTor) kinase during hypoxia/anoxia (Fig. 3; Brugarolas et al. 2004; Sofer et al. 2005; Li et al. 2007). Consistent with this expectation, we found a pronounced shift from hyper- to hypophosphorylated 4E-BP1 species in hypoxic and anoxic Hep3B and HeLa cells but not MCF7 cells (not shown). In conclusion, some cancer cells show a phosphosignature that indicates a flylike activated translation repression via 4E-BP1 controls when deoxygenated.

To understand the relevance of this checkpoint for the hypoxia adaptation, we collaborated with molecular oncologist Brad Wouters (Ontario Cancer Institute, Toronto) and radiologist Philippe Lambin (Maastro Clinics, Maastricht) to analyze how the RNAi-based loss-of-function of 4E-BP1 relates to hypoxic survival and treatment resistance of human cancer cells and tumor models. Expression of short hairpin–interfering RNA (shRNA) specific for 4E-BP1 yielded stable knockdown (kd) transformants of two human cancer cells (HeLa and glioblastoma U87) whose viability during prolonged and severe hypoxia exposure was significantly compromised. Moreover, a single 10-Gy dose irradiation of U87 tumor xenografts made from 4E-BP1 kd cells versus empty-vector (ev) transfected control cells revealed a differential radio sensitivity. The postradiation specific growth delay, a measure thought to closely reflect the relative number of cells killed by treatment, was always significantly higher in the kd tumors than in the ev control tumors, regardless of tumor end volume (Dubois et al. 2009). These data strongly suggest a reduction in the viable fraction of radio-resistant hypoxic cells in the kd tumors, which was further evidenced by increased levels of cleaved caspase-3 despite similar extent or severity of intratumoral hypoxia in both tumor types (pimonidazole IHC staining). Thus, 4E-BP1 loss-of-function increases radio sensitivity by decreasing the hypoxia tolerance of tumor xenografts without affecting the hypoxic fractions of the xenografts. Diminished hypoxia viability in cells of 4E-BP1 kd tumors was, however, associated with a threefold drop in steady-state ATP concentrations (Dubois et al. 2009).

Regulating the rates of protein synthesis via the control of the cap-dependent, eIF4F-driven translation is required, from fly to cancer cells, to facilitate energy conservation and to gain hypoxia tolerance. For tumors, this tolerance acquisition is associated with radioresistance. The results of this collaborative effort show that targeting translational controls represents an effective new way to sensitize cells to hypoxia and solid cancers to radiotherapy.

Strategies of Anoxia Tolerance in Ectothermic Vertebrates

While most vertebrates die within a few minutes of anoxia, a few can survive without any oxygen for days to months, depending on temperature (Fig. 4). Some of these tolerant ectotherms actively seek out colder ambient temperatures to lower their metabolism in order to better cope with lack of oxygen (HIBH, above). Anoxia-tolerance champions among vertebrates include the crucian carp (*Carassius carassius*) and some North American freshwater turtles of the genera *Trachemys* and *Chrysemys*. In these animals, anoxia tolerance has evolved to allow survival in small lakes and ponds that may become anoxic for several months during the winter when thick ice cover blocks both oxygen diffusion from the atmosphere and the light needed for photosynthetic oxygen release (Holopainen and Hyvärinen 1985; Ultsch 1989).

The strategies utilized for anoxic survival by crucian carp and turtles differ in several aspects (Lutz and Nilsson 1997; Nilsson and Lutz 2004). However, most importantly, they all defend their brain ATP levels by matching ATP use with glycolytic ATP production during anoxia. As such, they are able to maintain ion homeostasis and avoid cell membrane depolarization, which, when it occurs in the brains of anoxia-intolerant animals such as mammals, initiates many of the events that finally lead to anoxic cell death (Lutz et al. 2003).

While crucian carp maintain physical activity in anoxia (Nilsson et al. 1993), some turtles enter into a near-comatose state as they strongly depress both brain and heart activity. For example, the crucian carp maintains cardiac output during anoxia (Stecyk et al. 2004), while the heart rate of cold anoxic turtles falls to only about one beat per minute (Stecyk et al. 2008). Initially, the neural depression in turtles appears to be mediated by adenosine release (Nilsson and Lutz 1992) and subsequently maintained by a massive release of the inhibitory neurotransmitter gamma-amino butyric acid (GABA; Nilsson and Lutz 1991). This is combined with a downregulation of ion channels with conductance for Na^+ and Ca^{2+}, including glutamate receptors of the N-methyl-D-aspartate (NMDA) family (which in mammals is responsible for much of the detrimental Ca^{2+} influx during anoxia; Bickler et al. 2002). The extracellular level of GABA has been found to rise some 80 times in the anoxic turtle brain (Nilsson and Lutz 1991), suggesting that the turtle is virtually anesthetizing itself with GABA.

In contrast to the turtles, the crucian carp does not appear to suppress ion conductance in the brain, possibly with the exception of the NMDA receptor (Ellefsen et al. 2008). However, the carp downregulates selected neural functions, including vision and hearing, which are probably of little importance during the dark anoxic winter (Suzue et al. 1987; Johansson et

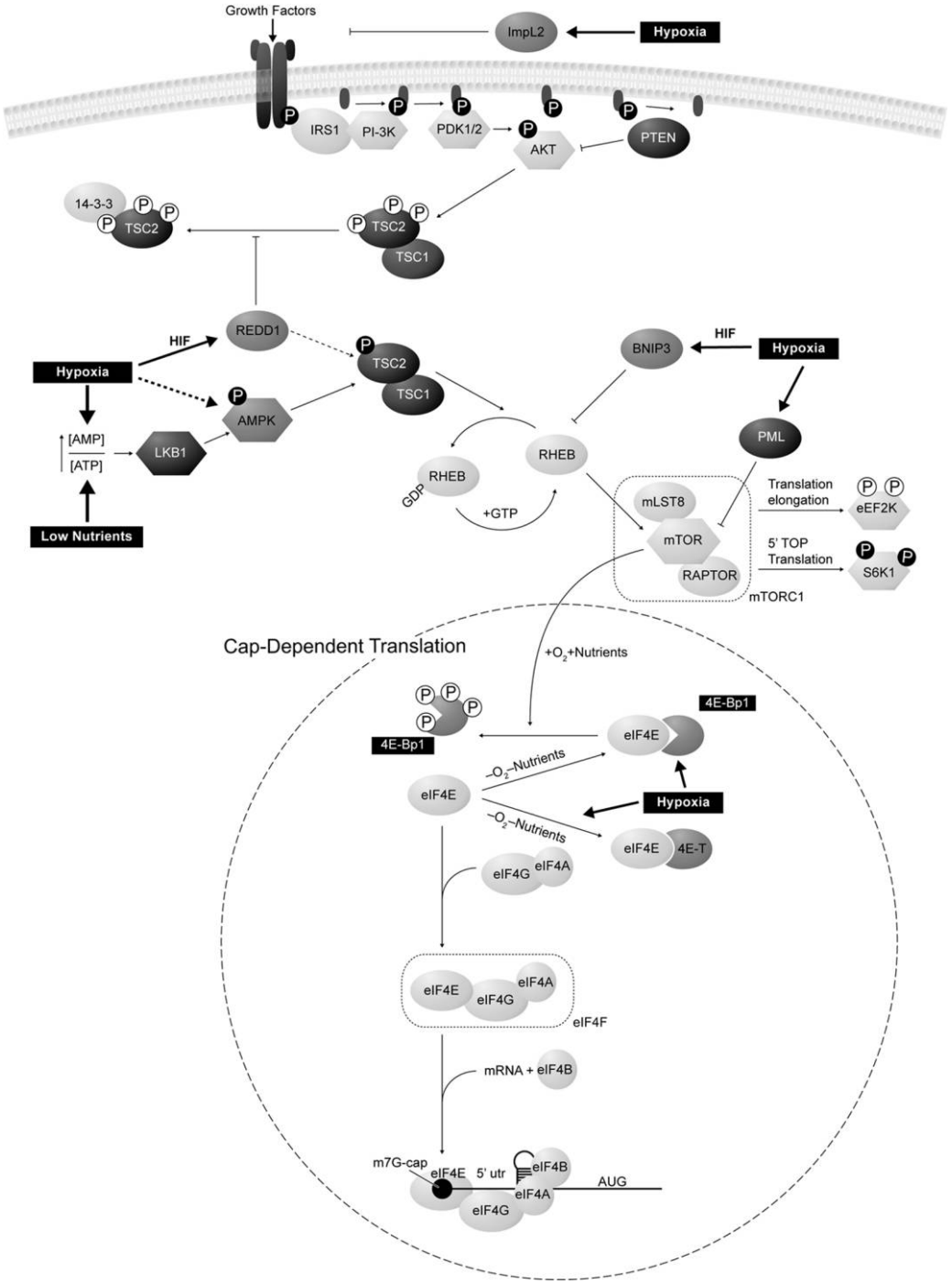

Figure 3. Mammalian target of rapamycin (mTOR) signaling pathway and its various points of inhibition exerted by hypoxia ($-O_2$) or nutrient shortage (−nutrients). When nutrients (e.g., amino acids) and/or oxygen are sufficiently available, the kinase mTOR transmits positive growth signals (e.g., binding of insulin/IGFs to the insulin receptor InR) to the translational machinery through its ability to phosphorylate (1) the p70 subunit of the ribosomal protein S6 kinase 1 (S6K1), (2) the eukaryotic elongation factor 2 kinase (eEF2K), and (3) the eukaryotic translation initiation factor 4E (eIF4E)–binding protein 1 (4E-BP1). These stimulating (*white P in black circle*) and inactivating (*black P in white circle*) phosphorylations cumulatively activate the translational initiation of cap-dependent (target 4E-BP1) or 5′ terminal oligopyrimidine tract (TOP)–carrying (target S6K1) RNAs and stimulate their translational elongation as well (target eEF2K). Hyperphosphorylation of the translational repressor 4E-BP1 releases the rate-limiting cap-binding protein eIF4E, allowing it to bind to the scaffolding protein eIF4G along with the eIF4A helicase. Once the functional eIF4F trimer (i.e., eIF4E + eIF4G + eIF4A) is formed, nascent mRNAs are bound via the 5′ cap/eIF4E interaction and transported to ribosomal subunits for the initiation of the cap-dependent translation. Activation of the mTOR complex 1 (mTORC1: mLST8 + mTOR + Raptor) relies on the interaction with GTP-loaded Rheb. This interaction may be inhibited during hypoxia by Bnip3 and/or the promyelocytic leukemia tumor suppressor. Hypoxia also inhibits mTOR through AMP-activated protein kinase (AMPK), which is itself

al. 1997). Oddly, turtles appear to maintain vision during anoxia (Stensløkken et al. 2008*b*). A moderate increase in extracellular GABA levels (Hylland and Nilsson 1999), possibly mediated by a reduced expression of GABA reuptake proteins (Ellefsen et al. 2009), is probably involved in depressing energy use in the crucian carp (Nilsson 1992), but only to a level that is compatible with some physical activity. Other mechanisms that may be involved in defending brain and heart energy charge in crucian carp include phosphorylation of AMP-activated kinase that in turn will initiate mechanisms that increase energy supply and reduce energy use (Stensløkken et al. 2008*a*).

Like other vertebrates, anoxic turtles produce lactate as the main anaerobic end product of anaerobic glycolysis. The crucian carp, on the other hand, has the exotic ability to produce ethanol during anoxia (Johnston and Bernard 1983; Nilsson 1988), thereby avoiding the enormous lactic acid load that turtles have to deal with. The capacity for ethanol production is backed up by enormous glycogen stores (Nilsson 1990; Vornanen and Paajanen 2006), and ethanol production is probably the key to the crucian carp's ability to maintain activity in anoxia. The advantage of retaining mobility during anoxia could be that it allows the crucian carp to seek out oxygen in the spring, thereby shortening the anoxic period (Nilsson 2001). The turtles, on the other hand, must reduce their metabolism and physical activity to a minimum to minimize lactate load.

Hypoxia, Erythropoeitin, Carotid Body, and Gender Differences

Inherently hypoxia-sensitive mammals can be engineered to somewhat modify or increase their adaptability to oxygen deprivation. This point is exemplified through the overexpression of erythropoietin (Epo) in mice. In 2000, our group introduced the transgenic mouse line tg6 (Ruschitzka et al. 2000; Wagner et al. 2001), which constitutively overexpresses the human Epo transgene in every tissue and, as a result, develops an average hematocrit of 0.80–0.85 within the first 2 mo of the mouse's life. The tg6 model is better protected from chronic alveolar hypoxia, a known inducer of pulmonary hypertension, than are wild-type (wt) animals. Hypoxia-induced pulmonary hypertension (PH) is characterized by pulmonary vascular remodeling with increased muscularization of the pulmonary vasculature, polycythemia, altered reactivity of the pulmonary vasculature, and subsequently, right ventricular (RV) hypertrophy. When wt mice were subjected to hypoxia, a pronounced increase in the degree of pulmonary vascular muscularization was observed. In contrast, such enhanced muscularization was not observed in tg6 mice kept under equivalent hypoxia (Weissmann et al. 2005). Thus, congenital Epo overexpression exerts an overall "antihypertensive" effect on the pulmonary vasculature by reducing vasoreactivity and muscularization during vascular remodeling. Consistent with these data, our recent studies showed a much attenuated increase of functional RV parameters (e.g., RV systolic pressure [RVSP]) in tg6 animals compared with wt animals during progressive inspired hypoxia (A. Deten, C. Schürmann-Huber, J. Hu, J. Soliz, T. A. Gorr, and M. Gassmann, unpublished manuscript). Furthermore, hypoxic lungs of wt mice reflected unabated inflammatory cell recruitment and vascular remodeling through a specific mRNA induction of the proinflammatory interleukin 6 (IL-6) marker. In sharp contrast, the IL-6 mRNA titer remained at basal levels and could no longer be stimulated by hypoxia in hypoxic lungs of tg6 mice or after acute injection of recombinant Epo (rEpo) into wt mice before their exposure to hypoxia (C. Schürmann-Huber, J. Hu, M. Gassmann, A. Deten, and T. A. Gorr, unpublished manuscript). It seems that overexpressed or acutely administered Epo mediates its antihypertensive property by counteracting IL-6-driven inflammation in the alveolar vasculature.

Interestingly, the dose response curve of RVSP to decreasing inspired oxygen peaked at higher O_2 concentrations in tg6 animals than in control animals, which could indicate an increased sensitivity to hypoxia in the transgenics. This was surprising, because decreased vasoreactivity and increased arterial oxygen content, which might follow from the increased hematocrit and blood volume in tg6 mice, would instead suggest a delayed maximum in the functional response curves along with an increased tolerance to reduced concentrations of inspiratory oxygen. To further analyze this conundrum, we set out to compare the ventilation of transgenic and wt mice, specifically looking at the breathing patterns of female and male animals during inspiratory hypoxia. Here we summarize both the background of the ventilatory response and our own findings with regard to the gender-specific role of Epo in controlling breathing during low oxygen levels.

The main peripheral chemoreceptor identified in mammals is the carotid body (Gonzalez et al. 1994), a small bilateral organ situated near the carotid bifurcation, where it permanently measures oxygen arterial pressure and controls ventilatory activity. While the main stimulus of carotid bodies is the decline of the arterial oxygen partial pressure (Pao_2), carotid bodies are also stimulated by other physical or chemical com-

activated by the tumor-suppressor kinase LKB1 during a fall in energy charge—that is, a rise in the [AMP] : [ATP] ratio—and the Redd1-dependent regulation of the TSC1–TSC2 complex. The TSC1–TSC2 complex displays GTPase-activating protein activity against Rheb, resulting in negative regulation of mTOR. Finally, hypoxia can directly influence the translation initiation machinery by sequestering eIF4E and its nuclear import factor 4E-T to the nucleus and cytoplasmic processing bodies. Redd1 and Bnip3 are hypoxia-inducible transcription factor (HIF)–dependent controls and mTOR antagonists. *Thin arrow* = pathway stimulation; *T-bar* = pathway inhibition; *thick arrow* = external signal of dominant influence; *dotted arrow* = unclear signal transmission; *hexagon* = kinase function. *Ellipse* = all other functions. *Black name on light gray* (e.g., Akt) = mTOR activator + oncogene, on gain-of-function mutation; *black name on dark gray* (e.g., AMPK, Bnip3) = generic mTOR antagonist; *white name on black* (e.g., PTEN, TSC1/2) = mTOR antagonist + tumor suppressor until loss-of-function mutation.

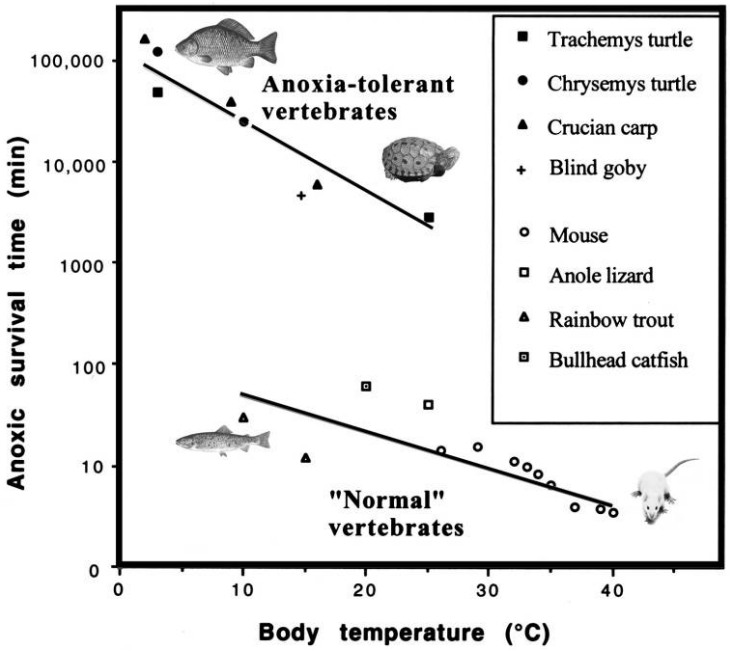

Figure 4. The 1,000-fold difference in anoxic survival time between "normal" vertebrates and anoxia-tolerant vertebrates. Because metabolic rate falls with temperature, animals will survive anoxia longer in the cold. For anoxia-tolerant vertebrates, a low temperature means that their glycogen stores last longer; that is, they will survive anoxia as long as they have glucose available. By contrast, for anoxia-intolerant vertebrates, a low temperature means only a slower loss of cellular energy charge and therefore a slower death process. Redrawn from Lutz et al. (2003).

ponents in blood, such as arterial carbon dioxide partial pressure ($Paco_2$), pH, temperature, arterial flow and pressure, or osmolarity. The carotid bodies are among the most vascularized organs in the body (five times more vascularized than the brain) and are highly innervated by afferent sensorial fibers and fibers from the autonomic nervous system (Gonzalez et al. 1994). When mammals are exposed to hypoxia, the carotid body afferent fibers are immediately stimulated and forward this information mainly via nonrespiratory neurons localized in the caudal obex, a region of the nucleus tractus solitarii (nTS; Housley and Sinclair 1988; Finley and Katz 1992). The modulation of the activity in the respiratory neurons determine the discharge frequency of the phrenic nerve-motor neurons located in the fifth cervical segment (de Burgh Daly 2000). Thus, the nTS neuron projection activated by the stimulation of the carotid bodies determines the parallel activation of the respiratory network and the sympathetic nervous system (Loewy and Burton 1978; Otake et al. 1992; Sun 1995; de Burgh Daly 2000).

The carotid bodies produce a large diversity of neurotransmitters, including acetylcholine, catecholamines (dopamine and noradrenalin), and serotonin, as well as several neuropeptides (substance P, enkephalines, atrial natriuretic peptide, galanine, calcitonine-gene-related peptide, endothelin, vasoactive intestinal peptide) and the gases nitric oxide (NO) and carbon monoxide (CO; Verna et al. 1995; Tamayo et al. 1997; Zapata 1997; Eyzaguirre and Abudara 1999). These substances are all produced in the Type I or glomus cells as well as in the nerve endings. It is known that on a natural hypoxic stimulus, the carotid body secretes different neurotransmitters that depolarize the Hering-membrane nerve, producing a nervous activation. From this cocktail of substances, it seems that dopamine, acetylcholine, ATP, substance P, and NO have a predominant role in glomus cell signal modulation (Prabhakar 1994; Verna et al. 1995; Tamayo et al. 1997; Zapata 1997; Eyzaguirre and Abudara 1999; Nurse and Zhang 1999; Vicario et al. 2000). Furthermore, under hypoxia it seems that dopamine in particular plays a major role in the neurochemistry of the carotid body and signal chemotransduction (Finley and Katz 1992). In fact, glomus cells are essentially catecholaminergic and represent the structures with the highest catecholamine content in the organism (10 times higher than in the striatum; Gonzalez et al. 1995). However, the role of dopamine at the synapse-chemosensitive level is still not understood.

Does blood-borne circulating Epo, especially when its synthesis and secretion is stimulated to greatly elevated levels by the HIF-mediated transactivation of the gene in the kidney, also participate in the regulation of the carotid body stimulation during hypoxia? This question arises from the fact that on exposure to Epo, nonerythroid cells displaying neuronal characteristics and carrying Epo, such as the rat pheochromocytomal cell PC12, showed increased intracellular Ca^{2+} concentration, dopamine release, tyrosine hydroxylase (TH) activity, and membrane depolarization (Assandri et al. 1999). As PC12 cells mimic carotid body Type I cells (Tanaka et al. 2001), we hypothesized that these peripheral chemoreceptors were also activated by Epo. To address this question, we measured the hypoxic ventilator response (HVR) in wt mice after injection

of 2,000 U/kg rEpo. Control wt animals received an injection of 0.9% NaCl solution. While there were no alterations observed at moderate hypoxia, Epo-injected animals showed lower tidal volume (VT) but higher respiratory frequency (fR) than saline-injected controls under severe hypoxia (6% inspiratory O_2; Soliz et al. 2005). These data are consistent with an effect of Epo on carotid body cells, most probably by binding to the Epo receptor (EpoR). To determine whether EpoR is present in the carotid body, we performed immunostaining in serial lateral sections from the carotid bifurcation. Staining of TH was used to identify the glomus cells on one section, and EpoR staining was performed on the subsequent one. We found a dense staining of EpoR in the carotid body, apparently localized within islets of chemosensitive cells, suggesting that peripheral chemoreceptors can be activated by plasma Epo on its binding to EpoR (Soliz et al. 2005). Together, our results suggested that Epo controls ventilation at central (brain stem) and peripheral (carotid body) levels.

Interestingly, we observed gender-dependent differences in the Epo-modulated HVR. Of note, female mammals, including women, exhibit better acclimatization to hypoxia at high altitudes. Furthermore, human and animal females are less susceptible to a number of hypoxia-associated sickness and syndromes (Joseph et al. 2000, 2002). This evidence draws attention to the importance of female sexual hormones in coping with hypoxia, especially because it was demonstrated that female sexual hormones can influence the expression of hypoxia-inducible genes such as Epo (Suzuma et al. 1999; Bausero et al. 2000; Earley and Resta 2002) and are able to modulate the cytokine regulation in PC12 cells (Koski et al. 2004). We thus hypothesized that the Epo-enhanced ventilator response via carotid bodies or the respiratory center in mice exposed to hypoxia is a gender-dependent process. To test this hypothesis, we used males and females from tg6 and tg21 mouse lines. In tg21 transgenics, the expression of Epo is confined to brain cells, therefore yielding, in contrast to tg6 animals (above), normal serum levels of Epo and normal blood hematocrit levels (42%; Wiessner et al. 2001). In tg6 mice, again, Epo is expressed ubiquitously, thus triggering an excessive erythrocytosis with enormously elevated hematocrit values (Ruschitzka et al. 2000; Shibata et al. 2003; Wenger and Katschinski 2005). We found that brain-derived Epo increases ventilation in female mice as it does in males; however, the Epo modulation of the ventilatory pattern was different between genders (Soliz et al. 2009). In addition, we found that the excessive erythrocytosis of tg6 male mice counterbalanced the stimulatory effect of brain-derived Epo (Soliz et al. 2007). Surprisingly, in contrast to what happens in males, excessive erythrocytosis and brain-derived Epo in tg6 females synergistically stimulated the HVR, resulting in a twofold increase of hypoxic ventilation in comparison with corresponding wt females (Soliz et al. 2009).

Assuming that plasma Epo does not efficiently cross the blood-brain barrier (Marti et al. 2000; Brines et al. 2004; Marti 2004), we also injected rEpo in male and female wt mice (2,000 U/kg body weight; intravenous). This model allowed us to test the effects of Epo on carotid bodies without the influence of cerebrally produced Epo. Although Epo injection did not affect the HVR of male animals, it dramatically increased the hypoxic ventilation in females (Soliz et al. 2009). Finally, to test whether our data in mice reflect the situation in humans, we evaluated the HVR in volunteers exposed to 10% O_2 for 15 min following intravenous injection of rEpo. Indeed, we observed that increased plasma Epo concentration altered HVR in all tested subjects. However, while HVR was decreased in men because of a significant decrease in fR rather than in VT, HVR was augmented in women because of a significant increase in VT rather than in fR (Soliz et al. 2009). Because we do not expect significant amounts of Epo to cross the blood-brain barrier in this short period of time, our results suggest that elevated Epo plasma levels influence the peripheral chemosensitivity in both mice and human beings. Taken together, these results show that carotid bodies in females are much more sensitive to Epo stimulation than are those in males. Thus, Epo plays a crucial role in the female capacity to adapt to hypoxia as well as improve protection against hypoxia-associated sickness and syndromes.

Conclusions

The models presented here, whether inherently tolerant to hypoxia or anoxia (e.g., *Drosophila*, crucian carp, etc.) or engineered to be better protected from inadequate O_2 availabilities (Epo-overexpressing mice), are beginning to reveal their potential values for understanding environmental responses to low O_2 and for the translation of findings onto human pathologies. Counteracting the effects of anoxia and ischemia during organ transplantation, myocardial infarction, or wound healing, for example, has been hampered by disappointments and slow progress. Here we present the first evidence that the tg6 mouse line, with its ubiquitous and constitutive overexpression of Epo, is a valuable model with which to study gender-specific ventilatory adaptations to normo- and hypobaric hypoxia. This will certainly redirect research activities toward the thus far largely ignored crosstalk between oxygen and steroid hormone signaling pathways. Beyond engineered rodents, fresh views on the translational bench-to-bedside path can be provided by studies on the profound tolerance toward little or no oxygen observed in many invertebrates and ectothermic vertebrates. In these stress-tolerant models, changes observed in physiological, biochemical, and molecular functions during a period of low/no oxygen are likely to be adaptive rather than pathological. To that end, protective mechanisms in the anoxic yet conductance-maintaining brain of the crucian carp can teach us key requirements to aid in the survival of a stroking area in the human cerebral cortex or how to safely and reversibly switch off the central circuits necessary for vision and hearing during surgery of the respective brain areas. Targeting tolerance-mediating and ATP-saving gene functions, like inhibitors for the cap-dependent mRNA translation, has begun to translate from these models (i.e., *Drosophila*) into innovative treatment concepts for the sensitization of radio-resistant hypoxic human malignancies. By identifying and interfering with the molecular processes underlying hypoxia and anoxia tolerance, more se-

lective diagnostic markers and anticancer therapies can be anticipated because almost all healthy tissues in the human body are homeostatic in their oxygenation and, arguably, are less likely to recruit hypometabolic defenses when deoxygenated. In this sense, invertebrate and selected vertebrate animals have proven to be very useful models, and they will continue to be in order to arrive at new diagnostic and therapeutic approaches against hypoxia-associated human pathologies. The value of these studies goes beyond directed applications for human health. The phyletic approach illustrates how regulatory molecules such as HIF that have been identified as stress-response factors in some species may function during normally encountered physiological processes in other organisms and under different conditions. Increased understanding of how individual species exhibit diverse modes of dealing with environmental challenges, such as the increasingly frequent episodes of hypoxic zones in estuaries and nearshore waters, may reveal strategies for an ecosystem-wide recording of the responses to global climate change.

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