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Review

Adaptations to environmental change: Globin superfamily evolution in Antarctic fishes

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ABSTRACT

The ancient origins and functional versatility of globins make them ideal subjects for studying physiological adaptation to environmental change. Our goals in this review are to describe the evolution of the vertebrate globin gene superfamily and to explore the structure/function relationships of hemoglobin, myoglobin, neuroglobin and cytoglobin in teleost fishes. We focus on the globins of Antarctic notothenioids, emphasizing their adaptive features as inferred from comparisons with human proteins.

We dedicate this review to Guido di Prisco, our co-author, colleague, friend, and husband of C.V. Ever thoughtful, creative, and enthusiastic, Guido spearheaded study of the structure, function, and evolution of the hemoglobins of polar fishes – this review is testimony to his wide-ranging contributions. Throughout his career, Guido inspired younger scientists to embrace polar biological research, and he challenged researchers of all ages to explore evolutionary adaptation in the context of global climate change. Beyond his scientific contributions, we will miss his warmth, his culture, and his great intellect. Guido has left an outstanding legacy, one that will continue to inspire us and our research.

1. Foreword

Molecular oxygen today plays a central role in powering cellular metabolism in the wide spectrum of aerobic organisms. Given its high reactivity, oxygen can be both a blessing and a curse, so much so that detecting it and managing its use through evolution of a family of oxygen-binding proteins, the globins, occurred early in the history of life. Between two and four billion years ago (bya), atmospheric oxygen levels on the planet were low, and an ancestral globin probably functioned as a chemotactic sensor/signal transducer to enable anaerobic organisms to avoid oxygen-rich environments (Vinogradov et al., 2007) or as enzyme for scavenging oxygen and nitric oxide (NO), or both (Vinogradov and Moens, 2008). Following the *Great Oxidation* event approximately 2.45 bya (Holland, 2006), which caused the first mass extinction in the history of life, globins evolved from protective functions to roles that enabled the exploitation of oxygen for enhanced energy production, thereby "fueling" vast increases in organismal complexity.

Globins are found in all kingdoms of life. There are three basic globin families: 1) the myoglobin-like (Mb) family displaying the classical 3-over-3 (3/3) α-helical sandwich fold (including flavo-hemoglobins and single-domain globins only found in bacteria and some eukaryotes); 2) the sensor globin family found only in prokaryotes; and 3) the truncated hemoglobin family characterized by a 2-over-2 (2/2) α-helical sandwich fold that is present in cyanobacteria, green algae, bacteria, fungi and plants (Vinogradov et al., 2013). Vertebrates have evolved a particularly complex superfamily of globins, including not only the well-known tetrameric hemoglobin (Hb) of red blood cells and monomeric myoglobin (Mb) of muscle, but also androglobin (Adgb) (Hoogewijs et al., 2012), cytoglobin (Cygb) (Burmester et al., 2002; Trent and Hargrove, 2002), globin E (GbE) (Kugelstadt et al., 2004), globin Y (GbY) (Fuchs et al., 2006), globin X (GbX) (Roesner et al., 2005) and neuroglobin (Ngb) (Burmester et al., 2000).

One of the major constraints that shaped the evolution of life in the oceans of our planet was the ability to accommodate to environmentally variable oxygen levels. Dissolved oxygen concentrations in water are highly dependent on temperature, currents, salinity and other parameters. This variability challenges aquatic organisms to adapt to profound differences in oxygen availability. For example, various fish taxa have evolved to live at diverse levels of oxygen

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saturation, ranging from the nearly anoxic deep sea to high altitude lakes and from the poorly oxygenated pools of warm desert springs to the highly oxygenated frigid waters of the frozen poles. Such adaptability makes fishes exciting model systems to understand how respiratory physiology can respond to the demands of environmental change.

One particularly intriguing example of globin adaptation is found in fishes belonging to the largely Antarctic suborder Notothenioidei. The Southern Ocean (SO), perennially at or near freezing, has high oxygen solubility with little seasonal variation (Eastman, 1993). As a result, High-Antarctic notothenioids differ from Sub-Antarctic, temperate, and tropical fish species in the evolutionary constraints placed upon the respiratory system by limiting oxygen levels. In their icy environment, Antarctic notothenioids evolved unusual cardiovascular traits that are unique among vertebrates, including at the extreme the loss of Hb and red blood cells by the clade of icefishes (Eastman, 1993).

Our goal in this review is to describe the evolution of vertebrate globin genes and the functions of the encoded proteins in general terms, with emphasis on fish globins, then to focus specifically on the molecular adaptation of the globin system in Antarctic notothenioids. Finally, we describe in detail the structural and functional properties of Hb, Mb, Ngb and Cygb of Antarctic notothenioids and their adaptive features inferred from comparisons with human proteins. Because structural and functional data on Antarctic fish GbX is lacking, we limit our consideration of this globin to its ancient origin and important role as a nitrite reductase in blood.

2. The vertebrate globin protein/gene superfamily – function and evolutionary conservation

Though highly divergent in function, all vertebrate globins share a similar underlying structure. Globins typically contain ~150 amino acids organized as eight α-helical segments (named A through H), which fold to give a 3/3 α-helical sandwich that surrounds the heme prosthetic group.

Globin functions are made possible by heme, the main ligand-binding site in these proteins. Heme contains an iron atom (Fe) in the reduced state. Coordination between heme-pocket side chains and the heme iron influences both the equilibrium and kinetic properties of these proteins. Only the ferrous state (Fe^{II}) binds oxygen reversibly, and multiple mechanisms prevent or reverse the oxidation of iron to the Fe^{III} (ferric) state.

In the deoxygenated state, Hb, Mb and GbE are classic penta-coordinated globins, with the sixth binding site of the ferrous iron free to bind oxygen reversibly. Ngb, GbX, Adgb and Cygb, by contrast, are hexa-coordinated. In the absence of exogenous ligands, the distal His E7 of Ngb (Pesce et al., 2004) and of Cygb (de Sanctis et al., 2004) interacts directly with the heme iron, whether ferrous or ferric. Therefore, exogenous ligands must compete with the His E7 of Ngb and Cygb to bind to heme iron (Smagghe et al., 2008; Trent et al., 2001). An invariant His at position F8 is present in all globins, providing the proximal ligand to the iron of the heme. While the secondary, tertiary and quaternary structures of globins are conserved among vertebrates, the primary structures often are not. The ability of globins to function under different environmental conditions resides in strategic regions of their primary structures that modify the intrinsic ligand affinity of the protein, its regulation by allosteric effectors, and its redox potential (Bonaventura et al., 2012). Although heme binds diatomic gaseous ligands covalently through its iron atom, the globin fold provides the environment that facilitates selective and reversible ligand binding. Phylogenetic surveys of vertebrate globins indicate that cyclostome Hbs are orthologous

and closely related to gnathostome hexa-coordinated Cygb (see Section 4.4). Thus, the oxygen-transport function appears to have been coopted in parallel in cyclostome and gnathostome Hbs from hexa-coordinated ancestral states (Hoffmann et al., 2010).

3. Vertebrate globin repertoire and the dynamic genome

A major theme in the history of vertebrate globins is evolution through repeated gene duplication and sub-functionalization (Storz, 2016). Gene duplication as a source of phenotypic novelty is a classic paradigm in evolutionary genetics (Ohno, 1970). The evolution of a single-copy gene is frequently constrained because it performs an essential function, which, if perturbed by mutation, may cause reduced organismal fitness (Ohno, 1970). However, after duplication, one copy of a gene can continue to produce the original protein, whereas the other is free to accumulate novel mutations, which may generate a new expression pattern and/or altered protein function. In the globin superfamily, duplications have provided substrates for the evolution of novel mutations, for diversification of paralogous genes, and for alterations in expression patterns that have facilitated adaptation to changing environments (Storz, 2016).

Whole-genome duplication (WGD) provides a particularly adaptive substrate for the diversification of paralogous genes. Non-reciprocal recombination between paralogs of a tandem duplication can result in homogenization of the duplicate genes, a process called gene conversion. This process has been reported in tandemly duplicated globin genes (Storz et al., 2011). By contrast, paralogous genes that result from whole genome duplication are thought to be more likely to escape gene conversion and, therefore, to evolve novel functions (Opazo et al., 2012). Much of vertebrate globin diversity can be traced back to a series of two WGD events that occurred early in vertebrate evolution. Prior to WGD, the ancestral vertebrate genome likely contained single copies of *Ngb* and of *Adgb*, four copies of *GbX*, and a vertebrate-specific *globin* gene (Opazo et al., 2015). The latter evolved through duplication to give rise to *Cygb*, *Mb*, *Hb*, *GbY* and *GbE* genes (Opazo et al., 2015; Schwarze et al., 2014). *Ngb* and *GbX* derive from duplications that predate the origin of deuterostomes (Ebner et al., 2010; Roesner et al., 2005), thus suggesting that the corresponding globins may be involved in physiological functions related to fundamental aspects of cellular metabolism. Other than the ancestrally shared *globin*-gene repertoire, the only vertebrate-specific ortholog shared between the jawless vertebrates (cyclostomes) and the jawed vertebrates (gnathostomes) is thought to be *Cygb* (Opazo et al., 2015; Schwarze et al., 2014). Reflecting the remarkable adaptability of the globin-gene superfamily, the cyclostomes have independently evolved functionally analogous *Mb* and *Hb* genes from an ancestral *globin* that is not orthologous to the *Mb* and *Hb* genes of gnathostomes (Schwarze et al., 2014). In cyclostomes (hagfish and lampreys), oxygen transport function is derived by cooption of the *Cygb* gene (Opazo et al., 2015; Schwarze et al., 2014).

After whole genome and local duplication events, the vertebrate globin gene repertoire has evolved to provide numerous tissue-specific roles. Expression patterns of clearly orthologous globins are often highly variable between species (Gallagher and Macqueen, 2016). For example, the *Mb* gene is typically most highly expressed in the heart, where the Mb protein plays a critical role in oxygen storage and NO homeostasis (Flögel et al., 2010). However, some species, such as threespine stickleback (*Gasterosteus aculeatus*), have lost the ability to produce Mb but do show a compensatory increase in production of Cygb-1 and Ngb in the heart (Gallagher and Macqueen, 2016). Similarly, Mb is largely absent from the heart of the African butterflyfish (*Pantodon buchholzi*) but is expressed highly in swim bladder (Gallagher and Macqueen, 2016). As the African butterflyfish is an obligate air breather that uses a modified swim bladder as an air-breathing organ, elevated Mb levels in this tissue may be an adaptation for capturing and storing oxygen from air. Thus, physiological adaptation of oxygen-dependent metabolism in vertebrates has occurred, in part, both through gene regulatory changes that alter the tissue specificity of *globin*-gene expression and through evolution of globin proteins with unique oxygen-binding properties.

Fig. 1 illustrates the organization of several *globin* genes within the genomes of teleost fishes representing diverse taxa, including the

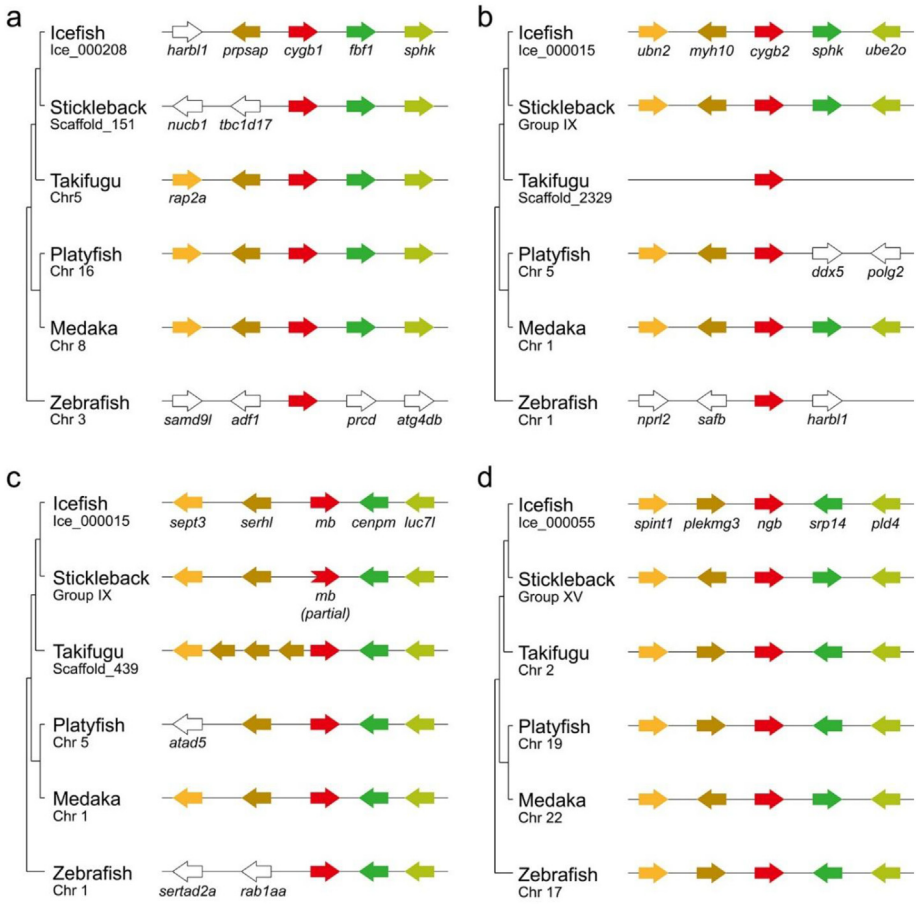

Fig. 1. Syntenic comparisons of several globin genes in the genomes of teleost fishes. Genomic neighborhoods and syntenic relationships for a. *Cygb1* b. *Cygb2*, c. *Mb* and d. *Ngb* genes in teleosts. Blackfin icefish (Perciformes): *Chaenocephalus aceratus*; Stickleback: *G. aculeatus*; Takifugu: *Takifugu rubripes*; Platyfish: *Xiphophorus maculatus*; Medaka: *Oryzias latipes*; Zebrafish: *Danio rerio*. Reprinted with permission from Kim et al. (2019).

Antarctic notothenioids (Kim et al., 2019). The syntenic relationships of two *Cygbs* (Fig. 1a-b), *Mb* (Fig. 1c), and *Ngb* (Fig. 1d) are generally well conserved among these teleost fishes with the exception of the cypriniform, the zebrafish. Conservation of synteny for *Ngb* is particularly striking. Consideration of the genomic status of *Hb* genes in teleosts is reserved for Sections 4.1, 4.2 and 4.3.

4. Hemoglobin – the major oxygen carrier in blood

4.1. Hemoglobin gene evolution in teleosts

The evolution of Hb is considered a key innovation in the success of vertebrate taxa, as the improved efficiency in oxygen distribution by Hb-filled erythrocytes resolved physiological constraints that limited body size and activity in non-vertebrate chordates (Nikinmaa et al., 2019 and references within). In vertebrate evolution, tandem duplication of a proto-*Hb* gene in the stem gnathostome created two *Hb* genes, *α-globin* and *β-globin* (Storz, 2016). This key event enabled the synthesis of α- and β-globins, which through hetero-oligomerization give rise to Hb tetramers with novel oxygen-binding properties (Storz, 2016). The ancestral state of *globin* genes in the teleost genome, determined through gene tree reconstruction and analysis of synteny with the spotted gar (*Lepisosteus oculatus*), was likely two *α*- and two *β*-genes. The teleost-specific WGD then expanded the *Hb* locus further, leading to two main *Hb* clusters. The two *Hb* clusters are named after the genes that flank each: the "MN" cluster (encompassed by *mpg/nprl3*) and the "LA" cluster (flanked by *lcmt1/aqp8*) (Hardison, 2008).

Teleost *Hb* genes have undergone repeated expansions and contractions in both the MN and the LA clusters. Comparative analysis of seven teleost genomes revealed MN clusters that ranged in overall size from 3.4 kb (*T. rubripes*) to 68.5 kb (*D. rerio*) and LA clusters between 3.4 kb (*G. aculeatus*) and 17.2 kb (*D. rerio*) (Opazo et al., 2012). Duplication of globin genes within each cluster affect both individual *α*- or *β-globin* genes and entire blocks of *α*- and *β-globin* pairs (Opazo et al., 2012). As a result, *globin* copy number within each cluster is highly variable and species-specific, ranging from as few as two *globin* genes in the MN locus of Tetraodontiformes and the LA locus of the threespine stickleback (*G. aculeatus*) to at least 13 *globin* genes in the MN locus of zebrafish and tilapia (*Oreochromis niloticus*) (Opazo et al., 2012). In the extreme case of salmonids, which have undergone an additional WGD compared to other teleosts, all LA-linked *Hb* clusters have been lost, whereas the duplicated MN clusters were retained (Quinn et al., 2010). Thus, both the MN and LA clusters have rapidly and repeatedly evolved through gene duplication and loss across the teleost phylogeny.

Duplicate *Hb* gene paralogs have frequently and independently evolved to produce specialized Hb chains. Teleosts frequently express two Hb isoforms named after their relative positions in a electrophoretic mobility assay, an “anodic” isoform that has a decreased oxygen affinity, particularly at low pH, and a “cathodic” isoform that has a high oxygen-binding affinity that increases with pH (Jensen et al., 1998; Weber, 2000; Weber et al., 2000; Weber and Jensen, 1988; Wells, 2009). The unique and divergent biochemical properties of these isoforms are thought to have facilitated teleost diversification and acclimation to environmental change (Rutjes et al., 2007). Cathodic and anodic Hb isoforms do not form monophyletic groups in phylogenetic gene trees. For example, cathodic Hbs of the dusky notothen (*Trematomus newnesi*) derived from the LA cluster, whereas the cathodic Hbs of European eel (*Anguilla anguilla*) originated from the MN cluster (Opazo et al., 2012). The molecular mechanisms behind convergent evolution of globins are not well understood for fishes. Given the multiplicity of fish Hbs and the wide range of oxygen concentrations in aquatic environments, there should be many opportunities to explore mechanisms

of convergent molecular evolution using the *globin* genes of fishes as a model system.

Much of the adaptive functional divergence in Hbs is facilitated through alterations in gene expression patterns of the multiple *globin* paralogs to meet stage- and environment-specific oxygen demands by production of heteromeric Hb isoforms with unique oxygen-binding properties. *Hb* gene regulation is complex and is subject to long-distance *cis*-regulatory elements that can be many kilobases away from transcription start sites (Palstra et al., 2008). In amniotes, *Hb* genes are arranged on chromosomes in the order in which they are expressed during development, such that there is a progressive activation of embryonic to adult *Hb* genes in a 5' to 3' direction (Grosveld et al., 1993; Hardison, 2001). This occurs in part through a long-range chromosome looping mechanism (Noordermeer and De Laat, 2008) that sequentially applies enhancer elements to activate transcription of the *Hb* genes. Although long-range *cis*-acting *Hb* enhancer elements exist in teleosts (Ganis et al., 2012), there does not appear to be a consistent trend between chromosomal position and activation of *Hb* gene expression during ontogeny (Opazo et al., 2012). Furthermore, orthologous relationships between *Hbs* with restricted expression patterns appear to be absent in teleosts (Opazo et al., 2012). Rather, orthologous genes have frequently diverged in expression patterns during teleost evolution whereas paralogous genes have often converged (Opazo et al., 2012). The expression of fish *Hb* genes is also regulated in response to environmental stresses such as hypoxia (Campo et al., 2008; Feng et al., 2014; Rutjes et al., 2007; Van Den Thillart et al., 2018). Thus, *Hb* gene switching in fish provides plastic cardiovascular compensations to meet the challenges of a dynamic environment, although the mechanisms underlying adaptive switching remain poorly understood.

According to Perutz (1984), adaptive changes in the functional properties of vertebrate Hbs may result from small numbers of amino acid replacements in key positions of the primary structure. For example, evolved changes in oxygen-binding properties are caused by amino acid mutations that increase or decrease intrinsic oxygen affinity and/or by mutations that change the sensitivity of Hb to physiological effectors of Hb allostery. Such changes generally occur at Hb subunit interfaces, distant from the ligand-binding site, and they change the energy required for the conformational transitions associated with ligand binding (Perutz, 1984). Because sites of adaptive functional change in Hb are limited, one finds that widely diverged species, when subject to similar environmental oxygen constraints, have independently evolved the same amino acid substitutions (Storz, 2018).

4.2. Hemoglobin genes in Antarctic notothenioid fishes – relaxed selection in a novel environment

The evolution of *Hb* genes in notothenioid fishes occurred in the unique environmental context of the SO. Since the mid-Miocene, the SO cooled from 15-20 °C to its current range of −2 to +2 °C (Eastman, 1993). South of the Antarctic Polar Front, the SO is thermally stable (typically < 0°C) (Littlepage, 1965), rich in oxygen, and well mixed by currents and storms. As a result, the concentration of oxygen in the water is consistently high. This has enabled a rare evolutionary experiment for any vertebrate taxon: relaxed constraint on globin evolution.

Red-blooded notothenioid fishes, like many teleosts, have *Hb* genes organized as *αβ* pairs in

alternating orientation (i.e., 5'-to-5' or head-to-head) (Opazo et al., 2012), as shown in Fig. 2 for the LA clusters of the bullhead notothen *Notothenia coriiceps* and Charcot's dragonfish *Parachaenichthys charcoti*. Between the *α*- and *β-globin* genes of the three clusters are short intergenic regions (2-4 kb) that contain *cis*-acting promoter and enhancer elements that drive bidirectional transcription (Lau et al., 2012).

These intergenic regions contain many predicted binding-site motifs for erythroid transcription factors, including Eklf, Gata1, C-myb, Nfe2, and Sp1 (Lau et al., 2001, 2012). Using luciferase reporter assays, Lau et al. (2012) identified two ~550-bp enhancer elements in the *N. coriiceps* intergenic region that were direct repeats (Fig. 2, NcDR1 and 2) (Lau et al., 2001). These enhancers were necessary for both *α*- and *β-globin* expression (Lau et al., 2001). However, comparisons with the intergenes of *N. angustata*, *Dissostichus mawsoni*, and other notothenioid species indicated that a single copy of the enhancer, designated NcDR (*N. coriiceps* Direct Repeat), was likely the ancestral state for the Antarctic radiation (Lau et al., 2012).

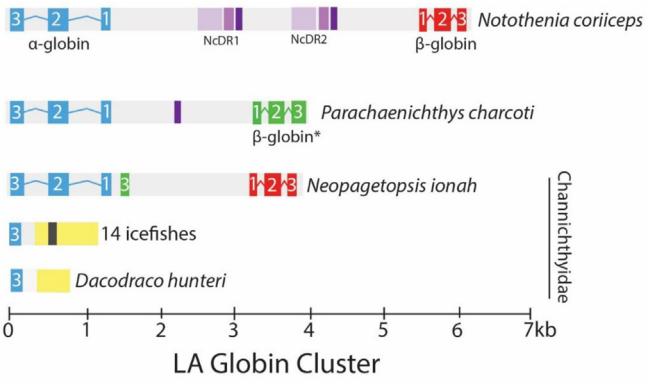

Fig. 2. The LA *globin* clusters of red-blooded notothenioid fishes and the LA pseudogenes of the white-blooded icefishes. Top two cartoons: organization of the adult LA *αβ-globin* gene complexes of two red-blooded notothenioids, *N. coriiceps*, and *P. charcoti*, respectively. The three exons of the orthologous *α-globin* genes are shown in blue and the two introns in blue chevrons. The three exons and two introns of the *N. coriiceps β-globin* gene are presented in red and red chevrons, respectively, whereas those of the non-orthologous *P. charcoti β-globin* gene are colored green. The intergenic regions, which stretch between exons 1 of the *α*- and *β-globin* genes, contain binding sites (purple) for transcription factors (see main text for explanation). Bottom three cartoons: structures of the *globin* LA pseudogenes of icefishes. The *α-globin* gene of *Neopagetopsis ionah* is colored blue to indicate orthology to the red-blooded fishes. The two *N. ionah β-globin* pseudogenes are colored red and green to indicate their phylogenetic relationships to the notothen and dragonfish *β-globin* genes, respectively. Fourteen icefish species retain a portion of intron 2 and exon 3 of the notothenioid *α-globin* gene abutted by an orthologous tRNA gene-containing chromosomal fragment (black tRNA gene embedded in yellow). The tRNA gene is deleted from the *Dacodraco hunteri* pseudogene. Lengths of sequence components can be estimated from the scale at bottom. Adapted from Near et al. (2006) and Lau et al. (2001, 2012).

During notothenioid evolution, there is a gradual winnowing of the intergenic DNA of LA *αβ-globin* gene pairs, consistent with the general reduction in Hb levels during the notothenioid adaptive radiation (Lau et al., 2012). At the extreme, some dragonfishes have lost 40-60% of the intergenic sequence found in more basal notothenioids (Lau et al., 2012), and their single NcDR enhancer has been reduced to a 91-104-bp fragment (e.g., *P. charcoti* of the subfamily Cygnodraconinae; Fig. 2). Nevertheless, this reduced enhancer, which contains Gata1 motifs, drives *globin* gene transcription at levels comparable to other notothenioids (Lau et al., 2012). Therefore, the lower Hb concentrations in the blood of dragonfishes, compared to other notothenioids, are probably due to evolution of reduced hematocrits under relaxed selection, rather than to a decrease in *Hb* gene transcription during erythroid differentiation.

4.3. Is hemoglobin synthesis a vertebrate synapomorphy? – the unusual case of the icefishes

Reflecting the utility of Hbs in adaptation to environmental change, virtually all vertebrate species, which number at least 60,000, have repeatedly evolved specialized Hbs through genetic duplication and subfunctionalization. Surprisingly, the 16 Antarctic icefish species of the notothenioid family Channichthyidae neither express Hb nor

produce erythrocytes – they are called “white-blooded” – whereas the remaining seven families of the suborder are red-blooded. Delivery of oxygen in icefishes is accomplished through circulation of a large volume of blood in which oxygen is physically dissolved. Although the volume-based oxygen-carrying capacity of icefish blood is < 10% that of red-blooded notothenioids (Holeton, 1970), compensatory cardiac and circulatory adaptations (e.g., large hearts, expanded vascular and capillary networks and blood volume, cutaneous respiration) offset the apparent deficit in oxygen transport (Sidell and O’Brien, 2006).

Given the lack of Hb synthesis and terminal erythropoiesis in icefishes, it is natural to ask about the evolutionary fate of genes underlying these processes. Cocca et al. (1995) were the first to address the status of icefish *Hb* genes in a study that examined what we now recognize as the LA *Hb* cluster. They reported that the genomes of icefish species from three different genera retained transcriptionally inactive remnants of the *α-globin* gene but apparently had lost the *β-globin* gene, probably via deletion. Zhao et al. (1998) extended these results by showing that *C. aceratus* and *C. rastrospinosus* shared a common genomic deletion that removed the 5’ end of the LA *α-globin* gene (including exons one and two) and the entirety of the linked LA *β-globin*. The mechanism underlying the loss of *Hb* gene expression by the entire icefish clade (Fig. 2) was revealed by a comprehensive survey of the LA locus of the 16 recognized species (Near et al., 2006; Zhao et al., 1998). Fifteen species retain the 3’ *α-globin* fragment but have lost the *β-globin* gene, as originally described by Zhao et al. (Zhao et al., 1998). This apparently simple scenario, *Hb* gene loss in the icefish clade via a single ancestral deletion, was confounded by the discovery of an evolutionary intermediate in the 16th, and final, icefish species to be examined, *Neopagetopsis ionah*. The “smoking gun” genetic fossil of the *N. ionah* LA locus is an *αβ-globin* complex that contains an intact *α-globin* gene linked to two phylogenetically distinct *β* pseudogenes whose origins encompass the entire Antarctic notothenioid radiation. Because *N. ionah* is a phylogenetically derived icefish species, Near et al. (Near et al., 2006) proposed that the initial event that compromised *Hb* gene expression occurred through interspecific introgression of a notothenid *β-globin* gene into the ancestral channichthyid LA locus. In the absence of selective pressure for *Hb* gene expression, subsequent deletions generated the majority allele, the 3’ *α-globin* fragment. Retention of the *N. ionah* and majority alleles is hypothesized to have resulted from incomplete lineage sorting during the rapid icefish radiation (Near et al., 2006).

Recently, a targeted-sequencing approach of the exomes of 44 notothenioid species and the genome assembly of *C. aceratus* have confirmed the genetic history of the icefish LA gene cluster. In addition, both studies demonstrated loss of all *Hb* genes of the MN cluster in icefishes (Daane et al., 2019; Kim et al., 2019). Thus, the same genomic instability that gave rise to the remarkable copy number variation of *Hb* genes, thereby facilitating vertebrate diversification, may also have led to their elimination when these genes were no longer necessary.

The loss of erythrocytes and Hbs by icefishes is an extreme physiological response to the cooling of the SO. The close taxonomic relationship between dragonfishes and icefishes suggests there may have been a stepwise reduction both in hematocrit and in the *Hb* gene regulatory apparatus that preceded their loss in icefishes. This hypothesis is consistent with a general trend of relaxed selection on notothenioid *Hb* clusters in the highly stable and oxygenated environment in the Antarctic SO and with the robustness of red-blooded notothenioids to experimentally-induced anemia in this environment (Borley et al., 2010; di Prisco et al., 1992; Hemmingsen, 1991). Thus, icefishes may have evolved from anemic ancestors prior to losing erythrocytes and Hb entirely. Whether loss of the conventional oxygen transport system of vertebrates was adaptive remains controversial (Sidell and O’Brien, 2006). Although absence of erythrocytes does reduce the viscosity of their blood fluid, icefishes have evolved large hearts and blood volumes that require twice the cardiac energy expended by red-blooded notothenioids (Sidell and O’Brien, 2006). The hearts of icefishes consume a staggering 22% of resting energy compared to 0.5-5% in temperate fishes (Farrell and Jones, 1992; Hemmingsen, 1991). Rather than being adaptive, the loss of erythrocytes by icefishes may simply be deleterious, but not lethal, in their unusual, richly oxygenated environment.

Because dissolved oxygen concentration is inversely proportional to water temperature, oceanic warming due to global climate change is predicted to have devastating consequences for marine organisms, in particular those that have evolved over long periods in cold, oxygen-rich waters (Beers and Jayasundara, 2015). Will icefishes be able to adapt to a warming climate through re-evolution of *Hb* genes and oxygen-transporting cells? This prospect appears to be very unlikely given the rapidity of warming with respect to the long generation times of icefish species. Although cyclostomes have evolved Hb-like function independently (Schwarze et al., 2014) and other fishes have repurposed globins in novel ways (Gallagher and Macqueen, 2016), the additional loss of the complex cellular machinery necessary for oxygen transport almost certainly precludes survival of icefishes at the elevated temperatures that are predicted to occur over the next several centuries, (0.6–2 °C rise in temperature by 2100, Collins et al., 2013).

4.4. Biochemistry of notothenioid hemoglobins

The equilibrium between the penta- and hexa-coordinated state plays an important role in shaping the reactivity and the function of globins. The physiological roles of hexa-coordinated globins are not well understood. These globins may (i) scavenge oxygen under hypoxic conditions (Greenberg et al., 2008); (ii) act as terminal oxidases by oxidizing NADH under hypoxic conditions, thereby enhancing ATP production by glycolysis (Sowa et al., 1999); (iii) function as oxygen sensors (Hargrove et al., 2000; Kriegl et al., 2002); and/or (iv) participate in NO metabolism (Smagghe et al., 2008). In Mb and Hb, the distal HisE7 may potentially hexa-coordinate with the heme, but this interaction is constrained by the protein matrix under physiological conditions. Under pathological conditions the formation of a reversible bis-histidyl complex may occur in Mb and Hb (Robinson et al., 2003; Svistunenko et al., 2000).

Tetrameric Hbs are composed of two α and two β homologous subunits, each displaying a 3/3 globin fold with eight α helices (A–H) and a heme group, able to reversibly bind oxygen, and in some cases, other ligands (Bolognesi et al., 1997; Perutz, 1979). The binding of oxygen is cooperative and depends on the allosteric equilibrium between a low-affinity T (deoxy)-state and a high-affinity R (oxy)-state.

Hb is primarily expressed in red blood cells, but recent studies describe expression of α and β subunits in atypical sites, including the brain (Russo et al., 2013), alveolar cells as determined by Western blotting (Newton et al., 2006), mesangial cells of the kidney (Nishi et al., 2008) as demonstrated by immunoblotting experiments, retinal ganglion cells (Tezel et al., 2010) by proteomic analyses, and hepatocytes (Liu et al., 2011) as shown by immunofluorescence. Neurons were shown to express transcripts for α- and β-chains (Biagioli et al., 2009). The functional implications of “ectopic” Hb expression are unclear but have been related to defense against reactive oxygen species (ROS) or regulation of NO signaling (Russo et al., 2013). Delivery of oxygen by Hb is modulated in a species-specific manner by several mechanisms, including (*i*) the expression of multiple isoforms; (*ii*) alterations in amino acid sequence or posttranslational modifications; and (*iii*) changes in the concentrations of allosteric ligands (ATP for most teleost fish) (Giordano et al., 2015).

Red-blooded Antarctic notothenioids have reduced hematocrits, cellular Hb concentrations, and Hb chain multiplicity (Eastman, 1993) compared to other teleosts. In contrast, non-Antarctic notothenioids commonly exhibit high Hb multiplicity to cope with environmental fluctuations (di Prisco et al., 2007). Most adult notothenioids (Nototheniidae) express a single major Hb (Hb 1) accompanied by minor or “embryonic” components Hb C (in traces) and Hb 2 (approx. 5% of the total) (Fuchs et al., 2004). Three exceptions are *T. newnesi* (D’Avino et al., 1994), *Pagothenia borchgrevinki* (Riccio et al., 2000) and

Pleuragramma antarcticum (Tamburrini et al., 1996), in which "embryonic" Hbs are expressed at significant levels (approx. 25% of the total). Species belonging to the families Artedidraconidae and Bathydraconidae lack the minor or "embryonic" Hbs (Tamburrini et al., 1992).

In addition to differences in isoform diversity and concentrations, the Hbs of Antarctic teleosts also have a peculiar biochemistry. The Hbs of many Antarctic species have a low affinity for oxygen, as indicated by larger values of P_{50} (the oxygen partial pressure required to achieve half saturation) (di Prisco et al., 1988). This reduction in oxygen-binding affinity likely evolved in response to the high oxygen concentration in the cold Antarctic waters. The affinity of non-Antarctic notothenioid Hbs is typically higher, but *D. eleginoides* Hbs have lower oxygen-binding affinity than the Hb of the Antarctic species *T. bernacchii* (Coppola et al., 2015). The blood Hbs of Antarctic fishes also oxidize via an unusual pathway (Vitagliano et al., 2008). The β chains of the Antarctic fish Hbs normally have a strong propensity to form hexa-coordinated bis-histidyl adducts (Riccio et al., 2002). Upon oxidation of the β iron, however, penta-coordination was observed (Vitagliano et al., 2008), a state not reported for other tetrameric Hbs under physiological conditions. Crystallographic and spectroscopic analyses of the major Hb of Antarctic *T. newnesi* showed that the protein adopts peculiar oxidation states under native-like conditions, which indicates potential involvement of the protein in functional redox processes yet to be identified and/or in scavenging ROS.

4.4.1. Physiological regulation of fish hemoglobins

The functional properties of fish Hbs are modulated by several environmental factors, with regulation of oxygen binding affinity by pH among the most important. The reduced oxygen affinity of Hbs at acidic pH, known as the Bohr effect, enables Hb to offload oxygen efficiently to respiring tissues. Although many studies indicate that the general structure of fish Hbs is similar to that of human Hb (Camardella et al., 1992; Coppola et al., 2012; Ito et al., 1995; Mazzarella et al., 2006a, 2006b, 1999; Tame et al., 1996; Vergara et al., 2010; Yokoyama et al., 2004), they generally display a greater dependence of oxygen affinity and cooperativity on proton concentration. The Root-effect Hbs of fishes have evolved an exaggerated Bohr effect, and the structural features that are responsible for their extreme sensitivities to proton concentration are not well understood. The Root effect is thought to have enabled the evolution of the swim bladder in fishes, as oxygen needs to be pumped against a concentration gradient into this organ (Berenbrink et al., 2005).

The original function of the Root effect was probably to facilitate oxygen unloading in the working muscle of teleosts. Later the mechanism was exalted to enhance oxygen secretion to the eye and swim bladder (Harter and Brauner, 2017; Nikinmaa et al., 2019). Thus, the Root effect has been critical in the evolutionary success and diversification of fishes.

Of the 13 His residues that contribute in the Bohr effect of human Hb, only three are conserved in the Root-effect Hbs of temperate fishes such as trout (Tame et al., 1996), tuna (Yokoyama et al., 2004), carp, and eel (Okonjo, 2018), as well as the cold-living Antarctic notothenioids *Trematomus bernacchii* (Camardella et al., 1992; Ito et al., 1995) and *Bathydraco marri* (Caruso et al., 1992). These three His residues are located in regions of Hb that are very sensitive to quaternary structural transitions: (*i*) the CD corner of the α-chain (Ronda et al., 2013; Vergara et al., 2010); and (*ii*) the $\alpha_1\beta_2/\alpha_2\beta_1$ subunit interface (Mazzarella et al., 2006a, 2006b; Yusuff et al., 2012). Formation of the His147β/Glu94β salt-bridge observed in carp Hb and anodic eel Hb also plays a role in the Root effect (Okonjo, 2018). The molecular bases of the Root effect have been extensively studied in Antarctic fish Hbs. X-ray crystallographic analysis of Hbs from *T. bernacchii* and *T. newnesi* (Mazzarella et al., 2006a, 2006b; Vergara et al., 2010) revealed that the aspartic acid triad at the $\alpha_1\beta_2$ interface and the CD corner of the α chain were critical for pH modulation of oxygen affinity and cooperativity. Binding of two protons per tetramer at the $\alpha_1\beta_2$ interfaces of these Hbs promotes formation of a hydrogen bond between Asp95α and Asp101β, thereby causing release of oxygen (Ito et al., 1995; Mazzarella et al., 2006a, 2006b).

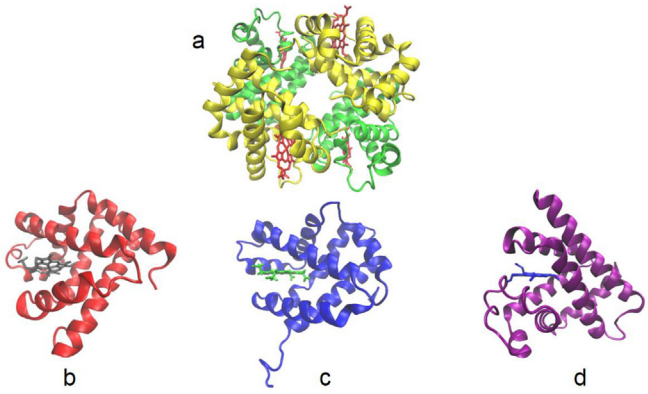

Fig. 3. 3D-structures of (a) *T. bernacchii* deoxy Hb (PDB 2H8D): α chains are in yellow, β chains in green and heme in red; (b) Sperm whale Mb (PDB 1VXA): heme is in gray; (c) hexa-coordinated Ngb of *D. mawsoni*: heme is in green; (d) *C. aceratus* Cygb-1: heme is in blue.

The allosteric effector ATP plays a significant role in modulating the Root effect in sub-Antarctic notothenioids. For example, the Root effect of *E. maclovinus* Hb 1 is largely dependent on ATP, whereas *T. bernacchii* Hb exhibits a strong Root effect in the absence of ATP. Thus, the mechanisms underlying ATP regulation and the Root effect in non-Antarctic notothenioid Hbs must differ in essential ways from those of High-Antarctic species.

The high-oxygen affinity of *E. maclovinus* Hb 1 in the absence of ATP indicates that its T-liganded state is less stable compared to that of *T. bernacchii* Hb. *E. maclovinus* Hb 1 may have additional ATP binding sites, and the occupancy of these sites by ATP could stabilize the low-affinity T-liganded structure (Coppola et al., 2012). The relationship between oxygen affinity/regulation and habitat features is still an open question.

The 3D structure of *T. bernacchii* deoxy Hb (PDB 2H8D) is shown in the Fig. 3a.

5. Myoglobin

5.1. Evolution and function of myoglobin

Mb is present in cardiac and aerobic skeletal muscle of vertebrates, where it functions in the short- and long-term buffering of oxygen and in facilitating oxygen diffusion from blood to mitochondria (Wittenberg and Wittenberg, 2003). The protein is also found at lower concentrations in smooth muscle, endothelial, and tumor cells (Cossins et al., 2009; Gorr et al., 2011; Helbo et al., 2013), thus suggesting that the protein plays a more complex and versatile role than previously thought.

Almost all vertebrates possess a single *Mb* gene in their genomes. Some vertebrates lack the *Mb* gene, including the threespine stickleback, *G. aculeatus*, and the African butterflyfish, *P. buchholzi* (Pantodontidae) (Macqueen et al., 2014). In contrast, two cyprinids, carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), have two *Mb* isoforms (*Mb1* and *Mb2*), which are differentially expressed and perform different functions (Fraser et al., 2006; Roesner et al., 2008). *In vitro* evidence suggests that Mb1 is endowed with the classic roles in oxygen supply and NO production, whereas Mb2 is actively involved in ROS scavenging (Helbo et al., 2012). Recently, three distinct *Mb* genes were found in two arowana species which share no more than 69% amino acid identity. Despite this sequence divergence, there is yet no evidence for their functional diversification and/or differential

expression of these *Mbs* (Gallagher and Macqueen, 2016). The frequency with which high *Mb* expression was lost in heart muscle during teleost evolution highlights that the function of Mb may be compensated by other globins or other mechanisms (Gallagher and Macqueen, 2016; Macqueen et al., 2014). The *Mb* gene is also deleted in anuran amphibians (Fuchs et al., 2006; Hoffmann et al., 2011), and the current hypothesis is that Cygb may compensate for the oxygen storage function in the skeletal muscle of these species (Opazo et al., 2015). In the elephant shark, *Mb* is expressed at a high level only in heart, but not in skeletal muscle, which may be the ancestral site of Mb function. The dominant expression of *Mb* in the skeletal muscle of many gnathostomes may have been a later innovation caused by an increase in tissue oxygen demands (Opazo et al., 2015).

5.2. Biochemistry of myoglobin

Mb is a 17-kDa monomeric, penta-coordinated globin with the classical 3/3 globin fold (Fig. 3b). In heart and skeletal muscle, it is present at concentrations of less than 0.5 mM, whereas in marine mammals Mb concentrations range from 4 to 5 mM (Wright and Davis, 2015). In diving marine mammals, the high concentrations of Mb store oxygen during prolonged apnea (Davis, 2014). Although the overall structure of the protein is conserved among terrestrial and marine species, the primary structure varies in regions of the protein (Mirceta et al., 2013; Naylor and Gerstein, 2000). The proximal (H93) and distal (H64) His are notably conserved in all Mb structures, whereas the interspecific variability of Mb primary structure may generate phenotypes with different ligand-affinity properties and stability that are subject to natural selection (Dasmeh et al., 2013; Naylor and Gerstein, 2000; Wittenberg and Wittenberg, 2003). Because variations in Mb oxygen affinity have a greater impact on oxygen storage and transport when pO_2 is low, hypoxia is probably the main evolutionary driver to preserve Mb function (Dasmeh and Kepp, 2012). Teleosts generally express lower levels of Mb whose oxygen dissociation rates are significantly faster than in mammalian Mbs. This allows Antarctic teleost Mbs to function at extreme low temperatures (Cashon et al., 1997).

Recent papers on knockout mice have revealed that Mb may be involved in (*i*) cardiac protection by scavenging NO and ROS (Flögel et al., 2010) in the oxy-Mb form; and (*ii*) ischemia-damage protection by producing NO from nitrite as deoxy Mb in response to cellular hypoxia (Hendgen-Cotta et al., 2008). Mb may also interact with fatty acids (Shih et al., 2014). In 2019 Park et al. (2019) demonstrated that nitrate levels in skeletal muscle of Mb-deficient mice are only moderately reduced, thus implying that other mechanisms sustain the NO pathway in the absence of Mb.

5.3. Myoglobin in Notothenioidei

As described above, there are many species with cardiac Mb deficit, which suggests that other biological/physiological mechanisms, still uncharacterized, intervene when the selective pressure on Mb-assisted oxygen supply to heart is relaxed. High levels of Mb are correlated with lifestyle or environmental conditions that require efficient oxygen delivery. Conversely, relaxed selection on Mb levels may occur in environments rich in oxygen. The hemoprotein-deficient Antarctic icefishes, which survive in the freezing water of the SO, provide an excellent system for evaluating the function of Mb under relaxed selection.

Six of the 16 species of icefishes lack expression of Mb in the heart ventricle due to four independent mutations (Moylan and Sidell, 2000; Sidell et al., 1997). Moreover, the oxidative skeletal muscle of all Antarctic notothenioids is devoid of Mb (Moylan and Sidell, 2000). Sidell and O'Brien (2006) and Beers et al. (2010) propose that the loss of expression of hemoproteins during evolution of the Antarctic icefishes resulted in increased steady-state NO levels that mediated modification of their vascular systems through a self-rectifying readaptation, thereby maintaining oxygen delivery at levels sufficient for normal physiological activities (reviewed by Detrich, (2007)). Greater NO levels in icefishes would be expected to induce vascular expansion, cardiomegaly, and other characters that increase blood volume and the capacity to delivery oxygen (Sidell and O'Brien, 2006). Nevertheless, icefishes are more sensitive to temperature elevation than their red-blooded relatives (Beers and Sidell, 2011) because their overall capacities to deliver (Hb), and in some species store (Mb), oxygen are reduced, which makes it likely that they will be the first "losers" among notothenioid fishes as the temperature of the SO rises as part of global climate change.

6. Neuroglobin – the nervous system globin

6.1. Evolution and function of neuroglobin

Ngb is predominantly expressed in neurons of the central and peripheral nervous systems, the gastrointestinal tract, and in endocrine organs (Brunori and Vallone, 2006; Burmester et al., 2000; Burmester and Hankeln, 2009; Emara et al., 2010; Hankeln et al., 2005; Hundahl et al., 2010; Wystub et al., 2003; Yu et al., 2012). Since its discovery in mammals, *Ngb* has been identified in all vertebrates except the cyclostomes (hagfish and lampreys) and Chondrichthyes (sharks and rays) (Opazo et al., 2015). *Ngb* is highly conserved, with an evolutionary rate of change that is about threefold slower than that of *Mb* and *Hb* (Burmester and Hankeln, 2014 and references within).

In mammals, Ngb mRNA and protein levels vary across different regions of the brain (Fabrizius et al., 2016; Reuss et al., 2016; Vorasubin et al., 2016). In most neural tissues Ngb is expressed at micromolar concentrations, which argues against a role in oxygen transport. However, Ngb concentrations are high (0.1–0.2 mM) in retinal rod cells, where the protein may facilitate oxygen supply to mitochondria during visual activity (Wystub et al., 2003). Increased production of Ngb protects against the deleterious effects of hypoxia and ischemia in the knockout mouse model (Sun et al., 2003, 2001) and against ROS and reactive nitrogen species (RNS) (Brunori et al., 2005; Herold et al., 2004; Khan et al., 2007; Wakasugi et al., 2003) by modulating the activation of the apoptotic cascade (Fago et al., 2008, 2006; Yu et al., 2012). In fact, steady state levels of Ngb in brain are positively correlated with tolerance of hypoxia (Avivi et al., 2010; Mitz et al., 2009; Schneuer et al., 2012). Zebrafish *Ngb* is specifically expressed in amacrine cells and is upregulated after optic nerve injury, suggesting a role for Ngb in the early stages of optic nerve regeneration (Kamioka et al., 2013).

6.2. Biochemistry of neuroglobin

Ngb functions as a 17 kDa monomer with a high oxygen affinity in the range of a typical Mb (P_{50} = 0.9 to 2.2 Torr (0.12–0.29 kPa)) (Burmester et al., 2000; Dewilde et al., 2001). Ngb is hexa-coordinated and displays the classical 3/3 globin fold with an elongated cavity that facilitates ligand diffusion to the heme (Burmester et al., 2000; Pesce et al., 2003). In Ngb, regulation of oxygen affinity is controlled by intermolecular disulfide bond formation. Experiments with high pressure suggest that the conformation flexibility of the CD region of Ngb is similar to that of *T. newnesi* Hbs and is important in modulating the equilibrium between the two coordinated states (Capece et al., 2009; Hamdane et al., 2005).

The current hypothesis suggests that the ability of Ngb (as well as Cygb) to perform multiple functions is linked to a tunnel system and multiple internal cavities surrounding the heme pocket. Internal cavities are essential for conformational flexibility of proteins and modulating their activity within cells and tissues. Characterization of globin cavities and tunnels is revealing ligand pathways to and from the heme pocket. In human NGB, an unusually large and dynamic network of cavities has been observed (Abbruzzetti et al., 2009) and confirmed by

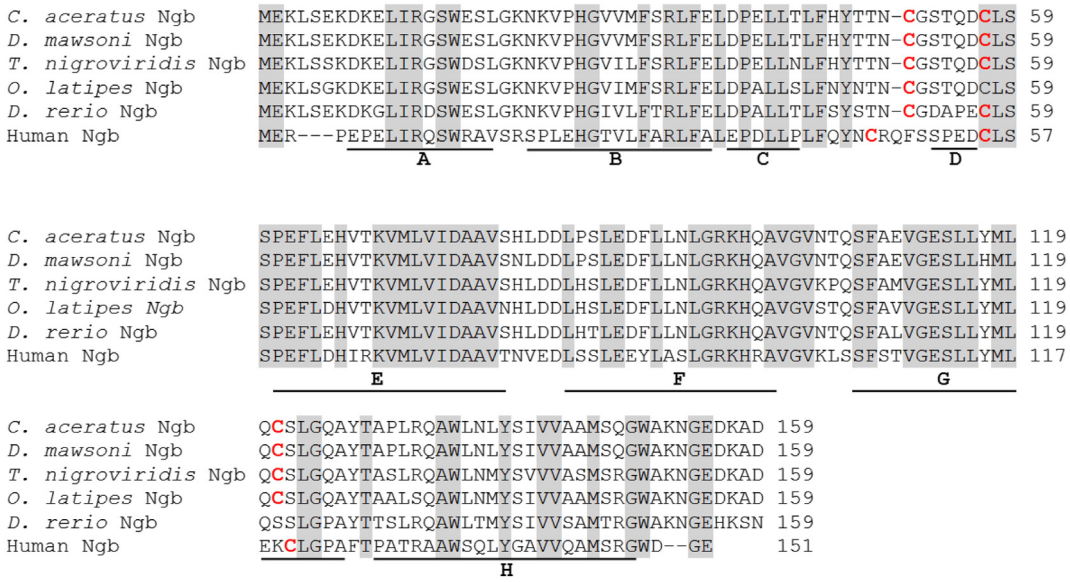

Fig. 4. Sequence alignment of the Ngbs of Antarctic fish with those of human, *D. rerio*, *T. nigroviridis* and *O. latipes*. Conserved residues are shaded; conserved Cys residues are in red and bold. Positions of helices A–H are underlined according to the secondary structure of human NGB. Adapted from Boron et al. (2011).

the crystal structures of unliganded and CO-bound NGB (Vallone et al., 2004).

6.3. Neuroglobin in Notothenioidei

The *Ngb* gene was identified in the brain of red-blooded notothenioids (*D. mawsoni, Gymnodraco acuticeps, and Bovichtus variegatus*) and in 13 of the 16 channichthyids (Cheng et al., 2009a, 2009b). Thus, athough Hbs and Mbs are dispensable in most notothenioids, Ngb appears to be conserved across the phylogeny. This high conservation suggests that there are important roles for Ngb in vertebrate physiology even in the context of high environmental oxygen. A detailed structural and functional analysis was carried out on Ngb from the brain of *C. aceratus* and from the retina of the closely related red-blooded notothenioid *D. mawsoni*. The 3D model of hexa-coordinated Ngb of *D. mawsoni*, using the human X-ray structure (PDB: 1OJ6) as a template (Boron et al., 2011), is shown in the Fig. 3c.

The two Antarctic proteins share 98% amino-acid sequence identity but only 55% sequence identity with human NGB (Boron et al., 2011; Giordano et al., 2012). The sequence alignment of human and fish Ngbs is shown in Fig. 4. Among teleosts, Ngbs from Antarctic species share > 80% of sequence identity with zebrafish and 90% with *Tetraodon nigroviridis* and *O. latipes* Ngbs. Compared to mammalian Ngb, fish Ngbs show an extension of charged residues at the N- and C-termini and the CD region is shorter by one residue. In zebrafish Ngb, the presence of Lys residues at the N-terminus, also present in other fish, appears crucial for functioning as a cell membrane interacting protein (Watanabe and Wakasugi, 2011, 2010, 2008). The shorter loop in the CD region of teleosts brings the two Cys residues, which form the disulfide bridge in human NGB, close to each other so that the Cys–Cys distance is shorter by several Å (Boron et al., 2011). In human NGB, the internal disulfide bond modulates the oxygen affinity of the protein by controlling exogenous ligand binding (Hamdane et al., 2003). Under oxidizing conditions, Cys 46 and Cys 55 form an internal disulfide bond whose cleavage induces a rearrangement of the CD loop that strengthens the bond between the heme iron and the distal His.

Similar to the human protein, Antarctic fish Ngbs can reversibly bind oxygen and CO in the Fe^{II} form and show hexa-coordination by the distal His in the absence of exogenous ligands (Giordano et al., 2012). When compared to human and zebrafish Ngbs, large cavities were discovered in Antarctic fish Ngbs, which enables retention of ligands for a longer intervals (Giordano et al., 2012). CO binding to zebrafish Ngb occurs with values that are almost identical to the values found for human NGB (Fuchs et al., 2004). Interestingly, CO rebinding in Antarctic fish Ngbs occurs significantly faster than in human NGB, suggesting that the biological function of Ngb is optimized in the cold-adapted fish. In contrast to red-blooded fish Ngb, the icefish protein shows slower migration of ligands into and within the cavities, leading to more efficient accumulation of ligands within the protein matrix (Giordano et al., 2012). The presence of these multiple binding sites allows for temporary docking of small gaseous ligands for relatively long times and is consistent with the involvement of the protein in NO-dependent processes, as proposed for human and mouse Ngbs (Brunori et al., 2005). As icefish have elevated levels of NO, the enlarged icefish cavities may be important in sequestering the excess circulating NO levels found in these species.

7. Cytoglobin – the cytoplasmic globin

7.1. Evolution and function of cytoglobin

Cygb is present as a single gene in tetrapods, cartilaginous fish, and non-teleost ray-finned fish (Hoffmann et al., 2011; Storz et al., 2013). Teleost fish have two *Cygb* gene copies resulting from the teleost WGD (Fuchs et al., 2005). *Cygb-1* is transcribed in many tissues, whereas *Cygb-2* mRNA is predominantly found in neuronal tissues (Fuchs et al., 2005). However, as with other globins the Cygbs have evolved numerous differential tissue-specific expression patterns, with some species (e.g., stickleback) expressing only one of the two paralogs (Gallagher and Macqueen, 2016). In most cell types Cygb is localized in the cytoplasm, although some studies indicate that Cygb is also present in the nuclei of neurons (Oleksiewicz et al., 2011).

The putative function of Cygb is protection of tissues under conditions of hypoxia, ischemia, and oxidative stress. Cygb is associated with (*i*) NO scavenging through its dioxygenase activity (Halligan et al., 2009), (*ii*) nitrite reduction and NO production under anaerobic conditions (Li et al., 2012), (*iii*) modulation of NO levels and metabolism in vascular walls (Liu et al., 2012), (*iv*) cell protection against ROS and RNS (Li et al., 2007), and (*vi*) fibrotic disorders (Bholah et al., 2015; Cui et al., 2012; Nakatani et al., 2004; Nishi et al., 2011; Okayasu et al., 2011; Schmidt et al., 2004; Stagner et al., 2009). Among the many functions hypothesized for Cygb, the reaction with NO is probably the

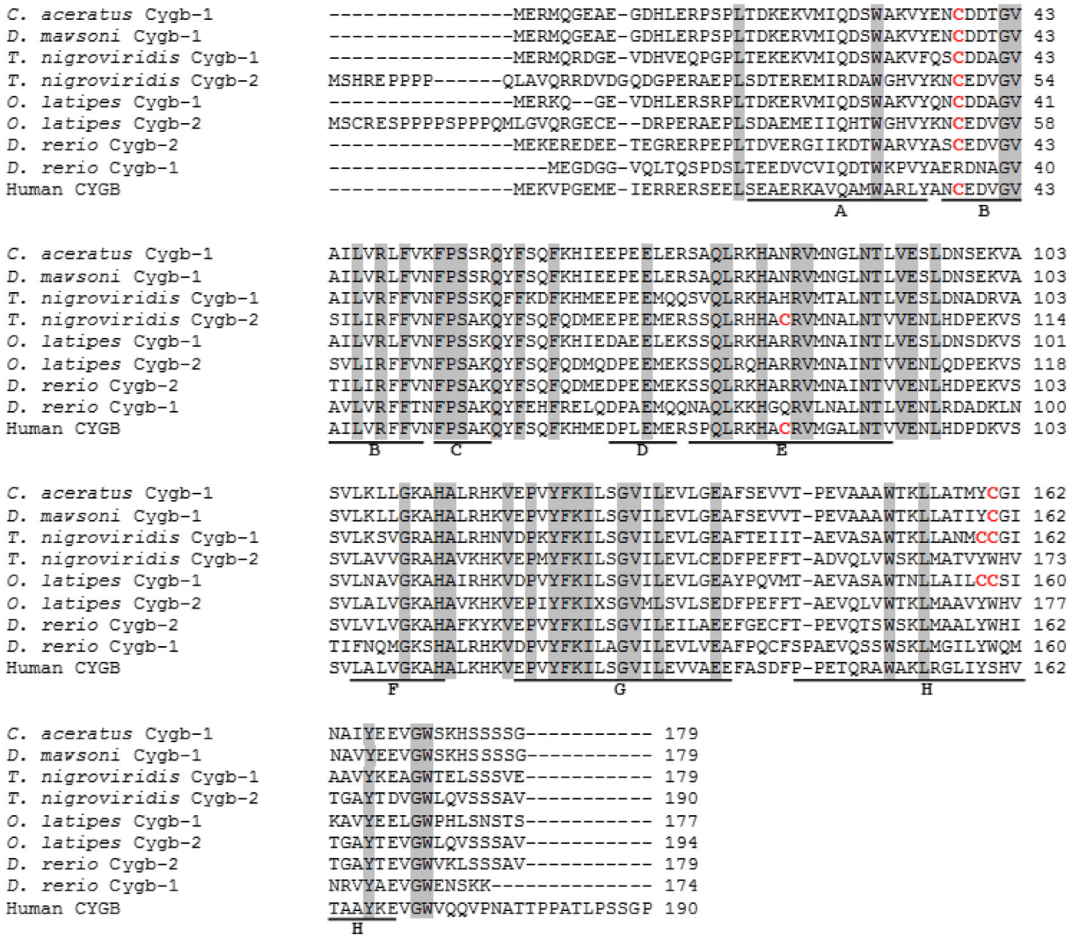

Fig. 5. Sequence alignment of Cygbs of Antarctic fish with human, *D. rerio*, *T. nigroviridis* and *O. latipes* Cygbs. Conserved residues are in grey; conserved Cys residues are in red and bold. Positions of helices A–H are underlined according to the secondary structure of human CYGB (de Sanctis et al., 2004). Adapted from Cuypers et al. (2017).

key for understanding the physiological role of the protein. NO dioxygenation occurs when oxygen-bound Cygb reacts with NO. This significantly contributes to NO metabolism *in vivo* and regulates vascular tone (Liu et al., 2012, 2017). However, the nature of the physiological reducing systems for Cygb that are necessary to regenerate reduced heme is unknown. One hypothesis is that Cygb reduction may be driven by Cytochrome b5 rather than over ascorbate in cell types, including hepatocytes, fibroblasts, and neurons, that are characterized by high ascorbate levels (Amdahl et al., 2017).

7.2. Biochemistry of cytoglobin

Cygb is a hexa-coordinated, 21-kDa protein that is ubiquitously expressed in vertebrate tissues, although it is found at highest concentrations in the brain, eyes, liver, heart, and skeletal muscles (Burmester et al., 2002; Fordel et al., 2004; Kawada et al., 2001). The oxygen affinity of Cygb is typically around 1 Torr (0.14 kPa) (Trent and Hargrove, 2002). Cygb is present under physiological conditions as a homodimer stabilized by electrostatic interactions, hydrogen bonds, and an inter-subunit disulfide bridge (Hamdane et al., 2003). In Cygb, a histidyl residue coordinates directly with the heme iron in both the ferrous (Fe^{II}) and ferric (Fe^{III}) states. The tertiary structure contains eight α-helices, numbered A to H, that display the classic 3/3 α-helix fold (de Sanctis et al., 2004; Makino et al., 2006; Sugimoto et al., 2004).

In human CYGB, like Ngb, the surface-exposed cysteine residues form intramolecular disulfide bonds that are important for ligand binding and enzymatic properties. There are three dominant forms of recombinant Cygb depending on the oxidation state of the protein: (*i*) dimers with an intermolecular disulfide, (*ii*) monomers with reduced cysteinyl residues, and (iii) monomers with an intramolecular disulfide in the same protein chain. These three forms have different properties in ligand binding and in the coordination states induced by binding to lipids (Astudillo et al., 2013; Beckerson et al., 2015). There is no evidence regarding the existence and physiological relevance of the dimeric form of the human protein. Moreover, the concentration of the protein within tissues is not compatible with the *in vivo* production of the dimer (Beckerson et al., 2015).

7.3. Cytoglobin in Notothenioidei

While there have been many recent studies on the structure and chemical reactivity of mammalian Cygb (de Sanctis et al., 2004; Gabba et al., 2013; Lechauve et al., 2010; Makino et al., 2011), fish Cygb remains largely uncharacterized. As in mammals, fish Cygbs are able to bind oxygen and NO (Corti et al., 2016a). The *cygb1* and *cygb2* genes of *D. rerio* encode proteins that show significant functional differences, with the activity of Cygb2 more closely aligned to that of the Cygb encoded by the single mammalian gene (Fuchs et al., 2005). Like their mammalian orthologs, both fish Cygbs are expressed in several tissues, with Cygb2 detected at highest levels in neuronal tissues (Fuchs et al., 2005).

Both the *Cygb1* and *Cygb2* genes are found in the genomes of the red-blooded Antarctic fish *N. coriiceps* (Shin et al., 2014) and the white-blooded icefish *C. aceratus* (Kim et al., 2019). Although surveys of

expression are incomplete, these genes are expressed in Antarctic fishes: *Cygb1* in the brain of *C. aceratus* and in the retina of *D. mawsoni* (Cuypers et al., 2017); *Cygb*1 and *Cygb*2 in the retina, brain and gills of the red-blooded *T. bernacchii* and the icefish *Chionodraco hamatus* (Daniela Giordano, personal communication).

A physico-chemical analysis of *C. aceratus* and *D. mawsoni* Cygb1s highlighted similarities and differences with the human ortholog (Cuypers et al., 2017). The homology model of *C. aceratus* Cygb1, using the 3D-structure (PDB: 1V5H) of human CYGB as template and built using SwissModel (https://swissmodel.expasy.org/), is shown in the Fig. 3d. As with human CYGB, electron paramagnetic resonance, resonance Raman and optical absorption spectroscopy reveal that Antarctic fish Cygb1 is hexa-coordinated (Cuypers et al., 2017). In contrast, Corti et al. (2016a, 2016b) reported that zebrafish Cygb2 is hexa-coordinated whereas Cygb1 has a penta-coordinate heme with slower autoxidation and biochemical properties that resemble oxygen transport carriers.

The sequence alignment between human and fish Cygb is shown in Fig. 5. *C. aceratus* and *D. mawsoni* Cygb1, which share 98% sequence identity, have only 70% sequence identity with Cygb1 of temperate fish and as little as 54 to 59% sequence identity with fish Cygb2 and human CYGB (Cuypers et al., 2017). Thus, there may be considerable functional differentiation of Cygbs in Antarctic teleosts.

Human CYGB contains conserved Cys residues at B2 and E9 positions, which form an intramolecular disulfide bridge *in vitro* (Hamdane et al., 2003). However, with the exception of *T. nigroviridis* Cygb2, the CysE9 residue is not conserved in teleosts and Cys H17 is too far from the B2 Cys to form a disulfide bridge (Fig. 5) (Cuypers et al., 2017). Therefore, unlike in human CYGB, the oxygen affinity of fish Cygb1 is not expected to depend on the redox state of the Cys residues. Although the Cys residues are too far apart to form disulfide bridges, biochemical characterization of Antarctic fish Cygb1 reveals non-covalent multimers (up to pentamers) at low protein concentrations. The ability of Antarctic fish Cygb1 to form stable multimers is intriguing, but evidence of functional differentiation among these forms and of their existence *in vivo* is lacking (Cuypers et al., 2017).

8. Globin X – the membrane-bound globin

The gene encoding GbX is found in fishes, amphibians, and reptiles, but has been lost independently by mammals and birds (Dröge and Makałowski, 2011; Roesner et al., 2005). GbX is synthesized in specific regions of the brain and retina of fish, associated with the olfactory and visual systems, respectively (Blank et al., 2011). Recently, the protein was also found in fish erythrocytes, where it may regulate NO production and platelet activation. GbX may also act as a fast nitrite reductase, up to 200-fold faster than human Hb and up to 50-fold faster than Ngb or Cygb (Corti et al., 2016b).

The GbX amino-acid sequence is longer than that of a typical globin (~200 amino acids) and differs from other globins in having *N*-terminal acylation (myristoylation at Gly2 and palmitoylation at Cys3) that enables GbX to bind to the plasma membrane (Blank et al., 2011). Therefore, GbX may be involved in protection of membrane lipids or in membrane-related cellular signaling process. The protein is also involved in RNS metabolism and protection of cell membrane from ROS stress (Koch and Burmester, 2016; Tejero and Gladwin, 2014).

GbX mRNA has been recently identified in the retina, brain and gills of the red-blooded *T. bernacchii* and the icefish *C. hamatus* (Daniela Giordano, personal communication). However, much work remains to determine the function and biochemistry of GbX in these species.

9. Globins, NO scavenging and the case for Antarctic fishes

The original and most important function of globins is likely their ability to react with NO. The ability of oxygen-bound Hb to scavenge NO, thus limiting its diffusion and toxicity, was the key discovery that identified NO as the endothelium-derived relaxing factor (Ignarro et al., 1987). Furthermore, nitrite reductase activities, which form NO under hypoxic conditions, have been clearly shown for Mb (Hendgen-Cotta et al., 2008) and for deoxy-Hb (Gladwin and Kim-Shapiro, 2008).

Supporting a central role for Hb in NO degradation, NO levels are 1.5-2-fold higher in icefishes (Borley et al., 2010), which lack the genes encoding Hb and, in many species, Mb. Experimentally-induced anemia via treatment of the red-blooded notothenioid *N. coriiceps* with the hemolytic agent phenylhydrazine (PHZ) resulted in an increase in circulating NO (Beers et al., 2010). This increase appears to be associated with the reduction in Hb, as there is no increase in nitric oxide synthase activity in the tissues of PHZ-treated *N. coriiceps* or of *C. aceratus* (Beers et al., 2010; Borley et al., 2010). Thus, it appears the loss of Hb scavenging of NO leads to elevated NO in icefishes.

NO signaling can induce systemic physiological changes, including an increase in vascular branching, muscle hypertrophy, mitochondrial biogenesis and vasculature luminal diameter (reviewed in Sidell and O'Brien (2006)). Intriguingly, all of these traits are observed in the icefishes. These results support an elegant model whereby the loss of Hb in icefishes directly leads to an increase in circulating NO, which is then able to facilitate coordinated physiological compensation in the rest of the cardiovascular system (Sidell and O'Brien, 2006). Whether all, or any, of these phenotypes are directly caused by increased circulating NO in icefishes remains unresolved.

10. Summary

As global climate change continues, there is intense interest in understanding whether, and how, species will adapt their physiologies under new environmental conditions. From the earliest origins of life, globins have been critical in facilitating adaptation, novelty and the diversification of species. The huge diversity of globins, their many functions, and their precise regulation make them excellent models for comparative studies to explore biochemical adaptations and to dissect the roles of genome architecture in evolution. Antarctic icefishes have discarded Hb and Mb, which once were considered synonymous with vertebrate survival and diversification. Nevertheless, the retention of other globins, such as Ngb and Cygb, suggests that there are essential roles for these proteins in an environment of abundant oxygen. By examining the biochemistry and function of globins that have evolved in extreme environments, such as the SO, we may be able to tease apart underappreciated mechanistic roles for these proteins in vertebrate biology and to understand better the roles of globins in facilitating physiological adaptation.

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