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Review

Gene duplication, genome duplication, and the functional diversification of vertebrate globins

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ABSTRACT

The functional diversification of the vertebrate globin gene superfamily provides an especially vivid illustration of the role of gene duplication and whole-genome duplication in promoting evolutionary innovation. For example, key globin proteins that evolved specialized functions in various aspects of oxidative metabolism and oxygen signaling pathways (hemoglobin [Hb], myoglobin [Mb], and cytoglobin [Cygb]) trace their origins to two whole-genome duplication events in the stem lineage of vertebrates. The retention of the proto-*Hb* and *Mb* genes in the ancestor of jawed vertebrates permitted a physiological division of labor between the oxygen-carrier function of Hb and the oxygen-storage function of Mb. In the *Hb* gene lineage, a subsequent tandem gene duplication gave rise to the proto α- and β-globin genes, which permitted the formation of multimeric Hbs composed of unlike subunits ($\alpha_2\beta_2$). The evolution of this heteromeric quaternary structure was central to the emergence of Hb as a specialized oxygen-transport protein because it provided a mechanism for cooperative oxygen-binding and allosteric regulatory control. Subsequent rounds of duplication and divergence have produced diverse repertoires of α- and β-like globin genes that are ontogenetically regulated such that functionally distinct Hb isoforms are expressed during different stages of prenatal development and postnatal life. In the ancestor of jawless fishes, the proto *Mb* and *Hb* genes appear to have been secondarily lost, and the Cygb homolog evolved a specialized respiratory function in blood-oxygen transport. Phylogenetic and comparative genomic analyses of the vertebrate globin gene superfamily have revealed numerous instances in which paralogous globins have convergently evolved similar expression patterns and/or similar functional specializations in different organismal lineages.

Contents

1. The functional diversification of vertebrate globins

In the 1970s and 80s, Morris Goodman and colleagues conducted a number of pioneering studies of hemoglobin (Hb) evolution that exploited a rich database of amino acid sequences (Goodman et al., 1975, 1987; Goodman, 1981; Czelusniak et al., 1982). Since the time of those seminal studies, a number of new globins have been discovered and conventional wisdom about homologous relationships among vertebrate globins has been revised as more and more genomic sequence data have become available (reviewed by Storz et al., 2011a). All jawed vertebrates

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(gnathostomes) that have been examined to date possess copies of neuroglobin (*Ngb*), cytoglobin (*Cygb*), and androglobin (*Adgb*; Awenius et al., 2001; Burmester et al., 2000, 2002, 2004; Burmester and Hankeln, 2009; Fuchs et al., 2004, 2005; Hankeln et al., 2005; Hankeln and Burmester, 2008; Hoffmann et al., 2010a; Hoogewijs et al., 2012; Kugelstadt et al., 2004; Pesce et al., 2002; Trent and Hargrove, 2002; Roesner et al., 2005; Wystub et al., 2004). The monomeric Ngb protein and the homodimeric Cygb protein have been subjected to intensive experimental scrutiny since the time of their discovery, but their physiological functions are still not clearly understood (Burmester and Hankeln, 2009; Hankeln and Burmester, 2008; Kakar et al., 2010).

Ngb is expressed in the retina, in neurons of the central and peripheral nervous system, and in some endocrine tissues, whereas Cygb is expressed in fibroblasts and related cell types and in distinct nerve cells in the central and peripheral nervous system (reviewed by Burmester and Hankeln, 2009; Hankeln and Burmester, 2008). The recently discovered *Adgb* gene is a chimeric fusion gene with a unique modular architecture. The encoded protein has an N-terminal calpain-like domain, an internal globin domain (which has undergone internal shuffling of α-helical subdomains), and an IQ calmodulin-binding motif. In mammals, the *Adgb* gene is preferentially expressed in testis (Hoogewijs et al., 2012). The heme-coordination chemistries and other features of Adgb, Ngb, and Cygb suggest that these globins may perform redox-regulated signalling functions or oxygen-sensing functions that mediate oxygen-dependent protein activities (Fago et al., 2004; Gardner et al., 2010; Hankeln et al., 2005; Hankeln and Burmester, 2008; Hoogewijs et al., 2012; Kakar et al., 2010; Li et al., 2011; Tiso et al., 2011).

In gnathostomes, hemoglobin (Hb) and myoglobin (Mb) appear to be indispensible globins that play critical roles in the maintenance of cellular oxygen supply in support of aerobic metabolism. One remarkable exception is provided by the Notothenioid icefish that inhabit the ice-laden waters surrounding the continental shelf of Antarctica. Notothenioid fish in the family Channichthyidae do not express Hb and many species do not express Mb either (Sidell and O'Brien, 2006). The Mb gene also appears to have been deleted in amphibians (Fuchs et al., 2006; Maeda and Fitch, 1982; Xi et al., 2007; Hoffmann et al., 2011).

In contrast to the *Ngb*, *Cygb*, *Adgb*, *Hb*, and *Mb* genes that have been retained in all or nearly all gnathostome lineages, a number of paralogous globins have been discovered that have far more restricted phyletic distributions. The *globin X* (*GbX*) gene has been documented in the genomes of some cyclostomes, elasmobranchs, teleost fishes, amphibians, and reptiles (Dröge and Makalowski, 2011; Roesner et al., 2005). This gene encodes a membrane-bound, hexacoordinate globin that appears to perform an antioxidant function (Blank et al., 2011b). The *globin Y* (*GbY*) gene has only been documented in the genomes of teleost fishes, *Xenopus*, the green anole lizard, and platypus (Fuchs et al., 2006; Hoffmann et al., 2010b; Patel et al., 2008). Experiments in *Xenopus* demonstrated that this gene is expressed in a diverse range of tissues and cell types (Fuchs et al., 2006), but its physiological function remains a mystery. Finally, the *globin E* (*GbE*) gene has so far only been documented in the genomes of birds (Blank et al., 2011a; Hoffmann et al., 2010b, 2011; Kugelstadt et al., 2004). This bird-specific protein appears to perform a Mb-like function in regulating oxygen supply to photoreceptor cells in the avascular avian retina (Blank et al., 2011a), although a role in regulating cellular redox homeostasis is also possible.

Whereas the *Ngb*, *Adgb*, and *GbX* genes originated prior to the divergence between deuterostomes and protostomes, the remaining members of the vertebrate globin gene repertoire are all products of vertebrate-specific duplication events (Ebner et al., 2003, 2010; Hoffmann et al., 2011, 2012a; Storz et al., 2011a; Fig. 1).

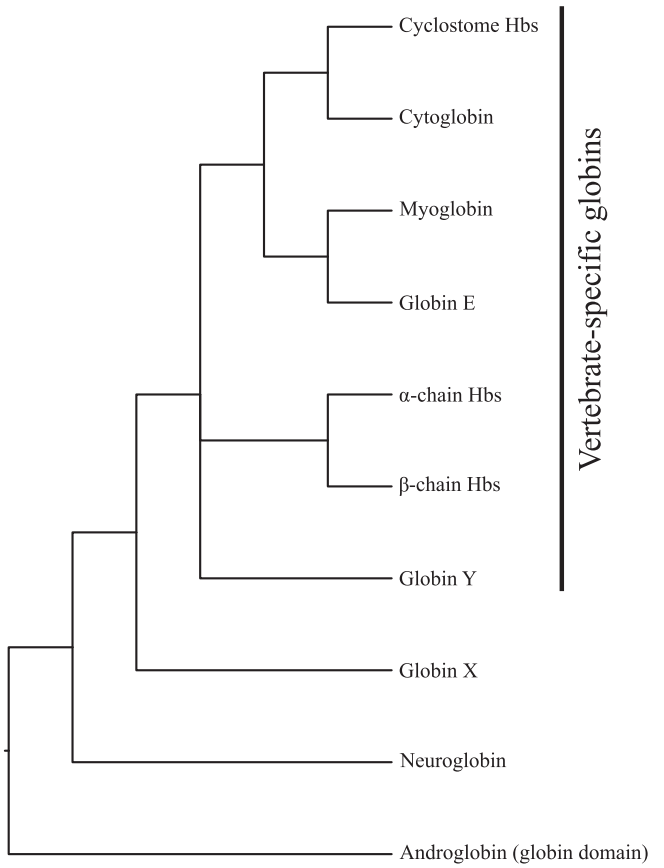

Fig. 1. Cladogram describing phylogenetic relationships among vertebrate globins.

Globins that have very restricted phyletic distributions within the vertebrates, like the *GbY* and *GbE* genes, invariably represent the products of ancient duplication events dating back to the stem lineage of gnathostomes (Hoffmann et al., 2011, 2012a). For example, even though the *GbE* gene is found exclusively in the genomes of birds – and possibly other archosaurs – it is clearly not the product of a bird-specific or archosaur-specific duplication event. Phylogenetic topology tests and patterns of conserved synteny clearly demonstrate that the *GbE* and *Mb* genes represent the paralogous products of a tandem gene duplication in the stem lineage of gnathostomes. Whereas the *Mb* gene was retained in all major gnathostome lineages other than amphibians, the paralogous *GbE* gene appears to have been lost independently in teleost fish, amphibians, mammals, and nonavian reptiles (Hoffmann et al., 2011).

The human genome contains copies of *Ngb*, *Cygb*, *Adgb*, *Mb*, and multiple α- and β-chain *Hb* genes, but the full panoply of vertebrate-specific globins (including *GbX*, *GbY*, and *GbE*) was only unveiled after examination of complete genome sequences from representatives of other tetrapod vertebrates and teleost fishes (Fuchs et al., 2005, 2006; Kugelstadt et al., 2004; Roesner et al., 2005). Even within vertebrates, it is possible that additional globin genes still await discovery as more taxa are added to the list of species with completely sequenced genomes.

2. The role of hemoglobin and myoglobin in blood-gas transport

Hb is responsible for transporting oxygen from the respiratory surfaces (lungs, gills, or skin surface) to the cells of respiring tissues throughout the body. After unloading oxygen in the tissue capillaries, Hb also facilitates the transport of the carbon dioxide

by-product of oxidation back to the respiratory surfaces to get rid of it. The Hb protein is a heterotetramer composed of two α-chain subunits and two β-chain subunits. Each of these subunit polypeptides contains a heme group – an iron atom at the center of a porphyrin ring – which reversibly binds a single dioxygen molecule in the ferrous state (Fe^{2+}). The related myoglobin (Mb) protein has an oxygen-storage function and is primarily expressed in myocytes of cardiac and skeletal muscle. Mb plays a role in regulating cellular oxygen tension in respiring tissues and it also regulates the bioavailability of the signaling molecule, nitric oxide (Wittenberg and Wittenberg, 2003). In contrast to the tetrameric Hb protein, Mb is a monomer, and is therefore structurally similar to a single heme-bearing subunit of Hb. Mb and the individual Hb subunits have similar heme-coordination chemistries, but Mb has a much higher ligand affinity than Hb. This fulfills an important requirement of an efficient oxygen-transporting system, as the storage molecule (Mb) should have a higher oxygen-affinity than the carrier molecule (Hb) at the low oxygen tensions that prevail in the tissue capillaries.

The evolution of Hb and Mb as specialized oxygen-transport and oxygen-storage proteins, respectively, played a key role in the evolution of aerobic energy metabolism in early vertebrates. In the absence of Hb and Mb, oxygen delivery to all the cells of the body could only be achieved by means of gaseous diffusion in the blood plasma. A reliance on passive oxygen diffusion to sustain aerobic metabolism is only possible for single-celled organisms and very small animals (e.g., arthropods that have elaborate tracheal systems to facilitate the diffusive conductance of oxygen throughout the body), but with the exception of notothenioid ice fish mentioned above, such a strategy is not tenable for relatively large, mobile vertebrate animals.

Nonvertebrate chordates like amphioxus possess a rudimentary circulatory system that is laid out on the same general pattern as that in vertebrates except that blood is pumped by the coordinated contraction of blood vessels lined with specialized myoepithelial cells. However, the blood is a colorless plasma that lacks any type of oxygen-carrying pigment to enhance solubility. The evolution of a true oxygen-transport protein represented a key innovation in early vertebrate evolution because it freed active, free-swimming animals from strict physiological constraints on maximum attainable body size.

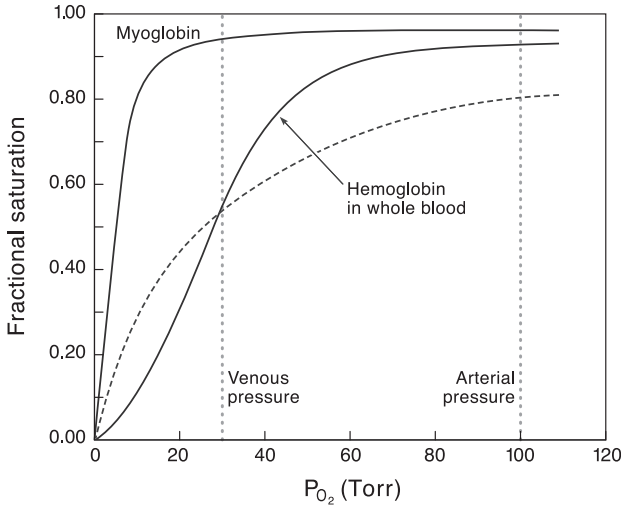

Fig. 2. Oxygen equilibrium curves for human Mb and Hb in whole blood. The dashed curve is a hyperbolic oxygen equilibrium curve with the same P_{50} (the partial pressure of oxygen at which Hb is half saturated) as human Hb (26 torr).

The efficiency of Hb as a specialized oxygen-carrier molecule stems from its multisubunit quaternary structure. The interaction between unlike subunits gives rise to the cooperativity of oxygen binding, whereby the first oxygen bound to a heme iron in deoxy Hb facilitates the binding of subsequent oxygen molecules at the three remaining unliganded hemes, and conversely, the first oxygen liberated by oxy Hb facilitates the unloading of oxygen molecules from the three remaining liganded hemes. The physiological significance of cooperativity is that it permits rapid and efficient oxygen-unloading over a relative narrow range of blood-oxygen tensions. The cooperativity of oxygen-binding is reflected by the sigmoid shape of the oxygen equilibrium curve for Hb, which contrasts with the hyperbolic curve for the monomeric Mb protein (Fig. 2). In addition to cooperativity, which results from interactions between subunits, the oxygen-affinity of Hb is also modulated by the binding of allosteric ligands at sites remote from the heme iron (Perutz, 2001; Weber and Fago, 2004). In most vertebrate taxa, Hb-oxygen affinity is inversely related to the intracellular concentration of carbon dioxide, protons, chloride ions, and various organic phosphates, all of which preferentially bind and stabilize the low-affinity deoxy conformation of the Hb tetramer.

3. Gene duplication, genome duplication, and the evolution of key innovations in the vertebrate oxygen-transport system

Gene duplication is known to play an extremely important role in the evolution of new protein functions. The role of gene duplication in promoting phenotypic novelty has been the subject of especially intense speculation in the context of vertebrate origins and evolution (Ohno, 1970; Holland, 2003). It has been hypothesized that two-rounds of whole-genome duplication (WGD) in the stem lineage of vertebrates provided genetic raw materials for the innovation of numerous vertebrate-specific features (Braasch et al., 2009a,b; Holland et al., 1994; Larhammar et al., 2009; Meyer, 1998; Ohno, 1970; Shimeld and Holland, 2000; Van de Peer et al., 2009; Wada, 2001; Wada and Makabe, 2006; Zhang and Cohn, 2008). In addition to the 1R and 2R WGDs in the stem lineage of vertebrates, an additional '3R' WGD occurred in the stem lineage of teleost fish (Meyer and Schartl, 1999; Taylor et al., 2001, 2003). Genomic evidence suggests that this teleost-specific WGD may have helped fuel the phenotypic diversification of this speciose vertebrate group (Braasch et al., 2006, 2007, 2009a,b; Meyer and Van de Peer, 2005; Sato et al., 2009). Although it has proven difficult to document causal links between vertebrate-specific or teleost-specific innovations and specific WGD events (Van de Peer et al., 2009), it was recently demonstrated that precursors of key globin proteins that evolved specialized functions in different aspects of oxidative metabolism and oxygen signaling pathways (Hb, Mb, and Cygb) represent paralogous products of the successive 1R and 2R WGDs in the vertebrate common ancestor (Hoffmann et al., 2011, 2012a). The physiological division of labor between the oxygen-transport function of Hb and the oxygen-storage function of Mb played an especially pivotal role in the evolution of aerobic energy metabolism in early vertebrates, supporting the hypothesis that WGDs helped fuel key innovations in vertebrate evolution.

The first clue that WGDs may have spurred the diversification of vertebrate globins was provided by an analysis of phylogenetic relationships among vertebrate globins and the complete globin gene repertoires of two nonvertebrate chordates: the sea squirt, *Ciona intestinalis* (subphylum Urochordata; Ebner et al., 2003) and amphioxus, *Branchiostoma floridae* (subphylum Cephalochordata; Ebner et al., 2010). This phylogenetic analysis revealed several distinct clades of vertebrate-specific globins that are sister to a single clade of globins in the amphioxus genome (Hoffmann et al., 2011, 2012a; Storz et al., 2011a; Fig. 3). The four main clades

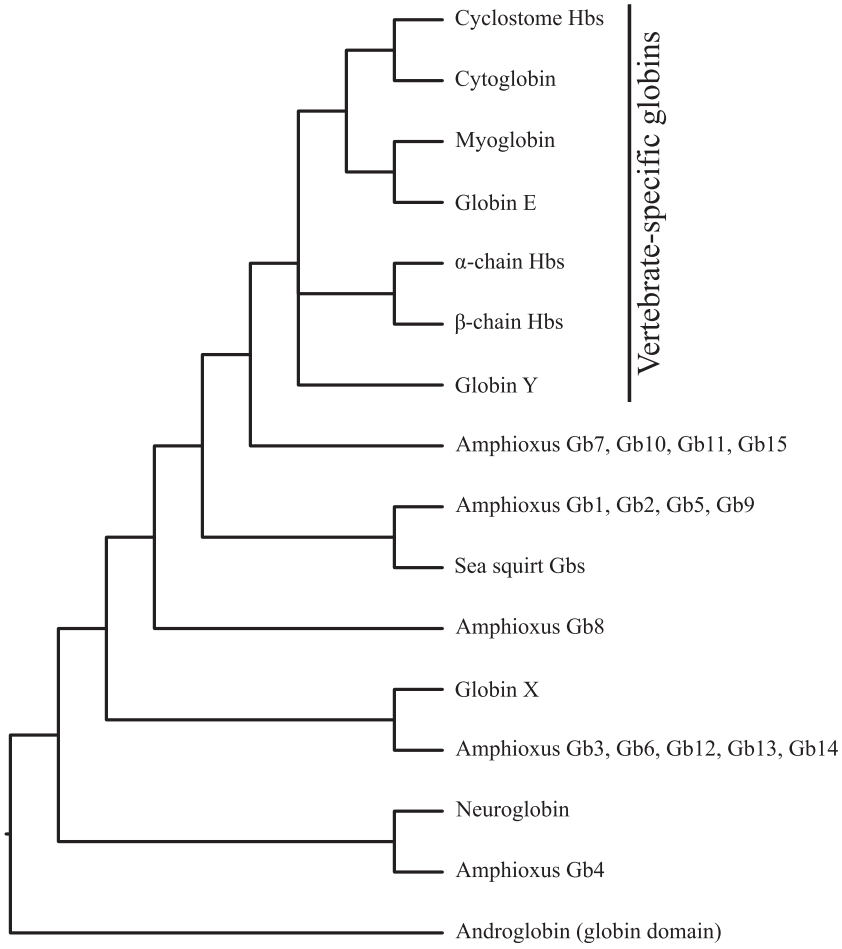

Fig. 3. Cladogram describing phylogenetic relationships among members of the globin gene superfamily in chordates.

of vertebrate-specific globins include: (i) *Cygb* and cyclostome *Hbs*; (ii) *Mb* + *GbE*; (iii) the α- and β-chain *Hb* subunits of gnathostomes; and (iv) *GbY*. In the absence of gene turnover, each clade of orthologous globin sequences would be expected to recapitulate the known organismal phylogeny. Accordingly, a phylogeny of orthologous globin genes from representatives of the three chordate subphyla (Craniata, Urochordata, and Cephalochordata) would be expected to place the sea squirt globins sister to the vertebrate-specific globins (Delsuc et al., 2006; Putnam et al., 2008). Contrary to this expectation, the phylogeny shown in Fig. 3 places a single clade of amphioxus globins sister to the vertebrate-specific globins, and indicates that orthologs of *GbX*, *Ngb*, and the pro-ortholog of all vertebrate-specific globins were secondarily lost from the sea squirt genome (it remains to be seen if this is true for all urochordates). Likewise, the pro-ortholog of the sea squirt globins appears to have been lost in the evolutionary line leading to vertebrates.

The 4:1 correspondence between the vertebrate-specific globins and their amphioxus homologs is consistent with the idea that they represent the paralogous products of two rounds of WGD in the vertebrate common ancestor, as postulated by the so-called '2R' hypothesis (for 'two rounds' of WGD; Dehal and Boore, 2005; Meyer and Schartl, 1999; Putnam et al., 2008). However, following two rounds of WGD, only a small minority of gene families would be expected to retain all four of the newly created paralogs. Thus, the 4:1 phylogenetic pattern is gradually obscured by small-scale gene duplications and deletions that occur after each round of WGD. For this reason, conclusive inferences about the role of WGDs in fueling the expansion of multigene families typically require the integration of molecular phylogenetic analyses with comparative genomic analyses of conserved macrosynteny (Abi-Rached et al., 2002; Braasch et al., 2006, 2007, 2009a,b; Dehal and Boore, 2005; Hoffmann et al., 2012a; Horton et al., 2003; Pébusque et al., 1998). Fig. 4 illustrates how the effects of two successive WGDs should be reflected in the physical linkage arrangement of paralogous genes – specifically, the fourfold pattern of intragenomic macrosynteny among paralogous chromosomal segments.

To assess whether the four main clades of vertebrate-specific globins shown in Fig. 3 represent the products of two successive WGDs (as predicted by the 2R hypothesis), Hoffmann et al. (2012a) used an integrated genomic/phylogenetic approach to test the following predictions:

(1) Representatives of the four clades of vertebrate-specific globins should be embedded in unlinked chromosomal segments that share similar, interdigitated arrangements of paralogous genes ('paralogons').
(2) The globin-defined paralogons should be united by 4:1 gene families and – in various combinations – by 3:1 and 2:1 gene families that trace their duplicative origins to the stem lineage of vertebrates.
(3) The globin-defined paralogons identified in vertebrate genomes should exhibit a fourfold pattern of conserved macrosynteny relative to the genomes of nonvertebrate chordates

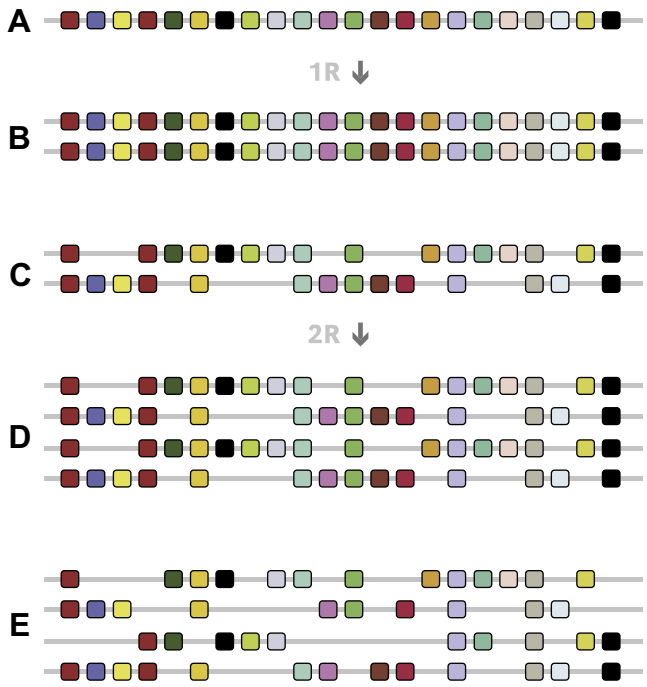

Fig. 4. Diagram depicting the hypothetical effects of two consecutive genome duplications (1R and 2R), as reflected in the physical linkage arrangement of paralogous genes – specifically, the fourfold pattern of intragenomic synteny. (A) Hypothetical chromosome in the vertebrate common ancestor; (B) the first genome duplication produces a complete set of paralogs in identical order; (C) many paralogous gene copies are subsequently deleted from the genome; (D) the second genome duplication produces yet another set of paralogs in identical order, with multigene families that retained two copies now present in four; (E) with the passage of time, additional gene losses ensue, thereby obscuring the fourfold pattern of synteny. Modified from Dehal and Boore (2005).

like amphioxus. This fourfold pattern would reflect the fact that the globin-defined paralogons represent the quadruplicated products of the same proto-chromosome in the chordate common ancestor.

The patterns described by predictions 1 and 2 could potentially be produced by large-scale segmental duplications as well as WGDs, but the pattern described by prediction 3 would be difficult to reconcile with any alternative to the 2R hypothesis. To test the above predictions, Hoffmann et al. (2012a) examined the genomic locations of the vertebrate-specific globin genes and characterized large-scale patterns of conserved macrosynteny in complete genome sequences from a number of representative vertebrate taxa. These analyses revealed that the *Cygb* gene, the *Mb*/*GbE* gene pair, and the α-*Hb*/*GbY* gene pair are each embedded in clearly demarcated paralogons. The '*Hb*' paralogon is defined by the α-globin gene cluster of amniotes and is defined by the tandemly linked α- and β-globin gene sets in teleost fishes and amphibians. The synteny analyses demonstrated that the *GbY* gene and the proto-*Hb* gene represent the products of an ancient tandem gene duplication that occurred prior to one or both rounds of WGD in the stem lineage of vertebrates (Hoffmann et al., 2012a). In fact, the ancestral linkage arrangement of these genes is still retained in the genomes of *Xenopus*, anole lizard, and platypus, as *GbY* is located downstream from the 3' end of the α-globin gene cluster in each of these taxa (Fuchs et al., 2006; Hoffmann et al., 2010b; Patel et al., 2008). Likewise, comparisons of conserved synteny revealed that the *GbE* and *Mb* genes also represent the paralogous products of a tandem gene duplication that occurred prior to the diversification of gnathostome vertebrates, and the ancestral linkage arrangement of these two genes is still retained in the avian genome (Blank et al., 2011a; Hoffmann et al., 2011).

The observed threefold pattern of conserved macrosynteny involving the *Cygb*, *Mb*, and *Hb* paralogons can be reconciled with the expected fourfold 'tetraparalogon' pattern predicted by the 2R hypothesis by invoking the secondary loss of one of the four paralogous globin genes that would have been produced by two successive WGDs. Consistent with this secondary-loss scenario, a detailed bioinformatic analysis of the human genome identified a clearly demarcated segment of human chromosome 19 that shares multiple gene duplicates with one or more of the other three globin-defined paralogons (Hoffmann et al., 2012a; Fig. 5). Thus, the sets of linked genes comprising these 4:1, 3:1, and 2:1 gene families appear to have co-duplicated with the *Cygb*, *Mb*, and *Hb* genes, and as predicted by the 2R hypothesis, the duplications occurred prior to the divergence of tetrapods and teleost fishes. These results implicate human chromosome 19 as the genomic location of the missing fourth paralogon, dubbed the 'globin-minus' (Gb^-) paralogon since the associated globin gene must have been secondarily lost. The identification of the Gb^- paralogon reveals the tell-tale pattern of fourfold conserved macrosynteny that is predicted by the 2R hypothesis (Hoffmann et al., 2012a).

The final line of evidence that WGD played a role in the diversification of vertebrate globins is provided by a comparative analysis of conserved synteny between the genomes of human and amphioxus. This comparison revealed that the *Hb*, *Mb*, *Cygb*, and Gb^- paralogons represent the quadruplicated products of the same linkage group in the reconstructed proto-karyotype of the chordate common ancestor (Fig. 6). In combination with the phylogenetic reconstructions and the observed linkage arrangements of paralogous genes, the fact that the globin-defined paralogons trace their duplicative origins to the same ancestral chordate 'proto-chromosome' provides conclusive evidence that three of the four main lineages of vertebrate-specific globins (*Hb*, *Mb*, and *Cygb*) originated via two successive WGD events in the stem lineage of vertebrates.

As mentioned above, the retention of the proto-*Hb* and *Mb* genes in the ancestor of jawed vertebrates permitted a physiological division of labor between the oxygen-carrier function of the Hb protein and the oxygen-storage function of the Mb protein. In the *Hb* gene lineage, a subsequent tandem gene duplication gave rise to the proto α- and β-globin genes. This duplication event appears to have occured roughly 450–500 million years ago in the Ordovician, before the ancestor of cartilaginous fish split from the lineage leading to the common ancestor of ray-finned fishes and tetrapods (Goodman et al., 1987; Hoffmann et al., 2012a). In the common ancestor of jawed vertebrates, functional divergence of the proto α- and β-globin genes permitted the formation of multimeric Hbs composed of unlike subunits ($\alpha_2\beta_2$).

The ancestral linkage arrangement of the proto α- and β-globin genes is still retained in the genomes of some modern-day amphibians and teleost fish (e.g., Gillemans et al., 2003; Jeffreys et al., 1980; Wetten et al., 2010; Opazo et al., in press). In amniote vertebrates, by contrast, the α- and β-like globin genes are located on different chromosomes, reflecting the fact that the ancestral β-globin gene was transposed to a new chromosomal location in the amniote common ancestor (Hardison, 2008; Patel et al., 2008, 2010). Even in mammals, the ancestral linkage arrangement of the proto α- and β-globin genes is reflected by the fact that an 'orphaned' β-like globin gene (ω-globin) is found in association with the tandemly linked α-like globin genes in the genomes of monotremes and marsupials (De Leo et al., 2005; Hoffmann et al., 2008a; Opazo et al., 2008a; Wheeler et al., 2001, 2004).

Whereas all tetrapod vertebrates examined to date possess a single copy of the *Cygb* gene, most teleost fishes possess two copies, and zebra fish possess three copies. Analysis of conserved synteny revealed that the two *Cygb* paralogs possessed by all

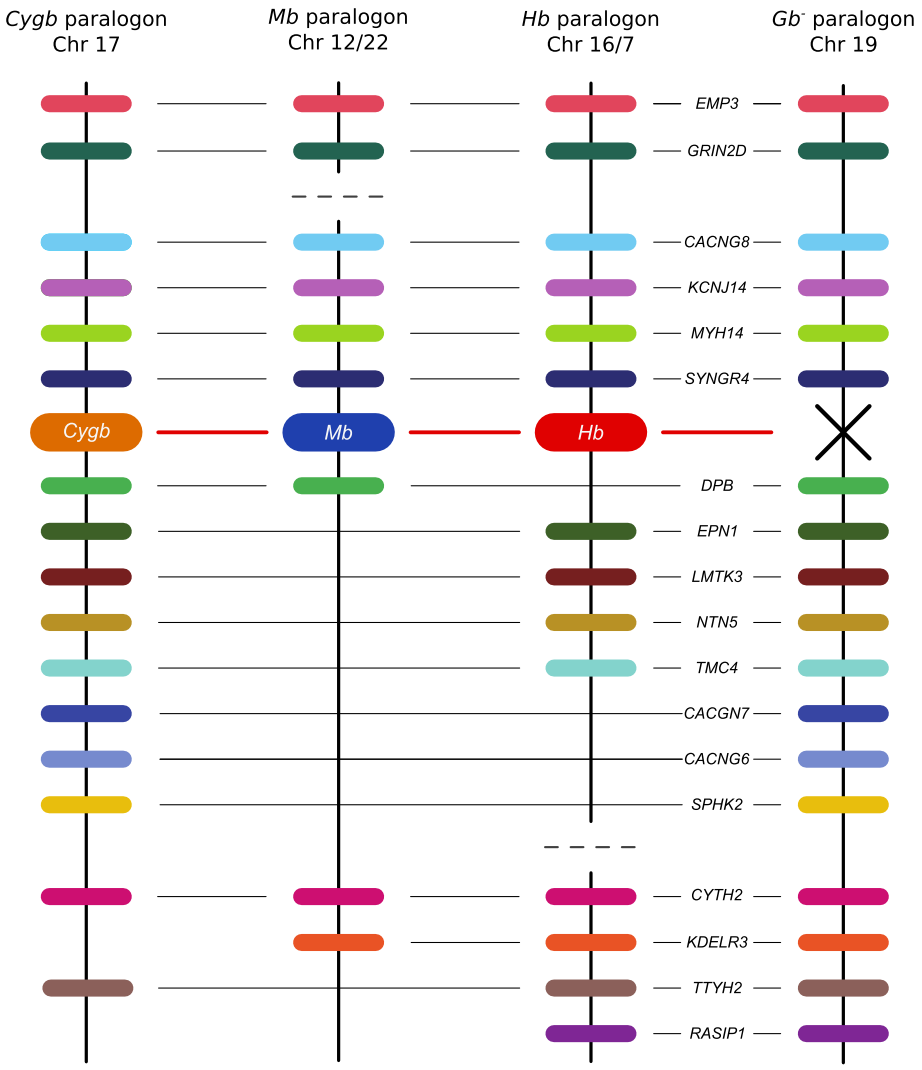

Fig. 5. Graphical depiction of gene duplicates that are shared between the *Gb*⁻ paralogon and the remaining three globin-defined paralogons (*Cygb*, *Mb*, and *Hb*) in the human genome. There are seven 4:1 gene families that unite the *Gb*⁻ paralogon with the *Cygb*, *Mb*, and *Hb* paralogons, there are seven 3:1 gene families that unite the *Gb*⁻ paralogon with two of the three globin-defined paralogons, and there are four 2:1 gene families that unite the *Gb*⁻ paralogon with a single globin-defined paralogon. On each chromosome, annotated genes are depicted as colored bars. The 'missing' globin gene on the *Gb*⁻ paralogon is denoted by an 'X'. The shared paralogs are depicted in colinear arrays for display purposes only, as there is substantial variation in gene order among the four paralogons. For clarity of presentation, genes that are not shared between the *Gb*⁻ paralogon and any of the three globin-defined paralogons are not shown. In the human genome, the *Gb*⁻ paralogon on chromosome 19 shares multiple gene duplicates with fragments of the *Hb* paralogon on chromosomes 16 and 7, and fragments of the *Mb* paralogon on Chromosomes 12 and 22. Members of the *EPN1*, *LMTK3*, and *KCNJ14* gene families that map to the *Hb* paralogon have been secondarily translocated from chromosome 16. From Hoffmann et al. (2012a).

teleosts, *Cygb-1* and *Cygb-2*, are embedded in clearly demarcated paralogons that derive from the 3R fish-specific WGD (Fuchs et al., 2005; Hoffmann et al., 2011; Fig. 7). In fact, comparative genomic analyses of conserved synteny (Hoffmann et al., 2011; Opazo et al., in press) demonstrated that both of the *Cygb*-defined paralogons descend from chromosome 'e' in the reconstructed proto-karyotype of the teleost ancestor (Nakatani et al., 2007). The additional round of WGD in teleost fishes also spurred the diversification of the α- and β-globin gene clusters (Opazo et al., in press), but available data suggest that the *Ngb*, *GbX*, *Cygb*, and *Mb* genes reverted to the single-copy state in all or most teleost lineages.

4. Convergent co-option of paralogous genes for similar functions

Remarkably, phylogenetic evidence indicates that erythroid-specific, oxygen-transport Hbs evolved independently from different ancestral precursor proteins in the two deepest branches of the vertebrate family tree: gnathostomes (jawed vertebrates) and cyclostomes (jawless fishes, represented by lampreys and hagfish; Hoffmann et al., 2010a). Phylogenetic analysis of vertebrate globins revealed that the erythroid Hbs of cyclostomes are orthologous to the Cygb protein of gnathostomes, a structurally distinct globin that has a heme-coordination chemistry unsuited to an oxygen-transport function. Thus, the independent evolution of oxygen-transport Hbs in these two anciently diverged vertebrate lineages involved the convergent co-option of two distinct precursor proteins to perform a similar respiratory function in circulating red blood cells. After being pressed into service as oxygen-transport proteins, the two paralogous globins convergently evolved distinct forms of cooperativity and allosteric regulation from ancestral precursor proteins that lacked these features. In the Hbs of both gnathostomes and cyclostomes, multisubunit quaternary structures provided the basis for cooperative oxygen-binding and allosteric regulation. However, in the Hbs of gnathostomes, cooperativity stems from an oxygenation-linked transition in quaternary structure. In the Hbs of cyclostomes, by contrast, cooperativity stems

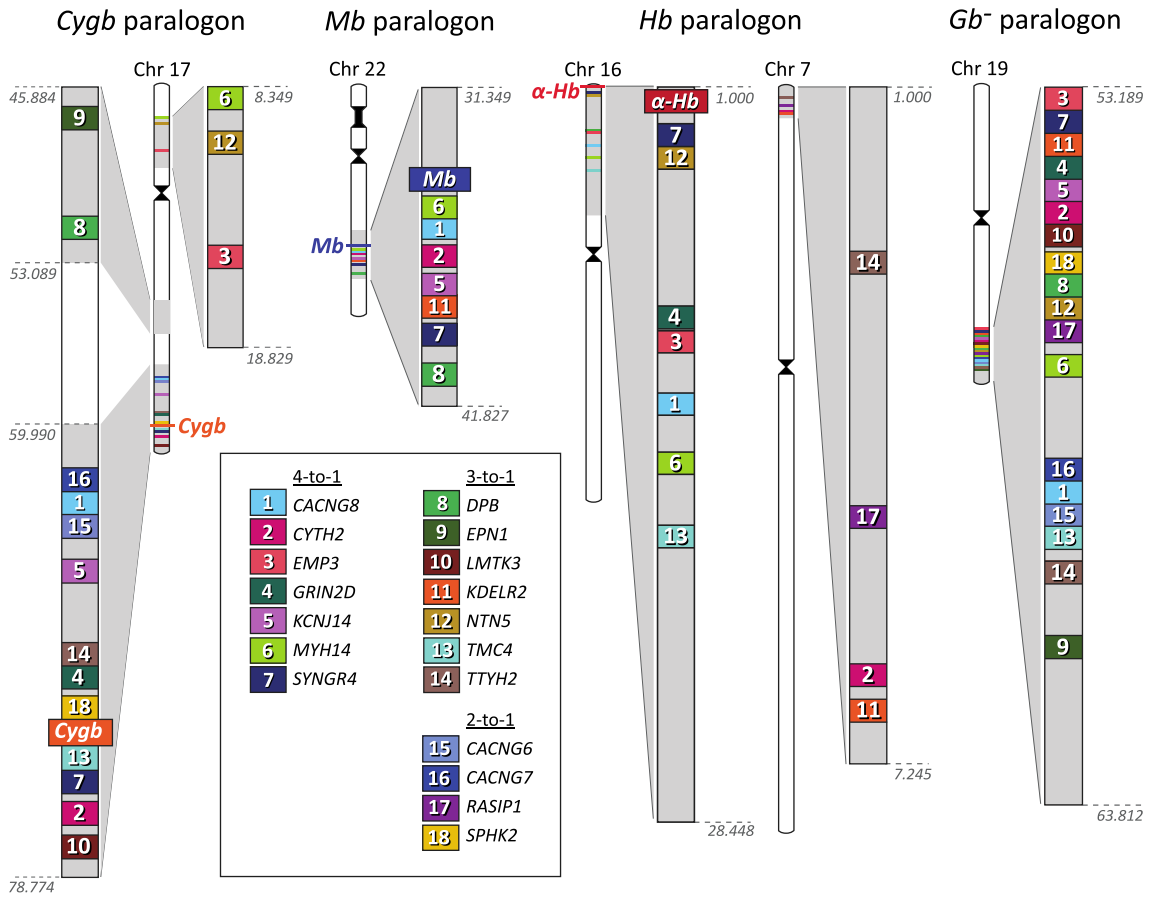

Fig. 6. Fourfold pattern of conserved macrosynteny between the four globin-defined paralogons in the human genome (including the *Gb*⁻ paralogon) and 'linkage group 15' of the reconstructed proto-karyotype of the chordate common ancestor (Putnam et al., 2008; shaded regions). This pattern of conserved macrosynteny demonstrates that the *Cygb*, *Mb*, *Hb*, and *Gb*⁻ paralogons trace their duplicative origins to the same proto-chromosome of the chordate common ancestor, and provides conclusive evidence that each of the four paralogons are products of a genome quadruplication in the stem lineage of vertebrates. Shared gene duplicates that map to secondarily translocated segments of the *Mb* paralogon (on chromosome 7) and the *Hb* paralogon (on chromosomes 12 and 17) are not pictured. From Hoffmann et al. (2012a).

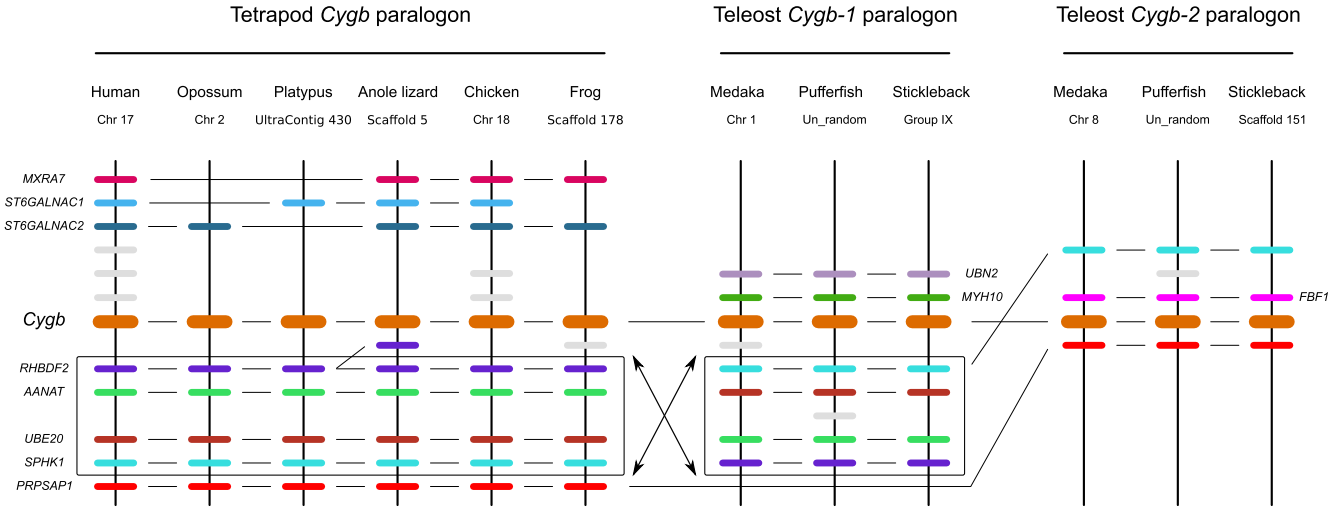

Fig. 7. Patterns of conserved synteny in the chromosomal region that harbors the *Cygb* gene in gnathostome vertebrates. Horizontal lines denote orthologous relationships. From Hoffmann et al. (2011).

from an oxygenation-linked dissociation of multimers into ligated monomers. Thus, the oxygen-transport Hbs of gnathostomes and cyclostomes represent superficially similar but structurally distinct design solutions to the challenge of maintaining a sufficient cellular oxygen supply to sustain aerobic metabolism (Hoffmann et al., 2010a).

In addition to the convergent evolution of oxygen-transport Hbs in gnathostomes and cyclostomes, phylogenetic analyses have also

revealed that the developmental regulation of Hb synthesis has evolved multiple times independently in different vertebrate lineages (reviewed by Storz et al., 2011a). Multiple rounds of duplication and divergence have produced diverse repertoires of α- and β-like globin genes that are ontogenetically regulated such that functionally distinct Hb isoforms are expressed during different stages of prenatal development and postnatal life (Alev et al., 2009; Brittain, 2002; Hardison, 2001; Hoffmann et al., 2008a,b; Opazo et al., 2008a,b, 2009; Storz et al., 2011b). Surprisingly, however, phylogenetic analyses of the α- and β-globin gene families have revealed that genes with similar stage-specific expression patterns in different species do not necessarily represent 1:1 orthologs that were inherited from a common ancestor (Czelusniak et al., 1982; Hoffmann et al., 2010b; Opazo et al., 2008a; Storz et al., 2011a; Opazo et al., in press). In many cases, the genes represent the products of independent, lineage-specific duplication events, and their similar expression patterns and functional properties are attributable to convergent evolution.

Evolutionary changes in the developmental timing of expression are typically associated with functional changes in ligand affinities and/or mechanisms of allosteric regulation, as the different Hb isoforms are adapted to perform distinct oxygen-scavenging/oxygen-transport tasks during different stages of development (Brittain, 2002; Nagel and Steinberg, 2001). In humans, for example, fetally expressed Hb (HbF; $\alpha_2\gamma_2$), is characterized by a higher oxygen affinity than adult Hb (HbA; $\alpha_2\beta_2$) due to a reduced sensitivity to the inhibitory effects of the organic phosphate, 2,3-diphosphoglycerate (DPG; a metabolite of red cell glycolysis). During pregnancy, the resultant oxygen-affinity differential between HbF in the fetal circulation and HbA in the maternal circulation facilitates oxygen-exchange across the placental barrier. Suppressed sensitivity to DPG and other allosteric polyanions is also responsible for the elevated oxygen-affinity of adult Hbs in different species of mammals that are adapted to the chronic oxygen deprivation of subterranean burrow systems (Campbell et al., 2010; Jelkmann et al., 1981) and high-altitude environments (Storz and Moriyama, 2008; Storz et al., 2007, 2009, 2010; Weber, 2007). This indicates that evolution has fashioned similar solutions to the physiological challenges associated with life underground, life at high-altitude, and prenatal development in the hypoxic intrauterine environment.

5. Summary

During the course of vertebrate evolution, the duplication event that gave rise to the progenitors of the Hb, Mb, and Cygb proteins opened up new opportunities for the evolution of aerobic energy metabolism in both jawed and jawless vertebrates. Moreover, the capacity to synthesize functionally distinct Hbs at different stages of development was made possible by repeated rounds of gene duplication in which newly created paralogs evolved new biochemical properties in conjunction with changes in the ontogenetic timing of expression. These changes in functional specialization often involved the convergent evolution of paralogous globins in different organismal lineages. The pervasive convergence in expression patterns and functional properties among vertebrate globins reflects a broader trend in the evolution of animal globins (Hoffmann et al., 2012b; Weber and Vinogradov, 2001). The highly conserved tertiary structure of globin proteins masks an extraordinary functional versatility that has been exploited for myriad different respiratory and non-respiratory functions during the course of animal evolution.

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