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Ontogeny of globin expression in zebrafish (*Danio rerio*)

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Abstract Respiratory proteins are responsible for transport and storage of oxygen. It is well established that specific requirements for oxygen during vertebrate ontogeny cause switches of hemoglobin chain expression. Here, we characterize the developmental profiles of zebrafish (*Danio rerio*) globins by means of quantitative real-time reverse transcription PCR. The total mRNA levels of the hemoglobin chains, including a newly identified embryonic α-chain, as well as myoglobin, neuroglobin, cytoglobin 1 and 2, and globin X were estimated. mRNAs of all globins were detectable in unfertilized eggs, suggesting maternal storage. Embryonic α- and β-hemoglobin mRNA peaked at hatching and the switch to adult hemoglobin expression occurred 16 dpf. Enhanced myoglobin mRNA levels were detected ~ 31 h post-fertilization (hpf), coinciding with the heart and the muscle development, while neuroglobin mRNA expression pattern correlates with the formation of the nervous system. Amounts of myoglobin and neuroglobin mRNA were similar within an order of magnitude throughout the ontogeny, tentatively supporting a respiratory role of neuroglobin. Cytoglobin 2 mRNA levels increased gradually, whereas cytoglobin 1 mRNA levels increased strongly after ~ 31 hpf, which is in agreement with a function in cell proliferation. Globin X mRNA level was highest in oocytes, but low in later stages. Together, these data suggest a specific role for each globin, which are also associated with certain events in fish development.

Keywords Cytoglobin · *Danio rerio* · Embryogenesis · Hemoglobin · Myoglobin · Neuroglobin · Oxygen · Zebrafish

Abbreviations
ARP	Acidic ribosomal protein
Cygb	Cytoglobin
Ef1α	Elongation factor 1 α
GbX	Globin X
Dpf	Days post-fertilization
Hb	Hemoglobin
His 2h3c1	Histone 2h3c1
Hpf	Hours post-fertilization
Ldha	Lactate dehydrogenase A
Mb	Myoglobin
Ngb	Neuroglobin
Ppia	Peptidylprolyl isomerase A (cyclophilin A)
ROS	Reactive oxygen species

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Introduction

Oxygen transport and storage in vertebrates is essentially mediated by globins, small heme-containing proteins that

reversibly bind an O_2 molecule between the iron of the porphyrin ring and a histidine of the polypeptide chain (Dickerson and Geis 1983). Recently, it has been demonstrated that vertebrates possess distinct types of globins that differ in terms of structure, tissue distribution, and function. Five distinct globin types are present in fish (Roesner et al. 2005). Hemoglobin (Hb) is located in the erythrocytes and is certainly the best investigated protein in biological sciences. Hb consists of two α- and two β-type chains and delivers O_2 in the blood to metabolically active tissues, but also transports CO_2 to the respiratory surfaces (Dickerson and Geis 1983; Perutz 1960).

During vertebrate development, the demand for oxygen changes and distinct Hb chains are expressed in certain stages. In fact, the ontogeny of Hb chains has become a prime model to study developmentally controlled gene expression. For example, humans possess six *Hb* genes (α, β, γ, δ, ε, and ζ) (Dickerson and Geis 1983). Their differential expression results in embryonic, fetal, and adult forms of hemoglobin tetramers (Dickerson and Geis 1983; Stamatoyannopoulos 1991). Embryonic and fetal Hb possesses a higher oxygen affinity than adult Hb, an essential requirement for overcoming the placental barrier in mammals (Bauer et al. 1975).

Like in mammals, distinct Hb chains are expressed during fish ontogeny (Brownlie et al. 2003; Wilkins 1985). Many teleost species are tetraploid and thus have a even larger diversity of globin genes (Hartley and Horne 1984; Johnson et al. 1987; Larhammar and Risinger 1994). Switching of globin expression during the teleost development has already been described in various species (Brunori 1975; Iuchi 1973; Wilkins 1985). For example, in the rainbow trout primitive hematopoiesis initially occurs about six to seven days after fertilization in an intra-embryonic structure known as the intermediate cell mass. Adult Hb is expressed in erythroid cells in blood, spleen and kidney during definitive hematopoiesis after hatching (Maruyama et al. 1999).

Myoglobin (Mb) sustains the oxidative metabolism of heart and muscle by facilitating oxygen diffusion to the respiratory chain and storing oxygen (Wittenberg and Wittenberg 2003). In mammals, Mb is expressed in muscle and cardiac muscles already in early embryonic stages and strongly increases in the later development (Weller et al. 1986). Mb has an essential function in embryogenesis and the majority of myoglobin-deficient knock-out mice die in utero with signs of cardiac failure (Meeson et al. 2001). In zebrafish (*Danio rerio*), maternal Mb mRNA is present in the early embryos, and ubiquitous muscular and non-muscular Mb mRNA expression was observed in the later stages of development (Vlecken et al. 2009). Silencing of the Mb gene causes various muscular and non-muscular defects, suggesting an important role of Mb in zebrafish development (Vlecken et al. 2009).

Neuroglobin (Ngb), cytoglobin (Cygb) and globin X (GbX) are three recently discovered members of the vertebrate globin family. There is still little information on their physiological roles (Burmester and Hankeln 2009; Hankeln et al. 2005), and—with a single exception (Mammen et al. 2006)—there are no data on the expression of these genes during development. Ngb is restricted to the central and peripheral nervous systems (Burmester et al. 2000), as well as some endocrine tissues (Reuss et al. 2002). There is conclusive evidence that Ngb is associated with the oxidative metabolism (Bentmann et al. 2005; Mitz et al. 2009), which hints to a function in oxygen supply (Roesner et al. 2006; Roesner et al. 2008) or, alternatively, protection from reactive oxygen species (ROS) (Burmester and Hankeln 2009). To the best of our knowledge, expression of Ngb in distinct developmental stages has not been investigated so far. Cygb is expressed in various tissues, mainly in fibroblast-related cells of connective tissues, as well as in distinct populations of brain neurons (Burmester et al. 2002; Schmidt et al. 2004). It is unlikely that Cygb plays an important role in oxygen supply, but may be involved in ROS detoxification or oxygen supply to certain enzymes (Hankeln et al. 2005; Schmidt et al. 2004). There are two paralogous Cygb genes in fish (Cygb1 and Cygb2), which differ in expression levels in neuronal- and non-neuronal tissues (Fuchs et al. 2005). Studying mouse development, Mammen et al. (2006) found that at the beginning of embryogenesis Cygb is only weakly expressed in neuronal tissue. Cygb mRNA increased in early neonatal stages and was highest in adult mice. GbX is a globin that is apparently restricted to amphibians and fishes (Roesner et al. 2005).

We have employed zebrafish (*D. rerio*) as model system to study the changes of globin expression throughout the development. These data provide extensive information on developmental specific expression patterns of globins concerning fish ontogeny and, additionally, provide clues for their potential physiological functions.

Materials and methods

Zebrafish maintenance

Inbred zebrafishes (*D. rerio*; Cypriniformes) were obtained from the Max-Planck Institute for Developmental Biology, laboratory of Dr. Brigitte Walderich, Tübingen, Germany. Fish were kept at 24–26°C in aquaria with standard day/night light cycles (12.5 h/11.5 h) and fed with *Artemia*. For breeding, adult fish were kept separately in a micromesh bottomed box. Eggs were isolated immediately after spawning and were kept separately in an aquarium at 26°C. Larvae were fed with Interpet Liquifry (Interpet, Viersen,

Germany). For the investigation of gene expression during zebrafish development, 26 different developmental stages from unfertilized egg cell to adult fish were collected.

RNA-isolation

Total RNA was isolated using peqGOLD Trifast^{TM} according to the manufacturer's instruction (peqLAB Biotechnologie GmbH). Total animals or tissue (~ 30 mg per developmental stage) were homogenized in a 1.5 ml reaction tube with a micropistil. RNA was further purified with RNeasy$^®$ Mini Kit (Qiagen), including an on-column digestion with RNase-Free DNase (Qiagen). Quantity and quality of the RNA were checked photometrically and by gel electrophoresis.

Reverse transcription polymerase chain reaction and cloning

Reverse transcription was performed with 500 ng of total RNA, oligo-(dT)$_{18}$ oligonucleotides (10 μM) and 200 U SuperScript^{TM} II RNase H$^-$ reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Short coding sequence fragments (80–250 bp) of each gene were amplified with Taq DNA-Polymerase (Invitrogen) and purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). After cloning into the standard vectors (pGEM$^®$ T-Easy Vector Systems, Promega, Mannheim, Germany; TOPO TA Cloning$^®$ Kit, Invitrogen), plasmids were sequenced by Genterprise Genomics (Mainz, Germany). The recombinant plasmids were used for generating standard curves for the quantitative real-time RT-PCR (see below).

Estimation of mRNA levels

Quantitative real-time RT-PCR (qRT-PCR) experiments were carried out on an ABI 7300 real-time PCR System (Applied Biosystems, Darmstadt, Germany) with the "ABI Power SYBR Green Master Mix" (Applied Biosystems, Darmstadt) employing a two step protocol. We used cDNA equivalent to 8.3 ng of total RNA for each PCR. Quantitative real-time PCR was carried out using 40 amplification cycles (95°C/15 s, 60°C/15 s, 72°C/30 s). Oligonucleotide primers are given in Supplemental Table 1. Fluorescence was measured at the end of each amplification cycle. Ten globin genes (embryonic Hbα, embryonic Hbβ, Hbα, Hbβ, HbαX, Mb, Ngb, Cygb1, Cygb2, GbX) were investigated; elongation factor 1 α (Ef1α), lactate dehydrogenase (Ldha), β-actin (β-Act), and acidic ribosomal protein (Arbp), Histon 2h3c1 (His 2h3c1), peptidylprolyl isomerase A (Ppia) were employed as putatively non-regulated reference genes. Most of the genes were amplified employing gene-

specific, intron-spanning oligonucleotides (10 μmol) (Supplemental Table 1). For the genes encoding embryonic hemoglobin chains, we used four fold degenerated primers that amplify all chains in a single reaction. Success and specificity of amplification was evaluated using dissociation curves with a temperature range from 60 to 95°C. All the reactions were performed in triplicates. For standard curves, duplicates were run using a tenfold dilution of plasmids containing the cDNAs of the genes of interest.

First evaluation of qRT-PCR data was performed with the ABI 7300 Sequence Detection Software V.1.3.1 (Applied Biosystems, Darmstadt). Reaction efficiency was calculated by the slope of the standard curve for each gene. Data were normalized according to the amount of total RNA. Further analyses were carried out by Microsoft Office Excel 2003. For comparison of mRNA levels, C_t-values of technical triplicates were averaged. Calculation of absolute copies numbers was carried out using plasmid standards in serial dilutions.

Phylogenetic methods

Hb-α amino acid sequences from the selected fish species (see Supplemental Table 2) were aligned by hand employing GeneDoc 2.6 (Nicholas and Nicholas 1997). The appropriate model of amino acid sequence evolution was selected by ProtTest (Abascal et al. 2005) using the Akaike Information Criterion (AIC). MrBayes 3.1.2 was employed for Bayesian phylogenetic analyses (Huelsenbeck and Ronquist 2001), assuming the WAG model with gamma distribution of rates (four gamma categories) and the "covarion model". The analyses were run for 2,000,000 generations with random starting trees. The final average standard deviations of split frequencies were stationary and <0.02. Trees were sampled every 100th generation and posterior probabilities were estimated on the last 15,000 trees (burnin = 5,000).

Results

Total RNA was extracted from 26 developmental stages of *D. rerio* and used for the estimation of mRNA levels by qRT-PCR. For better comparisons between the developmental stages and to avoid differences caused by the dissection of animals, we used total eggs, embryos, larvae and adult fishes in all the experiments. We initially tested six different genes (Ef1α, Ldha, β-Act, Arbp, His 2h3c1, Ppia) as putatively non-regulated reference genes for standardization of mRNA expression. However, none of these genes showed constant levels throughout zebrafish development. Therefore, mRNA levels of each gene were eventually

standardized according to total RNA amounts, an appropriate method used when no reference gene is available (Bustin 2002).

Expression pattern of hemoglobin genes

Analyses of the *D. rerio* genome revealed three adult and two embryonic α-Hb chain genes, and two adult and three embryonic β-Hb genes. An additional α-Hb chain was identified, which is described below. Because there is only minor sequence variation among the different genes, it was not possible to discriminate distinct adult and embryonic α- and β-Hbs, respectively. Therefore, the oligonucleotides used for qRT-PCR supposedly bound to all the members of a particular Hb type.

qRT-PCR data showed that mRNA of all the globins was detectable in unfertilized eggs, suggesting maternal storage. During embryogenesis (4–31 h post-fertilization; hpf) the

expression levels of embryonic hemoglobin genes were notably lower (5- to 1,000-fold) than in unfertilized egg cell (Fig. 1a). During late embryogenesis, expression levels of embryonic hemoglobin commenced to increase 12 hpf and peaked around hatching. Interestingly, embryonic Hb mRNA levels remained constantly high until adulthood.

In contrast to the embryonic Hb chains, adult Hb mRNA showed low levels after fertilization and until juvenile stages ($\sim$1,000-fold lower compared to the unfertilized egg) (Fig. 1b). Relative expression levels of adult α- and β-Hb chain mRNA reached the level of oocytes only at 16 dpf (days post-fertilization) and were highest around 35 dpf (100-fold compared to the unfertilized eggs). There was also a notable increase of adult α- and β Hb mRNA around the hatching period, which, however, did not reach the levels of oocytes.

Adult and embryonic α- and β-chains showed parallel expression pattern. In addition, total mRNA copy numbers

Fig. 1 Relative mRNA levels of Hb chains during zebrafish development. *Bar diagram* shows qRT-PCR results of different developmental stages from unfertilized eggs (0 hpf) to sexually mature adult zebrafish at 54 dpf (n = 3). The amounts of mRNA in the unfertilized eggs were set to one, the other stages were related to this value. The *y*-axis has a logarithmic scale. **a** Relative mRNA levels of embryonic Hbα (*white columns*), Hbβ (*black columns*) and HbαX (*gray columns*). **b** The relative expression of adult Hbα (*white columns*) and Hbβ (*black columns*). *Bars* represent standard errors of the means. *Asterisk* indicates missing data
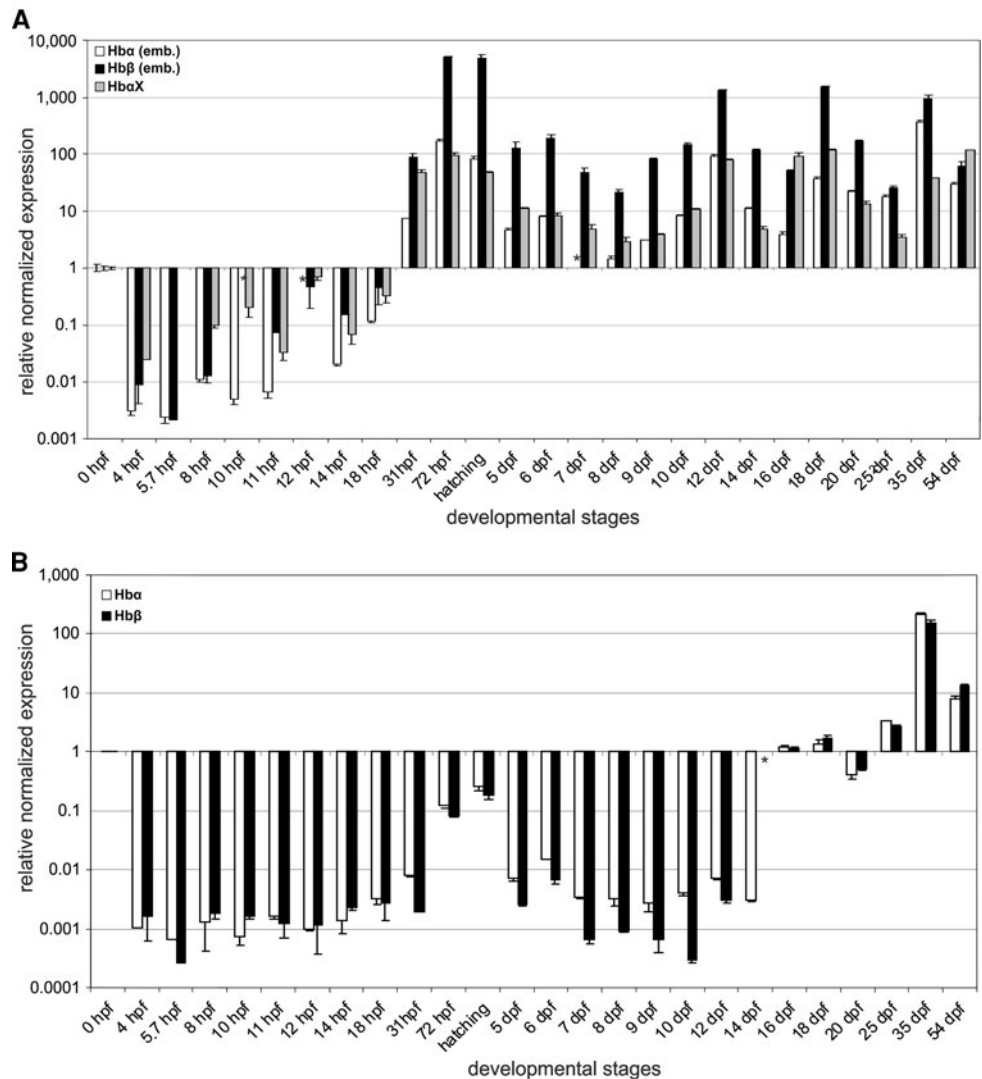
of α- and β-Hb were similar within an order of magnitude (Supplemental Fig. 1). Although we have no information on translation efficiencies or protein levels, these observations roughly validate our approach and methods. Notably, embryonic α-Hb mRNA levels were lower than that of embryonic β-Hb (Supplemental Fig. 2). However, this can be explained by the presence of an additional embryonic α-Hb chain (see below).

Identification of HbαX as embryonic hemoglobin

By searching the zebrafish genome of Hb sequences, we identified a previously unknown Hb α-chain (Acc. No. NM_001082834). To investigate its phylogenetic position, we added the amino acid sequence of this *D. rerio* α-Hb gene, which was tentatively termed as HbαX, to an alignment of 65 other fish α-chains (Supplemental Table 2). Human, mouse and rat Hbα sequences were added as outgroup. Bayesian phylogenetic analyses showed that *D. rerio* HbαX is a member of a group of other fish α-Hb chains, which form a monophyletic clade at the base of the teleost Hbα chains (Fig. 2). Embryonic and adult Hbs form distinct well-supported monophyletic clades, although in many cases the embryonic expression has only been inferred by orthology. There is a fourth clade consisting of various Hbα chains, which are according to the database

Fig. 2 Phylogenetic analyses of fish Hbα. The relationship among 64 teleost and two shark Hbα amino acid sequences was inferred by MrBayes 3.1.2, employing 3 mammalian Hbα sequences as outgroup
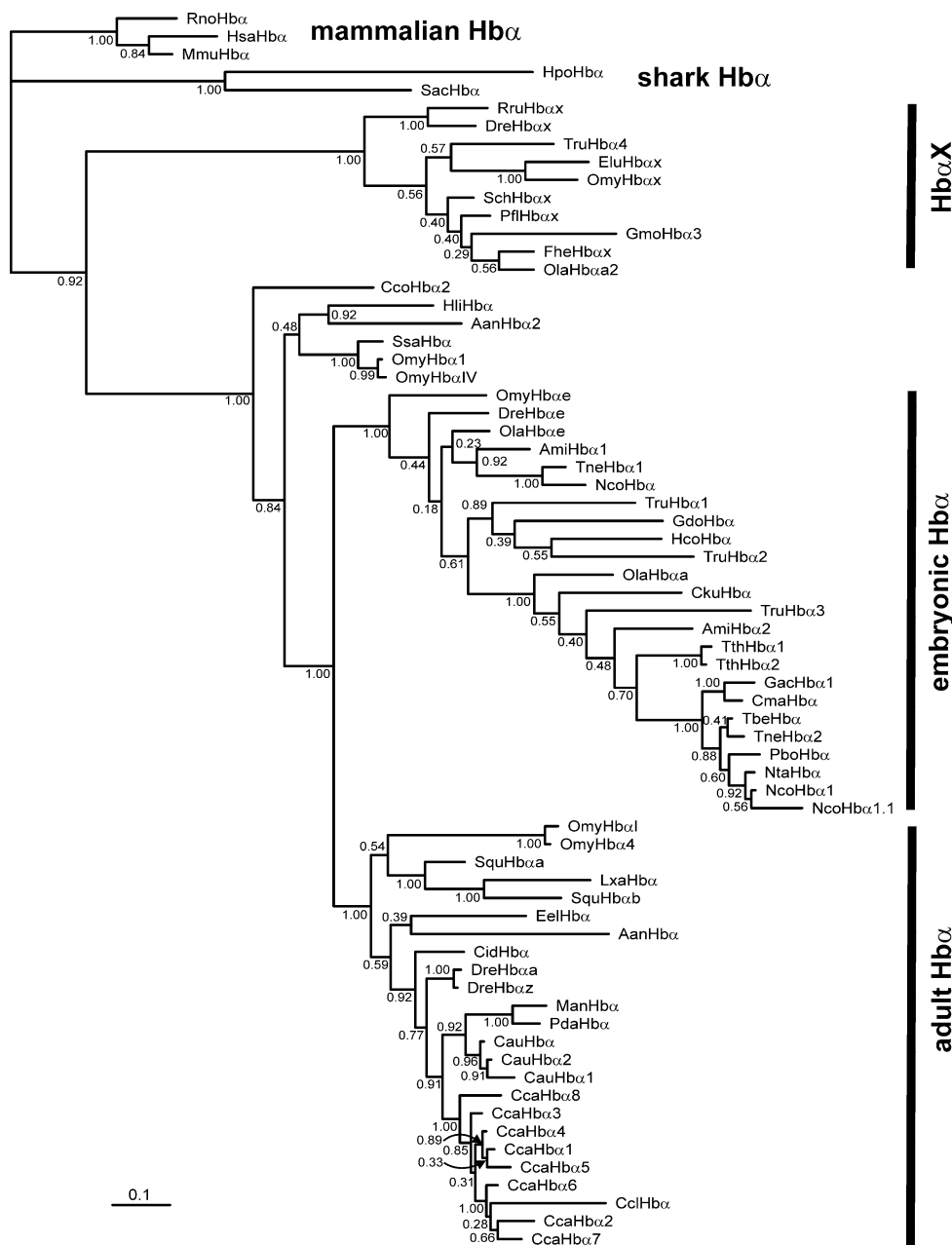
Fig. 3 Relative and total mRNA levels of Mb (*white columns*) and Ngb (*black columns*) during zebrafish development. *Bar diagram* shows qRT-PCR results of different developmental stages from unfertilized eggs (0 hpf) to sexually mature adult zebrafish at 54 dpf ($n = 3$). The amounts of mRNA in unfertilized eggs were set to one, the other stages were related to this value. The *y*-axis has a logarithmic scale. **a** Relative mRNA levels of Mb and Ngb. **b** Total mRNA copy numbers of Mb and Ngb. *Bars* represent standard errors of the means. *Asterisk* indicates missing data
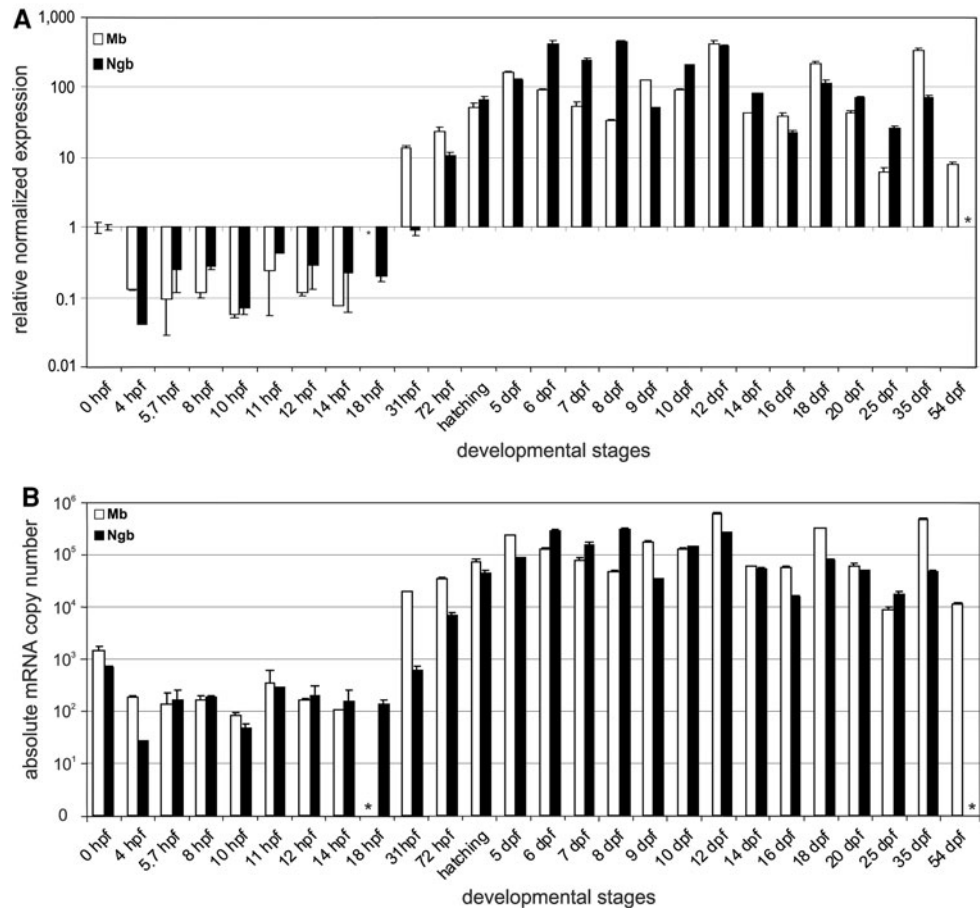
entries present in adult stages of various Salmoniformes. The Hbα2 of the European conger (*Conger conger*) could not be readily assigned to any clade.

The developmental pattern of HbαX was analyzed by qRT-PCR (Fig. 1a). These data identify HbαX as a novel embryonic globin chain, which essentially parallels the patterns of embryonic Hbα and Hbβ. The accumulated copy numbers of embryonic Hbα and HbαX on the one hand and embryonic Hbβ on the other were similar within an order of magnitude (Supplemental Fig. 2).

Relative and absolute expression of Mb and Ngb

During zebrafish development, Mb and Ngb showed largely similar trends in terms of relative and absolute levels (Fig. 3a, b). For both the genes, relatively low amounts of mRNA were detected after fertilization, being about a tenth compared to those in unfertilized eggs. Mb mRNA levels first peaked around late embryogenesis (31 hpf), whereas the amount of Ngb mRNA increased somewhat later around the hatching period (72 hpf). The mRNA levels of both the genes remained high in the adult stage. We further determined the absolute mRNA copy numbers of Mb and

Ngb in total eggs, embryos, larvae and adult fish (Fig. 3b). Because we used the same samples, expression changes of Mb and Ngb were the same as described above. Throughout the development, copy numbers of both genes were similar, essentially within an order of magnitude.

Expression changes of Cygb1, Cygb2 and GbX

Relative expression levels of Cygb1 and Cygb2 are strikingly different during zebrafish embryogenesis (Fig. 4a). Amounts of Cygb1 mRNA are persistently higher compared to Cygb2 with up to 150-fold higher expression levels in the adult zebrafish (Supplemental Fig. 3). Until hatching, mRNA levels of Cygb2 did not notably differ from those in oocytes, whereas the Cygb1 levels had decreased ∼10- to 100-fold. Amounts of Cygb2 mRNA slowly increased beginning at 14 hpf on, whereas levels of Cygb1 strongly increased in late embryogenesis up to 70-fold. Both Cygb1 and Cygb2 showed maximum amounts of mRNA at 12 dpf (∼100-fold). In contrast to the other globins, we found GbX mRNA levels were highest in the unfertilized eggs, but 10- to 100-fold lower in all other investigated stages of zebrafish development (Fig. 3b).

Discussion

It is well established that vertebrates have multiple Hb genes that are differentially expressed in ontogeny. Distinct compositions of Hb chains result in proteins of different oxygen affinities, which match the specific requirements of the organism at that particular stage (Coates 1975). Such switch in the expression of Hb genes has also been described in zebrafish (Brownlie et al. 2003). Much less is known about the other globins, although there is evidence for a critical function of Mb in zebrafish development except serving as oxygen supply protein of the heart (Vlecken et al. 2009).

Stored globin mRNA in the oocyte

Synthesis and accumulation of mRNA are essential functions of oogenesis. After midblastula transition of zebrafish (2.75 hpf), the available amount of maternally deposited mRNA decreased and embryonic transcription commenced (Kimmel et al. 1995). We used mRNA levels in oocytes as reference values and took next samples after 4 hpf, thus, immediately after midblastula transition. With the exception of Cygb2, mRNA levels of all investigated genes were higher in oocyte than in zygote, and the following

developmental stages. This agrees, for example, with observations by Vlecken et al. (2009), who found maternally stored Mb mRNA. Based on the function of at least some globins as respiratory proteins, it is conceivable that they play a role in oxygen supply in the oocyte, which displays a high metabolic rate during maturation (Van Blerkom et al. 1995).

Compared to the other globins, GbX is unique and shows the highest amount of mRNA in unfertilized eggs, but much lower levels in all other investigated developmental stages (Fig. 4). This finding agrees with the expressed sequence tags (ESTs) databases: all four available ESTs of GbX actually derive from cDNA libraries constructed either from ovaries or from mixed stages. Low expression levels of GbX have previously been reported from adult goldfish (*Carassius auratus*) and zebrafish (Roesner et al. 2005). To date, the function of GbX is unknown, but the present data suggest that GbX may have a particular role in oocytes.

Correlating hemoglobin expression and zebrafish development

As long as the distance is short and an adequate oxygen gradient applies, simple diffusion is sufficient for oxygen

Fig. 4 Relative expression levels of Cygb1, Cygb2 and GbX during zebrafish development. *Bar diagram* shows qRT-PCR results of different developmental stages from the unfertilized eggs (0 hpf) to sexually mature adult zebrafish at 54 dpf (*n* = 3). The amounts of mRNA in unfertilized eggs were set to one, the other stages were related to this value. The *y*-axis has a logarithmic scale. **a** Relative mRNA levels of Cygb1 (*white columns*) and Cygb2 (*black columns*). **b** Relative mRNA levels of GbX (*black columns*). *Bars* represent standard errors of the means. *Asterisk* indicates missing data
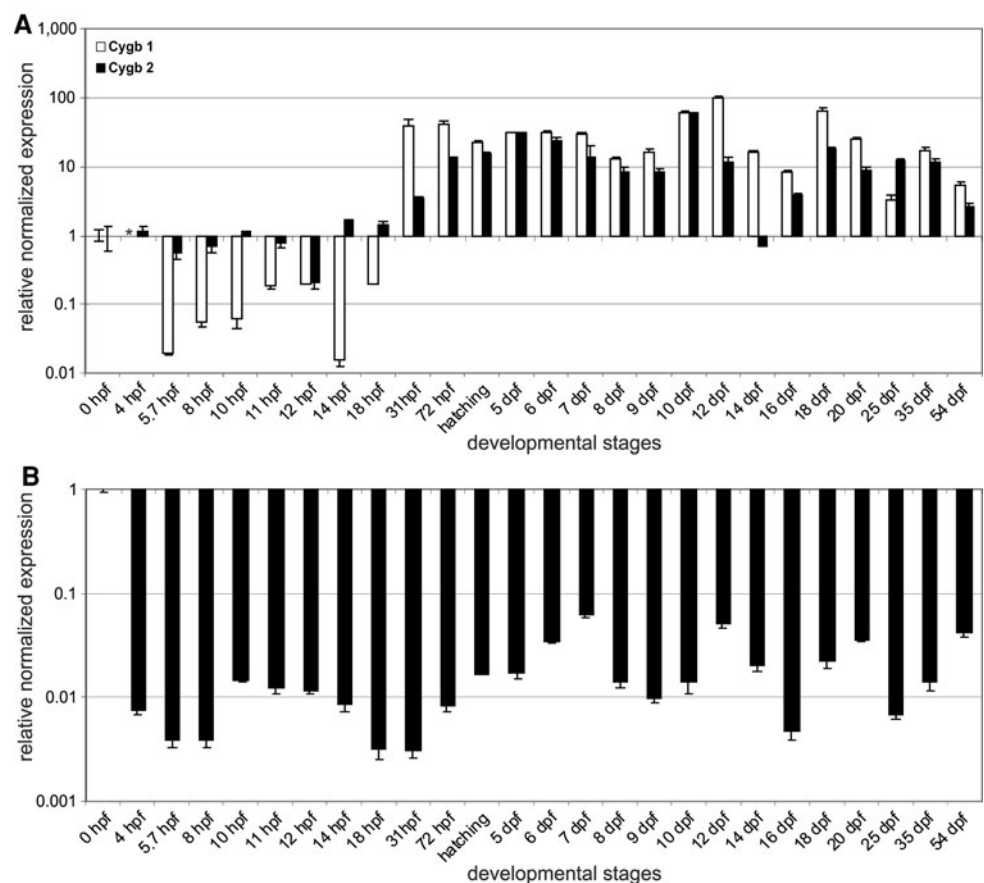
supply. Consequently, in the first 4–5 days of zebrafish development, the aerobic metabolism in embryos and larvae may be sustained by oxygen diffusion (Grillitsch et al. 2005; Pelster and Burggren 1996). Nevertheless, it has been demonstrated that at this time zebrafish already possesses a completely developed circulatory system and a functional Hb (Brownlie et al. 2003; Pelster and Burggren 1996). These observations agree well with our qRT-PCR data, which showed that the amount of embryonic Hb mRNA commenced to increase around 12–18 hpf (Fig. 1). However, circulatory oxygen supply is required only at later developmental stages. Erythrogenesis commenced around 15 hpf and Hb-containing red blood cells are present in the circulation system by 24 hpf, well before hatching (Brownlie et al. 2003). A respiratory function of Hb at that stage is uncertain. Pelster and Burggren (1996) showed that disruption of the oxygen transport function of Hb by carbon monoxide does not impair metabolic rate and whole-animal oxygen consumption. A recent study using larval and early adult zebrafish 5–42 dpf showed that Hb was only required in the periods of extreme hypoxia (Rombough and Drader 2009). Thus, Hb may rather supply O_2 for swim bladder inflation, which typically occurs between 4 and 6 dpf (Pelster and Burggren 1996).

To date the exact composition of zebrafish Hb tetramers, as well as oxygen affinities of distinct Hbs in zebrafish are unknown. However, on the genetic level there is clear evidence for a switch of distinct Hb chains. Probably due to genome polyploidization, there are multiple distinct adult and embryonic Hb genes. During the larval period of zebrafish development, the switch from embryonic to adult Hb commences ∼10 dpf and definitive hematopoiesis is initiated (Chan et al. 1997). Our qRT-PCR data roughly agree with these findings (Fig. 1) and showed the increase of adult Hb mRNA levels 18 dpf. We do not know whether and at what time of development the expression of embryonic Hbs ceases, but at least in the cDNA libraries from adult zebrafish no EST data are available. This suggests a termination of embryonic Hbs synthesis >54 dpf.

A novel embryonic hemoglobin α- chain of ancient evolutionary origin

BLAST searches of the *D. rerio* genome sequences revealed the presence of a novel α-globin that could not be readily assigned to either the adult or the embryonic Hb type and which was therefore designated DreHbαX. It is represented by six ESTs and in GenBank by the reference NM_001082834 [ZFIN (Sprague et al. 2006) gene zgc:163057]. Further similarity searches of the public databases revealed similar genes in the other fish species. The orthology of these globins was confirmed by the molecular phylogenetic studies (Fig. 2). The phylogenetic

tree also showed that this branch of Hbα chains is in sister group position to all other fish Hbα proteins. Thus, this type of Hbα emerged early in the evolution of bony fishes, before the major teleost families diverged.

Changes of mRNA levels throughout the zebrafish ontogeny clearly show that HbαX displays an expression pattern similar to that of the embryonic α- and β-chains (Fig. 1a). This observation agrees with the EST data, which actually derive from either embryonic or mixed-stages libraries. Together with the phylogenetic tree, now there is evidence for a twofold independent origin of embryonic α-Hb chains (provided that the orthologs are also expressed in embryos). The particular role of this globin in development must at present remain uncertain.

Myoglobin and the development of cardiac and striated muscles

Mb has an essential function in the supply of oxygen to muscle tissue. The first notable event in muscle development of zebrafish is the beginning of heartbeat at about 22 hpf (Kimmel et al. 1995). During late embryogenesis trunk myotomes produce first muscular contractions, which consistently grow to contractile swimming movements of zebrafish larvae (Kimmel et al. 1995; Stainier et al. 1993). The observed accumulation of Mb mRNA may be associated with these events (Fig. 3). An approximately 50-fold increase of Mb mRNA was observed from 18 to 31 hpf, which, thus, can be related to the heart development. These levels further increased by 10- to 20-fold in later stages, most likely representing further growth of the heart and development of skeletal muscles. The higher Mb levels at those stages also agree with the onset of circulatory oxygen supply 4–5 dpf (Grillitsch et al. 2005; Pelster and Burggren 1996).

While there had been a consensus that Mb is a typical muscle protein, recent data have demonstrated a much broader expression pattern. A microarray study identified Mb in the gills of adult zebrafish (van der Meer et al. 2005), whereas in adult carp there is a widespread expression of Mb in a broad range of tissues, including liver and brain (Fraser et al. 2006). Recently, Vlecken et al. (2009) reported Mb mRNA in the developing zebrafish in the non-muscular tissues. Furthermore, they showed that Mb knock-down led to various muscular defects, but also impairment of other tissues. By inference, we propose that the changes in Mb mRNA are mainly driven by heart and muscle development, but may also be influenced by the non-muscular expression of Mb.

Support for a respiratory role of neuroglobin

Like in most mammals, Ngb is preferentially expressed in the nervous system of zebrafish (Fuchs et al. 2004; Roesner

et al. 2006). Ngb mRNA levels were low during the early stages of zebrafish development and exceeded the oocyte level at 31 hpf. At that time, formation of the neural tube and initial brain development has already been completed (Kimmel et al. 1995). During the hatching period (~60 hpf), the majority of neuronal cells in zebrafish retina form and the typical retinal layers may be identified (Malicki et al. 2005). Neurogenesis continues after hatching and is completed in most parts of the brain by 6 weeks after fertilization, but occurs in the optic tectum and cerebellum even in adult fish (Miyamura and Nakayasu 2001). Accordingly, we observed ~2000-fold increase of Ngb mRNA 18 hpf to 6 dpf (Fig. 3). It is unlikely that Ngb plays a particular role in regulation of development. Rather the increase in Ngb mRNA levels correlates with the higher number of neurons that are formed during ontogeny.

Various functions for Ngb have been put forward, including that of a respiratory protein in neurons with an Mb-like role (for review: Burmester and Hankeln 2009; Hankeln et al. 2005). However, it has been repeatedly proposed that Ngb levels in the brain were much too low to sustain oxygen flow to mitochondria (Brunori et al. 2005; Fago et al. 2004). We therefore determined the absolute copy number of Ngb in relation to Mb mRNA. In fact, we found the mRNA copy numbers for Mb and Ngb to be similar within the range of one order of magnitude, with even being higher for Ngb in some periods, e.g., 6–8 dpf (Fig. 3b). Considering that in total animals, the mass of muscle tissue clearly exceeds that of neurons, these data show that Ngb is not at all a lowly expressed gene, at least in zebrafish. These data do not provide true evidence for protein amounts, a respiratory function of Ngb cannot a priori be denied on the basis of its expression level. In addition, our results provide a further tentative link of Ngb to the oxidative metabolism: Ngb levels are low in early stages when simple oxygen diffusion is sufficient, and rise along with the levels of Hb and Mb at the time 4–5 dpf when circulatory oxygen supply starts (Grillitsch et al. 2005; Pelster and Burggren 1996).

Cytoglobin expression correlates with organogenesis

Cygb is the only "novel" globin for which developmental data exist (Mammen et al. 2006). Mammen et al. (2006) basically showed a steady increase of Cygb mRNA during mouse development. We found essentially a similar trend for both Cygb1 and Cygb2 (Fig. 4). Both the genes are expressed in a broad range of tissues (Fuchs et al. 2005). In mammals, which possess only a single *Cygb* gene (Burmester et al. 2002), Cygb is preferentially expressed in the fibroblast lineage, where it is associated with cell proliferation, and some neuronal populations (Schmidt et al. 2004). We do not know the exact sites of expression of Cygb1 and

Cygb2, but current data suggest that Cygb2 is most strongly expressed in brain and eye, pointing to a neuronal role, whereas Cygb1 shows a more widespread distribution, which is in line with a location in the fibroblast cells (Fuchs et al. 2005). We found 50- to 150-fold stronger expression of Cygb1 mRNA during zebrafish development, which corresponds with Cygb2 being mainly restricted to neuronal tissue (Fuchs et al. 2005). It is difficult to reconcile the changes in Cygb2 expression in zebrafish with neurogenesis, although one has to consider that, at least in mammals, only a small fraction of neurons with currently unknown function contain Cygb (Schmidt et al. 2004). Moreover, the neuron-specific expression of Cygb2 has only been inferred from the tissue distribution (Fuchs et al. 2005). By contrast, the strong increase of Cygb1 mRNA levels from 18 hpf onwards might be associated with organogenesis (Jonz and Nurse 2005; Malicki et al. 2005). Again, this fits to previous observations in the mice of an association of Cygb with cell proliferation, e.g., chondroblasts and osteoblasts (Schmidt et al. 2004).

Conclusions

Globins play an important role in the physiology of developing animals and are a prime model to study the developmentally controlled changes of gene expression in vertebrates. While the switch of hemoglobin chains during mammalian ontogeny and the underlying mechanisms have been investigated in detail, much less has been known from fishes. Previous studies have focused mainly on the role and regulation of Hb in early stages of zebrafish (Brownlie et al. 2003; Pelster and Burggren 1996; Ransom et al. 1996; Rombough and Drader 2009). Much less is known about Mb (Vlecken et al. 2009) and there had been no data on the "novel" globin types, Ngb, Cygb and GbX. The expression patterns of globin genes during the first 8 weeks of zebrafish development presented in this study can be related to major events of the ontogeny of zebrafish. For example, the onset of expression of embryonic Hbs may be correlated with the inflation of the swim bladder and adult Hb expression coincides with definitive hematopoiesis. Induction of Mb expression can be associated with the heart and the muscle development, Ngb with neurogenesis, and Cygb1 with organogenesis.

Our results provide comprehensive knowledge on the developmental-specific globin expression in zebrafish. The data also suggest a specific role for each globin. While Hb can readily be associated with circulatory oxygen transport and Mb with oxygen supply to muscles, such function may apply for Ngb, while such function is unlikely for Cygb.

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