Evolution by Genome Duplication:
Insights from Vertebrate Neural Crest Signaling and Pigmentation Pathways in Teleost Fishes

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The problems of this world are only truly solved in two ways: by extinction or duplication.

Susan Sontag

The only interesting thing about vertebrates is the neural crest.

Peter Thorogood

Color is the place where our mind joins the universe.

Paul Cézanne

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1. SUMMARY

Gene and genome duplications are major mechanisms of eukaryotic genome evolution. Three rounds of genome duplication have occurred in the vertebrate lineage, two rounds (1R, 2R) during early vertebrate evolution and a third round, the fish-specific genome duplication (FSGD), in ray-finned fishes at the base of the teleost lineage. Whole genome duplications (WGDs) are considered to facilitate speciation processes and to provide the genetic raw material for major evolutionary transitions and increases in morphological complexity. In the present study, I have used comparative genomic approaches combining molecular phylogenetic reconstructions, synteny analyses as well as gene function studies (expression analyses and knockdown experiments) to investigate the evolutionary consequences and significance of the three vertebrate WGDs.

First, the evolutionary history of the endothelin signaling system consisting of endothelin ligands and receptors was reconstructed. The endothelin system is a key component for the development of a major vertebrate innovation, the neural crest. This analysis shows that the endothelin system emerged in an ancestor of the vertebrate lineage and that its members in extant vertebrate genomes are derived from the vertebrate WGDs. Each round of WGD was followed by co-evolution of the expanding endothelin ligand and receptor repertoires. This supports the importance of genome duplications for the origin and diversification of the neural crest, but also underlines a major role for the co-option of new genes into the neural crest regulatory network.

Next, I have studied the impact of the FSGD on the evolution of teleost pigment cell development and differentiation. The investigation of 128 genes showed that pigmentation genes have been preferentially retained in duplicate after the FSGD so that extant teleost genomes contain around 30% more putative pigmentation genes than tetrapods. Large parts of pigment cell regulatory pathways are present in duplicate being potentially involved in teleost pigmentary innovations. There are also important differences in the retention of duplicated pigmentation genes among divergent teleost lineages. Functional studies of pigment synthesis enzymes in zebrafish and medaka, particularly of the tyrosinase family, revealed lineage-specific functional evolution of duplicated pigmentation genes in teleosts, but also pointed to anciently conserved gene functions in vertebrates. These results suggest that the FSGD has facilitated the evolution of the teleost pigmentary system, which is the most complex and diverse among vertebrates.

In conclusion, the present study supports a major role of WGDs for phenotypic evolution and biodiversity in vertebrates, particularly in fish.

ZUSAMMENFASSUNG

Gen- und Genomverdopplungen sind wichtige Mechanismen der Genomevolution in Eukaryonten. Im Verlauf der Evolution der Wirbeltiere gab es drei wichtige Genomduplikationen. Zwei Genomverdopplungen (1R, 2R) fanden während der sehr frühen Vertebratenevolution statt. In der Linie der Fische kam es an der Basis der Teleostier zu einer weiteren, fischspezifischen Genomduplikation (FSGD). Man nimmt an, dass Genomduplizierungen Artbildungsprozesse begünstigen und dass sie zusätzliches genetisches Material für wichtige evolutionäre Übergänge und für die Steigerung morphologischer Komplexität erzeugen. In der vorliegenden Arbeit wurden Methoden der vergleichenden und funktionellen Genomik gewählt, um die Auswirkungen und die Bedeutung der drei Genomverdopplungen bei Vertebraten zu untersuchen. Dazu wurden molekularphylogenetische Stammbaumanalysen und Synteniedaten mit Genexpressionsstudien und Knockdown-Experimenten kombiniert.

Zunächst wurde die Evolution des Endothelin-Signalsystems rekonstruiert. Dieses besteht aus Endothelin-Liganden und -Rezeptoren und hat eine Schlüsselrolle in die Entwicklung der Neuralleiste. Die Neuralleiste und die von ihr abgeleiteten Zelltypen sind wirbeltierspezifische Innovationen. Die Analyse zeigt, dass das Endothelin-System in einem gemeinsamen Vorfahren der Vertebraten entstanden ist. Die in den Genomen rezenter Vertebraten vorkommenden Komponenten des Endothelin-Systems sind durch die drei Genomverdoppelungen entstanden. Nach jeder der Duplizierungen kam es zur Ko-Evolution der Liganden- und Rezeptorenfamilien. Die Evolution des Endothelin-System unterstreicht daher die Bedeutung der Genomduplizierungen für den Ursprung und die Diversifizierung der Neuralleiste. Sie weist aber auch auf eine wichtige Rolle für die Integrierung neuer Gene in das regulatorische Netzwerk der Neuralleiste hin.

Im Weiteren wurde der Einfluss der FSGD auf die Evolution der Pigmentzellentwicklung und -differenzierung in Teleostiern untersucht. Die evolutionäre Analyse von 128 Genen zeigte, dass Pigmentierungsgene nach der FSGD bevorzugt in zwei Kopien erhalten geblieben sind. Daher besitzen rezente Teleostier im Vergleich zu Landwirbeltieren zusätzlich ca. 30% mehr Gene mit potentiellen Funktionen für die Pigmentierung. Große Teile der regulatorischen Signalwege in den Pigmentzellen liegen daher als zwei Kopien vor. Diese waren möglicherweise an der Evolution von Innovationen in der Körperfärbung von Teleostiern beteiligt. In der vorliegenden Arbeit wurden auch wichtige Unterschiede zwischen verschiedenen Fischgruppen im Erhalt duplizierter Pigmentierungsgene gefunden. Funktionelle Studien bei Zebrafish und bei Medaka an Enzymen der Pigmentsynthese, insbesondere der Tyrosinase-Familie, gaben Hinweise darauf, dass die funktionelle Evolution duplizierter Pigmentierungsgene in Fischen linienspezifisch verlaufen kann. Die Studien ergaben außerdem, dass bestimmte Funktionen der Pigmentsyntheseenzyme innerhalb der Vertebraten konserviert sind. Die Evolution des Pigmentierungssystems der Fische, welches das vielfältigste und komplexeste innerhalb der Wirbeltiere ist, wurde somit maßgeblich durch die FSGD beeinflusst.

Zusammenfassend weisen die Ergebnisse der vorliegenden Arbeit darauf hin, dass die Verdopplung ganzer Genome ein wichtiger Mechanismus der phänotypische Evolution bei Vertebraten ist und damit in besonderem Maße zur ihrer Biodiversität beiträgt.

2. INTRODUCTION

"As buds give rise by growth to fresh buds, and these, if vigorous, branch out and overtop on all sides many a feebler branch, so by generation I believe it has been with the great Tree of Life, which fills with its dead and broken branches the crust of the earth, and covers the surface with its ever branching and beautiful ramifications." Charles Darwin, 1859

2.1 Vertebrate genomics reveal the gene forest in our genomes

The publication of *The Origin of Species* (Darwin 1859) gave modern biological science its unifying theory of evolution. It included the concept of the common descent of all organisms, connected with each other in the *great Tree of Life*. Darwin realized that new species and higher taxa evolve in a pattern of successive branching. However, he was not aware of the molecular genetic basis of inheritance and it took almost another one hundred years until genetics had been established on a solid scientific ground (see Mayr 1982 for a historical review) and the structure of DNA had been elucidated (Watson and Crick 1953). Soon thereafter, protein and nucleotide sequences became recognized as markers for evolutionary history (Zuckerkandl and Pauling 1965). This stimulated the formation of the new research field of molecular evolution aiming to reconstruct the *Tree of Life* based on molecular sequence data.

An important characteristic of phylogenetic trees reconstructed from gene or protein sequences is that gene duplications introduce additional branches into molecular phylogenies. A clear distinction has to be made between orthologs, i.e. sequences that diverge following speciation events, and paralogs, i.e. sequences that diverge following gene duplication events (Fitch 1970). Thus, a gene tree will not necessarily reveal the species tree. The true phylogeny of a group of organisms is derived when only orthologs are used for its reconstruction. In contrast, using paralogs from a single species will reveal the branching order of multigene or protein families.

The occurrence of gene, chromosome and whole genome duplications has been recognized since the early 20th century (see Taylor and Raes 2004 for a historical review). This led to the intriguing hypothesis that gene and genome duplications are a major mechanism to generate new gene functions providing the material for major leaps in evolution such as the origin of the vertebrate lineage (Ohno 1970). With the rise of the genomic era in the last decade the significance of gene and genome duplication became fully appreciated. In fact, gene duplications have frequently occurred in the evolution of eukaryotes (Lynch and Conery 2000) so that deep forests of gene family trees are found

within our genomes. The significance of gene and genome duplications for phenotypic change and biodiversity, however, remains matter of ongoing debate.

Comparative genomic analyses can be applied to the detailed reconstruction of gene family trees. In addition to phylogenetic tree inference, synteny information, i.e. the order of genes along chromosomes, is extremely powerful to determine the order of branching events in gene family evolution.

Since the publication of the human genome sequence in 2001 (Lander et al. 2001; Venter et al. 2001), genome assemblies from all major vertebrate lineages have become available including numerous mammals, birds, lizard, frog, several teleost fishes, shark and lamprey (Fig. 1), and more vertebrate genomes are soon to come (Margulies and Birney 2008). Thus, it can now be investigated on a genome-wide scale, whether particular branching points are found repeatedly in numerous multigene families and whether these nodes correlate with important branching events in the underlying vertebrate species tree. Based on this information it is possible to analyze the interplay of divergence between paralogs or entire paranomes (the ensemble of duplicated genes) and phenotypic evolution.

Therefore, today – one hundred fifty years after the publication of the *Origin of Species* – it is for the first time possible to investigate in-depth the impact of gene and genome duplications on the evolution of the vertebrate branch in the *Tree of Life*.

2.2 Early vertebrate genome duplications

The evolution of vertebrates from an invertebrate protochordate is one of the major transitions in the animal kingdom. This transition was accompanied with fundamental changes in both anatomy and genome structure. At the morphological level, neural crest cells, placodes, a complex brain and the endoskeleton are key innovations of the vertebrate bauplan (Gans and Northcutt 1983; Hall 1999; Shimeld and Holland 2000). At the genomic level, an increase in genome size from invertebrate chordates to vertebrates has occurred (Ohno 1970) that was accompanied by substantial expansion of numerous gene families (Spring 1997).

In his seminal book *Evolution by Gene Duplication*, Susumo Ohno (1970) proposed that the extra portion of genetic material found in extant vertebrate genomes has been acquired through the duplication of genes and, even more importantly, during successive whole genome duplications (WGDs). In his honor, duplicate genes derived from WGDs are termed ‘ohnologs’ (Wolfe 2001).

Genome duplication (polyploidization) may occur within a species (autopolyploidization) or through the hybridization of two different species (allopolyploidization). Distinguishing between auto- and allopolyploidization events is particularly challenging for ancient WGDs (Otto 2007).

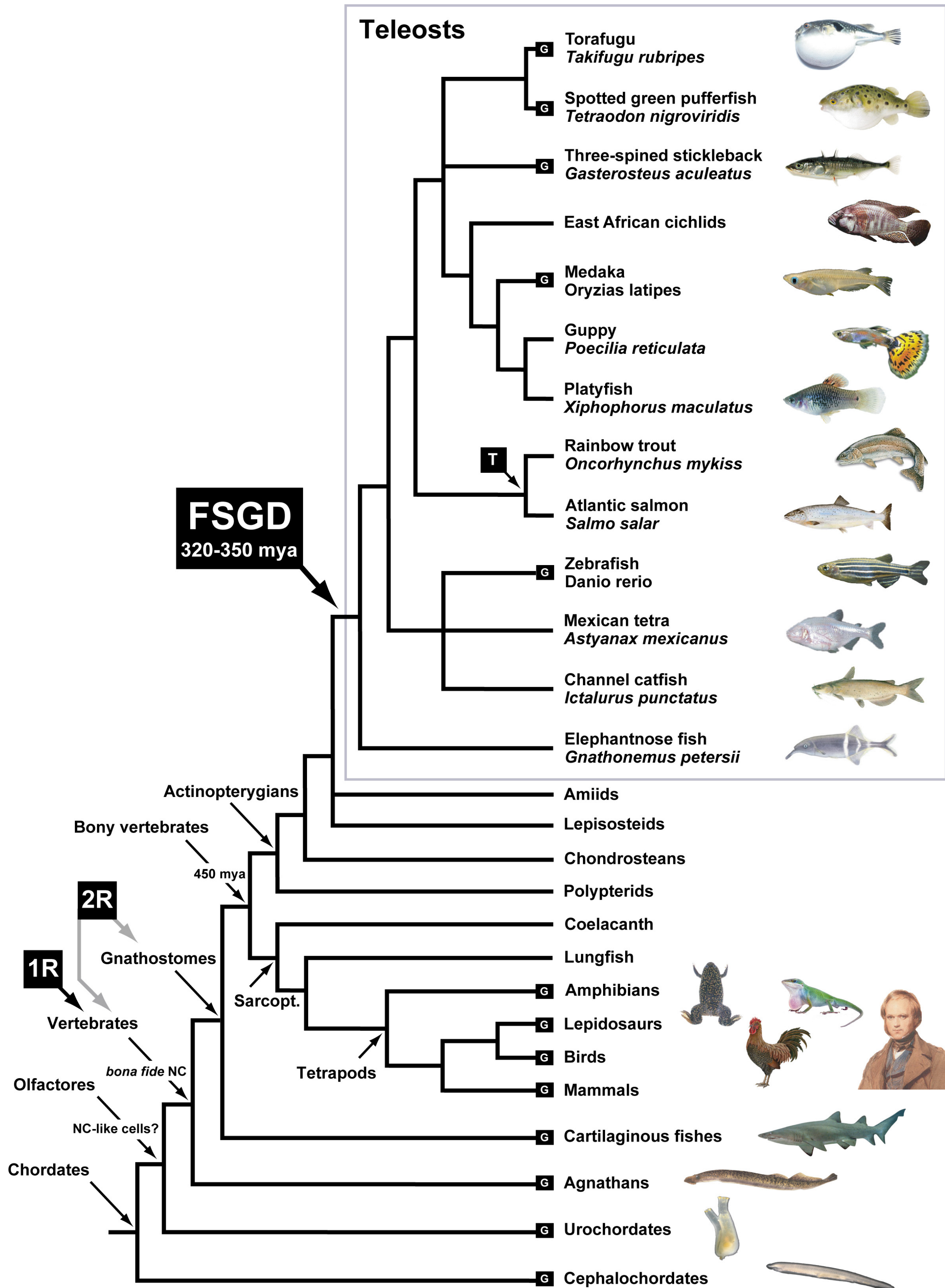


Figure 1 – Chordate phylogeny with emphasis on teleost fishes
The timing of the early vertebrate genome duplications (1R, 2R), the fish-specific genome duplication (FSGD) and the salmonid-specific autotetraploidization (T) are indicated. The timing of 2R is controversial and might have taken place before or after the divergence of agnathans. G denotes species and lineages for which a genome sequence assembly is available. mya: million years ago; NC: neural crest; Sarcopt.: sarcopterygians. Modified from Braasch et al. (2008).

Although hotly debated in the last decade (Panopoulou and Poustka 2005), it is now generally accepted that several rounds of polyploidization have occurred in vertebrates including two WGDs (1R/2R duplications) at the base of the vertebrate lineage (Ohno 1970; Furlong and Holland 2002; Dehal and Boore 2005; Putnam et al. 2008). An additional WGD, the fish-specific genome duplication (FSGD), has taken place in ray-finned fishes before the radiation of teleost fishes (Taylor et al. 2003[1]; Jaillon et al. 2004; Meyer and Van de Peer 2005; Kasahara et al. 2007). Additional more recent, lineage-specific WGDs have occurred in numerous vertebrate groups (reviewed in Otto 2007).

WGDs become evident by the identification of so-called paralogons, i.e. chromosomal blocks of duplicated genes showing conserved synteny within a genome. The most prominent examples of such paralogons are the Hox clusters. While there is only one Hox cluster in vertebrates' nearest invertebrate relatives, the cephalochordates and the urochrodates (Fig. 1), four Hox clusters are found in a typical vertebrate genome (reviewed in Hoegg and Meyer 2005). The fourfold paralogy of vertebrate Hox clusters is due to the twofold duplication of the single invertebrate Hox cluster. Fourfold paralogy is also found for other vertebrate genomic clusters such as the ParaHox clusters (Ferrier et al. 2005) and the adjacent receptor tyrosine kinase clusters (Siegel et al. 2007) or the Fox clusters (Wotton and Shimeld 2006). Examples for fourfold paralogy of chromosomal blocks consisting of unrelated genes are the major histocompatibility complex regions (Kasahara 2007), the neuropeptide Y receptor paralogons (Larsson et al. 2008) and the opioid receptor regions (Dreborg et al. 2008). The comparison of genome sequences of the urochordate *Ciona intestinalis* and of the cephalochordate amphioxus to vertebrate genomes, finally, revealed a genome-wide fourfold paralogy between invertebrate and vertebrate genomes providing definite evidence for two early WGDs in vertebrates (Dehal and Boore 2005; Putnam et al. 2008). Paralogons have also been used to reconstruct the ancestral vertebrate, pre-1R/2R karyotype (Nakatani et al. 2007; Putnam et al. 2008).

The phylogenetic timing of the two vertebrate WGDs, however, is not fully resolved. As shown in Fig. 1, the first round of WGD (1R) occurred concomitantly with the rise of vertebrates (Panopoulou and Poustka 2005). The timing of the second round (2R), in contrast, remains controversial and might have occurred in a common ancestor of agnathans (lamprey and hagfish) and gnathostomes (Kuraku, Meyer, and Kuratani 2008) or later in the gnathostome lineage after the split from agnathans (Panopoulou and Poustka 2005).

2.3 The fish-specific genome duplication

Ohno (1970) also hypothesized that a WGD had taken place in the lineage of ray-finned fishes (actinopterygians). First genomic evidence for this fish-specific genome duplication (FSGD) came with the identification of seven Hox clusters in teleosts (Amores et al. 1998; Hoegg and Meyer 2005). Subsequently, further evidence came through the identification of many additional duplicated genes in

[1] Own contributions are underlined.

divergent euteleost lineages such as zebrafish, pufferfishes and medaka (Fig. 1). These gene duplicates showed the phylogenetic topology, genome-wide chromosomal distribution and evolutionary age indicative of a large-scale genomic duplication event in a common ancestor (Wittbrodt et al. 1998; Meyer and Schartl 1999; Postlethwait et al. 2000; Taylor et al. 2001a; Taylor et al. 2003; Naruse et al. 2004). Finally, comparative analyses of teleost genome assemblies (Fig. 1) have provided conclusive evidence for the FSGD (Christoffels et al. 2004; Jaillon et al. 2004; Vandepoele et al. 2004; Woods et al. 2005; Kasahara et al. 2007), which is now generally accepted (Postlethwait et al. 2004; Meyer and Van de Peer 2005; Volff 2005; Froschauer et al. 2006). The genome assemblies of Tetraodon, medaka and zebrafish have been used to reconstruct the ancestral, pre-FSGD protokaryotype, which led to the definition of 13 protochromosomes (Jaillon et al. 2004; Woods et al. 2005; Kohn et al. 2006; Nakatani et al. 2007).

Studies including basal actinopterygian lineages further revealed that the FSGD event roughly coincided with the rise of the teleost lineage approximately 320-350 million years ago (Fig. 1), after its split from more basal ray-finned fish lineages, but before the divergence of the major teleost groups (Hoegg et al. 2004; Crow et al. 2006; Hurley et al. 2007).

In some fish lineages, further polyploidizations have occurred (reviewed in Le Comber and Smith 2004; Leggatt and Iwama 2004), for example the autotetraploidization in salmonids around 100 million years ago (Fig. 1) (Allendorf and Thorgaard 1984) or the allotetraploidization in the carp 12 million years ago (David et al. 2003). Sturgeons, which diverged from teleosts before the FSGD (Hoegg et al. 2004), have undergone several rounds of lineage specific genome duplications (Ludwig et al. 2001). Ray-finned fishes therefore offer a unique opportunity to study genome duplications of different ages.

2.4 Fates of duplicated genes and genomes

After duplication, paralogs are initially functionally redundant. In the short term, this increase in gene dosage may be compensated by gene-copy silencing (Adams and Wendel 2005; Pala et al. 2008). In the long term, the most likely fate of a pair of duplicates is nonfunctionalization, i.e. that one of the paralogs is lost (Fig. 2). Consequently, most gene duplicates have a rather short lifetime of a few million years only (Lynch and Conery 2000). However, many gene duplicates survive much longer periods and there are several models explaining how both duplicates can be retained and persist in the genome over long evolutionary periods. According to the classical neofunctionalization model (Ohno 1970), one of the two paralogs might acquire a new, positively selected function, while the other fulfils the ancestral gene functions (Fig. 2). Another possible fate of a duplicate gene pair is subfunctionalization (Fig. 2), i.e. the ancestral gene functions become distributed among the two paralogs, which are thus both retained (Force et al. 1999; Stoltzfus 1999). As proposed in the duplication-degeneration-complementation model (Force et al. 1999), subfunctionalization can be an explicitly neutral, degenerative process, but it is also possible that each of the two paralogs is

positively selected to specialize in one of the ancestral gene's functions (Conant and Wolfe 2008). Sub- and neofunctionalization are not mutually exclusive and might occur successively during paralog evolution (Fig. 2). Sub-neofunctionalization might be particularly observed in ancient duplicates (He and Zhang 2005). Nevertheless, many duplicates retain at least some functional overlap, thereby providing robustness to the system to withstand detrimental mutations (Wagner 2008). In addition, there are many more possible mechanisms for the retention of gene duplicates (see e.g. Taylor and Raes 2004; Semon and Wolfe 2007b; Conant and Wolfe 2008 for reviews). For example, a backup circuit between the duplicates may be establish after duplication to buffer against inactivation of one of the paralogs (Klüver et al. 2009) or duplicates can be rewired within the gene regulatory network by the acquisition of new transcription factor binding sites through insertion of transposable elements into their promoters (Herpin et al. submitted).

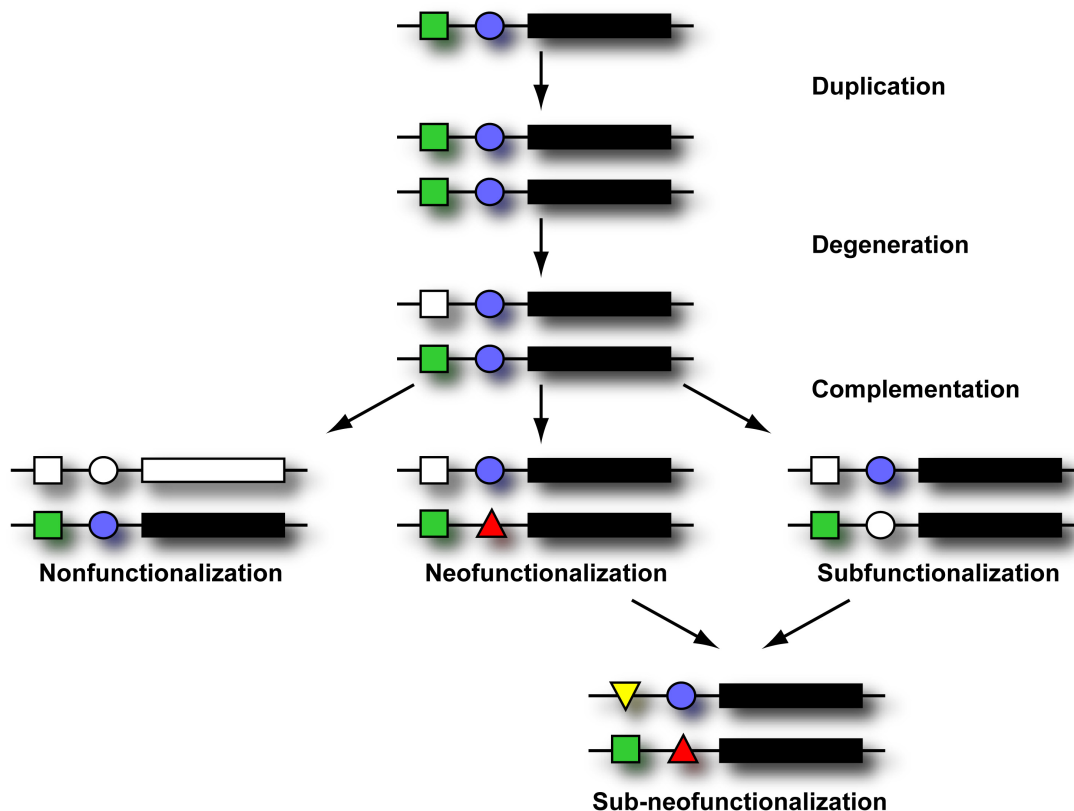


Figure 2 – Evolutionary fates of duplicated genes
An ancestral gene (black box) has two functions (green box, blue circle). Duplication generates two redundant copies of the gene. Subsequently, one of the genes looses a function (degeneration). As long as redundancy persists at least partially, the duplication may provide genetic robustness, i.e. the ability to withstand mutations. Under certain conditions, functional redundancy is evolutionary stable (Nowak et al. 1997). Further evolution of duplicate gene pairs may involve the three following fates: one of the two genes fulfils the ancestral functions, while the other gets lost (nonfunctionalization) or evolves a new function (neofunctionalization), represented by a triangle, or both genes partition the ancestral functions (subfunctionalization). Duplicate gene pairs might pass through subfunctionalization accompanied by neofunctionalization (sub-neofunctionalization). After Force et al. (1999) and He and Zhang (2005).

After genome duplication, the polyploid genome returns to the diploid state through massive nonfunctionalization of gene duplicates and genomic rearrangements (Wolfe 2001). Thus, at some point after WGD the genome becomes paleopolyploid, i.e. it is diploid, but still contains large proportions of duplicated genes that have survived the rediploidization process. This is particularly true for the vertebrate lineage, where the majority of duplicated genes in extant genomes are derived from the WGDs and not from small-scale, more local duplications (Blomme et al. 2006).
An important question is, which classes of genes are preferentially retained in duplicate. Recent studies on WGDs in eukaryotes as divergent as yeasts (Scannell et al. 2007; Wapinski et al. 2007), ciliates (Aury et al. 2006), plants (Maere et al. 2005), vertebrates in general (Putnam et al. 2008), teleosts (Brunet et al. 2006) and the frog *Xenopus leavis* (Semon and Wolfe 2008) revealed that slowly evolving as well as highly expressed genes are preferentially retained and gene dosage within protein complexes or metabolic pathways is generally kept. Furthermore, in multicellular organisms, genes involved in developmental processes, regulation of transcription and signal transduction are maintained at a high rate after WGDs. The retention of such genes is rather uncommon for paralogs generated through small-scale duplications (Maere et al. 2005; Blomme et al. 2006; Brunet et al. 2006; Putnam et al. 2008).

2.5 Evolutionary consequences of vertebrate genome duplications

The significance of WGDs for the long term evolutionary success of paleopolyploid species is not well understood (Otto 2007). For the early two rounds of vertebrate WGDs, it has been postulated that they have been necessary for the morphological novelties and the increased phenotypic complexity seen in vertebrates (Ohno 1970; Holland et al. 1994). For example, it has been proposed that 1R and 2R were genomic prerequisites for the evolution of the vertebrate endoskeleton (Zhang and Cohn 2008), the neural crest (Shimeld and Holland 2000; Wada 2001; Wada and Makabe 2006), jaws (Ohno 1970), or the adaptive immune system (Kasahara 2007; Okada and Asai 2008). However, detailed studies supporting these hypotheses are sparse and mostly restricted to few gene families.
The FSGD on the other hand has been proposed to be an important factor contributing to the amazing biodiversity and species richness of teleost fishes (Meyer and Schartl 1999; Postlethwait et al. 2004; Volff 2005). With around 23,500 species, teleosts represent approximately one half of all extant vertebrate species (Nelson 2006). The duplication-diversification hypothesis predicts that gen(om)e duplication and subsequent reciprocal gene loss or differential paralog evolution in divergent populations can lead to genomic incompatibilities between isolated populations and ultimately to speciation (Werth and Windham 1991; Lynch and Force 2000). Based on this so-called divergent resolution process, the FSGD might have facilitated the teleost radiation (Taylor et al. 2001b; Postlethwait et al. 2004; Volff 2005). Empirical support for this hypothesis has been provided by a recent comparative genome-wide analysis of paralog loss after the FSGD in zebrafish and Tetraodon (Semon and Wolfe 2007a).

Although teleost fishes are generally not considered to be phenotypically more complex than tetrapods, some aspects of their physiology and morphology are outstanding among vertebrates. To these belong, for example, the complexity and diversity of their body pigmentation and color patterning (Braasch et al. 2008; Kelsh et al. 2008). Based on the observation that several important genes in the development of pigment cells have been retained in teleosts after the FSGD, it was proposed that this genome duplication had an important impact on the evolution of their pigmentary system (Mellgren and Johnson 2002; Braasch et al. 2006).
Despite the possible correlation between genome duplications and increases in morphological complexity and/or biodiversity in vertebrates and other organisms, several authors disagree that gene and genome duplications are important mechanisms for morphological evolution (e.g. Donoghue and Purnell 2005; Carroll 2008). In the present study, I have investigated this important question by analyzing the influence of the vertebrate genome duplications on the evolution of signaling pathways involved in neural crest development as well as the impact of the FSGD on the evolution of the pigmentary system in teleost fishes.

2.6 Development and evolution of the vertebrate neural crest

During the development of vertebrate embryos, the neural crest is a multipotent stem cell population that originates at the neural plate border, the region at the junction of the neural plate and the prospective epidermis. After closure of the neural tube, neural crest cells delaminate and migrate to diverse regions of the embryo. Neural crest development involves a progressive series of fate restrictions such as the distinction into ectomesenchymal and non-ectomesenchymal neural crest (Fig. 3A), which finally leads to the differentiation into around 50 different cell types as diverse as pigment cells, craniofacial skeleton or enteric neurons (Hall 1999; LaBonne and Bronner-Fraser 1999; Le Douarin and Kalcheim 1999; Vickaryous and Hall 2006). It is due to the diversity of its derivatives that the neural crest is considered to constitute a “fourth germ layer” (Hall 2000).
The acquisition of the neural crest has been of major importance for the evolutionary success of the vertebrate lineage (Gans and Northcutt 1983; Hall 1999; Donoghue et al. 2008). Neural crest-derived structures are involved in the evolution of vertebrate-specific features, such as the transition from a sessile filter feeder to an active, jawed predator (“new head hypothesis”, Gans and Northcutt 1983).
The neural crest is absent from the most basal living group of chordates, the cephalochordates (Bourlat et al. 2006; Delsuc et al. 2006; Yu et al. 2008). In contrast, migratory ‘neural crest-like’ cells that develop into pigment cells have been found recently in urochordates, the sister clade of vertebrates (Jeffery et al. 2004; Jeffery 2007). A *bona fide* neural crest, however, is a synapomorphy of vertebrates including lampreys (Fig. 1) (Donoghue et al. 2008; Sauka-Spengler and Bronner-Fraser 2008b).
The “genome duplication model” underlines the importance of the two WGDs early in the vertebrate lineage (1R, 2R) for providing the genetic raw material necessary for the evolution of the neural crest

and its derivatives (Ohno 1970; Holland et al. 1994; Shimeld and Holland 2000; Wada 2001; Wada and Makabe 2006). However, this is not the only model proposed to explain the origin and subsequent evolution of the neural crest (reviewed in Donoghue, Graham, and Kelsh 2008). According to the "gene regulatory co-option model", the origin of the neural crest is based on the recruitment of neural crest specifier genes into a pre-existing gene regulatory network at the neural plate border (Meulemans and Bronner-Fraser 2005; Sauka-Spengler and Bronner-Fraser 2008a; Yu et al. 2008). The "new genes model" proposes that the evolution of the neural crest has been relying on the emergence of genes *de novo*, particularly signaling molecules, in the vertebrate lineage (Martinez-Morales et al. 2007). Importantly, the three models are not mutually exclusive but put emphasis on different aspects of neural crest evolution at the molecular level. Therefore, to further dissect the molecular basis of neural crest evolution, to differentiate between ancestral states and evolutionary novelties, and to evaluate the three models of neural crest evolution, it is necessary to reconstruct the emergence and evolution of key components in the neural crest regulatory network.

In the present study, I have reconstructed the evolutionary history of such a key component, the endothelin system (Braasch et al. 2009). The endothelin system consists of G protein-coupled endothelin receptors (Ednr) that are activated by endothelin (Edn) signaling peptides (Fig. 4) (Masaki 2004). Besides its importance for blood pressure regulation, the endothelin system plays a major role in the determination, migration, proliferation, survival and differentiation of neural crest cells and their derivatives (reviewed in Pla and Larue 2003). Disruption of endothelin signaling leads to malformations of neural crest derivatives like the craniofacial cartilage, enteric neurons and pigment cells. However, despite its important functions in vertebrate physiology and development relatively little is known so far about the evolution of the endothelin system in chordates.

2.7 Vertebrate pigment cells

Coloration and color patterning of skin, scales, feathers and hair belong to the most diverse phenotypic traits in vertebrates and have a plethora of functions such as camouflage, warning or threatening of predators, mate choice and species recognition (Braasch et al. 2008; Protas and Patel 2008). The different vertebrate groups are equipped with varying sets of pigment cell types derived from the neural crest (Fig. 3) (Bagnara and Matsumoto 2006). Common to all vertebrates are the dark melanophores (termed melanocytes in mammals and birds). The yellow to red xantho-/erythrophores and the silvery-reflecting iridophores are found in teleost fishes, amphibians and reptiles, but not in the dermis of mammals or birds (Fig. 3B, C). This suggests that the fish-like last common ancestor of bony vertebrates possessed melanophores, xantho-/erythrophores and iridophores. The latter two pigment cell types have been lost secondarily in the lineages leading to mammals and to birds, respectively, probably due to the evolution of an outer coat of hair or feather (Oliphant et al. 1992).

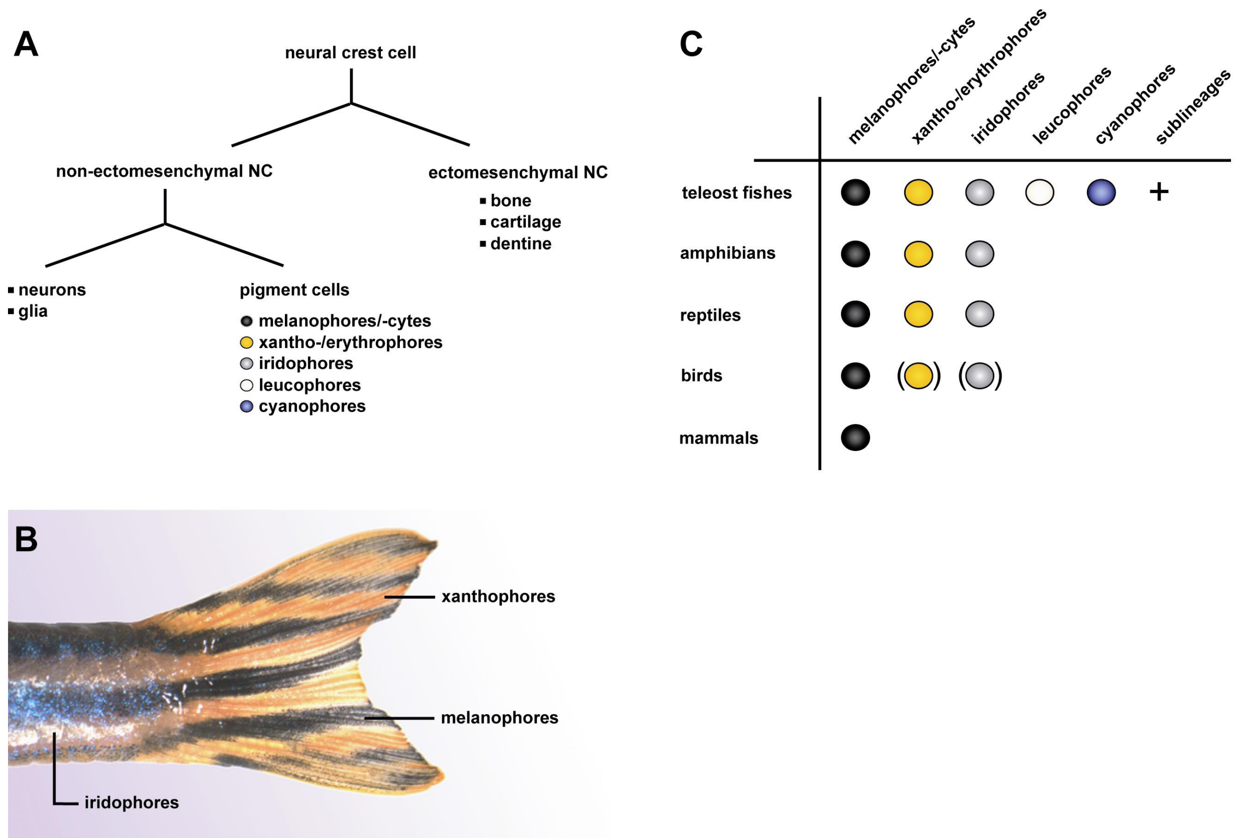


Figure 3 – Neural crest-derived pigment cells in vertebrates
A) Neural crest development involves a progressive series of fate restrictions. The multipotent neural crest cell population is partitioned into ectomesenchymal and non-ectomesenchymal neural crest (NC). Afterwards, cells become further fate-restricted, finally leading to the differentiation into around 50 different cell types. B) Three different types of pigment cells, melanophores, iridophores and xanthophores, build the stripe pattern on the tail of an adult zebrafish. C) Presence of dermal pigment cell types in different vertebrate lineages. In birds, iridophores and xanthophore/erythrophore-like cells are found in the iris (brackets), but not in the dermis.

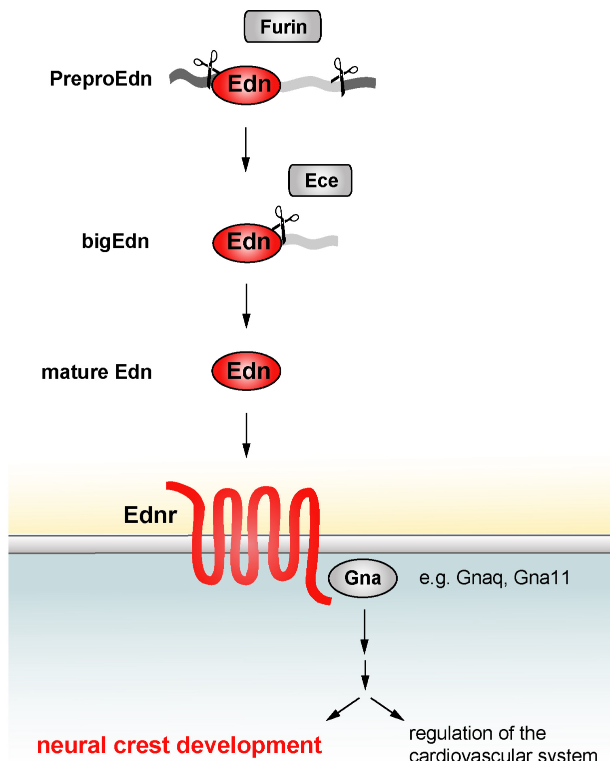


Figure 4 – The endothelin system
The big endothelin (Edn) peptide is cleaved from preproendothelin protein by the furin peptidase and further processed by the endothelin-converting enzyme (Ece) into the endothelin ligand peptide. Endothelins bind to the G protein-coupled endothelin receptors (Ednr) and regulate blood pressure as well as neural crest development.

The whitish leucophores have been found so far only in teleosts (Fujii 1993a) and the blue cyanophores only in some teleost families (Goda and Fujii 1995; Bagnara et al. 2007). In teleosts, several of the major pigment cell types are even further partitioned into distinct sublineages that are under different genetic controls (Kallman 1975; Johnson et al. 1995; Odenthal et al. 1996; Mills et al. 2007).

Each chromatophore has its characteristic pigments that reside in specialized pigmentary organelles (Tab. 1; Fujii 1993a; Bagnara and Matsumoto 2006). The relationships between the different chromatophore types remain largely unknown, but the occurrence of more than one type of pigmentary organelle or pigment in mosaic pigment cells suggests a common developmental origin of chromatophores from a stem cell population (Bagnara et al. 1979).

Table 1 – Vertebrate pigment cell characteristics (after Bagnara and Matsumoto 2006)

chromatophore	pigmentary organelle	pigment (chromophore)	coloration
1. melanophore	melanosome	melanin	black, brown
2. iridophore	reflecting platelet (iridosome)	purines, especially guanine; some pteridine crystals	structural colors and iridescence
3. leucophore	leucosome	uric acid (?), other purines (?)	structural white
4.a. xanthophore	pterinosome (xanthosome) carotenoid vesicles	pteridines carotenoids	yellow to orange
4.b. erythrophore	pterinosome (erythrothosome) carotenoid vesicles	pteridines carotenoids	orange to red
5. cyanophore	cyanosome	?	blue

The plethora of pigment patterns found in teleosts is achieved through variations in the spatial arrangement of the different pigment cell types, their combinatory effects, differences in the concentration and subcellular distribution of pigments within the chromatophores and the orientation of reflecting platelets in iridophores generating diverse structural colors (Hirata et al. 2003; Grether et al. 2004; Hirata et al. 2005).

Taken together, with the innovation of new pigment cell types (leucophores, cyanophores) and pigment cell sublineages the teleost pigmentary system has evolved a higher level of complexity than any other vertebrate group (Braasch et al. 2008; Kelsh et al. 2008). Furthermore, teleost pigmentation and color patterning are extremely polymorphic, subject to rapid evolutionary changes and are

involved in many adaptive processes and speciation events (Streelman et al. 2007). For example, sexual selection on male nuptial colors is a major mechanism in the adaptive radiations of cichlids in the East African Great Lakes (Salzburger et al. 2007; Salzburger 2008; Seehausen et al. 2008) or in the rapid speciation processes in sticklebacks (Boughman 2001; McKinnon and Rundle 2002). Poeciliids (guppies, platyfishes, swordtails) as well as coral-reef fish communities are further examples for particularly colorful fish assemblages. Pigment cell regression in the blind cave forms of the Mexican tetra is a fascinating example for the evolution in extreme environments (Protas et al. 2006; Gross et al. 2009).

2.8 Pigmentation pathways in teleost fishes and other vertebrates

Teleost pigmentation genes, i.e. genes involved in the different aspects of pigment cell development, have been identified recently through the systematic investigation of the large collections of natural and induced mutants of medaka and zebrafish. The Tomita collection includes more than 40 medaka pigmentation mutants (Tomita 1975; Kelsh et al. 2004), while over 100 pigmentation loci have been identified in the large-scale mutant screens in zebrafish (Kelsh et al. 1996; Odenthal et al. 1996; Parichy 2007). More than twenty teleost pigmentation genes have been identified (see www.zfin.org for zebrafish, and Fukamachi et al. 2001; Fukamachi et al. 2004a; Fukamachi et al. 2004b; Yu et al. 2006 for medaka). In mouse, in comparison, over 60 coat color genes involved in melanocyte development have been cloned so far (Silvers 1979; Bennett and Lamoreux 2003; www.espcr.org/micemut).

The identification of pigmentation genes in different vertebrates revealed that the genetic basis of melanophore development and differentiation is largely conserved between mammals and teleosts (Fig. 5), and that similar transcriptional networks, signaling pathways and pigment synthesis pathways are involved (Rawls et al. 2001; Kelsh et al. 2008). For instance, the function of the *Sox10* transcription factor gene to specify the non-ectomesenchymal neural crest including chromatophores is conserved between zebrafish and mouse (Dutton et al. 2001; Elworthy et al. 2003). Sox10 regulates the expression of the *Mitf* gene, which is the master regulator of melanophore/melanocyte differentiation in teleosts and mammals (Lister et al. 1999; Béjar et al. 2003). The involvement of the Kit and Ednrb transmembrane receptors in melanophore development is also conserved between fish and mammals (Parichy et al. 1999; Parichy et al. 2000a). Thus, 'vertebrate pigmentation genes' are defined here as those genes that were shown in at least one vertebrate species to be involved in the development and differentiation of neural crest-derived pigment cells.

Only very few genes involved in the development and differentiation of non-melanophore pigment cell types have been identified so far. For example, the *csf1r* receptor tyrosine kinase gene is essential for xanthophore development (Parichy et al. 2000b). Iridophore fate specification requires the expression of the *leukocyte tyrosine kinase* (*ltk*) gene (Lopes et al. 2008).

The presence of two *mitf* genes in teleosts is a primary example for the retention of a FSGD-derived duplicated pigmentation gene by subfunction partitioning at the transcriptional level. The teleost *mitf* duplicates encode two different Mitf isoforms, which are produced by a single gene in mammals. Subfunction partitioning of teleost *mitf* paralogs has been accomplished by reciprocal degeneration of regulatory elements leading to functional specialization for the development of the retinal pigment epithelium (RPE) or the melanophores (Lister et al. 2001; Altschmied et al. 2002).

2.9 Pigment synthesis pathways

The investigation of teleost pigmentation gene family evolution by the FSGD in the course of the present study was particularly focused on two pigment synthesis pathways, the pteridine and the melanin pathways.

The yellow to reddish pteridine pigments of xanthophores are synthesized in the pteridine synthesis pathway (reviewed in Ziegler 2003). The pteridine pathway is composed of three component pathways. The first one leads to the *de novo* synthesis of tetrahydrobiopterin (H_4biopterin) from GTP. H_4biopterin is a cofactor for neurotransmitter synthesis and tyrosinase activity in melanophores. The second component is the regeneration pathway of H_4biopterin. The third pathway shares several steps with the first one and leads to the formation of the yellow pigments, sepiapterin and its derivatives, as well as probably to the reddish drosopterin, which is also found in teleost fishes (Henze et al. 1977).

The first, rate limiting step in pteridine synthesis is catalyzed by the GTP cyclohydrolase I (GchI). *GchI* expression is an initial step for melanophore and xanthophore differentiation due to its involvement in the different component pathways (Ziegler 2003). During initial shotgun sequencing of the sex chromosomes of the platyfish (*Xiphophorus maculatus*) by our group, partial coding sequences of a *gchI* gene were found on the Y chromosome closely linked to the sex-determining locus (Froschauer 2003; Schultheis et al. 2006; Schultheis 2007). Since GchI is involved in the formation of reddish pigments, it is a major candidate for the sex-linked red-yellow pigment patterning locus (*RY*). The *RY* locus is responsible for yellow to red color patterns in the iris, on the body and fins of platyfish (Öktay 1964; Kallman 1975). The platyfish sex chromosomes are hot spots for segmental duplications and carry several genes in multiple copies (Schultheis et al. 2006) suggesting that additional *gchI* genes may be present in the platyfish genome.

Melanosomes of melanophores produce the dark eumelanin pigment. For teleosts, there is so far no evidence for the presence of the lighter pheomelanin, which is found in mammalian and avian melanosomes (Fujii 1993b). As shown in Fig. 6, disruption of melanin pigment synthesis leads to reduced pigmentation intensity culminating in oculocutaneous albinism (Spritz et al. 2003). Melanogenesis mainly involves the members of the tyrosinase gene family: *tyrosinase* (*tyr*), *tyrosinase-related protein 1* (*tyrp1*) and *dopachrome tautomerase* (*dct*; also known as *tyrosinase-related protein 2*) (Hearing and Tsukamoto 1991; del Marmol and Beermann 1996). During the early evolution of the chordate lineage, an ancestral *tyrosinase* gene was duplicated before the divergence of

urochordates and vertebrates leading to *tyrosinase* and a *tyrosinase-related* gene. The latter was subsequently duplicated in the vertebrate lineage giving rise to *tyrp1* and *dct* (Sato et al. 1999; Takeuchi et al. 2005). It was also shown that the *tyr* gene has been retained in duplicate after the FSGD by some teleost lineages (Hoegg et al. 2004).

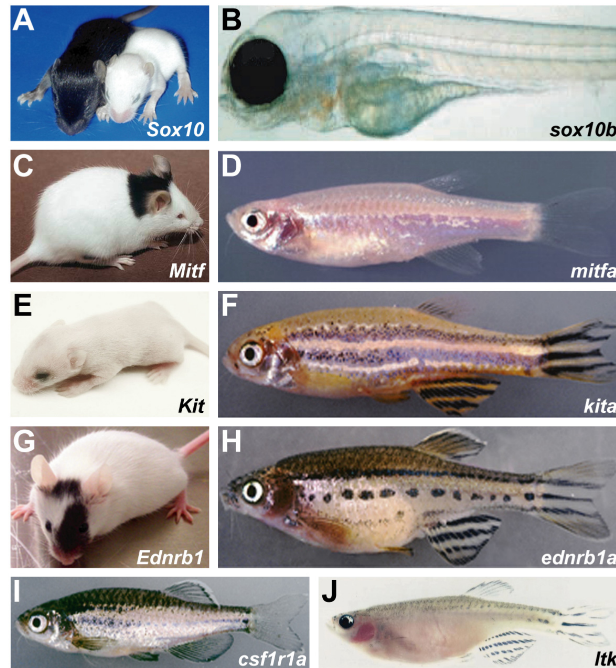


Figure 5 – Pigmentation mutants in mouse and zebrafish
Mutations in orthologous genes lead to similar melanocyte/-phore defects in mouse and zebrafish. *Dominant megacolon* (A, right) and *colorless* (B) are *Sox10* mutants lacking all melanocytes/-phores. *Microphthalmia* (C) and *nacre* (D) are affected in *Mitf*. *Dominant-white spotting* (E) and *sparse* (F) are mutated in *Kit*. *Piebald spotting* (G) and *rose* are *Ednrb1* mutants. Note that in zebrafish *kita* (F) and *ednrb1a* (H) mutants, only certain lineages of stripe melanophores are lost. The zebrafish *panther* mutant affected in *csf1ra* (I) is devoid of xanthophores. Mutation in the *ltk* gene leads to the *shady* phenotype in zebrafish (J). Mouse pictures from http://www.espcr.org/micemut/, zebrafish pictures from Parichy (2006), except B from Kelsh et al. (1996) and J from Haffter et al. (1996).

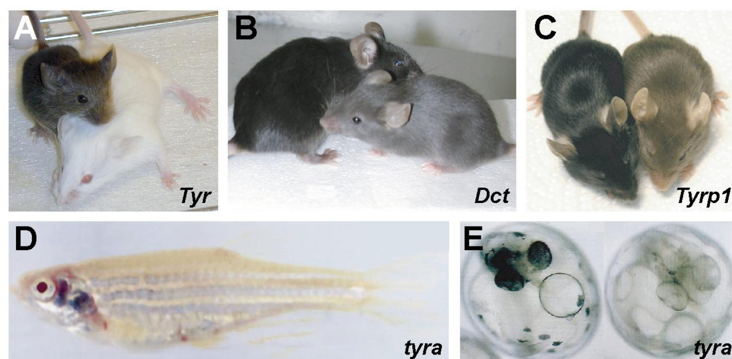


Figure 6 – Tyrosinase family mutants in mouse and teleosts
Mutations in *Tyr* genes lead to albino phenotypes in mouse (A, *albino* mutant), zebrafish (D, *sandy* mutant) and medaka (E, i^1 mutant). *Slaty* is a *Dct* mutant (B), *brown* a *Tyrp1* mutant (C) in mouse. No mutants for teleost *dct* or *tyrp1* genes have been identified yet. (A-C, F) Wildtype individuals to the left, mutants to the right. Mouse pictures from http://www.espcr.org/micemut/, D from Haffter et al. (1996), E from Hong et al. (1998).

3. AIMS OF THE STUDY

Whole genome duplications have been a recurrent phenomenon in the vertebrate lineage and are postulated to have facilitated major transitions and radiations during vertebrate evolution. The aim of the present study was to gain more insights into the significance of vertebrate genome duplications for the evolution of morphological complexity and diversity. The investigation should analyze duplicated genes derived from WGDs in the context of signaling pathways and developmental regulatory networks from both the comparative genomic perspective as well as on the functional, organismal level.

Firstly, the influence of the early 1R/2R vertebrate genome duplications for the origin and evolution of the neural crest should be analyzed. To this end, the evolutionary history and functional divergence of the endothelin signaling system as a key component of the neural crest regulatory network were to be reconstructed. This should lead to the evaluation of the "genome duplication model" and other models of neural crest evolution.

Secondly, the impact of the FSGD and other types of gene duplication for the evolution and diversity of the pigmentary system in teleosts should be examined. Thereby, it should be answered i) whether pigmentation genes have been preferentially retained in two copies after the FSGD; ii) which components of pigmentation pathways have been retained in duplicate; iii) which evolutionary fates duplicated pathways of pigment cell development have been met in teleosts; iv) what the phenotypic consequences of functional change in duplicated pigmentation genes have been; and v) whether there has been differential evolution of duplicated pigmentation genes in divergent teleost lineages that might be involved in the diversity of the teleost pigmentary system.

The more functional aspects of these questions should be analyzed focusing on the teleost pigment synthesis pathways, because they are specific to pigment cells. In the context of the pteridine synthesis pathway, the platyfish *gchI* gene should be investigated as a candidate for the sex-linked *RY* pigmentation locus. The evolution of teleost melanogenesis was to be investigated by functional analysis of duplicated tyrosinase gene family members in the two major fish models, medaka and zebrafish.

4. RESULTS AND DISCUSSION

4.1 Reconstructing the evolutionary history of vertebrate gene families

In the present study, the evolution of gene families was reconstructed using complementary approaches combining molecular phylogeny inference and synteny analyses. Sequence information from the genome assemblies of five teleosts (zebrafish, medaka, stickleback, Tetraodon, fugu), tetrapods (frog, chicken, lizard, mouse, human and other mammals), shark, lamprey and several invertebrate outgroups (Ciona, amphioxus, sea urchin, fruitfly, nematode) was included. This comparative genomic approach is particularly powerful to infer the duplication history of gene families in case of ambiguous tree topologies, incomplete genome assemblies and/or species-specific gene order rearrangements (Braasch et al. 2008). Distinguishing duplicates generated by the three vertebrate WGDs (1R, 2R, FSGD) or by local gene duplications is essential because duplicates of varying age and type show different patterns of duplicate retention and functional evolution (Maere et al. 2005; Aury et al. 2006; Wapinski et al. 2007).
Criteria that were used to conclude that pairs of duplicated genes were derived from a particular WGD are illustrated by the duplication of teleost *mitf* and *sox10* genes as result of the FSGD (Fig. 7). According to the tree topology, the duplication of the teleosts paralogs should date back to a common fish ancestor of the five teleost species (Fig. 7A, C). Furthermore, the gene duplicates should be part of paralogons (Fig. 7B, D) that show conserved synteny with each other and with an outgroup species, here e.g. human (double conserved synteny). For the 1R and 2R genome duplications, the duplicates should date back to the common ancestor of vertebrates and their chromosomal regions should show a pattern of fourfold paralogy.

4.2 The endothelin system in neural crest evolution

To gain more insight into the contribution of the early vertebrate genome duplications (1R, 2R) to the evolution of the neural crest, I have studied the endothelin core system (Fig. 4) as a key component of the neural crest regulatory network. Despite its important functions in vertebrate physiology and development, relatively little was known about the evolution of the endothelin system in chordates. Particularly, it has remained unclear (i) when and from which preexisting system the endothelin system emerged, (ii) whether the expansion of the endothelin system is based on vertebrate genome duplications or more local duplication events, (iii) how it has evolved in the different vertebrate lineages, (iv) whether expanding ligand and receptor repertoires have co-evolved, and finally (v) how the expansion of the endothelin system has contributed to the evolution of the neural crest.

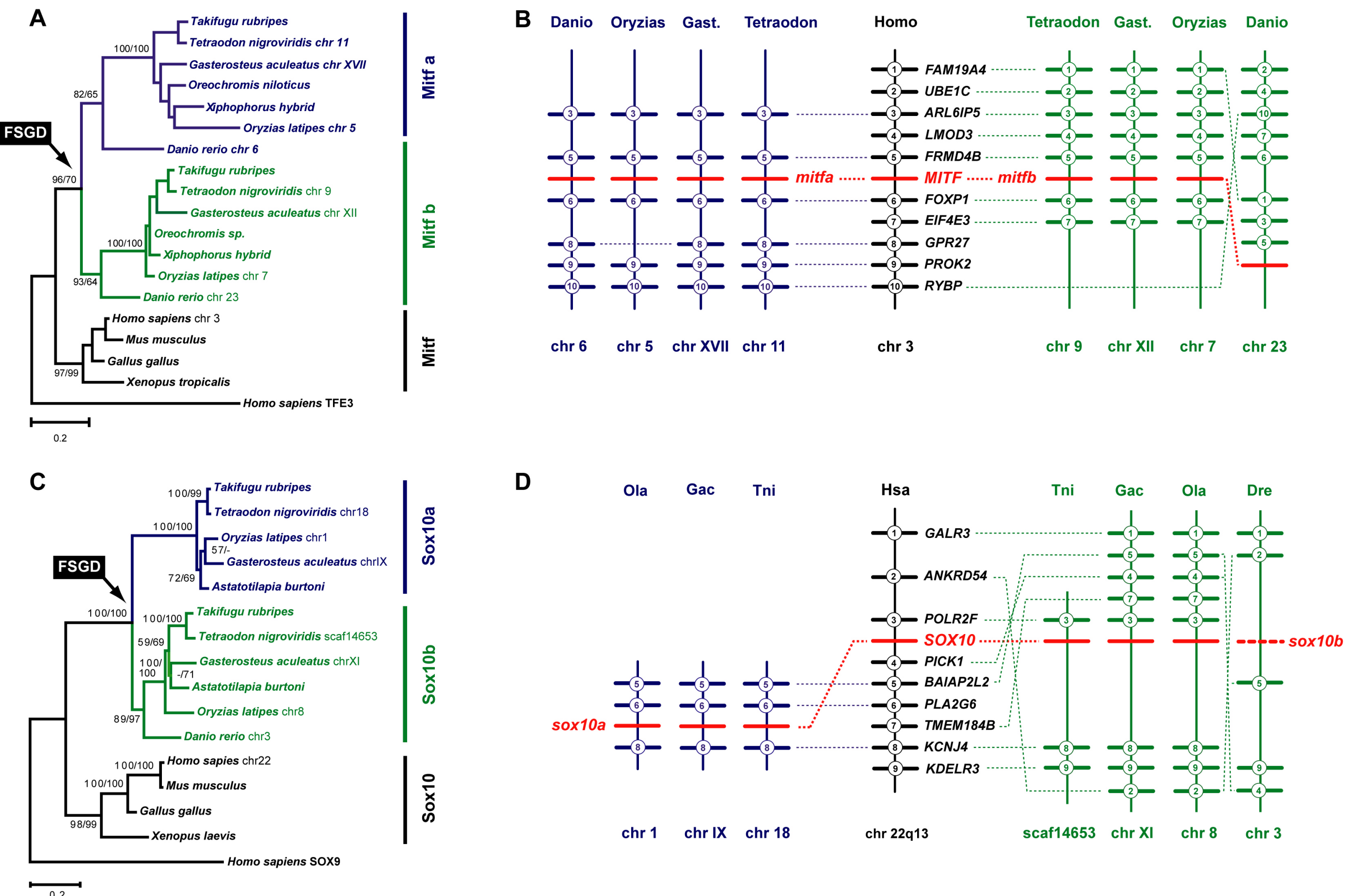


Figure 7 – Reconstructing the evolution of teleost *mitf* and *sox10* genes
Identification of teleost-specific paralogons containing two duplicated pigmentation genes, *mitf* (A,B) and *sox10* (C,D), as result of the fish-specific genome duplication (FSGD) by means of phylogenetic reconstruction (A, C) and synteny analyses (B, D). The topology of the maximum likelihood (ML) protein phylogenies is indicative of a duplication of the respective gene in the actinopterygian lineage after its split from the tetrapod lineage, where a single gene is found. As predicted for a whole genome duplication such as the FSGD, the pigmentation paralogues are part of larger paralogons within teleost genomes that show conserved synteny to each other and to a single chromosomal region in the human genome (B, D). A *sox10a* paralogon could not be identified in zebrafish. The *sox10b* gene (dotted red bar) is not included into the current zebrafish genome assembly but has been mapped to chr3. Bootstrap values (ML/Neighbor Joining method) above 50% are shown. Numbered bars represent genes contributing to conserved synteny, intervening genes that do not contribute to conserved synteny are not shown. Dotted lines connect orthologous genes. A-B) from Braasch et al. 2008, C-D) from Braasch et al. submitted.

4.2.1 Evolution of endothelin ligands

Preproendothelin genes have been not been found so far in lineages basal to tetrapods and teleost fishes (Hyndman and Evans 2007; Martinez-Morales et al. 2007). Consistently, in the present study no preproendothelin gene was identified in the genome assemblies of Ciona, amphioxus or more distantly related invertebrates. In contrast, multiple endothelin sequences were found here for the first time in lamprey and shark. Thus, the endothelin family emerged most likely in vertebrates and was found to be larger than previously thought (Fig. 8A), containing more members than the three known genes (*Edn1-3*). In teleost fishes, a completely new endothelin family member, *Edn4*, was identified, which seems to have already been present in the last common ancestor of all gnathostomes (Braasch et al. 2009).

A putative scenario for the evolution of the vertebrate endothelin gene family derived from the present study is shown in Fig. 8B (Braasch et al. 2009). The ancestral endothelin gene newly appeared in an ancestral vertebrate as part of a paralogon including the *Hivep* and *Phactr* genes. Phylogenies of the *Hivep* and *Phactr* gene families were used to reconstruct the evolutionary history of the Edn paralogons. The initial Edn paralogon was doubled twice during the 1R and 2R genome duplications, leading to four endothelin genes in gnathostomes. In the lineage leading to tetrapods, *Edn4* was lost. In teleost fishes, on the other hand, eight endothelin paralogons were generated by the FSGD. Both duplicates of *edn2* and *edn3* were retained, but one copy of *edn1* and *edn4* was lost, so that up to six endothelins are present in teleosts. Subsequent differential loss of endothelin duplicates has occurred during the teleost radiation: medaka has lost *edn2b* and pufferfishes *edn3a*. Such divergent resolution can be an important mechanism leading to speciation (Volff 2005 and references therein).

4.2.2 Evolution of endothelin receptors

No *Ednr* genes have so far been found outside vertebrates (Hyndman and Evans 2007). In the present study, survey of invertebrate genomes generally did not reveal any *Ednr* sequences except for two putative, '*Ednr*-like' genes from amphioxus. Since there is so far no evidence for conserved synteny between the *Ednr*-related gene regions and the vertebrate *Ednr* regions and because the ligand-binding domains of vertebrate Ednr proteins and of the putative amphioxus Ednr-like proteins are very divergent, a *bona fide* receptor for endothelin ligands seems to be indeed a vertebrate innovation. In contrast, partial *bona fide* Ednr proteins are present in lamprey, showing that this gene family was already present in the common ancestor of extant vertebrates (Braasch et al. 2009).

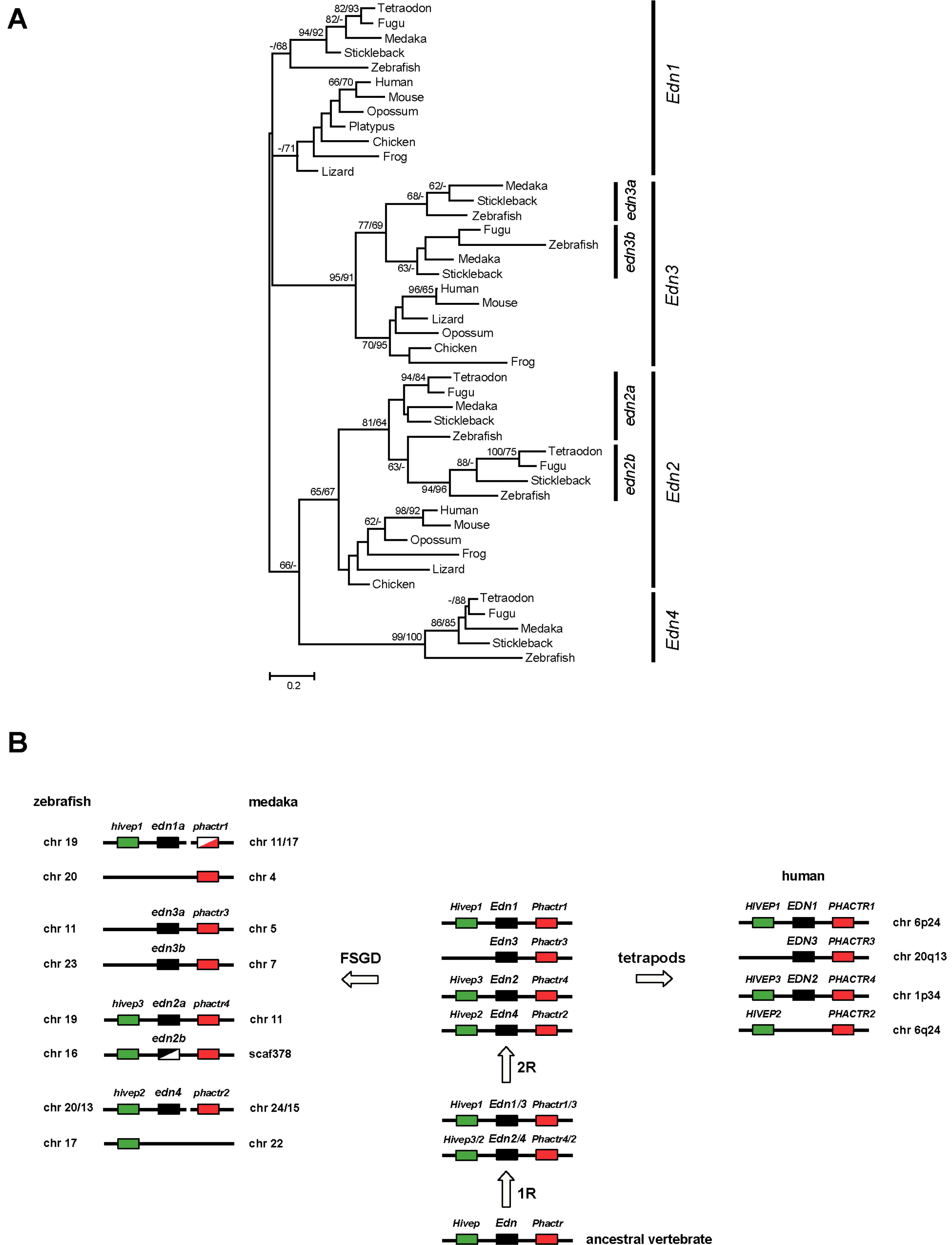
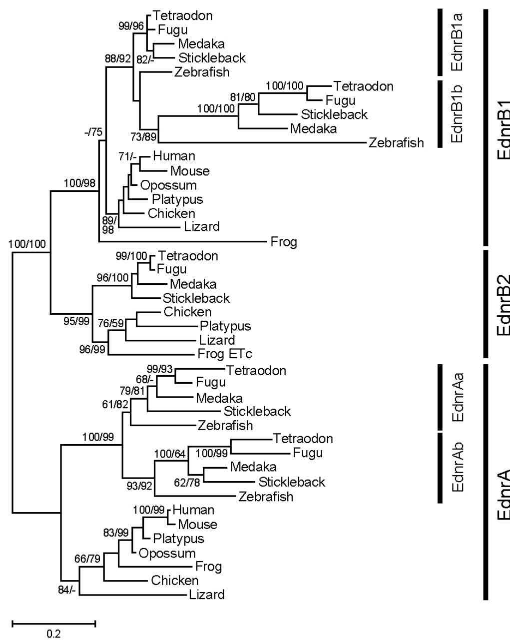


Figure 8 – Evolution of vertebrate endothelin genes
A) ML phylogeny of endothelin ligand nucleotide sequences. Bootstrap values above 60% (ML/NJ) are indicated. B) Putative evolution of endothelin ligand paralogons. Three genes, *Hivep*, *Ednr* and *Phactr*, have been linked in the ancestral vertebrate. This paralogon was duplicated twice during the 1R and 2R genome duplications. *Edn4* was lost in the tetrapod lineage, but kept in teleost fishes, which also have retained two copies of *edn2* and *edn3* after the fish-specific genome duplication (FSGD). From Braasch et al. (2009).

Two endothelin receptor genes, *EdnrA* and *EdnrB1*, had been found in mammals so far (Arai et al. 1990; Sakurai et al. 1990). A third gene reported to encode an amphibian-specific receptor, *ETc*, is present in Xenopus (Karne et al. 1993). *EdnrB2* found in birds was reported to be an avian-specific paralog of *EdnrB1* (Lecoin et al. 1998). In the present study, two (opossum and placental mammals), three (shark, frog, lizard, chicken, platypus), five (zebrafish) or six (medaka, stickleback, pufferfish) *Ednr* genes were identified. It further shows that three major clades of vertebrate *Ednr* genes exist: *EdnrA*, *EdnrB1*, and *EdnrB2* (Fig. 9A). The *EdnrB2* group contains the avian *EdnrB2* and the amphibian *ETc* gene and is also present in shark, teleosts, reptiles and monotreme mammals. However, *EdnrB2* has been lost in the lineage of therian mammals (marsupials and placentals) concomitantly with the rise of their sex chromosomes (Braasch et al. 2009).
Synteny analyses revealed that vertebrate *Ednr* genes are genetically linked to members of the *Spry*, *Brn3* and *Slain* gene families as well as to the ParaHox paralogons, which contain the ParaHox genes and a receptor tyrosinase kinase cluster (Ferrier et al. 2005; Braasch et al. 2006; Siegel et al. 2007). A model for the evolution of the endothelin receptor paralogons is shown in Fig. 9B. In early vertebrates, the newly arising *Ednr* gene was added to a preexisting chromosomal block containing *Spry*, *Brn3*, *Slain* and the ParaHox genes. Likewise, the ParaHox paralogon was expanded with receptor tyrosine kinase genes. Importantly, among these was the precursor of the *Kit*/*Csf1r* and *Pdgfr* type III receptor tyrosine kinase genes, which encode important vertebrate-specific regulators for the migration and survival of neural crest derivatives like pigment cells, craniofacial cartilage and others (Parichy et al. 1999; Parichy et al. 2000b; Hoch and Soriano 2003). Thus, it appears that a new chromosomal block was built in the very early vertebrate, from which many important neural crest genes are derived. Presumably, these singleton genes were already involved in the development of a basal type of neural crest in an early vertebrate ancestor.
The Ednr-ParaHox paralogon was then duplicated during 1R giving rise to *EdnrA* and *EdnrB*. After 2R, both copies of *EdnrB* were kept, while one of the two *EdnrA* genes was lost. In teleosts, the *ednr* paralogons were further doubled by the FSGD. Only the second copy of *ednrB2* was lost before the teleost radiation, so that up to five *ednr* genes (*ednrAa*, *ednrAb*, *ednrB1a*, *ednrB1b*, *endrB2*) are found in teleosts.
Further bioinformatic analyses provided evidence for functional divergence in the endothelin system following each round of genome duplication (1R, 2R, FSGD). Importantly, functional divergence after each WGD has occurred in the ligand-binding domains of the endothelin receptor proteins, modulating different aspects of ligand-receptor interactions. With progressive expansion of the ligand repertoire, changing the ligand selectivity became predominant. Therefore, co-evolution of the expanding endothelin receptor and ligand repertoires seems to have occurred (Braasch et al. 2009).

A



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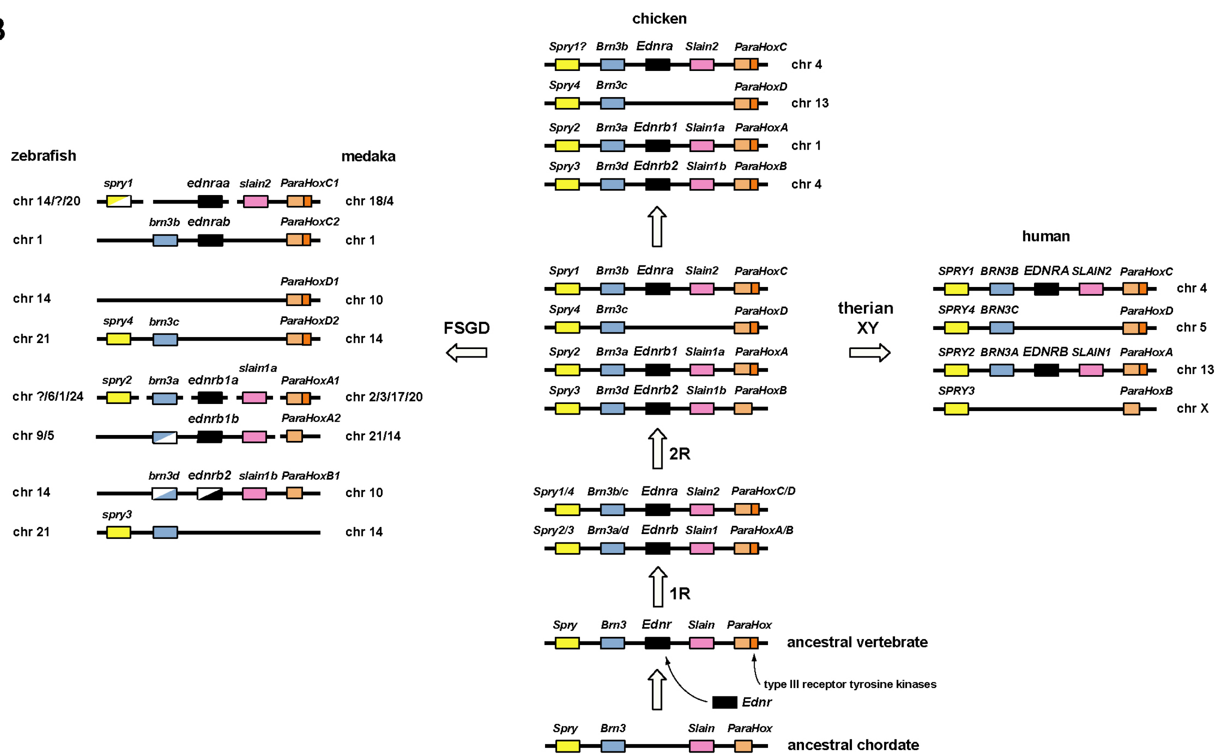

Figure 9 – Evolution of vertebrate endothelin receptor genes
A) ML phylogeny of endothelin receptor protein sequences. Bootstrap values above 60% (ML/NJ) are indicated. B) Putative evolution of endothelin receptor paralogons. A chromosomal block containing *Spry*, *Brn3*, *Slain* and the *ParaHox* cluster present in an ancestral chordate was expanded in an ancestral vertebrate with the newly arising *Ednr* and type III receptor tyrosine kinase genes. After 1R and 2R, four *Ednr* paralogons were present in gnathostomes. In the teleost lineage, after FSGD and subsequent gene losses, eight *Ednr* paralogons with six *ednr* genes are found. In therian mammals, degeneration of the fourth *Ednr* paralogon including the loss of *EdnrB2* occurred during the evolution of sex chromosomes (XY). From Braasch et al. (2009).

4.2.3 The endothelin system supports the three major models of neural crest evolution

During the specification and development of the vertebrate neural crest and its derivatives, the endothelin system is required at different time points. The Edn1/EdnrA system plays a role during early neural crest induction and maintenance (Bonano et al. 2008). In the following steps of development, the fate of neural crest cells is determined by their position along the anterior-posterior axis. The anterior neural crest gives rise to the ectomesenchymal neural crest, while the more posterior neural crest gives rise to the non-ectomesenchymal neural crest (Pla and Larue 2003; Vickaryous and Hall 2006). This emerged before the origin of lampreys (Donoghue et al. 2008) and as the endothelin system is required for the development of both of these neural crest groups, the first Edn/Ednr pair seems to have evolved its role in neural crest development in the very early vertebrate. After 1R and 2R, subfunctionalization appears to have occurred so that the Edn1/EdnrA system became specialized for the ectomesenchymal neural crest, particularly jaw structures. The Edn3/EdnrB system became specialized for the non-ectomesenchymal neural crest, in particular for pigment cells and enteric neurons (Pla and Larue 2003). Further subfunctionalization between EdnrB1 and EdrnB2 has occurred regarding the migratory routes of non-ectomesenchymal neural crest cells (Pla et al. 2005). After the FSGD, finally, the teleost *ednrA* paralogs have retained partially redundant roles for lower jaw formation (Nair et al. 2007). To summarize, after each round of vertebrate WGD, the endothelin system has become progressively specialized for particularly neural crest derivatives.

The diversification of the endothelin system following 1R, 2R and FSGD strongly supports the “genome duplication model” of neural crest evolution. Furthermore, the endothelin system is a vertebrate-specific signaling pathway and its emergence in an early ancestor of all vertebrates might have been a key event in neural crest evolution. Thus, it also supports the “new genes model”. Similarly, other signaling pathways involved in neural crest development with peptide ligands and G protein-coupled receptors such as the melanocortin system emerged at first in vertebrates and became diversified by the three vertebrate WGDs (Selz et al. 2007).

Finally, the initial function of the endothelin system might have been the regulation of muscle contractions (Zhang et al. 2001) and the neural crest function could have been acquired afterwards. Thus, also the “gene regulatory co-option model” might be supported. In conclusion, the present study suggests that at the molecular level the emergence and diversification of vertebrate neural crest has to be considered as a rewiring of gene regulatory networks that were supplemented by the integration of new components and expanded through whole genome duplications (Braasch et al. 2009). Future studies of neural crest-related gene families should take into account their specific functions, the phylogenetic timing of their appearance as well as their subsequent amplification during vertebrate WGDs.

4.3 Evolution of teleost pigment synthesis pathways by gene and genome duplication

4.3.1 Evolution of the pteridine synthesis pathway

For the pteridine synthesis pathway (Fig. 10A), only two genes, *spr* and *dhpr*, were found to have been retained in two copies after the FSGD. Interestingly, sepiapterin reductase (Spr) is involved in multiple steps of the pathway (Ziegler 2003). Several lineage-specific duplications were observed, such as the duplication of *gchfr* in the salmonid-specific tetraploidization or the duplication of *pcbd1* by retrotransposition in fugu (Braasch et al. 2007).

The most interesting result from the analysis of the pteridine synthesis pathway is the diversity of *gchI* genes in vertebrates. Until recently, a single *GchI* gene was known in mammals and zebrafish and these genes were considered to be orthologous. However, as shown in Fig. 10B, the vertebrate *GchI* gene family in fact consists of three members and it turned out that the known *GchI* genes from mammals and zebrafish are paralogous (Braasch et al. 2007). The first gene, *GchIa*, is present in all gnathostome groups analyzed so far (teleosts, amphibians, lizard, birds and mammals). The second gene, *GchIb*, has been found in teleosts (including the previously known zebrafish *gchI* gene) and amphibians, but not in mammals or birds. The third gene has so far been detected only in some teleosts (e.g., pufferfishes and stickleback). To rule out that teleost *gchI* genes were paralogs originating from the FSGD, zebrafish *gchIa* and *gchIb* genes were physically mapped to chromosomes Dre17 and Dre12, respectively, using a radiation hybrid panel (Braasch et al. 2007). Dre17 and Dre12 are not known to be derived from a common pre-FSGD protochromosome (Woods et al. 2005; Kasahara et al. 2007). The phylogenetic distribution of GchI genes (Fig. 10B) and further synteny analyses rather suggest that *GchIa* and *GchIb* were duplicated during the vertebrate 1R/2R genome duplications and that *GchIb* is a missing ohnolog in mammals and birds. It must have been deleted independently in these two lineages because of the presence of *GchIb* in the lizard genome (Braasch et al. 2008). *GchIc* might also be a 1R/2R ohnologue that was lost secondarily in more vertebrate lineages. The involvement of the individual GchI enzymes in the different branches of the pteridine synthesis pathways remains elusive, but the independent loss of *GchIb* in mammals and birds correlates well with the loss of dermal xanthophores in these lineages (Braasch et al. 2007; Braasch et al. 2008). This points to a function of *GchIb* specifically performed in xanthophores, i.e. the synthesis of pteridine pigment precursors, which is further supported by its expression in zebrafish xanthophores (Parichy et al. 2000b; Pelletier et al. 2001).

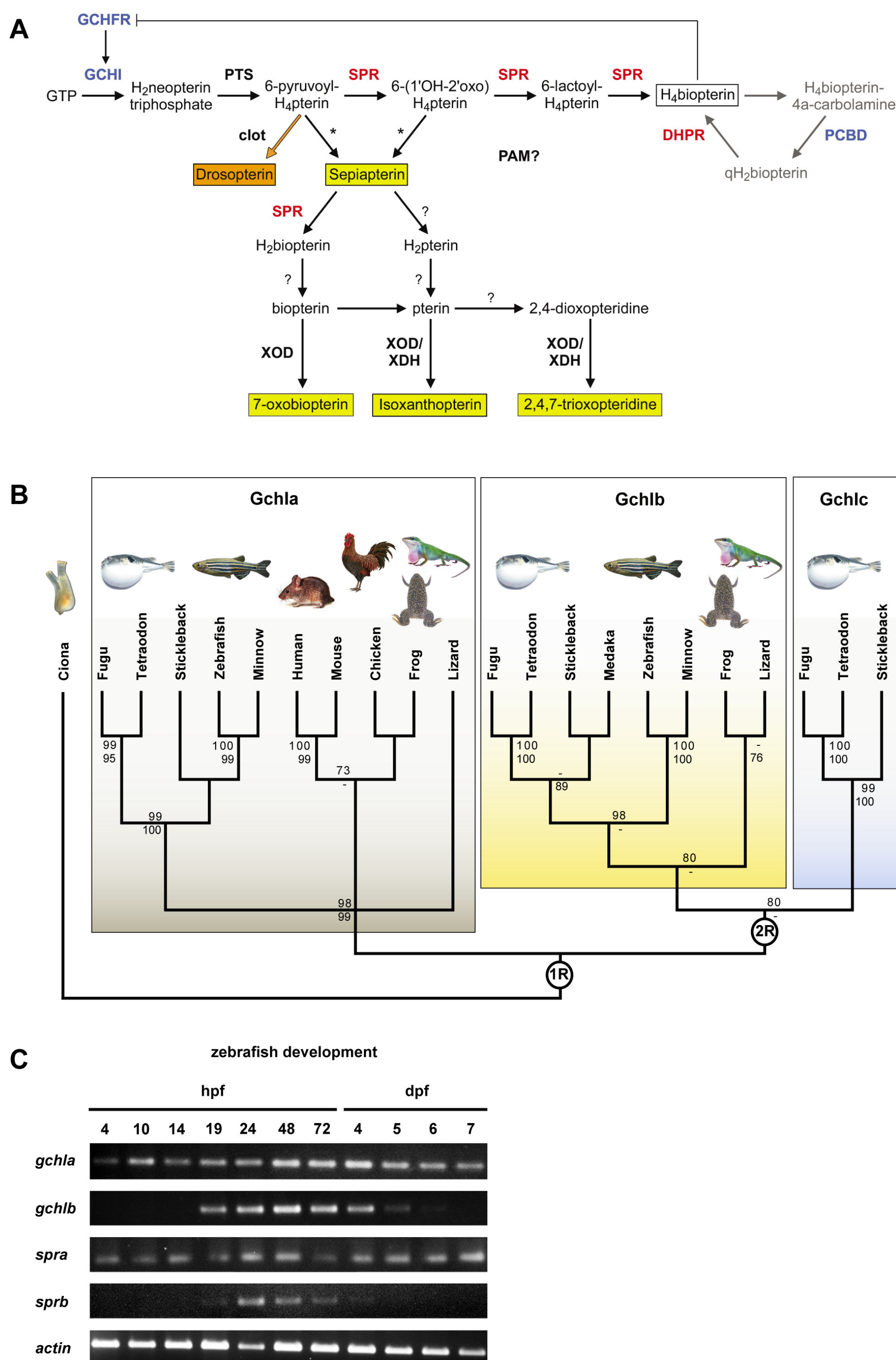

Figure 10 – Evolution of the pteridine synthesis pathway
A) Pteridine synthesis pathway and gene duplications in vertebrates. Pteridine synthesis contains three component pathways: the *de novo* synthesis of H_4biopterin from GTP (top line), the H_4biopterin regeneration pathway (grey) and the synthesis of yellow pteridine pigments. Asterisks indicate hypothetical reactions and question marks unidentified enzymes. Red indicates duplications during the fish-specific genome duplication, blue other types of duplication. From Braasch et al. (2007). B) ML bootstrap consensus phylogeny of vertebrate GchI proteins, rooted with the ortholog from Ciona. Bootstrap values above 60% (ML/NJ) are shown. Three groups, GchIa, GchIb, and GchIc are present that were most likely generated by the 1R and 2R WGDs. GchIb is absent from mammals and birds, which also lack dermal xanthophores. GchIc is only present in teleosts. Modified from Braasch et al. (2007). C) RT-PCR of *gchI* and *spr* genes during zebrafish development. hpf: hours post fertilization; dpf: days post fertilization.

Interestingly, *gchIb* is only transiently expressed during zebrafish development (Fig. 10C, I. Braasch, M. Fackelmann, J.-N. Volff, unpublished data) despite ongoing pteridine synthesis (Pelletier et al. 2001). Zebrafish *gchIa*, in contrast, is expressed continuously. A transient expression overlapping with the *gchIb* expression period is also found for *sprb*, a downstream target in the pteridine synthesis pathway. Its FSGD paralog, *spra*, is continuously expressed just like *gchIa* (Fig. 10C). Thus, it appears that members of pteridine synthesis enzyme families are co-expressed in certain combinations, (e.g. *gchIb* together with *sprb*), which are required differentially in time and potentially perform distinct functions.

4.3.2 The *gchIb* gene as a candidate for the red-yellow pigment patterning locus of the platyfish

Sequence fragments of a *gchI* gene had been found in shotgun sequencing fragments of the platyfish sex chromosomes (Schultheis et al. 2006; Schultheis 2007). As part of the present study, this Y chromosomal *gchI* gene was analyzed as a candidate for *RY* locus of the platyfish (I. Braasch, M. Schartl, J.-N. Volff, unpublished data; see also Appendix 1). The sequence fragments were found to belong to a *gchIb* gene. Its expression in fins with red pigment patterns further substantiates the hypothesis that *gchIb* might represent *RY*. Screening a platyfish BAC library (Froschauer et al. 2002) identified a second, autosomal *gchIb* gene. The *gchIb* gene appears to have been duplicated onto the Y chromosome as part of a larger chromosomal block. However, no *gchIb* sequences were identified on the platyfish X chromosomal BAC contig, which would be required if *gchIb* was indeed *RY*. It is, however, unclear if this is due to a gap in the library. In addition, the coding sequence of Y chromosomal *gchIb* gene lacks exon 1, so that it might constitute a pseudogene (Appendix 1).
In conclusion, some aspects such as its classification and expression make the platyfish Y chromosomal *gchI* gene a reasonable candidate for *RY*, while other aspects contradict this hypothesis. The ongoing platyfish genome sequencing project will help to clarify this question.

4.3.3 Evolution of the melanin synthesis pathway

The melanin synthesis pathway involves four enzymes. Three of them were found to be retained in duplicate in teleosts after the FSGD. For all of the melanosomal transporter genes, the second FSGD-derived duplicate has been lost (Fig. 11; Braasch et al. 2007).
In the tyrosinase gene family, FSGD duplicates were identified for *tyr* and *tyrp1*, while *dct* was present as a single copy gene in all lineages analyzed (Fig. 12). However, the retention of tyrosinase gene family members after the FSGD is variable between the different lineages. While medaka and stickleback have retained both copies of *tyr* and *tyrp1, tyra* was lost in the zebrafish lineage and *tyrp1b* in pufferfishes. The latter loss must have been quite recent as a *tyrp1b* pseudogene was amplified by PCR and sequenced from the Tetraodon genome (Braasch et al. 2007). Thus, the tyrosinase gene family is another good example for divergent resolution after the FSGD.

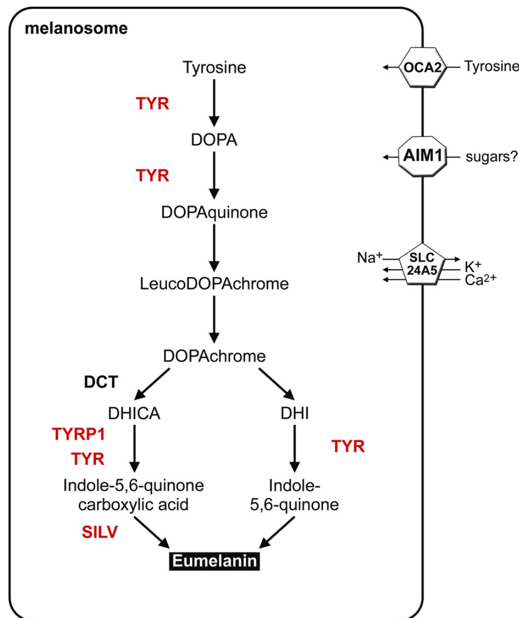

Figure 11 – Eumelanin synthesis pathway and gene duplications in vertebrates. Eumelanin is synthesized from tyrosine within the melanosome of melanophores. This requires members of the Tyrosinase family (TYR, DCT, TYRP1) and probably Silver (SILV). Three melanosomal transporters (OCA2, AIM1 and SLC24A5) are crucial for proper melanin synthesis. Red indicates duplications during the FSGD. From Braasch et al. (2007).

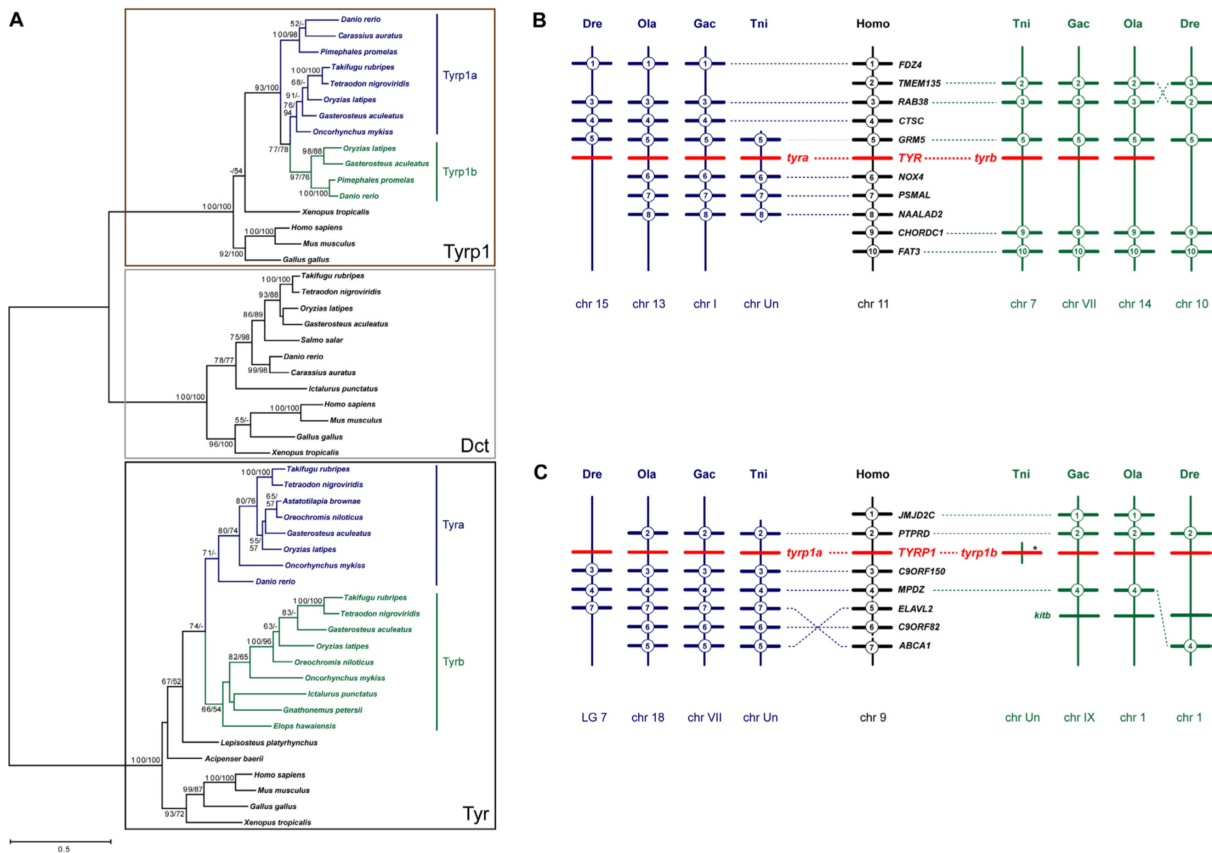

Figure 12 – Evolution of the tyrosinase gene family in vertebrates

A) ML phylogeny of protein sequences from the tyrosinase family. The tree is mid-point rooted. Bootstrap values above 50% (ML/NJ) are shown. Tyrp1a and Tyrp1b are assigned according to the analysis of their genomic environment. B) Synteny of *tyr*-containing regions in vertebrate genomes. The human *TYR* region is syntenic to two *tyr* paralogons in Tetraodon (Tni), stickleback (Gac) and medaka (Ola). *Tyrb* was apparently lost in the zebrafish (Dre). C) Synteny of *tyrp1*-containing regions in vertebrate genomes. The human *TYRP1* region is syntenic to two *tyrp1* paralogons in stickleback, medaka and zebrafish. In the course of the present study, the zebrafish *tyrp1a* gene was mapped to linkage group (LG) 7 using a radiation hybrid panel. A *tyrp1b* pseudogene is found in Tetraodon (asterisk). Numbered bars represent genes contributing to conserved synteny, genes that do not contribute to conserved synteny are not shown. Dotted lines connect orthologous genes. From Braasch et al. (2007).

The *silver* gene has also been retained in duplicate after the FSGD (Braasch et al. 2007). In zebrafish, *silva* is expressed in melanophores and the retinal pigment epithelium (RPE) of the eye, while *silvb* expression is restricted to the RPE (Schonthaler et al. 2005). The expression of *silv* paralogs is similar to the expression of the duplicated *mitf* transcription factor genes (Lister et al. 1999; Lister et al. 2001). In mammals, *Silv* transcription is dependent on Mitf (Baxter and Pavan 2003; Du et al. 2003).
It has been proposed that in chordates the molecular network controlling the expression of melanogenic genes with Mitf as its master regulator evolved initially in the pigmented cells of the eye and has then been recruited by the neural-crest-derived melanophores (Martinez-Morales et al. 2004). Due to this gene co-option, the *Mitf* gene and its downstream targets might have been under certain functional constraints so that the melanogenic networks of melanophores and of the RPE cannot evolve independently. In teleosts, however, the tight linkage of melanogenesis in melanophores and RPE could have been dissolved due to functional specialization of duplicated melanogenic network members for either melanophores or RPE (Braasch et al. 2008). Thereby, the FSGD could have provided new opportunities for variation of melanin-based body pigmentation without perturbing melanogenesis in the eye. Thus, I was interested whether other downstream targets of Mitf such as the duplicated members of the tyrosinase family also show functional specialization for melanogenesis in the RPE or in melanophores.

4.4 Functional evolution of the teleost tyrosinase gene family

Protein alignments and comparison to known loss-of-function mutations in tetrapods did not indicate fundamental changes at functionally important sites after the FSGD for Tyr or for Tyrp1 proteins (Braasch et al. in preparation and data not shown). Thus, teleost Tyra and Tyrb as well as Tyrp1a and Tyrp1b proteins most likely still constitute *bona fide* melanogenic enzymes.

4.4.1 Evolution of teleost *tyrosinase* genes

In zebrafish, only one *tyr* paralog has been retained and loss of *tyra* function leads to albinism (Fig. 6D). In the medaka, several albino mutants were identified (Fig. 6E) that are also affected in the *tyra* paralog (Koga et al. 1995; Iida et al. 2004). However, the present study has provided first evidence for the presence of *tyrb* in the medaka (Braasch et al. 2007). No *tyrb* mutant is available at present in fish. The fact that some *tyra* mutations in the medaka lead to a complete oculocutaneous albino phenotype (Koga et al. 1995; Iida et al. 2004) suggests that *tyrb* cannot substitute for *tyra*.
In situ hybridization experiments show that both *tyr* paralogs are expressed as expected for melanogenic enzymes, i.e. in RPE cells and melanoblasts/-phores (Fig. 13; I. Braasch, J.-N. Volff, M. Schartl, unpublished data). Expression of *tyr* genes in melanoblasts on the yolk and the developing embryo starts earlier than in the eyes. This spatio-temporal progression of expression is also seen for *dct* (Fig. 13) and *tyrp1* genes (Braasch et al. in preparation). Likewise, the following progression of

visible melanin formation is seen first in yolk and body melanophores and later in the eye (Lynn Lamoreux et al. 2005). In contrast, in zebrafish, melanin becomes first visible in the eye and shortly thereafter in melanophores. Expression of *dct* and *tyra* also starts first in the eye (Kelsh et al. 2000; Camp and Lardelli 2001). Therefore, the onset and relative timing of melanogenic enzyme expression and melanin formation in melanophores and RPE are quite different between medaka and zebrafish. It will be interesting to analyze in more detail how differences in melanogenesis between teleost species are related to the mode of their development and its interplay with the environment. Early differentiation of melanophores could for example be important for camouflage. Accordingly, in lifebearing poeciliids that develop within the mother, melanogenesis starts in the eyes and is seen rather late on the body (Tavolga 1949).

The similar expression of *tyra* and *tyrb* in medaka suggests that both are still involved in melanogenesis. This is in agreement with functional studies of the two *tyr* duplicates in the rainbow trout. Morpholino knockdown of both paralogs reduced pigmentation in the eye and the skin (Boonanuntanasarn et al. 2004), but it is unclear, how many *tyr* genes are actually present in the tetraploid trout. Initial morpholino knockdown experiments for medaka *tyrb* as part of the present study did not show any visible effect on melanin formation (data not shown). Thus, the function of *tyrb* in medaka and in teleosts in general remains elusive. Other putative functions of tyrosinase like in dopamine metabolism (Eisenhofer et al. 2003) or during light adaptation of the retinal neural network (Page-McCaw et al. 2004) might be starting points for future analyses of teleost *tyrb* functions.

4.4.2 Evolution of teleost *tyrp1* genes

Both *tyrp1* paralogs have been retained in zebrafish and medaka (Braasch et al. 2007) and so far no teleost *tyrp1* mutant has been identified. During zebrafish embryonic and larval development, both *tyrp1* paralogs are expressed in melanoblasts/-phores and the RPE (Fig. 14A-B). In the adult zebrafish, *tyrp1b* expression is found in melanogenic organs, i.e. in the eyes as well as in skin and fins, which contain melanophores. In contrast, *tyrp1a* is not expressed in adult zebrafish. Therefore, although expression of *tyrp1* duplicates in zebrafish largely overlaps, the *tyrp1b* gene has a broader expression spectrum than the *tyrp1a* gene.

During medaka development, expression of both *tyrp1* genes is seen in melanoblasts/-phores (Fig. 14C-D). Furthermore, expression of *tyrp1a*, but not of *tyrp1b*, is found in the eye. In the adult medaka, expression of both *tyrp1* duplicates is found in the eye. In skin and fin, weak expression of *tyrp1a* is detected, but *tyrp1b* expression appears to be restricted to the eyes. Thus, *tyrp1b* appears to have the minor role for melanogenesis in medaka. Consistently, the *tyrp1b* genes of medaka and stickleback have an elevated rate of molecular evolution and gained an additional intron. This could indicate relaxed functional constraints on *tyrp1b*, which might have become progressively less important in the acanthomorph lineage (medaka, stickleback, pufferfishes) and, at the extreme, have been rendered into a pseudogene or completely lost as in pufferfishes (Braasch et al. 2007).

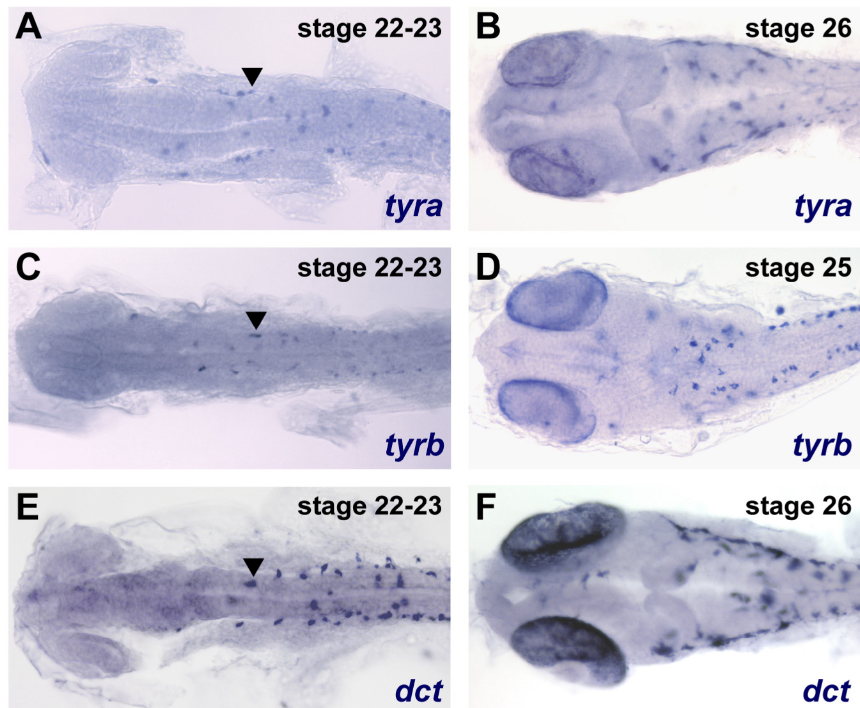

Figure 13 – Expression of *tyr* and *dct* genes in medaka
Whole-mount *in situ* hybridizations on medaka embryos show that expression of *tyra* (A, B), *tyrb* (C, D), *dct* (E, F) starts in melanoblasts on the body (A, C, E; arrowheads) and is later also found in the developing eye (B, D, F). Dorsal view, anterior is to the left.

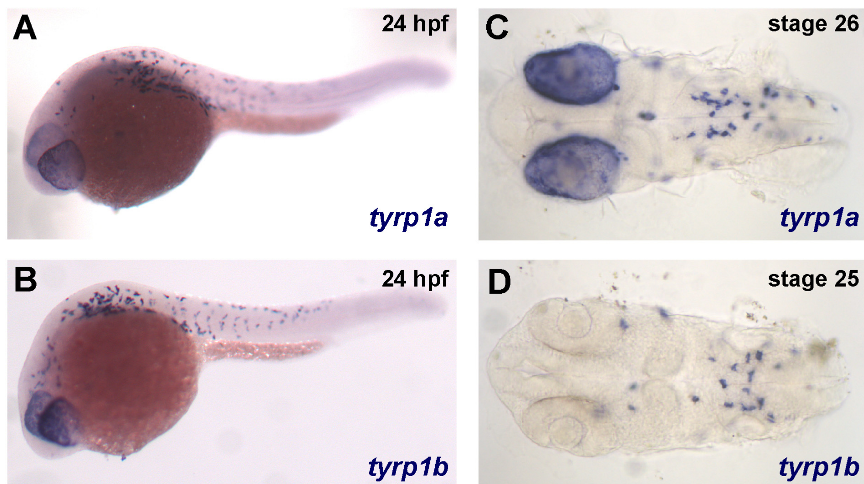

Figure 14 – Expression of *tyrp1* duplicates in zebrafish and medaka
Whole-mount *in situ* hybridizations show that both *tyrp1* paralogs in zebrafish embryos (A, B) are expressed in the eyes and melanoblasts. In medaka (C, D), *tyrp1a* is expressed in the eyes and melanoblasts (C), but *tyrp1b* is expressed in melanoblasts only (D). Anterior is to the left. A, B: lateral view; C, D: dorsal view. Experiments C and D by D. Liedtke.

The present results indicate that teleost *tyrp1* genes are unlikely to have acquired new functions after duplication. At the expressional level, the *tyrp1* duplicates in medaka and zebrafish obviously have met differential evolutionary fates since the divergence of the two species. Although differential evolution of duplicated genes in divergent lineages has been proposed to be a key mechanism for the biodiversity of teleost fishes (Postlethwait et al. 2004; Volff 2005; Froschauer et al. 2006), there have been only few studies demonstrating lineage-specific evolution of paralogous gene expression patterns. The present study adds duplicates of an enzyme gene to this list (Braasch et al. in preparation).

Next, I used morpholino (MO) antisense oligonucleotides to analyze the phenotypic effect of knocking down *tyrp1* paralogs in zebrafish. Morpholinos are nucleic acid analogs that can be used to specifically block the translation or splicing of mRNAs (Eisen and Smith 2008). Knockdown of only one *tyrp1* paralog did not lead to any obvious phenotypic effect with respect to pigmentation or any other developmental process. In contrast, a change of pigmentation was observed when MOs against both paralogs were co-injected (Fig. 15). This suggests that the *tyrp1* paralogs are functionally redundant during early zebrafish development increasing their robustness of melanogenesis (Braasch et al. in preparation).

At 2 dpf, double MO-injected embryos are characterized by severely hypopigmented melanophores and RPE. Melanophore pigmentation was hardly visible, and brownish instead of black pigment was found in the RPE (Fig. 15). At 3 dpf, the brown pigment became also apparent in the melanophores on the body (Fig. 16). Importantly, the brownish hue is based on a shift in the pigment color and not just the result of lower amount of black pigments. Melanophores with low amounts of black melanin are grayish in color. It will be interesting to analyze if knockdown of the *tyrp1* paralogs in medaka generates a similar brown pigment, and if the knockdown of *tyrp1a* in this species in contrast to zebrafish leads to morphants with brown eyes but black melanophores.

A brownish color is also seen in melanosomes of Tyrp1-deficient mammals (Fig. 6C; (Jackson 1988; Schmutz et al. 2002; Berryere et al. 2003; Schmidt-Kuntzel et al. 2005; Gratten et al. 2007) and birds (Nadeau et al. 2007). It has been previously proposed that the brown skin color of Africans with *TYRP1* mutations is caused by a increased pheomelanin/eumelanin ratio (Kidson et al. 1993). However, as pheomelanin is not found in teleosts (Fujii 1993b), the present study strongly suggests that the brown melanosome color in Tyrp1-deficient fish is an effect of altered eumelanin synthesis and does not depend on the presence of pheomelanin. This is in agreement with biochemical analyses, which have shown that polymerization of eumelanin monomers into black eumelanin polymers is impaired in Tyrp1-deficient mammals (Ozeki et al. 1995; Ozeki et al. 1997). Hence, it is highly likely that the brown melanosome color in Tyrp1-deficient mammals, birds and fish is due to a similar biochemical defect in eumelanin formation.

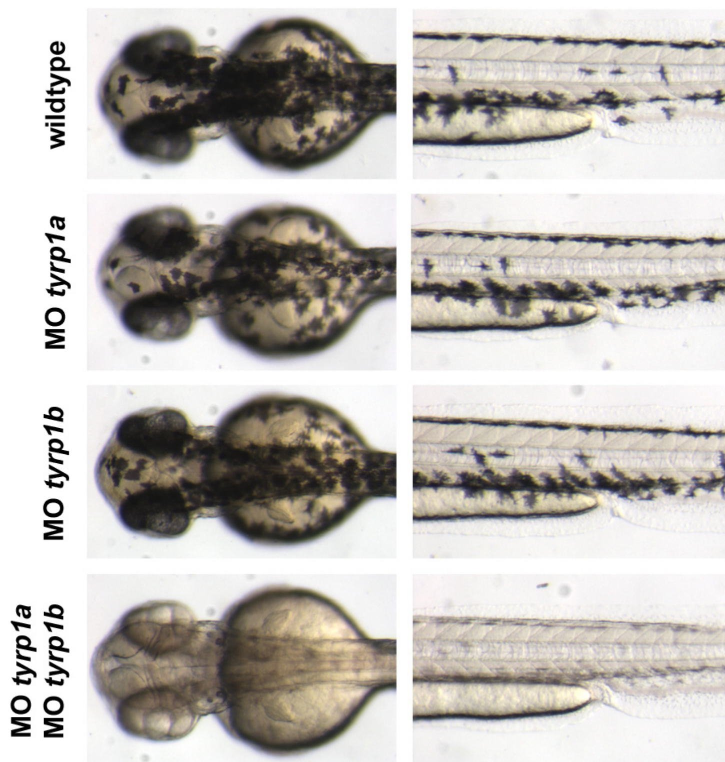

Figure 15 – Morpholino knockdown of *tyrp1* genes in zebrafish (2 dpf)
Dorsal view on head region (left column) and lateral view on trunk region (right column) of *tyrp1* splice morpholino (MO) -injected zebrafish embryos. A pigmentary defect is only seen in double MO-injected fish (bottom). Anterior is to the left.

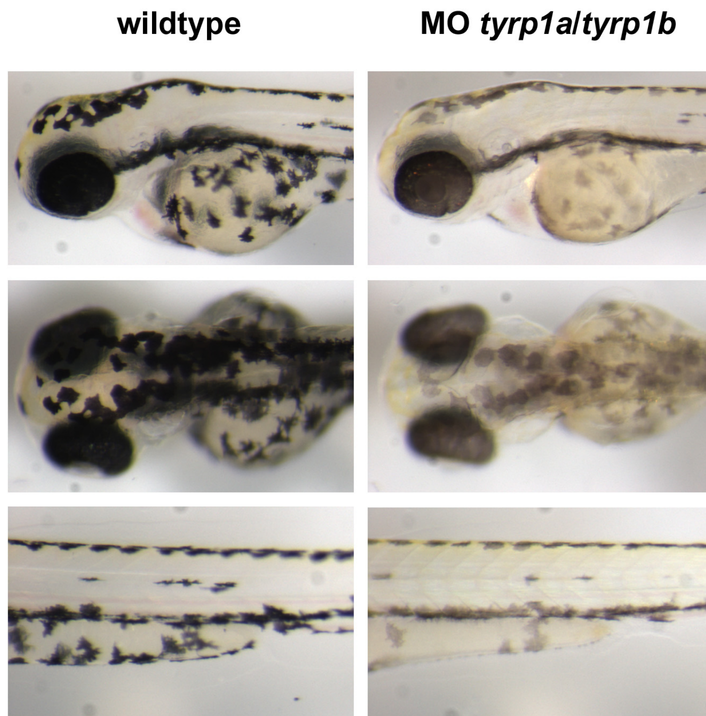

Figure 16 – Morpholino knockdown of *tyrp1* genes in zebrafish (3 dpf)
In comparison to black pigmented wildtype zebrafish embryos (left column), *tyrp1* double splice MO injected individuals show brown pigmentation in the eye and the melanophores on the body. From top to bottom: lateral view on head region, dorsal view on head region, lateral view on trunk region. Anterior is to the left.

At the ultrastructural level, double MO injected zebrafish showed severe defects in the structure, shape, size and pigment density of melanosomes. Similar melanosome defects and disruption of melanosome integrity have also been observed in Tyrp1-deficient mice (Moyer 1966) and humans (Kidson et al. 1993). Such defects might be explained by the participation of Tyrp1 in the melanogenic protein complex on the internal surface of the melanosomal membrane (Kobayashi and Hearing 2007). Thus, the present study provides further evidence for the hypothesis that Tyrp1 is essential for melanosome structure (Rittenhouse 1968; Sarangarajan and Boissy 2001). At the extreme, as seen in mice carrying the Tyrp1 *light* allele, hazardous melanin precursors can leak from the melanosome, leading to premature melanocyte death (Johnson and Jackson 1992). The duplication of *tyrp1* in teleost might have provided the genetic robustness to withstand such mutations.
In conclusion, the insights gained from knockdown of *tyrp1* genes in zebrafish suggests that the pigmentary readout of Tyrp1 function, i.e. the formation of black eumelanin, is conserved between teleosts and tetrapods and therefore was a feature of melanogenesis in their last common ancestor (Braasch et al. in preparation).

4.5 Evolution of pigmentation pathways by the FSGD

After investigating the impact of the FSGD on the evolution of the teleost pigment synthesis pathways, the analysis was extended to all known vertebrate pigmentation genes. To this end, I undertook database and literature surveys and generated a list of vertebrate pigmentation genes that included 128 genes involved in pigmentation in mouse, human and other mammals, birds, zebrafish and medaka (Braasch et al. submitted).
Importantly, many of the vertebrate pigmentation genes have other functions not related to pigmentation and in some cases pigmentation functions could become separated from other functions after duplication by subfunctionalization. In this case, the duplication would not increase the number of pigmentation genes *per se* as only one paralog retains the pigmentation function. However, since the paralog that keeps the pigmentation function will be released from some functional constraints imposed by other essential functions present in the ancestral gene, the duplication still might facilitate evolution of pigmentation by specializing one of the paralogs for the pigmentation. Thus, in the present study, the term 'pigmentation gene' corresponds to the presumed ancestral gene's pigmentation function at the time of its duplication.

4.5.1 Expansion of the teleost pigmentation gene repertoire in teleosts by the FSGD

Of the 128 pigmentation genes analyzed, 46 genes (35.9%) were retained in two copies after the FSGD in at least one of the five available teleost genomes, while for 82 genes (64.1%) one of the paralogs has been lost in all five teleosts. In a few cases, lineage-specific single gene duplications were found (Braasch et al. submitted).

On the genome-wide scale, the FSGD duplicated pigmentation genes take part in a pattern of macrosynteny connecting paralogous chromosomes (Fig. 17; F. Brunet, I. Braasch, J.-N. Volff, M. Schartl, unpublished data). The 46 pigmentation gene paralogs are derived from the reconstructed pre-FSGD karyotype (Kasahara et al. 2007) with at least two pairs of duplicates descending from 12 of the 13 protochromosomes. These data show that the duplication and retention of pigmentation genes are genome-wide and not restricted to a particular chromosomal subset. Using the established paralogy/orthology assignment of teleost chromosomes (Kasahara et al. 2007), four cases of reciprocal gene loss (RGL) after the divergence of zebrafish from acanthomorphs were identified.

Fig. 18 shows a putative scenario for the evolution of pigmentation genes in teleosts after the FSGD. At least around 40% of FSGD duplicated pigmentation genes had been retained in the last common euteleost ancestor of zebrafish, medaka, stickleback and pufferfishes. Afterwards, independent loss of several duplicates has occurred in the different teleost lineages, but to a similar extent. Thus, a similar FSGD duplicated retention rate of ≈ 30% is observed for pigmentation genes across the euteleost phylogeny (Fig. 18). This FSGD duplicate retention rate for teleost pigmentation genes is higher than different genome-wide estimations for pufferfishes and zebrafish, which range between 15-24% based on different approaches and data sets (Postlethwait et al. 2000; Jaillon et al. 2004; Woods et al. 2005; Brunet et al. 2006). For instance, 29.7% (38/128) of FSGD pigmentation gene duplicates have been retained in Tetraodon. This is significantly higher (χ^2-test, $p<0.001$) than the most thorough estimate of 15.4% for the genome-wide FSGD duplicate retention rate in Tetraodon (Brunet et al. 2006). Thus, pigmentation genes seem to have been preferentially retained in two copies in teleosts following the FSGD, which has added a significant proportion of additional genes potentially involved in pigmentation to teleost genomes (Braasch et al. submitted).

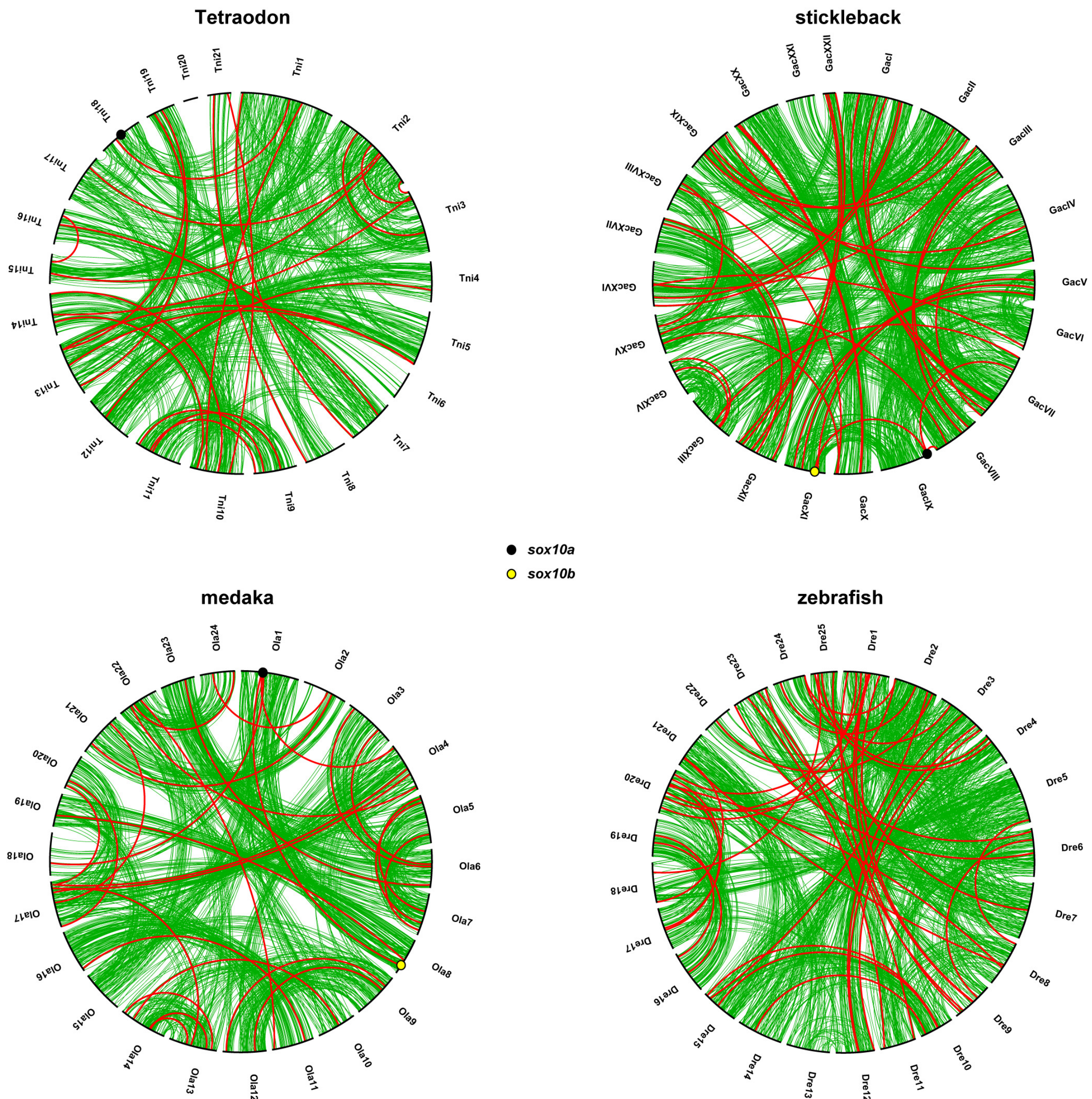

Figure 17 – Intragenomic macrosynteny patterns of duplicated teleost pigmentation genes
Green lines connect paralogous genes in the genomes of Tetraodon (Tni), stickleback (Gac), medaka (Ola) and zebrafish (Dre). Red lines connect paralogous pigmentation gene duplicates, showing that they mostly follow the major routes of FSGD duplication. The example of *sox10* paralogs illustrates common genome assembly problems that my be overcome with a comparative approach including information from several teleost genomes. The pufferfish *sox10b* gene has not been mapped to a chromosome. In zebrafish, *sox10a* is absent and *sox10b* is not included in the present genome assembly. In medaka and stickleback, however, both sox10 genes are present, included in the genome assembly and located to paralogous chromosomes derived from the FSGD. Tetraodon data modified from Braasch et al. submitted; other fish unpublished data by F. Brunet, I. Braasch.

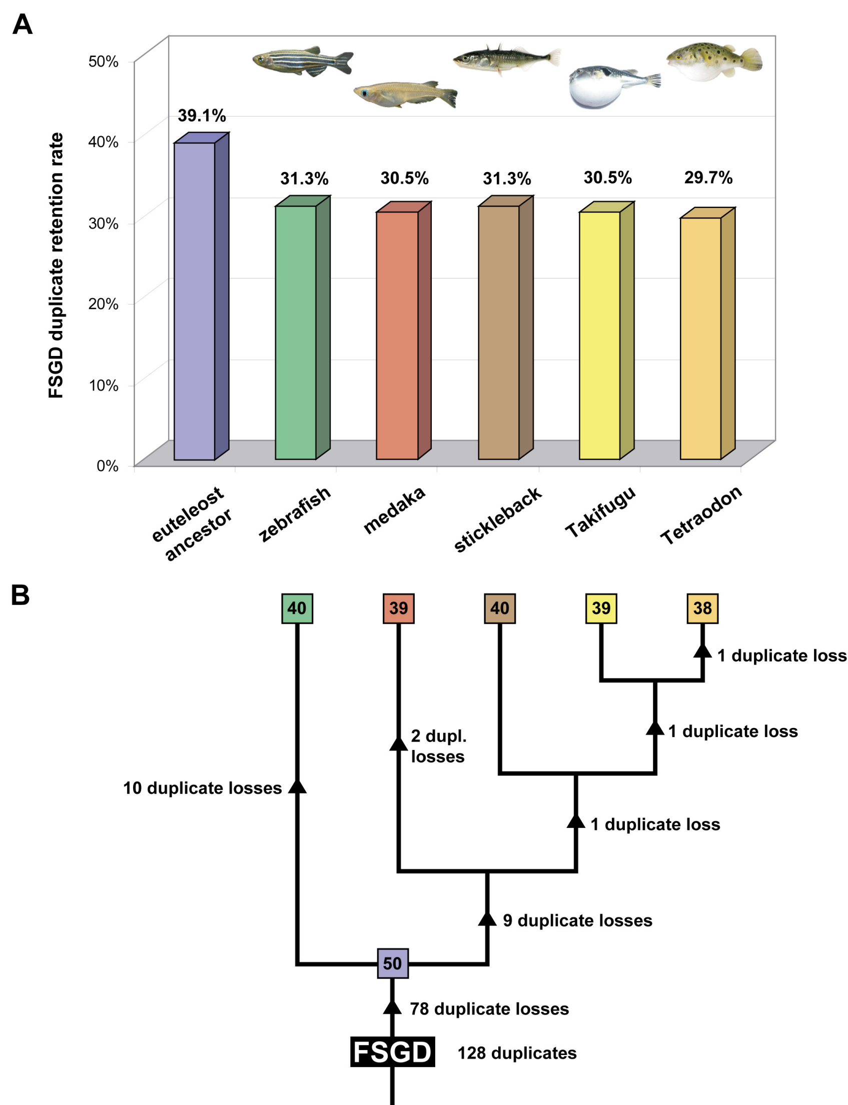

Figure 18 – Evolution of teleost pigmentation gene repertoires following FSGD
A) In the five extant teleost genomes, around 30% of FSGD duplicated pigmentation genes have been retained. B) Gene losses, mapped onto the teleost phylogeny (Setiamarga et al. 2008), have mainly occurred soon after the FSGD. Within acanthomorphs (medaka, stickleback, pufferfishes), further losses have occurred shortly after the split from the zebrafish lineage. The number of genes retained in duplicate are given in the boxes. From Braasch et al. submitted.

4.5.2 Pigmentation genes follow general patterns of WGD duplicate retention

There are important differences in the rate of FSGD duplicate retention among pigment cell types as well as among different functional categories for a given cell type (Tab. 2) and several interesting trends become apparent.

For example, almost all genes involved in melanosome construction have been reverted to a single gene status after FSGD. Genes of this category encode subunits of large protein complexes involved in the biogenesis of melanosomes and other lysosome-related organelles (Raposo and Marks 2007). Thus, these genes seem to have co-evolved to avoid detrimental imbalances and to keep stoichiometry of complex units, following the predictions of the 'gene balance hypothesis' (Papp et al. 2003).

Table 2 – FSGD duplicated retention rates according to pigmentation gene functions

category	# genes	FSGD duplicates	retention rate
all pigmentation genes	128	46	35.9%
melanophore genes	100	37	37.0%
melanophore development	45	22	48.9%
melanosome components	10	6	60.0%
melanosome construction	22	2	9.1%
melanosome transport	4	3	75.0%
regulation of melanogenesis	7	3	42.0%
systemic effects	13	2	15.4%
xanthophore genes	22	11	50.0%
xanthophore development	11	9	81.8%
pteridine synthesis	11	2	18.2%
iridophore genes	11	4	36.4%
uncategorized	10	3	30.0%
genes included in multiple categories	11	7	63.6%

Genes involved in melanosome transport within the pigment cell might also have been preferentially retained after the FSGD. Although this observation is based on four genes only, it might reveal important innovations in physiological color change. Physiological color change by moving melanosomes from the cell center to the periphery (darkening) or *vice versa* (lightening) is very important for many behavioral aspects and is highly sophisticated in teleosts compared to other vertebrates (Fujii 1993a; Fujii 2000).

Genes involved in the development of pigment cells are retained in duplicate at a high rate. This is the case for genes required for the development of melanophores and particularly for xanthophore development. These two categories contain many transcription factors and signaling pathway components, which are generally over-represented among FSGD duplicates in teleosts (Brunet et al. 2006) and duplicates generated by earlier vertebrate genome duplications (Putnam et al. 2008). Retention and functional divergence of duplicated developmental regulatory genes would be expected if the FSGD has made an important contribution to teleost-specific pigment cell evolution.

Most striking is the high retention rate for genes involved in xanthophore development of around 82%. This category consists mainly of genes for signaling and transcriptional control, reflecting the general high retention rate of developmental genes after the FSGD (Brunet et al. 2006). Furthermore, most of them (*Edn3*, *Ednrb1*, *Sox10*, *Pax3*, etc.) are involved in the development of more than one pigment cell type. Thus, it remains open whether the high FSGD retention rate for xanthophore developmental genes is indeed a xanthophore-specific character. If so, one would expect also an exceptional high

FSGD retention rate for xanthophore-specific genes, of which, unfortunately, only few have been identified so far.

In conclusion, the comparison of functional categories of pigmentation genes reveals that the retention of FSGD duplicates follows more general WGD duplicate retention patterns, i.e. preservation of protein complex stoichiometries and overrepresentation of developmental genes among retained duplicates. Genes with multiple functions for pigment cell development also seem to be preferentially retained.

4.5.3 Duplicated key players and functional modules of pigment cell development in teleosts

The current understanding of the signaling network in vertebrate melanocyte/-phore development with the Mitf transcription factor as master regulator in the central position (Béjar et al. 2003; Tachibana et al. 2003; Levy et al. 2006; Lin and Fisher 2007) is shown in Fig. 19. Nearly all known key players of this network, i.e. *Sox10, Pax3, Kit, Kitl, Ednrb1, Edn3* and *Mitf*, have been retained in duplicate after FSGD. Thus, it appears that large parts of the melanophore development network are present in two copies in teleosts and is an indication that this is also true for the developmental pathways of other pigment cells, particularly the xanthophore population.

Furthermore, there are subtle differences between teleost species in the retention of pathway component duplicates, such as the absence of the *sox10a* duplicate in zebrafish. Such modifications might be the genomic basis of species-specific differences in pigment cell repertoires like the absence of leucophores from larval pigment patterns in the zebrafish (Johnson et al. 1995).

According to a model recently developed by Freeling and Thomas (2006), increasing morphological complexity in the evolution of plants and animals is passively driven by recurrent WGDs. As result of WGDs, duplicated functional modules would accumulate in the gene pool and provide the genetic fundament for morphological innovations. Despite these and other hypotheses based on mathematical models (e.g. Wagner 1994; Evlampiev and Isambert 2008), the understanding of duplicated functional modules, pathways and networks in living organisms is rudimentary. The starch biosynthesis pathway in rice might be an example of a WGD-derived functional module or pathway (Wu et al. 2008).

The investigation of developmental and cellular evolution relies progressively on systems biology approaches integrating data from complex regulatory networks (Wagner et al. 2007; Arendt 2008; Chouard 2008). The teleost melanophore pathway may be an excellent model system to study in more detail the significance of duplication for the evolution of such networks at the level of multicellular organisms.

It largely remains to be investigated how the components of the pigment cell pathways have evolved after duplication with respect to functional divergence. One evolutionary fate could have been subfunctionalization of ancestral functions, as observed for the *mitf* paralogs (Lister et al. 2001; Altschmied et al. 2002). Since duplicated pathway components tend to become specialized for sister cell types (Arendt 2008), the different stripe melanophore populations in zebrafish might depend on

duplicated parts of the melanophore pathway (Mellgren and Johnson 2004). Another possible evolutionary route is the separation of pigmentary functions from other functions. This is seen for the Kit ligand-receptor pair. After FSGD, only *kitla* and *kita* are still involved in melanophore development, while *kitlb* and *kitb* have different, yet unknown functions not related to pigmentation (Mellgren and Johnson 2005; Hultman et al. 2007). Such cases can increase the evolvability of the pigmentary system since it releases it from functional constraints imposed by other essential functions of the ancestral genes. Finally, the duplicated pigmentation pathway components could have evolved new functions (neofunctionalization) required for the emergence of novelties in the teleost pigmentary system such as leucophores. Whatever the evolutionary fate of the individual pigmentation gene duplicates have been, the increase of complexity in the teleost pigmentary system is correlated with an increase in the repertoire of pigmentation pathway components that was generated in the FSGD.

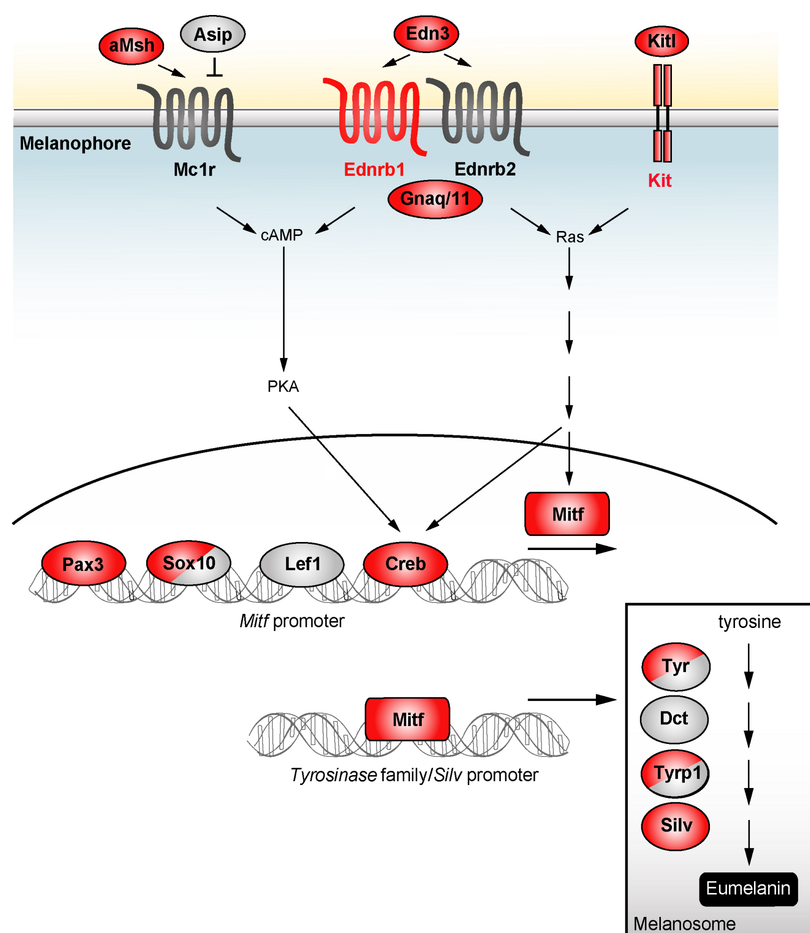

Figure 19 – Impact of the FSGD on the melanocyte/-phore signaling network
The signaling network is adapted from Tachibana et al. (2003); Levy et al. (2006); Lin and Fisher (2007). Diverse external signals act on the promoter of the *Mitf* transcription factor gene. Mitf is the master regulator of melanophore development that regulates the expression of melanogenic enzymes, which catalyze the biosynthesis of melanin in melanosomes. Red shading indicates duplications as result of the FSGD present in all five teleost genomes analyzed, gray shading indicates singleton genes. Molecules with divided red/gray shading indicate genes retained in duplicate in some teleost lineages but singleton in others. Most of the key regulators of the melanophore signaling network have been retained in two copies in teleosts. From Braasch et al. submitted.

5. CONCLUSIONS AND PERSPECTIVES

In the present study, I have investigated the impact of vertebrate genome duplications on the evolution of morphological structures and cell types that are important for the complexity and diversity of vertebrate life.

The analysis of the endothelin signaling system revealed that the early two WGDs had major influence on the components of the neural crest regulatory network. Furthermore, the timing of their emergence during chordate evolution as well as their co-option into the regulatory network are important aspects for the origin and diversification of the neural crest. Thereby, the relative timing of the emergence of the *bona fide* neural crest and the vertebrates WGDs is of central significance. Two scenarios can be proposed for the evolution of the neural crest by the two WGDs. In the subfunctionalization scenario, the neural crest gene regulatory network had been assembled before the WGDs, which then might have facilitated the diversification of the neural crest and its derivatives. In the neofunctionalization scenario, the duplication of transcription factors and signaling cascade members in the course of the WGDs released them from functional constraints, which in turn enabled their co-option into the newly establishing neural crest regulatory network. In this case, the WGDs were genomic prerequisites for the origin of the neural crest. The evaluation of these two scenarios will be a challenging task for future studies.

The investigation of pigmentation pathways shows that in teleosts the increase of complexity in the pigmentary system is correlated with a 30% increase in pigmentation genes. Large blocks of the functional modules of pigment cell development and differentiation have been retained after the FSGD, presumably providing the genetic raw material for teleost-specific pigment cell innovations. Importantly, WGDs enable at the same time the duplication of all genes found in a given, fine-tuned pathway, generating a complete copy of the ancestral network upon which selection can act. Such duplication of entire pathways would not be possible by succeeding simple gene duplications. The present study shows that entire duplicates of developmental networks may indeed exist in teleosts. For future studies of duplicated genes it will be essential to take into account their integration into such regulatory networks. In addition, it will be important to analyze if duplicated network blocks evolve independently or if interconnections between duplicated blocks exist. Finally, the functional analysis of the tyrosinase gene family in teleosts suggests that duplicated functional modules evolve in a lineage-specific manner. Together with the observed divergent resolution of duplicated pigmentation genes, the present study therefore strongly supports the duplication-diversification hypothesis of the teleost radiation.

In conclusion, the present study provides evidence for a major role of WGDs for phenotypic evolution in vertebrates. It therefore suggests that whole genome duplication events have been essential for the budding of some of the major branches in the *great Tree of Life*.

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7. ORIGINAL ARTICLES

7.1 Ingo Braasch, Jean-Nicolas Volff, and Manfred Schartl. 2009.
The endothelin system: evolution of vertebrate-specific ligand-receptor interactions by three rounds of genome duplication
Molecular Biology and Evolution, in press

7.2 Ingo Braasch, Manfred Schartl, and Jean-Nicolas Volff. 2007.
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7.4 Ingo Braasch, Daniel Liedtke, Jean-Nicolas Volff, and Manfred Schartl.
Functional evolution of *tyrp1* genes involved in melanin synthesis after duplication in fish
manuscript

7.5 Ingo Braasch, Frédéric Brunet, Jean-Nicolas Volff, and Manfred Schartl.
Evolution by genome duplication in vertebrates: insights from pigmentation pathways in teleost fishes
submitted manuscript

APPENDIX 1

The *GTP cyclohydrolase Ib* gene as a candidate for the red-yellow pigment patterning locus on the sex chromosomes of the platyfish *Xiphophorus maculatus*

Introduction

In numerous fish species, pigmentation genes evolve under sexual selection and are located on the sex chromosomes, especially in poeciliids (Lindholm and Breden 2002; Basolo 2006). Linkage of sex determination and pigmentation genes might facilitate speciation (Streelman et al. 2007). A primary example for this phenomenon is the guppy (*Poecilia reticulata*), where many conspicuous color patterns are linked to the sex chromosomes (Lindholm and Breden 2002). Several highly polymorphic color patterns are also encoded by loci located on the sex chromosomes of another poeciliid, the southern platyfish *Xiphophorus maculatus* (Kallman 1975; Basolo 2006). All these pigmentation loci are present in the pseudoautosomal region and are not sex-specific, but some are more strongly expressed in males (Basolo 2006). Some of the pigmentation loci appear to be under sexual selection (Fernandez and Morris 2008). Although the genetic basis of color patterns has been investigated for almost one century in *X. maculatus*, none of the responsible loci has been characterized at the molecular level yet.

The aim of the present study was to analyze a gene candidate for the red-yellow pigment patterning (*RY*) locus, which is located on the platyfish sex chromosomes in close proximity to the sex determination (*SD*) locus and to another pigmentation locus, the macromelanophore-determining (*MDL*) locus (Fig. A1; Weis and Schartl 1998). Multiple alleles have been described for the *RY* locus in natural platyfish populations influencing color patterning of the iris, the body and fins (Kallman 1975). For example in the platyfish strain analyzed in the present study (WLC 1235), two alleles of the *RY* locus are present (Fig. A2): *Dorsal red* (*Dr*) is located on the X chromosome and leads to orange-red pigmentation of the dorsal fin. *Anal red* (*Ar*) is present on the Y chromosome and causes orange-red coloration of the anal fin. Therefore, females of this platyfish strain (genotype X^{Dr}/X^{Dr}) have a red dorsal fin, but an inconspicuous, dull anal fin. Male individuals (genotype X^{Dr}/Y^{Ar}), in contrast, have a red dorsal fin as well as a red colored gonopodium, which is a specialized form of the anal fin in male poeciliids used as copulatory organ for internal fertilization.

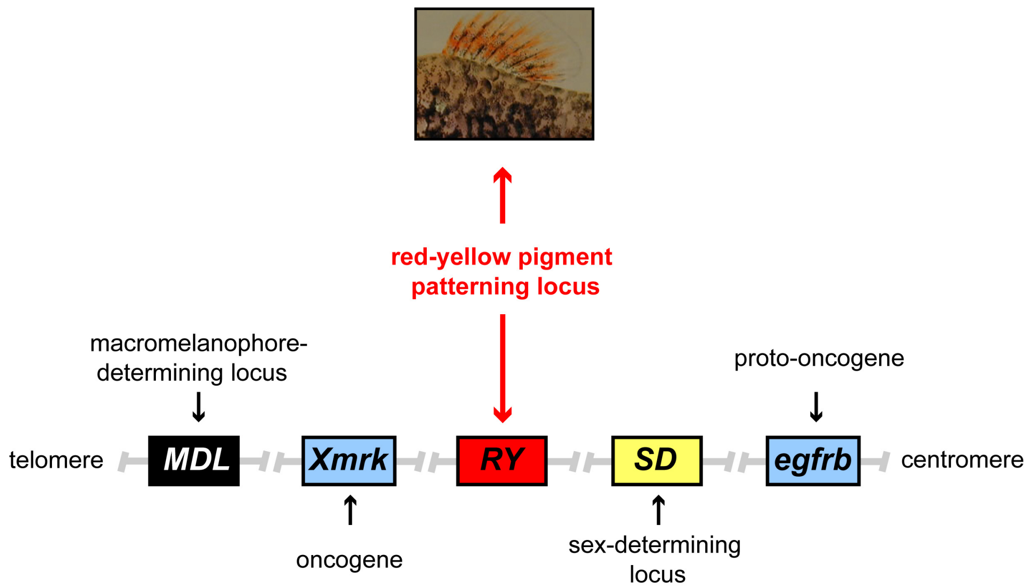

Figure A1 – The red-yellow pigment patterning (*RY*) locus on the platyfish sex chromosomes
RY is located in the subtelomeric region of the sex chromosomes in close proximity to the sex determining (SD) locus and another pigmentation locus, the macromelanophore-determining locus. *RY* and *SD* are flanked by the *egfrb* proto-oncogene and its duplicate, the *Xmrk* oncogene, which have been used as starting points to establish BAC contigs (Froschauer et al. 2002).

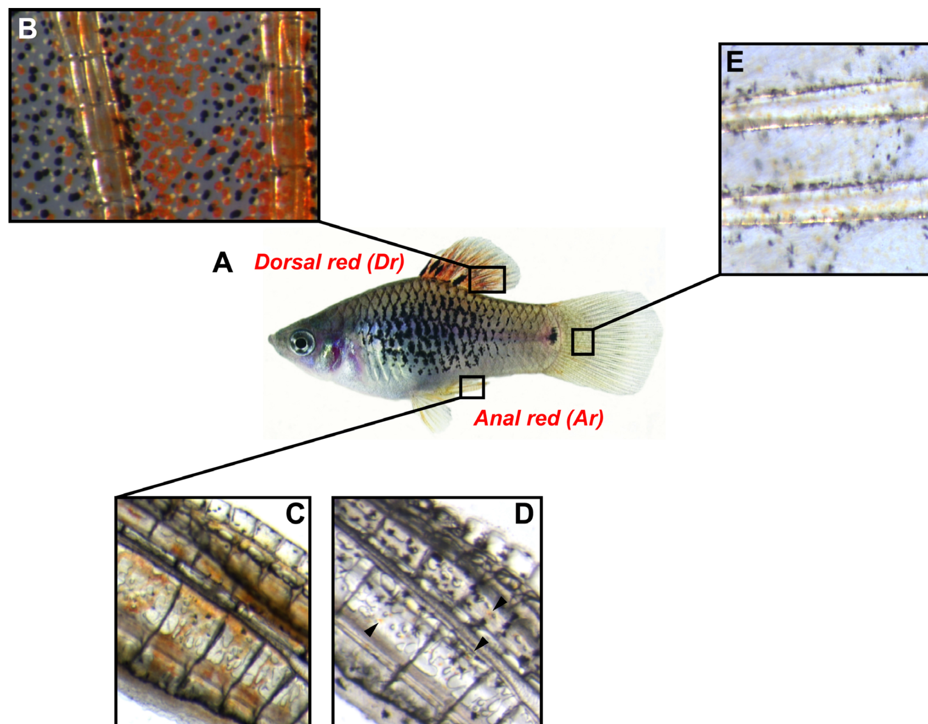

Figure A2 – Red-yellow pigment patterns in the platyfish
A) Male platyfish genotype X^{Dr}/Y^{Ar} (strain WLC 1235) showing the dorsal red pattern in the dorsal fin and the anal red pattern in the gonopodium. B) Close-up of the dorsal fin shows dark red xantho-erythrophores, yellow xanthophores and black melanophores surrounding two fin rays. C) Close-up of the gonopodium with red xantho-erythrophores and black melanophores. D) In a strain not bearing the *Ar* allele (*X. maculatus* WLC 1724), yellow xanthophores (arrowheads), but no xantho-erythrophores are found. E) Close-up of the caudal fin shows yellow xanthophores and black melanophores surrounding two fin rays. The anal fin of females from this strain (WLC 1235) as well as the dorsal fin of strains not bearing the *Dr* allele (e.g. WLC 1724) similarly only display yellow xanthophores.

The orange-reddish pigment patterns encoded by the *RY* locus are made up of xantho-erythrophore pigment cells (Goodrich et al. 1941; Matsumoto 1965). Importantly, the red pigment patterns are not due to a higher local density of pigment cells, but caused by the maturation of ubiquitously present yellow xanthophores into reddish xantho-erythrophores (Öktay 1964). During this maturation process, additional red pigment is synthesized and deposited surrounding the yellow pigment in the center of the cell (Öktay 1964; Matsumoto 1965). Biochemical studies revealed that this additional red pigment is drosopterin (Matsumoto 1965; Henze et al. 1977), a pteridine pigment synthesized starting from GTP (reviewed in Ziegler 2003).
It can be postulated that *RY* encodes a factor that regulates or initiates the synthesis of drosopterin in particular yellow xanthophores in a tightly controlled spatio-temporal pattern. Therefore, it was highly promising to identify sequence fragments of a *GTP cyclohydrolase I* (*gchI*) gene during the process of partial shotgun sequencing of the platyfish sex chromosomes (Schultheis et al. 2006; Schultheis 2007). GchI is the initial and rate-limiting enzyme of the pteridine synthesis pathway, which leads to the formation of pteridine pigments in xanthophores including drosopterins (Ziegler 2003).
In the present study, I have further analyzed this platyfish *gchI* gene. Although some aspects such as its classification to the xanthophore-related *gchIb* genes or its expression in red-pigmented fins make it an excellent candidate for *RY*, other aspects like the absence of an X chromosomal allele contradict this hypothesis.

Material and Methods

Fish strains

Platyfish strains WLC 1235 and WLC 1724 were kept under standard conditions (Kallman 1975) at the Biocenter of the University of Würzburg. WLC 1235 is a substrain of *X. maculatus* derived from the Jp163A inbred strain. The fish originate from the Rio Jamapa. They carry the following sex chromosomal pigmentation loci: Mdl^{Sr}-$xmrk^{A}$, RY^{Ar} on the Y and Mdl^{Sd}-$xmrk^{B}$, RY^{Dr} on the X chromosome. WLC 1724 is a strain of X. maculatus, origin Rio Jamapa. The fish carry the pigmentation locus Mdl^{Sp}-$Xmrk^{B}$ on the Y chromosome. The X chromosome is devoid of pigmentation loci. No *RY* allele has been identified in this strain.

RT-PCR expression analysis

Total RNA was extracted from fins of one male platyfish individual (strain WLC 1235) using the Nucleospin RNAII Kit (Machery & Nagel). cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Fermentas). RT-PCRs targeting *gchIb* were performed with the Xma_gchI-

ex2F/Xma_gchI-ex6R primer combination (Tab. A1). Primers to amplify platyfish *mc1r* (Mc1r-Xiph-F2/Mc1r-Xiph-R2) and *mc4r* (Xmcr1-F2/Xmcr1-R5) genes were a generous gift of Yvonne Selz.

BAC library screening

340bp coding sequence of the platyfish *gchIb* gene was PCR amplified from a cDNA pool of different adult platyfish tissues using primers Xma_gchI-ex2F and Xma_gchI-ex6R (Tab. A1) and blunt-end cloned into the pBS vector. Plasmid DNA was then used as probe to screen the platyfish BAC library twice by southern blot hybridization. The BAC library and the screening method are described in Froschauer et al. (2002).

BAC sequence analyses

Shotgun reads generated in an initial analysis of the platyfish sex chromosome BAC contigs (Schultheis et al. 2006), contigs provided by the platyfish sex chromosome sequencing initiative of Genoscope (http://www.genoscope.cns.fr/spip/Xiphophorus-maculatus-sex.html) and sequence reads derived by PCR amplification from BAC clones B14 (Y chromosomal) and C14 (autosomal) were assembled with the ContigExpress program of the Vector NTI Advance 10 suite (Invitrogen). For primer sequences see Tab. A1. Phylogenetic and synteny analyses were performed as described in (Braasch et al. 2007). The crystal structure of human GchIa (mmdbId:10183; Auerbach et al. 2000) was downloaded from the Molecular Modeling DataBase (http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml) and visualized using Cn3D 4.1 (Hogue 1997).

Table A1 – Primers used in the present study

Primer	**Sequence** (5’→3’)
gchIb-ex1F	ACCAARGGCTACCAYGAGAC
Xma_gchI-ex2F	CCACGATGAGATGGTGATTG
Xma-gchIb-ex2R	CAAATGATGTTCGCACAGG
Xmac-gchIB-ex3F	CAAACAAAAAAGTGGTGGGAC
Xmac-gchIB-ex4F	ATCTTCAGCCGCCGACTTC
Xmac-gchIB-ex4R	GAAGTCGGCGGCTGAAGAT
Xmac-gchIB-ex5R	CTTCTATAACCACAGCCACGC
Xma_gchI-ex6R	GGGTCCTCGAGGTAAACTCC
Mc1r-Xiph-F2	GCGGAACTTGTCGAGCTGCGT
Mc1r-Xiph-R2	CTGGCGCCTGCCTTTGTGAAAC
Xmcr1-F2	ATTCCTCTGCTGGCTGCTAC
Xmcr1-R5	GACATTTCAGGCTCTTCATCC
Xma-Actin1	GTAGGTGATGAAGCCCAGAGC
Xma-Actin2	GAGAAGAGCTACGAGCTCCCT

Results and Discussion

A *gchIb* gene is present on the platyfish Y chromosome

Previously, partial coding sequence of a *gchI* gene (exons 2, 3, 5, 6) was found in shotgun reads from BAC clones B14 and B29 from the platyfish Y chromosome BAC contig (Fig. A3) (Schultheis 2007). This region ("*ps-criptY* contig") is closely linked to the sex-determining (*SD*) locus (Froschauer 2003) as would be expected for a gene candidate for the *RY* locus (Fig. A1).

In teleosts, three distinct *gchI* genes are present: *gchIa*, *gchIb*, *gchIc* (Braasch et al. 2007). *GchIa* and *GchIb* were already present in the ancestor of gnathostomes, but *GchIb* has been lost subsequently and independently in mammals and birds (Braasch et al. 2008). *GchIc* has only been found so far in some teleost lineages (Braasch et al. 2007).

A phylogeny of vertebrate *GchI* genes (Fig. A4) shows that the platyfish Y chromosomal *gchI* sequence belong to the *GchIb* clade. This is further supported by synteny analysis, as platyfish *gchIbY* is linked to *criptY* and *msh2*, which are also linked to *GchIb* in other vertebrates (Fig. A3-A4).

The functions of the different teleost *gchI* genes remain unclear. The *gchIb* gene is expressed in xanthophores in zebrafish (Pelletier et al. 2001) suggesting that this paralog is indeed involved in pteridine pigment synthesis. Strikingly, *GchIb* has been lost in those vertebrate lineages that have lost xanthophores secondarily, i.e. mammals and birds, also pointing to a xanthophore function of this paralog (Braasch et al. 2008). Thus, *gchIbY* is an outstanding gene candidate for the platyfish *RY* locus.

Expression of platyfish *gchIb* correlates with *RY* fin patterns

Next, I asked about the expression of *gchIb* in different fins of male platyfish (genotype X^{Dr}/Y^{Ar}) displaying xantho-erythrophores in the dorsal fin and the gonopodium but not in the caudal and pectoral fins (Fig. A2). As shown in Fig. A5, *gchIb* expression indeed correlates with red-yellow pigment patterns. In contrast, expression of other putative pigmentation genes such as the melanocortin receptor genes *mc4r* and *mc1r* does not follow presence/absence of xantho-erythropohres. Thus, *gchIb* expression patterns further substantiates the hypothesis that *gchIb* might represent *RY*.

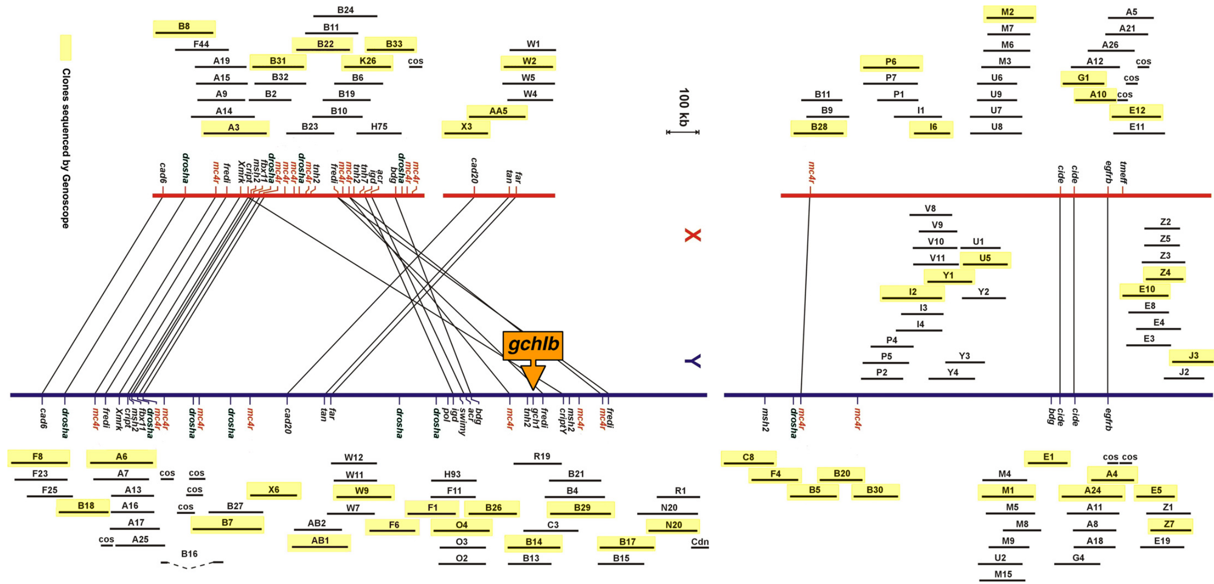

Figure A3 – BAC contig map of the platyfish sex chromosomes
The BAC contigs cover large parts of both sex chromosomes (X, Y) and have been established starting from *Xmrk*- and *egfrb*-containing BAC clones. A *gchIb* gene is present close to *ps-criptY* on the Y chromosome. BAC clones sequenced in collaboration with Genoscope are indicated in yellow. Multiple copies of another putative pigmentation gene, *mc4r* (red), are also present on both sex chromosomes. Modified from Schultheis et al. (2007) and unpublished results.

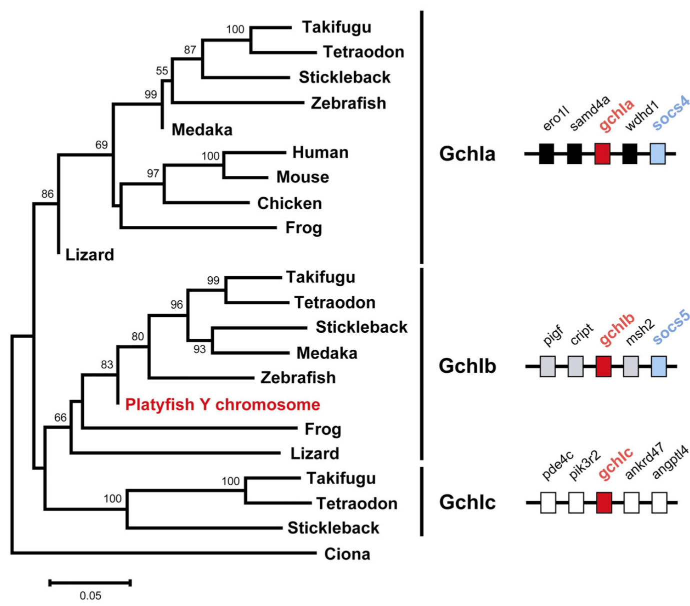

Figure A4 – Phylogenetic classification of the platyfish Y chromosomal GchI sequence
The vertebrate GchI neighbor-joining phylogeny is based on 269 amino acid positions and rooted with the single GchI protein from the urochordate Ciona. Bootstrap values above 50% based on 10,000 replications are shown. Three clades, GchIa, GchIb and GchIc can be identified. Syntenies for GchI clades are shown to the right. The platyfish Y chromosomal GchI sequence belongs to the GchIb clade. Further information on GchI evolution, synteny analysis and accession numbers is given in Braasch et al. (2007).

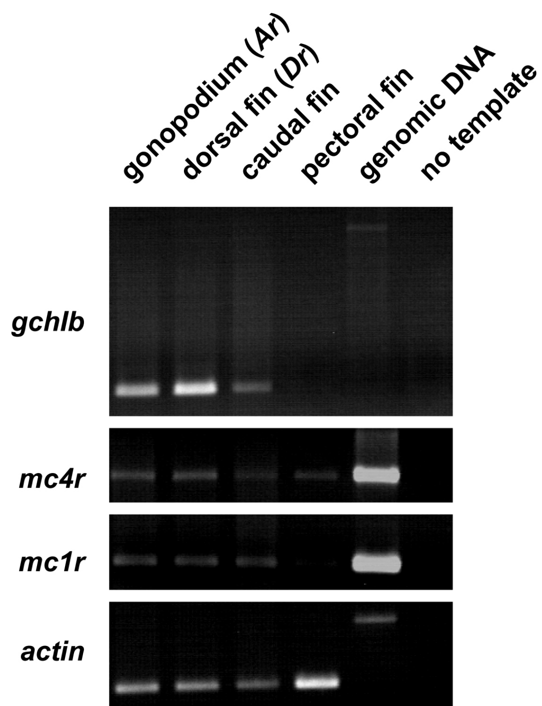

Figure A5 – Expression of platyfish *gchIb*
gchIb is strongly expressed in the gonopodium and the dorsal fin of strain WLC 1235 containing xantho-erythrophores. Expression at a lower level is also found in the caudal fin, which displays yellow xanthophores (Fig. A2), but not in the hyaline pectoral fin. Note that the primer set used here might amplify different *gchIb* gene copies simultaneously. Expression of two other pigmentation genes (*mc1r*, *mc4r*) does not correlate with the *RY* pigment pattern.

gchIb is present in at least two copies in the platyfish genome

Under the hypothesis that *gchIb* represents *RY*, another *gchIb* allele, the *Dr* associated allele, must be present on the X chromosome in the strain used for BAC library construction (genotype X^{Dr}/Y^{Ar}). Many genes are found in multiple copies on the platyfish sex chromosomes (Schultheis et al. 2006), including those linked to *gchIbY* (*cript*, *msh*), which are also present on the X chromosome (Fig. A3). By screening the BAC library for additional *gchIb* copies, in total nine *gchIb*-positive BAC clones were identified (Tab. A2). All clones had been previously characterized by BAC library screens with other probes.

Table A2 – Characterization of *gchIb*-positive BAC clones

BAC clone	location	further sequences present[a]
B13	Y chromosome : *ps-criptY* contig	*Xmcr, dc2*
B14	Y chromosome : *ps-criptY* contig	*Xmcr, dc2, fredi, helicase, protease*
R19	Y chromosome : *ps-criptY* contig	*fredi, helicase*
C1	autosomal *cript* contig	*Xcript, msh2*
C2	autosomal *cript* contig	*Xcript, msh2*
C4	autosomal *cript* contig	*Xcript, msh2*
C5	autosomal *cript* contig	*Xcript, msh2*
C6	autosomal *cript* contig	*Xcript, msh2*
C7	autosomal *cript* contig	*Xcript, msh2*

[a] identified by previous BAC library hybridizations (Froschauer 2003; Schultheis 2007)

Three of the BAC clones are part of the *ps-criptY* contig on the Y chromosome, and another six clones belong to the *cript* contig. The *cript* contig has been previously mapped to an autosome by fluorescence *in situ* hybridization (FISH) (Froschauer 2003). Thus, *gchIb* is present in at least two copies in the platyfish genome. No X chromosomal BAC clone was recovered in our *gchIb* screen. This might be due to a gap in the BAC library covering the platyfish X chromosome. Otherwise, absence of a *gchIb* allele from the X would make this gene unlikely to represent *RY*. Another possible explanation for the absence of *gchIb* from the X chromosome may be that *Ar* and *Dr* are not allelic but are encoded by different genes.

Sequence analysis of *gchIbY* and its autosomal paralog

The *gchIbY* gene and its autosomal paralog are clearly derived from a common precursor that was duplicated *en bloc* with several surrounding genes. However, so far only partial sequences of *gchIbY*, most importantly missing exon 1, have been obtained. Thus, I assembled scaffolds integrating all available sequence information including contigs provided by Genoscope and resequenced parts of BAC B14 with *gchIbY* derived primers (Tab. A1). These primers were also used to sequence the paralogous sequence of the autosomal *gchIb* gene from BAC C4 (Fig. A6A). In the BAC B14 scaffold, no *gchIb* exon 1-like sequence (as judged from *gchIb* sequences from other teleosts) was found in 38.3kb upstream of *gchIbY* exon 2. No PCR fragment could be amplified using a degenerate forward primer in exon 1 (Tab. A1) derived from an alignment of teleost *gchIb* sequences, neither from the Y-chromosomal BAC B14 nor the autosomal BAC C4. In addition, no putative alternative first exon for *gchIbY* was predicted using gene prediction software tools (data not shown). Therefore, *gchIbY* might well be a pseudogene just like many of its neighboring genes (e.g. *ps-criptY*).

The comparison of *gchIbY* and its autosomal paralog revealed their high similarity (Fig. A6). One obvious difference is the integration of a *TX-1* transposable element into intron 4 of *gchIbY* (Fig. A6A). This type of element is found in multiple copies on the platyfish sex chromosomes (Schartl et al. 1999). In the coding sequence, three single nucleotide polymorphisms (SNPs) between the two *gchIb* duplicates are present (i.e. 99.5% nucleotide sequence identity), and all three are non-synonymous (Fig. A6). Two of these substitutions occur in positions generally conserved in teleosts. The autosomal *gchIb* sequence, in contrast, is identical to the other teleosts at this position. On the one hand, these non-synonymous changes (and the absence of synonymous substitutions) might indicate that the *gchIbY* paralog is under positive selection. On the other hand, the second SNP leads to a conservative amino acid substitution from histidine to arginine (Fig. A6B). This histidine residue is strictly conserved among eu- and prokaryote GchI proteins and is in close proximity to the active site (Fig. A6C), which contains an essential zinc ion (Nar et al. 1995; Auerbach et al. 2000; Rebelo et al. 2003). The mutation of this site might thus be another sign that *gchIbY* is a pseudogene.

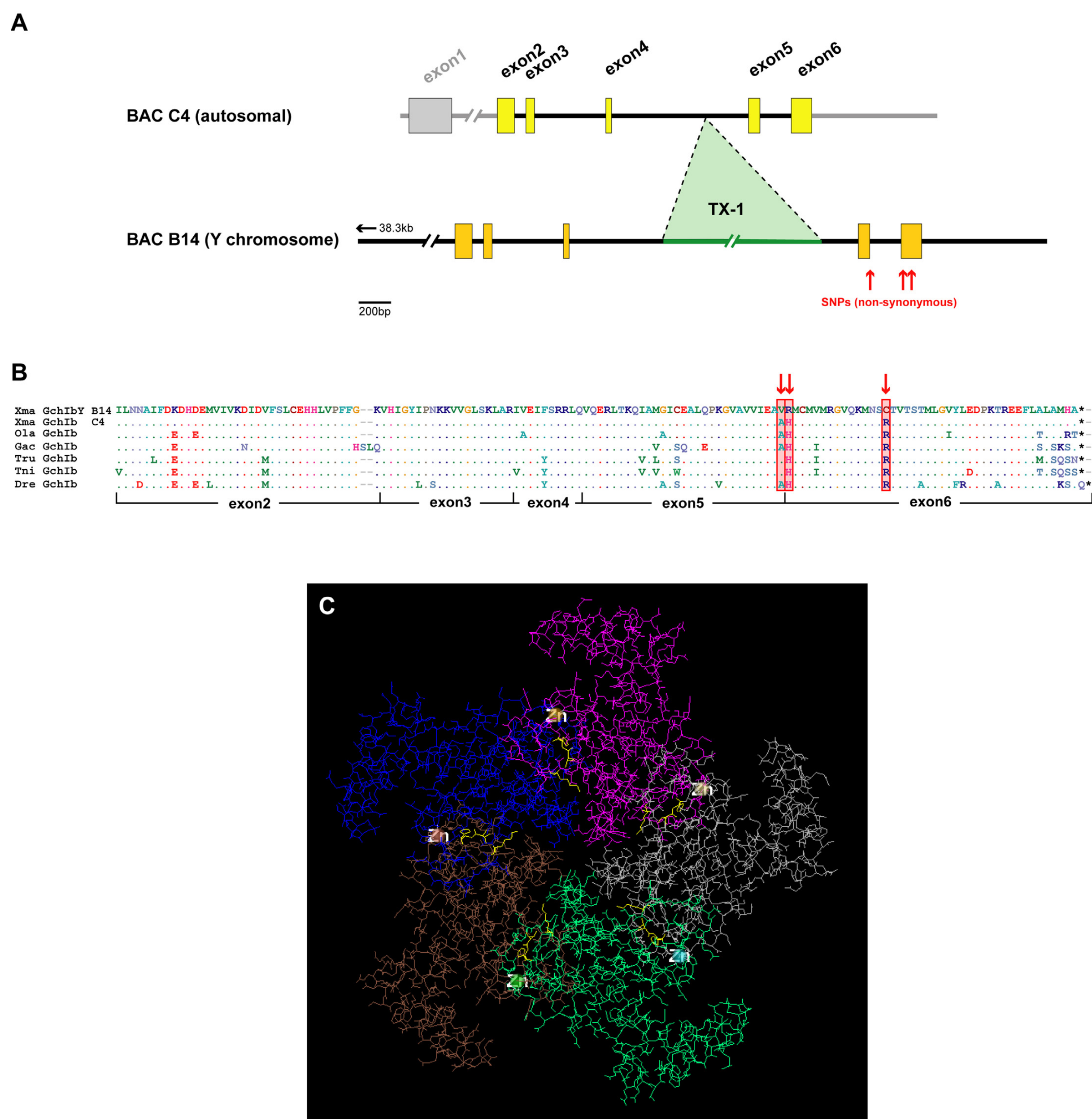

Figure A6 – Comparison of *gchIbY* and its autosomal precursor
A) Structural comparison of platyfish *gchIb* paralogs. Unsequenced parts of BAC C4 are gray. Exon 1 of *gchIb* in C4 is predicted from an alignment of teleost *gchIb* sequences, but its presence remains to be demonstrated. B) Alignment of teleost GchIb proteins. Arrows indicate amino acid substitutions between platyfish GchIb copies. C) Three dimensional structure of the human GchIa pentamer (Auerbach et al. 2000). Residues homologous to positions differing between platyfish GchIb copies are highlighted in yellow. They are in close proximity to the active site of the GchI enzyme harboring a zinc ion.

Conclusions and Perspectives

Its location on the platyfish Y chromosome, its putative function as initial enzyme for the synthesis of pteridine pigments, and the *gchIb* expression in fins containing the red xantho-erythrophores make *gchIbY* an outstanding candidate for the *RY* locus. However, I did not find evidence for the presence of another *gchIb* copy on the X chromosome, which would be expected if *gchIb* were indeed *RY*. This might also be due to a gap in the BAC library. In addition, there is also so far no evidence for a complete coding sequence of the Y chromosomal *gchIbY* copy, which could be an artifact of the sequence assembly in this highly repetitive chromosomal region. It thus remains elusive, whether *gchIbY* constitutes a functional gene.

FISH experiments with a *gchIb* probe on platyfish karyotypes should be used to check for the presence or absence of another putative *gchIb* gene on the X chromosome or somewhere else in the genome. Primer walking on *gchIbY* positive BAC clones should further be performed to confirm the sequence assembly upstream of *gchIbY* exon 2. 5'-RACE-PCR could be used to identify the sequence of the first exon of the platyfish *gchIb* genes. These additional experiments could help to clarify whether or not *gchIbY* must be excluded as a candidate gene for *RY*.

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APPENDIX 2 : CURRICULUM VITAE

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Supervisors: Prof. Dr. Dr. Manfred Schartl, Prof. Dr. Jean-Nicolas Volff (Lyon)

Internships and Courses

03/2000 – 07/2002	Internship: ***Genome duplication in fish*** Lehrstuhl für Zoologie/Evolutionsbiologie, University of Konstanz Supervisor: Prof. Axel Meyer
09/2002 – 11/2002	Internship: ***Mapping of pancreas mutants in the zebrafish*** Department of Developmental Biology, University of Freiburg, Germany Supervisor: Prof. Wolfgang Driever
11/2002 – 3/2003	Internship: ***Sequencing and analysis of cichlid EST libraries*** Lehrstuhl für Zoologie/Evolutionsbiologie, University of Konstanz Supervisor: Prof. Axel Meyer
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Teaching Experience

2003	**Teaching Assistant in Zoology** dissection course for biology students, University of Konstanz
2004 – 2006	**Supervision of Student Interships** University of Konstanz and University of Würzburg
2005 -2008	**Teaching Assistant in Biochemistry** practical course for medical students, University of Würzburg

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Professional Memberships

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European Society for Evolutionary Biology (ESEB)
European Society for Evolutionary Developmental Biology (EED)

Conference Presentations

2004 — ***Genome evolution of cichlid fishes – insights from receptor tyrosine kinases***
Poster at *Genomes & Evolution 2004* (SMBE/AGA meeting)
Pennsylvania State University, USA

2005 — ***Expression and adaptive evolution of the receptor tyrosine kinase csf1ra in egg-mimicking color patterns of East African cichlid fishes***

Evolution of pigmentation by gene and genome duplication in fish

Posters at the *4th European Zebrafish Genetics and Development Meeting*
Dresden, Germany

Comparative genomic investigation of the pdgfrβ-csf1r locus in cichlid and other teleost fishes and its implications for the evolution of teleost coloration
Poster at *ESEBX*
Krakow, Poland

2006 — ***Evolution of pigmentation pathways by gene and genome duplication in fish***
Poster at the *First and Founding Meeting of the EED*
Prague, Czech Republic

2007 — ***Evolution of pigmentation pathways by gene and genome duplication in fish***
Poster at *ESEBXI*
Uppsala, Sweden

2008 — ***Evolutionary developmental genomics of pigmentation pathways in fish***
Talk at *SMBE08*
Barcelona, Spain

APPENDIX 3: LIST OF PUBLICATIONS

1. Taylor, J. S., Y. Van de Peer, I. Braasch, and A. Meyer. 2001. **Comparative genomics provides evidence for an ancient genome duplication event in fish.** Philos Trans R Soc Lond B Biol Sci **356**:1661-1679.

2. Van de Peer, Y., J. S. Taylor, I. Braasch, and A. Meyer. 2001. **The ghost of selection past: rates of evolution and functional divergence of anciently duplicated genes**. J Mol Evol **53**:436-446.

3. Taylor, J. S., I. Braasch, T. Frickey, A. Meyer, and Y. Van de Peer. 2003. **Genome duplication, a trait shared by 22000 species of ray-finned fish**. Genome Res **13**:382-390.

4. Lang, M., T. Miyake, I. Braasch, D. Tinnemore, N. Siegel, W. Salzburger, C. T. Amemiya, and A. Meyer. 2006. **A BAC library of the East African haplochromine cichlid fish Astatotilapia burtoni.** J Exp Zoolog B Mol Dev Evol **306**:35-44.

5. Steinke, D., W. Salzburger, I. Braasch, and A. Meyer. 2006. **Many genes in fish have species-specific asymmetric rates of molecular evolution.** BMC Genomics **7**:20.

6. Froschauer, A., I. Braasch, and J. N. Volff. 2006. **Fish genomes, comparative genomics and vertebrate evolution.** Current Genomics **7**:43-57.

7. Braasch, I., W. Salzburger, and A. Meyer. 2006. **Asymmetric evolution in two fish-specifically duplicated receptor tyrosine kinase paralogons involved in teleost coloration**. Mol Biol Evol **23**:1192-1202.

8. Schultheis, C., Q. Zhou, A. Froschauer, I. Nanda, Y. Selz, C. Schmidt, S. Matschl, M. Wenning, A.-M. Veith, M. Naciri, R. Hanel, I. Braasch, A. Dettai, A. Böhne, C. Ozouf-Costaz, S. Chilmonczyk, B. Ségurens, A. Couloux, S. Bernard-Samain, M. Schmid, M. Schartl, and J. N. Volff. 2006. **Molecular analysis of the sex-determining region of the platyfish *Xiphophorus maculatus*.** Zebrafish **3**:299-303.

9. Braasch, I., M. Schartl, and J. N. Volff. 2007. **Evolution of pigment synthesis pathways by gene and genome duplication in fish.** BMC Evol Biol **7**:74.

10. Selz, Y., I. Braasch, C. Hoffmann, C. Schmidt, C. Schultheis, M. Schartl, and J. N. Volff. 2007. **Evolution of melanocortin receptors in teleost fish: The melanocortin type 1 receptor.** Gene **401**:114-122.

11. Siegel, N., S. Hoegg, W. Salzburger, I. Braasch, and A. Meyer. 2007. **Comparative genomics of ParaHox clusters of teleost fishes: gene cluster breakup and the retention of gene sets following whole genome duplications.** BMC Genomics **8**:312.

12. Salzburger, W.*, I. Braasch*, and A. Meyer. 2007. **Adaptive sequence evolution in a color gene involved in the formation of the characteristic egg-dummies of male haplochromine cichlid fishes.** BMC Biol **5**:51.

13. Salzburger, W., S. C. Renn, D. Steinke, I. Braasch, H. A. Hofmann, and A. Meyer. 2008. **Annotation of expressed sequence tags for the East African cichlid fish Astatotilapia burtoni and evolutionary analyses of cichlid ORFs.** BMC Genomics **9**:96.

14. Böhne, A., C. Schultheis, Q. Zhou, A. Froschauer, C. Schmidt, Y. Selz, I. Braasch, C. Ozouf-Costaz, A. Dettai, B. Ségurens, A. Couloux, S. Bernard-Samain, S. Chilmonczyk, A. Gannouni, K. Madani, F. Brunet, D. Galiana-Arnoux, M. Schartl, and J. N. Volff. 2008. **Identification of new gene candidates on the sex chromosomes of the platyfish *Xiphophorus maculatus*.** Cybium **32**:suppl. 69-71.

15. Braasch, I., J. N. Volff, and M. Schartl. 2008. **The evolution of teleost pigmentation and the fish-specific genome duplication.** Journal of Fish Biology **73**:1891-1918.

16. Klüver, N., A. Herpin, I. Braasch, J. Drießle, and M. Schartl. 2009. **Regulatory back-up circuit of medaka *wt1* co-orthologs ensures PGC maintenance.** Dev Biol **325**:179-188.

17. Braasch, I., J. N. Volff, and M. Schartl. 2009. **The endothelin system: evolution of vertebrate-specific ligand-receptor interactions by three rounds of genome duplication.** Mol Biol Evol, in press

18. Braasch, I., F. Brunet, J. N. Volff, and M. Schartl. **Evolution by genome duplication: insights from pigmentation pathways in teleost fishes.** submitted

19. Herpin, A.*, I. Braasch*, A. Krais, E. Thoma, and M. Schartl. **Transcriptional rewiring of the sex determining *dmrt1* gene duplicate by transposable elements.** submitted

20. Salzburger, W.*, D. Steinke*, I. Braasch*, and A. Meyer. **Genome desertification in mammals: gene deserts explain the uneven distribution of genes in placental mammalian genomes.** submitted

21. Braasch, I., D. Liedtke, J. N. Volff, and M. Schartl. **Functional evolution of *tyrp1* genes involved in melanin synthesis after duplication in fish.** manuscript in preparation

* equal contribution

APPENDIX 4: ERKLÄRUNG

Erklärung gemäß § 4, Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität Würzburg vom 15. März 1999

Hiermit erkläre ich, die vorgelegte Dissertation selbstständig angefertigt zu haben und keine anderen als die von mir angegeben Quellen und Hilfsmittel benutzt zu haben. Alle aus der Literatur entnommenen Stellen sind als solche kenntlich gemacht.

Des Weiteren erkläre ich, dass die vorliegende Arbeit weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen hat.

Zuvor habe ich keine akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 12. Januar 2009

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Ingo Braasch