Gene Amplification and Functional Diversification of Melanocortin 4 Receptor at an Extremely Polymorphic Locus Controlling Sexual Maturation in the Platyfish

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ABSTRACT In two swordtail species of the genus *Xiphophorus*, the onset of puberty has been shown to be modulated at the *P* locus by sequence polymorphism and gene copy-number variation affecting the type 4 melanocortin hormone receptor *Mc4r*. The system works through the interaction of two allelic types, one encoding wild type and the other dominant-negative receptors. We have analyzed the structure and evolution of the *P* locus in the platyfish *Xiphophorus maculatus*, where as many as nine alleles of *P* determining the onset of sexual maturity in males and females, fecundity in females, and adult size in males are located on both the X and Y chromosomes in a region linked to the master sex-determining locus. In this species, *mc4r* has been amplified to up to 10 copies on both the X and Y chromosomes through recent large serial duplications. Subsequently, *mc4r* paralogues have diverged considerably into many different subtypes. Certain copies have acquired new untranslated regions through genomic rearrangements, and transposable element insertions and other mutations have accumulated in promoter regions, possibly explaining observed deviations from the classical *mc4r* transcriptional pattern. In the *mc4r*-coding sequence, in-frame insertions and deletions as well as nonsense and missense mutations have generated a high diversity of *Mc4r*-predicted proteins. Most of these variants are expressed in embryos, adults, and/or tumors. Functional receptor characterization demonstrated major divergence in pharmacological behavior for *Mc4r* receptors encoded by different copies of platyfish *mc4r*, with differences in constitutive activity as well as binding and stimulation by hormones. The high degree of allelic and copy-number variation observed between individuals can explain the high level of polymorphism for sexual maturation, fecundity, and body size in the platyfish: multiple combinations of *Mc4r* variants with different biochemical properties might interact to modulate the melanocortin signaling that regulates the hypothalamus–pituitary–gonadal axis.

TELEOST fish of the genus *Xiphophorus*, which comprise two major and morphologically quite divergent groups, the platyfish and swordtails, are prominent models for the study of polymorphic traits such as sexual development, re-

production, pigmentation, and cancer (Kallman 1975, 1984; Anders and Anders 1978; Volff and Scharl 2002; Meierjohann and Scharl 2006; Scharl 2008; Schultheis *et al.* 2009). In the platyfish *Xiphophorus maculatus* and in other *Xiphophorus* species, many genetic loci involved in the control of these traits are located on the sex chromosomes, where they are linked to the sex-determining region (Gutbrod and Scharl 1999 and references therein).

Three types of sex chromosomes have been described in *X. maculatus*: X, Y, and W. Males can be XY or YY, females XX, WX, or WY (Kallman 1984). Many genetic loci have been

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mapped to the X and Y chromosomes of the platyfish, but information on the W chromosome is still scarce. The master sex-determining locus *SD*, also referred to as *SEX*, which determines the sexual phenotype of individuals (Kallman 1984), has not been identified so far at the molecular level. One well-studied locus linked to *SD* is the so-called *Tumor* locus (*Tu*), which is responsible for the formation of spontaneous melanoma in certain *Xiphophorus* interspecific hybrids (Gordon 1927; Kosswig 1928; Meierjohann and Scharl 2006; Scharl 2008). The critical constituent of this locus is a gene, *xmrk*, which encodes a mutationally activated version of the epidermal growth factor receptor (Wittbrodt *et al.* 1989).

Another important locus in this region on the sex chromosomes of the platyfish is the *Pituitary* (or *Puberty*) locus *P*, which controls the onset of sexual maturity in males and females, as well as adult size in males and fecundity in females (Kallman and Schreibman 1973; Kallman and Borkoski 1977; Schreibman *et al.* 1994). The *P* locus is highly polymorphic. As many as nine different alleles have been identified that determine different timing of puberty and adult male size (Kallman 1989). Cosegregation analyses mapped them all to both the X and Y chromosomes of the platyfish. As a consequence, *X. maculatus* shows a high phenotypic diversity with respect to the onset of sexual maturation, from early maturing to late-maturing animals with a continuum of intermediate phenotypes (Kallman 1989). Since males stop growing when they reach puberty, this polymorphism is also associated with great variation in adult body size: early maturing males are smaller and late-maturing animals are larger, with many intermediates in onset of puberty and, consequently, body size. In several *Xiphophorus* species, it has been shown that smaller vs. larger fishes display different reproductive behaviors: larger fishes defend territories visited by females and court them in a ritualized manner, while smaller fishes search for “sneak” mating opportunities by moving between territories and directly approach females (Zimmerer 1982; Ryan and Causey 1989; Ryan *et al.* 1990; Ryan and Rosenthal 2001). Hence, the *P* locus also controls reproductive behavior variation in *Xiphophorus*.

Due to the multiplicity of *P* alleles and associated phenotypes, genetic analyses are complicated to conduct in *X. maculatus*. In contrast, *X. nigrensis* and *X. multilineatus*, two swordtail species distantly related to *X. maculatus* within the genus *Xiphophorus* (Kang *et al.* 2013), present only three major *P*-determined male phenotypes (small/early maturing, large/late-maturing, and one intermediate phenotype). In these species, it has been shown that puberty onset, body size, and reproductive behavior are modulated by sequence polymorphism and gene copy-number variation affecting the type 4 receptor for melanocortin hormones, Mc4r (Lampert *et al.* 2010).

Melanocortins are neuromodulatory peptide hormones produced through post-translational processing of the precursor pro-opiomelanocortin. They include the melanocyte-stimulating hormones (α -, β -, and γ -MSH) and the adrenocorticotrophic

hormone ACTH. Melanocortins are expressed mainly but not exclusively in the pituitary gland and the central nervous system. These hormones are involved in diverse physiological processes including pigmentation, steroidogenesis, appetite and body weight regulation, inflammatory responses, cardiovascular system regulation, stimulation of exocrine gland secretion, and sexual function (Cone 2006). In vertebrates, melanocortin hormones mediate their effects through interaction with five types of G-protein-coupled receptors, Mc1r–Mc5r. The Mc4r receptor is involved in the control of food intake, energy balance, and body weight in mammals with some direct and indirect reported functions in reproduction (Adan *et al.* 2006; Van der Ploeg *et al.* 2002; Tao 2010; Israel *et al.* 2012 and references therein).

In *X. nigrensis* and *X. multilineatus*, both functional and defective *mc4r* copies have been identified. Defective copies have lost their capability for signal transduction because microdeletions have removed important cysteine residues from the carboxy-terminal region of the receptor. Such defective copies were detected only in males, being located on the Y chromosome (Lampert *et al.* 2010). Copy-number variation correlating with *mc4r* expression levels was observed for the defective *mc4r* copies in males: the highest copy-number and expression levels were found in large late-maturing males, while small early maturing males showed low copy number and low levels of expression. According to results from cell culture experiments, defective versions might compete with functional receptors to form Mc4r dimers. High expression of nonfunctional receptors would reduce the formation of functional Mc4r dimers. This would consequently inhibit melanocortin signaling, delay puberty, and allow further growth of the fish.

Through genetic analysis it was shown that *mc4r* copies are located on the sex chromosomes of *X. nigrensis* and *X. multilineatus* (Lampert *et al.* 2010). However, no genomic data are available to assess the origin, structure, and evolution of the *P* locus in any *Xiphophorus* species. In addition, characterization of the multiple *P* locus alleles involved in the very high polymorphism affecting body size and onset of puberty in *X. maculatus* requires a precise molecular characterization of the different *mc4r* copies involved. The genome sequence of a platyfish female ($n = 28$, XX) has been recently published (Scharl *et al.* 2013), but the structural complexity of the sex-determining region, with many recent large duplications and a high concentration of transposable elements and other repeat sequences, makes it very difficult to assemble from shotgun genomic data. In addition, this genome project, performed on female DNA, does not provide any insight into Y-linked *mc4r* copies in *X. maculatus*.

To characterize the structure of the *P* locus in the platyfish, a bacterial artificial chromosome (BAC) library has been established from XY platyfish males (Froschauer *et al.* 2002). BAC clones linked to the sex-determining region of the sex chromosomes have been isolated, assembled, and partially sequenced (Froschauer *et al.* 2002; Volff *et al.* 2003; Schultheis *et al.* 2006). We report here that as many

as 10 copies of the *mc4r* gene are present on each X and Y chromosome of the platyfish. Expression and pharmacological analyses strongly support functional diversification of Mc4r receptors after duplication through mutation of both regulatory and coding regions.

Materials and Methods

Fish and cell culture

Fish were reared at the Plateau de Recherche Expérimentale de Criblage in Vivo of the SFR BioSciences Gerland-Lyon Sud (US8/UMS3444, Lyon, France) and at the fish facility of Biozentrum at the University of Würzburg (Germany). Tumor tissues were obtained from backcross hybrids between *X. maculatus* and *X. hellerii*. *Xiphophorus* melanoma cell line PSM (Wakamatsu 1981) and embryonic cell line A2 (Kuhn *et al.* 1979) were used to test *mc4r* expression.

For receptor assays, human embryonic kidney (HEK)-293 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/liter glucose, 10% fetal calf serum (FCS), 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37°, 5% CO₂. All cells were routinely passaged every 2–3 days. Culture medium for cells stably expressing Mc4r receptors was additionally supplemented with 200 µg/ml G-418.

DNA and RNA analyses

DNA extraction was performed according to standard protocols (Sambrook *et al.* 1989). Southern blot hybridization was done as described using PCR-amplified fragments as probes (Selz *et al.* 2007). Small-scale sequencing reactions were performed using the CEQ-DTCS dye terminator cycle sequencing kit and run on a CEQ 8000 DNA sequencing system (Beckmann Coulter). Sanger-based shotgun sequencing of BAC clones was done at Genoscope (Evry, France).

Total RNA was extracted using the TRIZOL Reagent (Invitrogen Life Technologies). After reverse transcription, PCR was performed using primer F2 (attcctctgctgctgctac) in combination with either primer R3 (agaagatctctctaaaggtc, no UTR specificity), primer R5 (gacatttcaggctcttcatcc, specific for the “wild-type” 3' UTR), and primer 2-R1 (gtttgactgaaataaaaaacagg, specific for the alternative 3' UTR*).

In Silico DNA sequence analysis

Multiple sequence alignments were generated using ClustalX (Thompson *et al.* 1997). Mc4r phylogeny was determined using MrBayes (Huelsenbeck and Ronquist 2001). Maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ) analyses were also performed. The ML tree was obtained with PhyML (Guindon and Gascuel 2003) using a JTT model, a discrete γ -model with four categories. The γ -shape parameter and the proportion of invariable sites were calculated by ML. Both MP and NJ trees were constructed with Phylo_Win (Galtier *et al.* 1996) using pairwise gap removal. Selection parameter ω (dN/dS) was estimated using CodeML from the PAML 3.15 package

(Yang 1997). Recombination rate was calculated with DnaSP (Librado and Rozas 2009). Mc4r sequences from public databases were retrieved using the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) and ENSEMBL (<http://www.ensembl.org/Multi/blastview>) BLAST servers. *X. maculatus mc4r* sequences have been deposited in GenBank under accession nos. KF650640–KF650658.

Transient transfection of HEK-293 cells and selection of stable cell lines

HEK-293 cells were seeded on 10-cm culture plates to ~50% confluence. After attachment of the cells (4–6 hr), the cells were transfected with 6 µg of DNA using Effectene according to the manufacturer's instructions. All constructs were cloned in pcDNA3, and the empty pcDNA3 vector was used for control experiments. Medium was changed 12–16 hr later, and cells were harvested 48 hr after transfection. Positive clones were selected with 600 µg/ml of the neomycin analog G-418, and single clonal lines were isolated by limiting dilution. Cell lines were maintained at 200 µg/ml G-418. Expression of the receptor was verified by radioligand binding.

Membrane preparation

HEK-293 cells transiently transfected with Mc4r receptor constructs or stably expressing Mc4r receptors were grown adherently and cultivated in DMEM with 4.5 g/liter glucose, 10% FCS, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37°, 5% CO₂, as described above. Cells stably expressing Mc4r receptor constructs were split two or three times per week at a ratio between 1:5 and 1:10. For harvesting cells, culture medium was removed, cells were washed twice with phosphate buffered saline puffer, and membranes were prepared or cells were frozen on the dishes for later preparation of membranes. Crude membrane fractions were prepared from fresh (measurement of adenylyl cyclase) or frozen cells (radioligand binding) according to two different protocols, which have been previously described (Klotz *et al.* 1998). The resulting membrane pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, to give a final protein concentration of 1–2 mg/ml.

Adenylyl cyclase assay

Adenylyl cyclase activity in cell membranes was determined according to Jakobs *et al.* (1976). Fifty micrograms of membrane protein was added to an incubation mixture with final concentrations of 50 mM Tris-HCl, pH 7.4, 100 µM cAMP, 0.2% BSA, 10 µM GTP, 100 µM ATP, 1 mM MgCl₂, 100 µM isobutylmethylxanthine, 15 mM phosphocreatine, and 300 units/ml of creatine kinase. Membranes were incubated with ~25 kBq of [α -³²P]ATP for 20 min in the incubation mixture as described (Hoffmann *et al.* 2004). Accumulation of [α -³²P]cAMP was linear over at least 20 min under all conditions. The reaction was stopped by addition of 400 µl 125 mM zinc acetate solution and 500 µl 144 mM Na₂CO₃.

Samples were centrifuged for 5 min at $14,000 \times g$. A total of 800 μl of the resulting supernatant was finally applied to alumina WN-6 (Sigma) columns that were eluted twice with 2 ml 100 mM Tris-HCl, pH 7.4. Eluates were counted in a β -counter (Beckmann LS 1801). Data were analyzed using Origin 6.1 (OriginLab).

Radioligand binding

Radioligand-binding experiments were performed with membranes prepared as described above. Assays were done in a volume of 200 μl in 25 mM HEPES (pH 7.4) containing 2.5 mM CaCl_2 and 1 mM MgCl_2 in the presence of 100 μM GTP to ensure monophasic binding for agonists. For saturation binding experiments with different platyfish, we used Mc4r constructs up to 1600 pM ^{125}I -NDP-MSH. Nonspecific binding was determined in the presence of 2 μM of unlabeled NDP-MSH. For competition binding, we used between 120 and 200 pM ^{125}I -NDP-MSH and serial dilutions of competing unlabeled ligands: α -MSH, NDP-MSH, or ACTH. Membranes were incubated for 120 min at 30° , filtered through Whatman GF/C filters, and washed three times with ice-cold assay buffer. Samples were counted in a γ -counter (Wallac 1480 wizard 3'). K_D values for ^{125}I -NDP-MSH were calculated by nonlinear curve fitting with the program SCTFIT (De Lean *et al.* 1982). Ligand IC_{50} values were calculated using Origin 6.1 (OriginLab) and were transformed to K_i values according to Cheng and Prusoff (1973).

Results

Multiple copies of the *mc4r* gene in the platyfish *X. maculatus*

To obtain a first estimate on *mc4r* copy number in fish by Southern blot hybridization analysis, a molecular probe originating from one *mc4r* sequence linked to *xmrk* (Vollf *et al.* 2003) was used against genomic DNA from different poeciliid and non-poeciliid fish species (Figure 1). Even under high-stringency conditions, several hybridizing fragments were observed in both males and females of *X. maculatus* as well as in some other *Xiphophorus* species including *X. clemenciae* and *X. meyeri* (Figure 1A). In most other poeciliid species, as well as in *Fundulus*, in the medaka *Oryzias latipes* and in the Nile tilapia *Oreochromis niloticus*, only one strong intensity fragment was observed (additional fainter signals were identified as being due to cross-hybridization with other *mcr* genes such as *mc1r*) (Selz *et al.* 2007 and data not shown). The presence of a single copy *mc4r* gene was confirmed in different *Poecilia* species and in other non-poeciliid fish through PCR and BLAST analysis of publicly available genome assemblies. Unlike *mc4r*, another *mcr* gene, *mc1r*, is present as a single-copy gene in the platyfish (Figure 1B) (Selz *et al.* 2007). Hence, in contrast to other vertebrates analyzed so far, the platyfish *X. maculatus* and several other *Xiphophorus* species possess several copies of the *mc4r* gene (see also Lampert *et al.* 2010). Variations in the number and intensity of hybridizing fragments were observed even be-

tween individuals from the same natural population, suggesting intra- and interpopulation polymorphism for type and/or number of *mc4r* copies (tested for two natural populations, Rio Jamapa and Rio Papaloapan; Figure 1A). Hence, copy-number variation (CNV) of the *mc4r* gene is apparent in *X. maculatus* natural populations.

All *mc4r* copies are linked to *SD* and *Xmrk* on the sex chromosomes of *X. maculatus*

To isolate BAC clones containing the different *mc4r* copies present in the genome of the platyfish, a BAC library of XY males ($10\times$ genome coverage; Froschauer *et al.* 2002) was screened by Southern blot hybridization using a *mc4r*-specific molecular probe. The 42 positive BAC clones obtained were further analyzed by restriction fragment length polymorphism and Southern blot hybridization (data not shown). Some *mc4r*-positive BAC clones belonged to the same $\sim 800\text{-kb}$ large BAC contig containing the X-chromosomal allele of *Xmrk* (Froschauer *et al.* 2002) (Figure 2). As many as nine *mc4r* copies were localized in this X-linked contig. Five copies were detected in a BAC contig containing the Y-chromosomal allele of *Xmrk* (Froschauer *et al.* 2002). Five other copies were present in two other BAC contigs containing previously identified Y-chromosomal copies of pseudogenes (*rnase3L-ps*, also known as *droscha-ps*; *msh2-ps*; *cript-ps*) (Vollf *et al.* 2003; Figure 2). Sex chromosomal localization close to *xmrk* was confirmed by fluorescent *in situ* cohybridization on chromosome preparations of BAC clones from these contigs with BAC clones from the *xmrk* contigs (data not shown).

The group of BAC clones containing *mc4rS* could not be linked to *xmrk* contigs and did not present any informative Y-linked marker. However, fluorescent *in situ* cohybridization of a BAC clone containing *mc4rS* with clones from the *Xmrk* contigs showed that *mc4rS* is also sex chromosomal and located close to the *xmrk* contig (data not shown). Taken together, all BAC clones obtained through genomic library screening could be assigned either to the X or to the Y chromosome. Hence, all *mc4r* copies of the platyfish are sex chromosomal and linked to *xmrk* and *SD*, with 10 copies on the X and 10 copies on the Y chromosome.

Sequence diversity of duplicated *mc4r* copies

For each *mc4r* copy, one BAC clone was sequenced to completion. The sequence of the open reading frame of each individual *mc4r* copy was determined (Figure 3 and Figure 4). All *mc4r* copies were found to be intronless as in all other known vertebrate *mc4r* genes. Substitutions and insertions/deletions (indels) were identified in *mc4r*-coding regions through comparison with the single-copy *mc4r* gene from other species. Several identical mutations were found in different copies, suggesting either mutation occurrence before gene duplication or, alternatively, events of gene conversion or recombination between duplicated copies.

Indels observed in platyfish *mc4r* copies can be summarized as follows (Figure 3 and Figure 4): a deletion of 15

Figure 1 Southern blot hybridization analysis of *mc4r* in the platyfish *X. maculatus* and other fish species. Genomic DNA was cut with *HindIII* and hybridized with a *mc4r* (A) or *mc1r* (B) (Selz *et al.* 2007) internal fragment cloned from *X. maculatus*. All species belong to the family Poeciliidae except *Fundulus* sp. (Fundulidae), *O. latipes* (medaka, Adrianichthyidae), and *Oreochromis niloticus* (Nile tilapia, Cichlidae). The designation "1274" shows typical male and female patterns for the *X. maculatus* laboratory reference population WLC1274, which is derived from the Rio Jamapa natural population and has been used for the BAC genomic library (Froschauer *et al.* 2002). M, male; F, female.

nucleotides at the junction between transmembrane domain 2 and extracellular domain 2 (two copies on the X and three copies on the Y); a 500-nt deletion removing the coding sequence downstream from extracellular domain 3 (one copy on the X and one copy on the Y); a 6-nt insertion resulting in the addition of two alanine residues in intracellular domain 3 (one copy on the X and two copies on the Y); and a 15-nt deletion in intracellular domain 4 at the C-terminal end of the protein, removing 5 of the 11 carboxy-terminal amino-acid residues (five copies on the X and six copies on the Y). Most indels are in frame and therefore do not alter the primary sequence of the receptor downstream from the mutation. In addition, four copies on the X and two copies on the Y chromosome show a stop codon interrupting the coding region after transmembrane domain 4. Three copies on the X and two copies on the Y have a premature stop codon in the C-terminal coding region (intracellular domain 4), removing the last nine amino-acid residues of the protein. Strikingly, according to the Mc4R sequences from other fish species, including two swordtails of the genus *Xiphophorus* (Lampert *et al.* 2010), the wild-type *mc4r* sequence was no longer present in *X. maculatus*: all copies were affected by indels and/or premature stop codons at positions otherwise highly conserved in *mc4r* of vertebrates. No mutation in *mc4r* was found to be common to *X. nigrensis*/*X. multilineatus* (Lampert *et al.* 2010) and *X. maculatus*, suggesting independent mutational processes.

At the nucleotide sequence level, several *mc4r* copies from the X and the Y chromosome were identical (B and I; D and N; S and U) or very similar (A and H; G and P) (Figure 2). These copies might be included in discontinuous pseudoautosomal regions of the sex chromosomes, where recombination

between the X and the Y chromosomes still occurs. In contrast, other copies did not possess any clear allelic counterpart on the other sex chromosome. If these copies are expressed, they might hence represent an initiation of sex-chromosome-specific variations for Mc4r functions.

The sequence of the 5' UTR and 3' UTR regions was determined through the sequencing of *mc4r* complementary DNA (cDNA) clones from a melanoma cell line library. Surprisingly, the 5' region of all *mc4r* copies includes a short segment with high similarity to a gene called *dc2*, encoding a putative transmembrane protein reported as a component of the oligosaccharyltransferase complex in mammals (Shibatani *et al.* 2005). Sequence comparison with the platyfish *dc2* gene indicated that 218 nt from the ~500-nt *mc4r* UTR originated from *dc2*, this sequence being located only 38 nt upstream from the translational start codon of *mc4r*. Interestingly, the inserted *dc2*-like region corresponded to the 3' part of the last protein-coding exon of *dc2* and the 5' part of the next noncoding exon (the last exon), but without the intervening intron. Hence, the inserted segment was derived from the reverse transcription of a spliced *dc2*-mRNA. The *dc2*-related sequences were absent in the *mc4r* gene of other non-*Xiphophorus* fish species. All platyfish *mc4r* copies harbored the *dc2*-derived sequence, indicating that the *dc2* insertion occurred before *mc4r* serial duplication.

Two types of 3' UTR regions were detected in platyfish *mc4r* copies, presenting >90% identity of their first 114 nt and diverging completely after this breakpoint. One type of 3' UTR (~550 nt in length) was found in most copies (17/20) while the other, 3' UTR* (~370 nt), was found in only three copies (G on the X chromosome and P/R on the Y).

Figure 2 Localization of *mc4r* copies in BAC contigs from the X and Y sex chromosomes of *X. maculatus*. BAC clones are shown by horizontal lines. Identical *mc4r* copies are linked by continuous lines and very similar copies by dashed lines. The asterisk shows the position of a Y-specific pseudogene closely linked to the master sex-determining gene of *X. maculatus*. ps, pseudogene.

Amplification of *mc4r* by large serial sex chromosomal duplications

Molecular phylogeny based on the *mc4r*-coding sequence indicated that duplication events were relatively recent after divergence of the genus *Xiphophorus* and the related genus *Poecilia* (Figure 5). While several phylogenetic relationships between *X. maculatus* copies received strong statistical support, most of them could not be established unambiguously. In addition to the absence of an informative phylogenetic signal, one possible explanation might be gene conversion or recombination between copies obscuring the duplication history of *mc4r* genes. Accordingly, multiple recombination events were predicted between copies using DNAsP (Librado and Rozas 2009; data not shown). Using the program codeml from the PAML 3.15 package (Yang 1997), the natural selection parameter ω , corresponding to the ratio of nonsynonymous (dN) vs. synonymous (dS), was calculated. This indicated a relaxation of purifying selection probably reflecting a reduction of functional constraints ($\omega = 0.25286$) compared to the value globally observed for *mc4r* in other species ($\omega = 0.03766$).

Regions containing *mc4r* copies were compared over a sequence window including 10 kb upstream and 10 kb downstream from the open reading frame (Figure 6). In some cases, global nucleotide identities of >90% were observed between sequences on the same chromosome over the complete length of the region analyzed, with the exception of indels ranging from a few to several hundreds of nucleotides. Hence, certain events of *mc4r* duplication involved genomic regions of at least 20 kb in length.

Many indels were observed in duplicated regions; for example, a 3-kb sequence in *mc4rQ* was inserted directly 3' to the open reading frame. Shorter indels were also detected but are not shown in Figure 6. Some indels correspond to transposable elements, including DNA transposons and retrotransposons [long terminal repeat (LTR) and non-LTR retrotransposons]. Certain insertions arose before duplication. For example, the *Maui* non-LTR retrotransposon is located at the same position in 8 of 20 copies. Frequently, some indels and other mutations are found 5' close to the *mc4r* genes,

suggesting that they might have an effect on their transcriptional context. One 3-kb deletion removed the 3' part of the most common 3' UTR as well as several kilobases of neighboring DNA (in copies P, G, and R). Interestingly, this deletion generated a new 3' UTR region (3' UTR*; see above). Three copies (D, M, and N) are flanked by exons of the same (pseudo)gene (*rna3L*, aka *droscha*) on both sides, indicating that the duplicated *mc4r* was inserted into this gene.

Expression analysis of *mc4r* copies

Expression of *mc4r* copies was analyzed by RT-PCR and Northern blot hybridization (Figure 7). RT-PCR products were sequenced to determine which type of copy was expressed in different organs and tissues. Primers specific for each type of 3' UTR were also used in RT-PCR experiments. Results were compared with those obtained for the single-copy gene of the medaka.

In medaka adult tissues, expression of *mc4r* was predominantly observed in brain, testis, and ovary. This is consistent with expression patterns found in other teleosts (Kobayashi *et al.* 2008). In contrast, *mc4r* copies were ubiquitously expressed in adult platyfish (Figure 7), indicating that at least some copies have a modified transcriptional pattern. Expression was also detected in embryos and an embryo-derived cell line. Copies with an alternative 3' UTR region presented a more restricted expression pattern more similar to that observed for the single gene of the medaka. Notably, a very strong *mc4r* expression was observed in a melanoma cell line.

Sequencing of 171 cloned RT-PCR products obtained from different organs and tissues from *X. maculatus* males and females as well as from embryos and a melanoma cell line revealed detectable expression for most copies. No characteristic cDNA sequence could be identified only for copies P (Y) and E (X). Copies I (Y) and B (X) were not analyzed since the region matching the reverse PCR primer used for amplification is deleted in these copies. Expression was found for all structural types of copies (with different 3' UTRs, indels, or stop codons). Expression of several copies was detected in all tissues and organs tested (6 different

Figure 3 Structure of X-chromosomal and Y-chromosomal *mc4r* copies in *X. maculatus*. *Dc2-ps*, part of the 5' UTR derived from the *dc2* gene (in yellow). Del, deletion; ec, extracellular domain; ic, intracellular domain; ins, insertion; stop, stop codon; tm, transmembrane domain. See Figure 4 for a detailed description of the effect of mutations at the amino-acid level. UTRs are in black, alternative 3' UTR* in red, open reading frame in white.

copies among 14 cDNA sequences in eyes, 5/13 in brain, 5/15 in skin, 5/14 in gills, 3/8 in liver, 4/11 in muscle, 6/9 in testis, and 4/7 in ovary). At least 5 different copies were identified in melanoma, with strong expression of copy G. Copies D/N (with two additional alanine residues in intracellular domain 3) are the most widely expressed, with expression in all nonmelanoma tissues (41/96 sequenced cDNA molecules). The proportion of expression of copies with a stop codon interrupting the coding region after transmembrane domain 4 is variable (from 1/13 in brain to 6/7 in ovary).

Functional analysis of *mc4r* copies

Because all *mc4r* copies appeared to still encode complete or partial receptors, the question about their functionality arose. We chose representatives of major paralogue classes to test for their ability to elicit a hormone response by generation of cAMP and for ligand binding.

Of the 20 different *mc4r* copies that were cloned from *X. maculatus* (Figure 4), we selected 11 for further pharmacological evaluation in cell culture experiments. These 11 copies are representatives for each possible class of observed changes in the coding sequence.

The initial functional screening was done by transient transfection of HEK-293 cells. Membranes were prepared from transfected cells and were tested for receptor function by adenylyl cyclase stimulation. Vector-transfected cells served as negative controls, while cells transfected with human

mc1r, Tetraodon *mc1r*, and Tetraodon *mc4r* served as positive controls (Figure 8).

Membranes were stimulated with buffer, α -MSH, or ACTH, respectively. HEK-293 cells do not express endogenous functional Mcr receptors since no increase in cAMP production was observed upon stimulation using α -MSH or ACTH. In contrast, membranes prepared from cells transfected with cDNAs encoding the human Mc1r, Tetraodon Mc1r, or Tetraodon Mc4r clearly exhibited an increase in cAMP production upon agonist stimulation. These controls verified that HEK293 can be used to detect functional Mcr receptors.

For the platyfish Mc4r tested, of the 11 copies selected for testing, only the copy termed "Mc4r-S" exhibited a significant increase in cAMP production upon stimulation with α -MSH or ACTH. From the remaining clones, Mc4r-L, T, P, and O showed a higher level of basal cAMP production when compared to clone S. Since P, T, and O form a similarity cluster with respect to their changes in amino-acid sequence, we selected S, O, and L for further analysis.

HEK-293 cells were stably transfected with each corresponding construct. Since the cauMcr4 receptor from the goldfish *Carassius auratus* has been pharmacologically well characterized (Cerdeira-Reverte *et al.* 2003), a control cell line was generated using this receptor. Membranes were prepared and saturation binding experiments performed using 125 I-NDP-MSH as radioligand. Control HEK-293 did not exhibit any specific binding of 125 I-NDP-MSH (data not shown), while each clone tested exhibited specific saturable binding of 125 I-NDP-MSH (Figure 9). Next, competition binding experiments were performed to evaluate the ligand-binding properties of the selected clones (Table 1). The ligand-binding data show that all Mc4r copies tested have comparable binding affinities for NDP-MSH and α -MSH, while the affinity for ACTH varied more than fivefold between clone O and S. Differential binding affinity was also observed when the cauMc4r receptor was tested. The binding affinities for NDP-MSH and α -MSH are comparable between these species, while the cauMcr4 receptor has a 20- to 100-fold higher affinity for ACTH compared to platyfish.

Since clone S was the only receptor copy that clearly exhibited robust agonist-dependent adenylyl cyclase stimulation, we compared this receptor copy to cauMc4r for agonist-dependent adenylyl cyclase stimulation parameters. Concentration response curves for ligand-dependent cAMP production of cauMc4r in comparison to platyfish S are highly similar (Figure 10). For both receptors, the agonist rank order remained constant, with NDP-MSH being more potent than ACTH followed by α -MSH. The functional data observed are in good agreement with the binding data (Table 1) and show that receptor copy S is probably the (only) physiologically functional platyfish Mc4r receptor.

Discussion

Mc4r is well conserved with respect to primary structure in vertebrates (Schiöth *et al.* 2003, 2005; Haitina *et al.* 2004,

Figure 4 Sequence alignment of Mc4r conceptual translation products. Del, deletion; EC, extracellular domain; IC, intracellular domain; Ins, insertion; stop, stop codon; TM, transmembrane domain.

Figure 5 Molecular phylogeny of Mc4r sequences. Phylogeny was calculated on a 578-nucleotide sequence alignment using MrBayes (Huelsenbeck and Ronquist 2001). First or unique value gives the posterior probability value. For a given branch, when multiple values are available, a second value was obtained using maximum likelihood, a third value using maximum parsimony, and a final value using neighbor joining (see *Materials and Methods*). Selection parameter ω was calculated as the nonsynonymous substitution rate/synonymous substitution rate (dN/dS) ratio using CodeML from the PAML 3.15 package (Yang 1997).

2007a,b; Metz *et al.* 2006; Takahashi and Kawauchi 2006). In mammals, Mc4r is involved in the control of food intake, energy balance, and body weight (for review, Adan *et al.* 2006; Tao 2010). Over 150 distinct mutations in this gene have been associated with hereditary obesity in humans, and disruption of *mc4r* in mice leads to an obese phenotype, too (Stäubert *et al.* 2007 and references therein; for review: Adan *et al.* 2006; Tao 2010). In addition, roles have been reported for Mc4r in the regulation of skin pigmentation, in cardiovascular function, and in reproduction and sexual function (Van der Ploeg *et al.* 2002; Spencer and Schallreuter 2009; Israel *et al.* 2012). In adult mammals, *mc4r* is predominantly expressed in different regions of the brain

including the hypothalamus, septum, hippocampus, spinal cord, striatum, thalamus, brainstem, and cortex.

In teleost fish, the Mc4r receptor has been analyzed in different species (Ringholm *et al.* 2002; Cerdá-Reverter *et al.* 2003; Haitina *et al.* 2004; Klovins *et al.* 2004; Kobayashi *et al.* 2008; Sánchez *et al.* 2009; Lampert *et al.* 2010; Wan *et al.* 2012). From the pharmacological point of view, teleost Mc4r receptors have a much higher affinity for ACTH than mammalian receptors (Haitina *et al.* 2005). Several convergent observations support an important role of the melanocortin hormone system in the regulation of food intake and energy homeostasis in fish, probably mediated by the Mc4r receptor (Cerdá-Reverter *et al.* 2003; Song and Cone 2007;

Figure 6 Comparative structure analysis of *mc4r*-containing regions reveals large duplications on the sex chromosomes of the platyfish. Orange, *mc4r*; green, retrotransposons, with long terminal repeats in black; red, Tc1 DNA transposon. Boxes in white, blue, yellow, brown, and pink show regions present in some copies but absent from others. *Drosha* exons are indicated by arrowheads. Dashed lines indicate deletions.

Sánchez *et al.* 2009; Schjolden *et al.* 2009; Wan *et al.* 2012; Zhang *et al.* 2012; Sebag *et al.* 2013).

Interestingly, analysis of *mc4r* sequence polymorphism and copy-number variation in two swordtail species, *X. nigrensis* and *X. multilineatus*, has uncovered a new Mc4r-mediated physiological link between energy balance and reproduction. In these species, different versions of *mc4r* were found to modulate not only male adult size but also the onset of sexual maturity and mating behavior (Lampert *et al.*

2010). Large late-maturing fishes, which perform courtship mating and are preferred by females, display high copy number and high levels of expression of defective *mc4r* copies located on the Y chromosome. These copies encode receptors mutated in their C-terminal domain that are unable to transduce melanocortin-mediated signals. In contrast, small early maturing males that reproduce through “sneak” mating have a lower copy number and lower expression levels of these defective receptors. An intermediate

Figure 7 *Mc4r* expression analysis. (A) RT-PCR analysis in *Xiphophorus* using a reverse primer matching the "classical" 3' UTR ("*mc4r*") or the alternative 3' UTR ("*mc4r* (3' UTR*)"). Expression of the single copy *mc4r* of the medaka *O. latipes* was also analyzed. For calibration, the cDNA amount used in the PCR reaction was monitored with actin PCR. "no RT" indicates treatment without reverse transcriptase, indicating absence of contaminating genomic DNA. (B) Northern blot analysis in *Xiphophorus* using an internal *mc4r* fragment from *X. maculatus* as a probe.

male phenotype for body size, puberty onset, reproductive behavior, and defective *mc4r* expression is also observed in both swordtail species. Hence, *mc4r* receptor genes have been proposed to be major constituents of the sex chromosomal *P* locus, which modulates body size, sexual development, and reproductive behavior in *Xiphophorus* (Kallman and Schreibman 1973; Kallman and Borkoski 1977; Schreibman *et al.* 1994). However, no genomic data were available to analyze the structure, origin, and evolution of the *mc4r*-containing *P* locus in any species.

We report here the structure of the *mc4r*-containing loci on the X and Y chromosomes of the platyfish *X. maculatus*. In this species, which is relatively divergent from the two swordtails studied so far, the *P* locus controls the onset of sexual maturity not only in males but also in females and, in addition to adult size in males, also fecundity in females (Kallman and Borkoski 1977). In addition, the degree of phenotypic and genetic polymorphism is much higher than in *X. nigrensis* and *X. multilineatus*: as many as nine *P* alleles have been identified on both the X and Y chromosomes in association with a high diversity of phenotypes (Kallman 1989). In this study, we have identified 10 *mc4r* copies, which have been produced through large serial duplications on both the X and Y chromosomes of the platyfish.

Expression analysis suggested diversification of expression patterns for at least some copies after duplication. As observed in other fish, *mc4r* expression was found in brain, particularly for copies with the alternative 3' UTR. However, prominent *mc4r* expression was also detected in all other tissues and organs tested, with a surprisingly high expression in melanoma and a melanoma cell line. Several copies, for example, D/N, are expressed almost ubiquitously. Such deviations from classical expression patterns observed in other fish and vertebrates suggest that *mc4r* regulatory sequences have been modified in at least several of the

duplicated copies in *X. maculatus*. Certain rearrangements observed in this study might have effects on gene expression, for example, the different indels and other mutations found 5' from the *mc4r* genes. In some copies (P, G, and R), a 3' deletion has formed a new 3' UTR region. As this structure usually contains elements that regulate messenger RNA stability, the new 3' UTR may have consequences for gene expression levels or cell-type specificity. We do not know at the moment if such new expression patterns are associated with new functions for Mc4r in the platyfish.

In addition, *mc4r* copies have also diverged after duplication through different types of mutations in their coding sequence. Functional analysis of some of these copies revealed that they have different pharmacological properties, and almost all copies are expressed in at least one tissue type. Hence, sequence divergence in coding sequences might be relevant *in vivo* for the modulation of Mc4r-mediated signaling. From the sequence, no Mc4r was identified that contained all conserved amino acid positions usually shared between the known fish Mc4Rs and other vertebrates (Schiöth *et al.* 2003). At the pharmacological level, only one platyfish Mc4r version, Mc4r-S/U, was shown to behave as a classical Mc4r receptor. Therefore, Mc4r-S/U might correspond to the wild-type physiologically functional Mc4r receptor of *X. maculatus*. The *mc4rS/U* gene is located in a pseudoautosomal region of the sex chromosomes, where it might be less prone to mutations associated with the process of sex chromosome differentiation and degeneration. However, compared to the putative ancestral receptor before duplication, the Mc4r-S/U receptor open reading frame is interrupted by a premature stop codon having removed the nine C-terminal residues of the protein. This deletion occurred only one amino acid after a dicysteine motif highly conserved in vertebrate Mc4r proteins. This motif, likely to be essential for receptor activation, is probably modified by

Figure 8 Agonist-stimulated adenylyl cyclase activity in membranes isolated from HEK-293 cells transiently expressing different platyfish Mc4r receptors and wild-type melanocortin control receptors from other species. Adenylyl cyclase stimulation was calculated as fold stimulation of basal activity in untransfected control cells. A total of 100 μ g membranes were stimulated with H₂O, 100 μ M α -MSH, or 10 μ M ACTH. Open bars represent data for H₂O, light-gray bars represent data for stimulation with 100 μ M α -MSH, and dark-gray bars represent data for 10 μ M ACTH. Data are representative of four or five experiments done in duplicate.

palmitoylation, anchoring the receptor to the membrane and forming a fourth intracellular loop (Tao 2010). In fish, deletion of this dicysteine motif leads to inability to transduce the signal (Lampert *et al.* 2010). However, human receptors showing truncations after the C-C motif similar to Mc4r-S/U are fully functional (Ho and MacKenzie 1999; Yang *et al.* 2005). Hence, even with this short C-terminal truncation, Mc4r-S/U appears to function as the classical Mc4r receptor.

Due to premature stop codons or 3' large deletions, several *mc4r* genes such as copies A, C, F, M, H, Q, B, and I encode conceptual translation products with C-terminal truncation after the fourth transmembrane domain. No significant activity was detected for the tested representative receptors Mc4r-H and Mc4r-B. Human Mc4r receptor variants harboring truncations after the first five transmembrane domains are neither able to stimulate cAMP production nor able to bind agonists and do not localize correctly to the membrane (Ho and MacKenzie 1999). Hence, platyfish truncated receptors are most likely to be inactive. Transcripts were found for at least four of these receptors, particularly in testis and ovary. The fact that cDNAs for Mc4r-H and -B gave rise to protein products in HEK-293 cells may indicate that those transcripts are also translated in the platyfish. Consequences of this expression—for example, dominant negative effects through binding to a wild-type receptor that interfere with transport to the membrane—remain to be analyzed. While such an effect was not detected for truncated human Mc4r variants, despite truncation, biological activities have been reported for other types of G-protein-coupled receptors (Ho and MacKenzie 1999 and references therein).

Figure 9 125 I- α -NDP-MSH radioligand saturation binding to different platyfish Mc4r receptor constructs expressed in HEK-293 cells. The results of representative saturation binding experiments with the radioligand 125 I- α -NDP-MSH are shown as specific (square) and nonspecific binding (circle). (Top) Saturation binding at the platyfish Mc4r-S. (Middle) Saturation binding at platyfish Mc4r-L. (Bottom) Saturation binding at platyfish Mc4r-O. The results of the saturation binding experiments are summarized in Table 1.

In addition to Mc4r-S/U, some other receptors are full or almost full length and without important truncation at their C-terminal end. However, none of them could be stimulated

Table 1 Binding affinities from competition experiments for agonists at different Mc4r receptor subtypes

Receptor construct	ACTH		NDP-MSH		α -MSH	
	K_i (nM)	95% confidence limit	K_i (nM)	95% confidence limit	K_i (nM)	95% confidence limit
Cau Mc4r	3.72	2.54–5.44	0.47	0.37–0.61	260.1	155.7–434.4
Cau Mc4r (Lit)	ND		1.43		988	
Mc4r-S	466	214–1018	2.00	1.63–2.45	4083	2548–6543
Mc4r-L	116.6	69.4–196	4.09	2.20–7.58	5253	2595–10,633
Mc4r-O	76.6	52.1–112.7	2.44	1.18–5.04	2590	2041–3286

A total of 120–200 pM 125 I-NDP-MSH was used as radioligand. Experiments were done in the presence of 100 μ M GTP. K_i values were calculated from IC₅₀ values using the Cheng–Prussow equation and represent geometric mean values of at least three different experiments done in duplicate. “Lit” indicates data from the literature (Cerdá-Reverter *et al.* 2003).

by α -MSH or ACTH to produce cAMP, indicating mutational loss of the capacity to transmit agonist-induced signaling to the cell. This might be due to mutations occurring in the third intracellular loop. This region, which appears particularly variable between platyfish Mc4r proteins, has been shown to be involved in receptor signaling but not in ligand binding in human Mc4r. Indeed, some of these receptors are still able to bind agonists, as demonstrated for Mc4r-L and Mc4r-O. Accordingly, amino-acid residues shown to be involved in ligand binding in mammalian Mc4r are conserved in these receptors (for review, see Tao 2010). Since expression of Mc4r-L and Mc4r-O was detected in various organs, these receptors might correspond to dominant negative versions of Mc4r that reduce the formation of functional Mc4r dimers or sequester the MSH ligand, leading to reduction of agonist-mediated signaling. Accordingly, coexpression of swordtail functional and nonfunctional Mc4R alleles in cell culture experiments decreased signal transduction (Lampert *et al.* 2010). Interestingly, at least for receptors L, T, P, and O, the level of cAMP production without agonist stimulation is largely higher than for vector control or heavily truncated vectors such as Mc4r-H. Hence, these receptors might possess a constitutive activity to stimulate G proteins in the absence of agonists.

In contrast to the situation observed in *X. nigrensis* and *X. multilineatus*, where mutated *mc4r* genes were found only on the Y chromosome, genes with any kind of mutations were found on both the X and Y chromosomes in the platyfish. This is consistent with the fact that the *P* locus in the platyfish controls not only the onset of sexual maturity and adult body size in males, but also puberty and fecundity in females (Kallman and Borkoski 1977). In addition, many more *mc4r* copies were found in *X. maculatus*, reflecting the complexity and diversity of phenotypes observed in this species. In both *X. nigrensis* and *X. multilineatus*, in addition to a wild-type *mc4r* “A allele,” only two *mc4r* “B alleles” with microdeletions at their carboxyl terminus have been identified. Both B alleles have a 6-nt deletion particularly removing two adjacent cysteine residues. One B allele harbors an additional 4-nt deletion leading to a frameshift, which replaced the six last residues of the receptor by 13 new amino acids (Lampert *et al.* 2010). Comparison with mutations affecting the C-terminal region in predicted platyfish Mc4r sequences, in which at least one of the two adjacent

cysteines was always present, strongly suggested that these mutations have occurred independently in the *X. nigrensis*/*X. multilineatus* lineage, on the one hand, and in *X. maculatus*, on the other hand. It has been proposed in swordtails that nonsignal-transducing versions of Mc4r might reduce melanocortin signaling and delay the onset of puberty (Lampert *et al.* 2010). A similar mechanism, involving different combinations of the different Mc4r variants characterized in this work, might underlie polymorphism at the onset of sexual maturity in both males and females, adult size in males, and fecundity in females in *X. maculatus*. This might imply convergent evolution at the molecular level, where the same biochemical pathway is used to bring about a physiological signal leading to the onset of puberty, but through different changes in Mc4R.

Taken together, the data suggest that the sex-determining region of *X. maculatus* is a hotspot for large segmental duplications and show how sex chromosomal gene families can be formed. Y-specific duplications have been reported in other species, including threespine sticklebacks and humans (Peichel *et al.* 2004; Jobling 2008). However, in these cases, gene duplications were specific for one type of sex chromosome, the nonrecombining Y chromosome. In the platyfish, duplicated copies have been found on both the X and the Y chromosomes. Some copies are identical or extremely similar since they are located in pseudoautosomal regions, where X/Y recombination still occurs. Other copies are found in regions differing between the X and the Y chromosomes and may lead to the birth of new X- and Y-linked variations and even to new sex-specific Mc4r functions, with pseudoautosomal copies still working as classical receptors. Much more work is necessary to better characterize the physiological roles of each of these Mc4r variants and the *in vivo* consequences of their complex interactions.

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Figure 10 Concentration response curves for agonist-stimulated adenylyl cyclase activity in membranes isolated from HEK-293 cells stably expressing goldfish Mc4r or platyfish Mc4r-S. Adenylyl cyclase stimulation was calculated as a percentage of the stimulation of maximal agonist-induced stimulation. For all ligands, the maximal stimulation was similar. (Top) Data for goldfish Mc4r. (Bottom) Data for platyfish Mc4r-S. Solid squares represent data for stimulation with NDP-MSH, solid circles represent data for stimulation with ACTH, and solid triangles represent data for stimulation with α -MSH. Data are representative of four to seven experiments done in duplicate.

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