Dynamic regulation of the melanocortin 4 receptor system in body weight homeostasis and reproductive maturation in fish

Dynamische Regulation des Melanocortin-4-Rezeptor Systems bei der Körpergewichtshomöostase und der Fortpflanzungsreifung bei Fischen

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submitted by

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Summary

Puberty is an important period of life with physiological changes to enable animals to reproduce. *Xiphophorus* fish exhibit polymorphism in body size, puberty timing, and reproductive tactics. These phenotypical polymorphisms are controlled by the *Puberty* (*P*) locus. In *X. nigrensis* and *X. multilineatus*, the *P* locus encodes the melanocortin 4 receptor (Mc4r) with high genetic polymorphisms.

Mc4r is a member of the melanocortin receptors, belonging to class A G-protein coupled receptors. The Mc4r signaling system consists of Mc4r, the agonist Pomc (precursor of various MSH and of ACTH), the antagonist Agrp and accessory protein Mrap2. In humans, MC4R has a role in energy homeostasis. *MC4R* and *MRAP2* mutations are linked to human obesity but not to puberty.

Mc4rs in *X. nigrensis* and *X. multilineatus* are present in three allele classes, A, B1 and B2, of which the X-linked A alleles express functional receptors and the male-specific Y-linked B alleles encode defective receptors. Male body sizes are correlated with B allele type and B allele copy numbers. Late-maturing large males carry B alleles in high copy number while early-maturing small males carry B alleles in low copy number or only A alleles. Cell culture co-expression experiments indicated that B alleles may act as dominant negative receptor mutants on A alleles.

In this study, the main aim was to biochemically characterize the mechanism of puberty regulation by Mc4r in *X. nigrensis* and *X. multilineatus*, whether it is by Mc4r dimerization and/or Mrap2 interaction with Mc4r or other mechanisms. Furthermore, Mc4r in *X. hellerii* (another swordtail species) and medaka (a model organism phylogenetically close to *Xiphophorus*) were investigated to understand if the investigated mechanisms are conserved in other species.

In medaka, the Mc4r signaling system genes (*mc4r*, *mrap2*, *pomc*, *agrp1*) are expressed before hatching, with *agrp1* being highly upregulated during hatching and first feeding. These genes are mainly expressed in adult brain, and the transcripts of *mrap2* co-localize with *mc4r* indicating a function in modulating Mc4r signaling. Functional comparison between wild-type and *mc4r* knockout medaka showed that Mc4r knockout does not affect puberty timing but significantly delays hatching due to the retarded embryonic development of knockout medaka. Hence, the Mc4r system in medaka is involved in regulation of growth rather than puberty.

In *Xiphophorus*, expression co-localization of *mc4r* and *mrap2* in *X. nigrensis* and *X. hellerii* fish adult brains was characterized by *in situ* hybridization. In both species, large males exhibit strikingly high expression of *mc4r* while *mrap2* shows similar

expression level in the large and small male and female. Differently, *X. hellerii* has only A-type alleles indicating that the puberty regulation mechanisms evolved independently in *Xiphophorus* genus. Functional analysis of Mrap2 and Mc4r A/B1/B2 alleles of *X. multilineatus* showed that increased Mrap2 amounts induce higher cAMP response but EC50 values do not change much upon Mrap2 co-expression with Mc4r (expressing only A allele or A and B1 alleles). A and B1 alleles were expressed higher in large male brains, while B2 alleles were only barely expressed. Mc4r A-B1 cells have lower cAMP production than Mc4r A cells. Together, this indicates a role of Mc4r alleles, but not Mrap2, in puberty onset regulation signaling. Interaction studies by FRET approach evidenced that Mc4r A and B alleles can form heterodimers and homodimers *in vitro*, but only for a certain fraction of the expressed receptors. Single-molecule colocalization study using super-resolution microscope dSTORM confirmed that only few Mc4r A and B1 receptors co-localized on the membrane. Altogether, the species-specific puberty onset regulation in *X. nigrensis* and *X. multilineatus* is linked to the presence of Mc4r B alleles and to some extent to its interaction with A allele gene products. This is reasoned to result in certain levels of cAMP signaling which reaches the dynamic or static threshold to permit late puberty in large males.

In summary, puberty onset regulation by dominant negative effect of Mc4r mutant alleles is a special mechanism that is found so far only in *X. nigrensis* and *X. multilineatus*. Other *Xiphophorus* species obviously evolved the same function of the pathway by diverse mechanisms. Mc4r in other fish (medaka) has a role in regulation of growth, reminiscent of its role in energy homeostasis in humans. The results of this study will contribute to better understand the biochemical and physiological functions of the Mc4r system in vertebrates including human.

Zusammenfassung

Die Pubertät ist ein wichtiger Lebensabschnitt mit physiologischen Veränderungen, die die Fortpflanzung von Tieren ermöglichen. *Xiphophorus* Fische weisen einen Polymorphismus in Bezug auf Körpergröße, Pubertätszeit und Fortpflanzungstaktik auf. Diese phänotypischen Polymorphismen werden durch den *Pubertäts* (*P*) Locus gesteuert. In *X. nigrensis* und *X. multilineatus* kodiert der *P* Locus den Melanocortin-4-Rezeptor (Mc4r) mit hohen genetischen Polymorphismen.

Mc4r gehört zu den Melanocortin-Rezeptoren, die zur Klasse A der G-Protein-gekoppelten Rezeptoren gehören. Das Mc4r-Signalsystem besteht aus Mc4r, dem Agonisten Pomc (Prohormon der verschiedenen MSH und des ACTH), dem Antagonisten Agrp und dem akzessorischen Protein Mrap2. Beim Menschen spielt MC4R eine Rolle bei der Energiehomöostase. *MC4R* und *MRAP2* Mutationen stehen im Zusammenhang mit menschlicher Fettleibigkeit, jedoch nicht mit der Pubertät.

Mc4rs in *X. nigrensis* und *X. multilineatus* sind in drei Allelklassen vorhanden, A, B1 und B2, von denen die X-chromosomalen A Allele funktionelle Rezeptoren exprimieren und die spezifischen männlichen Y-chromosomalen B Allele für defekte Rezeptoren kodieren. Die männliche Körpergröße korreliert mit dem B Alleltyp und der Kopienzahl des B Allels. Spätreife große Männchen tragen B Allele in hoher Kopienzahl, während frühreife kleine Männchen B Allele in niedriger Kopienzahl oder nur A Allele tragen. Koexpressions-Experimente in Zellkultur zeigten, dass B Allele als dominant negative Mutanten-Rezeptor auf A Allele wirken können.

In dieser Studie war das Hauptziel die biochemische Charakterisierung des Mechanismus der Pubertätsregulation durch Mc4r in *X. nigrensis* und *X. multilineatus*. Dabei wurde untersucht, ob die Regulation durch eine Mc4r Dimerisierung und/oder Mrap2 Interaktion mit Mc4r oder durch andere Mechanismen erfolgt. Des Weiteren wurde Mc4r in *X. hellerii* (einer anderen Schwertträger Art) und Medaka (ein phylogenetisch naheliegender Modellorganismus von *Xiphophorus*) untersucht, um zu verstehen, ob die untersuchten Mechanismen in anderen Arten konserviert sind.

In Medaka werden die Gene des Mc4r Signalsystems (*mc4r*, *mrap2*, *pomc*, *agrp1*) vor dem Schlüpfen exprimiert, wobei *agrp1* während des Schlüpfens und der ersten Fütterung stark hochreguliert wird. Im adulten Medaka werden diese Gene hauptsächlich im Gehirn exprimiert und die Transkripte von *mrap2* und *mc4r* kolokalisieren, was auf eine Funktion bei der Modulation der Mc4r-Signaltransduktion hinweist. Ein funktionaler Vergleich zwischen Wildtyp- und

mc4r-Knockout Medaka zeigte, dass der Mc4r-Knockout das Pubertäts-Timing nicht beeinflusst, das Schlüpfen jedoch aufgrund der verzögerten embryonalen Entwicklung von Knockout-Medaka signifikant verzögert. Daher ist das Mc4r System in Medaka eher an der Regulation des Wachstums als an der Pubertät beteiligt.

Bei *Xiphophorus* wurde die Lokalisierung von *mc4r* und *mrap2* in erwachsenen Gehirnen von *X. nigrensis* und *X. hellerii* durch *in situ* Hybridisierung charakterisiert. Bei beiden Spezies zeigen große Männchen eine auffallend hohe Expression von *mc4r*, während *mrap2* bei großen und kleinen Männchen und Weibchen ein ähnliches Expressionsniveau zeigt. Im Gegensatz dazu weist *X. hellerii* nur Allele vom A-Typ auf, was darauf hinweist, dass sich die Pubertätsregulationsmechanismen in dem Genus *Xiphophorus* unabhängig voneinander entwickelt haben. Die funktionelle Analyse der Mrap2 und Mc4r A/B1/B2 Allele von *X. multilineatus* zeigte, dass erhöhte Mrap2-Mengen eine höhere cAMP-Antwort induzieren, die EC50-Werte sich jedoch bei der Mrap2-Coexpression mit Mc4r nicht wesentlich ändern (nur A Allel oder A und B1 Allele). A und B1 Allele wurden in großen männlichen Gehirnen höher exprimiert, während B2 Allele kaum exprimiert wurden. Mc4r A-B1 Zellen haben eine geringere cAMP-Produktion als Mc4r A Zellen. Zusammengenommen deutet dies auf eine Rolle von Mc4r-Allelen, jedoch nicht von Mrap2, bei der Signalgebung zur Regulation des Pubertätsbeginns hin. Interaktionsstudien mit den FRET-Methoden zeigten, dass Mc4r A und B Allele *in vitro* Heterodimere und Homodimere bilden können, jedoch nur für einen bestimmten Anteil der exprimierten Rezeptoren. Die Einzelmolekül-co-lokalisierungsstudie unter Verwendung von der hochauflösenden Mikroskopiemethode dSTORM bestätigte, dass nur wenige Mc4r A und B1 Rezeptoren auf der Membran co-lokalisiert sind. Insgesamt ist die artspezifische Regulation des Pubertätsbeginns bei *X. nigrensis* und *X. multilineatus* auf das Vorhandensein von Mc4r B Allelen und teilweise auf deren Interaktion mit Genprodukten des A Allels zurückzuführen. Dies wird dadurch begründet, dass ein bestimmtes cAMP Niveau (statische oder dynamische Schwelle) erreicht werden muss, um die Pubertät einzuleiten. In großen Männchen wird dieses cAMP Niveau später erreicht und so die Pubertät später eingeleitet.

Zusammenfassend ist die Regulation des Pubertätsbeginns durch die dominante negative Wirkung von mutierten Mc4r Allelen ein spezieller Mechanismus, der bisher nur bei *X. nigrensis* und *X. multilineatus* zu finden ist. Andere *Xiphophorus* Arten haben offensichtlich durch andere Mechanismen die gleiche Funktion des Signalwegs entwickelt. In anderen Fischen (Medaka) spielt Mc4r eine Rolle bei der Regulation des Wachstums und erinnert an seine Rolle bei der Energie-Homöostase beim Menschen. Die Ergebnisse dieser Studie werden dazu beitragen, die biochemischen und physiologischen Funktionen des Mc4r-Systems bei Wirbeltieren, einschließlich Menschen, besser zu verstehen.

Chapter 1

General Introduction: Puberty and the Melanocortin 4 Receptor (Mc4r) System

1. Puberty regulation in vertebrates

1.1. Hormonal regulation of puberty

Puberty is the period of physical changes, when an animal reaches sexual maturity and first becomes capable to reproduce. The main features of puberty for an organism is the maturation of reproductive organs and the development of secondary sexual characteristics. As a consequence of puberty, animals have complete sexual development, sufficient growth and enough energy storage to enable reproduction.

The hypothalamus-pituitary-gonad (HPG) axis is triggering puberty onset (**Figure 1.1**). The HPG axis is present in all vertebrates studied, from fish to mammals, and its function is conserved (Sower et al. 2009; MacManes et al. 2017). In the HPG axis, hypothalamus releases gonadotropin-releasing hormone (GnRH), which acts on the pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones circulate to ovaries and testes, and the cells in gonads respond to LH and FSH to produce androgens and estrogens, which initiate the male and female puberty. The sex steroids act back in the axis to generate feedback responses.
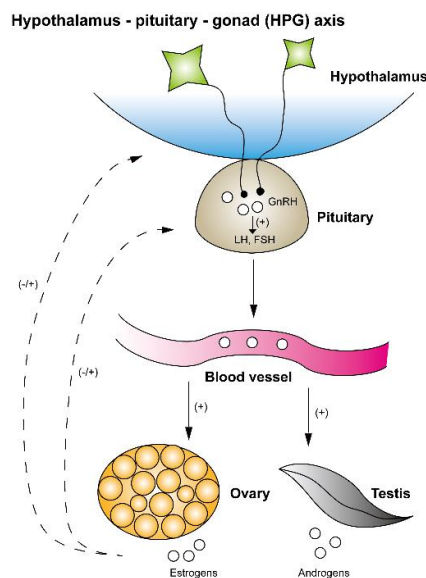
Figure 1.1 Schematic representation of hypothalamus – pituitary – gonad (HPG) axis.
HPG axis is conserved in vertebrates. Hypothalamus releases gonadotropin-releasing hormone (GnRH). GnRH stimulates the pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH circulate to ovaries and testes, and act on gonads to produce androgens and estrogens. Both hormones have feedback controls on brain function.

Upstream components of GnRH pathway include leptin-melanocortin-kisspeptin, which are considered the main regulators to transmit the permission on puberty in rodents (Manfredi-Lozano et al. 2016). Leptin and ghrelin regulate energy balance and target the neurons to produce melanocortins. Adequate energy status, like a metabolic gate, permits the process of reproductive maturation. Melanocortins act downstream on the Kiss neurons to produce kisspeptins, which further regulate puberty by functioning on GnRH neurons to release GnRH (**Figure 1.2**).
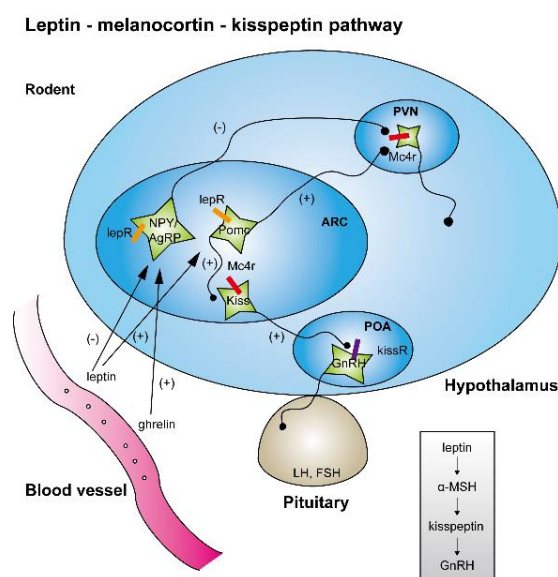
Figure 1.2 Schematic diagram of leptin – melanocortin – kisspeptin pathway in rodents.
The melanocortin 4 receptor (Mc4r) system complex is located in different subdomains of the hypothalamus. Proopiomelanocortin (Pomc) and agouti-related protein (Agrp) are expressed in a group of neurons in the hypothalamic arcuate nucleus (ARC). Mc4r is expressed in the paraventricular nucleus (PVN) of the hypothalamus. Leptin and ghrelin are acting upstream of Mc4r on Pomc neurons in ARC. In rodents, kisspeptin is the master puberty activating factor, and downstream effector of Mc4r. GnRH neuron in preoptic area (POA) acts downstream of Kiss neuron, which projects to pituitary and mediates the secretion of gonadotropin.

1.2. Puberty in mammals, fish and other vertebrates

Despite the diversity of reproductive strategies in different species, the HPG axis is commonly conserved in mammalian and non-mammalian vertebrates (Ball and Wade 2013) and GnRH is a conserved factor in this neurohormonal axis. Kisspeptins are well known to play an important role as the master puberty regulator in central control

of reproduction in mammals (Uenoyama et al. 2018). Kisspeptins are present in all classes of vertebrates except birds (Tena-Sempere et al. 2012). In other non-mammalian vertebrates, like fish, amphibians and reptiles, the kisspeptin system is also the major puberty regulator (Tena-Sempere et al. 2012). For fish in general, the same neuroendocrine circuit is applied. In addition to environmental factors influencing the puberty processes, other genes besides kisspeptin may affect the onset of puberty. Particularly, in a teleost fish, *Xiphophorus*, the melanocortin 4 receptor (*mc4r*) genes in the *Puberty* (*P*) locus are reported to be the genes that influence the HPG axis and determine the timing of puberty onset (Lampert et al. 2010).

2. Melanocortin 4 receptor (Mc4r)

2.1. The melanocortin 4 receptor system

The melanocortin system is a hormonal neuropeptidergic signaling system in vertebrates, that includes melanocortin receptors (Mcr), various agonists derived from proopiomelanocortin (Pomc), antagonists agouti and agouti-related protein (Agrp), and accessory proteins melanocortin 2 receptor accessory protein (Mrap) and melanocortin receptor accessory protein 2 (Mrap2).

The Mcr family comprises five receptors, Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r, that all belong to class A G-protein coupled receptors (GPCR). The five members are expressed in different tissues and function in various physiological processes (**Figure 1.3**). Mc1r is expressed in melanocytes and has a function in pigmentation. Mc2r is located in the adrenal cortex and has effects on adrenocortical steroidogenesis. Mc3r is in central nerve system, gastrointestinal tracts and kidney, playing a role in energy homeostasis and sodium balance. Mc4r is mainly expressed in brain and spinal cord which regulate energy homeostasis and autonomic function. Mc5r is detected in a range of tissues, enriched in exocrine cells, and functions in synthesis and secretion of exocrine products (Cone 2006).

In the Mc4r signaling system, Mrap2 is the accessory protein, Pomc is the precursor of agonists, and Agrp is the antagonist and as well as an inverse agonist (Cortes et al. 2014). Pomc is the precursor of α-, β- or γ- melanocyte-stimulating hormone (MSH) and the adrenocorticotropic hormone (ACTH) in mammals, while teleosts lack γ-MSH (**Figure 1.3**) (Cone 2006; Cortes et al. 2014). These Pomc-derived ligands

activate Mc4r, with β-MSH exhibiting highest affinity to Mc4r (Schioth et al. 1996). Other Mcrs also bind to the four ligands, except for Mc2r, which is specific for ACTH (Mountjoy et al. 1992).
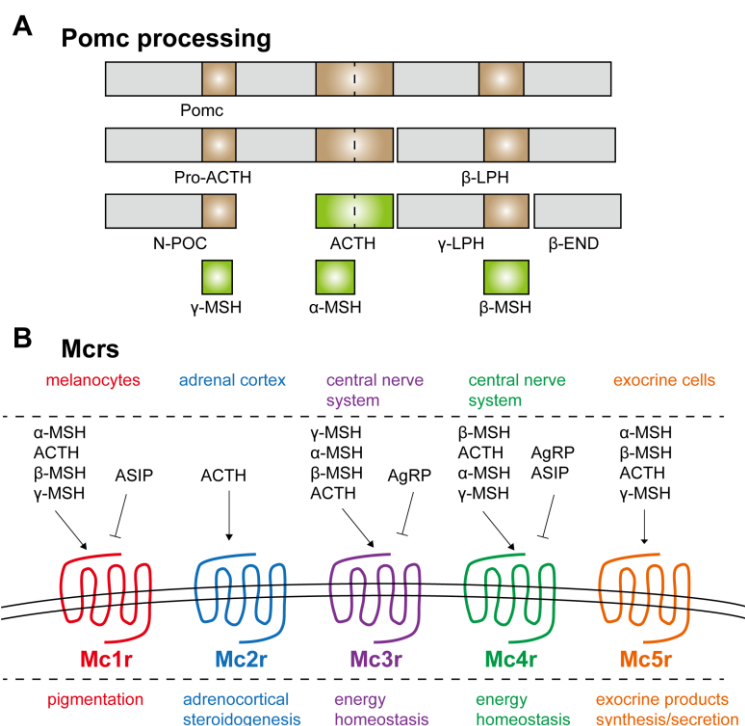
Figure 1.3 Post-translational processing of Pomc and melanocortin system in human.
(A) Proopiomelanocortin (Pomc) prohormone is modified through post-translational processing by prohormone convertases (PC1/3 and PC2). Melanocortins (ACTH, α-, β- and γ-MSHs) are derived from Pomc. **(B)** Melanocortin receptors (Mcrs) have five members, including Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r. The melanocortins are agonists to Mcrs, and agouti-signaling protein (ASIP) and agouti-related protein (AgRP) are antagonists to Mcrs. Agonists and antagonists bind to five Mcrs with various affinities. Mc2r uses only ACTH as agonist, while other Mcrs can react to several melanocortins. For simplification, only main tissues and functions are displayed. Abbreviations: ACTH, adrenocorticotropic hormone; LPH, lipotropin; N-POC, N-terminal peptide; END, endorphin; MSH, melanocyte stimulating hormone.

2.2. Mc4r biochemistry

Similar to other members in class A GPCRs, which includes well-studied rhodopsin, Mc4r is a seven-transmembrane domain (7TM) receptor. It consists of seven hydrophobic transmembrane (TM) helices, three intracellular loops (ILs), three extracellular loops (ELs), an extracellular amino-terminus (N-terminus), and an

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intracellular cytoplasmic carboxyl-terminus (C-terminus). The tertiary structure of Mc4r forms a barrel shape. The residues in the TM regions are important for ligand binding and signaling from the evidence of human MC4R studies, for example, TM3 is important for NDP-α-MSH binding and residues in TM5/6 affect α-MSH binding (Yang and Harmon 2017). The N-terminus of MC4R has agonistic effect and modulates its constitutive activity (Srinivasan et al. 2004; Ersoy et al. 2012). The C-terminus has a role in receptor trafficking and signaling. Truncation before the cysteine residues C318/C319 in the C-terminus of human MC4R impairs receptor membrane expression and function (Moore and Mirshahi 2018). Cell surface expression of MC4R is dependent on isoleucine residues I316/317 in the C-terminus (VanLeeuwen et al. 2003). TM3/4 and IL2 are the interface of human MC4R dimerization (Piechowski et al. 2013). A recent report also points out the importance of IL1, IL2, IL3 for G-protein coupling and activation (Heyder et al. 2019).

The downstream signaling in MC4R neurons is typically triggered by MC4R coupling to a stimulatory G protein (G_s) (Gantz et al. 1993). Binding of G_s regulates adenylyl cyclase (AC) activity. This increases cyclic 3',5'-adenosine monophosphate (cAMP) levels in the cell and enhances protein kinase A (PKA) activity. Some evidence also points to the coupling of MC4R to other G proteins (Breit et al. 2011), such as G_q in GT1-1 cells to contribute to calcium accumulation (Newman et al. 2006) or $G_{i/o}$ in HEK293 cells to mediate ERK activation (Chai et al. 2006).

Dimerization of many GPCRs can influence the signaling properties of the interacting protomers – such as ligand binding, G-protein coupling selectivity, or signal transduction mechanisms. Many GPCRs has been described as transient dimers though no specific interfaces are known. Regions of several TMs are reported to be involved by hydrophobic interaction for several class A GPCRs; and in some class C GPCRs other regions are crucial for interaction as well, like coiled-coil domains in C-terminus (for $GABA_B$) and disulphide bond in N-terminus (for $mGlu_1$) (Kunishima et al. 2000; Bowery et al. 2002; George et al. 2002; Milligan et al. 2019).

The first discovery of MC4R dimerization is from the MC4R D90N mutant (in TM2) in an early-onset obese patient (Biebermann et al. 2003). The mutant protein acts as a dominant negative mutant, and the mutant and wild-type protein form homo- or heterodimers. Another evidence shows that MC4R exists as a constitutive homodimer. This dimerization is not regulated by ligands binding (Nickolls and Maki 2006).

2.3. Mc4r effects on obesity and puberty in mammals

The MC4R system plays a key role in body weight homeostasis and is particularly relevant to human obesity. *MC4R* mutations can cause severe early-onset obesity in human, and the energy intake of inactive MC4R patients is as high as leptin deficiency patients (Farooqi et al. 2003). Furthermore, POMC loss-of-function mutations (Krude et al. 1998; Yaswen et al. 1999), AGRP overexpression (Graham et al. 1997) and MRAP2 mutations (Asai et al. 2013; Liu et al. 2013) all lead to obese phenotype in mammals (**Figure 1.4**).
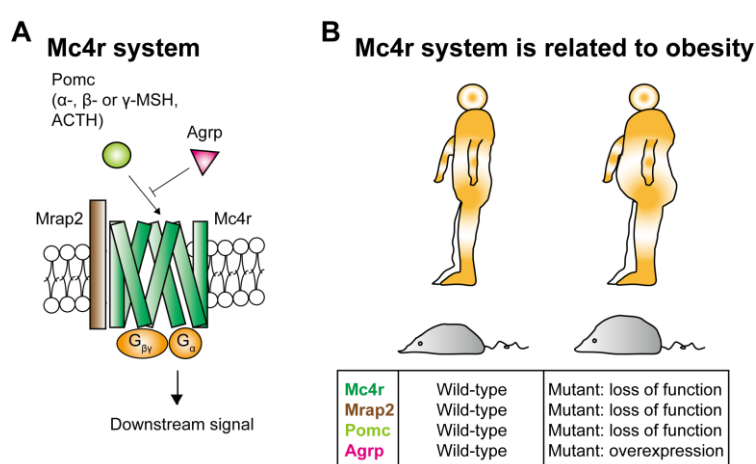
Figure 1.4 Mc4r system components and their relation to obesity in mammals.
(A) Mc4r is a G-protein coupled receptor and has seven transmembrane helices. Melanocortin receptor accessory protein 2 (Mrap2), Pomc-derived melanocortins as agonists, and Agrp as antagonists are other components in Mc4r system. Mc4r couples to G proteins and transduces downstream signaling. Mc4r mainly couples to the stimulatory G protein (G_s), resulting in increased adenylate cyclase (AC) activity and cAMP accumulation. **(B)** *MC4R* mutation causes severe early-onset obesity in human. MRAP2 mutations, POMC loss-of-function mutations, AGRP overexpression all lead to obese phenotype in mammals.

Several hormone molecules relate to MC4R, such as leptin, ghrelin, kisspeptin, and GnRH. Leptin and ghrelin are acting upstream of MC4R on Pomc neurons and/or Agrp neurons (Wilson and Enriori 2015). Kisspeptin is the master puberty activating factor (de Roux et al. 2003; Seminara et al. 2003), and a downstream effector of MC4R (Manfredi-Lozano et al. 2016). GnRH neurons act downstream of Kiss neurons, which project to the pituitary and mediate the secretion of gonadotropin.

Humans with loss-of-function mutations of MC4R signaling pathways develop obesity but have no reproductive deficits (Yeo et al. 1998). But mice with inactivated MC4R or POMC develop not only obesity but also subfertility (Elias and Purohit 2013; Hill and Elias 2018). Moreover, MC4R antagonist agouti overexpressing A^y mice are infertile (Granholm et al. 1986). In addition, in human, leptin is a metabolic gate for puberty onset (Cheung et al. 1997). This suggests possible a relevance of MC4R pathway in the metabolic control of puberty.

3. Mc4r and puberty in *Xiphophorus* fish

3.1. *Xiphophorus* fish

Teleost fish show an extremely high biodiversity, being almost half of the extant vertebrate species. This huge variety is credited to speciation triggered by such as gene gain/loss of function in genome duplication and sex chromosome plasticity (Volff 2005). During the evolution of vertebrates, two rounds of genome duplication (2R) have occurred. Furthermore, in the teleost fishes, a fish-specific genome duplication (3R) has led to more copies of ancestral genes (Meyer and Schartl 1999; Postlethwait et al. 2004; Meyer and Van de Peer 2005). Reproductive innovations are important for diversity and puberty is critical for reproduction adaptation.

Sex chromosomes are a hot spot of evolution, leading to sex- and reproduction-related traits. A highly polymorphic locus is located in the sex chromosomes of *Xiphophorus* fish, which controls male fish puberty, size and mating strategies polymorphism in wild population of these fish (Lampert et al. 2010). In this study, *Xiphophorus* and medaka fish were investigated to understand the regulation of sex maturation.

Fish in the genus *Xiphophorus* (family Poeciliidae, order Cyprinodontiformes), with known 26 species, are small freshwater fish that are native to Mexico and adjacent Central America (Rosen 1960; Kallman and Kazianis 2006). *Xiphophorus* fish, with common names as platyfish (Northern platy and Southern platy) and swordtails (Northern swordtail and Southern swordtail), are live-bearing fish, which have internal fertilization and ovoviviparity (**Figure 1.5**). A few examples of the fish in *Xiphophorus* genus include *Xiphophorus maculatus* (southern platyfish, genome available (Schartl et al. 2013)), *X. multilineatus* (transcriptome available (Lu et al. 2017)), *X. nigrensis* (Panuco swordtail), *X. hellerii* (green swordtail, genome available

(Shen et al. 2016)), *X. couchianus* (Monterrey platyfish, genome available (Shen et al. 2016)) and *X. montezumae* (Montezuma swordtail, genome available [unpublished data]) (Kang et al. 2013).
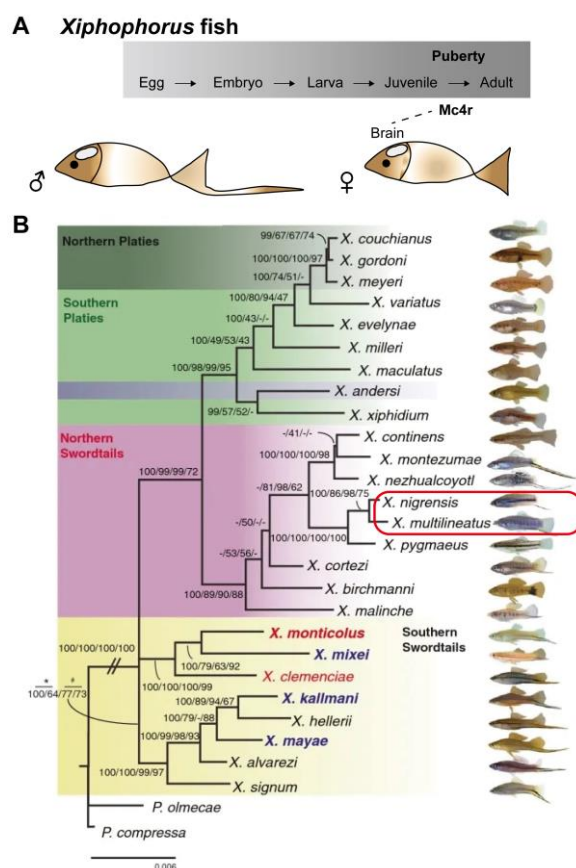
Figure 1.5 Puberty regulation by Mc4r and species phylogeny in *Xiphophorus* fish.

(A) Mc4r is a receptor mainly located in brains and is controlling the puberty onset regulation in some *Xiphophorus* fish species. **(B)** *Xiphophorus* fish are live-bearers and mainly categorized into platyfish (without swords) and swordtails (males with swords, and mainly used here). A previous study analyzed puberty and Mc4r in *X. nigrensis* and *X. multilineatus*. The phylogeny is modified from paper (Kang et al. 2013).

As a classic model, *Xiphophorus* fish have long been used as an important experimental animal model in genetics and evolution to understand genetic factors in quantitative traits, such as cancer, pigmentation, sex determination and reproduction (Volff and Schartl 2002; Meierjohann and Schartl 2006; Selz et al. 2007; Schultheis et al. 2009; Lampert et al. 2010; Volff et al. 2013; Schartl and Walter 2016; Kottler and Schartl 2018). In the 1970s, Kallman has discovered that in *X. maculatus*, a *P* locus

(namely "*Puberty*", yet previously known as "*Pituitary*" locus) on the sex chromosome is controlling the puberty onset in female and male, fecundity in females, and adult body size in males (Kallman and Schreibman 1973; Kallman and Borkoski 1978). The *P* locus is also associated with age and size at puberty in other *Xiphophorus* fish, nine *P* alleles being identified in *X. maculatus*, four in *X. multilineatus* and several in other *Xiphophorus* species (Kallman 1989; Schreibman et al. 1994). Male Poeciliids cease to grow largely upon sex maturation, therefore the body size at adulthood, in turn, reflects the puberty timing (Kallman and Schreibman 1973; Morris and Ryan 1990). Females continue to grow after puberty and do not have remarkable size differences among genotypes (Kallman and Schreibman 1973). Male sizes are distributed over a wide range: early-maturing males are small, while late-maturing males are large, with intermediate-maturing exhibiting intermediate size. Moreover, alternative mating tactics are applied by large and small *Xiphophorus* males: large males defend own territories and court to females by ritualized behavior, and small males swim between territories and grasp opportunities to sneak mate with females; females prefer the large males (Zimmerer 1982; Ryan and Causey 1989; Zimmerer and Kallman 1989; Ryan et al. 1990; Ryan and Rosenthal 2001).

The polymorphism in body size and puberty timing in these fish make them a good case to study puberty regulation. The *P* alleles controlling the phenotypic polymorphism encode melanocortin 4 receptor (*mc4r*) (Lampert et al. 2010; Volff et al. 2013). Although *X. maculatus* large-/intermediate-/small-size males covering a wide continuous size range can be explained by a high diversity of *mc4r* (10 copies on each X and Y chromosomes) in this species (Volff et al. 2013), a simpler case has been found in the *X. nigrensis* and *X. multilineatus* fish (Lampert et al. 2010).

Four size classes – small (s), intermediate-1 (I), intermediate-2 (II), and large (L) – determined by *P* alleles in the two species (Kallman 1989; Zimmerer and Kallman 1989) possess only three major *mc4r* alleles classes, functional allele A, and non-signal-transducing versions B1, B2; and furthermore, the copy number variation of B alleles is as the basis of the extreme polymorphism (Lampert et al. 2010). My investigation mainly bases on the Mc4r from *X. nigrensis*, *X. multilineatus* and *X. hellerii*.

3.2. Mc4r in *Xiphophorus maculatus*

In the *P* locus of *X. maculatus*, *mc4r* genes were suggested to be the *P* gene. Multiple copies of *mc4r* genes are present in the natural population. In *X. maculatus*, *mc4r* has 20 copies and all the copies are located in the sex chromosome X or Y (Volff et al. 2013). Among them, copy S/U appear to be the wild-type copy, being able to increase cAMP production upon agonists stimulation.

The sequence of *mc4r* copies are diversified (Volff et al. 2013). Like known *mc4r* genes in other vertebrates, all *mc4r* copies are intronless. Compared to *mc4r* genes from other species, substitution and insertions/deletions (indels) were identified and some mutations are identical in different copies. Although the wild-type copy S/U does not resemble a putative ancestral receptor, due to a short C-terminal truncation, the copy S/U may function as a classic Mc4r receptor deduced by the preservation of the dicysteine motif which is essential for receptor activation. Except for copy S/U, none of the other copies is predicted to transmit ligand-induced signaling. Some are able to bind agonists and could be the dominant negative versions of Mc4r. Some have high constitutive cAMP production, showing constitutive activity in the absence of agonists. This high level of variations was proposed to explain the polymorphism of puberty and body size in *X. maculatus*.

3.3. Mc4r in puberty onset regulation of *Xiphophorus nigrensis* and *Xiphophorus multilineatus*

In *X. nigrensis* and *X. multilineatus*, through mutational processes independent of that in *X. maculatus*, *mc4r* is represented by two allele groups. From biochemical evidence, A alleles, similar to *X. maculatus* S alleles, are perceived to be functional alleles, and are regarded as the wild-type allele; while B alleles (B1 and B2) lack the dicysteine motif, being the mutant alleles (**Figure 1.6**) (Lampert et al. 2010). B2 has another four-base deletion, resulting in an elongated C-terminus.

The signaling capacity of A and B differs largely. Receptors encoded by A alleles, but not B alleles, can be stimulated by agonists. Reporter gene expression of A alleles was reduced by a high level of B allele expression (Lampert et al. 2010). The B alleles might be a dominant negative version receptor acting on A alleles, diminishing the signals from the functional A alleles, and thereby delaying the onset of puberty.
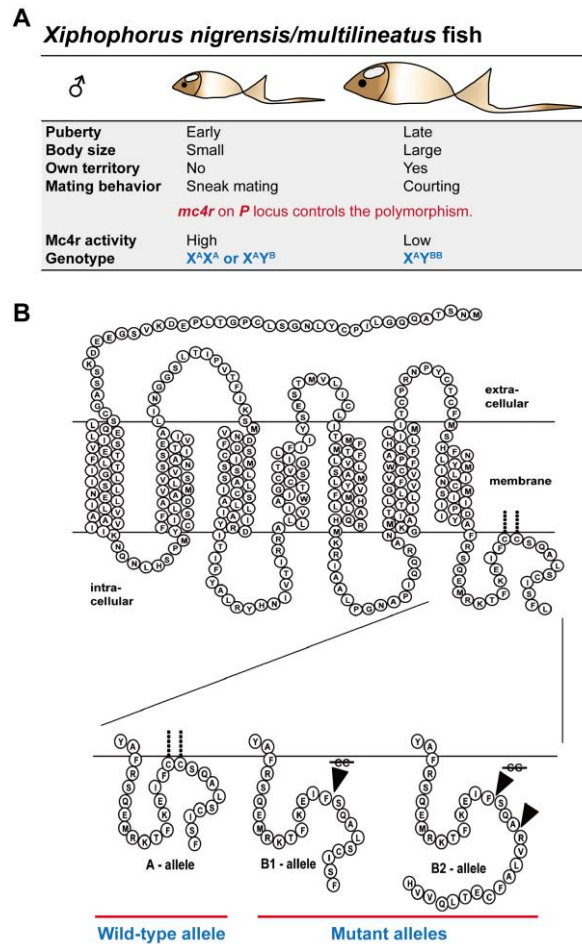
Figure 1.6 Puberty phenotypes and Mc4r receptors in *X. nigrensis/multilineatus* fish.

(A) In nature, *X. nigrensis/multilineatus* fish have small males and large males. They differ by puberty phenotypes and reproductive strategies. **(B)** In these fish, puberty controlling gene *mc4r* have A and B1/B2 alleles. A alleles are wild-type signal-transducing alleles, and B1/B2 alleles are mutant non-functional alleles. The differences among them are the dicysteine motif at cytoplasmic carboxyl-terminus. Large males possess B alleles and high B allele copy number, leading to low Mc4r activity, and consequently later puberty. The receptor scheme is modified from paper (Lampert et al. 2010). Copyright © 2010 by Elsevier. Reprinted with permission.

X. nigrensis and *X. multilineatus* have an XX/XY sex determination system. Mc4r A alleles are mainly assigned to X chromosomes and present in all animals, whereas B alleles are on Y chromosomes and can be found only in males. The male body size positively correlates with B alleles copy number. Moreover, Mc4r expression is higher in large and intermediate males than small males and females. This allelic and copy-number variation is responsible for the polymorphism of puberty and body size in *X. nigrensis* and *X. multilineatus* (Lampert et al. 2010).

4. Medaka model

Since live-bearers *Xiphophorus* fish are not applicable to reverse genetics, the phylogenetically close species medaka (Japanese rice fish) are used in the study as model animals (**Figure 1.7**) (Wittbrodt et al. 2002).
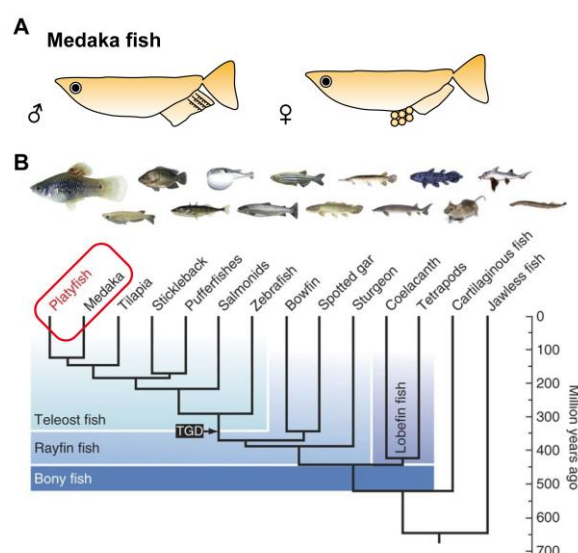
Figure 1.7 Medaka fish as a model organism.

(A) Medaka (*Oryzias latipes*) fish are egg-laying fish used in this study. The morphology of immature medaka males and females is similar and mature medaka show differences on their fin shape. **(B)** Medaka is phylogenetically related to *Xiphophorus* fish. The phylogeny is modified from paper (Schartl et al. 2013).

Medaka, *Oryzias latipes* (order Beloniformes, family Adrianichthyidae and subfamily Oryziinae), are small, oviparous freshwater fish that are distributed in Japan, Korea and China. They have been used widely as model animals in addition to zebrafish (*Danio rerio*) due to several advantages.

First and foremost, because medaka can spawn 30-50 fertilized eggs daily and eggs develop externally, known zebrafish techniques such as morpholino knockdown, CRISPR/Cas9 knockout/knockin and more others can be used in medaka (Kirchmaier et al. 2015). Second, the transparent eggs/embryos and synchronous early development, which has been described as 45 developmental stages (Iwamatsu 2004), allow easy observation under stereomicroscopes. Lastly, medaka are easily bred and

hardy: at 26 °C embryos hatch seven days post fertilization and reach sexual maturity around three months. Within a temperature range from 4 °C to 40 °C medaka can respond by altered developmental speed, and medaka are unaffected by common fish diseases.

Medaka has an XX/XY sex determination system, and 24 sets of chromosomes, in which chromosome one is the sex chromosome. The medaka genome size is around 800 million base pairs (Mb), much smaller than zebrafish's 1700 Mb. The lab strain used in this study is Carbio strain, obtained from Carolina Biological Supplies. The Mc4r system has not been studied in medaka fish. Hence, here we use medaka as a genetic model animal in addition to *Xiphophorus* fish.

5. Aim of the project

The first step towards successful sexual reproduction for vertebrates is to attain sexual maturity. In vertebrates, the HPG axis plays a key role in puberty regulation and the kisspeptin is the major puberty regulator. On top of the regulators, Mc4r has been demonstrated to be the determining factor of onset of puberty in *Xiphophorus* fish. To date, nevertheless, how Mc4r controls puberty onset and interacts with other components of the systems remains to be elucidated. In this context, the aim of the thesis is to understand Mc4r in the process of puberty onset in a mechanistic and comparative manner.

The main aim of the thesis is to biochemically characterize the Mc4r signaling system in *Xiphophorus nigrensis* and *Xiphophorus multilineatus*. To investigate the biochemical mechanisms of puberty regulation by Mc4r in these *Xiphophorus* fish, the hypothesis is that either Mc4r dimerization and/or Mrap2 interaction with Mc4r or other biochemical mechanisms are responsible for puberty regulation. Therefore, gene localization should be analyzed, interaction studies should be performed to test the hypothesis of Mc4r dimerization, and functional assays should be used to test the hypothesis of Mrap2 interaction with Mc4r. This is expected to shed light on the possible molecular mechanisms how Mc4r determines and regulates the puberty signaling pathway.

To investigate if the mechanisms of Mc4r puberty regulation in *X. nigrensis* and *X. multilineatus* is conserved in other species, another swordtail species and medaka

are studied. In *X. hellerii*, Mc4r should be analyzed on allele type and by transcript localizations. In medaka, gene expression and gene localization are planned for analysis using various strategies. The available Mc4r knockout (KO) medaka should be compared to wild-type (WT) fish in the study to describe and to characterize the function of medaka Mc4r. This is expected to provide a view on Mc4r signaling system in other representative fish species.

Altogether, the central aim of this work is to contribute to better understand the biochemical and physiological functions of the Mc4r system in vertebrates including human.

Chapter 2

Analysis of the Role of the Mc4r System in Development, Growth and Puberty of Medaka

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Author Contributions	
RL	performed the phylogeny, sampling, RT-qPCR, *in situ* hybridizations, KO adult and embryos functional analysis, and drafted the manuscript.
MK	established and provided the KO medaka line.
MA	supervised the experiments and helped to review the manuscript.
MS	defined and designed the study, coordinated all steps of the research and reviewed all versions of the manuscript.

Abstract

The melanocortin 4 receptor (Mc4r) signaling system mainly functions in energy homeostasis and appetite regulation in mammals. In fish of the genus *Xiphophorus* (platyfish and swordtails), *mc4r* encoded in *P* locus is the gene that determines the puberty onset. In wild populations of *Xiphophorus*, there are early- and late-maturing individuals; these polymorphisms are owing to copy number variation of different *mc4r* alleles. To reveal whether this is an adaptation of the Mc4r signaling system exceptional in the lineage of *Xiphophorus* or a more conserved mechanism commonly in teleosts, we investigated the role of Mc4r in the reproductive biology of medaka (*Oryzias latipes*). Medaka is a phylogenetically close relative to *Xiphophorus* and as well as a well-established model to study gonadal development. To better understand the potential role of Mc4r in medaka, we first characterized the Mc4r signaling system (*mc4r*, *mrap2*, *pomc*, *agrp1*). All these genes are expressed before hatching stages. In adults, they are primarily expressed in the brain. The transcript of *mc4r* and the receptor accessory protein *mrap2* co-localized in the hypothalamus in adult brains, indicating a conserved function of Mrap2 in modulating Mc4r signaling. Comparison of growth and puberty between wild-type and *mc4r* knockout medaka showed that the knockout of *mc4r* does not alter puberty timing but significantly delays hatching. Embryonic development of *mc4r* knockout fish is retarded compared to wild-types. In summary, the Mc4r signaling system in medaka is related to regulation of growth rather than puberty.

1. Introduction

Puberty, or sexual maturation, is the process through which an animal develops from an immature stage to the mature stage and acquires first time the capability to reproduce. This process is influenced by both the genetic background and the environmental factors (Palmert and Boepple 2001; Okuzawa 2002). Teleost fish of the genus *Xiphophorus* (platyfish and swordtails) exhibit polymorphisms in reproduction phenotypes, such as puberty onset timing, body size and reproduction strategies (Kallman and Borkoski 1978). The gene critically involved in establishing such polymorphisms is the melanocortin 4 receptor (Mc4r) encoded in the *P* (*Puberty*) locus (Lampert et al. 2010; Volff et al. 2013). The early or late puberty onset is determined by the *mc4r* alleles types and copy number variation of these *mc4r* alleles (Lampert et al. 2010).

Mc4r is a member of the melanocortin receptor family, which comprises five members, Mc1r to Mc5r; all belonging to the class A of G protein-coupled receptor (GPCR) (Cortes et al. 2014). Melanocortin receptors regulate various functions, for instance, pigmentation for Mc1r (Garcia-Borron et al. 2014), and energy homeostasis for Mc4r (Cone 2006). In addition to Mc4r receptor itself and its cognate ligands melanocortins, which are cleaved from the agonist precursor pro-opiomelanocortin (Pomc), the Mc4r signaling system consists of the antagonist agouti-related peptide (Agrp), and melanocortin receptor accessory protein 2 (Mrap2) (Cortes et al. 2014). Pomc in vertebrate is processed into physiological ligands α-MSH, β-MSH, γ-MSH, ACTH, however, teleosts lack γ-MSH (Alrubaian et al. 2003). Except for Mc2r, which interacts exclusively with ACTH; all other melanocortin receptors can bind the four ligands (Mountjoy et al. 1992). The four ligands have different affinities to each of the five receptors (Cone 2006). Mc4r shows the highest affinity for the β-MSH ligand and can be activated by synthetic ligand NDP-MSH, a highly potent α-MSH analog (Schioth et al. 1996). Agrp acts as an antagonist, inhibiting the activation of Mc4r by MSH; or as an inverse agonist, inhibiting the constitutive activity of Mc4r by its N-terminus, the intramolecular ligand (Ersoy et al. 2012).

In several fish species, Mc4r has been described to be involved in many physiological processes. The key physiological processes affected are energy balance and food intake, as reported in goldfish (Cerda-Reverter et al. 2003) and rainbow trout (Schjolden et al. 2009). In zebrafish, Mrap2 controls both larval and adult

development by regulating Mc4r signaling (Sebag et al. 2013). Zebrafish Mrap2 has two forms (Agulleiro et al. 2010): Mrap2a is the larval form, blocks Mc4r, and stimulates larval growth during development, and Mrap2b is the adult form, enhances Mc4r function, and controls adult somatic growth (Sebag et al. 2013).

In mammals, Mc4r is primarily expressed in the central nervous system. In human, *MC4R* mutations are the monogenic cause of severe early-onset obesity (Farooqi et al. 2003). In mice, *Mc4r* knockout is connected to hyperphagic obesity (Huszar et al. 1997), and the obese phenotype of these mice is similar to that of the *Agrp* overexpression mice (A^{vy}) (Graham et al. 1997). Involving in energy homeostasis, Mc4r acts as a potential target for pharmacological intervention with obesity (Farooqi et al. 2003). Leptin and ghrelin signaling pathways are acting upstream of Mc4r, while kisspeptin is downstream of Mc4r (Manfredi-Lozano et al. 2016). Due to its role in the center of this regulatory network, augmenting the knowledge about Mc4r is essential for a better understanding of metabolic disorders.

Fish of the genus *Xiphophorus* offers a valuable tool to study the genetic basis of puberty regulation and the molecular factors involved in this process (Lampert et al. 2010; Maderspacher 2010; Volff et al. 2013). However, reverse genetics cannot be applied to these fish for functional studies, because they are livebearing fish and have internal embryonic development. Medaka (*Oryzias latipes*) is a phylogenetically closely related species to *Xiphophorus* and an egg-laying fish. High-quality genomes have been available for both species (Kasahara et al. 2007; Schartl et al. 2013), and medaka is an established genetic model to study development and physiology (Wittbrodt et al. 2002). As an egg-laying fish, comparable to zebrafish, medaka is amenable to gene knockdown, knockout and knockin (Kirchmaier et al. 2015). Embryonic development can be readily observed because of the transparency of medaka embryos and the well-described normal embryonic development stages (Iwamatsu 2004). However, Mc4r and its physiological role have not yet been investigated in medaka.

Despite that the association of different male polymorphisms in puberty timing and its regulation by Mc4r in *Xiphophorus* fish have been shown, a similar role of Mc4r in other species remains unclear. To advance our knowledge of fish puberty, we investigated the Mc4r system in medaka fish. Through the temporal and spatial expression profile and functional studies analysis on *mc4r* mutants, we find that the

role of *mc4r* in medaka is related to development and growth, comparable to zebrafish, but not to controlling puberty timing, like in *Xiphophorus* fish.

2. Materials and Methods

2.1. Animals

Adult medaka (*Oryzias latipes*) were maintained under a standard light/dark cycle of 14/10 h at 26 °C in the fish facility of Biocenter at the University of Wuerzburg. Eggs of medaka fish were collected and cultured until hatching in Danieau's medium (NaCl 17.4 mM, KCl 0.21 mM, $MgSO_4$ 0.12 mM, $Ca(NO_3)_2$ 0.18 mM, HEPES 1.5 mM, methylene blue 0.0001 %). The wild-type medaka used in the study were from the Carbio strain. The knockout medaka *mc4r* mutant "-2/+3" was generated by TALEN technology; for details of the generation of the mutant refer to paper (Ansai et al. 2014).

All animals were kept and sampled in accordance with the applicable EU and national German legislation governing animal experimentation, in particular, all experimental protocols were approved through an authorization (568/300-1870/13) of the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law (TierSchG).

2.2. Phylogenetic analysis

The annotated Mc4r, Mrap2, Pomc, Agrp1 sequences from human and 14 fish species were retrieved from either National Center for Biotechnology Information nucleotide sequences database (NCBI, https://www.ncbi.nlm.nih.gov/) or Ensembl genome browser (http://www.ensembl.org/index.html). Protein sequences were used in the phylogenetic analysis. The sequences were aligned by ClustalW from the software package MEGA7 (Kumar et al. 2016). Evolutionary analyses were inferred with MEGA7 based on maximum likelihood method with 1000 bootstrap replicates.

The fish species used in the study were: eleven Actinopterygians (ray-finned fish) including Amazon molly (*Poecilia formosa*), cave fish (*Astyanax mexicanus*), cod (*Gadus morhua*), fugu (*Takifugu rubripres*), medaka (*Oryzias latipes*), Southern platyfish (*Xiphophorus maculatus*), spotted gar (*Lepisosteus oculatus*), stickleback

(*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridis*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*); one Sarcopterygian (lobe-finned fish), coelacanth (*Latimeria chalumnae*); one Chondrichthyan (cartilaginous fish), elephant shark (*Callorhinchus milii*); and one Agnathan (jawless fish), lamprey (*Petromyzon marinus*). Additionally, common carp (*Cyprinus carpio*) was used in Mrap2 phylogeny as a comparison to zebrafish. These were all compared with human (*Homo sapiens*). Accession numbers are listed in **Table S2.1**.

2.3. Quantitative gene expression analysis

Quantitative gene expression of Mc4r signaling system genes (*mc4r*, *mrap2*, *pomca*, *pomcb*, *agrp1*) was analyzed using medaka embryos and tissue samples. To determine the time course of expression of these genes, three pools of medaka embryos and larvae from 0, 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 days post fertilization (dpf) were sampled (0 dpf n=100, 1-4 dpf n=50, 5-8 dpf n=30, 10-20 dpf n=15). Due to the lack of proper normalization factors (expression level of housekeeping gene *ef1a* is not same in 0 dpf sample as in others), the exact amounts of transcripts of Mc4r signaling system genes could not be determined in the 0 dpf sample. To determine the tissue expression of Mc4r signaling system genes, three pools of male or female adult medaka fish tissues (n=3-4) of brain, eye, gill, kidney, liver, gonad, skin, and muscle were analyzed.

Samples were homogenized and total RNA was extracted using TRIZOL reagent (life technologies, Carlsbad, California, United States). After DNase treatment, first strand cDNA was synthesized by a reverse transcriptase with random hexamer primers, using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, United States) according to manufacturer's procedure. All cDNA was applied to Reverse Transcription Quantitative PCR (RT-qPCR) using SYBR Green reagent. The program used is a two-step PCR program: denaturation at 95 °C 15 s and annealing/elongation (with fluorescence detection) at 60 °C 30 s for 40 cycles. Amplification was detected on a Mastercycler realplex[2] Eppendorf machine (Eppendorf, Hamburg, Germany).

Primers for the genes *mc4r*, *mrap2*, *pomca*, *pomcb*, *agrp1* and housekeeping gene *ef1a* are listed in **Table S2.2**. Expression of target genes was analyzed by the ΔΔCt

method and normalized to medaka housekeeping gene *ef1a*. The relative expressions were presented as mean ± standard deviation (SD).

2.4. Whole mount *in situ* hybridization

Whole mount RNA *in situ* hybridization was used to analyze the spatial expression patterns of *mc4r* and *mrap2*, following procedures described previously (Thisse et al. 1993; Thisse and Thisse 2008; Berger et al. 2017).

To generate riboprobes, cDNA from medaka brain were used to PCR amplify both genes (primers listed in **Table S2.2**) and the amplicons were cloned into a pGEM-T Easy vector. The plasmids were verified by sequencing and were linearized using restriction enzymes. Riboprobes were synthesized by *in vitro* transcription using T7 or SP6 RNA polymerase (Roche, Basel, Switzerland) with digoxigenin or fluorescein RNA labeling mix (Roche).

Whole mount *in situ* hybridization was performed on dissected intact adult brains. The brains were fixed in 4 % PFA and dehydrated in methanol for storage. Before hybridization, brains were rehydrated with PBST, digested with 10 µg/ml proteinase K for 45 min, and prehybridized in hybridization mix (formamide 25 mL, 20×SSC 12.5 mL, heparin (25 mg/mL) 100 µL, 20 % Tween 20 250 µL, yeast RNA (Ribonucleic acid type IV) 25 mg, add H_2O to 50 mL, stored at -20 ℃.). Samples were hybridized with riboprobes in a humidified chamber overnight at 68 ℃. The brains were then subjected to stringent washes of SSC series (0.05X SSC used for washes of high stringency: 2XSSC/hybridization mix 25 %/75 %, 50 %/50 %, 75 %/25 %, 100 %/0 %, then 0.05XSSC twice, and 0.05XSSC/PBST 50 %/50 %,) and final wash by PBST.

After washing, the samples were embedded in 3 % agarose and cut into 100 µm section by TPI Vibratome Series 1000 Sectioning System (Technical Products International Inc., St. Louis, Missouri, United States). The sections were blocked with 5 % sheep serum and incubated with antibodies anti-digoxigenin-AP (1:5000) or anti-fluorescein-AP (1:2000) overnight at 4 ℃. After removing antibodies and repeated washing, sections were stained by NBT/BCIP solution (Roche) or FastRed Tablets (Sigma, St. Louis, Missouri, United States) to develop the signals by AP reaction according to manufacturer's instruction.

Double *in situ* hybridization was performed by simultaneously hybridizing two probes and sequentially visualizing probes: first fluorescein probes and thereafter digoxigenin probes. Sections were mounted in 80 % glycerol for microscopy (Zeiss AxioPhot, using AxioVision Rel 4.8 software, Oberkochen, Germany). Fluorescence pictures were imaged with a confocal microscope (Nikon Eclipse Ti C1, using NIS-Elements AR software, Minato, Tokyo, Japan). The transcript localization was determined according to the medaka brain atlas (Anken and Bourrat 1998).

2.5. Functional analysis of knockout fish

Medaka wild-type (WT) fish (Carbio strain) and Mc4r knockout (KO) -2/+3 TALEN-KO strain (Ansai et al. 2014) (*mc4r* WT and mutant sequences were provided in **Supplementary Data**) were compared with respect to their hatching time, puberty onset timing and growth.

Three groups of 60-200 wild-type and knockout embryos were collected, and the hatching time of each embryo was recorded. Embryo development from day 0 to day 7 was compared. Embryo development and linear growth (forehead to trunk terminus) of larvae were imaged by stereomicroscope (Nikon SMZ1000 microscope, Minato, Tokyo, Japan, with LEICA DFC450C to capture images, using LAS V4.1 software, Wetzlar, Germany). Linear growth was measured using ImageJ 1.51 (Schindelin et al. 2012; Schneider et al. 2012).

Three groups of 25 wild-type and 22-25 knockout fish were raised in a two-chamber aquarium (**Figure S2.3B**) to guarantee identical rearing conditions for the wild-type and knockout groups. Puberty is the stage when fish reach sexually mature and acquire for the first time the ability to reproduce (Okuzawa 2002). Since medaka has clear secondary sexual characteristics and a special courtship behavior during the mating processes, here the medaka puberty onset was determined for females as the day of the release of the first eggs and for males by the appearance of papillary processes in the anal fin (Kinoshita et al. 2009). All fish were observed every week after grown into juveniles. Females were additionally observed every day to monitor egg release. Three independent experiments were conducted. The growth of juveniles (the period when males and females are indistinguishable) was measured each week

until puberty onset and compared between KO and WT. The linear length and the age of adults were measured at the time of puberty, and KO and WT fish were compared.

Statistical analysis of the data was done with GraphPad Prism 6 software. Two-way *ANOVA* was applied for comparison of WT and KO females and males for the time of puberty onset and the size at puberty. To compare each sample with every other sample, corrections were made for multiple comparisons by Sidak's test. Multiple *t*-test with correction for multiple comparison by Holm-Sidak method was used to compare the WT and KO growth curves. One-way *ANOVA* was used for comparison of larval length. To compare KO day 7 and KO hatch with WT hatch, corrections were made for multiple comparisons by Dunnett's test. Above values were presented as the mean ± SD. Mann-Whitney test was used to compare WT and KO hatching time. Data are presented in box plots; whiskers are from min to max. In Kaplan-Meier plots for puberty, data were compared with the log-rank (Mantel-Cox) tests. GraphPad Prism 6 was used to display the results.

3. Results

3.1. Mc4r signaling system genes in fish

To get an overall picture of Mc4r signaling system in fish, the phylogenetic relationship of the genes was explored, and they all show in general a topology that follows the known organismic relationships (**Figure S2.1A**).

The phylogenetic tree of *mc4r* is in agreement with the current fish tree of life. Intriguingly, gene *mc4r* in Southern platyfish and blind cavefish have longer branches, indicating faster evolution of *mc4r* towards specialized functions in these lineages. Gene *mrap2* in zebrafish is different from all other species. Only in zebrafish, *mrap2* has two copies (**Figure S2.1B**) which have previously been assigned to different functions for growth regulation (Sebag et al. 2013). This seems to be a specialized species-specific duplication event in zebrafish, which cannot be generalized. However, search in common carp (*Cyprinus carpio*) genome reveals also two copies of *mrap2*, *mrap2a* and *mrap2b* (**Table S2.1**), indicating it as a lineage-specific duplication.

Two *pomca* and one *pomcb* sequences were found in several teleosts, including Southern platyfish and medaka (**Table S2.1**). On the other hand, more basal teleost groups, like zebrafish and cavefish, have only one *pomca*. Differently, gene *agrp1* in all analyzed ray-finned fish including medaka is present as a single copy gene (**Figure S2.1C**).

As a puberty gene determining sexually selected traits in *Xiphophorus*, an important feature of *mc4r* is sex-chromosome linkage. In Southern platyfish, *mc4r* is located on linkage group (LG) 21, which is the sex chromosome. In medaka, however, *mc4r* is on linkage group LG 20 and not on the sex chromosome (LG 1). None of the other Mc4r signaling system genes are sex-linked, neither in *Xiphophorus* nor in medaka (**Table S2.3**).

3.2. Developmental expression of Mc4r signaling system genes

Temporal expression pattern of Mc4r signaling system genes (*mc4r*, *mrap2*, *pomca*, *pomcb*, *agrp1*) during embryonic development was analyzed by RT-qPCR. The analysis revealed that transcripts of *mc4r* and *mrap2* were already present at 0 dpf (stage 10-11 blastula stage, developmental stages see **Table S2.4**). This indicates that *mc4r* and *mrap2* have maternal mRNA contribution (data not shown). Especially for *mrap2*, the maternal contribution appears to be rather high. Other genes are exclusively expressed from zygotic transcription.

Expression of *mc4r* increases steadily starting from 1 dpf and remains relatively constant from around 5 dpf on (**Figure 2.1A**). Expression of *mrap2* increases gradually from 1 dpf on (**Figure 2.1B**). Expression of *pomca* starts and rises from 3 dpf on (**Figure 2.1C**). Expression of *pomcb* initiates at 3 dpf at low levels and increases at 6-8 dpf (**Figure 2.1D**). Expression of *agrp1* starts with very low levels at 4-5 dpf and is highly upregulated at 8 dpf (**Figure 2.1E**), showing a dramatic upregulation after the fish start to feed.

In summary, expression of the whole *mc4r* signaling system genes during ontogenesis when the larvae start to feed agrees with their functions in appetite regulation and energy homeostasis in medaka.
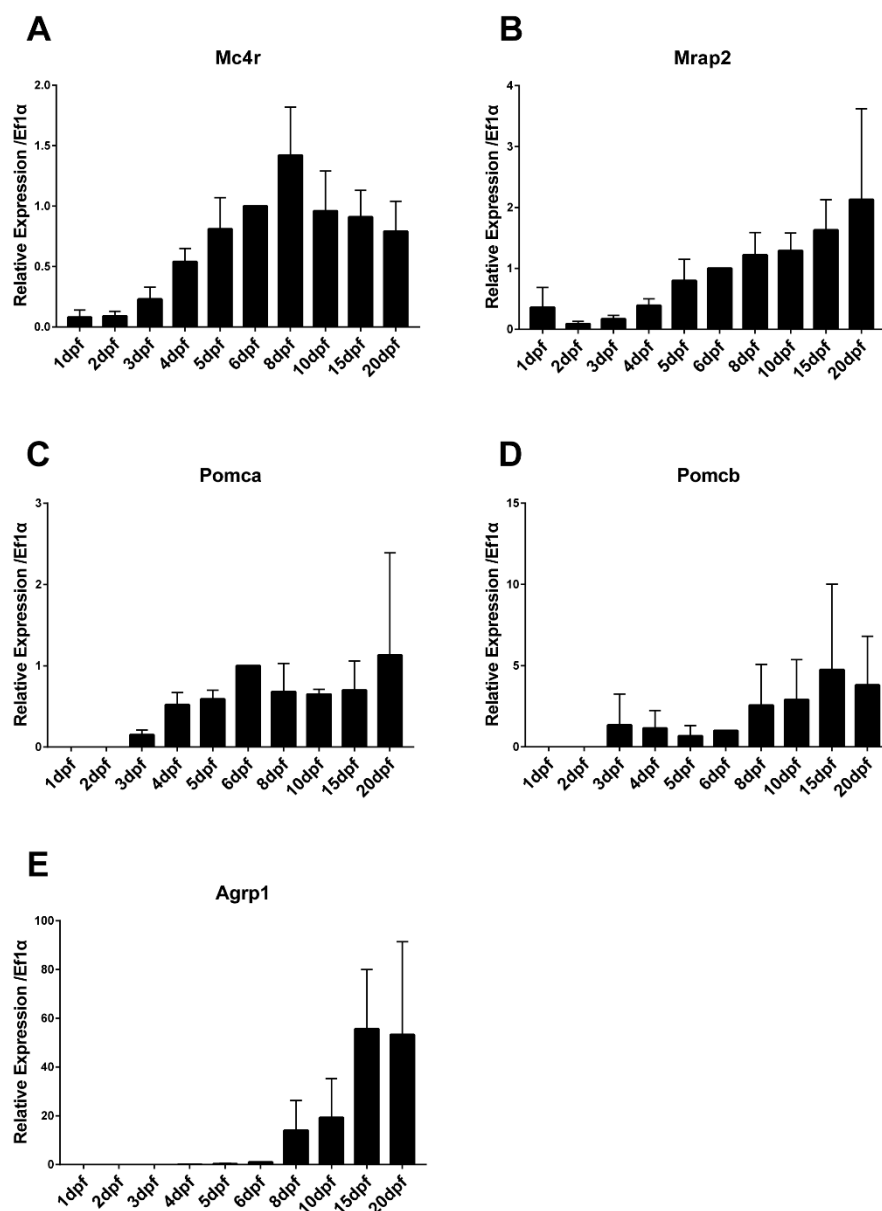
Figure 2.1 Time course of expression of Mc4r signaling system gene during medaka embryonic and larval development.

(A) Mc4r, **(B)** Mrap2, **(C)** Pomca, **(D)** Pomcb, **(E)** Agrp1. Expression levels are normalized to *ef1a* and relative to expression observed in 6 dpf. Relative expression levels are presented as mean ± SD. The figure is taken from paper (Liu et al. 2019).

3.3. Organ-specific expression of Mc4r signaling system genes

Differential expression of Mc4r signaling system genes (*mc4r*, *mrap2*, *pomca*, *pomcb*, *agrp1*) was analyzed using RT-qPCR to compare among tissues and between males and females. The *mc4r* and *mrap2* were highly expressed in the brains of both sexes (**Figure 2.2A, 2.2B**). High level of *mc4r* expression in brains of medaka is consistent

with the previous study (Volff et al. 2013). Brains of both sexes also highly express the *pomca*, *pomcb* and *agrp1* genes (**Figure 2.2C, 2.2D, 2.2E**). Among them, the expression of *agrp1* gene in female brain is higher (1.5-fold) than in male brain (**Figure 2.2E**).

In all tested tissues besides brain, *mc4r* is detected at low levels; except for skin, all the rest are background expression (**Figure 2.2A**). The *mrap2* gene also expresses in all tested tissues excluding female liver; and remarkably, it shows high expression in male kidney (12.6-fold higher than brain) and ovary (2.9-fold) (**Figure 2.2B**). Except for brains, no other tissues express *pomca*, but *pomcb* showed low expression in eyes, gills, gonads, skins and muscles (**Figure 2.2C, 2.2D**). The *agrp1* is highly expressed in testis in addition to brains, while there is only low expression in all other tissues (**Figure 2.2E**).

In brief, high expression of all *mc4r* signaling system genes in the brain of adult fish is consistent with their functions in central control of energy homeostasis in medaka. The sexually dimorphic expression pattern of *mrap2* (kidney, gonad) and *agrp1* (gonad) is intriguing and further studies are deserved to elucidate a sex-related function.

3.4. Co-localization of *mc4r* and *mrap2* in the adult brain

Mrap2 has been reported as Mc4r accessory protein which has important regulatory functions in zebrafish development (Sebag et al. 2013). Evidently, *mrap2* need to be co-expressed with *mc4r* in the same cells to achieve such functions.

To determine the localization of *mc4r* and *mrap2* expression, whole mount *in situ* hybridization was performed using intact brains from adult females and males. The *mc4r* was expressed in both the preoptic region and hypothalamus region, while the *mrap2* was only expressed in the hypothalamus (**Figure 2.3A, Figure S2.2A**). The expression region of *mc4r* in the hypothalamus is more anteriorly than that of *mrap2*. Importantly, two genes show overlapping expression region in the central part of the hypothalamus (**Figure 2.3B, Figure S2.2B**). The anatomical overlap of the expression domains may indicate that both genes are co-expressed on the cellular level. The expression patterns have no noticeable sex bias (**Figure 2.3C**). The fact that only a subset of *mc4r* expression domains overlap with that of *mrap2* suggests that the Mc4r

signaling system may function without the accessory protein Mrap2 in certain areas of the brain.
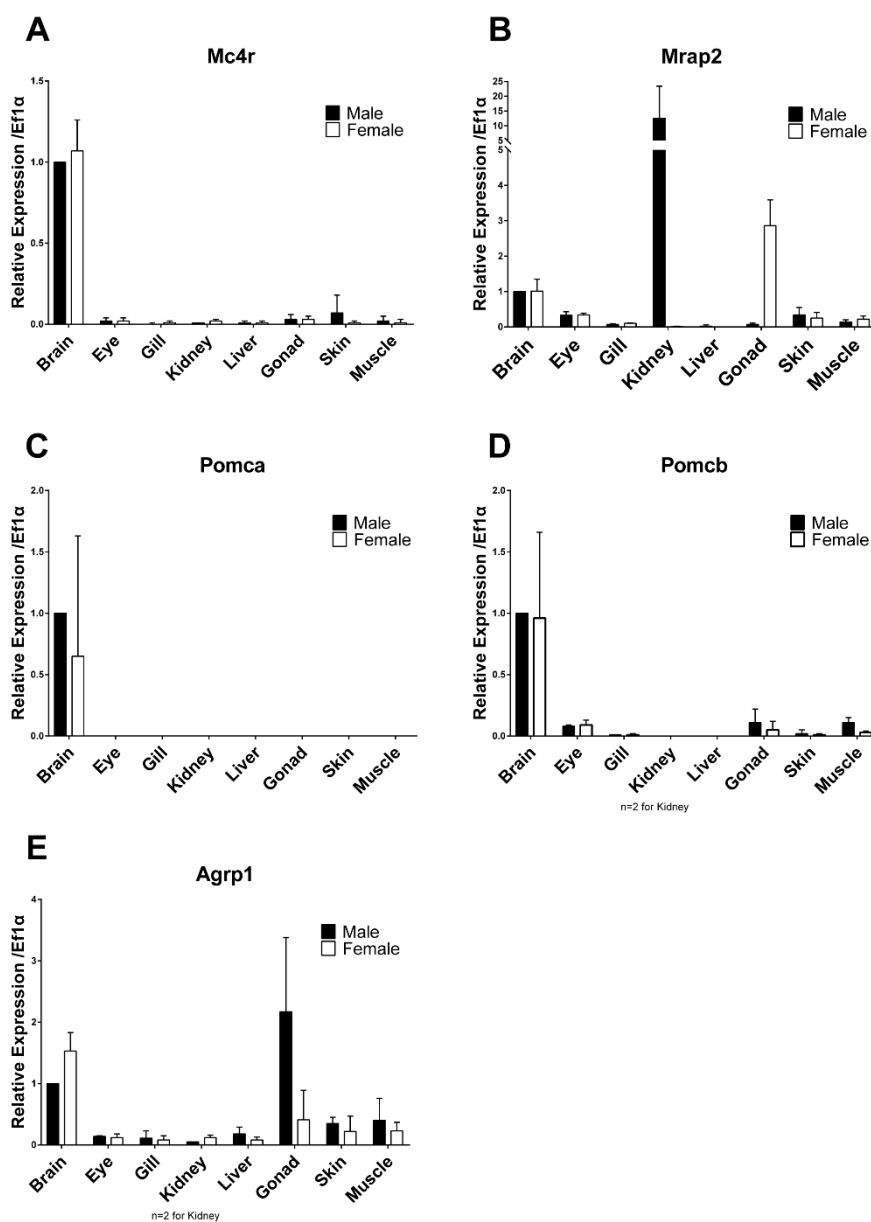
Figure 2.2 Differential tissues gene expression of Mc4r signaling system gene in adult males and females.

(A) Mc4r, **(B)** Mrap2, **(C)** Pomca, **(D)** Pomcb, **(E)** Agrp1. Expression levels are normalized to the expression level of *ef1a* and relative to expression observed in male brains. Relative expression levels are presented as mean ± SD. The figure is taken from paper (Liu et al. 2019).
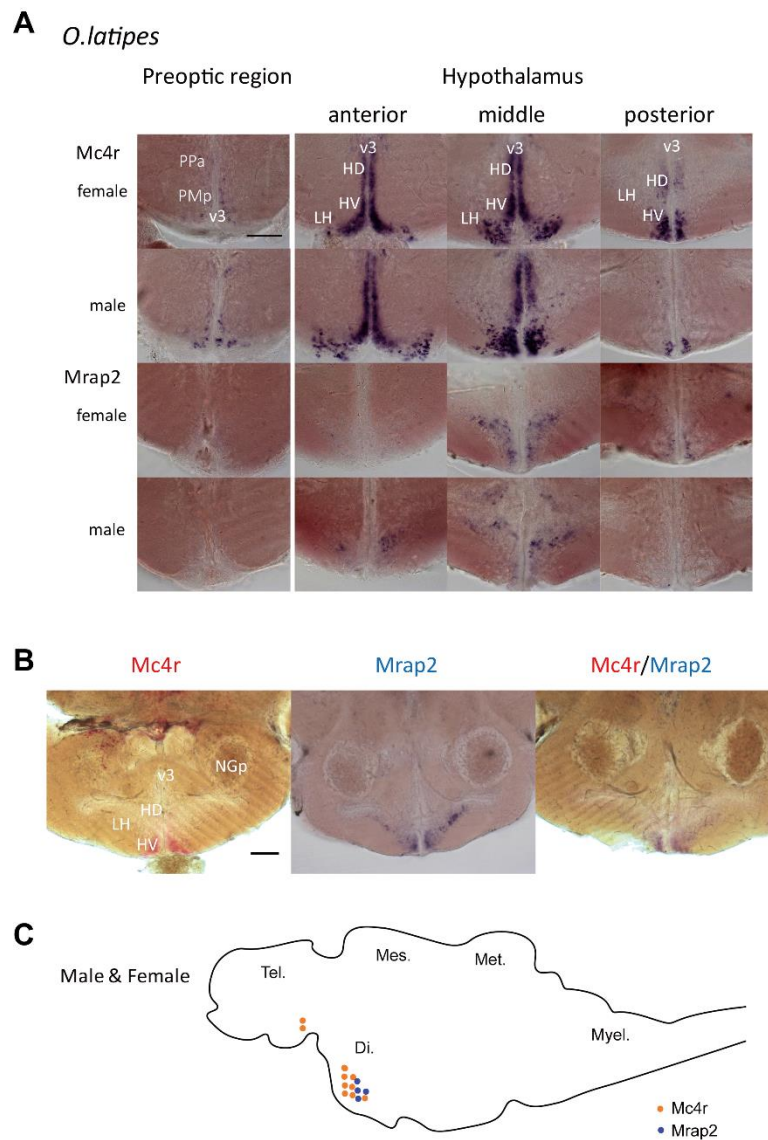
Figure 2.3 Whole mount *in situ* hybridization for mRNA localization of *mc4r* and *mrap2* in adult male and female brains.

(A) Expression localization of *mc4r* in the preoptic region and in the hypothalamus, and of *mrap2* in the medial hypothalamic region. Both genes presented the same expression pattern for males and females. Scale bar: 100 µm. **(B)** Double *in situ* hybridization in the hypothalamic region confirms the co-localization of *mc4r* (red) and *mrap2* (purple). Scale bar: 100 µm. **(C)** Schematic representation of *mc4r* (orange) and *mrap2* (blue) mRNA localization. Brain regions: Di.: diencephalon, Tel.: telencephalon, Mes.: mesencephalon, Met.: metencephalon, Myel.: myelencephalon. Brain areas in detail: v3: third ventricle, PPa: anterior parvocellular preoptic nucleus, PMp: parvocellular part, magnocellular preoptic nucleus, HD: dorsal periventricular hypothalamus, HV: ventral periventricular hypothalamus, LH: lateral nucleus of hypothalamus, NGp: posterior glomerular nucleus. The figure is taken from paper (Liu et al. 2019).

3.5. Effect of Mc4r knockout on linear growth

To examine whether *mc4r* gene would similarly determine puberty onset in medaka like in *Xiphophorus* fish, puberty onset was monitored in a medaka *mc4r* knockout line (Ansai et al. 2014). The medaka line used in the study carries -2/+3 mutation (*mc4r* sequences in **Figure S2.3A, Supplementary Data**, these indels in the *mc4r* gene were verified by genotyping and sequencing). The knockout line was compared to wild-type medaka.

Since male medaka develops upon puberty clear secondary sexual characteristics (anal fin papillary processes), which can be easily observed under a stereomicroscope, this feature was used to determine puberty onset in males. On the other hand, female medaka lays first eggs once puberty completes, thus this was used as the indicator of puberty onset in females (Sato et al. 2008).

No significant difference in puberty timing was found between KO and WT, neither in male fish nor in female fish (**Figure 2.4A, Figure S2.3C**). Body length at puberty also has no significant difference between KO and WT males. However, compared to WT females, KO females matured at shorter body length (**Figure 2.4A**). Moreover, the average standard body length of the KO fish had a trend to be shorter than that of WT. Although the difference is not significant, except at some later time points in trial 3 (**Figure 2.4B**), in general this shows that the knock-out fish grows at a slower linear growth rate. In summary, *mc4r* is not determining the onset of puberty in medaka.

3.6. Effect of Mc4r knockout on hatching timing

Another noticeable difference between WT and KO medaka is that the hatching time was delayed for KO fish. Compared to zebrafish, the developmental rate of medaka fish is much slower and the developmental stages until which they stay in the chorion are much more advanced (Kimmel et al. 1995; Iwamatsu 2004). This allowed to better distinguish changes in the timing of hatching in medaka.
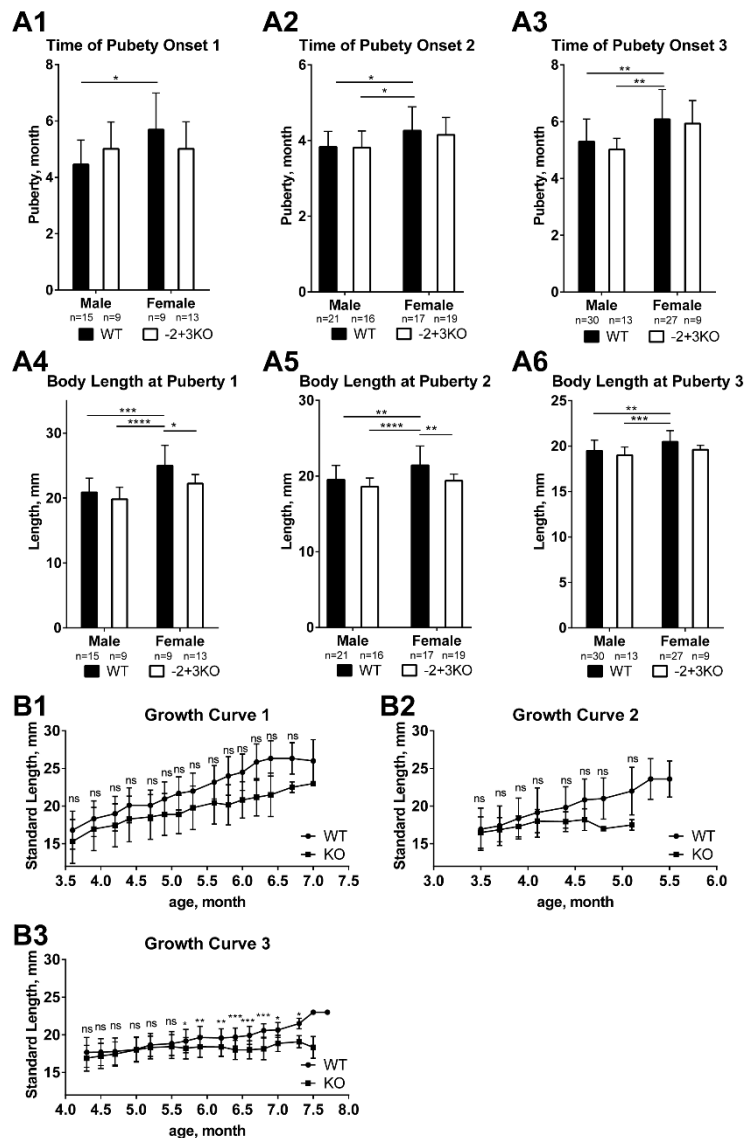
Figure 2.4 Effect of *mc4r* knockout on medaka growth and puberty.
(A) No differences in puberty onset timing in the three trials **(A1, A2, A3)**, but some differences in body length at puberty between WT and KO medaka females and males in three replicates **(A4, A5, A6)**. Data are presented as mean ± SD. Statistical significance after two-way *ANOVA* is indicated as asterisks (*). **(B)** The standard length of WT and KO during growth in the three experiments **(B1, B2, B3)**. Data are presented as mean ± SD. Statistical significance after multiple *t*-test is indicated as asterisks (*). For all statistics: ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. The figure is taken from paper (Liu et al. 2019).

From three independent experiments, hatching of the KO medaka is significantly delayed, clearly for at least one day (**Figure 2.5A**). The development of WT and KO embryos were followed to observe if the delay is because of a possible change in embryo development and growth. The KO embryos were about one stage slower than

WT starting already at day one (**Figure S2.4**). For embryos from day three on, the difference became more obvious. The body of KO fish was covering smaller portions of the yolk sphere than of WT fish. Afterwards, the standard body length of freshly hatched WT (day 7) and KO fish dechorionated on day 7 were compared, KO larvae at day 7 were significantly shorter than WT at day 7(**Figure 2.5B, 2.5C**). Finally, the body length of freshly hatched KO to WT were compared, the hatched KO despite being 1-4 days older had the equivalent size as the hatched WT (**Figure 2.5B, 2.5C**). All in all, these results show that *mc4r* is involved in regulating growth in medaka.
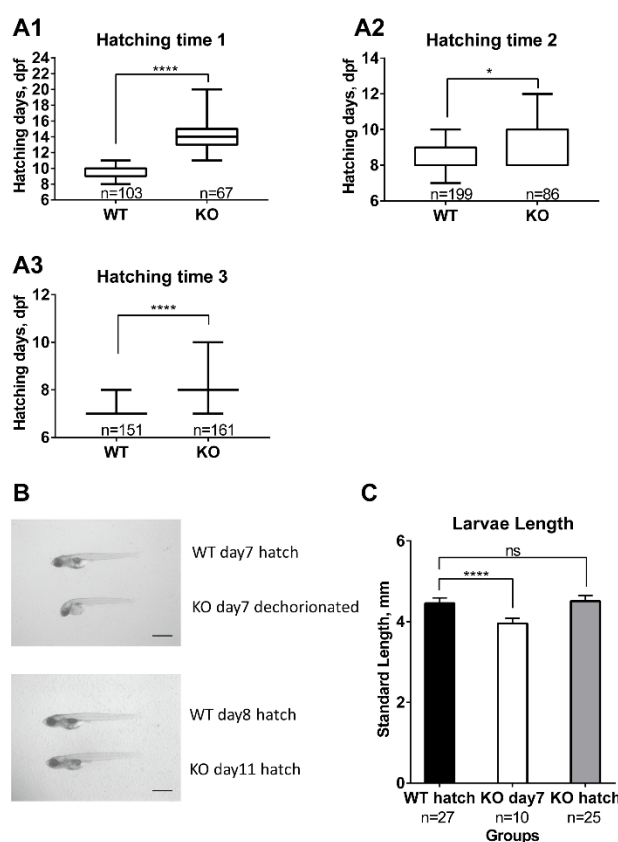
Figure 2.5 Effect of *mc4r* knockout on medaka development and hatching.

(**A**) Differences in hatching time of WT and KO medaka in the three trials (**A1, A2, A3**). Data are presented in box plots; whiskers are from min to max. Statistical significance after Mann-Whitney test is indicated as asterisks (*). (**B**) Photographs of larvae at 7 dpf and at hatch for WT and KO. Scale bar: 1 mm. (**C**) Graphic showing slower development of KO larvae compared to WT, apparent by a shorter length at 7 dpf. Data are presented as mean ± SD. Statistical significance after one-way *ANOVA* is indicated as asterisks (*). For all statistics: ns: not significant, *: p<0.05, ****: p<0.0001. The figure is taken from paper (Liu et al. 2019).

4. Discussion

4.1. Evolutionary characterization of the Mc4r signaling system

As the first step to investigate the Mc4r signaling system genes in medaka fish, an evolutionary analysis of Mc4r signaling system genes in various fish was performed.

In Southern platyfish, *mc4r* is present on the sex chromosome in multiple copies (Lampert et al. 2010; Volff et al. 2013). In contrast, medaka *mc4r* is not located on the sex chromosome. Moreover, medaka *mc4r* is a single copy gene, which confirms the earlier evidence from Southern blot data now on the whole genome sequence level (Volff et al. 2013). In the Mc4r phylogeny, the long branch for the Southern platyfish indicates considerable protein sequence divergence. It is hypothesized that Mc4r has special functions in regulating puberty onset that has evolved in the *Xiphophorus* fish lineage (Lampert et al. 2010; Volff et al. 2013). Likewise, cavefish also have long branch for Mc4r. That reflects a special adaptation of Mc4r in control of energy metabolism and adaptation to limited nutrients in the caves (Aspiras et al. 2015).

The *mrap2* gene has only one copy in most fish, except in zebrafish, being reported so far to have two copies (Sebag et al. 2013). Nevertheless, two copies of *mrap2* have been found also in common carp, indicating a lineage-specific duplication in cyprinids.

Since only one copy of *pomc* is found in other vertebrates than teleost, the two *pomc* paralogs (*pomca* and *pomcb*) probably arose during the 3rd round of whole genome duplication of the teleost. Two sequences of *pomca* (*pomca1* and *pomca2*) were present in most of Percomorpha teleost, which indicates a lineage-specific duplication of this gene.

The *agrp* gene has been reported in some species to exist in two versions, *agrp1* and *agrp2*. The *agrp1* (also named *agrp* previously) was described as an antagonist and inverse agonist of Mc4r and is involved in obesity (Ollmann et al. 1997; Shutter et al. 1997). On the other hand, the *agrp2* (in some fish named as *asip2b* (Braasch and Postlethwait 2011; Vastermark et al. 2012)) is related to background adaptation (Zhang et al. 2010) and pigment pattern formation (Kratochwil et al. 2018).

Taken together, on the *mc4r* signaling system gene level, the *Xiphophorus* fish and medaka are similar.

4.2. Physiological characterization of the Mc4r signaling system in medaka

In the next step towards a physiological characterization of the Mc4r signaling system of medaka, temporal and spatial expression analysis were performed (**Figure 2.6**).
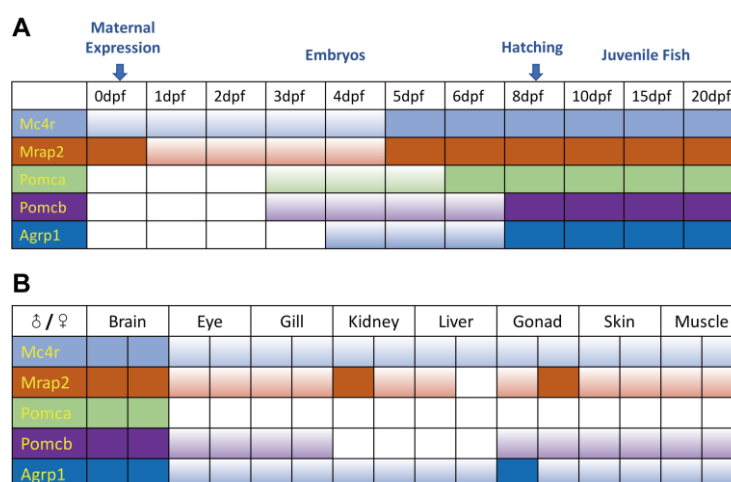
Figure 2.6 Expression pattern of the Mc4r signaling system genes in medaka.
(**A**) Summary table of temporal expression patterns. (**B**) Summary table of spatial expression patterns. Dark and light colors represent high and low expressions, respectively.

In temporal expression analysis, mRNA maternal contribution was present at low levels for *mc4r* and at high levels for *mrap2*. The *mc4r* and *mrap2* genes code for membrane proteins and are expressed early in development. This is concurrent with the late neurula stage and early brain development. Thus, *mc4r* and *mrap2* genes are expressed already in early and late embryonic stages, when the brain and integrated functional proteins in the neurons are developing.

Two days after expression of *mc4r* and *mrap2*, the expression of the *pomc* genes start, which encode precursors of Mc4r agonists. At the hatching stage, all genes are expressed, indicating that the whole system is in place for full function when the fish start feeding. Interestingly, after hatching, the *agrp1* expression is dramatically upregulated, coinciding with first feeding. This may be explained by the important role of *agrp1* in appetite regulation and could imply the role of the Mc4r signaling system in the regulation of energy metabolism from the very first feeding event (Song et al. 2003; Volkoff 2016).

In spatial expression analysis, the *mc4r* signaling pathway genes are expressed highly in the brains of both sexes. Both *mc4r* and *mrap2* express in the hypothalamus and *mc4r* also in neighboring regions of the brain, the preoptic area. These regions of expression are consistent with the center of food uptake regulation (Volkoff 2016).

The expression domains of *mc4r* and *mrap2* showed colocalization in certain areas in the hypothalamus, indicating interaction of Mc4r and Mrap2 in these cells. Since Mc4r and Mrap2 are both membrane proteins, their interaction could fine-tune intracellular signal transduction and signal strength as previously proposed for the zebrafish homologs (Josep Agulleiro et al. 2013). Yet, at some locations of *mc4r* expression, *mrap2* signal was not observed. This implies that in such regions *mc4r* may be able to transmit signals without its co-factor *mrap2*. Nevertheless, *mrap2* was expressed only in regions where the *mc4r* signal was present, supporting the specific role of Mrap2 in interacting with Mc4r in the brain (Sebag et al. 2013).

From the organ-specific expression analysis, the high transcript levels of *mrap2* were observed in ovary. This feature could be explained by the very high maternal RNA contribution. Inferred from maternal contribution level, mature eggs contain high levels of *mrap2*, and this is reflected in the high mRNA levels in the ovary.

High expression of *mrap2* in the male kidney has been reported before. In zebrafish, *mrap2* is highly expressed in the head kidney (Josep Agulleiro et al. 2013). Moreover, human *mrap2* is expressed mainly in brain and adrenal gland (Chan et al. 2009), and mice *mrap2* is widely expressed in tissues including kidney (Asai et al. 2013). In fish, *mrap2* might be similarly expressed in the interrenal gland on the kidney. A male-specific function of *mrap2* in kidney remains to be investigated.

4.3. Effect of Mc4r on puberty timing and adult growth

The expression data did not exclude that the Mc4r signaling system in medaka could have the same function in regulating puberty timing as in *Xiphophorus* fish. In *Xiphophorus*, *mc4r* expression is highest in the brain (Volff et al. 2013). In adult medaka, the Mc4r signaling system genes are found to be expressed mainly in the preoptic and hypothalamic region, similar as in zebrafish (Josep Agulleiro et al. 2013).

In spite of the similarity in expression patterns to *Xiphophorus*, the analysis of the Mc4r KO medaka fish revealed that males and females of the WT and Mc4r KO strain reach puberty at the similar time, on the contrary to *Xiphophorus*. Nevertheless, WT females reach puberty significantly later than WT males, while KO females and males do not show a significant difference on puberty timing. In zebrafish, it is difficult to monitor puberty onset (Uusi-Heikkila et al. 2012), hence an effect of *mc4r* on puberty timing was not noted. The results from KO medaka indicate that Mc4r may not be the critical puberty signal in these fish. Alternatively, the lack of a puberty phenotype in these TALEN KO fish might be due to the fact that other genes can compensate for the loss of Mc4r function (Trudeau 2018).

Although the timing of puberty onset is not altered in KO fish, body length at puberty in female KO fish is significantly shorter than in WT. WT females are significantly larger than WT males and KO males. KO females and males do not show a significant difference in body length at puberty. Since puberty is normally connected to animal size/weight, earlier puberty and shorter length at puberty in KO females implies an advanced pubertal process in KO females. This suggests that in medaka the Mc4r function is relevant for female growth, rather than male growth. This is unlike the situation in Southern platyfish and other *Xiphophorus* species, where Mc4r is involved in male size but has no noticeable effect on female growth and adult size.

Furthermore, KO fish are slightly shorter than WT, indicating a possible retarded growth in general in Mc4r KO fish. In zebrafish, Mc4r KO led to increased body length at 42 dpf (Zhang et al. 2012). Overexpression of the Mc4r inverse agonists, both Agrp1 and Asip1, increased linear growth in zebrafish (Song and Cone 2007; Guillot et al. 2016). Growth of Mc4r KO medaka surprisingly retarded suggests that eventually other receptors could compensate for the function of compromised Mc4r. Such an alternative could be the Mc3r, a receptor also related to energy balance and reacts with Pomc and Agrp1. It can respond to Pomc signals and may thus reduce the growth.

4.4. A role for Mc4r in medaka embryonic development and larval growth

Intriguingly, the hatching time of Mc4r KO medaka was affected. Development of the Mc4r KO fish was delayed from day one, coinciding with the onset of *mc4r*

expression. This effect became even more obvious from day three on. It could be speculated that Mc4r has a role in medaka development by influencing growth.

Zebrafish Mc4r KO, however, does not show changes in linear growth during embryonic and larval stages. Only at 42 dpf, an increased body length of Mc4r KO zebrafish was first detected (Zhang et al. 2012). The embryogenesis of zebrafish is fast, and the hatch stages of fish are as early as day 2-3, compared to day 7-9 in medaka. Thus, a delay in zebrafish development may have remained undetectable.

The overall growth of medaka was clearly slowed down in KO larvae. A point to note about the knockout of Mc4r in medaka is that Mc4r KO decreases growth rate, rather than increasing the body length. This is another difference to *Xiphophorus* fish (Lampert et al. 2010), in which the non-functional mutant Mc4r results in increased body size. Also, in zebrafish, the loss of *mc4r* led to increased body length at 42 dpf (Zhang et al. 2012).

To sum up, the regulatory network of Mc4r signaling system in medaka appears not to be involved in puberty regulation as in *Xiphophorus*, but rather in growth and development. These findings suggest that from an evolutionary perspective the role of *mc4r* as the critical *P* locus gene is a specific innovation in the *Xiphophorus* lineage.

5. Supplementary Information

5.1. Supplementary Figures
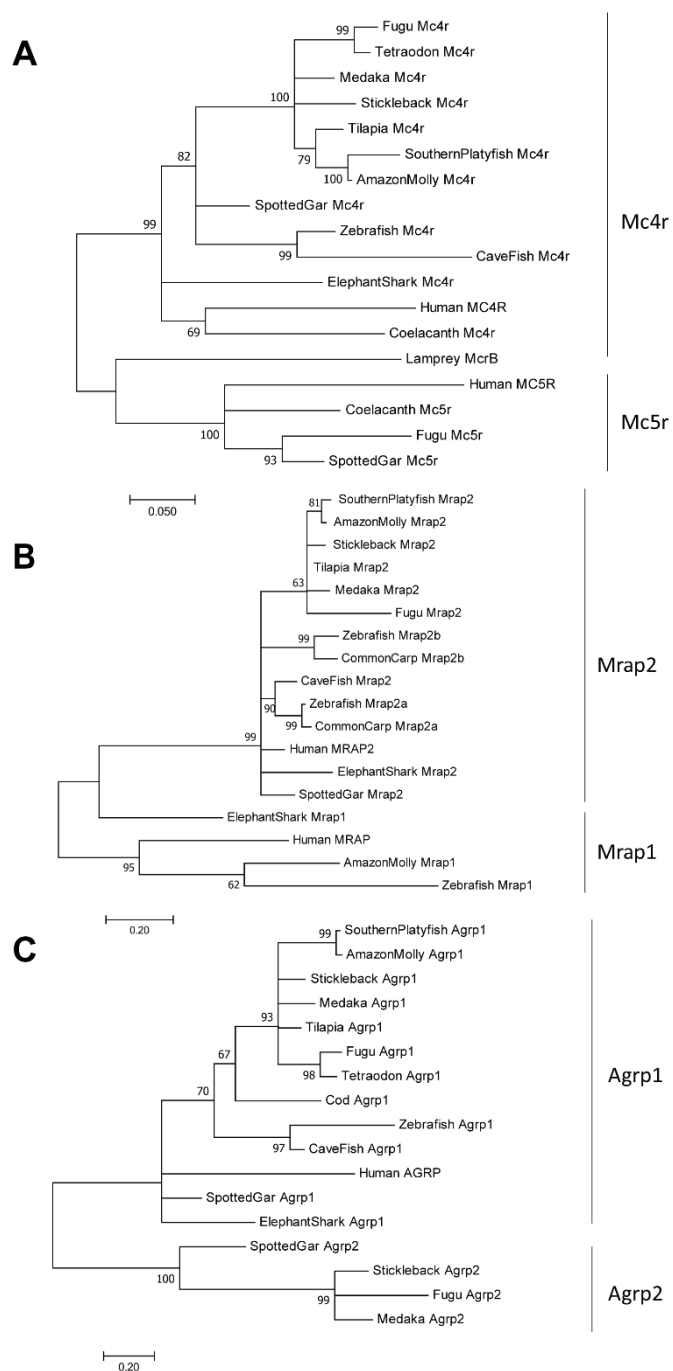
Figure S2.1 Phylogenetic analysis of fish species.

(A) Mc4r (Mc5r as outgroup), **(B)** Mrap2 (Mrap1 as outgroup), **(C)** Agrp1 (Agrp2 as outgroup). The phylogeny was inferred using the maximum-likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branches with bootstrap lower than 60 were collapsed. The figure is taken from paper (Liu et al. 2019).
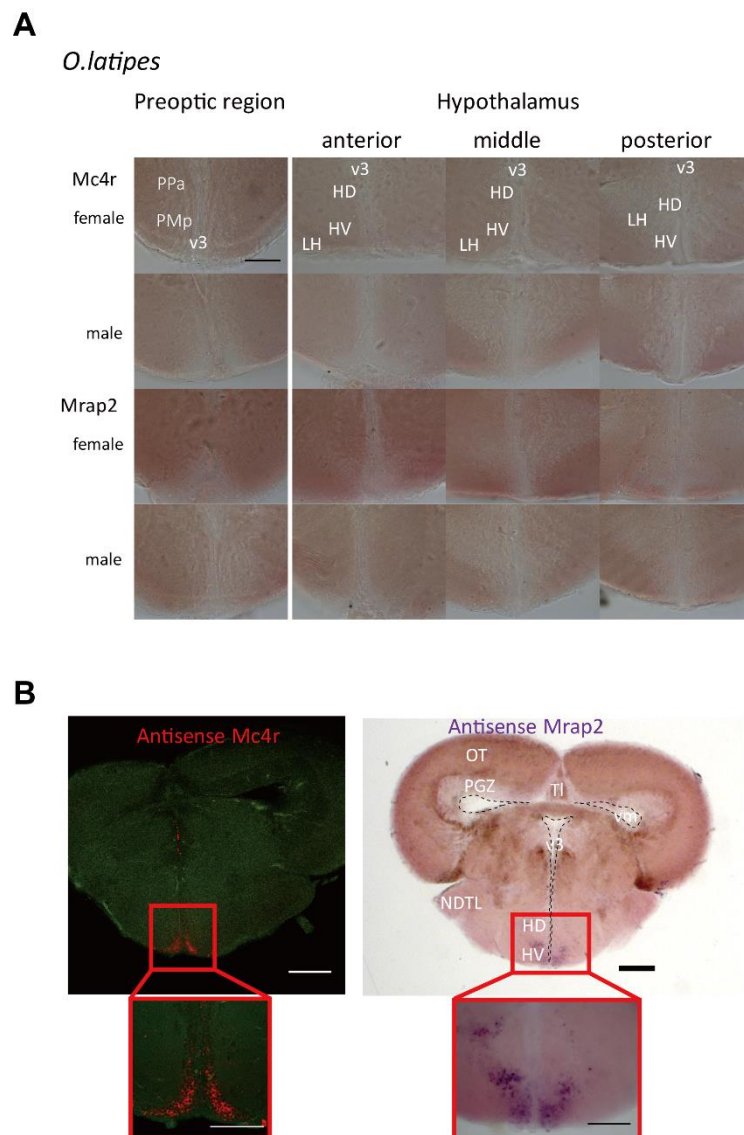
Figure S2.2 Whole mount *in situ* hybridization detection of *mc4r* and *mrap2* genes in adult brains.

(A) *mc4r* and *mrap2* sense probes used as negative control showing no background signals in male and female brains. Scale bar: 100 µm. **(B)** *mc4r* and *mrap2* were in part co-expressed in the same region of the hypothalamus. Scale bar: whole section 200 µm, magnified view 100 µm. Brain areas in detail: v3: third ventricle, PPa: anterior parvocellular preoptic nucleus, PMp: parvocellular part magnocellular preoptic nucleus, HD: dorsal periventricular hypothalamus, HV: ventral periventricular hypothalamus, LH: lateral nucleus of hypothalamus, OT: optic tectum, PGZ: periventricular grey zone, vm: mesencephalic ventricle, NDTL: diffuse nucleus of lateral torus, Tl: longitudinal torus. The figure is taken from paper (Liu et al. 2019).
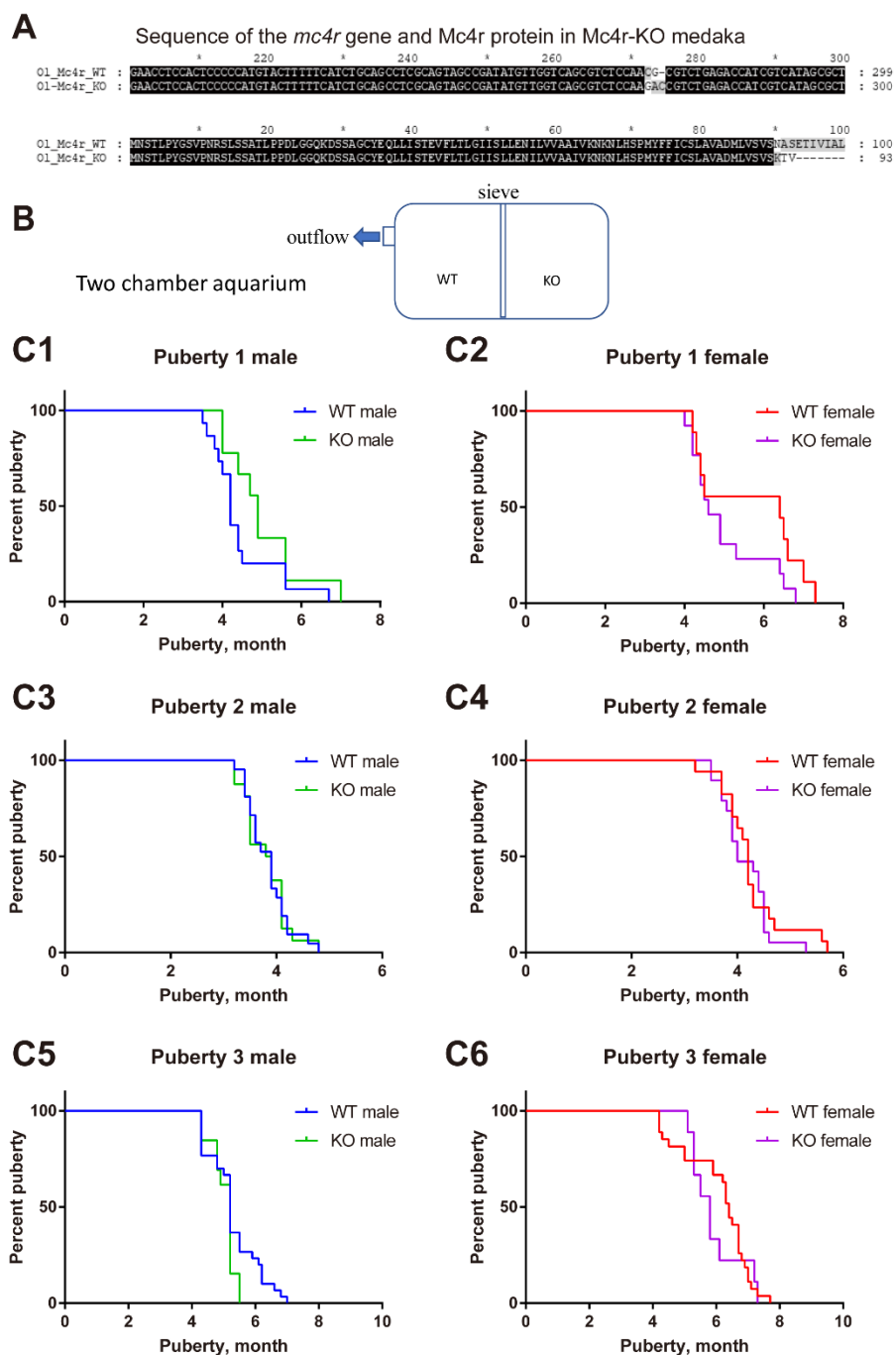
Figure S2.3 Effect of *mc4r* on medaka development and puberty.

(A) Mc4r knockout is a -2+3KO, which has a deletion of 2 nt and an addition of 3 nt at the TALEN cut site. This creates a frameshift mutation, resulting in a truncated protein. **(B)** Schematic drawing of the two-chamber aquarium, which warrants an identical environment for WT and KO. **(C)** Percentage of fish reaching puberty at a certain age. Note: males and females show no significant difference in puberty timing in three trials (males: **C1, C3, C5**; females: **C2, C4, C6**). The figure is taken from paper (Liu et al. 2019).
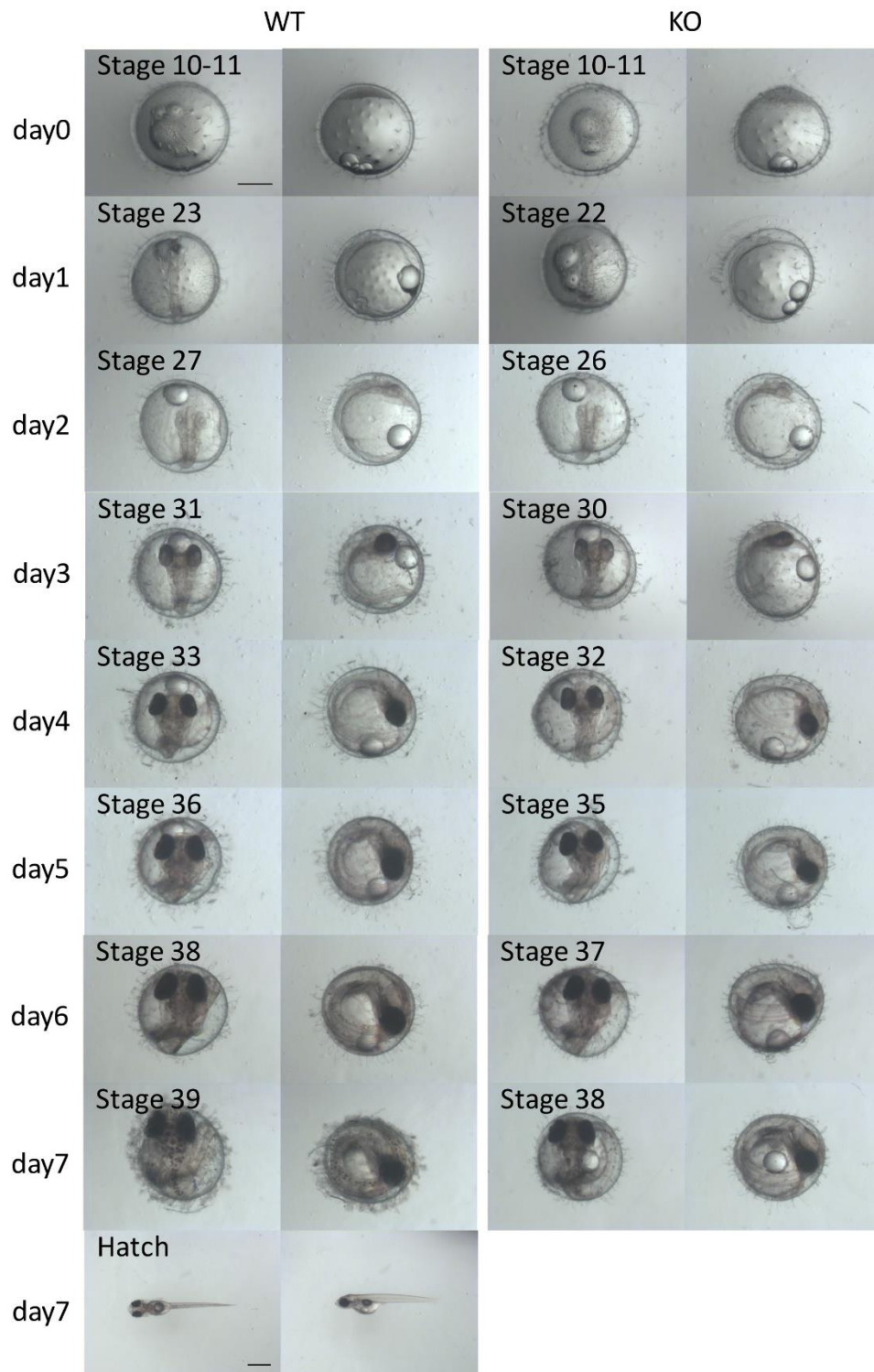
Figure S2.4 Effect of *mc4r* on medaka development.
Photomicrographs of WT and KO medaka embryos during development from day 0 to day 7 post fertilization. KO fish are at earlier developmental stages than the WT embryos. Scale bar: embryo 0.5 mm, hatched fish 1 mm.

5.2. Supplementary Tables

Table S2.1 List of accession numbers of sequences of various species used in the study.

All sequences are obtained from Ensembl Database or NCBI. The tables are taken from paper (Liu et al. 2019).

Mc4r

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Mc4r	Amazon molly	990	329aa	1	ENSPFOT00000021127	ENSPFOP00000021099.2
Mc4r	Cave fish	1008	335aa	1	ENSAMXT00000027076	ENSAMXP00000027055.1
Mc4r	Coelacanth	990	329aa	1	ENSLACT00000000489	ENSLACP00000000487.1
Mc4r	Elephant shark	1017	338aa	2	XM_007895520.1	XP_007893711.1
Mc4r	Fugu	969	322aa	1	NM_001032560.1	NP_001027732.1
MC4R	Human	1666	332aa	1	ENST00000299766	ENSP00000299766.3
McrB	Lamprey	1035	344aa	1	BK007095.1	DAA34034.1
Mc4r	Medaka	1941	321aa	1	XM_004081195.4	XP_004081243.1
Mc4r	Southern Platyfish	963	320aa	1	KF650657.1	AHC02892.1
Mc4r	Spotted gar	5875	333aa	1	XM_015354632.1	XP_015210118.1

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Mc4r	Stickleback	981	326aa	1	ENSGACT000000 07378	ENSGACP000000 07360.1
Mc4r	Tetraodon	972	323aa	1	ENSTNIT0000001 1720	ENSTNIP0000001 1538.1
Mc4r	Tilapia	984	327aa	1	ENSONIT000000 25784	ENSONIP0000002 5763.1
Mc4r	Zebrafish	1084	326aa	1	ENSDART000000 19555	ENSDARP000000 27547.2
MC5R	Human	1319	325aa	1	NM_005913.2	NP_005904.1
Mc5r	Fugu	1023	340aa	4	NM_001032765.1	NP_001027937.1
Mc5r	Spotted gar	2852	328aa	2	XM_015357781.1	XP_015213267.1
Mc5r	Coelacanth	981	326aa	1	XM_005996516.1	XP_005996578.1

Mrap2

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Mrap2	Amazon molly	2390	255aa	3	ENSPFOT0000 0019455	ENSPFOP0000001 9433.2
Mrap2	Cave fish	1511	218aa	3	XM_00723727 8.3	XP_007237340.2
Mrap2	Elephant shark	627	208aa	3	XM_00790843 3.1	XP_007906624.1
Mrap2	Fugu	687	228aa	3	XM_00397186 6.1	XP_003971915.1

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
MRAP2	Human	2153	205aa	4	ENST0000025 7776	ENSP0000025777 6.4
Mrap2	Medaka	2625	224aa	4	XM_02071471 2.2	XP_020570371.1
Mrap2	Southern Platyfish	1954	226aa	3	ENSXMAT000 00013733	ENSXMAP000000 13717.1
Mrap2	Spotted gar	1339	224aa	4	XM_00662602 7.2	XP_006626090.2
Mrap2	Stickleback	642	213aa	4	ENSGACT000 00012548	ENSGACP000000 12524.1
Mrap2	Tilapia	1725	264aa	4	ENSONIT0000 0018512	ENSONIP0000001 8495.1
Mrap2a	Zebrafish	1950	217aa	3	ENSDART000 00148801	ENSDARP000001 24181.1
Mrap2b	Zebrafish	600	199aa	3	ENSDART000 00168266	ENSDARP000001 32698.1
MRAP	Human	1023	172aa	5	NM_178817.3	NP_848932.1
Mrap1	Amazon molly	1359	124aa	5	XM_00756861 9.2	XP_007568681.1
Mrap1	Zebrafish	555	108aa	3	ENSDART000 00148193.3	ENSDARP000001 23129.2
Mrap1	Elephant shark	805	153aa	3	XM_00790355 0.1	XP_007901741.1
Mrap2a	Common carp	1949	214aa	3	XM_01911937 3.1	XP_018974918.1

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Mrap2b	Common carp	615	204aa	3	XM_019086530.1	XP_018942075.1

Pomc

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Pomca-1	Amazon molly	2246	249aa	3	XM_007569070.2	XP_007569132.2
Pomca-2	Amazon molly	794	183aa	3	XM_016681012.1	XP_016536498.1
Pomcb	Amazon molly	1025	236aa	3	ENSPFOT00000003135	ENSPFOP00000003131.1
Pomca	Cave fish	1003	221aa	3	XM_007244678.2	XP_007244740.2
Pomcb	Cave fish	878	197aa	3	XM_015607051.2	XP_015462537.2
Pomc	Coelacanth	768	255aa	2	ENSLACT00000013386	ENSLACP00000013290.1
Pomc	Elephant shark	1140	341aa	2	XM_007911414.1	XP_007909605.1
Pomca	Fugu	657	218aa	2	XM_011606335.1	XP_011604637.1
Pomcb	Fugu	2879	218aa	3	XM_003971727.2	XP_003971776.1

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
POMC	Human	1426	267aa	3	ENST00000405623	ENSP00000384092.1
Poc	Lamprey	1025	278aa	2	D55628.1	BAA09491.1
Pom	Lamprey	2045	245aa	2	D55629.1	BAA09492.1
Pomca-1 (Pomc-like)	Medaka	2350	265aa	3	XM_023959757.1	XP_023815525.1
Pomca-2	Medaka	898	212aa	3	XM_004066456.3	XP_004066504.1
Pomcb	Medaka	684	227aa	2	ENSORLT00000018657	ENSORLP000000018656.1
Pomca-1	Southern Platyfish	713	209aa	2	ENSXMAT00000001214	ENSXMAP00000001210.1
Pomca-2	Southern Platyfish	1442	257aa	3	ENSXMAT00000006432	ENSXMAP00000006424.1
Pomcb	Southern Platyfish	1408	236aa	3	ENSXMAT00000003692	ENSXMAP00000003687.1
Pomc	Spotted gar	1431	266aa	3	ENSLOCT00000020647	ENSLOCP000000020612.1
Pomca-1	Tilapia	1969	216aa	3	ENSONIT00000009130	ENSONIP000000009125.1
Pomca-2	Tilapia	814	208aa	2	ENSONIT00000023610	ENSONIP000000023589.1
Pomcb	Tilapia	1537	280aa	3	XM_005454720.3	XP_005454777.1
Pomca	Zebrafish	1224	222aa	3	ENSDART00000063333	ENSDARP000000063332.5

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Pomcb	Zebrafish	648	215aa	2	ENSDART000 00100751	ENSDARP0000 0091524.2

Agrp

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Agrp1	Amazon molly	767	141aa	3	ENSPFOT000000 02445	ENSPFOP00000 002441.1
Agrp1	Cave fish	1465	143aa	5	XM_022667066.1	XP_022522787.1
Agrp1	Cod	1364	128aa	3	BR000938.1	FAA00762.1
Agrp1	Elephant shark	588	155aa	4	XM_007889599.1	XP_007887790.1
Agrp1	Fugu	529	135aa	3	ENSTRUT000000 09422	ENSTRUP00000 009367.1
Agrp2	Fugu	907	115aa	3	ENSTRUT000000 09410	ENSTRUP00000 009355.1
AGRP	Human	764	132aa	4	ENST0000029095 3	ENSP000002909 53.2
Agrp1	Medaka	3293	141aa	3	XM_011487064.3	XP_011485366.1
Agrp2	Medaka	1737	114aa	3	XM_004078892.4	XP_004078940.1
Agrp1	Southern Platyfish	578	140aa	3	ENSXMAT000000 09960	ENSXMAP0000 0009946.1
Agrp1	Spotted gar	769	152aa	4	XM_006641519.2	XP_006641582.2

Gene	Species	Size (in bp)	Protein	Exon	Transcript ID	Protein ID
Agrp2	Spotted gar	3439	130aa	4	ENSLOCT000000 12669	ENSLOCP00000 012645.1
Agrp1	Stickleback	1130	132aa	3	BR000932.1	FAA00758.1
Agrp2	Stickleback	3507	115aa	3	BR000927.1	FAA00754.1
Agrp1	Tetraodon	393	130aa	3	ENSTNIT0000001 3316	ENSTNIP000000 13124.1
Agrp1	Tilapia	429	142aa	3	ENSONIT000000 04065	ENSONIP000000 04064.1
Agrp1	Zebrafish	676	126aa	4	ENSDART000001 35250	ENSDARP00000 116390.1

Table S2.2 Primers used in this study.

Primers. The table is taken from paper (Liu et al. 2019).

Primers	Sequences	Comments
MF_ef1a1-f01	GCCCCTGGACACAGAGACTTCATCA	RT-qPCR for Elf1a
MF_ef1a1-r01	AAGGGGGCTCGGTGGAGTCCAT	RT-qPCR for Elf1a
Mc4r_Ol_F	GGCAACCTGAGCATTCCTGTCA	RT-qPCR for Mc4r
Mc4r_Ol_R	ATGTAGCGGTCAACGGCAATGG	RT-qPCR for Mc4r
MRAP2a_Ol_F	CGCACGACGCAGTGACTATGT	RT-qPCR for Mrap2
MRAP2a_Ol_R	ACCGCCAGTCCAACCCAGAA	RT-qPCR for Mrap2
Pomca_Ol_F	TGGACTCTGAGAGCAGCATGAC	RT-qPCR for Pomca
Pomca_Ol_R	AAGGGATCTGAGGGAGGTGGAG	RT-qPCR for Pomca
Pomcb_Ol_F	TTGCTGGCTGTTGGTGGTTCT	RT-qPCR for Pomcb
Pomcb_Ol_R	AGGTCTGGGCTTTCAGGTTTGA	RT-qPCR for Pomcb
AgRP_Ol_F	CATCCCTCACCAGCAGTCCT	RT-qPCR for Agrp1
AgRP_Ol_R	GCGGCAGTAACAGATGGCATT	RT-qPCR for Agrp1
Mc4r_Ol_F2	CCTGGGAGGACAGAAAGA	PCR for Mc4r *in situ* hybridization probe synthesis
Mc4r_Ol_R2	ATGAAGAGGATACCCGACA	PCR for Mc4r *in situ* hybridization probe synthesis
MRAP2a_Ol_F2	ACGAGTATTATGACGACGAG	PCR for Mrap2 *in situ* hybridization probe synthesis
MRAP2a_Ol_R2	GATGGTGTATCCCTGCTT	PCR for Mrap2 *in situ* hybridization probe synthesis

Table S2.3 Chromosomal location of Mc4r signaling system genes.

Linkage group: LG. The table is taken from paper (Liu et al. 2019).

	Southern platyfish	medaka
sex chromosome	LG21	LG1
mc4r	LG21	LG 20
mrap2	LG15	LG24
pomca1	LG13	LG11
pomca2	LG24	LG2
pomcb	LG15	LG24
agrp1	LG4	LG3
agrp2	LG6 (*asip2b*)	LG17

Table S2.4 Medaka developmental stages.

Days post fertilization: dpf; stage: S. The table is taken from paper (Liu et al. 2019).

Days	Number of eggs in each pool	Stages
0dpf	100	S10-11
1dpf	50	S21-22
2dpf		S26
3dpf		S30
4dpf		S33
5dpf	30	S35
6dpf		S37
8dpf		8dpf Hatch
10dpf	15	10dpf
15dpf		15dpf
20dpf		20dpf

5.3. Supplementary sequences

Mc4r sequences in wild-type medaka.

>Ol_Mc4r_WT

```
ATGAACTCCACTCTGCCTTATGGGTCGGTCCCCAACAGAAGCCTCTCCTCGGCCACTCTCCCTCC
TGACCTGGGAGGACAGAAAGACTCGTCGGCGGGATGCTACGAGCAGCTTCTGATCTCCACTGAGG
TCTTCCTCACTTTGGGCATCATCAGCCTGCTGGAGAACATCCTGGTTGTTGCTGCGATCGTTAAA
AACAAGAACCTCCACTCCCCCATGTACTTTTTCATCTGCAGCCTCGCAGTAGCCGATATGTTGGT
CAGCGTCTCCAACGCGTCTGAGACCATCGTCATAGCGCTCATTAACGGAGGCAACCTGAGCATTC
CTGTCAGGCTCATCAAGAGCATGGACAATGTGTTTGACTCCATGATCTGCAGCTCTCTGCTGGCC
TCCATCTGCAGCTTGCTGGCCATTGCCGTTGACCGCTACATCACCATCTTCTACGCTCTGCGATA
CCACAACATCGTGACGCTGCGGCGAGCAGCCGTGGTCATCAGCAGCATCTGGACGTGCTGCATTG
TGTCGGGTATCCTCTTCATCATCTACTCGGAGAGTACCACGGTGCTCATCTGTCTCATCACCATG
TTCTTCACCATGCTGGTGCTCATGGCCTCCCTCTATGTCCACATGTTCCTGCTGGCACGTCTGCA
CATGAAGCGGATCGCGGCGCTGCCGGGCAACGCGCCCATCCACCAGCGGGCGAACATGAAGGGCG
CCATCACCCTCACCATCCTCCTCGGGGTGTTTGTGGTGTGCTGGGCGCCGTTCTTCCTCCACCTC
ATCCTCATGATCACCTGCCCCAGGAACCCTTACTGCACCTGCTTCATGTCGCACTTCAACATGTA
CCTCATTCTCATCATGTGCAACTCCGTCATCGACCCCATCATCTACGCTTTCCGGAGCCAGGAGA
TGAGGAAAACCTTCAAGGAGATCTTCTGCTGCTCCAACGCTCTCCTGTGTGTGTGA
```

>Ol_Mc4r_WT

```
MNSTLPYGSVPNRSLSSATLPPDLGGQKDSSAGCYEQLLISTEVFLTLGIISLLENILVVAAIVK
NKNLHSPMYFFICSLAVADMLVSVSNASETIVIALINGGNLSIPVRLIKSMDNVFDSMICSSLLA
SICSLLAIAVDRYITIFYALRYHNIVTLRRAAVVISSIWTCCIVSGILFIIYSESTTVLICLITM
FFTMLVLMASLYVHMFLLARLHMKRIAALPGNAPIHQRANMKGAITLTILLGVFVVCWAPFFLHL
ILMITCPRNPYCTCFMSHFNMYLILIMCNSVIDPIIYAFRSQEMRKTFKEIFCCSNALLCV
```

Mc4r sequences in -2/+3 TALEN-knockout medaka.

>Ol-Mc4r_KO-2/+3

```
ATGAACTCCACTCTGCCTTATGGGTCGGTCCCCAACAGAAGCCTCTCCTCGGCCACTCTCCCTCC
TGACCTGGGAGGACAGAAAGACTCGTCGGCGGGATGCTACGAGCAGCTTCTGATCTCCACTGAGG
TCTTCCTCACTTTGGGCATCATCAGCCTGCTGGAGAACATCCTGGTTGTTGCTGCGATCGTTAAA
AACAAGAACCTCCACTCCCCCATGTACTTTTTCATCTGCAGCCTCGCAGTAGCCGATATGTTGGT
CAGCGTCTCCAAGACCGTCTGAGACCATCGTCATAGCGCTCATTAACGGAGGCAACCTGAGCATT
CCTGTCAGGCTCATCAAGAGCATGGACAATGTGTTTGACTCCATGATCTGCAGCTCTCTGCTGGC
CTCCATCTGCAGCTTGCTGGCCATTGCCGTTGACCGCTACATCACCATCTTCTACGCTCTGCGAT
```

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ACCACAACATCGTGACGCTGCGGCGAGCAGCCGTGGTCATCAGCAGCATCTGGACGTGCTGCATT
GTGTCGGGTATCCTCTTCATCATCTACTCGGAGAGTACCACGGTGCTCATCTGTCTCATCACCAT
GTTCTTCACCATGCTGGTGCTCATGGCCTCCCTCTATGTCCACATGTTCCTGCTGGCACGTCTGC
ACATGAAGCGGATCGCGGCGCTGCCGGGCAACGCGCCCATCCACCAGCGGGCGAACATGAAGGGC
GCCATCACCCTCACCATCCTCCTCGGGGTGTTTGTGGTGTGCTGGGCGCCGTTCTTCCTCCACCT
CATCCTCATGATCACCTGCCCCAGGAACCCTTACTGCACCTGCTTCATGTCGCACTTCAACATGT
ACCTCATTCTCATCATGTGCAACTCCGTCATCGACCCCATCATCTACGCTTTCCGGAGCCAGGAG
ATGAGGAAAACCTTCAAGGAGATCTTCTGCTGCTCCAACGCTCTCCTGTGTGTGTGA
```

>Ol_Mc4r_KO-2/+3

```
MNSTLPYGSVPNRSLSSATLPPDLGGQKDSSAGCYEQLLISTEVFLTLGIISLLENILVVAAIVK
NKNLHSPMYFFICSLAVADMLVSVSKTV
```

Chapter 3

Melanocortin 4 Receptor Signaling and Puberty Onset Regulation in *Xiphophorus* Swordtails

(Article in preparation)

This chapter is based on a manuscript in preparation. Participants include:

Ruiqi Liu, Kang Du, Jenny Ormanns, Mateus C. Adolfi, Manfred Schartl

Participants Contributions	
RL	performed the fish sampling, *in situ* hybridizations, expression constructs cloning, luciferase assay, and drafted the manuscript.
KD	performed the transcriptome gene mapping analysis.
JO	performed identification of *mc4r* copies in *X. hellerii*.
MA	supervised the experiments and helped to review the manuscript.
MS	defined and designed the study, coordinated all steps of the research and reviewed all versions of the manuscript.

Abstract

Fish of the genus *Xiphophorus* provide a prominent example of genetic control of male body size and reproductive tactics. In *X. nigrensis* and *X. multilineatus*, puberty onset and body length are determined by melanocortin 4 receptor (Mc4r) allelic and copy number variations which were proposed to fine-tune the signaling output of the system. Accessory protein Mrap2 is required for growth across species by affecting Mc4r signaling. The molecular mechanism how Mc4r signaling controls the puberty regulation in *Xiphophorus* and whether the interaction with Mrap2 is also involved was so far unclear. Hence, we examined Mc4r and Mrap2 in *X. nigrensis* and *X. multilineatus*, in comparison to a more distantly related species, *X. hellerii*. Mc4r and Mrap2 transcripts co-localized in hypothalamus and preoptic regions in large males, small males and females of *X. nigrensis*, with similar signal strength for Mrap2 but higher expression of Mc4r in large males. This overexpression is constituted by wild-type and one subtype of mutant alleles. *In vitro* studies revealed that Mrap2 co-expressed with Mc4r increased cAMP production but did not change EC50. Cells co-expressing the wild-type and one mutant allele showed lower cAMP signaling than Mc4r wild-type cells. This indicates a role of Mc4r alleles, but not Mrap2, in puberty signaling. Different from *X. nigrensis* and *X. multilineatus*, *X. hellerii* has only wild-type alleles, but also shows a puberty onset and body length polymorphism, despite the absence of mutant alleles. Like in the two other species. *mc4r* and *mrap2* transcripts colocalized and *mc4r* is expressed at substantially highest levels in large males. These imply that the regulation mechanism may not be identical even within same genus.

1. Introduction

Puberty is the physiological process when an animal develops secondary sexual characteristics and becomes sexually matured, allowing for reproduction for the first time. Puberty is an essential process to be regulated. Precocious puberty and delayed puberty are among the puberty disorders in human (Palmert and Boepple 2001; Grumbach 2002). Early puberty is also a main problem in agriculture, for instance in farmed fish (Taranger et al. 2010). Conserved from fish to mammals, the onset of puberty is commonly triggered by the hypothalamus-pituitary-gonad (HPG) axis (Sower et al. 2009; MacManes et al. 2017). Kisspeptin is a key factor in reproduction regulation in mammals (Uenoyama et al. 2018), but may be a non-reproduction regulator in some fish, such as zebrafish and medaka (Tang et al. 2015; Nakajo et al. 2018).

Melanocortin 4 receptor (Mc4r) is related to onset of puberty in some *Xiphophorus* fish (Lampert et al. 2010). Mc4r is a class A G-protein coupled receptor involving in energy homeostasis. In human, *MC4R* mutations are the most common monogenic cause of severe early-onset obesity (Farooqi et al. 2003), and stimulation of Mc4r produces relative anorexia, which is often seen in some diseases like cancer cachexia (Marks et al. 2001). In rodents, *Mc4r* knockout causes obesity (Huszar et al. 1997) and overexpression of Mc4r leads to anorexia and weight loss (Gutierrez et al. 2009). Since nutrition affect pubertal development, energy homoeostasis is related to the puberty.

Melanocortins are agonists of Mc4r and are derived from proopiomelanocortin (Pomc) through post-translational processing (Cone 2006; Cortes et al. 2014). Pomc-derived ligands ACTH, α-, β- and γ-MSHs can all activate Mc4r, though γ-MSH is not present in teleosts (Alrubaian et al. 2003). β-MSH bind to Mc4r with highest affinity while α-MSH analog NDP-MSH, a synthetic ligand, activates Mc4r in a highly potent manner (Schioth et al. 1996). Agouti-related protein (Agrp) functions as antagonists and inverse agonists of Mc4r (Ersoy et al. 2012).

Melanocortin receptor accessory protein 2 (Mrap2) was described as an accessory protein of Mc4r and is required for growth and metabolism across species including human (Liu et al. 2013). Mrap, like other accessory proteins, is crucial for activity of the GPCR on the membrane. As an accessory protein of Mc2r, it is of essential

function for assisting the cell surface trafficking and increasing the cAMP response (Metherell et al. 2005). Interaction of Mrap2 with Mc4r regulates zebrafish developmental control (Sebag et al. 2013) and is associated with mammalian obesity (Asai et al. 2013). In zebrafish, two Mrap2 copies are found (Agulleiro et al. 2010). As a peculiarity of zebrafish Mrap2a and Mrap2b regulate Mc4r activity by inhibiting (Mrap2a, larval form) or activating (Mrap2b, adult form) Mc4r-dependent signaling (Sebag et al. 2013). In mice, deletion of *Mrap2* causes severe obesity at young ages; and in human, pathogenic genetic variants in *MRAP2* were found in severe, early onset obesity individuals (Asai et al. 2013; Liu et al. 2013).

Mc4r signaling system has been described to be of utmost importance for the puberty regulation in the laboratory model fish species *Xiphophorus* (Lampert et al. 2010; Volff et al. 2013).

The fish genus *Xiphophorus* comprises 26 species of life-bearing freshwater fish, whose geographical distribution spreads from the Atlantic drainages of Mexico to Belize and Guatemala (Rosen 1960; Kallman and Kazianis 2006). Males of some species (swordtails) develop extensions of lower rays in caudal fin, like a sword; for example, *X. nigrensis* and *X. multilineatus* (both belonging to the Northern swordtail clade), and *X. hellerii* (belonging to the Southern swordtail clade). Other species (platyfish) bear no swords; for instance, *X. maculatus* (Southern platyfish). Swords are attractive to females and render better reproductive success of males (Rosen 1960; Kang et al. 2013). Likewise, sizes of males influence their reproductive tactics and the mating success in the wild.

In several *Xiphophorus* species, large and small males exist. This polymorphism is due to the fact that males stop to grow upon puberty and some males mature late and some early. Large males are preferred by females and have greater mating success. In *Xiphophorus* fish a "puberty" locus (*P* locus), is involved in puberty onset regulation (Kallman and Schreibman 1973; Kallman and Borkoski 1978). For two species *X. nigrensis* and *X. multilineatus*, body size and puberty onset polymorphism have been associated with copy number and allelic variation of the melanocortin 4 receptor (*mc4r*) gene (Lampert et al. 2010; Volff et al. 2013).

In these species, *mc4r* alleles fall into three classes, A, B1 and B2. Mc4r A alleles mainly locate on the X chromosome and express functional receptors, while

male-specific Y-linked Mc4r B1 and B2 alleles are defective, lacking two cysteine residues at the carboxyl-terminus. The two cysteines are a motif that is frequently present in G-protein coupled receptor (GPCR) and is a site for palmitoylation (Qanbar and Bouvier 2003). This dicysteine motif is considered to anchor the carboxyl-terminal helix VIII into the plasma membranes, which appears to be vital for receptor activation (Garcia-Borron et al. 2005; Huynh et al. 2009). B2 has an additional four-base deletion at the carboxyl-terminus, which leads to a frameshift and read through of the stop codon, hence the carboxyl-terminus is elongated. Interestingly, copy numbers of B alleles correlate with male body size. Large males have high copy number. It was shown by *in vitro* experiments that B alleles elicit a decreased cAMP response and cell culture co-expression experiments indicated that B alleles may act as dominant negative receptor mutants (Lampert et al. 2010).

Like *X. nigrensis* and *X. multilineatus*, another swordtail species, *X. hellerii* shows a puberty onset and size polymorphism, but Mc4r and Mrap2 has not yet been investigated in this species.

Here we investigate the molecular mechanism how Mc4r and Mrap2 control puberty regulation in *X. nigrensis* and *X. multilineatus,* and compare this to *X. hellerii*. We tested if regulation of Mc4r signaling may be influenced through interaction with the accessory protein Mrap2. We find that in *X. nigrensis* and *X. multilineatus* fish, Mrap2 does not differ in expression in males of different sizes and does not affect the EC50 of Mc4r signaling. Meanwhile, in these fish, the elevated expression level of Mc4r in large males are crucial for differential puberty signaling. In *X. hellerii* fish, we observed only A-type alleles, but Mc4r also highly express in large males, while Mrap2 show same level of expression. Puberty regulation in *X. hellerii* is not due to alleles type but the conserved expression pattern and strength might indicate a conserved mechanism. The absence of different alleles implies the difference in the evolution of the mechanism within the same *Xiphophorus* genus.

2. Materials and Methods

2.1. Animals and cell culture

Xiphophorus nigrensis (WLC3760 for large males/females and WLC4715 for small males, from the nacimiento of the Río Choy) and *X. hellerii* (WLC1337, from Rio

Lancetilla) were reared under a standard light/dark cycle of 14/10 h at 26 °C at the fish facility of the Biocenter at the University of Wuerzburg, Germany. Standard length and total length of all animals and sword length of males used in this study were routinely determined. All animals were kept and sampled in accordance with the applicable EU and national German legislation governing animal experimentation, in particular, all experimental protocols were approved through an authorization (568/300-1870/13) of the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law (TierSchG).

Human embryonic kidney 293T (HEK293T) cells, and stable cell lines derived from HEK293T were cultured in Dulbecco's modified Eagle medium (DMEM) without sodium pyruvate (PAN Biotech, Aidenbach, Germany), with 10 % fetal calf serum (FCS) (Sigma, St. Louis, Missouri, United States) and penicillin 100 U/mL, streptomycin 100 μg/mL at 37 °C, 5 % CO_2. Stable cell lines used in the study expressing A or/and B alleles of *mc4r* from the *Xiphophorus multilineatus* fish are described previously (Lampert et al. 2010). Culture medium for the stable *mc4r* cell lines was supplemented with G418 200 μg/mL.

2.2. Cloning of *mc4r* genes from *X. hellerii*

To identify the alleles of *mc4r* in *X. hellerii* fish, *mc4r* was cloned form genomic DNA of one female, one small male and one large male fish. Genomic DNA was extracted from pooled organs of individual fish using standard phenol-chloroform extraction protocols for high molecular weight DNA (Blin and Stafford 1976; Sambrook et al. 1989).

The single exon of *mc4r* was amplified from genomic DNA using primer pair Xmcr1-F10 and Xmcr1-R5. The amplicon included 5'UTR of about 340 bp, the ORF of around 1000 bp and 3'UTR of up to 100 bp. Amplifications were performed with a proofreading Taq polymerase (ReproFast proofreading Taq Polymerase, Genaxxon BioScience GmbH, Germany). Cycling condition is: 35 cycles of 95 °C for 45 sec, 58 °C for 45 sec and 72 °C for 2 min. PCR products were column-purified and phosphorylated. Vector pBluescript II KS(+) was cut with EcoRV, column-purified and dephosphorylated. Vector and insert were blunt-end ligated at 16 °C for 18 h.

Constructs were transformed into DH5α competent cells and positive clones were screened by colony PCR using M13 primers. For each genomic DNA, five clones were sequenced with primers Xmcr1-F10/-R5. Primers for cloning are listed in **Table S3.1**.

The open reading frame of each sequence was determined. All the characterized *mc4r* have only one exon and no introns. The verified nucleotide sequences were translated into amino acid sequences of Mc4r. Multiple sequence alignments were generated with the 15 Mc4r amino acids sequences of *X. hellerii* and Mc4r A, B1, B2 alleles of *X. multilineatus* and Mc4 S allele of *X. maculatus*, using MUSCLE tool (Edgar 2004b; Edgar 2004a).

2.3. Whole mount *in situ* hybridization

To compare the spatial expression pattern of *mc4r* and *mrap2* genes in *X. nigrensis* fish, whole mount RNA *in situ* hybridization was performed in large males, small males and females of *X. nigrensis* brains following procedures described previously (Thisse et al. 1993; Thisse and Thisse 2008). To compare the spatial expression pattern of *mc4r* and *mrap2* in *X. hellerii* and *X. nigrensis*, whole mount RNA *in situ* hybridization of both genes was performed in large males, small males and females *X. hellerii* fish brains as in *X. nigrensis*.

Riboprobes were designed to match the gene sequences in *X. maculatus*, *X. multilineatus*, *X. nigrensis* and *X. hellerii*. Thus, the riboprobes of *mc4r* and *mrap2* for *X. hellerii* used in the study are the same as previously designed for *X. multilineatus* and *X. nigrensis*. Riboprobes were generated by first amplifying both genes using plasmids containing coding region of the respective genes (primers listed in **Table S3.1**), then cloning into pGEM-T Easy vector and verifying by sequencing, and finally linearizing and performing *in vitro* transcription by T7 or SP6 RNA polymerase (Roche, Basel, Switzerland) with digoxigenin RNA labeling mix (Roche).

Whole mount *in situ* hybridization was performed on dissected intact brains fixed in 4 % PFA and dehydrated in methanol for storage. Before hybridization, brains were rehydrated with PBST and digested with 10 µg/ml proteinase K for 45 min. Samples were prehybridized in hybridization mix (formamide 25 mL, 20×SSC 12.5 mL, heparin (25 mg/mL) 100 µL, 20 % Tween 20 250 µL, yeast RNA (Ribonucleic acid

type IV) 25 mg, add H$_2$O to 50 mL, stored at -20 °C), and then were hybridized with riboprobes overnight at 68 °C in a humidified chamber. The brains were then subjected to stringent washes of SSC series (0.05X SSC used for washes of high stringency: 2XSSC/hybridization mix 25 %/75 %, 50 %/50 %, 75 %/25 %, 100 %/0 %, then 0.05XSSC twice, and 0.05XSSC/PBST 50 %/50 %,) and final wash with PBST.

The samples were embedded in 3 % agarose and 100 μm sections were cut by TPI Vibratome Series 1000 Sectioning System (Technical Products International Inc., St. Louis, Missouri, United States). After blocking with 5 % sheep serum, anti-digoxigenin-AP (1:5000) (Roche) was applied to the sections overnight at 4 °C. After removing antibodies and repeated washing, NBT/BCIP solution (Roche) was used to develop the signals by AP reaction according to the manufacturer's instruction.

Sections were embedded in EPON or directly mounted in 80 % glycerol for microscopy (Zeiss AxioPhot, using AxioVision Rel 4.8 software, Oberkochen, Germany). Anatomical localization of transcripts was determined according to the *Xiphophorus hellerii* brain atlas (Anken and Rahmann 1994).

2.4. Gene expression analysis

To determine the expression of *mc4r* A, B1, and B2 alleles, we downloaded the RNA-seq data (BioProject Accession: PRJNA352550) of four Y-II size male *X. multilineatus* fish brains (GEO Accession of individuals a-d: a: GSM2375943, b: GSM2375944, c: GSM2375945, d: GSM2375946. The a/b data are from low quality juvenile environment fish, and the c/d data are from high quality juvenile environment fish, which were better fed than a/b.) (Lu et al. 2017). The reads were mapped to the sequences of *X. multilineatus mc4*r alleles A, B1, B2 using TopHat (Trapnell et al. 2012). *mc4r* allelic expression can be evaluated based on read counts regardless of the gene length, due to similar gene length among the three alleles. Reads were only kept and counted for each allele when they were exactly aligned.

2.5. Cloning of expression vectors of Mrap2

The conserved sequence of *mrap2* in *X. maculatus*, Amazon molly (*Poecilia formosa*), and guppy (*Poecilia reticulata*) was used to design primers upstream and downstream of the coding sequence. The amplicon was sequenced and then used for subcloning into the *mrap2* expression vector CMV-Mrap2-3XpolyA.

Each fragment (CMV, Mrap2, and 3XpolyA) was cloned in entry vectors of the Golden GATEway system. In a predefined order, the three fragments were simultaneously assembled into a destination vector by GoldenGATE reaction, using BsaI (enzyme Eco31I, 10 U/µl, Thermo Scientific, Waltham, Massachusetts, United States) and T4 DNA ligase (30 Weiss U/µl, Thermo Scientific) as described previously (Kirchmaier et al. 2013). Cycling condition were as follows: 1) 37 °C 30 min (Digestion); 2) 16 °C 30 min (Ligation); repeat 1)-2) 10X; 3) 50 °C 5 min (Redigest to eliminate plasmids still containing BsaI sites); 4) 80 °C 5 min (Inactivation of enzymes).

Primers for cloning are listed in **Table S3.1**.

2.6. Pharmacological characterization of Mc4r and Mrap2 functions

HEK293T cell lines, stably expressing Mc4r A or A-B1 (expression level A:B1=1:3.27) (Lampert et al. 2010) were subjected to adenylyl cyclase assays by luciferase reporter gene assays. Luciferase determination was performed as described previously (Sturm et al. 2003). Through transactivating genes containing cAMP response element (CRE) motifs in their regulatory regions by CRE binding protein (CREB), the assay measures elevation of intracellular cAMP concentration by enhanced transcription of the reporter gene.

Cells were seeded at 50 000 cells per well in 12-well plates and grown to 50-70 % confluency. Cells were transfected by polyethylenimine (PEI) with pSGIICRE4luc ("Experimental" Reporter with CRE elements, firefly), pGL4.74[hRluc/TK] ("Control" Reporter, Renilla) and pEGFP-N1 plasmids (Thiel et al. 2005). As positive control, plasmid pCMV-Flag-NLS-Cα (constitutive PKA) was additionally transfected. Plasmid pcmvMRAP2a was co-transfected at 0 ng, 100 ng, 200 ng, and 300 ng. After overnight transfection, cells were treated with NDP-MSH of 0 nM, 0.1 nM, 0.3 nM,

1 nM, 3 nM, 10 nM, 30 nM, 100 nM, and 300 nM for 8 h before harvesting the cells. Cell lysates were measured by Dual-Luciferase® Reporter Assay System kit (Promega, Madison, Wisconsin, United States) using BERTHOLD TriStar LB941 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) according to manufacturer's recommendations. Firefly luciferase was measured at 550-570 nm, and Renilla luciferase at 480 nm. Three independent experiments were performed, and data were presented as mean ± standard deviation (SD).

The data were analyzed as firefly/Renilla ratio and normalized to the negative control. The dose response curves were generated and fitted by log[agonist] vs. response in GraphPad Prism 6 using a variable slope model. Half maximal effective concentration (EC50), basal response and maximum response (Rmax) in dose response curves were calculated.

3. Results

3.1. Transcript localization of *mc4r* and *mrap2* in adult brains of *X. nigrensis* males and females

To characterize the spatial expression pattern of *mc4r* and *mrap2*, the localizations of expressing cells were determined by whole mount *in situ* hybridization on whole brains of large and small males and females *X. nigrensis* (for body length and weight of the fish see **Table S3.2**). In all three analyzed fish, *mc4r* and *mrap2* were expressed in the preoptic region and hypothalamus, with overlapping expression in both regions (**Figure 3.1A**, **3.1B**, **Figure S3.1A**).

Importantly, higher hybridization signals of *mc4r* was observed in large males (**Figure 3.1A**). As all samples were treated in the same conditions for *in situ* hybridization of each gene, we can therefore infer that *mc4r* has higher expression in large males, as consequence of enhanced transcription. This is in agreement with the earlier RT-qPCR results from whole brain extracts (Lampert et al. 2010). Females express *mc4r* in the same periventricular region as large males, but with lower intensity. Level of *mc4r* expression in small males is like in females. The expression is particularly low in preoptic region (a reproduction-regulating brain region) in small males, while females show a robust expression in the posterior part.
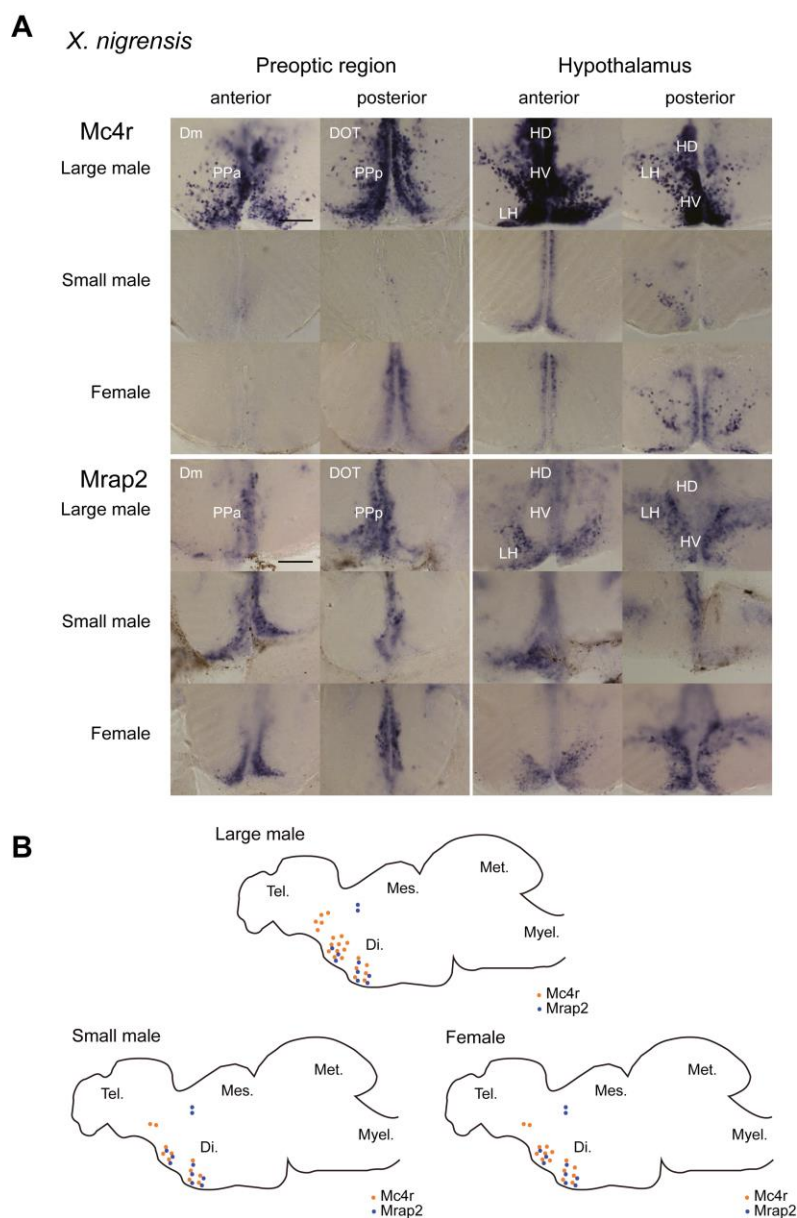
Figure 3.1 Expression domains of *mc4r* and *mrap2* in X. nigrensis adult brains.

(A) Expression of *mc4r* and *mrap2* were detected in cross sections of brains in the preoptic region and hypothalamus regions by whole mount *in situ* hybridization. *mc4r* presents different signal strength, showing high expression level in large males and lower in small males and females; while *mrap2* hybridization signals are of similar strength in the same regions. Scale bar: 100 µm.
(B) Schematic representation of *mc4r* (orange) and *mrap2* (blue) mRNA localization from sagittal sections. Brain regions: Di.: diencephalon, Tel.: telencephalon, Mes.: mesencephalon, Met.: metencephalon, Myel.: myelencephalon. Brain areas in detail: Dm, dorsomedial telencephalic zone; PPa, anterior parvocellular preoptic nucleus; DOT, dorsal optic tract; PPp posterior parvocellular preoptic nucleus; HD, dorsal hypothalamus; HV, ventral hypothalamus; LH, lateral hypothalamic nucleus.

On the contrary, *mrap2* expression was similar in both male morphs and female. We also observed an unexpected *mrap2* expression below the optic tectum region in the anterior hypothalamus (**Figure S3.1B**). This region was identified as the cortical nucleus and pretectal nucleus, where *mc4r* is not expressed. It indicates that another receptor expressed in these regions may interact with Mrap2 expressed here.

The expression domains of *mc4r* and *mrap2* are overlapping in the preoptic nucleus and periventricular hypothalamus. This indicates a possible relation of *mc4r* and *mrap2* functions. This co-localization importantly showed that the interaction seen in the *in vitro* studies is in fact occurring in the *in vivo* situation. On the other hand, *mc4r* or *mrap2* may also function independently from each other. Indeed, there are regions, where only *mc4r* or *mrap2* are expressed. Especially in large males, *mc4r* expressing cells are widely spread in the preoptic region and are only overlapping with *mrap2* in medial part. In such areas, *mc4r* might transduce signals without *mrap2*. The expression of *mrap2* in the ventral optic tectum is independent of *mc4r*, which is not expressed here. In conclusion, *mc4r* might function without its accessory protein and *mrap2* may function with other interacting partners.

3.2. Mc4r allelic expression levels in adult brains

As the level of expression of *mc4r* differs among large males, small males and females (**Figure 3.1A**), we asked if the high *mc4r* expression in large males is differential with respect to the three allele types, for example, due to the known presence of more B alleles in large males.

We analyzed the available transcriptome (Lu et al. 2017) from four male brains of *X. multilineatus* (Y-II size, large male) fish. Of note, two individuals (c, d) were better fed than a and b. In general, read counts (expression levels) of Mc4r A and B1 alleles were high while the B2 alleles were only lowly expressed (**Figure 3.2**). The group of better fed fish (c, d) shows higher Mc4r expression of both alleles than the other group (a, b). The higher expression of both alleles after better feeding indicates that A and B1 alleles respond equally to an external stimulus. Overall, the high *mc4r* expression in large males (Y-II) is from high levels of A and B1 alleles, while B2 alleles appear to be not expressed and thus would not contribute to a Mc4r effect on puberty regulation.
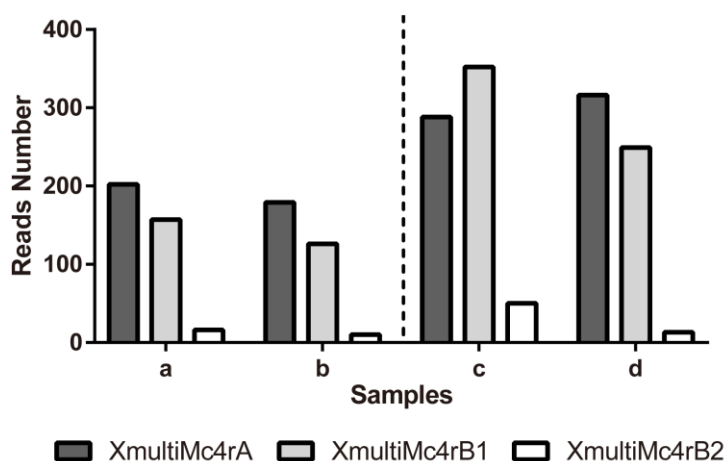
Figure 3.2 Differential gene expression of Mc4r A/B1/B2 alleles.

Mc4r A, B1, B2 allelic gene expression in four males (size class Y-II). A and B1 alleles are much higher expressed than B2. The better-fed individuals (c, d) show higher expression than the well-fed (a, b).

3.3. Effect of Mrap2 on Mc4r signaling

Mrap2 has been shown to be a Mc4r accessory protein important for its function in mammals and zebrafish (Asai et al. 2013; Sebag et al. 2013). To investigate the effect of Mrap2 on Mc4r in *Xiphophorus*, cAMP production of different alleles of Mc4r in the absence or presence of Mrap2 was determined.

HEK293T cells do not express endogenously a functional Mc4r as NDP-MSH does not stimulate and induce cAMP production (**Figure S3.2A**). Thus, the cAMP production from Mc4r expressing stable cell lines derived from HEK293T reliably monitors the signaling of function of Mc4r. At first, cAMP production in three stable cell lines expressing Mc4r but not Mrap2 were compared. In Mc4r A, Mc4r B1 and Mc4r A-B1 cell lines, the level of cAMP production without stimulation by NDP-MSH is higher than in HEK293T controls, indicating that both Mc4r A and B1 receptors have constitutive activity in the absence of agonists (**Figure S3.2A, S3.2B**). Moreover, the constitutive cAMP production for all three cell lines in the absence of Mrap2 did not differ (**Figure S3.2C**). Importantly, the dose response curves of the Mc4r A expressing cell line and Mc4r A-B1 cell line show that the potency of the A allele is reduced by B1 (**Figure 3.3A**). These data are in agreement with the earlier findings that A alleles but not B alleles can respond to NDP-MSH, and B alleles

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reduce signaling of A alleles (Lampert et al. 2010).

Since Mc4r B1 expressing cells cannot be stimulated by NDP-MSH (**Figure S3.2A**), it is not used in the following experiments. The signaling properties of Mc4r A and Mc4r A-B1 expressing cells were further determined with co-expression of Mrap2. Both cell lines respond to NDP-MSH in a dose-dependent manner.

In the cell line, which expresses only the *mc4r* A allele, EC50 was 4.00 nM in the absence of Mrap2; while in the Mrap2 co-transfected cells, from 100 ng to 300 ng, EC50 remained in a similar range around 2-4 nM (**Figure 3.3B**, **Table 3.1**). The potency (referred as quantity of NDP-MSH necessary to produce a cAMP effect determined here as EC50) did not change. However, the dose-response curve was shifted upwards with increasing Mrap2 expression level. The constitutive cAMP production and the induced cAMP production (under treatment of 300 nM NDP-MSH) in 300 ng Mrap2 cells was 1.9-fold higher and 2.2-fold higher, respectively than without Mrap2 (**Table 3.1**). This indicates that Mrap2 modulates Mc4r by increasing the efficacy of Mc4r.

In cells, which express *mc4r* A and B1 alleles in a 1:3.27 ratio (Lampert et al. 2010), EC50 value was 6.36 nM without Mrap2. Mrap2 co-transfected cells had higher EC50 values, around 6-12 nM (**Figure 3.3C**, **Table 3.1**). Similarly, constitutive and induced cAMP production increased 2.5-fold and 3.2-fold, respectively, with introduction of Mrap2 (**Table 3.1**).

In both Mc4r A and Mc4r A-B1 cell lines, increased Mrap2 amount induced a higher cAMP response, but the EC50 values did not change significantly upon Mrap2 co-expression. The EC50 values are in the same range as those reported for human and pig Mc4r (Kim et al. 2004; Fan et al. 2008; Chapman and Findlay 2013). This shows that the Mrap2 does not change the potency of Mc4r.
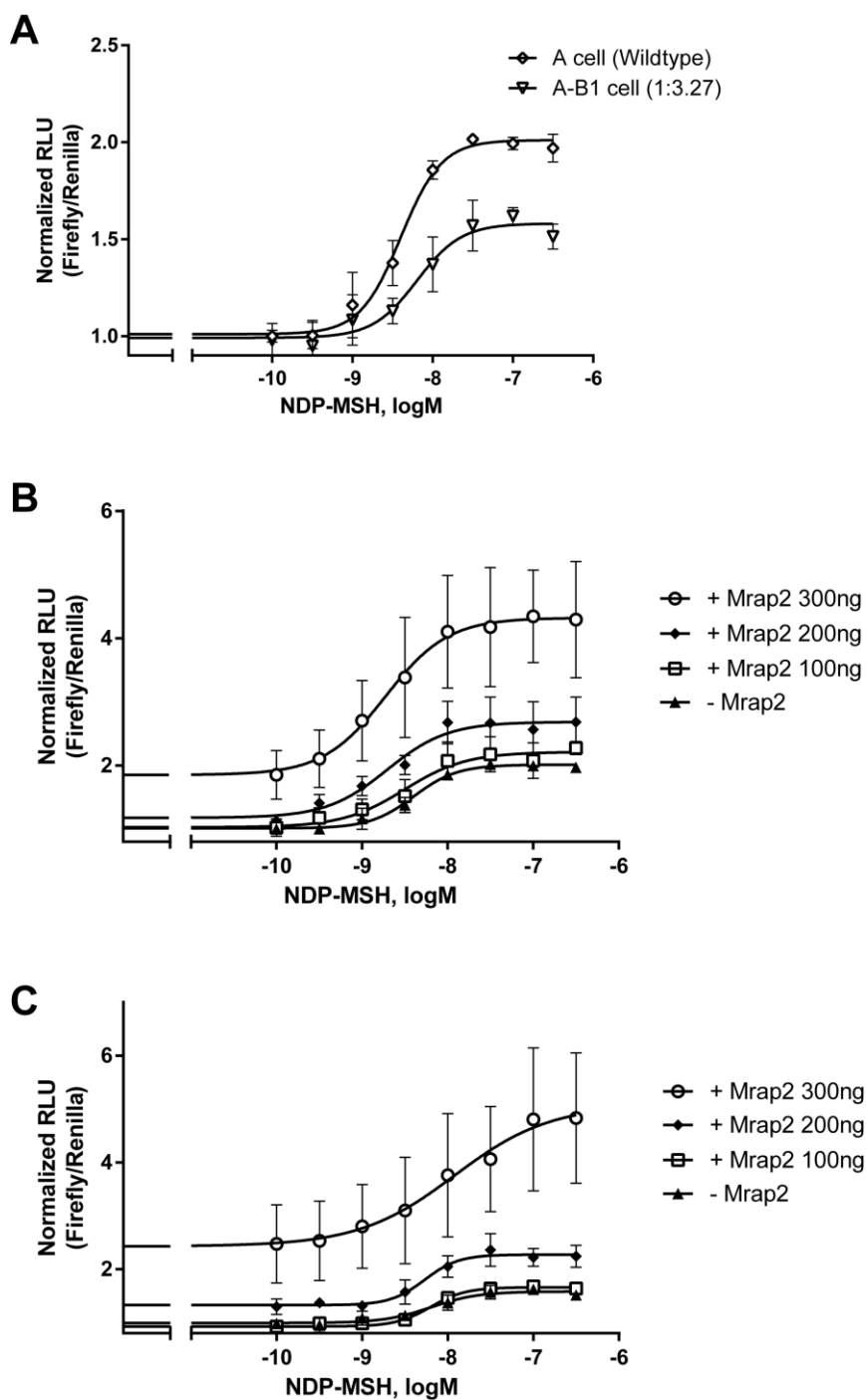
Figure 3.3 Pharmacological characterization of Mc4r receptors in presence or absence of Mrap2.

(A) Dose response curve of two stable cell lines showing that co-expression of B1 reduces potency of A. **(B)** Effect of Mrap2 on dose response curves of Mc4r in the A allele expressing cell line. **(C)** Effect of Mrap2 on dose response curves of receptor in Mc4r A-B1 (1:3.27) expressing cell line. Mrap2 did not change cAMP production. The data are presented as mean ± standard deviation (SD).

Table 3.1 Signaling properties of Mc4r alleles in absence or presence of Mrap2.

cAMP production was measured in Mc4r expressing cell lines in the absence or presence of Mrap2 upon stimulation with agonist NDP-MSH (0 nM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM). Mc4r expressing cell lines used include Mc4r A expressing cell (A) and Mc4r A-B1 expressing cells (A-B1 1:3.27). Mrap2 was either absent from Mc4r expressing cell lines (-Mrap2) or transfected into the Mc4r expressing cell lines with increasing amounts from 100 ng to 200 ng to 300 ng (+Mrap2).

Mc4r		-Mrap2	+Mrap2 100ng	+Mrap2 200ng	+Mrap2 300ng
A					
EC50*	Mean	4.001	3.126	1.958	1.881
(nM)	95 %CI	3.164 to 5.059	1.521 to 6.424	0.9872 to 3.883	0.6574 to 5.384
Basal	Mean	1.01	1.028	1.176	1.85
	95 %CI	0.9545 to 1.066	0.8453 to 1.21	0.9309 to 1.42	1.239 to 2.461
Rmax	Mean	2.011	2.21	2.684	4.32
	95 %CI	1.945 to 2.077	2.007 to 2.414	2.473 to 2.895	3.798 to 4.841
A-B1 (1:3.27)					
EC50	Mean	6.355	6.302	5.357	12.01
(nM)	95 %CI	3.728 to 10.84	4.692 to 8.463	3.365 to 8.527	0.8201 to 175.9
Basal	Mean	0.9911	0.9251	1.325	2.428
	95 %CI	0.9237 to 1.058	0.8732 to 0.9769	1.219 to 1.432	1.626 to 3.23
Rmax	Mean	1.581	1.659	2.273	5.083
	95 %CI	1.495 to 1.667	1.597 to 1.722	2.149 to 2.398	3.153 to 7.013

*EC50 represents the half maximal effective concentration in nM. Basal and Rmax indicate in normalized RLU the basal response and maximum response, respectively. All values are presented as mean and 95 %CI (95 % confidence interval).

3.4. Absence of B alleles of *mc4r* in *X. hellerii*

In *X. hellerii* fish, large and small males co-exist in wild population like in *X. nigrensis* and *X. multilineatus*. However, it is unknown if *mc4r* is similarly involved in determining the puberty onset timing as in the two previously studied species. To first identify *mc4r* copies present in the genome of *X. hellerii* fish, *mc4r* was cloned from genomic DNA of a female, a small male and a large male fish (**Table S3.3**). Five *mc4r* clones were sequenced from each animal.

Unexpectedly, despite the remarkable size differences of the fish, the copies found in

female, small and large male of *X. hellerii* belong to the same allele class. The two cysteine residues at the carboxyl-terminus missing in B-alleles are present in all the Mc4r sequences. There were also no other insertions/deletions (indels) or non-conservative substitutions in either the small or large male which would point to changes in protein structure and function. They are most similar to the A alleles class in the *X. nigrensis* and *X. multilineatus* and the wildtype S-allele of *X. maculatus* which also presents the CC motif (**Figure 3.4**) indicating that they are also functional wild-type alleles with uncompromised signal transducing capacity.
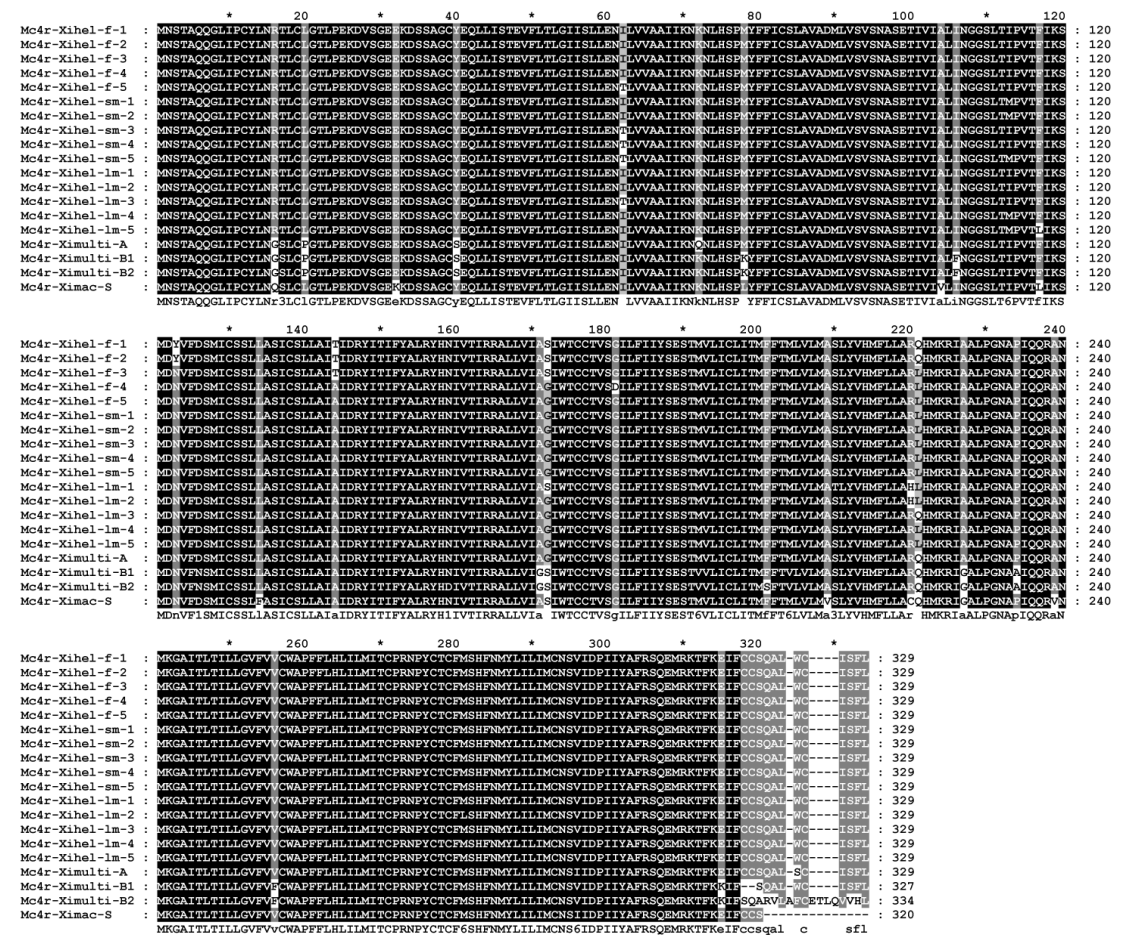
Figure 3.4 Sequence alignment of Mc4rs in *X. hellerii.*

Mc4r sequences from *X. hellerii* females (Mc4r-Xihel-f), small males (Mc4r-Xihel-sm) and large males (Mc4r-Xihel-lm) aligned and compared to Mc4r alleles (A, B1, B2) in *X. multilineatus* and S allele in *X. maculatus*. *X. hellerii* Mc4rs are most similar to Mc4r wild-type A alleles in *X. multilineatus*.

3.5. Localization of *mc4r* and *mrap2* mRNA in the brain of *X. hellerii* fish

In *X. nigrensis*, *mc4r* and *mrap2* are mainly expressed in the preoptic region and hypothalamus. To further characterize and compare the spatial expression pattern of both genes in *X. hellerii*, large males, small males and females (**Table S3.4**) fish were examined by whole mount *in situ* hybridization.

In all individuals independent of sex or size, *mc4r* and *mrap2* were expressed in the preoptic region and hypothalamus with overlapping domains in both regions (**Figure 3.5A, 3.5B, Figure S3.3**).

The *mc4r* expression in the large male was substantially higher than in small male and female (**Figure 3.5A**), indicating a high transcript level. The spatial expression pattern of *mc4r* in female was the same as in large male. In small male, the expression level was like in female in the hypothalamus, but no signal was detected in the posterior part of the preoptic region.

In contrast, the expression pattern of *mrap2* in large male, small male, and female was very similar (**Figure 3.5B**). The preoptic region showed high expression of *mrap2*, while in the hypothalamus it was expressed at a lower level.

In the preoptic nucleus and periventricular hypothalamus, the expression domains of *mc4r* and *mrap2* were anatomically overlapping. In addition, in some expression domains, only *mc4r* or *mrap2* were expressed, like in *X. nigrensis*.
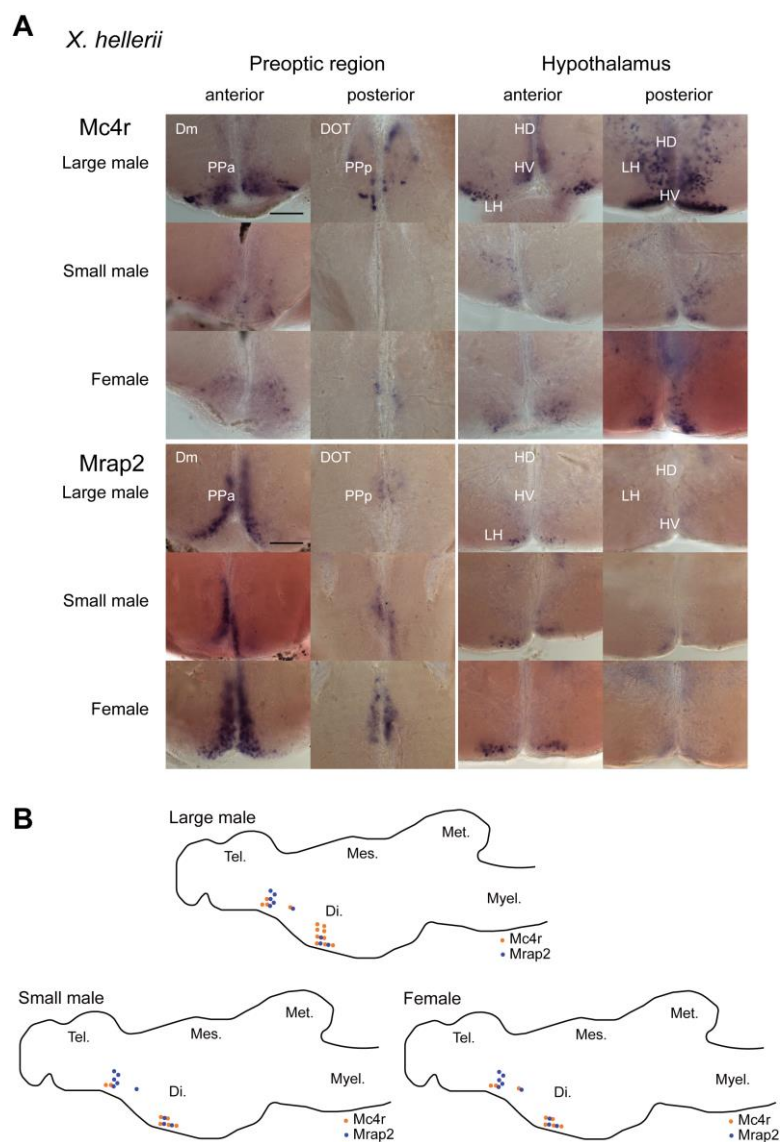
Figure 3.5 Whole mount in situ hybridization for *mc4r* and *mrap2* gene expression in *X. hellerii* adult brains.

(A) Expression of *mc4r* and *mrap2* in cross sections of brains in the preoptic and hypothalamus region. Large males have higher expression level of *mc4r* than the females and small males, while *mrap2* gene signal is of similar strength in all samples. Scale bar: 100 µm. **(B)** Schematic representation of *mc4r* (orange) and *mrap2* (blue) mRNA localization from sagittal sections. Brain regions: Di.: diencephalon, Tel.: telencephalon, Mes.: mesencephalon, Met.: metencephalon, Myel.: myelencephalon. Brain areas in detail: Dm, dorsomedial telencephalic zone; PPa, anterior parvocellular preoptic nucleus; DOT, dorsal optic tract; PPp posterior parvocellular preoptic nucleus; HD, dorsal hypothalamus; HV, ventral hypothalamus; LH, lateral hypothalamic nucleus.

4. Discussion

4.1. Mrap2 increases constitutive and ligand-induced Mc4r signaling

Large and small male *Xiphophorus nigrensis* and *Xiphophorus multilineatus* possess different Mc4r allele types with low and high signaling properties (Lampert et al. 2010). We found that Mrap2 co-expressed in the brain in most parts where Mc4r is expressed, indicating that Mrap2 is used as the accessory protein of Mc4r. Mc4r and Marp2 co-expressed in hypothalamus and preoptic regions, implying that these two proteins are able to interact in the *in vivo* situation. Hypothalamus has vital roles in HPG axis to regulate puberty onset, and preoptic region is a part of brain also expressing GnRH neurons (Xue et al. 2014), showing their roles in reproduction regulation. Mrap2 is also expressed in pretectal nucleus without Mc4r. In pretectal nucleus, orexin receptor OX2R is also partly expressed (Trivedi et al. 1998). Mrap2 has been reported to be required for the inhibition of orexin receptor signaling (Rouault et al. 2017).

It is unclear what is the functional importance of Mrap2 on the differential Mc4r signaling in *Xiphophorus*. Results show that increased Mrap2 expression levels in Mc4r expressing cell lines amplify both the constitutive activity without stimulation and NDP-MSH induced maximum cAMP response. This has been shown in Mc4r wild-type allele expressing cells and also in Mc4r A-B1 cells co-expressing a mutant, defective allele. However, Mrap2 does not change the potency and ligand sensitivity of Mc4r, but rather increases the efficacy of Mc4r signaling. This is different from the situation in zebrafish where Mrap2a, the zebrafish specific embryonic form, reduces efficacy while it does not change potency. It was suggested that Mrap2a stabilizes an inactive conformation of Mc4r (Sebag et al. 2013). Hence, our results suggest that the Mrap2 in *Xiphophorus* stabilizes an active conformation of Mc4r. In other fish species, Mrap2 has different roles in modulating Mc4r. In zebrafish, Mrap2b, the zebrafish specific adult form increases α-MSH ligand sensitivity to Mc4r (Sebag et al. 2013). In gar (*Lepisosteus oculatus*) Mrap2 increases the NDP-MSH induced stimulation on Mc4r (Wolverton et al. 2019). In sea lamprey (*Petromyzon marinus*), McrB is the ancestor of Mc4r in other fish, and Mrap2 can enhance the response of α-MSH to McrB (Zhu et al. 2019). However, in orange-spotted grouper (*Epinephelus coioides*), the Mrap2 decreases the constitutive and induced cAMP signaling of Mc4r (Rao et al.

2019). In other species, for example, in chicken, Mrap2 also reduces Mc4r responsiveness to α-MSH (Zhang et al. 2017). In humans Mrap2 decreases NDP-MSH induced signaling (Chan et al. 2009), but in mice it increases α-MSH ligand sensitivity (Asai et al. 2013). This demonstrates that Mrap2 can have different roles across species in modulating ligand selectivity of Mc4r.

Moreover, evidence from *in situ* hybridization shows that large male, small male and even female *X. nigrensis* express same level of *mrap2* in the same anatomical regions of adult brains. We could infer that such similar Mrap2 levels will cause similar levels of signal amplification of Mc4r. Hence, this will not lead to differential Mc4r signaling in large and small males. We hypothesize that although Mrap2 can amplify Mc4r signaling it is not mediating differential Mc4r signaling as the basis of large or small size male phenotypes.

In all studied *Xiphophorus* species, *mrap2* was expressed in some brain regions, which showed no detectable levels of Mc4r. This is different from medaka fish, in which *mrap2* is always expressed where also *mc4r* is present, while *mc4r* expression can be found in regions which are devoid of *mrap2* expression signals (Liu et al. 2019). In addition to the study about orexin receptor mentioned above, studies in mammals show that Mrap and Mrap2 can interact with all five melanocortin receptors (Chan et al. 2009) and even other GPCRs like ghrelin receptor (Srisai et al. 2017). It is likely that *Xiphophorus* Mrap2 not only regulates Mc4r signaling, but is also involved in other pathways. In zebrafish, Mc4r changes pharmacological profiles in response to ACTH when co-expressed with Mrap2a (Josep Agulleiro et al. 2013). Similar cases have been reported for the chicken Mc4r (Zhang et al. 2017) and human Mc4r (Soletto et al. 2019). This together indicates that Mrap2 and Mc4r not only vary between species, but also vary in response to different ligands (MSH or ACTH) in the same and in between species.

4.2. Elevated Mc4r expression in large males is correlated with differences in body size and late puberty

In *X. nigrensis* and *X. hellerii*, localization of *mc4r* and *mrap2* co-expression is found in those brain region of the food intake feeding centers, the hypothalamus and its neighbor preoptic regions, similar to zebrafish and medaka (Josep Agulleiro et al.

2013; Liu et al. 2019). Unlike in mammals, where feeding centers are restricted to the hypothalamus, those centers are more widespread in fish (Volkoff 2016). Puberty has been linked to nutritional and energy status determined by food intake. Thus, Mc4r signaling may mediate the hypothalamus-pituitary-gonad (HPG) axis and transmit a physiological signal of an appropriate status to induce puberty.

To investigate the effect of Mc4r signaling on puberty, considering that the Mrap2 are of same amount in three types fish (large male, small male, female), we should evaluate the Mc4r signaling properties with same Mrap2 levels. At comparable Mrap2 amounts, the potency (EC50) of Mc4r A-B1 for NDP-MSH was slightly lower. In addition, the Mc4r A-B1 situation showed a lower efficacy (maximum response). This could mean that a combination of Mc4r A wild-type and B1 mutant alleles causes weaker signaling. Conceivably, Mc4r B1 may act dominant negatively on Mc4r A.

Interestingly, *in situ* hybridization and expression analysis of Mc4r gave further hints on such a role. Large males express higher amounts of Mc4r compared to small males and females. Mc4r A and B1 are equally highly expressed alleles in large *X. multilineatus*. Such Mc4r B1 expression *in vivo* implies an effect of lower signaling and thereby may delay puberty in large males. Signaling activity of Mc4r B alleles is only basal. NDP-MSH cannot induce cAMP signaling in Mc4r B allele expressing cells. High expression of A and B1, particularly B1 alleles, could be the essential factor for lower signaling in large males. This correlates with the previous study that the copy number variation is the determining factor for differences in puberty timing (Lampert et al. 2010). A molecular mechanism how B alleles could act as dominant negative versions remains to be further elucidated.

4.3. Convergent evolution of *X. hellerii*, *X. nigrensis* and *X. multilineatus*

Mc4r is related to energy homeostasis and appetite, not only in mammals but also in fish species. The human gain-of-function mutations protect people from obesity (Lotta et al. 2019). In mice, the Mc4r KO has been reported to be obese (Huszar et al. 1997), and it has been also reported that such obese phenotype can be observed in Mc4r expressing cells $G_s\alpha$ KO mice, confirming G_s mediates Mc4r function (Podyma et al. 2018). In cave fish, the Mc4r has a role in regulation of appetite, allowing them to adapt to nutrients-poor conditions (Aspiras et al. 2015).

Mc4r and puberty have not been directly related in humans. Individuals with loss-of-function Mc4r mutations are obese (Farooqi et al. 2003), but do not have reproductive deficits (Yeo et al. 1998). Yet mice with inactivated Mc4r develop not only obesity but also subfertility (Elias and Purohit 2013; Hill and Elias 2018).

Even in teleosts, a role of Mc4r in puberty outside *Xiphophorus* has not been described so far. In zebrafish an effect of Mc4r knockout on the timing of reaching sexual maturity was not noted possible owing to the difficulty of monitoring puberty onset in these fish (Uusi-Heikkila et al. 2012; Sebag et al. 2013). Medaka Mc4r knockout animals reach puberty at similar time as wild-type animals, in contrast to *Xiphophorus* (Liu et al. 2019). Thus, regulation of the timing of puberty onset by Mc4r in *Xiphophorus* may be specific adaptation of this lineage. And even within this lineage, the molecular mechanism appears not to be conserved. In *X. hellerii*, even though large and small male exist like in *X. nigrensis* and *X. multilineatus*, surprisingly, both carry only one and the same type of Mc4r allele, which is grouped with the wild-type A allele in *X. nigrensis* and *X. multilineatus*. In *X. nigrensis*, Mc4r expression is much higher in large male than in small male. Such differential expression is also observed in *X. hellerii*. Since in contrast to *X. nigrensis* and *X. multilineatus*, in which Mc4r are present as A and B alleles, *X. hellerii* has only A allele Mc4r , the growth and puberty determinant of *X. hellerii* is not represented by different Mc4r alleles, but rather may be environmental or other genetic factors. The regulation of puberty onset in *X. nigrensis* and *X. multilineatus* is not only by genetic factor Mc4r but also by environmental factors. In the environment, it is also known that endocrine disruptors could affect pubertal timing of human (Fisher and Eugster 2014). Thus, the genetics of *X. hellerii* could only explain part of the polymorphism. The Mc4r copies of *X. maculatus,* which all map to region of the chromosome that harbors the *Puberty* locus*,* have many indels, including transposable elements, in upstream and downstream regions of the Mc4r open reading frames (ORF) (Volff et al. 2013). Such indels out of ORF in addition to the allelic variation could be an independent mechanism to determine puberty onset timing. In large and small *X. hellerii* males, the signaling levels may differ due to the diversity in the promoter regions, like in *X. maculatus*. The promoter regions of *mc4r* in *X. hellerii* remains to be elucidated. The high expression of Mc4r in large males is conserved between *X. nigrensis* and *X. hellerii*. It could be an ancestral situation in the *Xiphophorus*

genus, and some lineages, like *X. nigrensis*, *X. multilineatus* and *X. maculatus*, acquired different alleles in the coding region of Mc4r, even copy number variations. Furthermore, the G protein coupled to Mc4r may be different and differences in downstream effectors in Mc4r signaling between large and small may explain the polymorphism.

The mechanism of puberty onset regulation is not conserved even within the genus *Xiphophorus*. This could indicate that *Xiphophorus* fish have convergently evolved with same functional but distinct molecular mechanisms.

5. Supplementary Information

5.1. Supplementary Figures
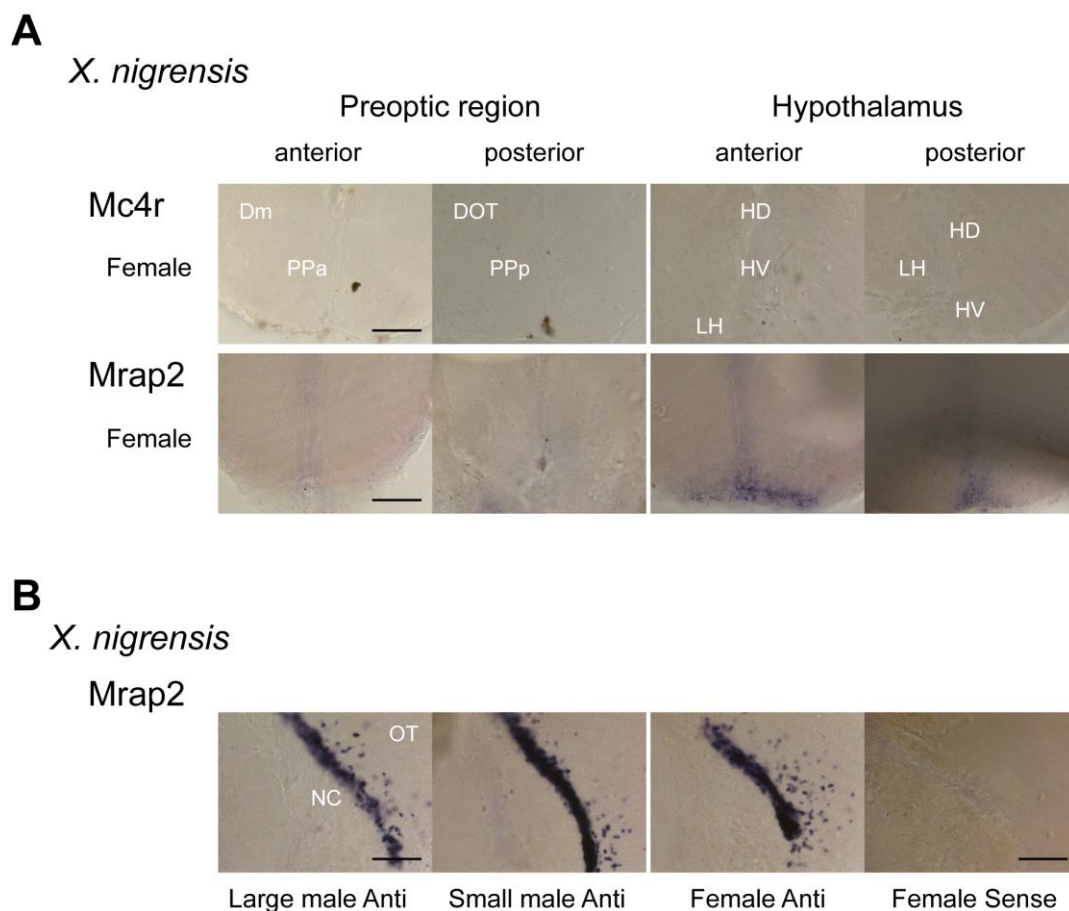
Figure S3.1 Whole mount *in situ* hybridization detection of *mc4r* and *mrap2* genes in *X. nigrensis* adult brains.

(A) *mc4r* and *mrap2* sense probes used as negative control showing no background signals in adult brains. Scale bar: 100 µm. **(B)** *mrap2* was expressed below the optic tectum region at the level of anterior hypothalamus. Scale bar: 100 µm. Brains were cut in cross sections. Brain areas in detail: Dm, dorsomedial telencephalic zone; PPa, anterior parvocellular preoptic nucleus; DOT, dorsal optic tract; PPp posterior parvocellular preoptic nucleus; HD, dorsal hypothalamus; HV, ventral hypothalamus; LH, lateral hypothalamic nucleus; OT, optic tectum; NC, cortical nucleus.
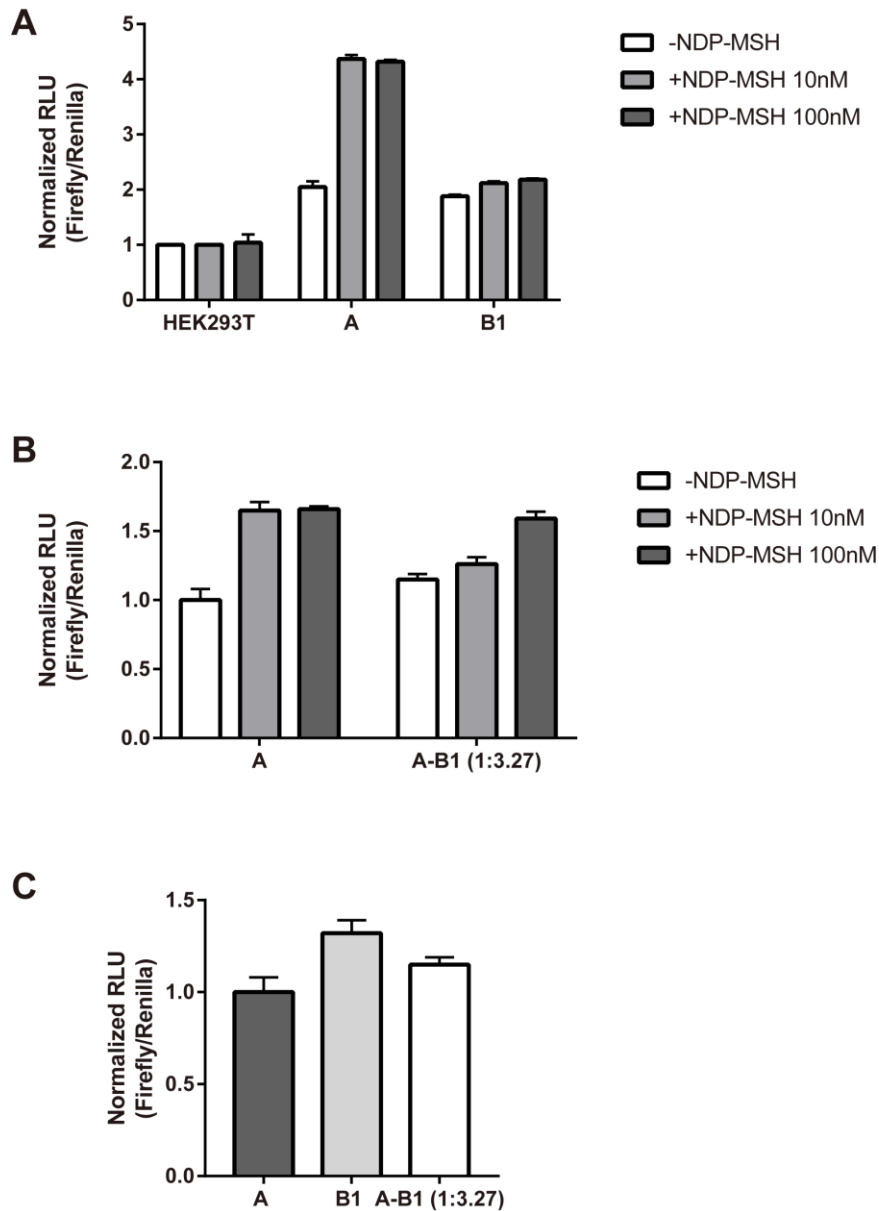
Figure S3.2. Pharmacological characterization of Mc4r receptors.

(A) Mc4r A and B1 have comparable constitutive activity. Mc4r A can be induced by NDP-MSH while Mc4r B is not responding to NDP-MSH. **(B)** Mc4r A and A-B1 (1:3.27) show constitutive and induced cAMP production. The data are presented as mean ± standard deviation (SD). **(C)** Similar constitutive cAMP production of Mc4r A, B1, and A-B1 expressing cells without Mrap2.
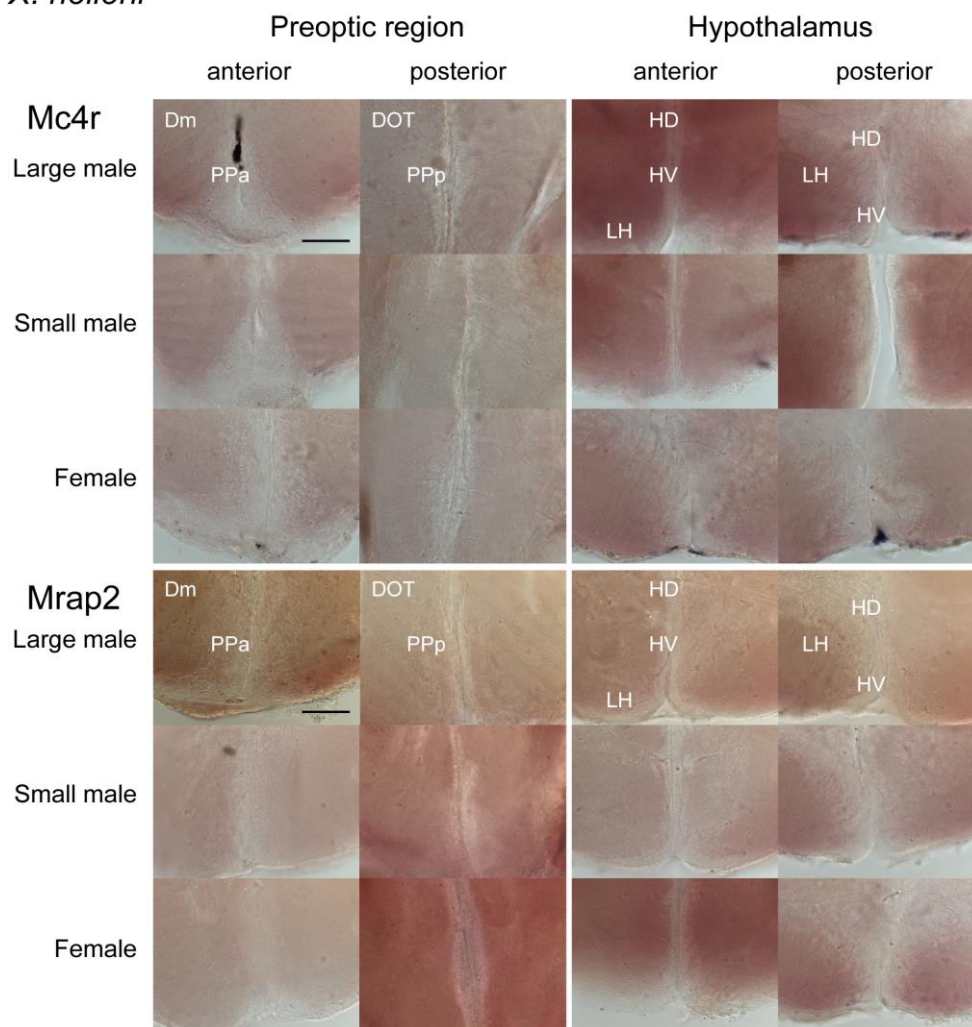
Figure S3.3 Whole mount *in situ* hybridization detection of *mc4r* and *mrap2* genes in *X. hellerii* adult brains.

mc4r and *mrap2* sense probes used as negative control showing no background signals in adult brains. Scale bar: 100 µm. Brains were cut in cross sections. Brain areas in detail: Dm, dorsomedial telencephalic zone; PPa, anterior parvocellular preoptic nucleus; DOT, dorsal optic tract; PPp posterior parvocellular preoptic nucleus; HD, dorsal hypothalamus; HV, ventral hypothalamus; LH, lateral hypothalamic nucleus.

5.2. Supplementary Tables

Table S3.1 Primers used in this study.

Primers.

Primers	Sequences	Comments
Mc4r_Xm_F2	ACTCCACGGCTCAGCAAG	PCR for Mc4r *in situ* hybridization probe synthesis
Mc4r_Xm_R2	CGCAGGGCGTAGAAGATG	PCR for Mc4r *in situ* hybridization probe synthesis
MRAP2a_Xm_F2	TGGCAGTATGAGTATTACGA	PCR for Mrap2 *in situ* hybridization probe synthesis
MRAP2a_Xm_R2	AGTTTGGGATGTTGAAGTG	PCR for Mrap2 *in situ* hybridization probe synthesis
CMV_BamHI_F	CACCGGATCCTAGTTATTAATAGTAATCAAT	GoldenGATE cloning for CMV
CMV_KpnI_R	TATAGGTACCGATCTGACGGTTCACTAAACC	GoldenGATE cloning for CMV
MRAP2a_Xm_BamHI_ATG_F	CACCGGATCCATGTCCGACTTCCACAACCGAAG	GoldenGATE cloning for Mrap2
MRAP2a_Xmulti_KpnI_STOP_R	TATAGGTACCTTAGTGGGTGTCACAGAAACCAT	GoldenGATE cloning for Mrap2
Xmcr1-F10	CCTTTCACCGTTTCTCCTC	Cloning for Mc4r
Xmcr1-R5	GACATTTCAGGCTCTTCATCC	Cloning for Mc4r
M13_F	GTAAAACGACGGCCAGT	Mc4r clones screening
M13_R	CAGGAAACAGCTATGAC	Mc4r clones screening

Table S3.2 Body length and weight of the *X. nigrensis* fish used for *in situ* hybridization.

				Length, cm				Weight, g
				Body	Tail	Total	Sword	
Mc4r	anti	*X. nigrensis*	Large Male	3.5	2.5	4.5	1.5	1.04
	anti	*X. nigrensis*	Small Male	2.2	1.3	2.8	0.7	0.22
	anti	*X. nigrensis*	Female	3.2	0.9	4.1	--	0.8
	sense	*X. nigrensis*	Female	3.2	0.9	4.1	--	0.8
Mrap2	anti	*X. nigrensis*	Large Male	2.9	0.8	3.7	--	0.62
	anti	*X. nigrensis*	Small Male	2.3	1.1	2.9	0.5	0.26
	anti	*X. nigrensis*	Female	2.7	0.7	3.4	--	0.48
	sense	*X. nigrensis*	Female	2.5	0.6	3.1	--	0.4

Body: body length is the standard length, from forehead to trunk terminus.

Tail: tail length is the length of tail including sword, from trunk terminus to tail terminus with sword.

Total: total length is the length from forehead to tail fin excluding the sword.

Sword: sword length is the length of swords.

Table S3.3 Body length of the *X. hellerii* fish used for *mc4r* cloning.

		Length, cm			
		Body	Tail	Total	Sword
X. hellerii	Large Male	4.7	3.6	6.1	2.2
X. hellerii	Small Male	2.8	1.9	3.7	1.0
X. hellerii	Female	4.2	1.1	5.3	--

Table S3.4 Body length and weight of the *X. hellerii* fish used for *in situ* hybridization.

				Length, cm				Weight, g
				Body	Tail	Total	Sword	
Mc4r	anti	*X. hellerii*	Large Male	4.8	3.1	6.3	1.6	2.42
	anti	*X. hellerii*	Small Male	3.2	2.5	4	1.7	0.44
	anti	*X. hellerii*	Female	4.4	1.2	5.6	--	2.5
	sense	*X. hellerii*	Large Male	4.7	4	5.9	2.8	2.22
	sense	*X. hellerii*	Small Male	3.5	2.6	4.4	1.7	0.77
	sense	*X. hellerii*	Female	4.7	1.3	6	--	3.08
Mrap2	anti	*X. hellerii*	Large Male	4.3	3.7	5.5	2.5	1.78
	anti	*X. hellerii*	Small Male	3.5	2.5	4.4	1.6	0.72
	anti	*X. hellerii*	Female	4.4	1.3	5.7	--	2.02
	sense	*X. hellerii*	Large Male	4.5	3.9	5.8	2.6	2.14
	sense	*X. hellerii*	Small Male	3	1.8	3.8	1	0.62
	sense	*X. hellerii*	Female	3.6	1.1	4.7	--	1.5

Chapter 4

Puberty Onset Regulation in *X. nigrensis* and *X. multilineatus* by Mc4r A and B Alleles

This chapter is based on experiments by several participants. Participants include:

Ruiqi Liu, Patrick Eiring, Mike Friedrich, Katherina Hemmen, Mateus C. Adolfi, Carsten Hoffmann, Katrin Heinze, Markus Sauer, Manfred Schartl

Participants Contributions	
RL	performed the expression constructs cloning, immunofluorescence on cells, FRET measurement, dSTORM sample preparations, and drafted the manuscript.
PE	completed the dSTORM imaging and analysis.
MF	guided and helped the FRET measurement.
KHM	performed the simulations of FRET analysis.
MA	supervised the experiments and reviewed the manuscript.
CH	designed and supervised the FRET project.
KH	supervised the FRET measurement study and analysis of data.
MKS	designed and supervised the dSTORM project.
MS	defined and designed the study, coordinated all steps of the research and reviewed all versions of the manuscript.

Abstract

Melanocortin 4 receptor (Mc4r) is a class A G-protein coupled receptor involved in metabolism and obesity in human and puberty onset regulation in *Xiphophorus nigrensis* and *Xiphophorus multilineatus*. In these fish, Mc4r allelic types and copy number variation determine the polymorphism. Mc4r A alleles are functional and B alleles (B1, B2) are defective in signaling. A-B co-expression cells have lower signaling than A. Thus, the B alleles may act on A alleles by a dominant negative mechanism. However, direct evidences of fish Mc4r dimerization is lacking. Here, we examined the possible interaction of Mc4r alleles. By fluorescence resonance energy transfer (FRET) approach, we analyzed the Mc4r A/B1/B2 homo- and heterodimerization. Sensitized emission detected FRET signals and acceptor photobleaching measured FRET efficiency of around 5 % for homodimers and heterodimers. Agonist treatment did not alter the FRET efficiency, showing the dimerization was not changed under this stimulation condition. Simulation of Mc4r dimers on the membrane calculated expected FRET efficiency to be around 24 %. These interaction analyses evidenced that Mc4r A and B form merely partial homo-/heterodimers. The single-molecule microscopy analysis confirmed that only few Mc4r A and B1 receptors co-localized, with an average percentage of colocalizing of 5.27 %. These together exhibit that such specialized puberty onset regulation in these two species are linked to Mc4r B allele through additive or other dominant negative mechanisms.

1. Introduction

G-protein coupled receptors (GPCRs) were perceived long to be functioning as monomer. In the recent 20 years, using engineered constructs in heterologous expressing system, many GPCRs were reported to form dimers or oligomers (Vischer et al. 2015). Such example is that class C GPCRs are fully functioning only as dimers to transduce signals (Kniazeff et al. 2011); for example, $GABA_B$ receptor are obligatory heterodimers (Marshall et al. 1999) and mGlu receptor are constitutive homodimers (Kunishima et al. 2000). Class A GPCRs can also dimerize, though the signaling transducing property may not require dimerization.

One of the important class A GPCR involved in metabolism and obesity in human is the melanocortin 4 receptor (Mc4r). In human, mutations in *MC4R* are the most common cause of early-onset obesity (Farooqi et al. 2003). Up to now, over 200 distinct mutations have been identified in various human patient cohorts, and the frequency of *MC4R* mutations in cohorts of obese patients is around 5 % (Tao 2009; Kuhnen et al. 2019). Furthermore, human MC4R can form dimers. The heterozygous D90N mutation is a loss-of-function mutation with normal cell surface expression and ligand binding. This D90N mutant receptor and wild-type MC4R form homodimers and heterodimers, and the dimerization implements the dominant negative effect by mutant MC4R (Biebermann et al. 2003).

In *Xiphophorus nigrensis* and *Xiphophorus multilineatus* fish, Mc4r is the *P* locus (*Puberty* locus) gene, which regulates polymorphism in reproductive phenotypes in these fish (Lampert et al. 2010). Wild population of these fish have large, late-maturing males, which have their own territories and display courtship rituals to females; and in the same time, there are small, early-maturing males, which have no territories and perform sneak mating. Intermediate males co-exist and exhibit intermediate phenotypes. The Mc4r gene polymorphism, namely allelic variation and copy number variation, determines the phenotype polymorphism (Lampert et al. 2010). Mc4r alleles were characterized into A, B1 and B2. A alleles are able to be stimulated and can transmit signals, while B alleles are defective and could not be stimulated. B alleles (B1 and B2) lack a dicysteine motif in the carboxyl-terminus (Lampert et al. 2010), which is a palmitoylation site anchoring the carboxyl-terminus to the plasma membrane (Qanbar and Bouvier 2003). B2 has an elongated carboxyl-terminus due to an additional four-base deletion. Cell culture co-expression

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of A and B1 showed decreased signaling compared to Mc4r A cells, indicating a dominant negative effect of B1 on the A (Lampert et al. 2010).

Nevertheless, the molecular mechanism of puberty regulation, by conceivable interaction of Mc4r alleles requires more evidence. Here we investigate the molecular mechanism of how Mc4r controls the puberty regulation in *X. nigrensis* and *X. multilineatus* fish. Regulation of Mc4r signaling may be through the Mc4r dimerization or other mechanisms. Here, our results indicate that unlike in human, wild-type Mc4r A alleles does not dimerize, and the possible dominant negative effect of Mc4r B allele on the Mc4r A allele may not be through dimerization.

2. Materials and Methods

2.1. Cell culture

Human embryonic kidney 293T (HEK293T) cells and Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM) without sodium pyruvate (PAN Biotech, Aidenbach, Germany), with 10 % fetal calf serum (FCS) (Sigma, St. Louis, Missouri, United States) and penicillin 100 U/mL, streptomycin 100 µg/mL at 37 °C, 5 % CO_2. HEK293 cells were cultured in DMEM (Gibco / Life Technologies, Carlsbad, California, United States) with 4.5 g/L glucose, 2 mM L-glutamine 10 % FCS, penicillin 100 U/mL, streptomycin 100 µg/mL at 37 °C, 5 % CO_2. All cells were routinely passaged every 3-4 days.

2.2. Cloning of expression vectors of Mc4r

Mc4r different alleles (A, B1, B2) of *X. multilineatus* were cloned and identified previously (Lampert et al. 2010). Expression vectors were designed as Mc4r genes driven by CMV promotors, tagged at N-terminus a cassette of signal peptide – FLAG tag – SNAP- or CLIP-tag, and terminated by 3XPolyA tails (**Figure S4.1A**). Each fragment was cloned in entry vectors of the Golden GATEway system. The fragments were simultaneously assembled in a predefined order into a destination vector by GoldenGATE reaction using BsaI (enzyme Eco31I, 10 U/µl, Thermo Scientific, Waltham, Massachusetts, United States) and T4 DNA ligase (30 Weiss U/µl, Thermo Scientific) as described previously (Kirchmaier et al. 2013). For the GoldenGATE

reaction, the cycling condition is as follows: 1) 37 °C 30 min (Digestion); 2) 16 °C 30 min (Ligation); repeat 1)-2) 10X; 3) 50 °C 5 min (Redigest to eliminate plasmids still containing BsaI sites); 4) 80 °C 5 min (Inactivation of enzymes). Mc4r A, B1, B2 with both SNAP- and CLIP-tags were assembled (six plasmids).

Primers for cloning are listed in **Table S4.1** and **Table S4.2**. The Golden GATEway plasmid kit was a gift from Prof. Joachim Wittbrodt (University of Heidelberg).

2.3. Immunofluorescence on cells

To verify Mc4r expression in cells, Mc4r constructs were expressed in cells and visualized by immunofluorescence assay.

HEK293T cells were seeded on 24 mm coverslips and grown till 50-70 % confluent. Cells were transiently transfected by PEI reagent to express expression vectors containing Mc4r different alleles tagged by FLAG tags. After 48 h expression, cells were fixed by 4 % PFA for 10-15 min, extensively washed by PBS, and permeabilized in 0.1 % Triton X-100/PBS for 10 min. Cells were blocked in 5 % BSA for 20 min and then subject to primary antibody anti-FLAG M2 (1:1000, Sigma) incubation overnight at 4 °C. Following extensive washing with PBS, cells were incubated with conjugated secondary antibodies anti-mouse Alexa 488 or anti-mouse Alexa 568 (1:1000, Invitrogen, Carlsbad, California, United States) at room temperature for 1 h. Cells were stained with Hoechst 33342 (1:500, Invitrogen) for 5 min and mounted in Mowiol 4-88 (Roth, Karlsruhe, Germany) / DABCO (Sigma). Fluorescence pictures were acquired with a confocal microscope (Nikon Eclipse Ti C1, using NIS-Elements AR software, Minato, Tokyo, Japan).

2.4. Fluorescence resonance energy transfer

To understand the molecular mechanism of the Mc4r alleles on receptor function, fluorescence resonance energy transfer (FRET) assay were performed to verify the homo-and/or heterodimerization of Mc4r alleles.

HEK293T cells were seeded 500 000 cells per well on 24 mm coverslips coated 20 min with 100 µg/ml poly-D-lysine in 6-well plates and grown till 50-70 % confluency. Cells were transiently transfected by PEI reagent to express expression

vectors containing Mc4r different alleles tagged by SNAP- or CLIP-tags. Single transfections with an either SNAP- or CLIP-tagged receptor were used as experimental controls, and double transfections by one SNAP- and one CLIP-tagged receptors with 1:1 and 1:5 ratios were performed in FRET assay.

After overnight transfection, SNAP-tagged receptors were labeled by SNAP-Surface®-549 dye (New England Biolabs, #S9112, Ipswich, Massachusetts, United States) at 37 °C for 30 min and CLIP-tagged receptors were labeled by CLIP-Surface™-647 dye (New England Biolabs, #S9234) at 37 °C for 1 h. Both dyes were diluted 1:2000 in complete medium and sequential labelings were performed.

In addition, SNAP-CD28 and SNAP-β1AR were used as positive and negative controls, respectively. Labeling for these two controls was performed using SNAP-Surface®-549 dye and SNAP-Surface®-Alexa-647 (New England Biolabs, #S9136) 1:2000 diluted in complete medium at 37 °C for 30 min.

To investigate the dimerization after agonist stimulation, following labeling, Mc4r expressing cells were treated with NDP-MSH at a final concentration of 100 nM for 1 min or 5 min.

Cells were fixed by 4 % PFA and mounted in Mowiol 4-88/DABCO. Fluorescence was monitored by Leica SP5 confocal microscope (Leica, Wetzlar, Germany, operated by LAS AF software), using 561 nm and 633 nm lasers. Emission of donor channel was recorded at 575-615 nm and emission of acceptor was recorded at 650-718 nm. Images were acquired at middle plane of the cells in 8-bit of 512x512 format at 400 Hz speed with line average of 8.

FRET measurements were performed by acceptor photobleaching approaches in manufacturer's wizard tools (FRET AB Wizard in LAS AF software). Bleach was done by 60 % laser in 2 frames.

FRET Efficiency (*E*) is calculated by **Equation 4.1**:

$$E = \frac{D_{post} - D_{pre}}{D_{post}}$$

D_{pre}, donor intensity pre-bleach; D_{post}, donor intensity post-bleach.

Three independent experiments were performed, and for each Mc4r allele combination, n=20-50 cells were measured. In saturation curve, the mean FRET

efficiency of controls was plotted against acceptor intensity before bleaching and fitted by GraphPad Prism 6.

Lastly, to verify FRET efficiency at various expression level and at different imaging planes, SNAP-CD28 and SNAP-β1AR were applied in live cell imaging. HEK293 cells were seeded 300 000 cells per well on coverslips in 6-well plate, grown overnight, transfected by Effectene transfection reagent (Qiagen, Hilden, Germany) overnight using 400 ng DNA per transfection, and labeled using 1:200 dyes mixture for 30 min at 37 °C. Fluorescence images were acquired on the middle plane and bottom membrane of the cells in 12-bit of 512x512 format at 400Hz speed with line average of 8 at a zoom factor of 5.5. FRET efficiency of cells with low/middle/high expression levels (brightness of acceptor before photobleaching) was compared.

2.5. Images processing

The images were processed with ImageJ 1.51 (Schindelin et al. 2012; Schneider et al. 2012). In FRET sensitized emission approach, the background was first subtracted from original images. Images were labeled using three-letter labels as in **Table S4.3** (Shyu et al. 2008). Afterwards, in single labeled donor or acceptor samples, cell membranes were selected and measured for mean intensity of donor/bleedthrough (Ddd/Dad) and acceptor/cross excitation (Aaa/Daa).

Correction ratio of donor C_D and of acceptor C_A were calculated from mean intensity ($\overline{F}$) of respective channels by **Equation 4.2** and **Equation 4.3**:

$$C_D = \frac{\overline{F}_{Dad}}{\overline{F}_{Ddd}}$$

$$C_A = \frac{\overline{F}_{Daa}}{\overline{F}_{Aaa}}$$

Corrected FRET images corrected from Daf ($FRET_{c(Daf)}$) were processed by image calculator from fluorescence images (F) of respective channels in ImageJ by **Equation 4.4**:

$$FRET_{c(Daf)} = F_{Daf} - \left(C_A \times F_{Aaf} + C_D \times F_{Ddf}\right)$$

It should be noted that the acquisition of Dad/Ddd images for C_D does not require

identical laser power as of FRET images, while the acquisition of Aaa/Daa images for C_A require identical laser power as of FRET images. All presented images were cropped and scaled to same size.

2.6. Simulation of expected FRET efficiency

To estimate the average FRET efficiency which we could expect from our homo- and heterodimers, we used the FRET Positioning and Screening tool (FPS) (Sindbert et al. 2011; Kalinin et al. 2012). This program models the accessible volume (AV) of the SNAP- and CLIP-tags linked to our either one of the MC4R variants or our dimeric control CD28. Here, the fluorophore is modeled as a hard sphere with radii rx, ry and rz, which is connected to the protein of interest via a flexible linker (cylindrical pipe). A spring-like model is then used to model all possible positions of the center of mass of each fluorophore, so that it may sample the conformational space that is restricted only by steric clashes with the surface of the biomolecule and the cell membrane. The result is that each dye forms a "cloud" of possible spatial coordinates.

We based our modeling on the structural information available from the human MC4R (PDB ID: 2IQP), the SNAP-tag (3KZY), the CD28 (1JYD) and the additional linking amino acids in between the SNAP-MC4R and SNAP-CD28. Please note that structure-wise SNAP- and CLIP-tag are identical (Gautier et al. 2008).

FPS needs as input parameter the size of the fluorophore (length, width, height), a structure or homology model of the receptor and the information how many flexible, linking residues were chosen to connect the SNAP-tag with the receptor.

Determining the size of the fluorophore is straight-forward by simply measuring its extension in all three dimensions. We obtained a height of 36 Å and width/length of 33 Å, resulting in dye radii of 18 Å and 16.5 Å, respectively.

A common problem in X-ray crystallography is that N- or C-termini of proteins stay unresolved due to their structural flexibility. However, for our case, this means we have to include them in our modeling of the connecting linker. For MC4R, the first 26 residues are missing, the last six of SNAP are missing and additional six linking residues are placed between SNAP and MC4R. This results in a total linker length of 136 Å (38 * 3.6 Å). The width of the linker was set to 4.5 Å.

Next, we confined the linker motion by homology modeling with the Phyre2 server (Kelley et al. 2015). As expected, the transmembrane region could be well defined by the human homolog. For the first 26 residues, the algorithm proposed that they form a kind of lid on top of the receptor and due to this only the first 15 residues are unstructured. We rerun our FPS simulation with this reduced linker length of 97 Å.

Finally, we performed FPS for our dimeric control CD28. Here, we had a total linker length of 26 residues (93 Å) based on a 20 residue long, unstructured signal peptide and additional six unresolved SNAP residues.

The average FRET-efficiency $\langle E \rangle$ was then calculated by **Equation 4.5**:

$$\langle E \rangle = \frac{1}{mn} \sum_{i=1}^{n} \sum_{j=1}^{m} \left(\frac{R_0^6}{R_0^6 + \left| \overrightarrow{R_D^{(i)}} - \overrightarrow{R_A^{(j)}} \right|^6} \right)$$

2.7. Colocalization analysis by single-molecule microscopy

The super-resolution microscopy was performed on direct stochastic optical reconstruction microscopy (dSTORM) as described in detail before (van de Linde et al. 2011) to analyze the colocalization of Mc4r alleles on the cell membrane.

Hela cells were seeded 50 000 cells per well in 8 chambered cover glass system (Lab-Tek® II in Nunc, Roskilde, Denmark; or Cellvis, Mountain View, California, United States) coated 1 h with 100 µg/ml poly-D-lysine. Cells were transfected by JetPRIME® reagent (Polyplus-transfection SA, Illkirch-Graffenstaden, France) with SNAP- and CLIP-tagged Mc4r expression vectors. After overnight transfection, cells were sequentially labeled with SNAP-Surface®-Alexa-647 (New England Biolabs, #S9136) at 37 °C 0.5 h and CLIP-Alexa-532 at 37 °C 1 h. The CLIP-Alexa-532 was synthesized from BC-NH$_2$ (New England Biolabs, #S9236) with Alexa Fluor 532 and purified by high-performance liquid chromatography (HPLC). Labeled cells were fixed with 2 % PFA+0.25 % glutaraldehyde 15 min at room temperature. Cells were observed by dSTORM super-resolution microscope to reveal Mc4r receptor single-molecules localizations.

2.8. dSTORM imaging, images reconstruction and data analysis

The one- and two-color dSTORM images were imaged on a homebuilt widefield setup with an inverted microscope (Olympus IX-71, Shinjuku, Tokyo, Japan), equipped with an oil immersion objective (Olympus APON 60xO TIRF, NA 1.49). The dyes were excited with semiconductor lasers of the wavelength 639 nm (Genesis MX639-1000, Coherent, Santa Clara, California, United States) and 514 nm (Genesis MX514-500, Coherent). The excitation light was spectrally filtered from the emitted fluorescence by a beam splitter (ZT405/514/635rpc, Chroma, Vermont, United States) and the emission filters BrightLine HC 679/41 (Semrock, Rochester, New York, United States) and BrightLine HC 582/75 (Semrock). Imaging was performed with an EMCCD camera (iXon Ultra 897, Andor, Belfast, United Kingdom) at a rate of 50 Hz for 20 000 frames at $\sim$ 5 kW/cm² in 100 mM β-mercaptoethylamine at pH 7.4 for Alexa Fluor 647 and at pH 6.8 for Alexa Fluor 532. The two-color images were imaged in 100 mM β-mercaptoethylamine at pH 7.0. The dSTORM images were reconstructed using the open source software rapidSTORM 3.3. and the reconstructed images were aligned using an elastic transformation of the ImageJ plugin bUnwarpJ (Arganda-Carreras et al. 2006) with at least 200 Tetraspecks as landmarks.

Aligned two color images were checked for colocalization by using the ImageJ plugin "Colocalization threshold".

3. Results

3.1. Interaction of different Mc4r alleles

Mc4r alleles have been shown to have different signaling strength, cAMP production of cells expressing A-B1 were lower than cells expressing A. (Lampert et al. 2010). Thus, it is hypothesized that the mutant allele B may inhibit the functionality of the A wild-type allele.

To understand the molecular mechanism of B acting on A, we speculated that alleles can physically interact, that is, fish Mc4r receptors dimerize as the mammalian. To prove the direct interaction of Mc4r in living cells, we performed the FRET assay mainly using acceptor photobleaching approach. As expected for membrane receptors, Mc4r A/B1/B2 receptors all expressed on the cell membrane (**Figure S4.1B**). In

FRET assay, dimerization can be visualized if SNAP-Surface®-549 dye labeled SNAP-tagged receptors (donor) are in close proximity to CLIP-Surface™-647 dye labeled CLIP-tagged receptors (acceptor) and can transfer the energy to the acceptor. The spectra of this FRET pair overlap sufficiently (**Figure S4.1C**), leading to an estimated Förster distance of 57 Å. By acceptor photobleaching approach, an increase of donor intensity upon photobleaching of acceptor can determine FRET efficiency. In sensitized emission approach, FRET images can be calculated by eliminating crosstalk from donor bleedthrough and acceptor cross excitation (**Figure S4.2A**, **S4.2B**, **S4.2C**).

FRET signals are readily detectable by either sensitized emission (**Figure S4.2C**) and acceptor photobleaching (**Figure 4.1**). Cells co-transfected with SNAP-A and CLIP-B1/B2 in both ratio 1:1 and 1:5 showed some FRET signals in corrected FRET images (**Figure S4.2C**).

In the acceptor photobleaching assays, CD28, a dimeric cell-surface receptor was used as a reference dimer (positive control) and β1-adrenergic receptor (β1AR) was used as a reference monomer (negative control) (Meral et al. 2018). CD28 showed an expression-dependent increased and saturated FRET efficiency (**Figure S4.3A**), with calculated maximum FRET efficiency = 34.5 % and a K_d = 37.4 relative fluorescent units (RFU). β1AR showed a low linear (unspecific) increased FRET efficiency (**Figure S4.3A**), with the highest obtained FRET efficiency of 5.2 % at an expression level of 41 RFUs (slope = 0.079 %/RFU).

In addition, to verify the approach of the measurement setup, we also compared FRET efficiency by low/middle/high expression level (brightness) in CD28 and β1AR, using middle plane of cells or bottom layer of membrane in live cell imaging. The FRET efficiency did not show differences among groups (**Figure S4.3B**).
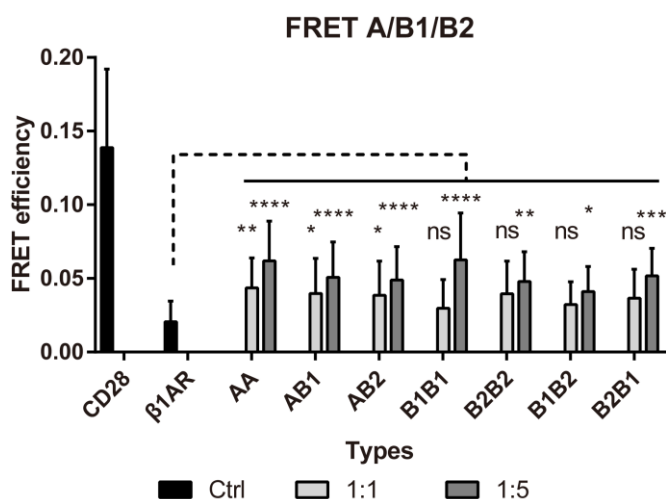
Figure 4.1 Interaction of Mc4r A/B1/B2 alleles.

Homodimerization and heterodimerization analysis by FRET analysis. Statistics were made by one-way *ANOVA* to compare all with β1AR, with correction made by Dunnett's multiple comparisons test. For statistics: ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

Expression of FRET pair SNAP-A/B1/B2 and CLIP-A/B1/B2 have FRET efficiency of 3-5 % and 4-6 % for ratio 1:1 and 1:5, respectively (**Figure 4.1**). FRET efficiency of A-B1, A-B2, A-A pair in ratio 1:1 and 1:5 are significantly higher than that of negative control β1AR. Pair B1-B1, B2-B2, B1-B2, B2-B1 in ratio 1:5, but not in 1:1, have significantly higher FRET than β1AR. This indicates that Mc4r A-B1, A-B2, B1-B2 alleles can form heterodimers, and A-A, B1-B1, B2-B2 can form homodimers. Among all, the A-A, A-B1, A-B2 dimers are more prominent. Nevertheless, the fraction of dimers could be low. Albeit existing, only partial dimers were formed, leading to a low FRET efficiency.

Among all the homodimer pairs, A-A makes certain amounts of homodimers, B1-B1 dimerizes when highly expressed, and B2-B2 makes few dimers when highly expressed. From the heterodimer pairs, A-B1 and A-B2 produce some heterodimers when highly expressed. B2-B1 pair have more B1 when highly expressed and are more likely to dimerize than B1-B2 pair, showing B1 may be more prone to dimerize than B2 (**Figure 4.1**). Thus, the inclination of the three receptor types to dimerize could be A>B1>B2.

To compare, accessible volume simulation of CD28 has an estimated FRET efficiency

of 28 %, while Mc4r dimers have around 24 %, considering 100 % labeling efficiency and 100 % dimer formations. This agrees with the experimental evidence that only a small part of Mc4r forms dimers.

We furthermore asked if agonist stimulation could alter the efficiency of Mc4r dimerization. Based on reporter gene luciferase assay, the signals induced by 100 nM of NDP-MSH is already at the plateau (**Chapter 3**), and therefore this concentration of NDP-MSH was used for stimulation. GPCRs activate at millisecond to second scales (Lohse et al. 2008), therefore agonist treatment time here was attempted to reduce to minimum duration, such as 1 min/5 min. A-A and A-B1 at ratio of 1:1 and 1:5 were stimulated for 1 min or 5 min, but no obvious change of FRET efficiency was measured (**Figure S4.3C**), indicating in this stimulation condition the dimerization of Mc4r does not increase or decrease.

In summary, the FRET data suggest that Mc4r A and B alleles form homo- and heterodimers, but only a fraction of available receptors dimerize.

3.2. Colocalization of Mc4r allelic variants

Confocal microscope has a diffraction limit of 200-300 nm. This prevents to visualize receptor localization on the membrane at a small scale. To prove if the Mc4r alleles co-localize in the cell membrane, we used super-resolution microscope dSTORM, which can achieve a resolution of 20-30 nm. To achieve this, we used the photo-switchable dyes, SNAP-Surface®-Alexa-647 and CLIP-Alexa-532. Both fluorophores are wildly used for dSTORM.

We performed two-color dSTORM imaging of Mc4r A and B1 with CLIP532 labeled A and SNAP647 labeled B1. Mc4r molecules were not distributed into large clusters but were scattered all over the membrane. The two-color images show that a small amount of Mc4r A and B1(**Figure 4.2A**, **4.2B**) colocalize at an average percentage of 5.27 % (**Table 4.1**).
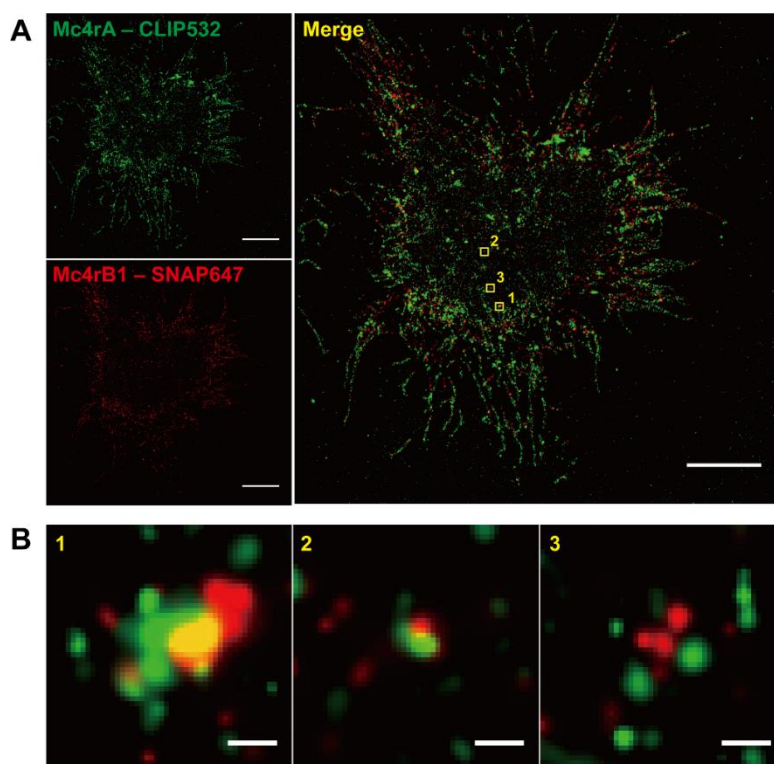
Figure 4.2 Colocalization analysis by dSTORM.

(A) dSTORM images of SNAP- labeled Mc4r B1 (red) and CLIP- labeled Mc4r A (green) on the bottom layer of cell membrane. Composite image shows two types of molecules are only partially colocalized. Scale bar: 5 µm. **(B)** Magnified images of the three yellow boxes in whole cell image displays colocalized domain (1), related domain (2) and independent domain (3). Scale bar: 100 nm.

Table 4.1 The dSTORM analysis: clustering density and percentage of colocalizing molecules.

Cells (N=4)	Total CLIP532	Density CLIP532	Total SNAP647	Density SNAP647	ROI (µm²)	Colocal. Spots	Colocal. (%)
HELA_1	680	13.19	748	14.52	51.55	33	4.89
HELA_2	171	3.06	417	7.48	55.88	8	5.16
HELA_4	610	8.45	1057	14.65	72.18	33	5.42
HELA_5	332	5.46	754	12.29	61.365	18	5.62

4. Discussion

4.1. Mc4r receptors work preferentially as monomers

Based on the expression and functional analysis, we proposed that the expression of variant alleles of Mc4r and their expression levels are the main cause for the effect of lower signaling and late puberty in large males. The hypothesis is that Mc4r dimerizes and the B1 dominant negatively acts on A, resulting in reduced puberty signaling, similar to human mutant MC4R (Biebermann et al. 2003).

To test for dimerization, several FRET approaches and co-localization imaging were used. FRET analysis of Mc4r dimerization demonstrated that A-A, A-B1, A-B2 are prominent dimers among all the pairs, and Mc4r A is most prone to dimerize, followed by B1 and then B2. However, the measured FRET efficiencies are lower compared to simulation analyses indicating only partial dimerization of Mc4r in *Xiphophorus*. The FRET efficiency measured with the fish Mc4r`s is one third or half of that in human Mc4r (Biebermann et al. 2003).

In the analysis, the FRET simulations are based on a few reasonable assumptions, which gives a rough guide about dimers formation, not quantitatively rather qualitatively. Ideally, quantitative analysis should require 0 % dimer (Mc4r with mutations in dimerization domains to prevent dimer formation) and 100 % dimer (two Mc4r covalently linked together as dimer: SNAP-Mc4r-linker-Mc4r-CLIP) controls. Here the analysis only gives an indication that SNAP/CLIP-tagged Mc4r constructs can form dimers in principle. The measured FRET efficiencies are lower compared to simulation analyses. This could be explained by that 100 % of receptor are calculated to form dimers in the simulation, but in realty we maybe do not achieve 100 % labeling efficiency and 100 % dimer formation. Furthermore, among all the measured Mc4r pairs, FRET efficiencies of 1:5 ratio of donor: acceptor pair are higher than that of 1:1 ratio pair. Higher amount of acceptor could increase the chance that donors are in close proximity to acceptor and initiate the energy transfer. Yet the 1:1 ratio may better reflect the physiological ratio in fish brain cells. Altogether, though the fraction of dimers cannot be quantified, it can be concluded that the FRET measurement shows partial dimerization of Mc4r.

In addition, evidence from super-resolution imaging showing mostly single-molecules localization on the cell membranes implicates that dimers are not prominent. Our data

suggest that the dominant negative effect of Mc4r B allele is not through the direct effect of the dimerization.

On the other hand, due to the differences of membrane conditions between mammalian and fish cells, the observed lack of dimerization might be a technical problem. Fish cells in culture need 28 °C while mammalian cells in culture requires 37 °C. This may cause differences of the membrane fluidity which can influence the protein dimerization. Further analysis with human MC4R is necessary.

4.2. Mc4r signaling models

Two models of Mc4r signaling can be proposed to explain the mechanism of puberty in *Xiphophorus* fish based on publication (Maderspacher 2010): a static threshold model and a dynamic threshold model (**Figure 4.3**).

In the first model, signaling of Mc4r in both small males and large males increases through time, but large males can only reach the threshold (fixed threshold) much later than small males, since signaling of large males increases slower. In the latter model, both small males and large males have a stable level of Mc4r signaling, which is higher in the large males than small males, but the threshold (dynamic threshold) of puberty permission may be also elevated in large males, causing delayed attainment of HPG axis activation.

Nevertheless, the dynamic threshold model may exclude the *mc4r* as determining gene for the puberty onset. General models for threshold effect base on a variable parameter and a constant parameter. The variable parameter has to be determined by the genotype. If threshold is variable, there should be a threshold gene. In the case of puberty regulation, the threshold gene should determine the variable puberty onset. This means that the threshold gene is a puberty gene and the constant *mc4r* is like a pre-puberty gene in this model. Only when threshold puberty gene overrides *mc4r*, the puberty happens. In this way, it is contradictory to that *mc4r* is the *P* locus gene, which determines the onset of puberty. Other genes have to be searched as future candidates.
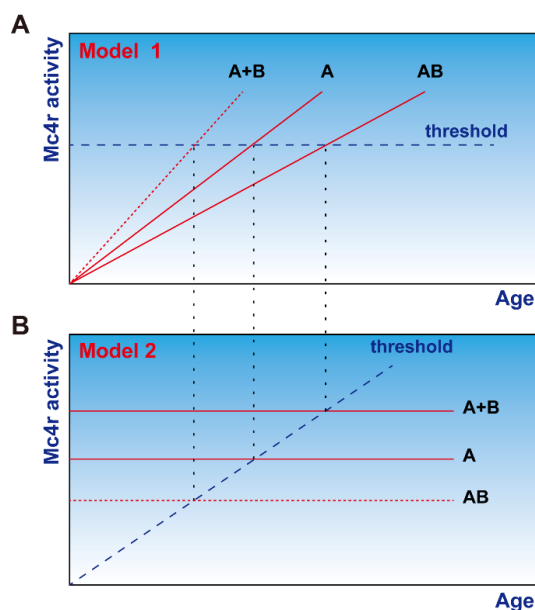
Figure 4.3 Two models for Mc4r function in male puberty onset determination.
(A) Static threshold model (model 1). Mc4r activity increases through time and the threshold that permits puberty onset is fixed. Mc4r activity is higher in A+B (additive of A and B) and lower in AB dimer (dominant negative of B on A) than A alone. The AB dimers scenario needs longer to reach the fixed threshold and could explain the delayed puberty in large males. The A+B scenario is unlikely in model 1 since it will pass the threshold early. **(B)** Dynamic threshold model (model 2). Mc4r activity is stable through time and the threshold permitting puberty onset is higher in the large males than small males. Mc4r activity is A+B>A>AB dimers. The A+B additive scenario has high Mc4r activity and passes the high threshold late, thus the puberty is delayed in large males. The AB dimers scenario is unlikely in model 2 since it reaches threshold early. In both figures, the red solid lines are the possible situations and red small-dotted lines are the unlikely situations. The blue dashed lines represent thresholds.

Besides direct interaction of A and B1 receptors, our data could be explained by other mechanism of dominant negative effects. For example, the B alleles may act as decoy receptors. Physiological concentration of ligands is in a proper range regulated by encoded genes. If B alleles compete with A alleles to bind the ligands, A alleles will have less ligands and less cAMP signaling, leading to a later puberty. Hence, the activity of the Mc4r A and B in large males may be reduced to a lower level compared to small males and females. More Mc4r B copy numbers cause even lower signaling leading to larger body size for the males. This explanation can be well fitted by fixed threshold model (model one), since the signaling of Mc4r is lower and rises slower in large male, thus reaching the fixed threshold later than small males. In large males,

although the expression of A and B1 are high, the B alleles may reduce largely the cAMP production, by mechanism such as ligands competition, which causes lower Mc4r activity, leading to increased food intake and growth, and late maturity.

If we consider the B alleles do merely additive effect on the signaling for A alleles rather than the dominant negative effect, our data can be in agreement with the dynamic threshold model (model two). Higher level of Mc4r signaling in large males causes later puberty than in small males; unlike in fixed threshold model (model one), the level of Mc4r signaling is necessary to be lower in the large males than small males. Lack of Mc4r A-B1 dimerization may exclude model one here, because dominant negative effect by the dimerization is important to cause a lower level of Mc4r signaling under a high expression of A and B1 alleles. Model two, however, can explain the monomers situation. In the large males, the Mc4r A and B1 are present equally at high levels and A-B1 dimers are only a very small portion from the whole A and B receptor population. B alleles show constitutive activity in the *in vitro* assay reflected by basal luciferase signals in Mc4r B allele expressing cells. Therefore, the Mc4r signaling of A and B together as monomers in large males additively could be higher than the Mc4r signaling of an equal amount of A alone in small males. It is possible that the measured constitutive activity of B alleles is due to overexpression of Mc4r in HEK293T cells. Even not considering signaling activity of B alleles, since large males have higher expression and more A and B1, the large males have more signaling from A than small males. The high level of signaling in large males may be sensed by an even higher threshold, the elevated dynamic threshold compared to small males. Consequently, the small males with low level of Mc4r signaling reach the low threshold early and mature early, while the large males with high level of Mc4r signaling reach the high threshold late and mature late. This is also a possible situation when considering the *X. hellerii* Mc4r signaling. For *X. nigrensis* and *X. multilineatus*, problem remained in this dynamic threshold model is that the function of B alleles cannot be explained, since no B alleles are found with premature stop, frameshifts or other non-functional alleles which are expected under conditions of neutral evolution, genetic drift and gene decay. It seems that the first model fits better for these two fish.

In summary, Mc4r B1 alleles are the main factor that causes differential signaling in large males, by itself and with Mc4r A alleles. Mc4r are preferentially present as monomers, and the overall Mc4r signaling in large males could be low due to other

dominant negative effect. Alternatively, the overall Mc4r signaling could be higher in large males than small males and sensed by the high dynamic threshold leading to later puberty.

5. Supplementary Information

5.1. Supplementary Figures
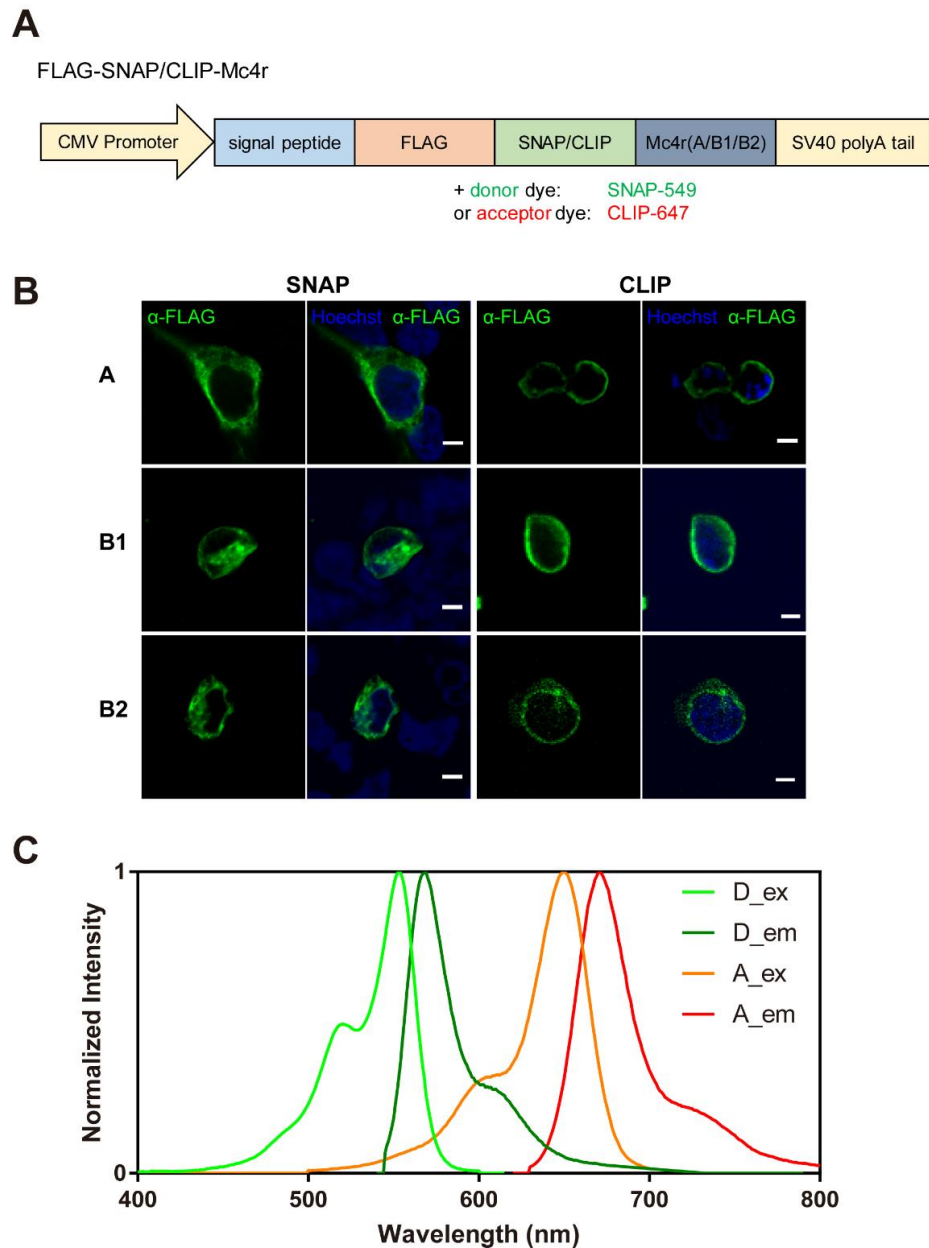
Figure S4.1 Expression constructs, membrane expression and spectral overlap of FRET pair.
(A) In expression construct, Mc4r A or B1 or B2 were tagged at N-terminus a FLAG tag and a SNAP/CLIP-tag. **(B)** Immunofluorescence on HEK293T cells transiently transfected with SNAP/CLIP-tagged Mc4r expression vectors showed membrane expression of the receptors. Scale bar: 5 μm. **(C)** Spectrum of FRET pair SNAP-Surface-549 and CLIP-Surface-647 dyes overlap sufficiently. SNAP-Surface-549 dye: excitation at 560 nm and emission at 575 nm; CLIP-Surface-647 dye: excitation at 660 nm and emission at 673 nm.

A1 Detector 575-615nm; Detector 650-718nm; Laser 561nm; Bleedthrough

D_ex; D_em

A2 Cross excitation; Detector 650-718nm; Laser 561nm; Laser 633nm

A_ex; A_em

B

Ddd; Dad; Aaa; Daa

Bleedthrough

$$C_D = \frac{\overline{F}_{Dad}}{\overline{F}_{Ddd}} \qquad C_A = \frac{\overline{F}_{Daa}}{\overline{F}_{Aaa}}$$

Cross excitation

C

$$FRET_{c(Daf)} = F_{Daf} - \left(C_A \times F_{Aaf} + C_D \times F_{Ddf} \right)$$

Ddf; Aaf; Daf; Daf corr. FRET

AB1_1:1; AB1_1:5; AB2_1:1; AB2_1:5
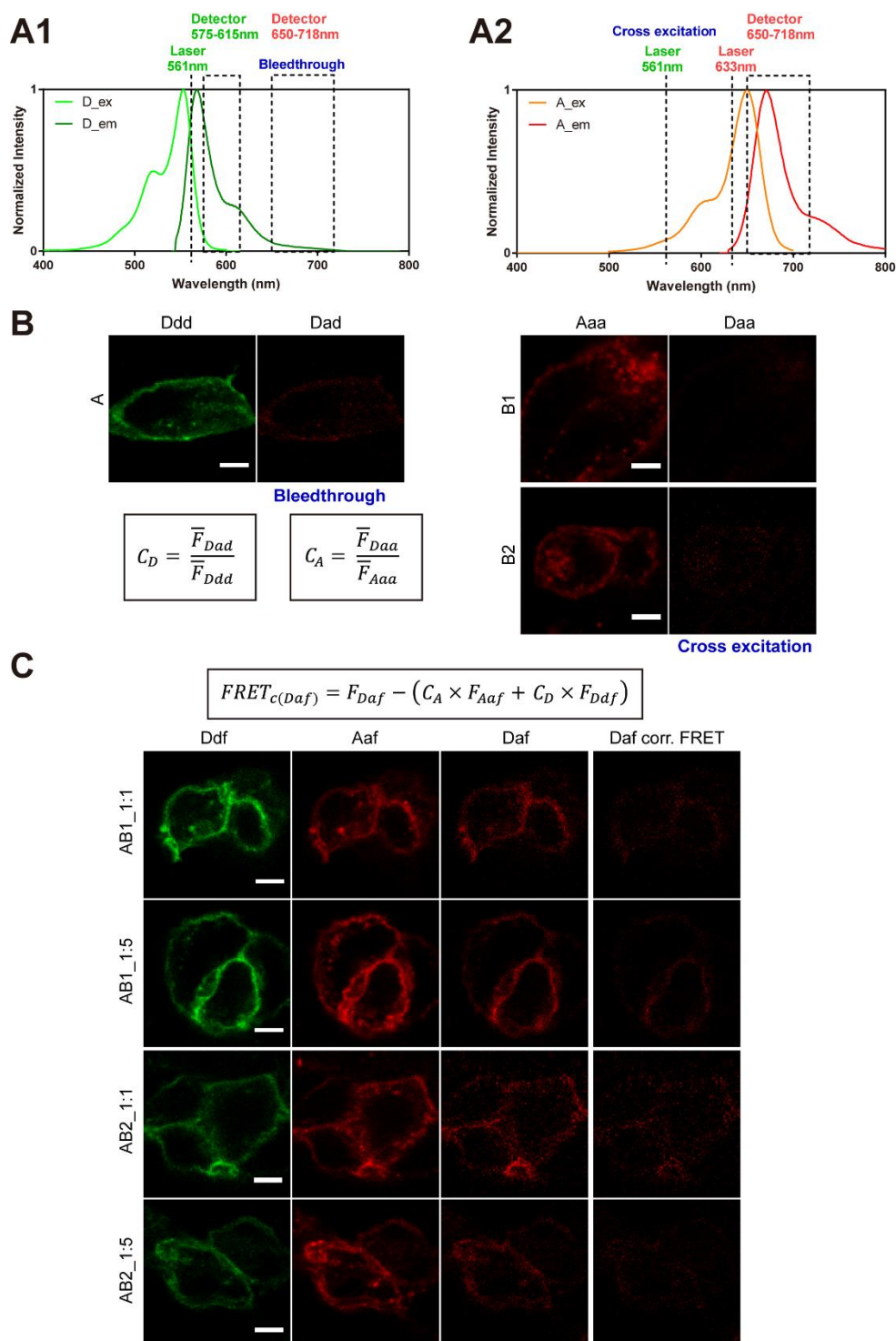
Figure S4.2 FRET analysis of Mc4r A/B1/B2 by sensitized emission approach.

(A) Spectra of SNAP-Surface-549 **(A1)** and CLIP-Surface-647 dyes **(A2)** explain possible crosstalk through channels. **(B)** Correction images by single labeled donor or acceptor samples. The crosstalk can be determined as correction ratios in order to correct FRET images. **(C)** Sensitized emission FRET approach reveals FRET signals after correction for crosstalk. Representative images by AB1 and AB2 show weak FRET emission after exciting donor. Scale bar: 5 µm.
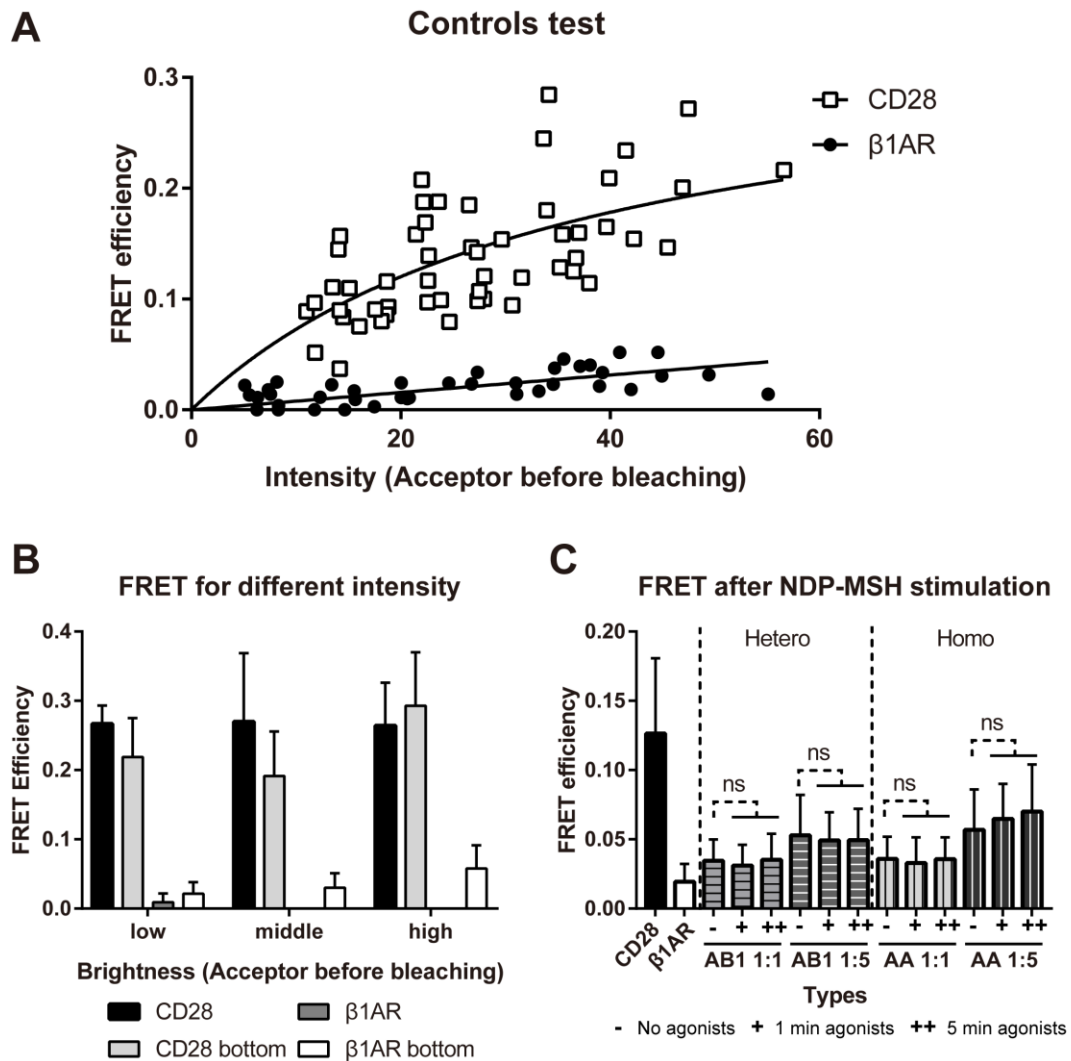
Figure S4.3 FRET analysis of Mc4r A/B1/B2 and controls.

(A) Mean FRET efficiency of controls plotted as a function of acceptor intensity (expression) before bleaching. **(B)** FRET measurement by live cell imaging using HEK293 cells expressing CD28 and β1AR (bottom membrane and middle plane) were compared regarding acceptor brightness before bleaching. Brightness of acceptor before bleaching were measured and sorted. Cells with brightness of the first, second, and last 1/3 fall into into low, middle and high categories, respectively. **(C)** Mc4r agonist NDP-MSH stimulation has no impact on FRET efficiency by acceptor photobleaching approach. Both heterodimer AB1 and homodimer AA were analyzed. Statistics were made by comparing NDP-MSH treated samples to their untreated cell counterpart, using Student's *t*-test. For statistics: ns: not significant.

5.2. Supplementary Tables

Table S4.1 Primers used in this study.

Primers.

Primers	Sequences	Comments
CMV_BamHI_F	CACCGGATCCTAGTTATTAAT AGTAATCAAT	GoldenGATE cloning for CMV
CMV_KpnI_R	TATAGGTACCGATCTGACGGT TCACTAAACC	GoldenGATE cloning for CMV
SFS_BamHI_kATG_ F	CACCGGATCCGCCGCCATGAA GACGATCATCGCCCTGAG	GoldenGATE cloning for signal peptide-FLAG-SNAP/CLIP
SFS_KpnI_R	TATAGGTACCGCCCAGCCCAG GCTTGCCCA	GoldenGATE cloning for signal peptide-FLAG-SNAP
SFC_KpnI_R	TATAGGTACCTCCCAAGCCTG GCTTCCCCA	GoldenGATE cloning for signal peptide-FLAG-CLIP
Mc4r_B2_Xmulti_Ba mHI_-ATG_F	CACCGGATCCAACTCCACGGC TCAGCAAGG	GoldenGATE cloning for Mc4r A/B1/B2
Mc4r_6_A_Xnig_Sto p_R	TCACAGAAAGCTAATACACGA GA	GoldenGATE cloning for Mc4r A
Mc4r_28_B1_Xnig_S top_R	TCACAGAAAGCTAATACACCA GA	GoldenGATE cloning for Mc4r B1
Mc4r_B2_Xmulti_Sto p_R	TTACAGATGAACTACCTGGAG CG	GoldenGATE cloning for Mc4r B2

Table S4.2 Primer pairs used in GoldenGATE molecular cloning.

Gene	length	Forward	Reverse
CMV	589bp	CMV_BamHI_F	CMV_KpnI_R
SFSK	621bp	SFS_BamHI_kATG_F	SFS_KpnI_R
SFCK	621bp	SFS_BamHI_kATG_F	SFC_KpnI_R
Mc4r_A_Xmulti	990bp	Mc4r_B2_Xmulti_BamHI_-ATG_F	Mc4r_6_A_Xnig_Stop_R
Mc4r_B1_Xmulti	984bp	Mc4r_B2_Xmulti_BamHI_-ATG_F	Mc4r_28_B1_Xnig_Stop_R
Mc4r_B2_Xmulti	1005bp	Mc4r_B2_Xmulti_BamHI_-ATG_F	Mc4r_B2_Xmulti_Stop_R

Table S4.3 Three-letter labeling of FRET images.

chromophores	Donor Ex/Em	Acceptor Ex/Em	FRET Ex/Em
Donor only	Ddd	--	Dad
Acceptor only	--	Aaa	Daa
Donor and acceptor	Ddf	Aaf	Daf

First letter: Excitation filter. Second letter: Emission filter.

Third letter: Chromophores. (f: FRET pair.)

Chapter 5

Integrative Discussion and Conclusion

1. Integrative discussion

Sexual maturation, or puberty, is the key process that enables animals to reproduce and to generate progeny. Commonly in vertebrates, the hypothalamus-pituitary-gonad (HPG) axis is crucial in initiating this process. In humans, MC4R is correlated with childhood obesity (Farooqi et al. 2003). In *Xiphophorus* fish, the onset of puberty is associated with the *P* locus, on which *mc4r* is the determining gene in the species *X. multilineatus* and *X. nigrensis* (Lampert et al. 2010). In several fish species, such as in goldfish (Cerda-Reverter et al. 2003) and rainbow trout (Schjolden et al. 2009), Mc4r is involved in energy homeostasis. However, it remains unclear through which mechanism Mc4r regulates puberty in *Xiphophorus*. Furthermore, it has not been studied so far how conserved a role of Mc4r in puberty onset regulation is in other species.

1.1. Molecular hypothesis for the mechanism of puberty regulation in *Xiphophorus* by Mc4r signaling

Previous study on Mc4r in *X. nigrensis* and *X. multilineatus* fish showed that in these fish Mc4r has three alleles classes: functional allele A, and non-signal-transducing alleles B1 and B2; moreover, cell assay showed that co-expression of B alleles with A alleles reduced the function of A alleles (Lampert et al. 2010). The large males possess more B alleles and high B allele copy numbers, which correlate to late puberty. Thus, the B alleles were hypothesized to act on A alleles by dominant negative effect.

Several lines of evidences were investigated for such a hypothesis (**Chapter 3**). Firstly, the Mc4r expression is mainly in the brain preoptic region and hypothalamus region, and the large males highly express Mc4r in both regions compared to small males and females. Such expression regions are in agreement with the expression domains in zebrafish (Josep Agulleiro et al. 2013), indicating a role of Mc4r in the central control of energy homeostasis and reproduction. The high Mc4r expression in large males suggests that the differential Mc4r expression could be related to the early/late puberty onset regulation. Secondly, the high expression of Mc4r in large male is mainly due to high levels of A and B1 alleles, providing further hints on B alleles as crucial factors for lower signaling in large males. Thirdly, the phenomenon of B alleles reducing the function of A alleles has been reported (Lampert et al. 2010).

Here, further gene reporter assay showed that the cells expressing Mc4r A-B1 has same constitutive activity like cells expressing Mc4r A, but after NDP-MSH stimulation the Mc4r A-B1 cells has lower cAMP production than Mc4r A cells. Compared to Mc4r A alleles, the amino acid sequence of Mc4r B alleles is known to have two cysteine residues deletion on the carboxyl-terminus (Lampert et al. 2010), which could drastically affect the function of the receptors. Therefore, B alleles are defective, and could even act on A alleles to reduce its function.

Another hypothesis which is not necessarily excluding the previous one is that Mrap2 accessory protein functions on Mc4r to regulate the downstream signaling (**Chapter 3**). First, the Mrap2 and Mc4r co-expressed in the brain preoptic region and hypothalamus region, supporting that Mrap2 functions on Mc4r. The level of Mrap2 expression in large male, small male and female were similar, suggesting Mrap2 may act on Mc4r in different fish similarly. Second, gene reporter assay showed that Mrap2 could elevate the dose response curve upwards. Both constitutive activity and agonist induced cAMP production were upregulated, suggesting Mrap2 may stabilize active conformation of Mc4r. That both Mc4r A cells and Mc4r A-B1 cells respond the same to Mrap2 indicates Mrap2 not as a factor to activate or repress preferentially one form of Mc4r. Combining the evidence together, Mrap2 can regulate the signaling, but cannot explain the signaling differences between large and small males.

Based on the above analysis, the explanation for late puberty that Mc4r B alleles act dominant negatively on A alleles to reduce the signaling in the large males still holds true. From this explanation, next question arises: do the Mc4r A alleles and B alleles physically interact to exert such an effect?

As Mc4r is a membrane receptor and may undergo dynamic association/dissociation, a better way to detect protein-protein interaction for Mc4r in intact cells is through engineered receptor molecules by fluorescence resonance energy transfer (**Chapter 4**). The dimerization of Mc4r A-A, A-B1, A-B2 seems to be more prominent among all pairs, though the overall FRET efficiency is lower than that in previous human MC4R study (Biebermann et al. 2003). This indicates that the Mc4r alleles undergo only partial dimerization in the cells. Other line of evidence was explored by super-resolution imaging which can show single-molecule localization on the cell membrane (**Chapter 4**). In dSTORM, however, negligible colocalization of Mc4r A and B1 could be detected on transfected human cells. This could mean that the

dominant negative effect may be not through the direct effect of the dimerization. On the other hand, lack of dimerization and colocalization could be a technical problem due to the expression of fish receptors in a mammalian cell line. Since fish cell requires lower temperature (about 10 °C lower) in culture, the membrane might be more rigid or less compared to mammalian cells, which can have an effect on biological processes.

From these results, two hypothetical models were proposed to provide possible explanations of the puberty regulation in the absence of clear dimerization (**Chapter 4**). One model is that large males have lower signaling due to other dominant negative effects of B1, for instance that the B alleles remain ligand binding without signaling properties, reducing availability of ligands for A alleles, which then in turn can only reach the fixed threshold late to enter puberty. The other model is that large males have a higher Mc4r signaling due to overall high expression of Mc4r but need to reach a higher dynamic threshold to enter puberty. These models warrant further investigations.

1.2. Micro-evolutionary analysis of puberty regulation in *Xiphophorus* by Mc4r signaling

The above-mentioned possible mechanism in puberty regulation in *Xiphophorus* is mainly based on the case of *X. nigrensis* and *X. multilineatus*. It was unknown what the situation is in other *Xiphophorus* species, whether Mc4r is also the main factor in puberty regulation and what the mechanism is.

X. hellerii, belonging to Southern swordtails, have similarly large and small males in wild populations. Thus *X. hellerii* was studied here to disclose a role of Mc4r and puberty regulation in a distantly related species (**Chapter 3**). First of all, the analysis of *X. hellerii* revealed that only A alleles for Mc4r exist despite of the coexistence of large and small males. Moreover, total *mc4r* expression of *X. hellerii* is like that in *X. nigrensis* and *X. multilineatus*, showing higher expression in preoptic region and hypothalamus of large males than of small males and females. The expression level of Mrap2 is identical among *X. hellerii* large males, small males and females. Such observation indicates that the growth and puberty determinant in large and small male of *X. hellerii* is not due to allelic and copy number variation of *mc4*r, but has a strong

environmental component and/or is due to other genetic factors, for instance, promoter variations in the *mc4r* gene or even other relevant genes.

In *X. maculatus*, *mc4r* has 20 identified copies which vary in sequences, both in the coding regions and the non-coding regions (Volff et al. 2013). The correlation of *mc4r* allele types with large and small males has not been proposed in *X. maculatus* owing to its complexity. It could be inferred that the mechanism of puberty onset regulation is not conserved even within *Xiphophorus* genus. This could indicate that Mc4r of *Xiphophorus* fish have convergently evolved with the same function in puberty regulation but employ different molecular mechanisms.

It is conserved between *X. nigrensis* and *X. hellerii* that *mc4r* expression is high in large males. Concerning that only A-type alleles (wild-type alleles) exist in *X. hellerii*, the Mc4r signaling might be high in large males because of high expression. The second model from the previous section may explain these common cases of *X. nigrensis* and *X. hellerii*, in which large males have a higher dynamic threshold, causing a later puberty. This is however not in agreement with *mc4r* being the *P* locus gene. The formal genetics leaves only the *P* locus on the sex chromosomes as the responsible locus for the differences in puberty timing. Therefore, it is necessary to search for other genes as future candidates. Furthermore, more alleles types and copy number variations arise in other *Xiphophorus* such as *X. nigrensis*, *X. multilineatus* and *X. maculatus*. This may change the signaling outcome and the significance of the changes may be to better genetically control and balance the phenotypes and behaviors in the fish population.

Of note, the Mc4r pathway gene analysis exhibited that *kiss1* is missing in Southern platyfish (*X. maculatus*) and Amazon molly (*Poecilia formosa*) (**Appendix**). Moreover, in *Xiphophorus couchianus* and *Xiphophorus hellerii*, the *kiss1* gene is also missing. Kisspepetin has a role in reproduction regulation. In mammals, kisspeptin is known as the master puberty regulator in central control of reproduction (Uenoyama et al. 2018). The *kiss1* is reported to be missing from several fish, including fugu (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*) and stickleback (*Gasterosteus aculeatus*) (Felip et al. 2009; Tena-Sempere et al. 2012). In zebrafish and medaka, *kiss1* and *kiss2* knockout animals have reproductive capacity, indicating them as non-reproduction regulators (Tang et al. 2015; Nakajo et al. 2018). Particularly the place of action for *kiss1* is assigned to the habenula in zebrafish and is related to other

physiological processes such as learning and fear response, mediated by intrinsic neuromodulation (Lupton et al. 2017; Ogawa and Parhar 2018). Thus, genes other than *kiss1* may have taken over the function in reproduction. The absence of *kiss1* gene in *X. maculatus* and others showed more flexibility across species and it might be related to the establishment of reproduction axis via Mc4r.

1.3. Macro-evolutionary analysis of puberty and growth regulation in medaka by Mc4r signaling

Mc4r is known to play a key role in energy homeostasis and the role in puberty regulation could be specific to *Xiphophorus*. Because genetic manipulation is easy to perform in medaka (*Oryzias latipes*) like in zebrafish, this model organism was used to better understand the role of Mc4r signaling system in reproduction and growth in a totally different lineage of teleost fish (**Chapter 2**). First on the genic level the macro-evolution of the Mc4r signaling system was analyzed among *Xiphophorus*, medaka and zebrafish.

Mc4r signaling system genes (*mc4r*, *mrap2*, *pomca*, *pomcb*, *agrp1*) in medaka are generally conserved with other vertebrates. In Southern platyfish, the *mc4r* has more copies compared to medaka (Volff et al. 2013), and in zebrafish, the *mrap2* has two copies: *mrap2a* and *mrap2b* (Agulleiro et al. 2010). In general, there is a good conservation of the gene repertoire of the Mc4r system.

On the expression level, in medaka, the Mc4r signaling system genes are all in place at the stage of first feeding during development and particularly *agrp1* is highly upregulated at hatching stages when first feeding happens. All these genes are mainly expressed in the brain of adult fish. This indicates a role of central control of the Mc4r signaling system on energy homeostasis starting early from the first feeding event. Expression of *mc4r* and *mrap2* in the brain is also in preoptic region and hypothalamus, and overlapping in both regions, similar to the pattern in *Xiphophorus*, despite the expression level being the same between females and males. In zebrafish, *pomc* and *agrp* are expressed 24 hours post fertilization (slightly earlier compared to medaka expression stages), and *agrp* is upregulated upon fasting (Song et al. 2003). The expression of *mc4r* and *mrap2a* overlap in the preoptic region and dorsal hypothalamus, while *mc4r* and *mrap2b* overlap in the tuberal hypothalamus (Josep

Agulleiro et al. 2013).

The function of Mc4r signaling in medaka is not related to puberty timing, shown by similar time reaching puberty between WT and Mc4r KO strains. The overall growth is slightly shorter in KO strains, and the embryonic development and larval growth is affected by KO of Mc4r (Liu et al. 2019). This is different from *Xiphophorus*, in which Mc4r alleles influence the growth and puberty, the non-functional mutant Mc4r leading to increased body size (Lampert et al. 2010). In zebrafish, no changes in growth were observed during embryonic and larval stages in Mc4r KO fish and an increased body length was detected at 42 dpf of Mc4r KO zebrafish (Zhang et al. 2012). Such discrepancy of the roles of Mc4r in body length regulation among these fish hints that they have a common function although by distinct regulation processes. For example, in zebrafish, the two copies of Mrap2 regulate the larval growth (Mrap2a) and adult growth (Mrap2b) through blocking Mc4r and enhancing function of Mc4r, respectively (Sebag et al. 2013).

These together agree with a conserved function of Mc4r signaling pathway in energy homeostasis and could indicate sub-/neo-functionalization of *mc4r* and *mrap2* in *Xiphophorus* and zebrafish, respectively, during evolution.

In addition, synteny analysis showed that *ghrl* is missing in medaka (**Appendix**). Ghrelin is known as hunger hormone acting with leptin to regulate energy balance (Nakazato et al. 2001). In zebrafish, *ghrl* is present and regulates feeding and metabolism (Eom et al. 2013). A report shows that in an agastric fish, ballan wrasse (*Labrus bergylta*), ghrelin is also absent, correlating with the lack of stomach in this fish (Lie et al. 2018). It implies that medaka, which also has no defined anatomical structure that correlates to a stomach, has no *ghrl* in the same sense, and may have acquired other genes in the evolution of species to play a ghrelin-like role.

2. Future directions

Further studies are necessary to disclose the molecular mechanism of puberty regulation in *X. nigrensis* and *X. multilineatus*. Interaction of Mc4r alleles from *X. multilineatus* have been studied in HEK293T cells by expressing only the expression vectors for A and/or B alleles. Further analysis could co-express Mrap2 as a regulatory factor, to understand if Mrap2 changes interaction processes. In some

cases, ligands (agonists or antagonists) may alter the interaction of GPCRs. Agonist Pomc (using NDP-MSH) can be applied to Mc4r while observing the interaction processes. The same applies for the antagonist Agrp. They may stabilize or disrupt the dimers. Moreover, due to the differences between human cell line and fish cell line, the interaction of Mc4r alleles can be investigated in fish cells, using human MC4R as positive controls.

The Mc4r in other *Xiphophorus* could be studied to unveil the conservation of such a regulatory mechanism. Mc4r signaling system in *X. hellerii* fish remains mysterious. In this fish, other components of Mc4r signaling could be investigated to find out the molecular basis of its puberty onset regulation. Other species more closely related to *X. nigrensis* and *X. multilineatus* can be characterized to discover new allelic variants. Mutations in *mc4r* genes in the potential ligand binding or G protein coupling domains could release different signaling strength and thereafter can be used to find another mechanism of puberty onset regulation. All allelic variants can be considered in a phylogenetic context to infer the Mc4r evolution in *Xiphophorus* fish.

To better characterize the Mc4r protein itself, receptor mutagenesis or natural occurring receptor isoforms may be used to find out the important residues in the peptide chains; to name a few, ligand binding domains, dimerization domains, and G-protein coupling domains. This can be used to identify fish Mc4r structure compared to known human MC4R structure information. Based on conserved amino acids sequence and important mutations or indels in Mc4 that lead to altered function, molecular mechanism of the Mc4r signaling can be better deduced. All the investigation could be an additive to the study of human MC4R and MC4R-related metabolic disorders.

3. Conclusion

Puberty has been long studied on the hormonal level in humans; yet, it requires more attention on molecular/biochemical level and as well in other species. The study of the mechanisms of puberty onset regulation in this thesis shows in *X. nigrensis* and *X. multilineatus*, a differential Mc4r signaling between large and small males, which determines late or early puberty. This is due mainly to the effect of defective B alleles, possibly by a dominant negative action on A alleles, although it may not be through

direct physical interaction of receptors. The investigation in *X. hellerii* revealed that the different size groups possess only the Mc4r A-type alleles but with different expression levels, which could explain puberty induced by high Mc4r signaling. This situation is in contrast to situation in *X. nigrensis* and *X. multilineatus*. Comparison with medaka indicates conservation of the Mc4r signaling genes as well as their expression pattern. However, the function is more related to growth rather than to puberty, which is like in zebrafish and many other vertebrates. Absence of some other relevant signaling genes in some fish species also indicates that Mc4r signaling and beyond has certain degree of flexibility. In summary, the overall analyses give an example (in *X. nigrensis* and *X. multilineatus*) of the possible mechanism how Mc4r is regulating puberty onset timing, extend to other *Xiphophorus* (*X. hellerii*) and expand into other fish species (medaka) to decipher the Mc4r signaling system on the level of evolution. The various roles of the different components of the Mc4r signaling system are not fully conserved across species, and especially their mechanisms might be even more diverse, both within the genus *Xiphophorus* and with other more distant species.

Appendices

Appendix 1 Methodologies

1. General strategies and concepts

1.1. Evolutionary analysis (phylogeny, synteny)

Evolutionary studies are profoundly important in biology to understand the origins and evolution of functions. This reveals the significance of mechanisms which animals have adapted through time.

Phylogenetic analysis is one of the main approaches in evolutionary studies. To put it simply, the principle is to infer the evolutionary history by analyzing the similarities and differences among biological entities. The processes of molecular (genes and proteins) phylogenetic analysis involve identification of a dataset, multiple sequence alignment, phylogenetic reconstruction, and graphical illustration of the inferred tree (Baldauf 2003). DNA and/or amino acid sequences can be used for phylogenetic analysis. DNA sequences are used often in closely related sequences. Amino acids sequences are applied in a distant relationship. Several methods have been developed to infer phylogeny: distance-matrix methods, such as neighbor-joining (NJ) (Saitou and Nei 1987), and discrete data methods, for examples, maximum likelihood (ML) (Felsenstein 1981) and Bayesian inference (Huelsenbeck and Ronquist 2001).

Some features of phylogenetic tree are important. First is the branch length. Structure of a phylogenetic tree includes branches and nodes. An internal node represents the last common ancestor of everything derived from it, while an external node is the sequence in the dataset. The width of nodes has no meaning, but the length of the branches resembles the amount of evolutional differences. Second is the root of the tree. A tree can be unrooted or rooted, where rooted tree uses an outgroup (not ingroups) as a reference to root. Lastly, bootstrap values are required. The test to evaluate how good a tree is the bootstrapping. Bootstrapping takes subsamples of the dataset to build trees and calculates how much of the trees from the subsamples can reproduce the parts of the original tree (Baldauf 2003).

In addition to phylogenetic analysis on the molecular level, synteny analysis enables the comparison of genome structures between different species to infer the orthology

relationships. The basis of synteny analysis is that organisms of recent divergence share conserved gene blocks and common gene orders. Different from alignment methods, which give information on sequence mutations, synteny can show some processes of evolution, for example, gene family expansion or loss, and chromosomal rearrangements (Liu et al. 2018). Genomicus, a genome browser for comparative genomics is frequently used to analyze the synteny (Louis et al. 2013; Louis et al. 2015; Nguyen et al. 2018).

Phylogeny was analyzed for components of the Mc4r signaling system and synteny was analyzed for other reproduction related genes to investigate the Mc4r evolution in this thesis.

1.2. Expression analysis (RT-qPCR, ISH)

Temporal and spatial expression of messenger RNA (mRNA) has been widely used to analyze gene expression and gene regulation. Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) is a powerful high-throughput technique for the quantification of gene expression in a more specific and sensitive way. RT-qPCR uses fluorescent dyes or fluorescently tagged oligonucleotide probes to detect low abundant mRNA from samples such as tissues. Two detection approaches include dye-based detection and probe-based detection. SYBR Green dye (a dye-based detection) binds and intercalates within double-stranded DNA and starts to fluoresce brightly upon binding. TaqMan probe (a probe-based detection), on the other hand, is a designed fluorescent reporter oligonucleotide probes that specifically target the DNA sequences.

During RT-qPCR, fluorescence intensity correlates to the amount of DNA formed in the reaction in a window when fluorescence increases exponentially (before the threshold level: background fluorescence, plateau phase: fluorescence saturates the detector). The threshold cycle or Ct value is the point when fluorescence signal is first detected above the background. A common quantification approach is comparative Ct method, or known as the ΔΔCt method. Ct values of test and calibrator samples are normalized to a reference gene (housekeep gene) to compensate for difference in samples amount. Such ΔCt values of the test samples are compared with ΔCt in calibrator sample (usually baseline sample) to determine the relative expression of

target gene. The relative amount (fold difference) of a target gene in test vs. calibrator sample normalized to reference gene is presented as expression ratio $2^{-\Delta\Delta Ct}$.

Gene expression analysis by RNA *in situ* hybridization (ISH) is widely used to localize a specific mRNA sequence by labeled complementary RNA probe in location (*in situ*). Patterns of differential gene expression in tissues can be observed. The probes are synthesized complementary RNA (riboprobe) labeled by digoxygenin (Dig) or fluorescein (Fluo). The signal detection uses alkaline phosphatase (AP)- and horseradish peroxidase (PO)-based immunoassays. AP chromogenic substrates, including nitro blue tetrazolium / 5-bromo-4chloro-3-indolyl phosphate (NBT/BCIP) and Fast Red, are commonly used and they can sequentially visualize two probes. In such an approach, ideally the Fluo labels the stronger probe and Dig labels the weaker probe (Schumacher et al. 2014). Moreover, multiplexing has also been developed to simultaneously detect multiple RNA by chromogenic and fluorescent ISH (Hauptmann et al. 2016).

1.3. Receptor functional analysis (Luciferase assay)

The purpose of the receptor functional assay is to study the ability of receptor to produce second messenger upon ligand stimulation. Mc4r function is measured by the amount of cAMP generated. The assay used is a cAMP response element (CRE)/luciferase reporter gene assay.

Luciferase reporter assay has been developed mainly to two forms: single reporter assay and dual reporter assay. In this thesis, dual-luciferase reporter assay is applied. Dual reporter assay can express simultaneously two reporter enzymes and measure two luciferases sequential in a single sample, considering that the two luciferases have different substrate requirements and distinct output light wavelength. Firefly luciferase from beetles (*Photinus pyralis*) produces a greenish yellow light in the range of 550-570 nm, while *Renilla* luciferase is from sea pansy (*Renilla reniformis*) and the light emitted is a blue light of 480nm. Firefly luciferase uses luciferin as substrate and requires the presence of oxygen, ATP and magnesium in the bioluminescence. *Renilla* luciferase uses substrate coelenterazine and requires only oxygen.

In this assay pSGIICRE4luc plasmid is used as "Experimental" Reporter, containing

CRE elements and encoding firefly luciferase. pGL4.74[hRluc/TK] plasmid is the "Control" Reporter which expresses *Renilla* luciferase. pCMV-Flag-NLS-Cα is a plasmid encoding catalytic subunit of protein kinase A (PKA) with a nuclear localization signal, which is a constitutive PKA and active even in the absence of cAMP (Thiel et al. 2005).

The mechanism of such CRE/luciferase reporter assay is that G_s activation can generate cAMP which activates PKA to phosphorylate CRE binding protein (CREB), and then CREB can transactive reporter luciferase genes containing CRE motifs. As a result, cAMP production can be measured by the luciferase readout. This assay can also be extended to generate dose-response curve and determine the half maximal effective concentration (EC50) value of the ligand on the receptor.

1.4. Fish physiology

Knockout (KO) animal has been widely applied in the study of gene functions. To characterize KO animals, it is important to determine their physiological characters relevant to the respective gene by comparing KO and wild-type (WT) animals. Different scenarios could be suggested. Some KO may be similar to WT, and then the gene could be not important for the function. Other KO may have decreased function observed, then the gene could determine this function. Still other KO may have increased functionality, then the gene could act on negative feedback of this function.

Relevant physiology of Mc4r in fish is the growth and possibly the puberty onset. Growth of fish is normally determined by standard linear length, the distance from forehead to trunk terminus. Puberty onset of medaka fish has noticeable characteristics although juveniles are indistinguishable between females and males. Upon puberty, females lay first eggs and males have grown papillary processes in the anal fins.

Other metabolism parameters, which has been used in mice, such as food intake and energy expenditure, are not relatively feasible to measure in the fish.

1.5. Golden Gate cloning

Gateway cloning system was developed and commercialized by Invitrogen since the

late 1990s. This invention leads to a paradigm shift of molecular cloning, from the classical multiple cloning sites (MCS) dependent cloning approach. It provides a highly efficient way to clone DNA into vectors for protein expression and functional analysis. It uses a set of recombination sequences, *att* sites, and two bacteriophage λ derived site-specific recombination enzymes (Landy 1989), LR Clonase, and BP Clonase. The first step in Gateway cloning is BP reaction: the reaction of *attB* containing PCR products with *attP* containing Gateway donor vector by BP Clonase, and the product is *attL* containing Gateway entry clone. Then LR reaction: *attL* containing Gateway entry vector recombines with *attR* containing Gateway destination vector, and the gene cassette is cloned into Gateway destination vector, generating *attB* containing expression clone (Hartley et al. 2000).

Another new cloning system is called Golden Gate cloning system, invented in 2008 (Engler et al. 2008) and designed to generate fusion and recombination constructs. The general principle of Golden Gate cloning is to clone DNA fragments efficiently in one tube in one step utilizing type IIs restriction endonucleases (Engler et al. 2008; Engler et al. 2009; Kirchmaier et al. 2013). Type IIs restriction endonucleases are distinct from normally applied restriction enzymes. Their recognition sites are distal to their cleavage sites. Such enzyme property results that the overhangs from cleavage sites can be freely designed and that the recognition sites have been removed from the assembly. The advantages of the approaches have several facets. First, overhang sequences are not restricted by the enzymes and no additional scar sequences are introduced. Second, free designed overhangs permit orderly assembling multiple fragments simultaneously. Lastly, due to the elimination of recognition sites from the assembly, simultaneous digestion and ligation are possible in one reaction.

The type IIs restriction endonuclease for Golden Gate reaction is BsaI. BsaI recognition site GGTCTC is not palindromic but directional. Outside the recognition site and separated by a single bp spacer is the four bp cleavage site / overhang. In the Golden Gate cloning, the first step is to generate a series of Golden Gate entry vectors using BamHI/KpnI or XcmI cloning, and afterward in a one-step one-tube Golden Gate reaction the fragments from Golden Gate entry vectors are orderly assembled into Golden Gate destination vector using BsaI and T4 ligase. In the Golden Gate cloning kit, up to eight fragments can be assembled, making use of eight positions of Golden Gate entry vectors (Kirchmaier et al. 2013). New constructs with similar gene

structures can be designed to reuse existing Golden Gate entry vectors and to exchange only a few modules. This gives high versatility of the assembly system.

Moreover, a combinatorial approach that combines Golden Gate cloning with Multisite Gateway™ cloning has also been developed, which allow possible 24 (8x3) fragments to be orderly assembled (Kirchmaier et al. 2013). Such cloning approaches give simple and modular design strategy to design more complex constructs.

1.6. Dimerization analysis (FRET)

Different approaches have been used to study GPCR dimerization / oligomerization, such as conventional method coimmunoprecipitation (co-IP), as well as new approach proximity ligation assay (PLA) and fluorescence-based method bimolecular fluorescence complementation (BiFC) (Vischer et al. 2015).

Discovery of green fluorescent protein (GFP) and the invention of GFP variants in the 1990s opens the era of dimerization study using another fluorescence-based method – fluorescence resonance energy transfer (or Förster resonance energy transfer, FRET). The labeled GPCR allows not only localization analysis by microscopes, but also interaction analysis by FRET technique. Despite large progress in how GPCRs function, signal, and exert physiological roles, direct proofs of GPCRs interaction is still limited. Indeed, FRET assay is also undirect interaction proof, however, it can detect in nanometer range to indicate rather close proximity, being an accepted approach to determine dimerization.

The principle of FRET is that excited donor can transfer non-radiative energy to appropriate acceptor in close proximity and result in partial acceptor emission. FRET requires several parameters. First of all, donor emission and acceptor excitation spectra should overlap. Secondly, distance between donor and acceptor should be less than 10 nm. Lastly, dipole orientation of donor and acceptor should be optimal, parallel dipole orientation has the highest FRET (Lohse et al. 2012).

A widely used FRET sensor pair is cyan fluorescent protein (CFP) / yellow fluorescent protein (YFP) pair. Another labeling strategy to visualize proteins in living cells is SNAP-tag and CLIP-tag (Maurel et al. 2008; Kolberg et al. 2013). These tags are based on a DNA repair protein human O^6-alkylguanine-DNA-alkyltransferase

(hAGT). The two tags differ in their specific substrates. SNAP-tag uses benzylguanine derivatives, whereas CLIP-tag uses benzylcytosine derivatives as substrates. In the labeling reaction, substrates of the tag can covalently bind to the tag through the benzyl group. A series of fluorescent substrates are available commercially, providing several choices in different applications. In the FRET study here, SNAP-Surface®-549 dye as the donor and CLIP-Surface™-647 dye as the acceptor are applied. They have overlapping spectra and are both cell impermeable dyes.

There are several techniques for measuring FRET in microscopy, for instance, acceptor photobleaching, sensitized emission, fluorescence lifetime imaging microscopy (Broussard et al. 2013). The basic approach used here is acceptor photobleaching. It measures the increase of donor emission after bleaching of the acceptor, and FRET efficiency can be calculated from the increase of donor emission. Its advantage is that it is a quick and straightforward measurement and disadvantage is that it is an irreversible endpoint assay.

In this thesis, Mc4r hetero- and homodimerization were analyzed by measuring the fluorescence of SNAP- or CLIP-tagged Mc4r expression proteins in acceptor photobleaching FRET approach.

1.7. Colocalization analysis microscope (dSTORM)

All conventional fluorescence microscopes including confocal microscopes face a resolution limit of approximately 200 nm in lateral dimensions. Although, in practice, higher resolution can be achieved by using shorter imaging wavelength, higher numerical aperture, and imaging medium with a larger refractive index, the resolution power is restricted by Abbe diffraction limit. With such techniques, small structures such as protein complexes of a few tens of nanometer size cannot be resolved.

Since the 1990s, imaging techniques have been developed to overcome the resolution limit imposed by the diffraction limit. These super-resolution microscopes have a lateral resolution of about 20 nm. There are two types of super-resolution strategies, one is illumination-based super-resolution and the other is probe-based super-resolution (Patterson et al. 2010). Illumination-based super-resolution can reduce the focal spot size by non-linear optical approaches. One example is the

stimulated emission depletion (STED) microscope. Probe-based super-resolution, on the other hand, can separate closely spaced molecules in the same diffraction-limited volume temporally by photoswitchable fluorescent probes. This resolves spatial difference by temporal separation. Examples are photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and direct STORM (dSTORM).

In this thesis, dSTORM was applied to analyze the colocalization of Mc4r A/B1 alleles. dSTORM experiments rely on synthetic organic fluorophores which can be reversibly photoswitched by light irradiation. These photoswitchable fluorophores possess two reversible states – a fluorescent ON state and a nonfluorescent OFF state. The first step in dSTORM experiment is to transfer fluorophores in bright fluorescent samples to reversible OFF state by light irradiation. Next, a sparse population of fluorophores is recovered to ON state spontaneously or photoinduced by light irradiation with a second wavelength. Lastly, emitted photons are precisely determined and used to reconstruct the image (van de Linde et al. 2011). To keep only a single fluorophore emitting within a diffraction-limited area, lifetime of the OFF state should be 100 milliseconds to several seconds, much longer than lifetime of the ON state.

Many commercially available fluorophores can be photoswitchable by adding reducing thiols in millimolar concentrations. The thiols are electron donor and assist triplet state fluorophores to form OFF state dye radical anion. In the study here, SNAP-Surface®-Alexa-647 and synthesized CLIP-Alexa-532 were used. In particular, Alexa Fluor® 647 dye is the extensively used dye for dSTORM because of its extremely good photoswitching properties.

To achieve high signal with low background, the microscope setup is based on a total internal reflection fluorescence (TIRF) microscopy mode (van de Linde et al. 2011). TIRF microscope can limit fluorophores excitation and detection to a very thin region of the specimen, which is called the evanescent field (100-200 nm). Light intensity decays exponentially with the distance from the surface, and detectable fluorophore excitation is restricted to the thin layer on the surface. Making use of these properties, single-molecule-based localization visualizes the small structures such as protein complexes inside of cells.

Appendix 2 Additional result

1. Molecular phylogenetic investigations of genes involved in energy homeostasis and regulation of reproduction in fish

1.1. Background

As a key component in regulating energy homeostasis, Mc4r could connect feeding and puberty. In rodents, the leptin – melanocortin – kisspeptin pathway transmits onset signals on puberty (Manfredi-Lozano et al. 2016). Leptin and ghrelin target the receptors expressed on Pomc and Agrp neurons to regulate the energy balance. When energy and nutrition are sufficiently high enough, the reproductive maturation is permitted. In rat, leptin acts as a metabolic gate for puberty onset (Cheung et al. 1997). Here the downstream effector kisspeptin functions as a master puberty regulator.

Leptin is a satiety signal and produced by adipose tissue (Zhang et al. 1994). Leptin functions in regulating energy homeostasis and reproduction in mammals. Failure of leptin signaling leads to hyperphagia and hypometabolism. Leptin deficient *ob/ob* mice (Pelleymounter et al. 1995) and leptin receptor deficient *db/db* mice (Chen et al. 1996) are obese and infertile. Leptin stimulates Pomc expression and inhibits Agrp expressing. This will decrease the food intake, increase energy expenditure and thereby reduce body weight. In fish, leptin has also been cloned. The amino acids sequence of leptin is not well conserved, only 13 % identity between human and fugu (*Takifugu rubripes*) (Kurokawa et al. 2005) and 19 % between human and zebrafish (Michel et al. 2016) were reported; nevertheless, the tertiary structure of the peptides are similar (Kurokawa et al. 2005). In teleosts, leptin is expressed mostly in liver rather in adipocytes (Kurokawa et al. 2005; Michel et al. 2016). Zebrafish leptin has been reported to be not required for adipostasis and reproduction but only glucose homeostasis (Michel et al. 2016). In medaka, knockout of leptin receptor causes a modest increase in food intake but does not influence final body size (Chisada et al. 2014).

Contrary to leptin as an anorexigenic hormone, ghrelin is an orexigenic hormone. The two hormones act together to maintain energy balance (Nakazato et al. 2001). Ghrelin

is a hunger signal and originates from the gastrointestinal tract (Date et al. 2000). It also has functions in growth hormone release and others. Ghrelin amino acids sequences also vary, showing great diversity at the C-terminus but retaining the highly conserved N-terminus motif GSSFLSP across species (Kaiya et al. 2014). In fish, stomach is also the main expressing organ and hormonal release, appetite regulation and reproduction are as well the main functions of ghrelin (Kaiya et al. 2008). However, newer evidence shows that the role of ghrelin in the regulation of feeding and metabolism in fish could be more complex and might be species- or lineage-specific (Volkoff 2016).

Kisspeptin is expressed in various brain regions in different species. It has a role in neuroendocrine regulation of reproduction. Human patients with loss-of-function KISS1R develop hypogonadotropic hypogonadism (de Roux et al. 2003). The *KISS1* gene product is processed to kisspeptin-54 and shorter peptides like kisspeptin-10, -13, -14, which all bind and activate the KISS1R (Kotani et al. 2001). In addition to *kiss1*, *kiss2* is cloned from medaka and zebrafish, regulating reproduction including puberty (Kitahashi et al. 2009). Both *kiss1* and *kiss2* are conserved in teleost, but *kiss1* is not present in some fish, such as fugu (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*) and stickleback (*Gasterosteus aculeatus*) (Felip et al. 2009; Tena-Sempere et al. 2012). The kisspeptin system is conserved in vertebrates except in the avian lineage (Tena-Sempere et al. 2012). The duplication leading to *kiss1* and *kiss2* occurred in the ancestral vertebrate and mammals have lost *kiss2* during evolution (Kanda and Oka 2012).

Here, to characterize the neuropeptide genes in the energy balance and puberty pathway, synteny analysis was performed on *leptin*, *ghrelin*, *kisspeptin* genes in some fish species.

1.2. Methods

Synteny relationships of *leptin*, *ghrelin*, *kisspeptin* were identified and compared using Ensembl genome browser (http://www.ensembl.org/index.html) and Genomicus genome browser, version 86.01 (http://www.genomicus.biologie.ens.fr/genomicus-86.01/cgi-bin/search.pl). Four teleosts of which high quality genomes are available were compared: medaka (*Oryzias latipes*) – HdrR, zebrafish (*Danio rerio*) – GRCz10,

Southern platyfish (*Xiphophorus maculatus*) – Xipmac4.4.2, Amazon molly (*Poecilia formosa*) – PoeFor_5.1.2. Not annotated genes were retrieved by various BLAST strategies from the NCBI database (https://www.ncbi.nlm.nih.gov), using the above listed genomic data, and confirmed with the new PacBio genomes of medaka – ASM223467v1, and Southern platyfish – X_maculatus-5.0-male.

1.3. Results

In addition to the Mc4r signaling pathway genes *Mc4r*, *Pomc*, *Agrp*, *Mrap2*, other signaling pathway genes known to be involved in energy metabolism and reproduction include *Lep*, *Lepr*, *Ghrl*, *Ghsr*, *Kiss1*, *Kiss1r* and *Gnrh1* in mouse (Manfredi-Lozano et al. 2016). The question arises as to whether the pathway genes are conserved between fish and mouse. In zebrafish, medaka, Southern platyfish and Amazon molly, *mc4r*, *pomc*, *agrp*, *mrap2*, *lepr*, *ghsr*, *kiss1r*, *gnrh* are all annotated. Nevertheless, *leptin*, *ghrelin* and *kisspeptin* are not present in the database in all the four fish. Zebrafish has all genes, annotated as *lepa*, *lepb*, *ghrl*, *kiss1* and *kiss2*. Medaka has annotated *lepa*, *lepb*, *kiss1* and *kiss2*. Amazon molly has only *lepa* and *lepb* annotated. Southern platyfish, however, has none of them annotated so far (**Table A2.1**).

To elucidate the presence of the "missing" genes, chromosome syntenic analysis and various blast analysis were performed. The synteny relationships were first identified according to known gene annotations. Afterwards, the annotated genes were used in blast analysis to identify the "missing" ortholog in other species. The search for unannotated genes were performed by blast (blastn or tblastn) on the regions between the conserved synteny: *lrrc4.2* to *rbm28* for *lepa*, *snd1* to *impdh1b* for *lepb*, *ccdc174* to *tatdn2* for *ghrl*, *golt1a* to *csf1a* for *kiss1*, and *spx* to *ldhba* for *kiss2*. The results with high similarity to annotated genes were taken as predicted genes. The results showing presence of the known conserved motifs were taken as predicted motifs, indicating presence of the orthologous genes. In the case of *kiss* genes, an additional blast (tblastn) was performed on the genome assembly of the Southern platyfish (*Xiphophorus maculatus*) (Schartl et al. 2013).

Chromosome syntenic analysis showed that the loci of *lepa*, *lepb* and *ghrl* are conserved between medaka and Southern platyfish (**Figure A2.1A, A2.1B, A2.1C**).

Low conservation was shown in *kiss1* and *kiss2* genes among medaka, zebrafish, Amazon molly and Southern platyfish (**Figure A2.1D, A2.1E**). The conserved synteny makes it possible to identify genes, such as *lepa* and *lepb*, by blasting in the predicted regions, as indicated above.

The *lepa*, *lepb*, *ghrl*, *kiss2* could be predicted to be present in Southern platyfish. In medaka, the two genes near *lepa* are *rbm28* and *lrrc4.2*, and they are conserved in Southern platyfish. The candidate region is therefore determined by the neighboring genes of *lepa*, that is *rbm28* and *lrrc4.2*, and this region is used to blast (blastn) the *leptin* gene in all species. In Amazon molly, *lepa* was found, taken as predicted gene. In this way, *lepa* was also predicted in Southern platyfish. In a similar way, *lepb* was predicted in Southern platyfish and Amazon molly.

The Southern platyfish and Amazon molly *ghrl* were searched by tblastn using tilapia *ghrl* as query and gene sequence starting from the beginning of *vhl* to the end of *ccdc174* as subject; and the blast result showed that the conserved motif SSFLSP is present in the conserved synteny. The overall sequences may vary largely among species; the existing core sequence indicates the presence of *ghrl* in Southern platyfish and Amazon molly. Using the same approach in medaka, no *ghrl* gene and no motif was predicted.

The *kiss2* gene has the same neighboring gene *ldhba* in medaka, zebrafish, Southern platyfish and Amazon molly (**Figure A2.1E**). Assembly of Southern platyfish and Amazon molly were analyzed by tblastn using medaka *kiss2* as query. A conserved motif FNYNPFGLRF was revealed in a conserved synteny region, which indicates the presence of *kiss2* in Southern platyfish.

Regarding the *kiss1* gene, both *kiss1* and the neighboring gene *golt1a* could not be found by blast in Amazon molly and Southern platyfish. Moreover, mapping of RNA-seq data from Southern platyfish brain, 15-stages whole fish, one-month-old whole fish showed that *kiss1* and *golt1a* did not predict such genes. The *kiss1* and the *golt1a* were also not found in Mosquitofish (*Gambusia*) (*G. affinis* and *G. holbrooki*) by blast (blastn and tblastn) on the genome assembly.
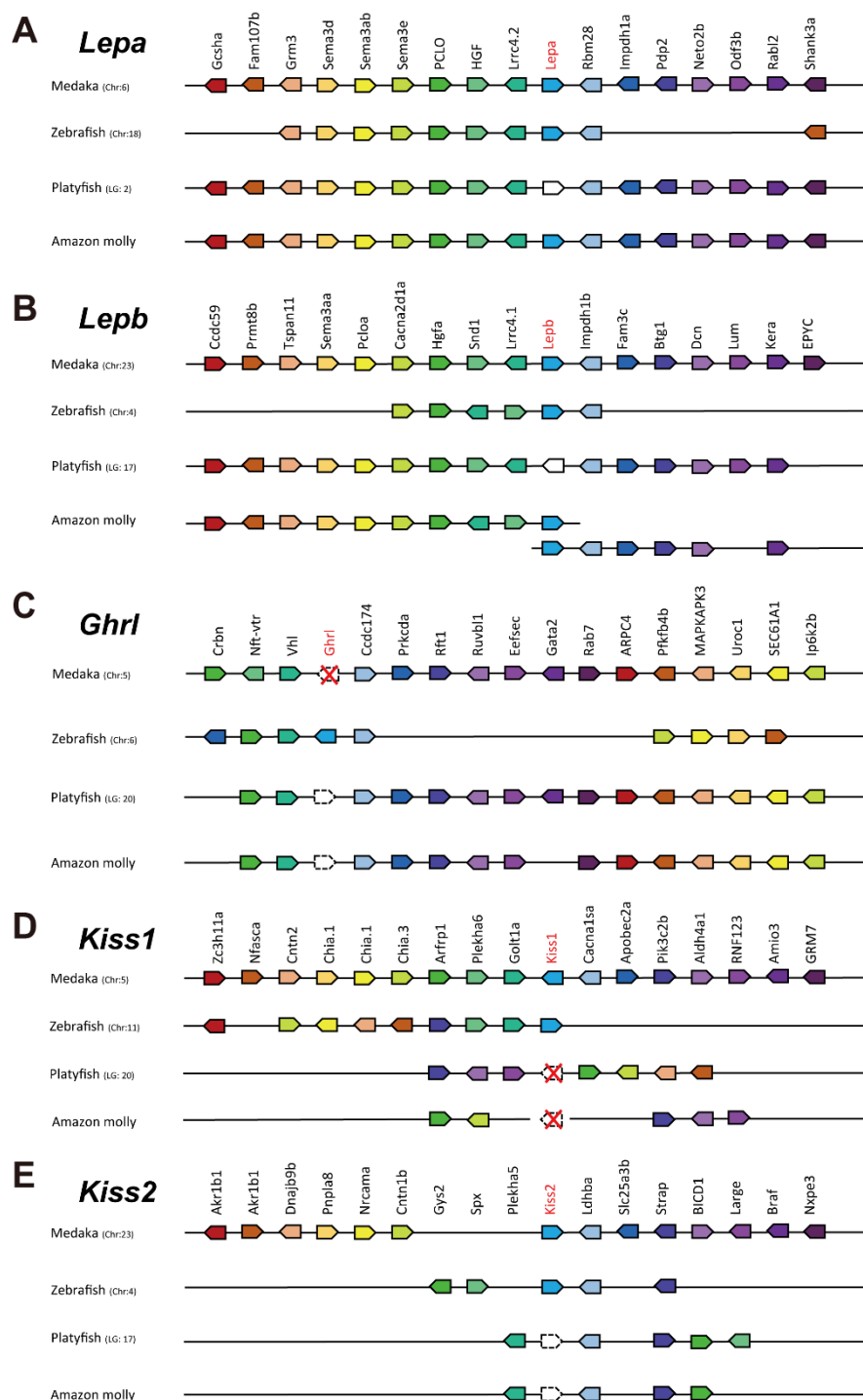
Figure A2.1 Conserved genomic synteny of fish peptide ligand genes.

(A) Lepa, **(B)** Lepb, **(C)** Ghrl, **(D)** Kiss1, **(E)** Kiss2. PhyloView of Genomicus v86.01 web site was used to establish the maps. Analysis was performed on the genome of medaka, zebrafish, platyfish and Amazon molly. Orthologs of each gene were compared and marked in genomic synteny maps by the same color. White polygons by continuous line indicates a predicted ortholog, a dotted line a predicted gene domain and with a red cross missing of the genes. The discontinuous line in Lepb and Kiss1 of Amazon molly indicates two scaffolds.

Furthermore, genome assembly of *Xiphophorus couchianus* and *Xiphophorus hellerii* were available (Shen et al. 2016), the *kiss1* gene was also searched by blast (tblastn) on the assembly of these two fish. The results show that *kiss1* is also missing in these two *Xiphophorus* fish. Taken together, *kiss1* may be lost during evolution in several *Xiphophorus*, Mosquitofish and Amazon molly, indicating a lineage-specific loss of *kiss1* in Poeciliids. All the results are summarized in **Table A2.1**.

Table A2.1 Genes information and the gene location.

Gene IDs are obtained from NCBI.

Specie	Gene	bp	Protein	Chr.	Gene ID
Medaka	Lepa	1097	155aa	6	100049298
	Lepb	588	158aa	23	100301595
	Ghrl			5	No gene
	Kiss1	392	100aa	5	100144371
	Kiss2	519	115aa	23	100301593
Zebrafish	Lepa	501	166aa	18	100150233
	Lepb	616	168aa	4	564348
	Ghrl	472	104aa	6	798613
	Kiss1	661	116aa	11	100134982
	Kiss2	588	125aa	4	100216472
Southern platyfish	Lepa			2	Predicted gene
	Lepb			17	Predicted gene
	Ghrl			20	Predicted motif
	Kiss1			20	No gene
	Kiss2			17	Predicted motif
Amazon molly	Lepa	4884	166aa		103133824
	Lepb	2841	200aa		103146251, 103146196
	Ghrl				Predicted motif
	Kiss1				No gene
	Kiss2				Predicted motif

Note: No gene, no gene were predicted by BLAST. Predicted gene, gene were predicted. Predicted motif, conserved motifs were predicted.

bp, base pair. Chr., chromosome.

1.4. Discussion

Mc4r is a conserved protein but shows distinct roles across species. In *Xiphophorus*, it is connected to puberty onset regulation (Lampert et al. 2010), while in medaka and zebrafish, it is related to growth and development (Sebag et al. 2013; Liu et al. 2019). Other genes of the Mc4r signaling pathway (*pomc*, *agrp*, *mrap2*) are also present and conserved in different fish species.

Comparing the other reproduction related signaling pathway genes among medaka, zebrafish, Southern platyfish and Amazon molly, it could be shown that the pathway is not fully conserved, and certain genes are most likely missing in some fish. This analysis shows low conservation of some genes and indicates the flexibility of Mc4r signaling pathway.

lepa and *lepb* could be predicted in Southern platyfish. *ghrl* is missing in medaka and predicted as motifs present in Southern platyfish and Amazon molly. *kiss1* is missing in Southern platyfish and Amazon molly; *kiss2* is predicted as motifs present in Southern platyfish and Amazon molly. These three genes encode peptide ligands, and the precursor peptide sequences could be considerably divergent in each species, thus increasing the difficulties to BLAST search them. The conserved motifs are very distinct sequences in ghrelin and kisspeptin (Oakley et al. 2009; Kaiya et al. 2011; Kaiya et al. 2014), and could verify the possible existence of the genes together with the evidence from synteny conservation. It should be noted that this could not identify if the genes are expressed or only present as a non-transcribed pseudogene, which needs further analysis.

In teleost, gene evolution has been shaped by a whole genome duplication (WGD). The 3R WGD is responsible for duplicate copies of the genes in teleost fish (Steinke et al. 2006). In the case of the missing genes, they may undergo lineage-specific gene loss in some species. Missing of *ghrl* in medaka may indicate that the function of *ghrl* is not conserved. *kiss2* is conserved in teleosts, but *kiss1* is absent in some fish (Felip et al. 2009; Tena-Sempere et al. 2012), like the case for missing of *kiss1* in Poeciliids including several *Xiphophorus*, Mosquitofish and Amazon molly. Kisspeptin is a key factor in reproduction regulation in mammals but may be not a regulator involved in reproduction in medaka (Nakajo et al. 2018). Other genes may have taken over the function of *kiss1* in reproduction in some fish.

Taken together, the Mc4r upstream and downstream signaling may have more flexibility across species. Particularly in *Xiphophorus* compared to medaka, the Mc4r signaling system evolved a special function in regulating puberty onset. This new function of *mc4r* may lead to that *kiss1* is not required in reproduction and got lost during evolution. The loss of *kiss1* may be involved in the establishment of growth and reproduction axis with small discrepancy.

Appendix 3 Primer list

Primer list

Primer	Sequence (5'-3')	Comment
MF_ef1a1-f01	GCCCCTGGACACAGAGACTTCATCA	RT-qPCR, medaka
MF_ef1a1-r01	AAGGGGGCTCGGTGGAGTCCAT	RT-qPCR, medaka
Mc4r_Ol_F	GGCAACCTGAGCATTCCTGTCA	RT-qPCR, medaka
Mc4r_Ol_R	ATGTAGCGGTCAACGGCAATGG	RT-qPCR, medaka
MRAP2a_Ol_F	CGCACGACGCAGTGACTATGT	RT-qPCR, medaka
MRAP2a_Ol_R	ACCGCCAGTCCAACCCAGAA	RT-qPCR, medaka
Pomca_Ol_F	TGGACTCTGAGAGCAGCATGAC	RT-qPCR, medaka
Pomca_Ol_R	AAGGGATCTGAGGGAGGTGGAG	RT-qPCR, medaka
Pomcb_Ol_F	TTGCTGGCTGTTGGTGGTTCT	RT-qPCR, medaka
Pomcb_Ol_R	AGGTCTGGGCTTTCAGGTTTGA	RT-qPCR, medaka
AgRP_Ol_F	CATCCCTCACCAGCAGTCCT	RT-qPCR, medaka
AgRP_Ol_R	GCGGCAGTAACAGATGGCATT	RT-qPCR, medaka
Mc4r_Ol_F2	CCTGGGAGGACAGAAAGA	*in situ* probe synthesis, medaka
Mc4r_Ol_R2	ATGAAGAGGATACCCGACA	*in situ* probe synthesis, medaka
MRAP2a_Ol_F2	ACGAGTATTATGACGACGAG	*in situ* probe synthesis, medaka
MRAP2a_Ol_R2	GATGGTGTATCCCTGCTT	*in situ* probe synthesis, medaka
Mc4r_Xm_F2	ACTCCACGGCTCAGCAAG	*in situ* probe synthesis, *Xiphophorus*
Mc4r_Xm_R2	CGCAGGGCGTAGAAGATG	*in situ* probe synthesis, *Xiphophorus*
MRAP2a_Xm_F2	TGGCAGTATGAGTATTACGA	*in situ* probe synthesis, *Xiphophorus*
MRAP2a_Xm_R2	AGTTTGGGATGTTGAAGTG	*in situ* probe synthesis, *Xiphophorus*
M13_F	GTAAAACGACGGCCAGT	pGEM-T colony PCR
M13_R	CAGGAAACAGCTATGAC	pGEM-T colony PCR

Primer	Sequence (5'-3')	Comment
CMV_BamHI_F	CACC**GGATCC**TAGTTATTAATAGTAATCAAT	GoldenGate Cloning, CMV promoter
CMV_KpnI_R	TATA**GGTACC**GATCTGACGGTTCACTAAACC	GoldenGate Cloning, CMV promoter
SFS_BamHI_kATG_F	CACC**GGATCC**GCCGCCATGAAGACGATCATCGCCCTGAG	GoldenGate Cloning, signal peptide-FLAG-SNAP/CLIP
SFS_KpnI_R	TATA**GGTACC**GCCCAGCCCAGGCTTGCCCA	GoldenGate Cloning, signal peptide-FLAG-SNAP
SFC_KpnI_R	TATA**GGTACC**TCCCAAGCCTGGCTTCCCCA	GoldenGate Cloning, signal peptide-FLAG-CLIP
Mc4r_B2_Xmulti_BamHI_-ATG_F	CACC**GGATCC**AACTCCACGGCTCAGCAAGG	GoldenGate Cloning, Mc4r A/B1/B2
Mc4r_6_A_Xnig_Stop_R	TCACAGAAAGCTAATACACGAGA	GoldenGate Cloning, Mc4r A
Mc4r_28_B1_Xnig_Stop_R	TCACAGAAAGCTAATACACCAGA	GoldenGate Cloning, Mc4r B1
Mc4r_B2_Xmulti_Stop_R	TTACAGATGAACTACCTGGAGCG	GoldenGate Cloning, Mc4r B2
MRAP2a_Xm_-_F	AACCGCAGAAATGTTCAG	Cloning, Mrap2
MRAP2a_Xm_-_R	TTCCCTCTGCCAGTCTTA	Cloning, Mrap2
MRAP2a_Xm_BamHI_ATG_F	CACC**GGATCC**ATGTCCGACTTCCACAACCGAAG	GoldenGate Cloning, Mrap2
MRAP2a_Xmulti_KpnI_STOP_R	TATA**GGTACC**TTAGTGGGTGTCACAGAAACCAT	GoldenGate Cloning, Mrap2
pGGEV_F	GGGGCAATGACGCAAATG	Entry vetors colony PCR
pGGEV_R	TCTTCCCATTCTAAACAACACCC	Entry vetors colony PCR
Mact1upper	TTCAACAGCCCTGCCATGTA	medaka actin control
Mact2lower	GCAGCTCATAGCTCTTCTCCAGGGAG	medaka actin control
DMTY_a	GGCCGGGTCCCCGGGTG	medaka XY male genotyping
DMTY_d	TTTGGGTGAACTCACATGG	medaka XY male genotyping
Med-Mc4r-KO_F3	GCTACGAGCAGCTTCTGATCTCCACTGAGG	TALEN2(Δ11, -2/+3)sequencing
Med-Mc4r-KO_F4	GCTCTGCTGGCTGGAGCACAGTGACG	TALEN KO genotyping, TALEN1 (Δ7)sequencing
Med-Mc4r-KO_R3	AGAAGATGGTGATGTAGCGGTCAACGGC	TALEN KO genotyping

Primer	Sequence (5'-3')	Comment
Med-Mc4r-WT_F1	ATGTTGGTCAGCGTCTCCAACG	TALEN KO genotyping HiDi (-2/+3)
Med-Mc4r_23KO_F2	ATGTTGGTCAGCGTCTCCAAGAC	TALEN KO genotyping HiDi (-2/+3)
h-Actin-f	GGCATCCTGACCCTGAAGTA	human actin control
h-Actin-r	GGGGTGTTGAAGGTCTCAAA	human actin control
myko-mgso	TGCACCATCTGTCACTCTGTTAACCTC	Mycoplasma PCR
myko-gpo-1	ACTCCTACGGGAGGCAGCAGTA	Mycoplasma PCR
Xmn_MC4R_f2	CAGCCAGGAGATGAGGAAGA	X.multi Mc4r cell genotyping
Xmn_MC4R_r1	GCACTGCACACGGATTACAG	X.multi Mc4r cell genotyping
Xmn_MC4R_f1	TCCTCATCATGTGCAACTCC	X.multi Mc4r cell genotyping
pCDNA3neo-rev1	CGAGCTCTAGCATTTAGGTGACA	pcDNA3neo cell genotyping
pCDNA3.1zeo-rev1	CTGATCAGCGGGTTTAAACG	pcDNA3.1zeo cell genotyping
Xmcr1-F10	CCTTTCACCGTTTCTCCTC	Cloning for Mc4r
Xmcr1-R5	GACATTTCAGGCTCTTCATCC	Cloning for Mc4r
XElf_for	GCAGGACGTCTACAAGATTGG	RT-qPCR, *Xiphophorus*
XElf_rev	GCATCTCCACGGATTTCACT	RT-qPCR, *Xiphophorus*
Mc4r_Xm_F	GCCTGTGCCTGGGAACTCT	RT-qPCR, *Xiphophorus* (Xm)
Mc4r_Xm_R	CAGGCTGATGATGCCCAAA	RT-qPCR, *Xiphophorus* (Xm)
Mc4r_AB_Xmn_F3	ATGTCGCACTTCAACATG	RT-qPCR, *Xiphophorus* (Xmn)
Mc4r_AB_Xmn_R15	AAGGTCTTCCTCATCTCC	RT-qPCR, *Xiphophorus* (Xmn)
Mc4r_A_Xhel_F	CCCATGCTACCTGAACCGG	RT-qPCR, *Xiphophorus* (Xhel)
Mc4r_A_Xhel_R	AGGCTGATGATGCCCAAAGT	RT-qPCR, *Xiphophorus* (Xhel)
MRAP2a_Xm_F	TGTCCGACTTCCACAACCG	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
MRAP2a_Xm_R	GCCAGTCCCACCCAGAAAC	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
Pomca1_Xi_F	GTGTTGCTATTGGTGGTTGCA	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)

Primer	Sequence (5'-3')	Comment
Pomca1_Xi_R	TGCTAAGAATGGAGGTCATCTCAT	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
Pomca2_Xi_F2	ATTGGTGGCCGTGGTGG	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
Pomca2_Xi_R2	TGGGACCAGGGGCGTCT	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
Pomcb_Xi_F	TCAGTGCTGGTTGTTTATGGTTC	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
Pomcb_Xi_R	CCTGGGTGGCTTGGTTTCA	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
AgRP_Xi_F	GAGGCGGGCTCCAACAAT	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)
AgRP_Xi_R	GCAGCGAGTAACCCAGACAGA	RT-qPCR, *Xiphophorus* (Xm, Xmn, Xhel)

Primer pair list

Gene	length, bp	Forward	Reverse	Comment
RT-qPCR for medaka				
Elf1a	211	MF_ef1a1-f01	MF_ef1a1-r01	RT-qPCR medaka
Mc4r	122	Mc4r_Ol_F	Mc4r_Ol_R	RT-qPCR medaka
Mrap2	123	MRAP2a_Ol_F	MRAP2a_Ol_R	RT-qPCR medaka
Pomca	118	Pomca_Ol_F	Pomca_Ol_R	RT-qPCR medaka
Pomcb	147	Pomcb_Ol_F	Pomcb_Ol_R	RT-qPCR medaka
Agrp	100	AgRP_Ol_F	AgRP_Ol_R	RT-qPCR medaka
in situ hybridization probe synthesis for medaka and *Xiphophorus*				
Mc4r	471	Mc4r_Ol_F2	Mc4r_Ol_R2	*in situ* probe synthesis
Mrap2	426	MRAP2a_Ol_F2	MRAP2a_Ol_R2	*in situ* probe synthesis
Mc4r	463	Mc4r_Xm_F2	Mc4r_Xm_R2	*in situ* probe synthesis
Mrap2	514	MRAP2a_Xm_F2	MRAP2a_Xm_R2	*in situ* probe synthesis
lacZ	232+insert	M13_F	M13_R	pGEM-T colony PCR
Expression vector construction by GoldenGate system				
cmv promoter	589	CMV_BamHI_F	CMV_KpnI_R	GoldenGate Cloning
signal peptide - FLAG - CLIP	621	SFS_BamHI_kATG_F	SFS_KpnI_R	GoldenGate Cloning
signal peptide - FLAG - SNAP	621	SFS_BamHI_kATG_F	SFC_KpnI_R	GoldenGate Cloning
Mc4rA	990	Mc4r_B2_Xmulti_BamHI_-ATG_F	Mc4r_6_A_Xnig_Stop_R	GoldenGate Cloning
Mc4rB1	984	Mc4r_B2_Xmulti_BamHI_-ATG_F	Mc4r_28_B1_Xnig_Stop_R	GoldenGate Cloning
Mc4rB2	1005	Mc4r_B2_Xmulti_BamHI_-ATG_F	Mc4r_B2_Xmulti_Stop_R	GoldenGate Cloning
Mrap2	864	MRAP2a_Xm_-_F	MRAP2a_Xm_-_R	Cloning
Mrap2	681	MRAP2a_Xm_BamHI_ATG_F	MRAP2a_Xmulti_KpnI_STOP_R	GoldenGate Cloning

Gene	length, bp	Forward	Reverse	Comment
Entry vector	338+insert	pGGEV_F	pGGEV_R	Entry vectors colony PCR
Medaka knockout line genotyping				
Actin	349	Mact1upper	Mact2lower	medaka actin control
DMRT1Y -HNI allele	282	DMTY_a	DMTY_d	medaka XY male genotyping
Mc4r	~660	Med-Mc4r-KO_F 4	Med-Mc4r-KO_R 3	TALEN KO genotyping
Mc4r		Med-Mc4r-KO_F 4		TALEN1 (Δ7)sequencing
Mc4r		Med-Mc4r-KO_F 3		TALEN2 (Δ11, -2/+3)sequencing
Mc4r WT	190	Med-Mc4r-WT_F 1	Med-Mc4r-KO_R 3	TALEN KO genotyping HiDi
Mc4r -2/+3KO	191	Med-Mc4r_23KO _F2	Med-Mc4r-KO_R 3	TALEN KO genotyping HiDi
HEK293T derived cell lines genotyping				
Actin	~200	h-Actin-f	h-Actin-r	human actin control
Mycoplas ma gene	~620	myko-mgso	myko-gpo-1	Mycoplasma PCR
Mc4r	~110	Xmn_MC4R_f2	Xmn_MC4R_r1	X.multi Mc4r cell genotyping
Mc4r	213	Xmn_MC4R_f1	pCDNA3neo-rev1	pcDNA3neo cell genotyping
Mc4r	190	Xmn_MC4R_f1	pCDNA3.1zeo-rev 1	pcDNA3.1zeo cell genotyping
X. hellerii mc4r Cloning				
Mc4r		Xmcr1-F10	Xmcr1-R5	Cloning for Mc4r
RT-qPCR for *Xiphophorus*				
Elf1a	134	XElf_for	XElf_rev	RT-qPCR *Xiphophorus*
RT-qPCR for *X. maculatus*				
Mc4r	125	Mc4r_Xm_F	Mc4r_Xm_R	RT-qPCR *X. maculatus*
Mrap2	151	MRAP2a_Xm_F	MRAP2a_Xm_R	RT-qPCR *X. maculatus*

Gene	length, bp	Forward	Reverse	Comment
Pomca_1	163	Pomca1_Xi_F	Pomca1_Xi_R	RT-qPCR *X. maculatus*
Pomca_2	154	Pomca2_Xi_F2	Pomca2_Xi_R2	RT-qPCR *X. maculatus*
Pomcb	166	Pomcb_Xi_F	Pomcb_Xi_R	RT-qPCR *X. maculatus*
Agrp	112	AgRP_Xi_F	AgRP_Xi_R	RT-qPCR *X. maculatus*

RT-qPCR for *X. nigrensis* / *X. multilineatus*

Gene	length, bp	Forward	Reverse	Comment
Mc4r	98	Mc4r_AB_Xmn_F3	Mc4r_AB_Xmn_R15	RT-qPCR *X. nigrensis* / *X. multilineatus*
Mrap2	151	MRAP2a_Xm_F	MRAP2a_Xm_R	RT-qPCR *X. nigrensis* / *X. multilineatus*
Pomca_1	163	Pomca1_Xi_F	Pomca1_Xi_R	RT-qPCR *X. nigrensis* / *X. multilineatus*
Pomca_2	154	Pomca2_Xi_F2	Pomca2_Xi_R2	RT-qPCR *X. nigrensis* / *X. multilineatus*
Pomcb	166	Pomcb_Xi_F	Pomcb_Xi_R	RT-qPCR *X. nigrensis* / *X. multilineatus*
Agrp	112	AgRP_Xi_F	AgRP_Xi_R	RT-qPCR *X. nigrensis* / *X. multilineatus*

RT-qPCR for *X. hellerii*

Gene	length, bp	Forward	Reverse	Comment
Mc4r	144	Mc4r_A_Xhel_F	Mc4r_A_Xhel_R	RT-qPCR *X. hellerii*
Mrap2	151	MRAP2a_Xm_F	MRAP2a_Xm_R	RT-qPCR *X. hellerii*
Pomca_1	163	Pomca1_Xi_F	Pomca1_Xi_R	RT-qPCR *X. hellerii*
Pomca_2	154	Pomca2_Xi_F2	Pomca2_Xi_R2	RT-qPCR *X. hellerii*
Pomcb	166	Pomcb_Xi_F	Pomcb_Xi_R	RT-qPCR *X. hellerii*
Agrp	112	AgRP_Xi_F	AgRP_Xi_R	RT-qPCR *X. hellerii*

Appendix 4 Abbreviations

2R	two rounds of genome duplication
3R	fish-specific genome duplication
7TM	seven-transmembrane domain
A	acceptor
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
Agrp	agouti-related peptide
AP	alkaline phosphatase
ARC	arcuate nucleus
ASIP	agouti-signaling protein
AV	accessible volume
BiFC	bimolecular fluorescence complementation
bp	base pair
cAMP	cyclic 3',5'-adenosine monophosphate
CFP	cyan fluorescent protein
Chr.	chromosome
co-IP	coimmunoprecipitation
CRE	cAMP response element
CREB	CRE binding protein
C-terminus	carboxyl-terminus
D	donor
Dig	digoxygenin
DMEM	Dulbecco's modified Eagle medium
dpf	days post fertilization
dSTORM	direct stochastic optical reconstruction microscopy
EC50	half maximal effective concentration
ELs	extracellular loops
END	endorphin
FCS	fetal calf serum
Fluo	fluorescein
FPS	FRET positioning and screening tool
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone

GPCR	G-protein coupled receptor
G_s	stimulatory G protein
hAGT	human O6-alkylguanine-DNA-alkyltransferase
HEK293T	human embryonic kidney 293T
HPG	hypothalamus-pituitary-gonad axis
HPLC	high-performance liquid chromatography
ILs	intracellular loops
indels	insertions/deletions
ISH	*in situ* hybridization
KO	knockout
LG	linkage group
LH	luteinizing hormone
LPH	lipotropin
Mb	million base pairs
Mc4r	melanocortin 4 receptor
Mcr	melanocortin receptors
MCS	multiple cloning sites
ML	maximum likelihood
Mrap	melanocortin 2 receptor accessory protein
Mrap2	melanocortin receptor accessory protein 2
mRNA	messenger RNA
MSH	melanocyte-stimulating hormone
NBT/BCIP	nitro blue tetrazolium / 5-bromo-4chloro-3-indolyl phosphate
NJ	neighbor-joining
N-POC	N-terminal peptide
N-terminus	amino-terminus
ORF	open reading frames
P	*puberty*
PALM	photoactivated localization microscopy
PC	prohormone convertases
PEI	polyethylenimine
PKA	protein kinase A
PLA	proximity ligation assay
PO	horseradish peroxidase
POA	preoptic area
Pomc	pro-opiomelanocortin
PVN	paraventricular nucleus
RFU	relative fluorescent units

Rmax	maximum response
RT-qPCR	quantitative real-time reverse transcription PCR
SD	standard deviation
STORM	stochastic optical reconstruction microscopy
TIRF	total internal reflection fluorescence
TM	transmembrane
WGD	whole genome duplication
WT	wild-type
YFP	yellow fluorescent protein

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Publications List

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