Pharmacological modulation of two melanocortin-5 receptors by MRAP2 proteins in zebrafish

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Running Title: Pharmacological modulation of MC5R by MRAP2
Melanocortin receptor accessory protein 2 (MRAP2) plays an important role in regulating melanocortin receptors. In zebrafish, MRAP2a and MRAP2b show distinct pharmacological effects on MC4R activity, but how MRAP2 protein regulates other zebrafish melanocortin receptors is barely studied. Zebrafish have two mc5r genes: mc5ra and mc5rb, it is still vague which one is the homologous isoform to the mammalian paralog. Here we utilize synteny and phylogenetic analysis to demonstrate the evolutionary conservation of zebrafish MC5Ra and MC5Rb among different species. We also show that MRAP2a and MRAP2b could interact and regulate surface expression of two MC5R receptors. Bimolecular fluorescence complementation (BiFC) studies suggest that zebrafish MC5Rs could form homo- and hetero-dimers, which are suppressed by co-expression with MRAP2 proteins. In comparison with mammalian MC5R-MRAP2 system and different pharmacological effects of zMRAP2 protein on MC5Rs, zmc5ra is identified as the evolutionary homologous paralog to the mammals and it is regulated by metabolic state in zebrafish brain region.

Key words:
Melanocortin 2 receptor accessory protein 2; melanocortin-5 receptor; zebrafish; pharmacology

1 INTRODUCTION
The melanocortin receptor family consists of five G-protein coupled receptors, MC1R~MC5R, which are regulated by pro-opiomelanocortin (POMC) and agouti protein
families (Logan, et al. 2003). MC1R plays a role in dermal pigmentation; MC2R mediates the effect of adrenocorticotropic hormone (ACTH) on steroid secretion; MC3R and MC4R regulate energy homeostasis in the central nervous system (Cone 2006). In chickens, MC3R and MC4R can be activated by α-MSH or ACTH. But AgRP can antagonize α-MSH or ACTH actions on MC3R/MC4R and lower the constitutive activity of MC3R and MC4R (Zhang, et al. 2017). In mice, knockout of MC5R exhibit a severe dysfunction of exocrine secretion (Chen, et al. 1997). In adipocytes, MC5R regulates lipolysis and re-esterification through the cAMP/PKA and MAPK/ERK1/2 pathways (Rodrigues, et al. 2013). MC5R also participates in the energy homeostasis since it can increase glucose uptake in skeletal muscle through the PKA pathway (Enriori, et al. 2016). MC5R showed a hepatic lipolysis function in sea bass (Sanchez, et al. 2009). Heterodimerization of MC1R and MC5R may be involved in the control of color change in teleosts (Kobayashi, et al. 2016). The mc5r gene is duplicated in zebrafish: mc5ra and mc5rb (Vastermark and Schioth 2011), while there is only one mc5r gene in fugu or goldfish(Cerda-Reverter, et al. 2003; Klovins, et al. 2004). In zebrafish, mc5ra is highly expressed in ovary, brain, gastrointestinal tract, while mc5rb is highly expressed in ovary, brain, gastrointestinal tract and eye, both of them can be stimulated by α-MSH (Ringholm, et al. 2002).

Melanocortin receptor accessory proteins (MRAPs) are single-transmembrane proteins, several studies confirmed that they could form antiparallel homo- and heterodimers (Metherell, et al. 2005; Sebag, et al. 2007). The MRAP system is involved in the regulation of trafficking and signaling of melanocortin receptors (Asai, et al. 2013; Cerda-Reverter, et al. 2013). Two main forms MRAP1 and MRAP2 exist in zebrafish genome. As in mammals, MRAP1 is essential for adrenocorticotropic hormone receptor (MC2R) trafficking to the cell membrane, ligand binding, and downstream signaling (Agulleiro, et al. 2010). MRAP2 (melanocortin receptor accessory protein 2) was found as a homologue of MRAP1. MRAP2 interacts with all five MCRs and modulates
receptors trafficking to the membrane, agonist binding and cAMP production (Cerda-Reverter et al. 2013; Chan, et al. 2009). Recent studies demonstrated that MRAP2 could also interact with non-melanocortin receptors and modulate their signaling (Chaly, et al. 2016) (Srisai, et al. 2017). In mammals, MRAP2 enhances MC4R activity and loss of function of MRAP2 causes early-onset obesity (Asai et al. 2013). The mrap2 gene is present in the genome of lampreys (Vastermark and Schioth 2011). In zebrafish, mrap2 has two homologues: mrap2a and mrap2b. Both of MRAP2a and MRAP2b can interact with zebrafish MC4R: MRAP2a inhibit the binding of MC4R to its α-MSH, whereas MRAP2b stimulates MC4R activation by increase ligand binding ability and surface expression (Sebag, et al. 2013). Josep et al.(Josep Agulleiro, et al. 2013) also found that MC4R could be activated by ACTH when co-expressed with MRAP2a. MRAP2b can decrease the constitutive activity of MC4R during fasting which suggests that the deletion of MRAP2b may induce growth changes or obesity. As in zebrafish, MRAP2 can enhance the sensitivity of MC4R for ACTH and block the constitutive activity of MC4R in chickens (Zhang et al. 2017). Unlike MC4R, the physiological functions between MC5Rs and MRAP2 proteins are largely unknown in zebrafish.

In this study, we examined the interaction and pharmacological modulation between two pairs of zebrafish proteins: MC5Ra, MC5Rb and MRAP2a, MRAP2b. MRAP2a prevents both MC5Ra and MC5Rb expression on cell membrane, thus inhibits their activity. MRAP2b inhibits the efficacy of MC5Ra but stimulates MC5Rb. In addition, we compared the regulation of MC5R and MRAP2 between zebrafish and mice. Our results revealed the fact that zebrafish MC5Ra was the functional homologous to mammals.

2 METHODS

2.1 Homology and phylogenetic analysis of MC5R

Preparatory multiple sequence alignments were performed using MUSCLE 3.8.31 with default parameters (Edgar 2004). The percentage of similarity between the amino
Acid sequences were calculated with DNAMAN. Putative TMDs of MC5Rs were predicted with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Phylogenetic tree based on the amino acid sequences was constructed by Neighbor-joining (NJ) methods with 1000 bootstrap resampling and Poisson correction model, pairwise deletion option using Mega 7.0 software. To determine whether mc5ra or mc5rb is more orthologous to mammals, synteny analysis was performed between zebrafish, medaka, spotted gar, human, mouse, and rat with ensemble (http://www.ensembl.org/index.html).

2.2 Animal Care

Wild type zebrafish (TU) were raised at 26~28 °C, with 14 hour light and 10 hour night cycle. Fish aged from 5 dpf to 10 dpf were fed twice a day with paramecia, Fish aged from 10 dpf to 15 dpf were fed with paramecia and brine shrimp, adult fish were fed with brine shrimp in system water. Zebrafish care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji University.

2.3 Plasmids and Peptides

3HA-zMC5Ra, 3HA-zMC5Rb, 2Flag-zMRAP2a and 2Flag-zMRAP2b, cloned from wild type zebrafish, were constructed into vector pcDNA3.1 (+) (Invitrogen). α-MSH and SHU9119 were purchased from GenScrpipt Corporation Ltd. (China). AgRP (83 - 132) were synthesized by Chinese Peptide Company (Hangzhou, China).

2.4 Cell Culture and Transfection

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium contain 10% fetal bovine serum and 1% penicillin/streptomycin. Chinese hamster ovary (CHO) cells were cultured in DMEM/F12 medium contain 5% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in a humidified atmosphere consisting of 5% CO₂ at 37°C. For transfection, when cells growth to 70% - 80% confluence, plasmids were transfected using ViaFect.
Transfection Reagent (Promega).

2.5 Co-Immunoprecipitation and Western Blotting

1×10^6 HEK 293T cells were seeded into 60mm dishes and transfected next day. After 48 hours of transfection, cells were washed with PBS and lysed with lysis buffer (0.75% Triton-X, 50 mM Tris-HCl pH 7.9, 150 mM NaCl and proteinase inhibitor cocktail from Roche) for 1 hour at 4 °C and centrifuged. Supernatants were incubated with anti-HA antibody (abcam) overnight at 4 °C. Protein A/G Agarose beads were added to cell lysate next day, and rotated for 4 hours at 4 °C, beads were washed three times using lysis buffer and centrifuged, suspended in loading buffer and boiled for 15 min. 10% gels were used for SDS-PAGE. Anti-HA (abcam) and anti-Flag (abelonal) antibodies were used at a dilute of 1:4000 for immunoblotting.

2.6 cAMP Assays

HEK 293T cells were plated into 24-well plates, and transfected with indicated plasmids next day. After 24 hours of transfection, cells were treated with α-MSH or SHU9119 in DMEM medium supplemented with 0.1% bovine serum albumin for 4 hours at 37 °C, concentration of α-MSH ranged from 10^{-12} M to 10^{-6} M, concentration of SHU9119 ranged from 10^{-13} M to 10^{-7} M signaling assay was tested in HEK 293T cells stimulated by 2×10^{-9} M α-MSH and AgRP with final concentration ranging from 10^{-13} M to 10^{-7} M. The cAMP level was measured using Dual-Glo Luciferase Assay System (Promega). Luminescence was measured using a Spectramax M5 plate reader. All data were normalized to Renilla luminescence.

2.7 Cell Surface ELISA

12-well plates were treated with Poly-L-lysine Solution before used, HEK 293T cells were seeded onto treated plates and transfected next day. On the third day, cells were washed with D-PBS three times, fixed with 4% poly-formaldehyde for 20 min at room temperature. After fixation, cells were washed, blocked for 30 min in 5% milk in D-PBS for surface groups or in 5% milk in lysis buffer (Beyotime) for whole cell groups,
and incubated with anti-HA antibody (abcam) for 2 hours at room temperature. After washed three times with D-PBS, cells were incubated with secondary antibody for 1 hour. Then cells were washed three times with D-PBS and incubated with TMB solution for 15-30 min, the reaction was stopped by adding 2 M H₂SO₄. Spectramax M5 plate reader was used to record OD at 450 nm. Then all liquid in plate wells were removed, and added 200μl 1× Janus Green per well to stain for 5 min, plates were washed three to five times with deionized water. After removed all the water, added 200 μl 0.5 M HCl to shake for 10 min, record OD at 595 nm. All data were normalized to OD 595nm before used. Statistical analyses were performed using Graph Pad Prism, version 6 (GraphPad Software, San Diego, CA, USA).

2.8 Immunofluorescence

YFP fragments YFP-F1 and YFP-F2 (Sebag and Hinkle 2009) were constructed into C-terminal of GPCRs respectively, CHO cells were seed into a 12-well plates with slides and transfected next day. After 24 hours of transfection, cells were fixed with paraformaldehyde for 20 minutes, then washed 3 times with PBS. To detect surface expression of MRAP2s, cells were incubated with anti-V5 antibody (abcam) at 1:1000 for 2 hours at 37 °C. Then washed 3 times and incubated with 1:1000 secondary antibody Alexa Fluor®594 (abcam) for 2h at 37 °C. For FACS analysis, MRAP2 : MC5R-F1 : MC5R-F2 were transfected at ratio 2 : 1 : 1, after 36 hours of transfection, cells were digested into single cell using 0.02% EDTA and centrifuged, 5×10⁵ cells were dispensed into 500 μl DMEM/F12 medium, filtered through a 40 μm mesh sieve into a 5 ml Polystyrene Round-Bottom Tube (FALCON), then analyzed by BD FACSVers.

2.9 Fasting and Real Time Quantitative PCR

Male wild type zebrafish (TU) were fasted 0, 2, 10, 15 days respectively, keeping water change every day. On indicated day, the brain RNA of adult zebrafish was extracted using RNAiso Plus (Takara), according to the manufacturer’s instructions. 1μg RNA was reverse transcribed using a TIANGEN FastKing RT kit (Beijing, China).
cDNA reaction was diluted 1:10 as a template for qPCR. Real-time PCR was performed using LightCycler 96 (Roche), the PCR conditions were: preincubation at 95°C for 600 s; 45 cycles of 95°C 10 s, 60°C for 10 s, and 72°C for 10 s; melting at 95°C for 10 s, 65°C for 60 s, 97°C for 1 s. Primers for qPCR: mc5ra, forward 5’-TCCTCATCCTTGGGATCCTGTCAGT-3’, reverse 5’-TGGCGATTGGTGAGCGAGGTA GAT-3’; mc5rb, forward 5’-AGAACAAGAAACCTCCACCAAGAATG-3’, reverse 5’-TTGCCAGCAGATGAATGACGATTGT-3’; ef1α, forward 5’-CCTGCCAGTGTTGCTTCGT-3’, reverse 5’-CCTCCTTGCGCTCAAATCCTTCC-3’. All reactions were run in triplicate and repeated three times.

2.10 Statistical analyses

All experiments were performed at least three times. Data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA). Results of cAMP assays were analyzed by two-way ANOVA with Tukey post-test. Results of surface ELISA and qPCR were analyzed by two-tailed t test. All Results were shown as mean ± SEM.

3 RESULTS

3.1 Amino acid sequences of MC5R

The amino acid sequences of MC5R consisted of seven transmembrane domains connected by alternating extracellular and intracellular loops with an extracellular N-terminus and an intracellular C-terminus (Supplementary figure 1). Alignment of the amino acid sequences of MC5R with that of other species demonstrated that zebrafish MC5Ra and MC5Rb were homologues to tetraodon (78.53% and 78.85%, respectively), and fugu (78.53% and 75.00%, respectively) MC5Rs, whereas the homology of zebrafish MC5R to mammalian MC5Rs, such as human and mouse MC5Rs, was lower (61.83% - 75%). As shown in Supplementary figure 1, the amino acid sequences in the transmembrane domains and intracellular loops of MC5Ra and MC5Rb were found to be highly identical to that of other species while lower homology was identified at the N-termini and extracellular loops. A NJ phylogenetic tree was analyzed based on amino
acid sequences of MC5Rs from different species and our results indicated that MC5Ra of zebrafish was more evolutionarily related to tetraodon and fugu MC5Rs. However, the zebrafish MC5Rb out-groups to all bony vertebrate MC5R orthologs (Figure 1A).

To determine which mc5r is more orthologous to mammals, synteny analysis was performed between zebrafish, spotted gar, human, mouse, and rat (Figure 1B). Our results showed that adjacent genes of mc5ra were highly conserved between zebrafish and human, mouse and rat MC5Rs. The adjacent genes of mc5ra, including rnmt, fam210aa, ldlrad4a, sehil, ptpn2b and psmg2 were identical with those of spotted gar, human, mouse and rat MC5Rs. Interestingly, the adjacent genes of mc5rb: ldlrad4b, fam210ab and especially mc2r were conserved between zebrafish mc5rb and mouse, rat and human MC5Rs.

### 3.2 Interaction of zebrafish MRAP2s with MC5Rs

To verify the protein-protein interactions between two MRAP2s and two MC5Rs, 4 groups of HEK 293T cells were transfected with 2Flag-zMRAP2a and 3HA-zMC5Ra, 2Flag-zMRAP2b and 3HA-zMC5Ra, 2Flag-zMRAP2a and 3HA-zMC5Rb, 2Flag-zMRAP2b and 3HA-zMC5Rb, respectively. Co-immunoprecipitation results showed that zebrafish MRAP2a (Figure 2, A and B) and MRAP2b (Figure 2, C and D) could interact with MC5Ra and MC5Rb, suggesting that MRAP2a and MRAP2b could modulate the signaling of MC5Ra and MC5Rb in zebrafish.

### 3.3 Surface expression of MC5Rs was influenced by MRAP2s

To investigate the influences of MRAP2s on the MC5R trafficking, we next measured the surface expression of MC5Ra and MC5Rb in the absence or presence of MRAP2a or MRAP2b. 293T cells were transfected with MC5R alone (1:0) and MRAP2 at progressive ratio (1:3, 1:6) as used in previous studies (Liang, et al. 2018). The results showed that MRAP2a decreased the surface expression of both MC5Ra and MC5Rb, whereas MRAP2b had no significant effect (Figure 2, E-H). Since the results were different from previous studies in human (Chan et al. 2009; Sebag and Hinkle 2009), then
we validated the surface expression of mammalian MC5Rs in the absence or presence of mammalian MRAP2s. These results showed that human MRAP2 decreased the surface expression of human MC5R like MRAP2a, but mouse MRAP2 had no significant influence on the surface expression of mouse MC5R, which was similar to the results of MRAP2b in zebrafish (Figure 2, I-J).

3.4 MRAP2a and MRAP2b modulate signaling of MC5Ra and MC5Rb in different ways

To determine how MRAP2a and MRAP2b affect MC5Rs signaling, CRE-luciferase reporter assay was performed to detect the cAMP level stimulated by α-MSH through MC5Rs. We found that MRAP2a dose-dependently inhibited the efficacy of both MC5Ra and MC5Rb, MRAP2b inhibited MC5Ra efficacy but increased the maximal stimulated efficacy of MC5Rb (Figure 3, A-D). To test whether this effect was stable with other agonist, similar results were seen when stimulated with SHU9119, another reported MC5R agonist (Figure 3, E-H). We also tested the effect of mouse MRAP2 on MC5R and found that MRAP2 dose-dependently inhibited the efficacy of mouse MC5R (Figure 3, I and J), which was similar to zebrafish MC5Ra. However, both MRAP2a and MRAP2b did not affect the agonists and antagonists signaling competing affinity (Figure 3, K-N). LogEC50 values of each curve were shown in Table 1.

3.5 Dimerization of zebrafish MC5Rs

Human MC5R has been reported to form homodimers, which could be disrupted by MRAP (Sebag and Hinkle 2009). To assess the ability of dimerization of two zebrafish MC5Rs, YFP fragments were fused to C-terminal of zMC5Ra and zMC5Rb. zMC5R-YFP-F1 and zMC5R-YFP-F2 were co-expressed in CHO cells and the YFP fluorescence indicated the presence of zMC5Ra and zMC5Rb homodimers on cell surface (Figure 4A-B). Unexpectedly, zMC5Ra and zMC5Rb could also form heterodimers (Figure 4C). However, when co-expressed with MRAP2a or MRAP2b, the YFP fluorescence could barely be detected (Figure 4, D-I). Thus we hypothesized that
MRAP2a and MRAP2b could disrupt dimerization of zMC5Rs just like the effect of MRAP on human MC5R. To validate our hypothesis, we counted the fluorescent cells by FACS (Fluorescence Activated Cell Sorter). Compared to control groups that co-expressed with RAMP3, another reported transmembrane protein that did not regulate melanocortin receptor trafficking (Sebag and Hinkle 2009), zMRAP2a and zMRAP2b significantly decreased the percentage of YFP fluorescent cells (Supplementary figure 2). Thus, zMRAP2a and zMRAP2b could disrupt the dimerization of both zMC5Rs homodimers and heterodimers, similar to the effect of MRAP on human MC5R.

3.6 Fasting increased the expression of mc5ra in zebrafish brain

The melanocortin system may be involved in regulating food intake of fish, but no experiments had been carried out to evaluate whether the progressive fasting could affect the expression of MC5Rs in zebrafish. We fasted wild type zebrafish for 2, 10 and 15 days, and detected the mRNA level by qRT-PCR. After 2 to 10 days fasting, the expression of mc5ra in zebrafish brain increased 4-fold, but mc5rb showed no significant differences (Figure 5).

4. DISCUSSION

The fish specific genome duplication (FSGD or 3R) occurred about 350 million years ago, thus zebrafish experienced an additional genome duplication than human (Meyer and Van de Peer 2005), which caused several gene duplications in zebrafish genome, such as mrap2a and mrap2b. Although MRAP2b seems more like mammalian MRAP2, MRAP2a can also regulate MC4R in a different way, both of them are functional in the control of zebrafish early development (Sebag et al. 2013). Following the three whole genome duplication event, chromosome reduction and gene loss, the only duplicated melanocortin receptor genes left in the zebrafish genome are the mc5ra and the mc5rb, and all the other zebrafish melanocortin receptor genes are present in single copy. Actually, the function of MC5Rs have not been elucidated in fish. Results of amino
acid sequences demonstrated that the MC5Ra was more homologous to mammals
(Supplementary figure 1). Zebrafish MC5Ra shared more than 78% amino acid identity
with several other teleost MC5Rs, including tetraodon and fugu MC5Rs, and it was
61.27% identical to hMC5R. The amino acid sequences of MC5Rs were highly conserved
at 7 hydrophobic transmembrane domains. Phylogenetic analysis based on amino acid
sequences revealed that MC5Ra was closer to hMC5R. Synteny analysis further
demonstrated that genes locating in the upstream region of mc5ra in zebrafish were
highly conserved compared with those in spotted gar, human, mouse and rat, we
hypothesized that the transcriptional regulation of mc5ra could be more conserved than
mc5rb. Sequence alignment of multiple MC5Rs could not differentiate them, thus we
attempted to find the differences between MC5Ra and MC5Rb by exploring the
regulating relationship between MRAP2s and MC5Rs. Previous studies have
demonstrated that MRAP and MRAP2 can interact with MC5R, suppress MC5R activity
by decreasing the cell surface expression level (Chan et al. 2009; Sebag and Hinkle
2009). Chan et al. observed that hMRAP2 decreased hMC5R trafficking while we
observed that mMRAP2 did not effect mMC5R trafficking. These data suggest that there
appears to be two types of MRAP2 in mammals, and the alternative effects are species
specific.

Our results suggested that the interactions between MRAP2s and MC5Rs were
conserved in zebrafish. Although both MRAP2a and MRAP2b could interact with
melanocortin receptors in zebrafish (Agulleiro et al. 2010; Cortes, et al. 2014; Sebag et al.
2013), they had distinct functions on modulating the signaling of receptors. As same as
mouse MRAP2, MRAP2b had no influence on the surface expression of MC5R. Previous
studies showed that MRAP2b could inhibit the adult zebrafish MC4R constitutively
activity but increase ligand-dependent activity (Sebag et al. 2013). Like MRAP2b, mouse
MRAP2 could increase the signaling of mouse MC4R, so MRAP2b was considered as
the zebrafish ortholog of MRAP2 (Asai et al. 2013).
We further verified that MRAP2a and MRAP2b could modulate the signaling of MC5Ra and MC5Rb in different ways. MRAP2a could inhibit maximal activity of both MC5Ra and MC5Rb, which might be explained by decreased cell surface expression. To our surprise, MRAP2b also inhibited MC5Ra activity in zebrafish without changing its surface expression, and unexpectedly increased MC5Rb activity. MC5R was reported to form homodimers and heterodimers with other GPCRs (Kobayashi et al. 2016; Sebag and Hinkle 2009). To elucidate the pharmacological mechanism of MC5Rs modulated by MRAP2s, we observed the dimerization of MC5Rs. zMC5Ra could form homodimers on cell membrane, but homodimers of zMC5Rb and heterodimers of zMC5Ra and zMC5Rb were trapped in endoplasmic reticulum. MRAP2 could disrupt MC5R homodimers (Sebag and Hinkle 2009), we observed the same phenomena, that both homodimers and heterodimers were decreased or disappeared when co-expressed with MRAP2a or MRAP2b. Thus MRAP2a or MRAP2b could modulate MC5Rs signaling by regulating their dimerization. Recent studies suggested that α-MSH could regulate muscle glucose intake via MC5R-PKA pathway (Enriori et al. 2016), which might help us understanding why mc5ra mRNA level increased 4-fold during fasting. Promoter region is crucial in the transcription regulation, synteny analysis suggested that mc5ra had a more conserved far upstream sequence compared to mc5rb.

In summary, by parallel comparison of the evolution and regulation relationship between MRAP2 and MCRs family, our studies suggested that mc5ra was more homologous to mammalian MC5R. Further work is needed to elucidate the physiological roles of each mc5r by cas9 specific knock-out in the zebrafish.

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Declaration of interest

The authors declare no conflict of interest.
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FIGURE LEGENDS

Figure 1: Phylogenetic and synteny analysis of zebrafish MC5R receptors.

(A) Phylogenetic tree of MC5Rs constructed by NJ method with Mega 6.0 software. Jones-Taylor-Thornton (JTT) model was used. The strength of branch relationships was assessed by bootstrap replication (N =1,000 replicates). Asterisk (*) indicates zebrafish mc5ra and mc5rb. GenBank or NCBI Reference Sequence accession numbers for MC5Rs: human Homo sapiens (AAH95531.1), western gorilla Gorilla gorilla (XP_004059266.1), chimpanzee Pan troglodytes (NP_001009119.1), hamadryas baboon Papio hamadryas (ACO90015.1), rabbit Oryctolagus cuniculus (CCX35395.1), American mink Neovison vison (AGT56096.1), red fox Vulpes vulpes (ABG48760.1), dog Canis lupus familiaris (NP_001074193.1), raccoon dog Nyctereutes procyonoides (ABO38182.1), Norway rat Rattus norvegicus (NP_037231.1), house mouse Mus musculus (NP_058673.2), pig Sus scrofa (NP_999338.1), sheep Ovis aries (NP_001119842.1), goat Capra hircus (NP_001272520.1), cattle Bos Taurus (NP_776535.1), water buffalo Bubalus bubalis (AHN49803.1), domestic yak Bos grunniens (ADH51715.1), wild yak Bos mutus (ADZ24287.1), American alligator Alligator mississippiensis (KYO35523.1), Ring-necked pheasant Phasianus colchicus (ACF35264.1), chicken Gallus gallus (NP_001026685.1), hazel grouse Tetrastes bonasia (ACF35263.1), common carp Cyprinus carpio (CBX89936.1), goldfish Carassius auratus (CAD58853.1), Ya-fish Schizothorax prenanti (AGF80338.1), zebrafish Danio rerio (NP_775387.1) and (NP_775386.1).

(B): Synteny analysis of MC5Rs. The adjacent genes of zebrafish mc5ra or mc5rb were as follow: rnmt, mRNA cap guanine-N7 methyltransferase; fam210aa, uncharacterized protein C18orf19 homolog A; cthrc1a, Collagen triple helix repeat containing 1a; scl25a32a, Scl25a32a protein; fam210ab, uncharacterized protein C18orf19 homolog B; ldlrad4b, low-density lipoprotein receptor class A domain-containing protein 4-like; mc2r, melanocortin 2 receptor; lmna, lamin. Genes in
blue color are conserved among zebrafish, spotted agar, human, mouse, and rat. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

**Figure 2: MRAP2s interact and regulate surface expressions of MC5Rs.**

Co-Immunoprecipitation assay exhibits the interactions between zMC5Ra and zMRAP2a (**A**), zMRAP2b (**B**), also between zMC5Rb and zMRAP2a (**C**), zMRAP2b (**D**). *, IgG heavy chain. Surface expression of zMC5Ra and zMC5Rb measured by whole-cell ELISA in 293T cells transfected with zMC5Ra or zMC5Rb and different amounts of zMRAP2a or zMRAP2b (**E-H**). Data are shown as mean ± SEM and analyzed by two-tailed t test. **, *p* < 0.01; ***, *p* < 0.001. Surface expression of mouse MC5R measured by whole-cell ELISA in 293T cells transfected with mouse MC5R and different amounts of mouse MRAP2 (**I**).

**Figure 3: Pharmacological modulation of MC5Rs by MRAP2s.**

Dose response curves of α-MSH induced cAMP production in 293T cells upon transfection with zMC5Ra (**A-B**) or zMC5Rb (**C-D**) in presence of different amount of zMRAP2a or zMRAP2b. Dose response curves of SHU9119 induced cAMP production in 293T cells transfected with zMC5Ra (**E-F**) or zMC5Rb (**G-H**) in presence of different amount of zMRAP2a or zMRAP2b. Dose response curves of α-MSH (**I**) and SHU9119 (**J**) induced cAMP production in 293T cells transfected with mouse MC5R and the indicated amount of mouse MRAP2. Binding competition of agonist (α-MSH) and antagonist (AgRP) of MC5Rs modulated by MRAP2s (**K-N**).

**Figure 4: Zebrafish MC5R forms homo- and hetero- dimers.**

YFP fluorescence (green) of zMC5Ra homodimers (**A**), zMC5Rb homodimers (**B**) and zMC5Ra zMC5Rb heterodimers (**C**) (Scale bar = 10 µm). Effect of zMRAP2a and
zMRAP2b on zMC5Rs dimerization. CHO cells co-expressing zMC5Ra homodimers (D-E), zMC5Rb homodimers (F-G) or zMC5Rs heterodimers (H-I) with zMRAP2a (D, F, H) or zMRAP2b (E, G, I). Surface expression of zMRAP2a and zMRAP2b is shown in red, detected by anti-V5 antibody and secondary anti-mouse Alexa594 (abcam). MRAP2 suppressed the dimer formation of MC5R proteins.

Figure 5: Fasting induced expression alternation of MC5R in zebrafish brain.

Adult zebrafish (TU) were fasted 0, 2, 10, 15 days and the mc5ra and mc5rb expression in the brain were analyzed by qRT-PCR. Data are analyzed by two-tailed t test compared with Day 0, graphs are shown as mean ± SEM. **, \( p < 0.01 \); ***, \( p < 0.001 \); ns, not significant.

Supplementary figure 1: Amino acid alignment of multiple MC5R sequences.

MC5Rs from multiples species were analyzed with MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). The alignment was colored with DNAMAN software (Lynnon Biosoft). ENSEMBLE or GenBank or NCBI Reference Sequence accession numbers for MC5Rs: human Homo sapiens (ENSP00000318077), house mouse Mus musculus (ENSMUSP00000130497), cow Bos taurus (ENSBTAP00000012049), chicken Gallus gallus (ENSGALP00000049188), xenopus Xenopus tropicalis (ENSXETP00000057015), fugu Takifugu rubripes (ENSTRUP00000032203), tetraodon Tetraodon nigroviridis (AAQ55179), zebrafish Danio rerio MC5Ra (ENSDARP00000040072) and MC5Rb (ENSDARP00000071694), elephant shark Callorhinchus milii (XP_007893314) and mud shark Squalus acanthias (AAS67890). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Supplementary figure 2: FACS analysis of YFP fluorescent cells.

Percent of YFP+ cells co-expressing zMC5Ra homodimers (A), zMC5Rb homodimers (B) and zMC5Ra zMC5Rb heterodimers (C) with RAMP3 (negative control), MRAP2a and MRAP2b. Data were analyzed by two-tailed t test, graphs are shown as mean ± SEM, ***, p < 0.001 compared with control group.
**mock zMC5Ra**

**mock zMC5Rb**

**Surface M C5Ra** (% MC5Ra alone)

**Surface M C5Rb** (% MC5Rb alone)

**Surface M C5R** (% hMC5Ra lone)

**Surface M C5R** (% hMC5Rb lone)
Fold change to day 0

Day

mc5ra
mc5rb

0 2 10 15

0 2 10 15

*** *** ** ns
Table 1. Analyze data in figure 3

<table>
<thead>
<tr>
<th>Analyze Data in Fig.3</th>
<th>LogEC50</th>
<th>P value for Vmax comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:0</td>
<td>1:3</td>
</tr>
<tr>
<td>A zMC5Ra : zMRAP2a</td>
<td>-9.84±0.16</td>
<td>-9.58±0.20</td>
</tr>
<tr>
<td>B zMC5Ra : zMRAP2b</td>
<td>-9.87±0.21</td>
<td>-9.09±0.18</td>
</tr>
<tr>
<td>C zMC5Rb : zMRAP2a</td>
<td>-9.53±0.12</td>
<td>-8.91±0.09</td>
</tr>
<tr>
<td>D zMC5Rb : zMRAP2b</td>
<td>-9.11±0.16</td>
<td>-9.28±0.22</td>
</tr>
<tr>
<td>E zMC5Ra : zMRAP2a</td>
<td>-10.8±0.32</td>
<td>-10.88±0.39</td>
</tr>
<tr>
<td>F zMC5Ra : zMRAP2b</td>
<td>-10.49±0.27</td>
<td>-10.65±0.30</td>
</tr>
<tr>
<td>G zMC5Rb : zMRAP2a</td>
<td>-11.09±0.12</td>
<td>-10.54±0.11</td>
</tr>
<tr>
<td>H zMC5Rb : zMRAP2b</td>
<td>-10.82±0.18</td>
<td>-10.59±0.30</td>
</tr>
<tr>
<td>I mMC5R : mMRAP2(a-MSH)</td>
<td>-10.12±0.41</td>
<td>-9.03±0.19</td>
</tr>
<tr>
<td>J mMC5R : mMRAP2(SHU9119)</td>
<td>-9.82±0.14</td>
<td>-9.01±0.09</td>
</tr>
<tr>
<td>K zMC5Ra : zMRAP2a</td>
<td>-8.02±0.17</td>
<td>-8.31±0.09</td>
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<tr>
<td>L zMC5Ra : zMRAP2b</td>
<td>-8.15±0.15</td>
<td>-8.03±0.12</td>
</tr>
<tr>
<td>M zMC5Rb : zMRAP2a</td>
<td>-7.50±0.29</td>
<td>-7.87±0.14</td>
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<tr>
<td>N zMC5Rb : zMRAP2b</td>
<td>-7.73±0.44</td>
<td>-7.30±0.42</td>
</tr>
</tbody>
</table>

Statistics were measured using a two-way ANOVA with Tukey post-test.