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Pharmacological modulation of melanocortin-4 receptor by melanocortin receptor accessory protein 2 in Nile tilapia

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ABSTRACT

The melanocortin-4 receptor (MC4R) acts as a member of G-protein coupled receptors and participate in food intake and energy expenditure. Melanocortin 2 receptor accessory protein 2 (MRAP2) plays a critical role in regulating MC4R signaling in mammals and zebrafish. However, evidence on their interaction in other teleost species remains elusive. Here, we cloned and assessed the evolutionary aspect and pharmacological modulation of MRAP2 on MC4R signaling in Nile tilapia (Oreochromis niloticus). Tissue distribution analysis of *tmc4r* and *tmrap2* confirmed their co-expression in the brain region. tMRAP2 protein could form antiparallel homo-dimer and directly interacted with tMC4R in vitro and presence of tMRAP2 led to the reduction of agonist response and surface expression of tMC4R. Overall, our findings provide a comparative overview on the evolutionary conservation, genomic distribution, tissue-specific expression and pharmacological profile of the MC4R and MRAP2 in another non-mammalian teleost.

1. Introduction

Melanocortins, derived from pro-opiomelanocortin (POMC) derived small peptides, are mainly composed of three melanocyte-stimulating hormones (α-, β- and γ-MSH) and adrenocorticotropic hormone (ACTH) (Nakanishi et al., 1979). The physiological functions of these peptides are mediated by a family of G-protein-coupled receptors that positively couple to adenylyl cyclase and cAMP signaling cascades (Cone, 2006). Generally, the genomes of some teleosts and most mammals encode five melanocortin receptors (MC1R–MC5R) (Zhang et al., 2017). However, zebrafish have six melanocortin receptors with two MC5Rs while fugu lacks MC3R (Cerda-Reverter et al., 2011; Logan et al., 2003). For the receptor stimulation, teleost and tetrapod MC2R orthologs are specific for ACTH (Agulleiro et al., 2010; Liang et al., 2011) while the other four receptors can all be activated by either ACTH or the MSH-sized ligands (Schiöth et al., 2005). Furthermore, MCR signaling is not only regulated by the binding of endogenous agonists, but also by naturally competing antagonists, agouti-signaling protein (ASIP) and agouti-related protein (AgRP) (Cone, 2006).

Each MCR has a tissue-specific expression pattern and binds to differing melanocortin peptides, which involves in multiple physiologic functions. For example, MC4R is expressed predominantly in the central nervous system and mediates the effects of melanocortins on the food intake as MC4R-knockout mice display severe alterations in energy homeostasis (Huszar et al., 1997). Interruption of central melanocortin signaling by ectopic constitutive expression of agouti gene (Ay) results in hyperphagia, hyperinsulinemia, increased linear growth, maturity-onset obesity and yellow fur (Lu et al., 1994). The same disease is also observed in transgenic mice with overexpressing AgRP (Ollmann et al., 1997), and in MC4R-knockout mice (Huszar et al., 1997). In addition, central administration of the C-terminal fragment of AgRP (Rossi et al., 1998) increases food intake in rodents, and intracerebroventricular (ICV) injections of the MCR agonist, MTII, produces a dose-dependent reduction in food intake (Fan et al., 1997). However, MC4R-deficient mice do not respond to the anorectic effects of MTII, suggesting that α-MSH inhibits feeding primarily by activating MC4R in the hypothalamus (Marsh et al., 1999).

Melanocortin 2 receptor accessory protein 2 (MRAP2), a small single transmembrane-domain protein, is a unique homologue of MRAP (Chan et al., 2009). Tetrapod species have two MRAP paralogues (MRAP1 and MRAP2). MRAPs evolutionarily first appear in the genome of sea lamprey (Vastermark and Schioth, 2011). Our previous study has showed that sea lamprey MRAP2 protein lacks the long carboxyl terminus and directly interacts and decreases the surface expression but enhances the α-MSH induced agonism of MCa and MCb of sea lamprey (Zhu et al., 2019). However, due to extra round of genomic duplication,

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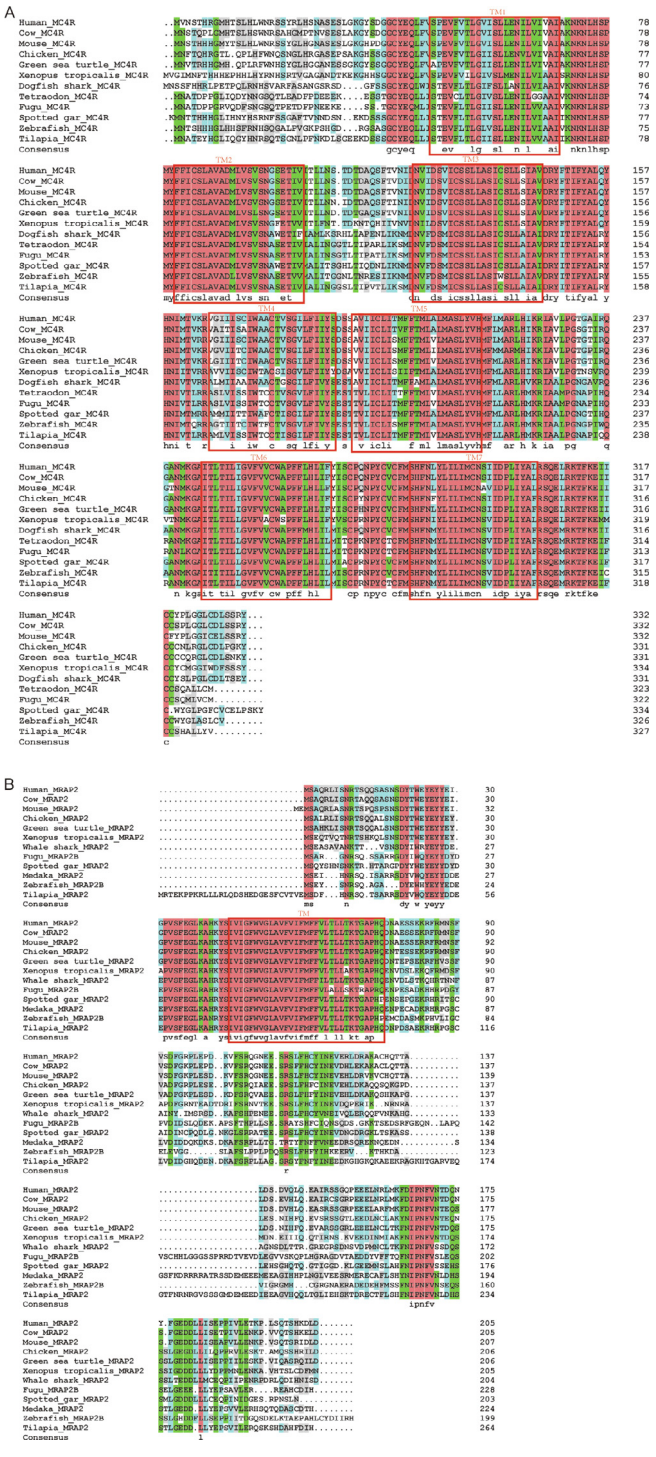

Fig. 1. Amino acid alignment of multiple MC4R protein sequences. MC4Rs from multiple species were analyzed with MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). The alignment was colored with DNAMAN software (Lynnon Biosoft). A: ENSEMBLE, GenBank or NCBI accession numbers for MC4Rs: human Homo sapiens (NP_005903.2), mouse Mus musculus (NP_058673.2), cow Bos taurus (NP_776535.1), chicken Gallus gallus (NP_001026685.1), Green sea turtle (XP_007066776.1), Xenopus tropicalis (XP_004915370.1), Dogfish shark (AAO39833.1), tetraodon (ENSTNIG00000008689), fugu Takifugu rubripes (NP_001027732.1), tilapia Oreochromis niloticus (XP_005449158.1), spotted gar (Lepisosteus oculatus) (ENSLOCG00000018161) and zebrafish Danio rerio (NP_775385.1). B: ENSEMBLE, GenBank or NCBI accession numbers for MRAP2s: human Homo sapiens (NP_612418), mouse Mus musculus (NP_001094952.2), cow Bos taurus (NP_001092863.1), chicken Gallus gallus (KT183012), Green sea turtle (XP_007052626.2), Frog Xenopus tropicalis (XP_002933963), Dogfish shark (XP_020377388.1), fugu Takifugu rubripes (ENSTRUG00000018262), medaka (XP_020570371.1), tilapia Oreochromis niloticus (XP_003458293.2), zebrafish Danio rerio MRAP2a (zfMRAP2a, F8W4H9.1) and MRAP2b (zfMRAP2b P0DM64) and spotted gar (XP_006626090.2).

some teleost species can have additional paralogues. For example, MRAP2 is duplicated in zebrafish as MRAP2a and MRAP2b (Sebag et al., 2013; Zhu et al., 2018). MRAP2a and MRAP2b show distinct pharmacological effects on MC4R and MC5Rs activity in zebrafish (Sebag et al., 2013; Zhu et al., 2018). MRAPs have been shown to modulate MCR functions in vitro and in vivo. For instance, MRAP2 can interact with MC4R and modulate MC4R-mediated cAMP signaling (Asai et al., 2013; Chan et al., 2009), while MRAP is crucial for MC2R trafficking, ACTH binding and downstream signaling (Dores et al., 2016; Sebag and Hinkle, 2007). Mice with whole-body and brain-specific deletion of MRAP2 develop early onset severe obesity, due to the impaired central MC4R signaling (Asai et al., 2013). Taken together, all these findings reveal a more complex interaction of MC4R with α-MSH, AgRP and MRAP2 in the regulation of energy homeostasis (Cone, 2006; Ghamari-Langroudi et al., 2015).

We are interested in the Nile tilapia (Oreochromis niloticus), an Africa originated and one of most consumed aquaculture species worldwide, because we find that the tilapia also lacks MC3R, the same as fugu. Since MC3R and MC4R are both involved in energy balance in many species, the absence of MC3R makes the function of tilapia MC4R appear to be more important and vague. Moreover, unlike zebrafish, only one MRAP2 is found in tilapia genome and the interaction between tMC4R and tMRAP2 is largely unknown, hence worth exploring. In this study, we first performed alignment and synteny analysis of MC4R and MRAP2 to map orthologs of MC4R and MRAP2 from tilapia to other species. Furthermore, mRNA expression revealed the relevance and coexistence of *tmc4r* and *tmrap2* in vivo. Immuno-precipitation and immuno-colocalization studies showed that the unique tMRAP2 anti-parallel homo-dimer physically interacted with tMC4R in vitro. In addition, we also illustrated the pharmacological regulation of tMC4R by tMRAP2. The α-MSH, SHU9119 or AgRP induced alternation of tMC4R activity appeared to be modulated by the co-expression of tMRAP2. Overall, our results provide a comparative insight into the pharmacological properties of MC4R and MRAP2 in Nile tilapia, which may contribute significantly to better understanding for physiological function of MC4R signaling in teleost evolution.

2. Methods and materials

2.1. Homology and phylogenetic analysis

Preparatory multiple sequence alignments of Tilapia MC4R and MRAP2 sequences were performed using MUSCLE 3.8.31 with default parameters (Edgar, 2004). Phylogenetic tree based on the amino acid sequences was constructed by Neighbor-joining (NJ) methods with 1000 bootstrap resampling and Poisson correction model, pairwise

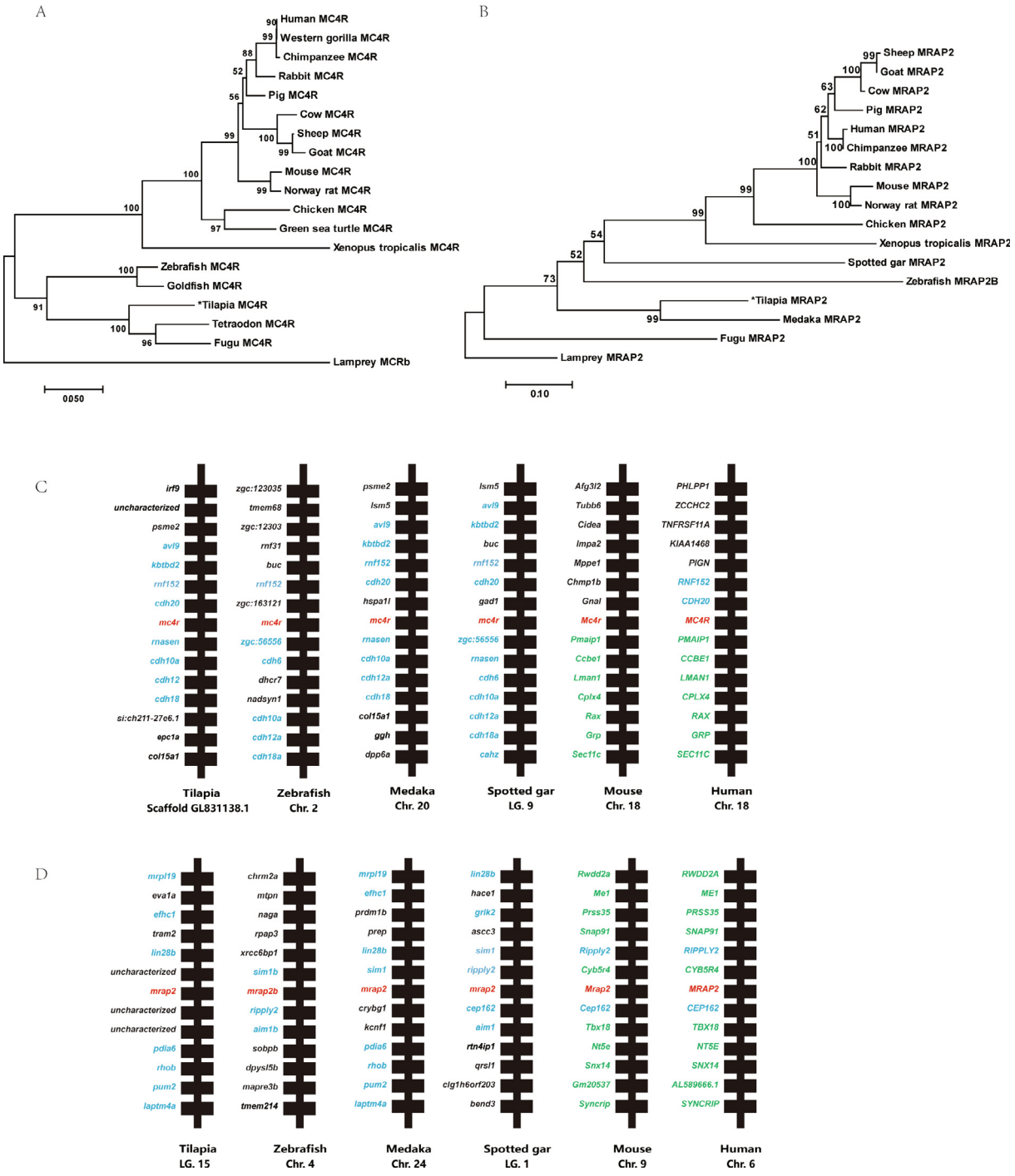

Fig. 2. Synteny and phylogenetic analysis of tilapia MC4R and MRAP2. Phylogenetic tree of MC4Rs (A) and MRAP2s (B) was constructed by NJ method and Jones-Taylor-Thornton (JTT) model with Mega 6.0 software. The strength of branch relationships was assessed by bootstrap replication (N = 1000 replicates). Asterisk (*) indicates tilapia MC4R and MRAP2. B: GenBank or NCBI accession numbers for MC4Rs: human Homo sapiens (NP_005903.2), western gorilla Gorilla gorilla (XP_004059534.1), chimpanzee Pan troglodytes (NP_001153207.1), rabbit Oryctolagus cuniculus (XP_002713662.1), pig Sus scrofa (NP_999338.1), cow Bos taurus (NP_776535.1), sheep Ovis aries (NP_001119842.1), goat Capra hircus (NP_001272520.1), mouse Mus musculus (NP_058673.2), Norway rat (NP_037231.1), chicken Gallus gallus (NP_001026685.1), Green sea turtle (XP_007066776.1), Frog Xenopus tropicalis (XP_004915370.1), zebrafish Danio rerio (NP_775385.1), goldfish Carassius auratus (CAD58853.1), Tilapia Oreochromis niloticus (ENSONIP00000025763), Tetraodon (AAQ55178.1), fugu Takifugu rubripes (NP_001027732.1), Lamprey (ABB36647.1). C: GenBank or NCBI accession numbers for MRAP2s: sheep Ovis aries (NP_001119842.1), goat Capra hircus (NP_001272520.1), cow Bos taurus (NP_001092863.1), pig Sus scrofa (NP_999338.1), human Homo sapiens (NP_001333471.1), chimpanzee Pan troglodytes (NP_001153207.1), rabbit Oryctolagus cuniculus (XP_002713662.1), mouse Mus musculus (NP_001171202.1), Norway rat (NP_037231.1), chicken Gallus gallus (ALO81626.1), Xenopus (XP_002933963.1), zebrafish Danio rerio mrap2a (ENSDARP00000124181), Spotted gar (ENSLOCP00000020464), Tilapia Oreochromis niloticus (XP_003458293.2), Medaka (ENSORLP00000016039), fugu Takifugu rubripes (ENSTRUP00000046738), Lamprey (FAA00710.1). Synteny analysis of MC4Rs (C) and MRAP2s (D). The adjacent genes of tilapia MC4R or MRAP2 were: cdh10a, cadherin 10; cdh12, cadherin 12; cdh18, cadherin 18; rnf152, ring finger protein 152; cdh20, cadherin 20. Zgc indicates zebrafish gene collection; sobpb, sine oculis binding protein homolog (Drosophila) b; kcnf1a, potassium voltage-gated channel, subfamily F, member 1a; grik2, glutamate ionotropic receptor kainate type subunit 2; lin28b, lin-28 homolog B (C. elegans); sim1a, single-minded family bHLH transcription factor 1a. Zgc indicates zebrafish gene collection. Genes in blue color are conserved among teleosts, and genes in green color are conserved between human and mouse.

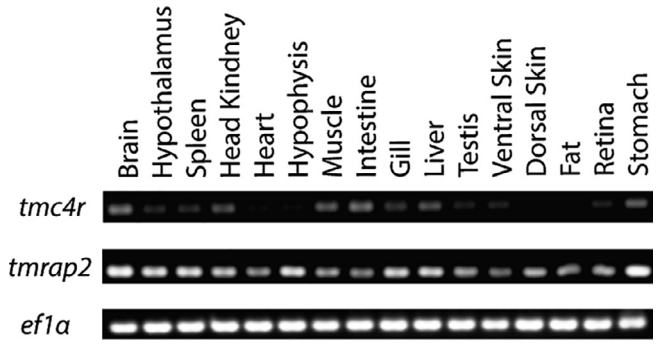

Fig. 3. Tissue distribution of *tmc4r* and *tmrap2 tmc4r* and *tmrap2* mRNA expression pattern from multiple tilapia tissues was determined by RT-PCR. The *ef1α* mRNA was used as an internal control.

deletion option using Mega 7.0 software. Synteny analysis was performed between tilapia, zebrafish, medaka, spotted gar, human and mouse with ensemble databases (http://www.ensembl.org/index.html).

2.2. Tissue expression analysis

Real-time quantitative reverse transcription PCR (RT-PCR) was carried out as previously described (Agulleiro et al., 2010). Briefly, cDNA was synthesized as above from RNA purified from fresh tissues (brain, hypothalamus, spleen, head kidney, heart, hypophysis, muscle, gills, liver, testis, ventral skin, dorsal skin, fat, retina and stomach) of adult tilapia. Total RNA was isolated using TRI-reagent (Sigma) and cDNA was synthesized using FastQuant RT Kit (with gDNase) (TIANGEN). Primers for targeted genes were synthesized from GENEWIZ and PCR fragments for each gene were separated on 1.5% agarose gel. PCR for elongation factor-1α (*ef1a*) mRNA was carried out as internal control. Primers used for RT-PCR cloning of tMC4R and tMRAP2. tMC4R_fw: TTCTATGCCCTGCGATAC;tMC4R_rev: CAGTGGTGCTTTCCGAGT;tMRAP2_fw: GCACCACATCAAGATAACC;tMRAP2_rev: GTGACCCTTGTCTTCCTC;EF1α_fw: GGCTCCTTCAAGTACGCCT;EF1α_rev: CGAACTCGCCAACACCAG.

2.3. 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. Cells were maintained in a humidified atmosphere consisting of 5% CO_2 at 37 °C. For transfection, when cells growth to 70%–80% confluence, plasmids were transfected using ViaFect Transfection Reagent (Promega).

2.4. Co-immunoprecipitation and western blotting

Proteins were N-terminally tagged with 2 × Flag (DYKDDDDKDDDDKC) or 3 × HA (YPYDVPDYAYPYDVPDYAYPYDVPDYAD) epitopes. 3HA-tMC4R and 2Flag-tMRAP2 were constructed into vector pcDNA3.1 (+) (Invitrogen). After 24 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 the cell lysates the 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% SDS-PAGE gels were applied and anti-HA (Abcam) and anti-Flag (Abclonal) antibodies were used at a dilution factor of 1:5000 for immunoblotting.

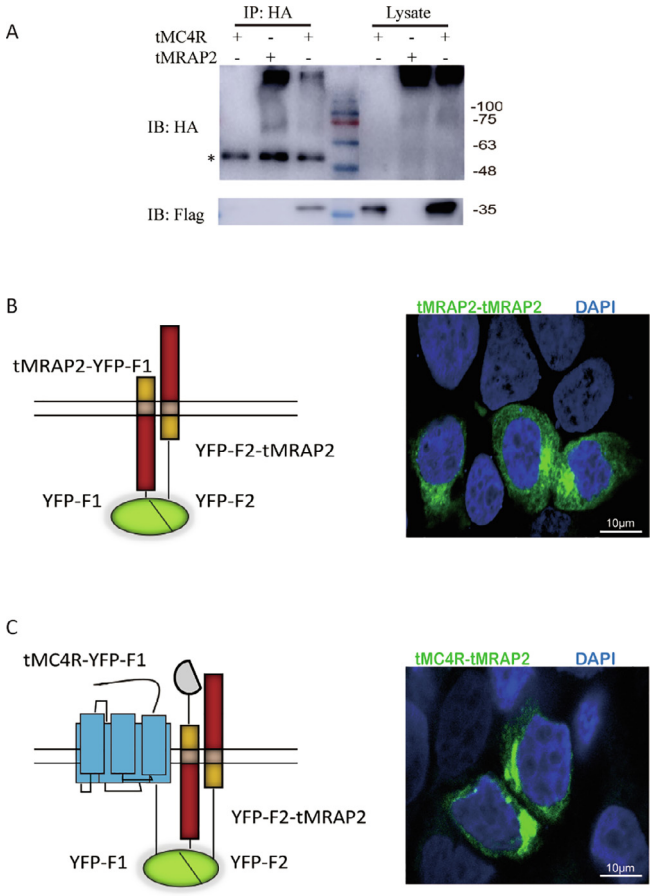

Fig. 4. Interaction of tMRAP2 protein with tMC4R. A: Co-Immunoprecipitation assay exhibited the interactions of tMC4R and tMRAP2. *, IgG heavy chain. YFP fluorescence imaging was performed in HEK293T cells transfected with plasmids encoding tMRAP2-YFP-F1 and YFP-F2-tMRAP2 (B) or tMC4R-YFP-F1 and F2-YFP-tMRAP2 (C). B:YFP fluorescence (green) of tMRAP2 antiparallel homodimer. C: YFP fluorescence (green) of tMC4R-tMRAP2 protein complex. (Scale bar = 10 μm) Nuclei were counterstained in blue (DAPI).

2.5. Fluorescence complementation assay

Proteins were N- or C-terminally tagged with two non-fluorescent fragments of YFP (F1 and F2). HEK293 cells grown onto poly-l-lysine-coated coverslips were transiently transfected with F1-tMC4R, F1-tMRAP2 and tMRAP2-F2. 24 hours later, cells were fixed with paraformaldehyde for 20 minutes. 2 μM DAPI (4′,6-diamidino-2-phenylindole) was used to stain nuclei for 10 min in dark. Cells were washed in phosphate saline buffer (PBS). Coverslips were then mounted in medium for fluorescence. Fluorescent signal were examined with a laser-scanning Zeiss confocal microscopy (LSM880) and a 60× oil objective.

2.6. Cell surface ELISA

To measure cell surface receptor expression, HEK293T cells were seeded in poly-l-lysine-coated 12-well plate and transfected independently with pcDNA3.1/3HA-tMC4R, pcDNA3.1/2Flag-tMRAP2 with different proportion (1:0, 1:3 and 1:6). 24 hours after transfection, cells were washed with PBS, fixed at room temperature for 20 min with 4% para-formaldehyde. After fixation, cells were washed, blocked for 30 min in 5% milk in D-PBS, incubated with anti-HA antibody (Abcam) for 2 hours at room temperature and 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

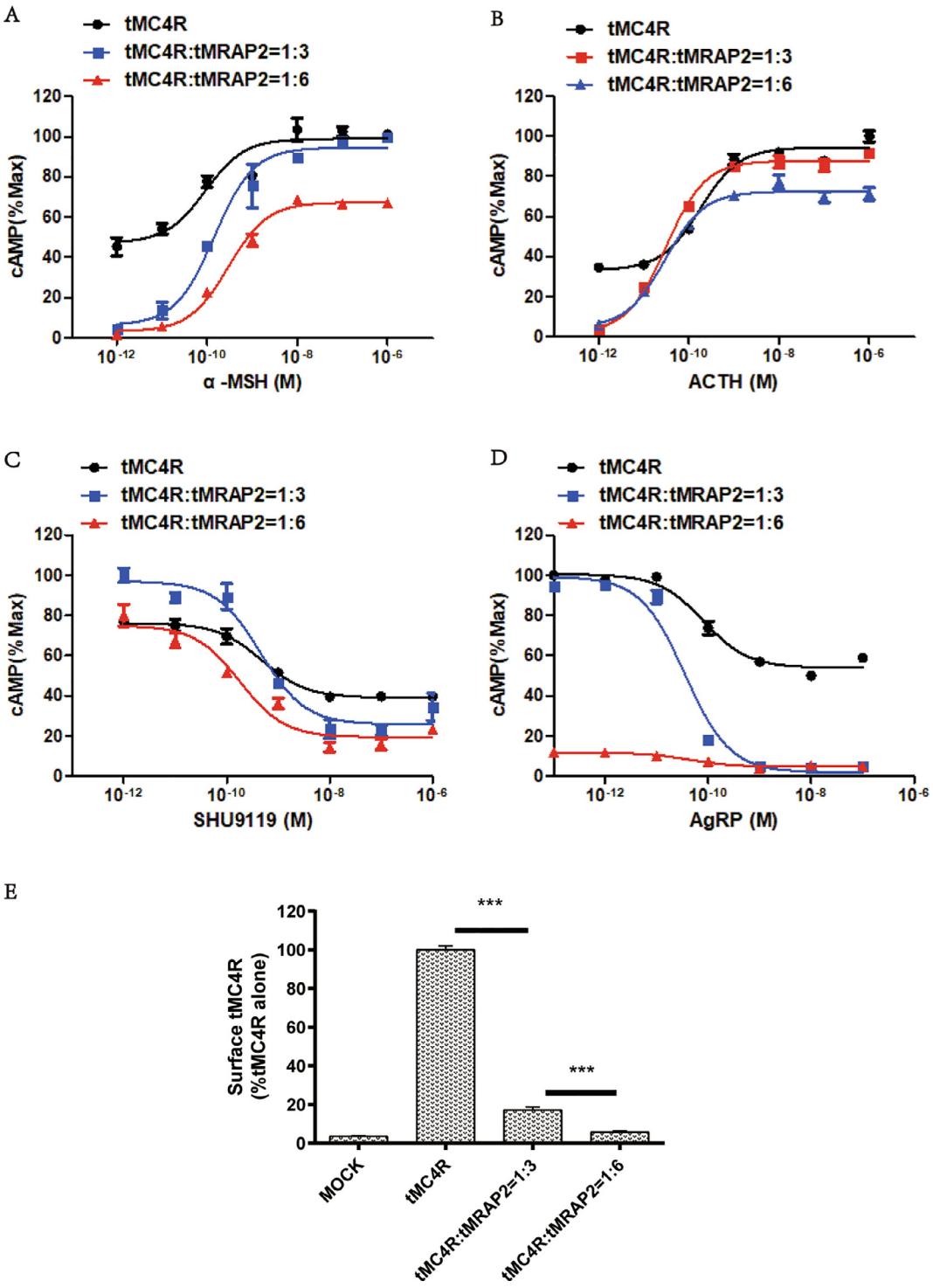

Fig. 5. Signaling modulation of tMC4R by tMRAP2. Concentration-response curves of α-MSH (A) or ACTH (B) induced cAMP production in HEK293T cells upon transfection with tMC4R in presence of different amount of tMRAP2. Binding competition of agonist (α-MSH) and antagonist, SHU9119 (C) or AgRP (D) of tMC4R modulated by tMRAP2. Surface expression of tMC4R was measured by whole-cell ELISA in HEK293T cells transfected with tMC4R and different amounts of tMRAP2 (E). Data are shown as mean ± SEM and analyzed by two-tailed t test. **, $p < 0.01$; ***, $p < 0.001$.

by adding 2 M H_2SO_4. Spectramax M5 plate reader was used to measure OD (optical density) at 450 nm. Experiments were repeated independently in triplicate.

2.7. cAMP measurement

HEK293T cell were plated into 24 well plates and transfected with different proportion (1:0, 1:3 and 1:6) of tMC4R and tMRAP2 plasmids in next day. Experiments were always started 24 h post-transfection. Cells were treated with α-MSH, ACTH(1–39), SHU9119(Ac-Nle-c[Asp-His-DNal (2′)-Arg-Trp-Lys]-NH2) or AgRP(83–132) in DMEM supplemented with 0.1% bovine serum albumin for 4 hours at 37 °C. Concentration of α-MSH or ACTH ranged from 10^{-12} M to 10^{-6} M, concentration of SHU9119 ranged from 10^{-13} M to 10^{-7} M. Inhibition assay was tested in HEK293T cells stimulated by 2×10^{-9} M α-MSH and AgRP with final concentration ranging from 10^{-13} M to 10^{-7} M.

Table 1
Statistical analysis of tMC4R-tMRAP2 in response to different ligands.

Statistical analysis of Fig. 3		EC50/IC50 (nM)			P value for Vmax comparison		
		1:0	1:3	1:6	1:0 vs. 1:3	1:0 vs. 1:6	1:3 vs. 1:6
A	tMC4R: tMRAP2 (α-MSH)	10.04 ± 0.40	−9.84 ± 0.23	−9.53 ± 0.16	< 0.0001	< 0.0001	< 0.0001
B	tMC4R: tMRAP2 (ACTH)	9.749 ± 0.20	10.51 ± 0.13	10.53 ± 0.18	< 0.0001	< 0.0001	< 0.0001
C	tMC4R: tMRAP2 (SHU9119)	−9.32 ± 0.22	−9.34 ± 0.32	−9.75 ± 0.34	< 0.0001	< 0.0001	< 0.0001
D	tMC4R: tMRAP2 (AgRP)	10.09 ± 0.21	10.45 ± 0.19	10.36 ± 0.67	< 0.0001	0.0001	0.0368

Values were expressed as the mean ± S.E.M. of at least three independent experiments. Two-way ANOVA with Tukey post-test was applied in the statistical analysis.

The cAMP level was measured using Dual-Glo Luciferase Assay System (Promega). Luminescence was detected with a Spectramax M5 plate reader. α-MSH ACTH and SHU9119 were purchased from GenScrpipt Corporation Ltd. (China). AgRP were synthesized by Chinese Peptide Company (Hangzhou, China).

2.8. Statistical analysis

The data from receptor surface expression and concentration response curves were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA). Results of cAMP assays were analyzed by one-way ANOVA with Tukey post-test. Results of surface ELISA were analyzed by two-tailed t test. All results were shown as mean ± SEM.

3. Results

3.1. Evolutionary conservation and synteny analysis of tMC4R and tMRAP2

We first performed the protein alignment of MC4R and MRAP2 to determine sequence conservation between tilapia and other species including human, mouse, cow, chicken, frog, fugu, medaka, zebrafish and spotted gar (Fig. 1A and B). The amino acid sequences of transmembrane domains of tMC4R were found to be highly conserved to other species. tMC4R was most identical to tetraodon (87.8%), fugu (87.16%) and least to Xenopus tropicalis (64.6%). tMRAP2, most identical to medaka (58.71%), showed consistent lower sequence conservation with other species compared to tMC4R. Analyzing NJ phylogenetic tree based on amino acid sequences of MC4R and MRAP2 from multiple species, we found that tMC4R was evolutionarily more related to tetraodon and fugu (Fig. 2A) and tMRAP2 was more related to medaka (Fig. 2B), which were consistent with alignment results. To further determine whether tMC4R and tMRAP2 are orthologous to mammals, synteny analysis was performed between tilapia, zebrafish, medaka, spotted gar, human and mouse. Our results showed that the adjacent genes of MC4R, including *avl9*, *kbtbd2*, *rnf152*, *cdh20*, *rnasen*, *cdh10a*, *cdh12*, *cdh18* (Fig. 2C) and the adjacent genes of MRAP2, including *lin28b*, *grik2*, *sim1a* (Fig. 2D) were highly identical between tilapia, medaka and spotted gar.

3.2. Tissue distribution of tmc4r and tmrap2

To characterize mRNA expression profiles of *tmc4r* and *tmrap2*, we next extended our RT-PCR analysis to a panel of 16 tissues from adult tilapia (Fig. 3). *tmrap2* showed universal expression in different tissues. In contrast, tMC4R was expressed highly in the brain, kidney, liver, muscle, intestine and stomach. These results indicate that the tMC4R and tMRAP2 may act together *in vivo*, even though a positive signal in RT-PCR does not necessarily imply a physiologically relevant level of expression of the receptor (Roberts et al., 2006), further studies are still necessary to analyze their expression at the protein level.

3.3. Direct interaction between tMC4R and tMRAP2

Since tMC4R and tMRAP2 are evolutionarily conserved and the mRNA of both is expressed in the brain, it spurs us to verify the protein interaction between tMRAP2 and tMC4R. Previous studies from multiple species showed that MRAP2 acted as accessory protein of MC4R. Therefore, we performed immunoprecipitation using HEK293 cells transfected with plasmid constructs (pcDNA3.1/3HA-tMC4R, pcDNA3.1/2Flag-tMRAP2). As we expected, protein complex between tMC4R and tMRAP2 was detected on the gel (Fig. 4A).

3.4. The co-localization of tMC4R-tMRAP2 on plasma membrane

It is known that MRAP2 can form antiparallel homo-dimer in mice (Sebag and Hinkle, 2010). However, the conformation of MRAP2 complex of tilapia is still unknown. To expound this point, we performed a bimolecular fluorescence complementation experiment (Kerppola, 2006) as shown in Fig. 4B and C. Here we expressed two MRAP2 proteins in CHO cells, one was MRAP2 fused with a fragment of YFP (YFP-F1) at the C terminus and the other was a MRAP2 protein with the complementary fragment (YFP-F2) at the N terminus. In this case, molecular complementation of YFP could only occur when the two proteins were inserted across the membrane in opposite directions and micro-close to each other. As expected, fluorescence was detected in cells that contained tMRAP2-YFP-F1 and YFP-F2-tMRAP2, demonstrating the presence of antiparallel homo-dimers of tMRAP2 (Fig. 4B). In order to visualize the tMC4R-tMRAP2 complex, tMC4R-YFP-F1 and YFP-F2-tMRAP2 plasmids were transfected into CHO cells. The tMC4R-tMRAP2 complex produced significant fluorescence and was found to localize in intracellular compartments (Fig. 4C). This bimolecular fluorescence complementation study also further confirmed the direct interaction of tMC4R and tMRAP2 dimers.

3.5. Pharmacological modulation of tMRAP2 on tMC4R signaling

To investigate the influence of tMRAP2 on tMC4R trafficking, we transfected HEK293T cells with 3HA-tMC4R and tMRAP2 at progressive ratios (1:0, 1:3, 1:6) and then examined the surface expression of tMC4R by ELISA assay. As shown in Fig. 5E, co-expression of tMRAP2 significantly decreased the surface expression of tMC4R, as previously reported in human (Chan et al., 2009). To further monitor the effect of tMRAP2 on tMC4R signaling, we employed pGL3-CRE (cAMP response element)-luciferase reporter system to detect the cAMP level produced by tMC4R activation. As shown in Fig. 5A–B, when induced by α-MSH and ACTH, tMRAP2 dose-dependently inhibited the efficacy of tMC4R. The lactam analogue, SHU-9119, was reported as a potent antagonist for human melanocortin 3 and 4 receptors and a partial agonist for the hMC5R (Grieco et al., 2007). Likewise, we found that SHU-9119 as an antagonist of tMC4R, could reduce the activity of tMC4R (Fig. 5C). By agonist and antagonist signaling competing affinity test, we further confirmed that tMRAP2 also dose-dependently inhibited the signaling of tMC4R (Fig. 5C–D). Taken together, these results demonstrated that tMRAP2 could suppress the agonist stimulated response of tMC4R.

4. Discussion

In this study, we have revealed two remarkable genomic features of tMC4R and tMRAP2. First, the phylogenetic analysis clearly showed that newly identified tMC4R was ortholog of tetraodon and fugu MC4R. However, tMRAP2 was more evolutionary related to medaka (Fig. 2A and B). The TMD1-TMD7 of tMC4R displayed the highest degree of conservation with MC4Rs of other species, whereas they showed almost no apparent similarity in the N-terminal and C-terminal regions (Fig. 1A and B). Furthermore, tilapia had been reported to have the highest genetic relationship with fugu and tetraodon. Besides, MC3R is missing in both tilapia and fugu (Klovins et al., 2004), which is virtually as same as our observations. Although the RT-PCR is not well suitable for quantitative analysis of gene expression, this approach is very effective in detecting expression of genes sets in a wide range of tissues. We have evaluated the relative expression levels of *tmc4r* and *tmrap2* mRNA in a subset of different tissue types. Like that of mouse MRAP2 (Asai et al., 2013), broad distribution of *tmrap2* mRNA was seen in almost all the tissues we collected and the expression of *tmc4r* is generally high in the brain, with certain amount of expression in some peripheral tissues (Fig. 3). In previous reports, *mc4r* mRNA of spotted scat was also found to be expressed in the pituitary and gonads (Li et al., 2016), similar to the results obtained in the snakeskin gourami, Ya-fish and zebrafish but not in lamprey (Haitina et al., 2007; Jangprai et al., 2011; Ringholm et al., 2002; Wei et al., 2013). It is interesting that in chicken, in addition of central nerve system, the MC4R is expressed in a wide variety of peripheral tissues, including the heart, adrenal glands, ovaries, testes, spleen and adipose tissues (Takeuchi and Takahashi, 1998). Taken together, these evidences show that the expression pattern of the MC4R in mammals has become much more confined to central regions, as compared with lower vertebrates. Widespread expression of *mc4r* mRNA in peripheral tissues in different fishes indicates that MC4R could be associated with various other unknown physiological functions.

Previous studies have demonstrated that MRAP system interacts with the melanocortin receptors to allow receptor trafficking to the plasma membrane (MC2R) (Agulleiro et al., 2010), modify the pharmacological profile or attenuate the constitutive activity of MC4R (Roy et al., 2012; Sebag et al., 2013). In this study, by co-immunoprecipitation and fluorescence complementation studies, we also demonstrated that tMRAP2 could form a unique antiparallel homo-dimers and interacted with the tMC4R on plasma membrane (Fig. 4). Luciferase reporter assay revealed that stimulation with α-MSH or ACTH increased tMC4R-mediated intracellular cAMP production in a dose-dependent manner in HEK293 cells transiently expressing tMC4R. Furthermore, the presence of tMRAP2 decreased tMC4R-mediated intracellular cAMP production (Fig. 5). Like zebrafish MC4R (Sebag et al., 2013; Zhu et al., 2018), tMC4R exhibited high constitutive activity and more importantly, tMC4R no longer showed spontaneous activity in the presence of tMRAP2 (Fig. 5A). These alterations are opposed to results from MRAP2 and MC4R experiments in humans (Schonnop et al., 2016). Unlike zebrafish MC4R, which became an ACTH receptor by co-expression of zMRAP2 (Josep Agulleiro et al., 2013), we did not observe the dramatic change of EC50 of tMC4R in response to α-MSH/ACTH in presence of 1:6 tMRAP2 (Fig. 5A–B and Table 1). In addition, performing α-MSH and SHU9119/AgRP signaling competing affinity test, we found that tMRAP2 dramatically attenuated the signaling of tMC4R as well (Fig. 5C–D). In general, our results indicate that tMC4R acts as α-MSH and ACTH receptor, and tMRAP2 could modulate the potency and efficacy of agonist response of tMC4R. In summary, this study systematically elucidated an intricate interaction network of MC4R with MRAP2 in another teleost species: Nile tilapia. It implies that tMRAP2, via its interaction with tMC4R, may be involved in the regulation of energy balance and other physiological processes. Overall, this is the first comprehensive genomic and functional characterization of MC4R and MRAP2 within the tilapia genome. Manipulation of this signaling pathway hopes to potentially benefit the aquaculture yield of tilapia stock in the future.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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