Supplementary Information

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

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Supplementary Material

Figure legends

Figure S1. 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.

Figure S2. 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.

Figure S3. 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).
Figure S1
**A**

*O. latipes*

| Preoptic region | Hypothalamus |
|-----------------|--------------|
|                 | anterior     | middle      | posterior |
| Mc4r            | v3           | v3          | v3        |
| female          | HD           | HD          |           |
|                 | LH           | LH          |           |
| male            |               |             |           |
| Mrap2           | v3           | v3          | v3        |
| female          | HD           | HD          |           |
|                 | LH           | LH          |           |
| male            |               |             |           |

**B**

Antisense Mc4r

Antisense Mrap2

**Figure S2**
Sequence of the *mc4r* gene and Mc4r protein in Mc4r-KO medaka

![Sequence of the mc4r gene and Mc4r protein in Mc4r-KO medaka](image)

Two chamber aquarium

![Two chamber aquarium](image)

**Figure S3**
### Supplementary Tables

#### Table S1. List of accession numbers of sequences of various species used in the study.
All sequences are obtained from Ensembl Database or NCBI.

| Gene | Specie     | 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    | ENSLACT0000000489           | 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    | ENST000000299766            | ENSP000000299766.3       |
| McrB | Lamprey     | 1035| 344aa   | 1    | BK007095.1                  | DAA34034.1               |
| Mc4r | Medaka      | 1941| 321aa   | 1    | XM_004081195.4              | XP_004081243.1           |
| Mc4r | Southern Platypush | 963 | 320aa   | 1    | KF650657.1                 | AHC02892.1               |
| Mc4r | Spotted gar | 5875| 333aa   | 1    | XM_015354632.1              | XP_015210118.1           |
| Mc4r | Tetraodon   | 981 | 326aa   | 1    | ENSTNIT000000017378         | ENSTNIP000000011538.1    |
| Mc4r | Tilapia     | 984 | 327aa   | 1    | ENSONIT000000025784         | ENSONIP000000025763.1    |
| Mc4r | Zebrafish   | 1084| 326aa   | 1    | ENSDART000000019555         | ENSDARP0000000257547.2   |
| 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           |

| Gene | Specie     | bp  | Protein | Exon | Transcript ID | Protein ID |
|------|------------|-----|---------|------|---------------|------------|
| Mrap2| Amazon molly | 2390 | 255aa   | 3    | ENSPFOT00000019455 | ENSPFOP00000019433.2   |
| Mrap2| Cave fish   | 1511| 218aa   | 3    | XM_007237278.3 | XP_007237340.2   |
| Gene | Specie          | bp   | Protein | Exon | Transcript ID        | Protein ID                  |
|------|----------------|------|---------|------|----------------------|-----------------------------|
| Pomca-1 | Amazon molly  | 224  | 249aa   | 3    | XM_007569070.2       | XP_007569132.2              |
| Pomca-2 | Amazon molly  | 794  | 183aa   | 3    | XM_016681012.1       | XP_016536498.1              |
| Pomcb | Amazon molly   | 102  | 236aa   | 3    | ENSPFOT0000003135    | ENSPFOP0000003131.1         |
| Pomca | Cave fish      | 100  | 221aa   | 3    | XM_007244678.2       | XP_007244740.2              |
| Pomcb | Cave fish      | 878  | 197aa   | 3    | XM_015607051.2       | XP_015462537.2              |
| gene  | species       | bp   | protein | exon | transcript ID                  | protein ID               |
|-------|---------------|------|---------|------|--------------------------------|--------------------------|
| Pomc  | Coelacanth    | 768  | 255aa   | 2    | ENSLACT00000013386             | ENSLACP00000013290.1     |
| Pomc  | Elephant shark| 114  | 341aa   | 2    | XM_007911414.1                 | XP_007909605.1           |
| Pomca | Fugu          | 657  | 218aa   | 2    | XM_011606335.1                 | XP_011604637.1           |
| Pomcb | Fugu          | 287  | 218aa   | 3    | XM_003971727.2                 | XP_003971776.1           |
| POMC  | Human         | 142  | 267aa   | 3    | ENST00000405623                | ENSP00000384092.1        |
| Poc   | Lamprey       | 102  | 278aa   | 2    | D55628.1                       | BAA09491.1               |
| Pom   | Lamprey       | 204  | 245aa   | 2    | D55629.1                       | BAA09492.1               |
| Pomca-1 (Pomc-like) | Medaka | 235  | 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    | ENSORLT0000001868657           | ENSORLP00000018656.1     |
| Pomca-1 | Southern Platytfish | 713  | 209aa   | 2    | ENSXMAT0000001214              | ENSXMAP0000001210.1      |
| Pomca-2 | Southern Platytfish | 144  | 257aa   | 3    | ENSXMAT00000006432             | ENSXMAP00000006424.1     |
| Pomcb | Southern Platytfish | 140  | 236aa   | 3    | ENSXMAT00000003692             | ENSXMAP00000003687.1     |
| Poc   | Spotted gar   | 143  | 266aa   | 3    | ENSLOCT0000002060647           | ENSLOC00000020612.1      |
| Pomca-1 | Tilapia  | 196  | 216aa   | 3    | ENSONIT00000009130             | ENSONIP00000009125.1     |
| Pomca-2 | Tilapia  | 814  | 208aa   | 2    | ENSONIT00000023610             | ENSONIP00000023589.1     |
| Pomcb | Tilapia       | 153  | 280aa   | 3    | XM_005454720.3                 | XP_005454777.1           |
| Pomca | Zebrasfish    | 122  | 222aa   | 3    | ENSDART000000063333            | ENSDARP00000006332.5     |
| Pomcb | Zebrasfish    | 648  | 215aa   | 2    | ENSDART0000000100751           | ENSDARP000000091524.2    |

| Aggrp | Gene | Specie | bp   | Protein | Exon | Transcript ID                  | Protein ID               |
|-------|------|--------|------|---------|------|--------------------------------|--------------------------|
| Aggrp1| Amazon | 767  | 141aa | 3     | ENSPFOT0000000 | ENSPFOP000000024        |
| Animal          | Species      | Length (aa) | Accession       | Percent Identity |
|----------------|--------------|-------------|-----------------|------------------|
| Agrp1 Cave fish| 2445         | 5           | XM_022667066.1  | XP_022522787.1   |
| Agrp1 Cod      | 1364         | 3           | BR000938.1      | FAA00762.1       |
| Agrp1 Elephant shark | 588       | 4           | XM_007889599.1  | XP_007887790.1   |
| Agrp1 Fugu     | 529          | 3           | ENSTRUT00000009422 | ENSTRUP0000009367.1 |
| Agrp2 Fugu     | 907          | 3           | ENSTRUT00000009410 | ENSTRUP0000009355.1 |
| AGRP Human     | 764          | 4           | ENST00000290953 | ENSP00000290953.2 |
| Agrp1 Medaka   | 3293         | 3           | XM_011487064.3  | XP_011485366.1   |
| Agrp2 Medaka   | 1737         | 3           | XM_004078892.4  | XP_004078940.1   |
| Agrp1 Southern Platypus | 578       | 3           | ENSXMAT00000009960 | ENSXMAP000000946.1 |
| Agrp1 Spotted gar | 769      | 4           | XM_006641519.2  | XP_006641582.2   |
| Agrp2 Spotted gar | 3439     | 4           | ENSLOCT00000012669 | ENSLCP00000012645.1 |
| Agrp1 Stickleback | 1130    | 3           | BR000932.1      | FAA00758.1       |
| Agrp2 Stickleback | 3507    | 3           | BR000927.1      | FAA00754.1       |
| Agrp1 Tetraodon | 393        | 3           | ENSTNIT00000013316 | ENSTNIP00000013124.1 |
| Agrp1 Tilapia | 429          | 3           | ENSONIT00000004065 | ENSONIP00000004064.1 |
| Agrp1 Zebrafish | 676         | 4           | ENSDART000000135250 | ENSDARP00000116390.1 |
### Table S2. Primers used in this study.

| Primers       | Sequences                                      | Comments                                      |
|---------------|------------------------------------------------|-----------------------------------------------|
| MF_ef1a1-f01  | GCCCTGGACACAGAGACTTCATCA                      | RT-qPCR for Elf1a                             |
| MF_ef1a1-r01  | AAGGGGCTCGGGTGGAGTCCAT                       | RT-qPCR for Elf1a                             |
| Mc4r_Ol_F     | GGCAACCTGAGCATTCCTGTCA                       | RT-qPCR for Mc4r                              |
| Mc4r_Ol_R     | ATGTAGCGGTCAACGGCAATGG                       | RT-qPCR for Mc4r                              |
| MRAP2a_Ol_F   | CGCACGACGCAGTGAATGT                          | RT-qPCR for Mrap2                             |
| MRAP2a_Ol_R   | ACCGCCAGTCCAACCCAGAA                        | RT-qPCR for Mrap2                             |
| Pomca_Ol_F    | TGGACTCTGAGAGCATGAC                          | RT-qPCR for Pomca                             |
| Pomca_Ol_R    | AAGGGATCTGAGGGAGGGAGGAG                     | RT-qPCR for Pomca                             |
| Pomcb_Ol_F    | TTGCTGGCTTGGTGTGTTCT                         | RT-qPCR for Pomcb                             |
| Pomcb_Ol_R    | AGGTCTGGGCTTTCCAGTTTGAG                     | RT-qPCR for Pomcb                             |
| AgRP_Ol_F     | CATCCCCTACACGACATCGCT                       | RT-qPCR for Agrp1                             |
| AgRP_Ol_R     | GCCGCAGTAACAGATGGGCATT                      | 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  | ACGAGTTATGACGACGAG                         | PCR for Mrap2 in situ hybridization probe synthesis |
| MRAP2a_Ol_R2  | GATGGTGTTACTCCCTGTT                         | PCR for Mrap2 in situ hybridization probe synthesis |
**Table S3.** Chromosomal location of Mc4r signaling system genes. Linkage group: LG.

| gene       | 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 S4.** Medaka developmental stages.
Days post fertilization: dpf; stage: S.

| 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    |
Supplementary sequences

Mc4r sequences in wild-type medaka.

>Ol_Mc4r_WT
ATGAACTCCACTCTGCTCCCTTATGGGTCGGTCCCCAACAGAACCTCCTCCTCGGCCACTCCTCCTCACCTGAGCTCTGCTGCTGGAGAACATCTGGTTGTTGCTGCGATCGTTAAAAACAAGAACCTCCTCCACCTCGGCTACATGATCTCCACTGAGGTCTTCCTCACTTTGGGCATCATCAGCCTGCTGGAGAACATCTGGTTGTTGCTGCGATCGTTAAAAACAAGAACCTCCACTCCCCCATGTACTTTTATCTGCAGCCTCGCAGTAGCCGATATGT

>TGGTCAGCGTCTCCAACGCGTCTGAGACC
ATCGTCATAGCGCTCATTAACGGAGGCAACCTGAGCATTCCTGTCAGGCTCATCAAGAGCATGGACAATGTGTTTGACTCCATGATCTGCAGCTCTCTGCTGGCCTCCATCTGCAGCTTGCTGGCCATTGCCGTTGACCGCTACATCACCATCTTCTACGCTCTGCGATACCCACAACATCGTGACGCTGCGGCGAGCAGCCGTGGTCATCAGCAGCATCTGGACGTGCTGCATTGTGTCGGGTATCCTCTTCATCATCTACTCGGAGAGTACCACGGTGCTCATCTGTCTCATCACCATGTTCTTCACCATGCTGGTGCTCATGGCCTCCCTCTATGTCCACTGTTCATCATGCCACTGCTCTGCTTCCACCTCATCCTGATCACCTGC

CCCAGGAACCCTTACTGCACCTGCTTCATGTCGCACTTCAACATGTACCTCATTCTCATCATGTGCAACTCCGTCATCGACCC CATCATCTACGCTTTCCGGAGCCAGGAGATGAGGAAAACCTTCAAGGAGATCTTCTGCTGCTCCAACGCTCTCCTGTGTGTGA

>Ol_Mc4r_WT
MNSTLPYGSVPNRSLSSATLPDDLGGKQKDSAGCYEQLLSTEVFLTGLISSLENI LVVAAIVKKNLHSPMYFFICSLAVADMLVSVSNASTIVIALINGNLSPVRLIK SMNVDFMICSLLASILSLAIAVDRYITIFILYALRYNHIVTLRRAAVSIWITC CIVSGILFIYYSESTTVCLICLITMFFTMVLVMLASYVMHFLARLHMOKRIAALPGNA PIHQRANMKGATILWIIILGVVVFVCWAPFHLILIMITCPGRNPYCTCFMSHFNMYLIL IMCNSVIDPIIYAFRSQEMRTKFEIFCSSNALLCV

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

>Ol-Mc4r_KO-2/+3
ATGAACTCCACTCTGCTCCCTTATGGGTCGGTCCCCAACACAGAACCTCCTCCTCCTCGGCCACTCCTCCTCACCTGAGCTCTGCTGCTGGAGAACATCTGGTTGTTGCTGCGATCGTTAAAAACAAGAACCTCCACTCCCCCATGTACTTTTATCTGCAGCCTCGCAGTAGCCGATATGT

>TGGTCAGCGTCTCCAACGCGTCTGAGACCATCGTCATAGCGCTCATTAACGGAGGCAACCTGAGCATTCCTGTCAGGCTCATCAAGAGCATGGACAATGTGTTTGACTCCATGATCTGCAGCTCTCTGCTGGCCTCCATCTGCAGCTTGCTGGCCATTGCCGTTGACCGCTACATCACCATCTTCTACGCTCTGCGATACCCACAACATCGTGACGCTGCGGCGAGCAGCCGTGGTCATCAGCAGCATCTGGACGTGCTGCATTGTGTCGGGTATCCTCTTCATCATCTACTCGGAGAGTACCACGGTGCTCATCTGTCTCATCACCATGTTCTTCACCATGCTGGTGCTCATGGCCTCCCTCTATGTCCACTGTTCATCATGCCACTGCTCTGCTTCCACCTCATCCTGATCACCTGC

CCCAGGAACCCTTACTGCACCTGCTTCATGTCGCACTTCAACATGTACCTCATTCTCATCATGTGCAACTCCGTCATCGACCCCATCATCTACGCTTTCCGGAGCCAGGAGATGAGGAAAACCTTCAAGGAGATCTTCTGCTGCTCCAACGCTCTCCTGTGTGTGA

>Ol-Mc4r_KO-2/+3
MNSTLPYGSVPNRSLSSATLPDDLGGKQKDSAGCYEQLLSTEVFLTGLISSLENI LVVAAIVKKNLHSPMYFFICSLAVADMLVSVSNASTIVIALINGNLSPVRLIK SMNVDFMICSLLASILSLAIAVDRYITIFILYALRYNHIVTLRRAAVSIWITC CIVSGILFIYYSESTTVCLICLITMFFTMVLVMLASYVMHFLARLHMOKRIAALPGNA PIHQRANMKGATILWIIILGVVVFVCWAPFHLILIMITCPGRNPYCTCFMSHFNMYLIL IMCNSVIDPIIYAFRSQEMRTKFEIFCSSNALLCV
CATTTCTGCTGGCACGTCTGCACATGAAGCGGATCGCGGCGCTGCCGGGCAACGC
GCCCATCCACCGGCGAACATGAAGGGCCTCCACCCTACCATCCTCTTCCGGGCTTGTTTTGTGGTGTGCTGGGCCGGCGTTCTTCTCCACCCTACCATCCTCAGATCACCTG
CCCAGGAACCCTTAACCTGACCTGTTCATGTGCACTTCAACATGTACCTCATTCT
CATCATGTGCAACTCGCTCATGACCCACCATCATCTACGCTTCCGGAGCCAGGAGAT
GAGAAAACTTTCAAGGAGATCTTCTTCATGCTGCTCCACGCTCTCCTGCTGTGTGTA

>Ol_Mc4r_KO-2/+3
MNSTLPYGSVPNRLSSATLPPDLGGQKDSSAGYEQLLISTEVFLTLGIISSLLENI
LVVAAIVKKNLHSPMYFFICSALAVMLVSVSKTV