# Supplemental information (SI)

## Materials & Methods

### Generation of KO lines of kisspeptin related genes

We generated *kiss1* gene KO medaka using TALEN (1), while we generated *kiss2*, *gpr54-1*, *gpr54-2* KO medaka using CRISPR/Cas9 (2). The specific TALEN and CRISPR/Cas9 target sites were identified using an online tool (ZiFiT: supplied by ZINC FINGER CONSORTIUM; http://www.zincfingers.org/default2.htm). Exon 3 of *kiss1* gene and exon 2 of *kiss2* gene, which code the core sequence of kisspeptin, were selected for target sites (Supplemental Figure 1, 2). Exon 4 of *gpr54-1* gene, which codes the transmembrane region, was selected for the CRISPR target site (Supplemental Figure 3). Approximately 50 bp downstream of the first methionine of *gpr54-2* was selected for the CRISPR target site (Supplemental Figure 4). Gene-specific TALEN constructs were assembled using Joung Lab REAL Assembly TALEN kit (Addgene, Cambridge, MA, USA) as described (1). TALEN coding region was transferred to pCold II (Takara), which contains the cold shock promoter, His-tag, and polyadenylation signal. NiCo21 (DE3) E. coli cells (NEB, Ipswich, MA, USA) were transfected by TALEN expression plasmid. TALEN proteins were purified by His-bind column (BIO-RAD, CA, USA) and by Heparin chromatography (QIAGEN, Hilden, Germany). For generation of gRNA, Oligo DNA (2μM) for gRNA listed in Table 1 were annealed and ligated with gRNA expression vector (DR274; Addgene) digested by BsaI (NEB) according to Hwang et al., 2013. After cloning and digestion by DraI (NEB), gRNA was transcribed by T7 polymerase (Roche, Molecular Biochemicals GmbH, Mannheim, Germany). The Cas9 mRNA was transcribed using PmeI-digested Cas9 expression vector (MLM 3613; Addgene) by mMessage mMachine T7 ULTRA kit (Life technologies). *EGFP* mRNA was transcribed using SP6 promoter in linearized pCS2+EGFP vector. TALEN solution containing left and right TALEN protein, or CRISPR/Cas9 solution containing gRNA (12.5 ng/μL) and Cas9 mRNA (300 ng/μL), with *EGFP* mRNA (1-5ng/μL; for validation of successful microinjection) and 0.02 % phenol red in 1 x PBS were injected into the cytoplasm of fertilized one-cell stage medaka eggs with intact chorion. Genomic DNA of F1 fish was extracted from the caudal fin using Mag Extractor -Genome- (TOYOBO CO, Tokyo, Japan) or prepGem-tissue (ZyGEM, Hamilton, New Zealand) according to the respective manufacturer's instructions. Amplicon that include the target region of each gene was generated by PCR using LightCycler 480 SYBER Green I Master (Roche), Thunderbird SYBR qPCR Mix (TOYOBO CO) and corresponding primers (Table 2). Candidate fish were chosen by comparing the peaks of the melting curves between wild type and F1 fish. After PCR reaction, primers were digested by Exonuclease I (Takara, Shiga, Japan), and dNTPs were dephosphorylated by Shrimp Alkaline Phosphatase (Takara). Amplicons of the candidate fish were sequenced by a commercial company (Fasmac, Kanagawa, Japan or Eurofins genomics, Tokyo, Japan). F1 fish that had mutation were intercrossed. Homozygous KO F2 fish were selected by genome sequence as described above.

### Luciferase Assays

The luciferase assay experiments were performed as described previously in Kanda et al., 2013. The cDNA fragments containing full-length open reading frames of *gpr54-1* and *gpr54-2* were subcloned into the expression vector pcDNA3.1 (Invitrogen). COS-7 cells were grown on 24-well plate at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum. The plasmid DNAs (100 ng/well) were transfected into monolayer culture cells with either pSRE-Luc or pCRE-Luc (100 ng/well; Clontech, Palo Alto, CA), and pRL-CMV containing the Renilla luciferase reporter gene (2.5 ng/well; Promega, Madison, WI), using Lipofectamine LTX (Invitrogen). The cells were maintained in a serum-free medium for 24 hours. After that, they were incubated with various concentrations (from 0 to $10^{-6}$ M) of medaka Kiss1, Kiss2, or FTM145 for six hours and then harvested and analyzed. Luciferase activity in the cell extract was measured using Dual-Glo Luciferase Assay System (Promega) with Lumat LB9507 (EB & G Berthold, Bad Wildbad, Germany). The transfection experiments were performed in more than triplicate and were repeated at least three times.

## *in vivo* administration of drugs

LHRH-A (synthetic Luteinizing Hormone-Releasing Hormone analog; [des-Gly$^{10}$, D-Ala$^{6}$]-LH-RH ethylamide acetate salt hydrate) was dissolved in a fish saline (0.7% NaCl solution) and was injected i.p. (0.1µg/g BW (body weight)). Pimozide (PIM) was suspended in a vehicle of 0.7% NaCl with 0.1% sodium metabisulfite and was injected i.p. at 10 µg/g BW. The control groups were given an equivalent volume of saline and/or the PIM vehicle. The decapeptide of medaka Kiss1 (Kiss1-10: YNLNSFGLRY-$NH_2$) and the dodecapeptide of medaka Kiss2 (Kiss2-12: SKFNYNPFGLRF-$NH_2$) were synthesized (Sigma-Aldrich Japan, Tokyo, Japan; Bonac Corporation, Kurume, Japan, respectively). Synthetic Kiss1-10 and FTM145 (4-fluorobenzoyl-Phe-Gly-ψ[(*E*)-CH=CH]-Leu-Arg-Trp-$NH_2$; GPR54 agonist (3-6) was dissolved in saline and injected i.p. (0.1 µg or 1 µg/g BW) or i.c.v. (0.1nmol). Note that we initially confirmed by luciferase assay that FTM145 possesses binding affinities and efficacies to both of medaka Gpr54-1 and Gpr54-2 (Supplemental Figure 5B, D). Synthetic Kiss2-12 was first dissolved in DMSO and diluted with saline (0.02% DMSO in final concentration) and was injected i.p. (0.1 µg /g BW) or i.c.v. (0.1 nmol).

In the experiments, the animals were divided into three groups: 1) those receiving i.p. administration on a short time scale, 2) those receiving i.p. administration on a long time scale, and 3) those receiving i.c.v. administration. Ovulation was checked by the release of a stream of ripe translucent oocytes from the ovipore following application of a slight pressure to the abdomen (7). In Group 1 (i.p. administration of a long time scale), PIM was injected 12 hours prior to the co-injection of peptide and PIM (0h) according to the previous study in goldfish using LHRH-A (8), and the blood was sampled just before the first injection of PIM (-12h), 24 hours and 48 hours after peptide injection (Figure 1A). In Group 2 (i.p. administration of a short time scale), PIM was injected three hours prior to the co-injection of peptide and PIM according to the previous study in goldfish using kisspeptin (9), and the blood was sampled just before the injection of peptide and PIM (0h), two hours and six hours after that (Supplemental Figure 8). In Group 3 (i.c.v. administration), PIM was injected i,p, 12 hours prior to the i.c.v. injection of peptide (0h), and the blood was sampled just before the first injection of PIM (-12h), just before the injection of peptide (0h), 0.5 hour and 3 hours after peptide injection. In all experiments, ovulation was checked until three days after the peptide injection (Supplemental Figure 9).

## Hematoxylin and eosin (HE) staining of gonads

The ovaries and testes (90 dph (days post-hatch)) were fixed with Bouin's fixative at 4°C overnight. After fixation, each tissue sample was routinely processed and embedded in paraffin, and sections of 8 µm thickness were stained with hematoxylin and eosin (HE). Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany) attached to an upright microscope (DM5000B; Leica Microsystems).

## Quantitative PCR

Three-month-old medaka were deeply anesthetized with 0.02% MS-222, and the pituitaries were collected for real-time PCR analysis at 10 A.M.. Total RNA was extracted from the pituitaries using the NucleoSpin RNA XS (Takara, Shiga, Japan) or the brain using the NucleoSpin II (Takara) according to the manufacture's protocol. Genomic DNA was removed by deoxyribonuclease I (Ambion, Applied Biosystems, Foster City, CA) treatment on a column membrane. Total RNA was reverse transcribed with High Capacity PrimeScript Reverse Transcriptase (Takara) according to the manufacturer's instructions. For real-time PCR, the cDNA was amplified using Thunderbird SYBR qPCR Mix (TOYOBO CO) or KAPA SYBR FAST qPCR Kit (Nippon Genetics Co, LTD, Tokyo, Japan) with the LightCycler 480 II system (Roche). The temperature profile of the reaction was 95°C for 5 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. The PCR product was verified using melting curve analysis. The data were normalized to a housekeeping gene, ribosomal protein s13 (*rps13*). The primer pairs used in the real-time PCR are listed in Table 2.

### $Ca^{2+}$ imaging of LH:IP cells

We performed $Ca^{2+}$ imaging using the whole brain-pituitary preparations from *lhb*:*IP* or *lhb*:*IP*;*gnrh1*:*EGFP* transgenic medaka (four males and five females) as we described previously (10). Positions of the LH cells were visually identified under epifluorescence illumination, and fluorescence images of IP were recorded (exposure: 100ms; interval: 5 s). In all experiments repetitively applying (2-3 times each) mdKiss1-10 and mdGnRH1 (medaka GnRH1) to one preparation for 5-6 min (Supplemental Figure 10), the intervals (washout time) between every two application trials were 10-30 minutes. For data analysis, we selected 27-50 ROIs (region of interest) and calculated their responses as follows. The time when drug reached the experimental chamber was defined as Frame 0 (F0). F0 was calculated as an average of five frames; Frame -8 to -4. The fluorescence intensity change (ΔF/F0) was calculated as (F0 -F)/F0, and the peak ΔF/F0 was picked up from 25 frames; Frame -3 to 21. As for the period before the first peptide application trial, we calculated F0 as average of Frame -52 to -48, and picked up peak ΔF/F0 from Frame -60 to -40. We excluded the data from one male fish because its LH cells showed severely weak IP fluorescence and did not respond to medaka GnRH1 application.

### Specificity of EGFP labeling on *gpr54-1* expressing cells

In order to confirm the specificity of EGFP expression in *gpr54-1* expressing cells, we performed dual labeling of EGFP immunohistochemistry (IHC) and *gpr54-1* mRNA *in situ* hybridization (ISH) on frozen brain sections of this transgenic medaka. A *gpr54-1*-specific digoxigenin (DIG)-labeled mRNA probe was prepared and applied to IHC and ISH procedures, following a standard protocol that we documented previously (10-12) with some minor modifications. Briefly, adult male and female medaka were anesthetized by immersion in 0.02% tricaine methanesulfonate (MS-222) (Sigma-Aldrich, Darmstadt, Germany) and quickly decapitated or fixed by perfusion with 4% paraformaldehyde (Nacalai Tesque, Japan) in PBS (Takara, Japan). The whole brains were quickly dissected out and fixed with 4% paraformaldehyde in PBS for 2-6 hours (without perfusion fixation) or 10-15 min (after perfusion fixation), and then substituted with 30 % (w/v) sucrose (Wako, Japan) in PBS overnight. Frontal sections (20-30 μm) were prepared by using a cryostat (CM 3050S; Leica Microsystems, Wetzlar, Germany), and mounted onto MAS-GP typeA-coated glass slides (Matsunami, Osaka, Japan). To detect EGFP-immunoreactive (EGFP-ir) cells, we used anti-EGFP antibody raised in rabbit (13) (1:1000; generous gift from Dr. Kaneko and Dr. Hioki, Kyoto University, Kyoto, Japan, or Thermo Fisher Scientific, A-11122). To detect *gpr54-1* mRNA, the sections were hybridized with 100-200 ng/ml DIG-labeled antisense cRNA probes prepared from the medaka brain cDNA samples using a labeling kit (Roche Applied Science, Mannheim, Germany) overnight at 58°C. A sense cRNA probe was used as negative controls. For dual-fluorescent visualization steps of IHC and ISH signals, AlexaFluor488 conjugated anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) and HNPP/FastRed (HNPP Fluorescent Detection Set);(Roche Applied Science, Mannheim, Germany) were applied respectively. The sections were observed by a DM5000 B fluorescence microscope (Leica) and a LSM-710 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) for the examination of co-localization for *gpr54-1* mRNA and EGFP.

### Deep sequencing of *gpr54-1*:*EGFP* expressing cells

The whole brains of adult male and female medaka were prepared as described above and placed in silicone-bottom dish filled with an artificial cerebrospinal fluid (ACSF) consisting of (in mM) 134 NaCl, 2.9 KCl, 1.2 $MgCl_2$, 2.1 $CaCl_2$, 10 HEPES, and 15 glucose (pH 7.4, adjusted with NaOH). Whole-brain *in vitro* preparations were carefully peeled off the meninges covering the telencephalon and hypothalamus in ACSF. Next, we identified *gpr54-1* expressing cells by their EGFP fluorescence under an upright fluorescent microscope with infrared-differential interference contrast optics (Eclipse E-600FN;

Nikon) and an infrared charge-coupled device camera (C3077–78; Hamamatsu Photonics). Then we collected five EGFP-positive cells in ventrolateral preoptic area (vPOA) by suction of the pipettes made from borosilicate glass capillaries of 1.5 mm outer diameter (GD-1.5; Narishige, Tokyo, Japan). The pipettes were pulled using a Flaming-Brown micropipette puller (P-97; Sutter Instruments, Novato, CA, USA) and we approached the tip of the pipette close to the cells by MP-225 micromanipulator (Sutter Instrument, California, USA). The resistances of the pipette tips for cell collection were approximately 2–5 MΩ. For collecting the cells in area ventralis telencephali pars dorsalis/supracommissuralis/posterior (Vd/Vs/Vp), nucleus preopticus pars magnocellularis/parvocellularis (POm, POp), and nucleus posterioris periventricularis (NPPv), we sectioned the whole brain frontally by vibratome and the cells were dissociated by Papain solution (Roche Applied Science, Mannheim, Germany) and collected likewise under an inverted fluorescence microscope. We prepared the mixed sample containing 10 cells collected from Vd/Vs/Vp, vPOA, and hypothalamus (mixed 5 cells each from male and female) and performed the following lysis, reverse transcription (RT), and purification steps using SuperScript III (Thermo Fisher Scientific, MA, USA) and Nucleospin Gel and PCR clean-up kit (Macherey-Nagel, Berlin, Germany). cDNA libraries were obtained by these procedures mainly based on the standard protocol provided by Life Technologies, and then applied for the next generation sequencer Ion PGM (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), following the standard protocol of Ion PGM system. We selected the candidate genes judging from reads per kilobase of exon per million mapped sequence reads (RPKM) for expression value in the obtained data.

## The nomenclature of the medaka brain nuclei

ca/ch/cp/ct, commissura anterior/horizontalis/posterior/transversa; CE, corpus cerebelli; Dc/Dm/Dl/Dp, area dorsalis telencephali pars centralis/medialis/lateralis/posterior; DM, nucleus dorsomedialis thalami; flm, fasciculus longitudinalis medialis; fr, fasciculus retroflexus; GR, corpus glomerulosum pars rotunda; HB, habenula; lfb, lateral forebrain bundle; mfb, medial forebrain bundle; NAT, nucleus anterior tuberis; NC, nucleus corticalis; NDTL, nucleus diffusus tori lateralis; NIP, the interpeduncular nucleus; NPPv, nucleus posterioris periventricularis; NVT, nucleus ventralis tuberis; PGm, nucleus preglomerulosus pars medialis; POm/POp, nucleus preopticus pars magnocellularis/parvocellularis; pTGN, preglomerular tertiary gustatory nucleus; PTH, nucleus prethalamicus; TO, tectum opticum; TL/TS, torus longitudinalis/semicircularis; Vd/Vs/Vp/Vi/Vv, area ventralis telencephali pars dorsalis/supracommissuralis/posterior/intermedia/ventralis VM, nucleus ventromedialis thalami.

## Supplemental Figure legends

**Supplemental Figure 1.** Genomic and deduced amino acid sequences of wild type (wt) and TALEN knockout (KO) *kiss1* gene. A, Alignment of genomic DNA sequences of *kiss1* open reading frame (ORF) region of wt and *kiss1* KO lines. Underlines indicate left and right TALEN targets. B, Alignment of deduced amino acid sequences of precursor of Kiss1 from genomic data of wt and *kiss1* KO lines. Shaded region indicates kisspeptin core-peptide sequence. D; deletion, I; insertion, R; replacement.

**Supplemental Figure 2.** Genomic and deduced amino acid sequences of wt and CRISPR/Cas9 KO *kiss2* gene. A, Alignment of genomic DNA sequences of *kiss2* ORF region of wt and *kiss2* KO lines. Underline indicates gRNA target. B, Alignment of deduced amino acid sequences of precursor of Kiss2 from genomic data of wt and *kiss2* KO lines. Shaded region indicates kisspeptin core-peptide sequence. D; deletion.

**Supplemental Figure 3.** Genomic and deduced amino acid sequences of wt and CRISPR/Cas9 KO *gpr54-1* gene. A, Alignment of genomic DNA sequences of *gpr54-1* ORF region of wt and a *gpr54-1* KO line. Underline indicates gRNA target. B, Alignment of deduced amino acid sequences of precursor of Gpr54-1 from genomic data of wt and a *gpr54-1* KO line. Shaded region indicates transmembrane domain (TM). D; deletion, R; replacement.

**Supplemental Figure 4.** Genomic and deduced amino acid sequences of wt and CRISPR/Cas9 KO *gpr54-2* gene. A, Alignment of genomic DNA sequences of *gpr54-2* ORF region of wt and *gpr54-2* KO lines. Underline indicates gRNA target. B, Alignment of deduced amino acid sequences of precursor of Gpr54-2 from genomic data of wt and *gpr54-2* KO lines. Shaded region indicates transmembrane domain (TM). D; deletion, I; insertion, R; replacement.

**Supplemental Figure 5.** Luciferase assays for the activation of two types of receptors, Gpr54-1 and Gpr54-2, by GPR54 agonist, FTM145 and synthetic kisspeptins showing their activation of Gpr54-1/2. A, Comparison of amino acid sequences of predicted mature kisspeptin in medaka and goldfish and FTM145. B-E, Medaka Gpr54-1 (B, C) or Gpr54-2 (D, E) cDNA was transfected to COS-7 cells with SRE-luc or CRE-luc vector. Various concentrations of FTM145 (B, D) and medaka Kiss1-10, and medaka Kiss2-12 (C, E) were applied to the culture medium, and the luciferase activity was measured. The results are indicated as mean±SEM, each of which was conducted in more than triplicates. The data are expressed as the ratio of changes in luciferase activity over the control renilla luciferase activity. (C) and (E) were modified from Kanda et al., 2013.

**Supplemental Figure 6.** Hematoxylin and eosin (HE)-stained sections of ovaries (90 dph, left column) or testes (90 dph, right column) of *kiss1*, *kiss2*, *gpr54-1*, or *gpr54-2* KO medaka. A-F, Ovaries (1) and testes (2) of wt (A), *kiss1*$^{-/-}$ (B), *kiss2*$^{-/-}$ (C), *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ (D), *gpr54-1*$^{-/-}$ (E), and *gpr54-2*$^{-/-}$ (F). PV; previtellogenic, LV; late vitellogenic, FG; full-grown, SG; spermatogonia, SC; spermatocyte, and SZ; spermatozoa. Scale bars, 500 μm (ovary) or 50 μm (testis).

**Supplemental Figure 7.** Normal expression levels of gonadotropin genes in the KO medaka. A-B, Relative expression levels of *lhb* (A) and *fshb* (B) mRNA in the pituitary of wt male, *kiss1*$^{-/-}$ male, wt female, and *kiss1*$^{-/-}$ female. C-D, Relative expression levels of *lhb* mRNA in the pituitary of *kiss1*$^{+/-}$;*kiss2*$^{-/-}$, *kiss1*$^{+/-}$;*kiss2*$^{+/-}$, *kiss1*$^{-/-}$ ;*kiss2*$^{-/-}$, *gpr54-1*$^{-/-}$, *gpr54-1*$^{+/-}$ (only in C), *gpr54-2*$^{-/-}$, *gpr54-2*$^{+/-}$, and wt male (C) and female (D). E-F, Relative expression levels of *fshb* mRNA in the pituitary of *kiss1*$^{+/-}$;*kiss2*$^{-/-}$, *kiss1*$^{+/-}$;*kiss2*$^{+/-}$, *kiss1*$^{-/-}$ ;*kiss2*$^{-/-}$, *gpr54-1*$^{-/-}$, *gpr54-1*$^{+/-}$ (only in E), *gpr54-2*$^{-/-}$, *gpr54-2*$^{+/-}$, and wt male (E) and female (F). All the values were normalized to *rps13*.

**Supplemental Figure 8.** Plasma LH levels and % ovulated fish after intraperitoneal (i.p.) administration of kisspeptins and LHRH-A on a short time scale in goldfish showing no effect of kisspeptins. A, Experimental procedure for i.p. administration on a short time scale. B, Plasma LH levels (ng/mL) at 0 hour (h), 2 h and 6 h from peptides administration and % ovulated fish 60 h after peptide administrations. Note that only LHRH-A increased plasma LH levels and induced ovulation. PIM; pimozide, LHRH-A; luteinizing hormone releasing hormone analog.

**Supplemental Figure 9.** Plasma LH levels and % ovulated fish after intracerebroventricular (i.c.v.) administration of kisspeptins in goldfish showing no effect of kisspeptins. A, Experimental procedure for i.c.v. administration. B, Plasma LH levels (ng/mL) at -12 h, 0 h, 0.5 h and 3 h from peptide administrations and % ovulated fish 60 h after peptide administrations.

**Supplemental Figure 10.** $Ca^{2+}$ imaging of LH:IP cells showing no effect of kisspeptin on $Ca^{2}$ rise for LH release. A, A representative picture of the analyzed LH cells in *lhb*:*IP* transgenic medaka (10 yellow circles, region of interest (ROI)) corresponding to the graphs shown in B. Scale bar, 50 μm. C, caudal; R, rostral. B, Representative traces of $Ca^{2+}$ responses to 1 μM mdKiss1-10 (left graph, blue) or 1 μM mdGnRH1 (right graph, red) application. The graphs show the traces from 10 ROIs (thin lines) and the averaged traces (thick lines). C, The graph showing the averaged peak amplitudes of $Ca^{2+}$ responses of LH cells to 1 μM mdKiss1-10 (blue) or 1 μM mdGnRH1 (red) applications in three male (Black circles) and five female (white circles) fish. Compared to the “Before” group (male: n=3, female: n=5), Kiss1-10 group (male: n=6, female: n=10) did not affect $Ca^{2+}$ responses of LH cells, whereas GnRH1 group (male: n=6, female: n=14) significantly facilitated them as we previously reported in Karigo et al., 2014. Note that there is no significant difference between male and female. ***: $p<0.001$, Steel’s multiple comparison test.

**Supplemental Movie.** Representative 3D movie of whole mount IHC using the *gpr54-1*:*EGFP* transgenic medaka showing heavy projection from POA to the pituitary. The dense EGFP-ir fibers are mainly localized in the pars distalis and intermedia of the pituitary. The orientation of the first frame is the same in Figure 3E.

## Supplemental references

1. Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, Yeh JR. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat Biotechnol 2011; 29:697-698
2. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 2013; 31:227-229
3. Tomita K, Oishi S, Cluzeau J, Ohno H, Navenot JM, Wang ZX, Peiper SC, Akamatsu M, Fujii N. SAR and QSAR studies on the N-terminally acylated pentapeptide agonists for GPR54. J Med Chem 2007; 50:3222-3228
4. Tomita K, Niida A, Oishi S, Ohno H, Cluzeau J, Navenot JM, Wang ZX, Peiper SC, Fujii N. Structure-activity relationship study on small peptidic GPR54 agonists. Bioorg Med Chem 2006; 14:7595-7603
5. Inoue N, Sasagawa K, Ikai K, Sasaki Y, Tomikawa J, Oishi S, Fujii N, Uenoyama Y, Ohmori Y, Yamamoto N, Hondo E, Maeda K, Tsukamura H. Kisspeptin neurons mediate reflex ovulation in the musk shrew (Suncus murinus). Proc Natl Acad Sci U S A 2011; 108:17527-17532
6. Tomita K, Oishi S, Ohno H, Peiper SC, Fujii N. Development of novel G-protein-coupled receptor 54 agonists with resistance to degradation by matrix metalloproteinase. J Med Chem 2008; 51:7645-7649
7. Stacey NE, Cook AF, Peter RE. Ovulatory surge of gonadotropin in the goldfish, Carassius auratus. Gen Comp Endocrinol 1979; 37:246-249
8. Chang JP, Peter RE. Effects of pimozide and des Gly10,[D-Ala6]luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, Carassius auratus. Gen Comp Endocrinol 1983; 52:30-37
9. Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, Zhu P, Shi Y, Cheng CH, Liu X, Lin H. Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (Carassius auratus). J Endocrinol 2009; 201:407-418
10. Karigo T, Aikawa M, Kondo C, Abe H, Kanda S, Oka Y. Whole brain-pituitary in vitro preparation of the transgenic medaka (Oryzias latipes) as a tool for analyzing the differential regulatory mechanisms of LH

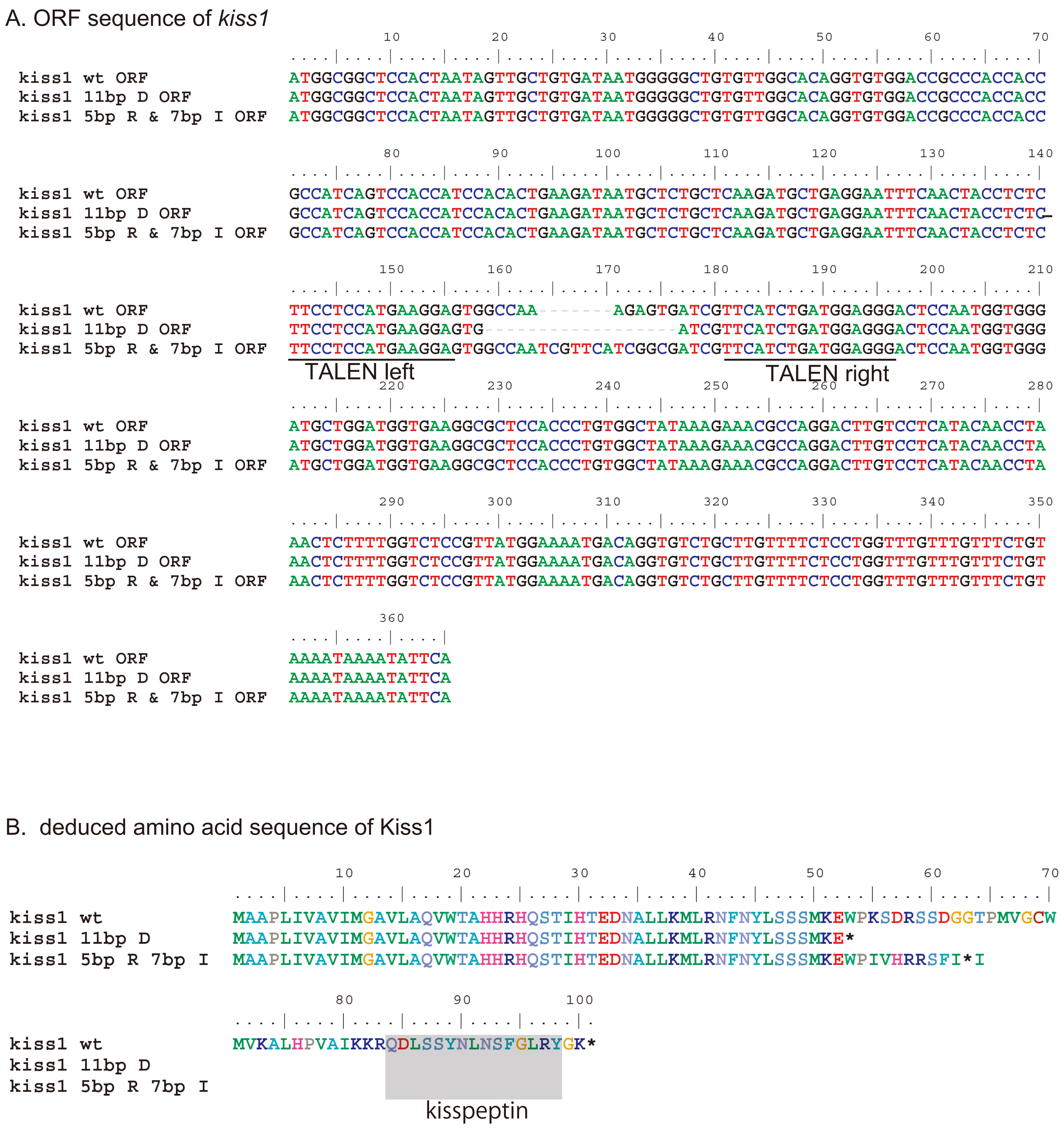
and FSH release. Endocrinology 2014; 155:536-547

**11.** Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, Maeda K, Oka Y. Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (Oryzias latipes). Endocrinology 2008; 149:2467-2476

**12.** Hasebe M, Kanda S, Shimada H, Akazome Y, Abe H, Oka Y. Kiss1 neurons drastically change their firing activity in accordance with the reproductive state: insights from a seasonal breeder. Endocrinology 2014; 155:4868-4880

**13.** Nakamura KC, Kameda H, Koshimizu Y, Yanagawa Y, Kaneko T. Production and histological application of affinity-purified antibodies to heat-denatured green fluorescent protein. J Histochem Cytochem 2008; 56:647-657

# Supplemental Figure 1



A. ORF sequence of *kiss1*

```
                                  10        20        30        40        50        60        70
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss1 wt ORF              ATGGCGGCTCCACTAATAGTTGCTGTGATAATGGGGGCTGTGTTGGCACAGGTGTGGACCGCCCACCACC
kiss1 11bp D ORF          ATGGCGGCTCCACTAATAGTTGCTGTGATAATGGGGGCTGTGTTGGCACAGGTGTGGACCGCCCACCACC
kiss1 5bp R & 7bp I ORF   ATGGCGGCTCCACTAATAGTTGCTGTGATAATGGGGGCTGTGTTGGCACAGGTGTGGACCGCCCACCACC

                                  80        90       100       110       120       130       140
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss1 wt ORF              GCCATCAGTCCACCATCCACACTGAAGATAATGCTCTGCTCAAGATGCTGAGGAATTTCAACTACCTCTC
kiss1 11bp D ORF          GCCATCAGTCCACCATCCACACTGAAGATAATGCTCTGCTCAAGATGCTGAGGAATTTCAACTACCTCTC-
kiss1 5bp R & 7bp I ORF   GCCATCAGTCCACCATCCACACTGAAGATAATGCTCTGCTCAAGATGCTGAGGAATTTCAACTACCTCTC

                                 150       160       170       180       190       200       210
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss1 wt ORF              TTCCTCCATGAAGGAGTGGCCAA-------AGAGTGATCGTTCATCTGATGGAGGGACTCCAATGGTGGG
kiss1 11bp D ORF          TTCCTCCATGAAGGAGTG------------------ATCGTTCATCTGATGGAGGGACTCCAATGGTGGG
kiss1 5bp R & 7bp I ORF   TTCCTCCATGAAGGAGTGGCCAATCGTTCATCGGCGATCGTTCATCTGATGGAGGGACTCCAATGGTGGG

                                 220       230       240       250       260       270       280
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss1 wt ORF              ATGCTGGATGGTGAAGGCGCTCCACCCTGTGGCTATAAAGAAACGCCAGGACTTGTCCTCATACAACCTA
kiss1 11bp D ORF          ATGCTGGATGGTGAAGGCGCTCCACCCTGTGGCTATAAAGAAACGCCAGGACTTGTCCTCATACAACCTA
kiss1 5bp R & 7bp I ORF   ATGCTGGATGGTGAAGGCGCTCCACCCTGTGGCTATAAAGAAACGCCAGGACTTGTCCTCATACAACCTA

                                 290       300       310       320       330       340       350
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss1 wt ORF              AACTCTTTTGGTCTCCGTTATGGAAAATGACAGGTGTCTGCTTGTTTTCTCCTGGTTTGTTTGTTTCTGT
kiss1 11bp D ORF          AACTCTTTTGGTCTCCGTTATGGAAAATGACAGGTGTCTGCTTGTTTTCTCCTGGTTTGTTTGTTTCTGT
kiss1 5bp R & 7bp I ORF   AACTCTTTTGGTCTCCGTTATGGAAAATGACAGGTGTCTGCTTGTTTTCTCCTGGTTTGTTTGTTTCTGT

                                 360
                          ....|....|....|
kiss1 wt ORF              AAAATAAAATATTCA
kiss1 11bp D ORF          AAAATAAAATATTCA
kiss1 5bp R & 7bp I ORF   AAAATAAAATATTCA
```

B. deduced amino acid sequence of Kiss1

```
                            10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss1 wt            MAAPLIVAVIMGAVLAQVWTAHHRHQSTIHTEDNALLKMLRNFNYLSSSMKEWPKSDRSSDGGTPMVGCW
kiss1 11bp D        MAAPLIVAVIMGAVLAQVWTAHHRHQSTIHTEDNALLKMLRNFNYLSSSMKE*
kiss1 5bp R 7bp I   MAAPLIVAVIMGAVLAQVWTAHHRHQSTIHTEDNALLKMLRNFNYLSSSMKEWPIVHRRSFI*I

                            80        90       100
                    ....|....|....|....|....|....|.
kiss1 wt            MVKALHPVAIKKRQDLSSYNLNSFGLRYGK*
kiss1 11bp D
kiss1 5bp R 7bp I
```

Supplemental Figure 2

## A. ORF sequence of *kiss2*

```
                          10        20        30        40        50        60        70
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt ORF      ATGACACGTGCGGTTGTGCTCGTGCTGTGCGCGCTGATCGCAGCTCAGGACGGGGGGCGCGCGGCTGCTG
kiss2 2bp D ORF   ATGACACGTGCGGTTGTGCTCGTGCTGTGCGCGCTGATCGCAGCTCAGGACGGGGGGCGCGCGGCTGCTG
kiss2 5bp D ORF   ATGACACGTGCGGTTGTGCTCGTGCTGTGCGCGCTGATCGCAGCTCAGGACGGGGGGCGCGCGGCTGCTG

                          80        90       100       110       120       130       140
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt ORF      GTCTGGCCGCGCGGGACTCTGGGCGCGGGACACACGCGACAGGTGTGCTGTGGATCCTCCGCAGGAGCGA
kiss2 2bp D ORF   GTCTGGCCGCGCGGGACTCTGGGCGCGGGACACACGCGACAGGTGTGCTGTGGATCCTCCGCAGGAGCGA
kiss2 5bp D ORF   GTCTGGCCGCGCGGGACTCTGGGCGCGGGACACACGCGACAGGTGTGCTGTGGATCCTCCGCAGGAGCGA

                         150       160       170       180       190       200       210
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt ORF      GGACGACTCTGCGGCAGGGGGGGCCGGGCTGTGCTCGTCCCTGCGGGAGGACGACGAGCAGCTGCTGTGC
kiss2 2bp D ORF   GGACGACTCTGCGGCA--GGGGGCCGGGCTGTGCTCGTCCCTGCGGGAGGACGACGAGCAGCTGCTGTGC
kiss2 5bp D ORF   GGACGACTCTGCGG-----GGGGCCGGGCTGTGCTCGTCCCTGCGGGAGGACGACGAGCAGCTGCTGTGC
                  __________________
                   gRNA target
                         220       230       240       250       260       270       280
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt ORF      GCCGACCGCCGCAGCAAGTTTAACTACAACCCGTTTGGGCTGCGCTTCGGGAAACGAGCTCCGCCCCCCA
kiss2 2bp D ORF   GCCGACCGCCGCAGCAAGTTTAACTACAACCCGTTTGGGCTGCGCTTCGGGAAACGAGCTCCGCCCCCCA
kiss2 5bp D ORF   GCCGACCGCCGCAGCAAGTTTAACTACAACCCGTTTGGGCTGCGCTTCGGGAAACGAGCTCCGCCCCCCA

                         290       300       310       320       330       340       350
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt ORF      GAGGAGCGCACCGAGCGCGCGCCATGAAGCTCCCTCTGATGTCCCTGTTTCAGGAGGTGCCCACCTGAAC
kiss2 2bp D ORF   GAGGAGCGCACCGAGCGCGCGCCATGAAGCTCCCTCTGATGTCCCTGTTTCAGGAGGTGCCCACCTGAAC
kiss2 5bp D ORF   GAGGAGCGCACCGAGCGCGCGCCATGAAGCTCCCTCTGATGTCCCTGTTTCAGGAGGTGCCCACCTGAAC

                         360       370       380       390       400       410       420
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt ORF      ACCCCCCCCCCCAGGATGTCAAGGACATGTGGGTGGGGAGGTGGGGGGTTAAAGGGTCACCCTTTTTGTA
kiss2 2bp D ORF   ACCCCCCCCCCCAGGATGTCAAGGACATGTGGGTGGGGAGGTGGGGGGTTAAAGGGTCACCCTTTTTGTA
kiss2 5bp D ORF   ACCCCCCCCCCCAGGATGTCAAGGACATGTGGGTGGGGAGGTGGGGGGTTAAAGGGTCACCCTTTTTGTA

                         430       440       450       460       470
                  ....|....|....|....|....|....|....|....|....|....|....|...
kiss2 wt ORF      CAGTGTTTGTGAAATTATTCCTAATCAAATCAACATGGAAATAAAAGAAAAAAAGTGA
kiss2 2bp D ORF   CAGTGTTTGTGAAATTATTCCTAATCAAATCAACATGGAAATAAAAGAAAAAAAGTGA
kiss2 5bp D ORF   CAGTGTTTGTGAAATTATTCCTAATCAAATCAACATGGAAATAAAAGAAAAAAAGTGA
```

## B. deduced amino acid sequence of Kiss2

```
                      10        20        30        40        50        60        70
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
kiss2 wt      MTRAVVLVLCALIAAQDGGRAAAGLAARDSGRGTHATGVLWILRRSEDDSAAGGAGLCSSLREDDEQLLC
kiss2 2bp D   MTRAVVLVLCALIAAQDGGRAAAGLAARDSGRGTHATGVLWILRRSEDDSAAGGRAVLVPAGGRRAAAVR
kiss2 5bp D   MTRAVVLVLCALIAAQDGGRAAAGLAARDSGRGTHATGVLWILRRSEDDSAGGRAVLVPAGGRRAAAVRR

                      80        90       100       110
              ....|....|....|....|....|....|....|....|....|.
kiss2 wt      ADRRSKFNYNPFGLRFGKRAPPPRGAHRARAMKLPLMSLFQEVPT*
kiss2 2bp D   RPPQQV*
kiss2 5bp D   PPQQV*
                  kisspeptin
```

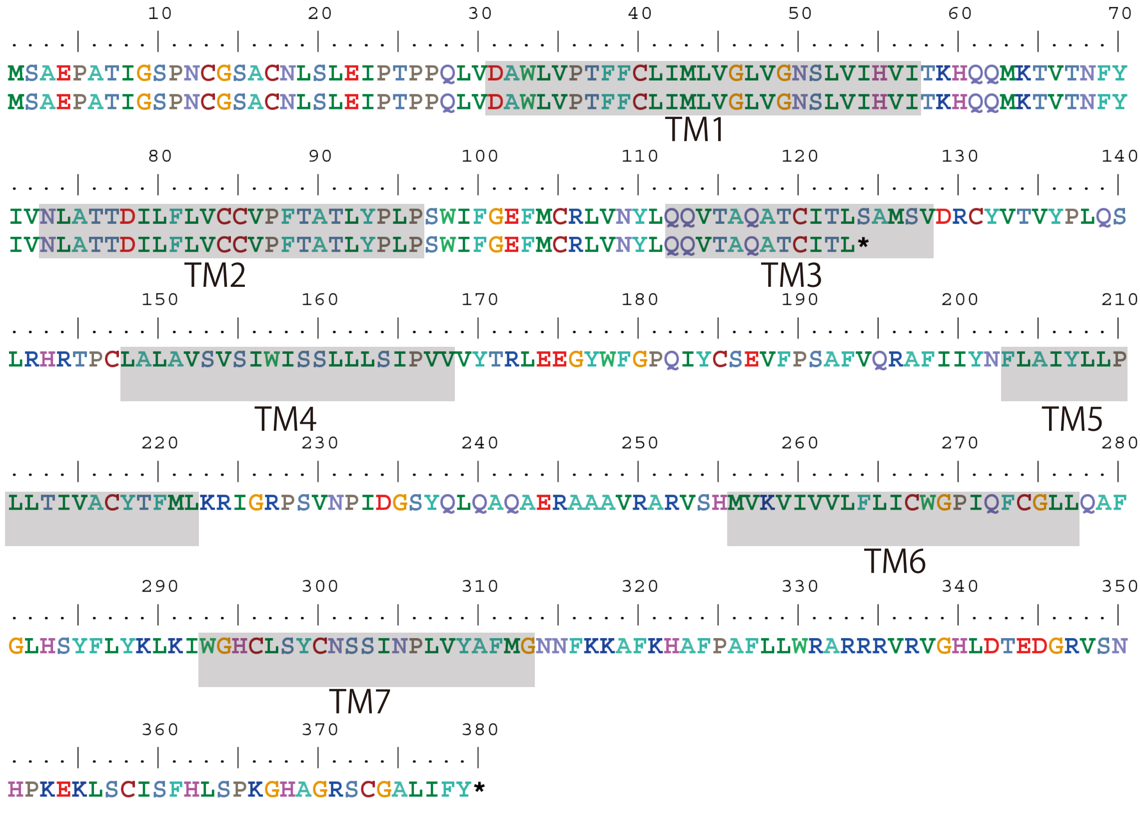
# Supplemental Figure 3

## A. ORF sequence of *gpr54-1*

```
                                  10        20        30        40        50        60        70
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            ATGTCTGCAGAACCGGCGACCATTGGGAGTCCGAACTGTGGCTCTGCGTGCAACCTTTCCCTGGAGATCC
gpr54-1 10bp D 2bp R ORF  ATGTCTGCAGAACCGGCGACCATTGGGAGTCCGAACTGTGGCTCTGCGTGCAACCTTTCCCTGGAGATCC

                                  80        90       100       110       120       130       140
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            CAACGCCACCGCAGCTGGTCGACGCCTGGTTGGTGCCCACTTTCTTCTGCCTCATCATGCTGGTCGGTCT
gpr54-1 10bp D 2bp R ORF  CAACGCCACCGCAGCTGGTCGACGCCTGGTTGGTGCCCACTTTCTTCTGCCTCATCATGCTGGTCGGTCT

                                 150       160       170       180       190       200       210
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            GGTCGGGAACTCGCTGGTCATACATGTGATCACGAAGCATCAGCAGATGAAGACTGTCACCAATTTCTAC
gpr54-1 10bp D 2bp R ORF  GGTCGGGAACTCGCTGGTCATACATGTGATCACGAAGCATCAGCAGATGAAGACTGTCACCAATTTCTAC

                                 220       230       240       250       260       270       280
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            ATAGTCAATCTGGCTACTACTGACATCTTGTTCCTGGTGTGCTGCGTTCCCTTCACCGCCACTCTGTACC
gpr54-1 10bp D 2bp R ORF  ATAGTCAATCTGGCTACTACTGACATCTTGTTCCTGGTGTGCTGCGTTCCCTTCACCGCCACTCTGTACC

                                 290       300       310       320       330       340       350
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            CTCTGCCCAGCTGGATCTTTGGGGAGTTCATGTGCCGTCTGGTCAATTATCTACAACAGGTGACTGCGCA
gpr54-1 10bp D 2bp R ORF  CTCTGCCCAGCTGGATCTTTGGGGAGTTCATGTGCCGTCTGGTCAATTATCTACAACAGGTGACTGCGCA

                                 360       370       380       390       400       410       420
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            GGCGACTTGCATCACCCTGTCTGCCATGAGCGTGGACCGCTGCTATGTGACGGTCTATCCTCTGCAGTCG
gpr54-1 10bp D 2bp R ORF  GGCGACTTGCATCACCCTGTG----------ATGGACCGCTGCTATGTGACGGTCTATCCTCTGCAGTCG
                                             gRNA target
                                 430       440       450       460       470       480       490
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            CTGCGACACCGCACCCCCTGCTTGGCTCTGGCCGTCTCTGTGTCCATCTGGATAAGCTCCTTGCTTCTGT
gpr54-1 10bp D 2bp R ORF  CTGCGACACCGCACCCCCTGCTTGGCTCTGGCCGTCTCTGTGTCCATCTGGATAAGCTCCTTGCTTCTGT

                                 500       510       520       530       540       550       560
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            CCATCCCTGTGGTCGTGTACACCCGTCTAGAGGAAGGATACTGGTTTGGCCCACAGATTTACTGCAGCGA
gpr54-1 10bp D 2bp R ORF  CCATCCCTGTGGTCGTGTACACCCGTCTAGAGGAAGGATACTGGTTTGGCCCACAGATTTACTGCAGCGA

                                 570       580       590       600       610       620       630
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            GGTCTTCCCCTCTGCTTTTGTCCAGAGAGCCTTCATCATTTACAACTTTTTGGCCATCTACCTCCTCCCC
gpr54-1 10bp D 2bp R ORF  GGTCTTCCCCTCTGCTTTTGTCCAGAGAGCCTTCATCATTTACAACTTTTTGGCCATCTACCTCCTCCCC

                                 640       650       660       670       680       690       700
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            CTTCTGACCATCGTTGCCTGTTACACCTTCATGCTCAAGCGCATTGGCCGACCCAGTGTGAATCCCATCG
gpr54-1 10bp D 2bp R ORF  CTTCTGACCATCGTTGCCTGTTACACCTTCATGCTCAAGCGCATTGGCCGACCCAGTGTGAATCCCATCG

                                 710       720       730       740       750       760       770
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            ACGGCAGCTACCAACTCCAGGCTCAGGCGGAGCGAGCAGCAGCCGTCCGAGCTCGAGTCTCCCACATGGT
gpr54-1 10bp D 2bp R ORF  ACGGCAGCTACCAACTCCAGGCTCAGGCGGAGCGAGCAGCAGCCGTCCGAGCTCGAGTCTCCCACATGGT

                                 780       790       800       810       820       830       840
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            GAAGGTTATAGTGGTCCTCTTCCTCATCTGCTGGGGCCCCATCCAGTTCTGTGGGCTGCTGCAAGCTTTT
gpr54-1 10bp D 2bp R ORF  GAAGGTTATAGTGGTCCTCTTCCTCATCTGCTGGGGCCCCATCCAGTTCTGTGGGCTGCTGCAAGCTTTT

                                 850       860       870       880       890       900       910
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            GGCCTCCACAGCTACTTTCTATACAAACTAAAGATTTGGGGCCACTGCTTGTCCTACTGCAACTCCTCCA
gpr54-1 10bp D 2bp R ORF  GGCCTCCACAGCTACTTTCTATACAAACTAAAGATTTGGGGCCACTGCTTGTCCTACTGCAACTCCTCCA

                                 920       930       940       950       960       970       980
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            TCAACCCACTGGTTTATGCCTTCATGGGCAACAACTTCAAGAAGGCTTTCAAACATGCTTTCCCAGCCTT
gpr54-1 10bp D 2bp R ORF  TCAACCCACTGGTTTATGCCTTCATGGGCAACAACTTCAAGAAGGCTTTCAAACATGCTTTCCCAGCCTT
```

```
gpr54-1 10bp D 2bp R ORF  TCTTCTGTGGCGCGCCAGGAGAAGAGTCCGGGTGGGACATTTAGACACGGAGGACGGCAGAGTCAGCAAC

                                    1060      1070      1080      1090      1100      1110      1120
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            CACCCAAAGGAGAAGCTGAGCTGCATTTCCTTTCATCTGAGTCCTAAAGGCCACGCAGGCCGTTCATGCG
gpr54-1 10bp D 2bp R ORF  CACCCAAAGGAGAAGCTGAGCTGCATTTCCTTTCATCTGAGTCCTAAAGGCCACGCAGGCCGTTCATGCG

                                    1130      1140      1150      1160      1170      1180      1190
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            GTGCGCTCATTTTTTATTAATAAACCTGCTCTGGAGTGGTGTGAACATAAACACATGATCAGAAAAGAGG
gpr54-1 10bp D 2bp R ORF  GTGCGCTCATTTTTTATTAATAAACCTGCTCTGGAGTGGTGTGAACATAAACACATGATCAGAAAAGAGG

                                    1200      1210      1220      1230      1240      1250      1260
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt ORF            AATATTTGTTCAAAGTTTCTAAAAGTCTGACAGACTTTAAGTCCTTGAAGCAGAAGCACACAGCCTCACA
gpr54-1 10bp D 2bp R ORF  AATATTTGTTCAAAGTTTCTAAAAGTCTGACAGACTTTAAGTCCTTGAAGCAGAAGCACACAGCCTCACA

                          ....|...
gpr54-1 wt ORF            TGATTCTT
gpr54-1 10bp D 2bp R ORF  TGATTCTT
```

## B. deduced amino acid sequence of Gpr54-1



```
                                 10        20        30        40        50        60        70
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt               MSAEPATIGSPNCGSACNLSLEIPTPPQLVDAWLVPTFFCLIMLVGLVGNSLVIHVITKHQQMKTVTNFY
gpr54-1 10bp D 2bp R     MSAEPATIGSPNCGSACNLSLEIPTPPQLVDAWLVPTFFCLIMLVGLVGNSLVIHVITKHQQMKTVTNFY

                                 80        90       100       110       120       130       140
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt               IVNLATTDILFLVCCVPFTATLYPLPSWIFGEFMCRLVNYLQQVTAQATCITLSAMSVDRCYVTVYPLQS
gpr54-1 10bp D 2bp R     IVNLATTDILFLVCCVPFTATLYPLPSWIFGEFMCRLVNYLQQVTAQATCITL*

                                150       160       170       180       190       200       210
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt               LRHRTPCLALAVSVSIWISSLLLSIPVVVYTRLEEGYWFGPQIYCSEVFPSAFVQRAFIIYNFLAIYLLP
gpr54-1 10bp D 2bp R

                                220       230       240       250       260       270       280
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt               LLTIVACYTFMLKRIGRPSVNPIDGSYQLQAQAERAAAVRARVSHMVKVIVVLFLICWGPIQFCGLLQAF
gpr54-1 10bp D 2bp R

                                290       300       310       320       330       340       350
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-1 wt               GLHSYFLYKLKIWGHCLSYCNSSINPLVYAFMGNNFKKAFKHAFPAFLLWRARRRVRVGHLDTEDGRVSN
gpr54-1 10bp D 2bp R

                                360       370       380
                         ....|....|....|....|....|....|
gpr54-1 wt               HPKEKLSCISFHLSPKGHAGRSCGALIFY*
gpr54-1 10bp D 2bp R
```

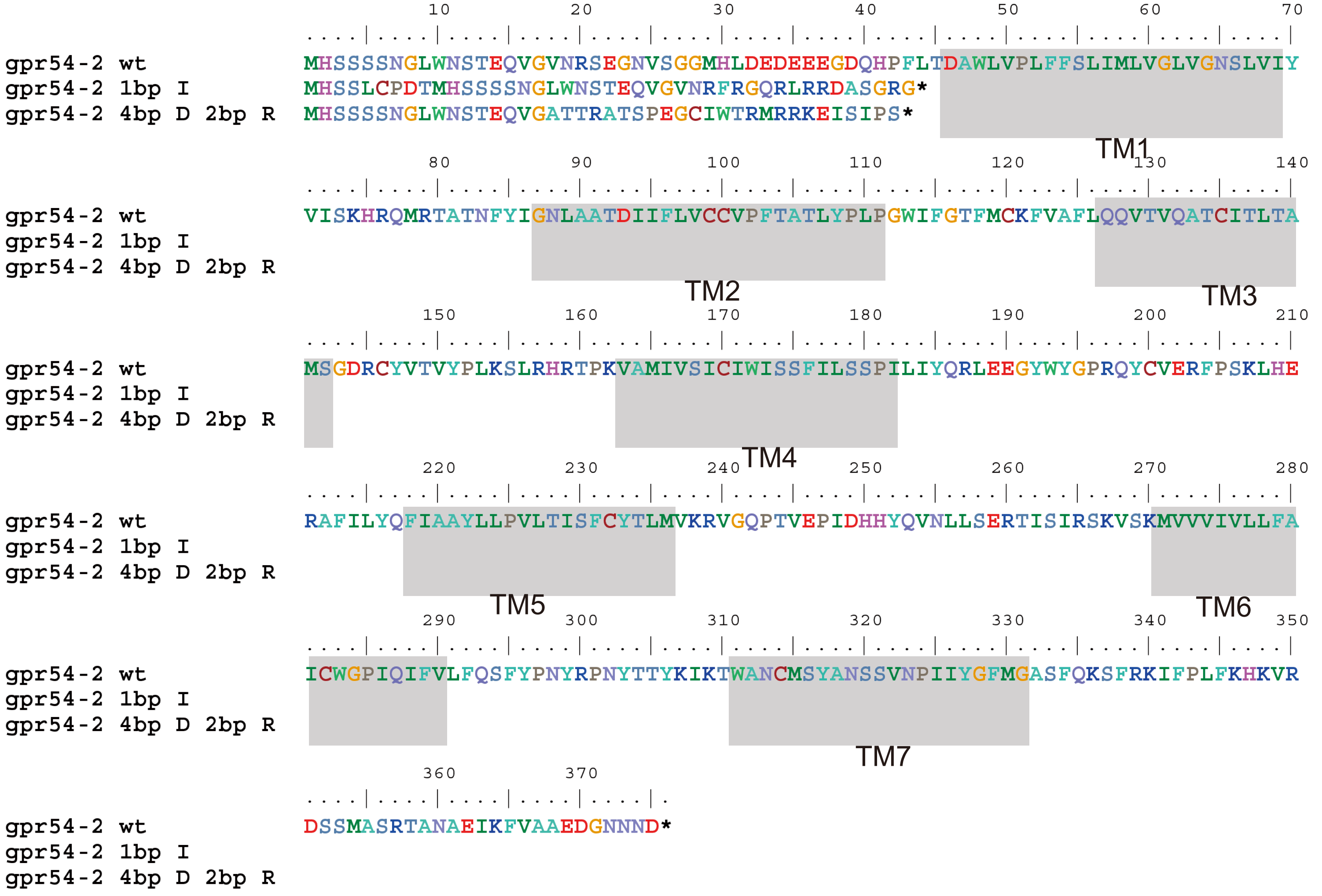
Supplemental Figure 4

ORF sequence of *gpr54-2*

```
                                       10        20        30        40        50        60        70
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  ATGCACTCCTCCTCCTCCAACGGGCTCTGGAACTCCACCGAGCAGGTGGGGGTCAACAGAT-CCGAGGGC
gpr54-2 1bp I ORF               ATGCACTCCTCCTCCTCCAACGGGCTCTGGAACTCCACCGAGCAGGTGGGGGTCAACAGATTCCGAGGGC
gpr54-2 4bp D & 2bp R ORF       ATGCACTCCTCCTCCTCCAACGGGCTCTGGAACTCCACCGAGCAGGTGGGGG-CAACGA----CGAGGGC
                                                                                 gRNA target

                                       80        90       100       110       120       130       140
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  AACGTCTCCGGAGGGATGCATCTGGACGAGGATGAGGAGGAAGGAGATCAGCATCCCTTCCTGACCGATG
gpr54-2 1bp I ORF               AACGTCTCCGGAGGGATGCATCTGGACGAGGATGAGGAGGAAGGAGATCAGCATCCCTTCCTGACCGATG
gpr54-2 4bp D & 2bp R ORF       AACGTCTCCGGAGGGATGCATCTGGACGAGGATGAGGAGGAAGGAGATCAGCATCCCTTCCTGACCGATG

                                      150       160       170       180       190       200       210
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  CCTGGCTGGTCCCTTTGTTCTTCTCGCTCATCATGCTGGTTGGACTTGTCGGCAACTCTCTGGTTATCTA
gpr54-2 1bp I ORF               CCTGGCTGGTCCCTTTGTTCTTCTCGCTCATCATGCTGGTTGGACTTGTCGGCAACTCTCTGGTTATCTA
gpr54-2 4bp D & 2bp R ORF       CCTGGCTGGTCCCTTTGTTCTTCTCGCTCATCATGCTGGTTGGACTTGTCGGCAACTCTCTGGTTATCTA

                                      220       230       240       250       260       270       280
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  TGTGATTTCTAAACACAGACAGATGAGGACGGCCACTAACTTCTACATAGCAAACCTGGCTGCCACTGAT
gpr54-2 1bp I ORF               TGTGATTTCTAAACACAGACAGATGAGGACGGCCACTAACTTCTACATAGCAAACCTGGCTGCCACTGAT
gpr54-2 4bp D & 2bp R ORF       TGTGATTTCTAAACACAGACAGATGAGGACGGCCACTAACTTCTACATAGCAAACCTGGCTGCCACTGAT

                                      290       300       310       320       330       340       350
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  ATCATCTTCTTGGTGTGCTGCGTCCCCTTCACCGCCACCCTGTACCCCCTACCTGGATGGATCTTCGGCA
gpr54-2 1bp I ORF               ATCATCTTCTTGGTGTGCTGCGTCCCCTTCACCGCCACCCTGTACCCCCTACCTGGATGGATCTTCGGCA
gpr54-2 4bp D & 2bp R ORF       ATCATCTTCTTGGTGTGCTGCGTCCCCTTCACCGCCACCCTGTACCCCCTACCTGGATGGATCTTCGGCA

                                      360       370       380       390       400       410       420
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  CATTTATGTGCAAGTTTGTTGCTTTTTTGCAGCAGGTGACAGTGCAGGCTACTTGTATCACCCTGACTGC
gpr54-2 1bp I ORF               CATTTATGTGCAAGTTTGTTGCTTTTTTGCAGCAGGTGACAGTGCAGGCTACTTGTATCACCCTGACTGC
gpr54-2 4bp D & 2bp R ORF       CATTTATGTGCAAGTTTGTTGCTTTTTTGCAGCAGGTGACAGTGCAGGCTACTTGTATCACCCTGACTGC

                                      430       440       450       460       470       480       490
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  AATGAGTGGCGATCGTTGTTATGTGACCGTCTACCCCCTGAAATCTCTCCGCCACCGTACCCCAAAAGTA
gpr54-2 1bp I ORF               AATGAGTGGCGATCGTTGTTATGTGACCGTCTACCCCCTGAAATCTCTCCGCCACCGTACCCCAAAAGTA
gpr54-2 4bp D & 2bp R ORF       AATGAGTGGCGATCGTTGTTATGTGACCGTCTACCCCCTGAAATCTCTCCGCCACCGTACCCCAAAAGTA

                                      500       510       520       530       540       550       560
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  GCCATGATTGTCAGTATCTGCATTTGGATAAGCTCTTTCATACTGTCATCTCCGATCCTAATTTACCAAC
gpr54-2 1bp I ORF               GCCATGATTGTCAGTATCTGCATTTGGATAAGCTCTTTCATACTGTCATCTCCGATCCTAATTTACCAAC
gpr54-2 4bp D & 2bp R ORF       GCCATGATTGTCAGTATCTGCATTTGGATAAGCTCTTTCATACTGTCATCTCCGATCCTAATTTACCAAC

                                      570       580       590       600       610       620       630
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  GACTAGAGGAGGGATATTGGTACGGCCCGAGACAATACTGCGTGGAAAGATTTCCCTCAAAGCTTCACGA
gpr54-2 1bp I ORF               GACTAGAGGAGGGATATTGGTACGGCCCGAGACAATACTGCGTGGAAAGATTTCCCTCAAAGCTTCACGA
gpr54-2 4bp D & 2bp R ORF       GACTAGAGGAGGGATATTGGTACGGCCCGAGACAATACTGCGTGGAAAGATTTCCCTCAAAGCTTCACGA

                                      640       650       660       670       680       690       700
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  GAGGGCCTTCATCCTCTACCAGTTCATAGCTGCGTACCTGCTGCCGGTGCTGACGATCTCCTTCTGCTAC
gpr54-2 1bp I ORF               GAGGGCCTTCATCCTCTACCAGTTCATAGCTGCGTACCTGCTGCCGGTGCTGACGATCTCCTTCTGCTAC
gpr54-2 4bp D & 2bp R ORF       GAGGGCCTTCATCCTCTACCAGTTCATAGCTGCGTACCTGCTGCCGGTGCTGACGATCTCCTTCTGCTAC

                                      710       720       730       740       750       760       770
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  ACTCTGATGGTAAAAAGGGTTGGTCAGCCCACAGTGGAACCAATAGACCACCACTATCAGGTCAACCTTC
gpr54-2 1bp I ORF               ACTCTGATGGTAAAAAGGGTTGGTCAGCCCACAGTGGAACCAATAGACCACCACTATCAGGTCAACCTTC
gpr54-2 4bp D & 2bp R ORF       ACTCTGATGGTAAAAAGGGTTGGTCAGCCCACAGTGGAACCAATAGACCACCACTATCAGGTCAACCTTC

                                      780       790       800       810       820       830       840
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                  TGTCTGAAAGAACAATCAGCATCAGGAGCAAAGTCTCCAAGATGGTGGTGGTGATCGTTCTACTCTTCGC
gpr54-2 1bp I ORF               TGTCTGAAAGAACAATCAGCATCAGGAGCAAAGTCTCCAAGATGGTGGTGGTGATCGTTCTACTCTTCGC
gpr54-2 4bp D & 2bp R ORF       TGTCTGAAAGAACAATCAGCATCAGGAGCAAAGTCTCCAAGATGGTGGTGGTGATCGTTCTACTCTTCGC

                                      850       860       870       880       890       900       910
```

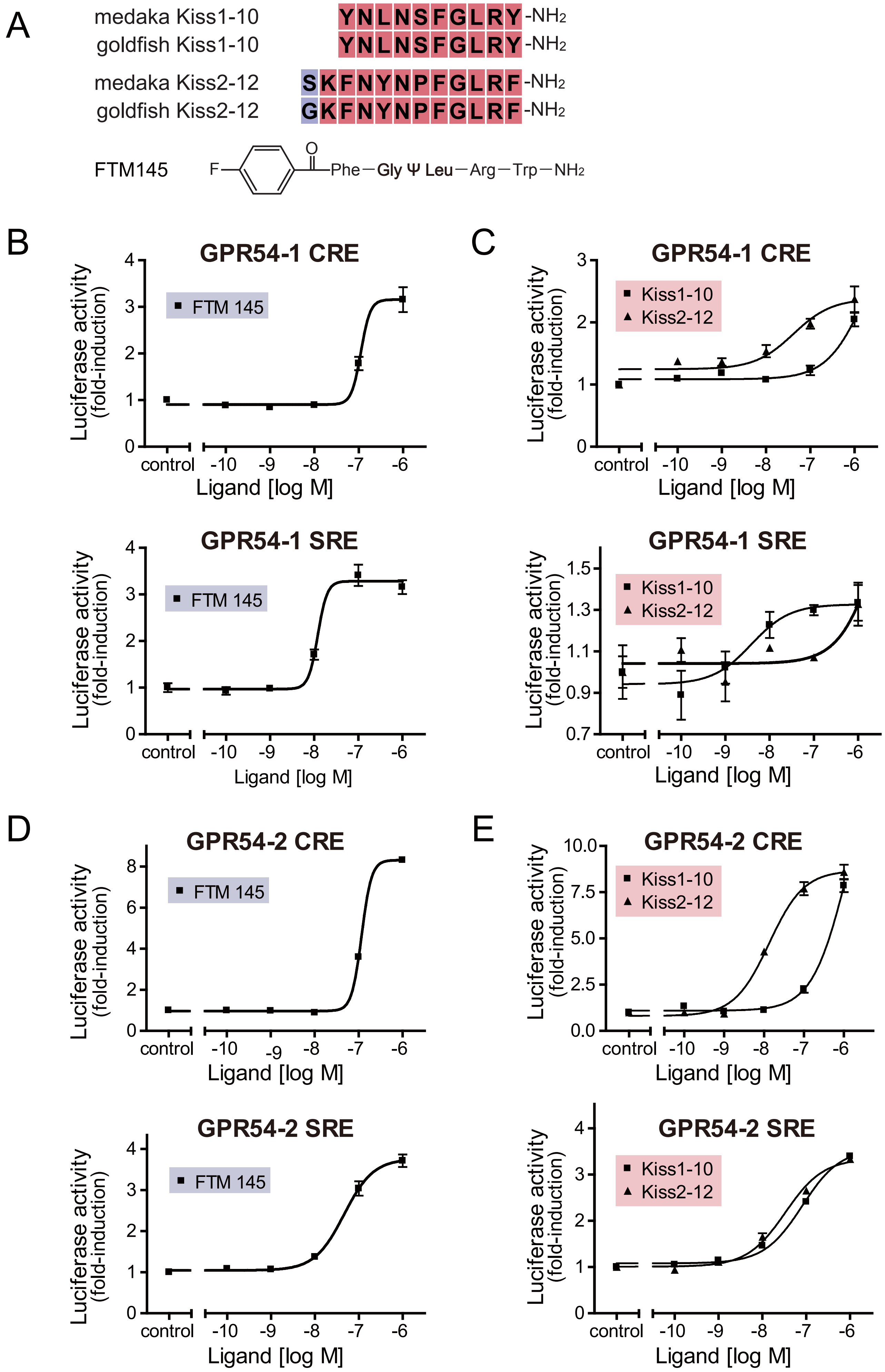
```
                              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                CATCTGCTGGGGTCCCATCCAGATATTTGTCCTTTTTCAATCCTTCTACCCAAACTACCGTCCAAACTAC
gpr54-2 1bp I ORF             CATCTGCTGGGGTCCCATCCAGATATTTGTCCTTTTTCAATCCTTCTACCCAAACTACCGTCCAAACTAC
gpr54-2 4bp D & 2bp R ORF     CATCTGCTGGGGTCCCATCCAGATATTTGTCCTTTTTCAATCCTTCTACCCAAACTACCGTCCAAACTAC

                                     920       930       940       950       960       970       980
                              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                ACAACCTACAAGATCAAGACATGGGCCAACTGCATGTCCTACGCTAACTCTTCAGTCAACCCCATCATCT
gpr54-2 1bp I ORF             ACAACCTACAAGATCAAGACATGGGCCAACTGCATGTCCTACGCTAACTCTTCAGTCAACCCCATCATCT
gpr54-2 4bp D & 2bp R ORF     ACAACCTACAAGATCAAGACATGGGCCAACTGCATGTCCTACGCTAACTCTTCAGTCAACCCCATCATCT

                                     990      1000      1010      1020      1030      1040      1050
                              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                ATGGATTCATGGGGGCCAGCTTTCAGAAGTCCTTCAGGAAAATCTTCCCCTTCCTGTTTAAGCACAAGGT
gpr54-2 1bp I ORF             ATGGATTCATGGGGGCCAGCTTTCAGAAGTCCTTCAGGAAAATCTTCCCCTTCCTGTTTAAGCACAAGGT
gpr54-2 4bp D & 2bp R ORF     ATGGATTCATGGGGGCCAGCTTTCAGAAGTCCTTCAGGAAAATCTTCCCCTTCCTGTTTAAGCACAAGGT

                                    1060      1070      1080      1090      1100      1110      1120
                              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt ORF                CCGAGATAGCAGCATGGCCTCAAGGACCGCCAATGCCGAAATCAAGTTTGTGGCTGCAGAGGACGGGAAC
gpr54-2 1bp I ORF             CCGAGATAGCAGCATGGCCTCAAGGACCGCCAATGCCGAAATCAAGTTTGTGGCTGCAGAGGACGGGAAC
gpr54-2 4bp D & 2bp R ORF     CCGAGATAGCAGCATGGCCTCAAGGACCGCCAATGCCGAAATCAAGTTTGTGGCTGCAGAGGACGGGAAC

                                    1130
                              ....|....|..
gpr54-2 wt ORF                AATAATGACTGA
gpr54-2 1bp I ORF             AATAATGACTGA
gpr54-2 4bp D & 2bp R ORF     AATAATGACTGA
```

## B. deduced amino acid sequence of Gpr54-2

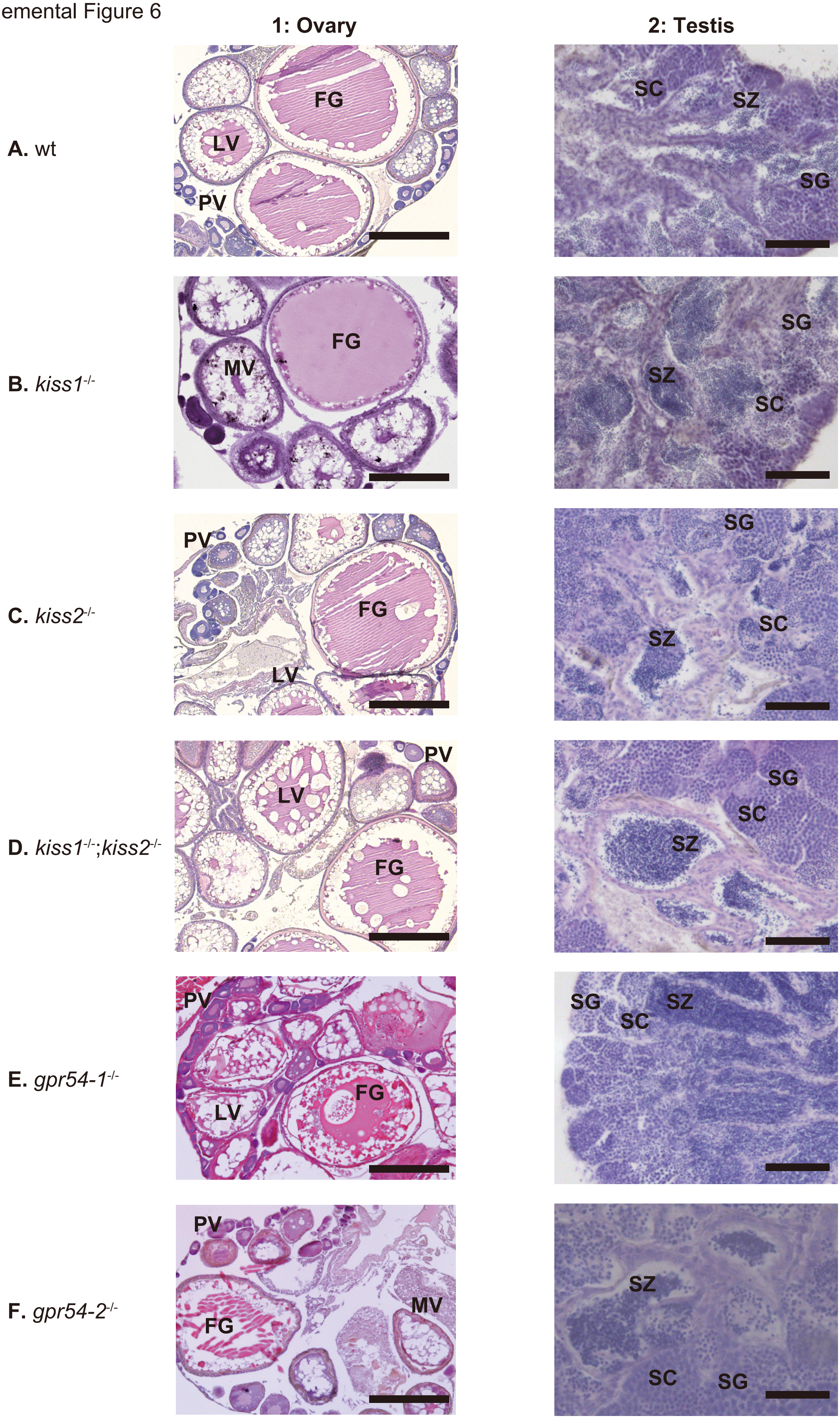


```
                               10        20        30        40        50        60        70
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt             MHSSSSNGLWNSTEQVGVNRSEGNVSGGMHLDEDEEEGDQHPFLTDAWLVPLFFSLIMLVGLVGNSLVIY
gpr54-2 1bp I          MHSSLCPDTMHSSSSNGLWNSTEQVGVNRFRGQRLRRDASGRG*
gpr54-2 4bp D 2bp R    MHSSSSNGLWNSTEQVGATTRATSPEGCIWTRMRRKEISIPS*

                               80        90       100       110       120       130       140
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt             VISKHRQMRTATNFYIGNLAATDIIFLVCCVPFTATLYPLPGWIFGTFMCKFVAFLQQVTVQATCITLTA
gpr54-2 1bp I
gpr54-2 4bp D 2bp R

                              150       160       170       180       190       200       210
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt             MSGDRCYVTVYPLKSLRHRTPKVAMIVSICIWISSFILSSPILIYQRLEEGYWYGPRQYCVERFPSKLHE
gpr54-2 1bp I
gpr54-2 4bp D 2bp R

                              220       230       240       250       260       270       280
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt             RAFILYQFIAAYLLPVLTISFCYTLMVKRVGQPTVEPIDHHYQVNLLSERTISIRSKVSKMVVVIVLLFA
gpr54-2 1bp I
gpr54-2 4bp D 2bp R

                              290       300       310       320       330       340       350
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gpr54-2 wt             ICWGPIQIFVLFQSFYPNYRPNYTTYKIKTWANCMSYANSSVNPIIYGFMGASFQKSFRKIFPLFKHKVR
gpr54-2 1bp I
gpr54-2 4bp D 2bp R

                              360       370
                       ....|....|....|....|....|.
gpr54-2 wt             DSSMASRTANAEIKFVAAEDGNNND*
gpr54-2 1bp I
gpr54-2 4bp D 2bp R
```

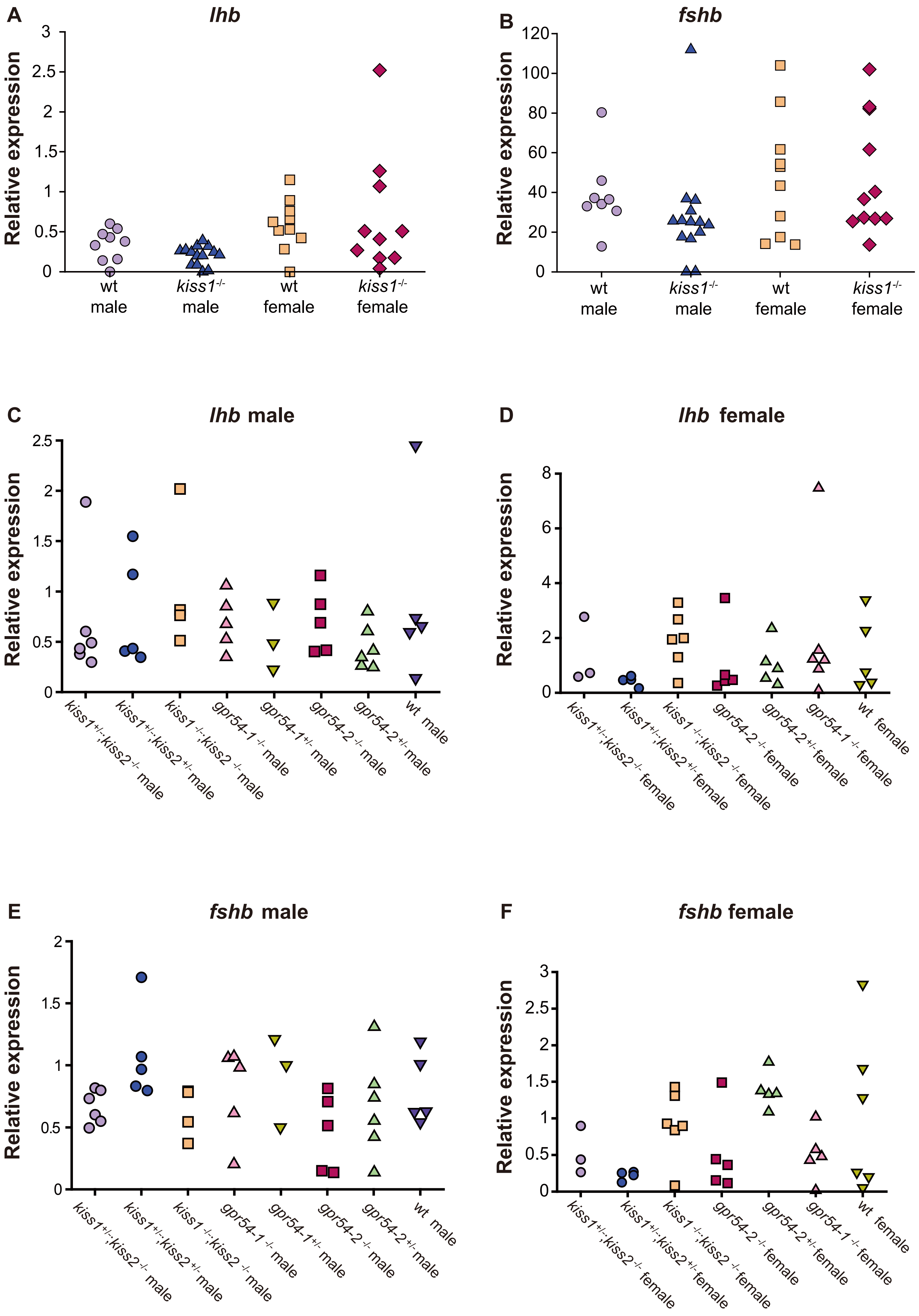
Supplemental Figure 5



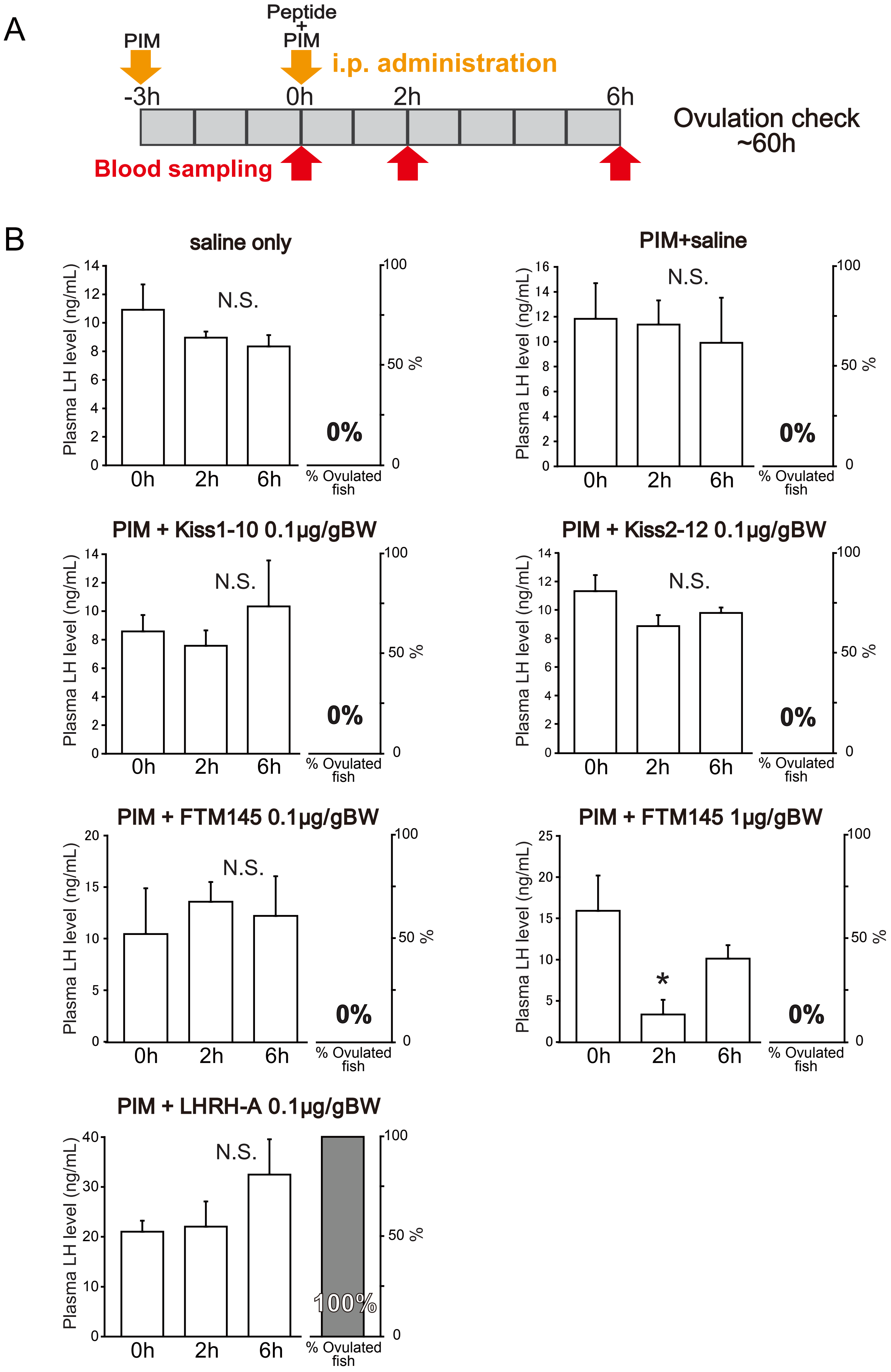
Supplemental Figure 6



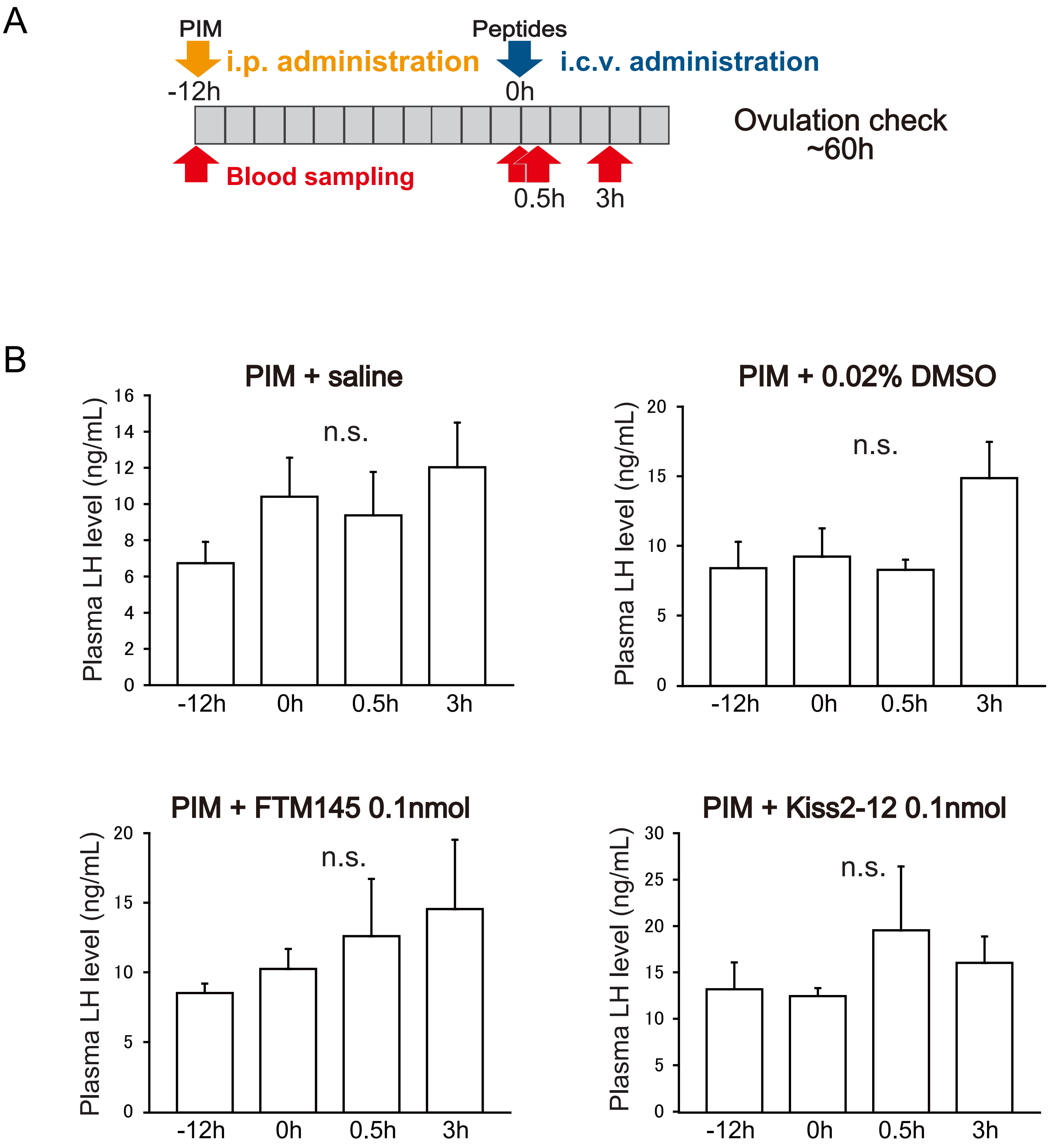
Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9



Supplemental Figure 10