Duplicated Kiss1 receptor genes in zebrafish: distinct gene expression patterns, different ligand selectivity, and a novel nuclear isoform with transactivating activity

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ABSTRACT **The kisspeptin (Kiss1) and Kiss1 receptor (Kiss1r) pathway plays a central role in the neuroendocrine control of reproduction. In contrast to humans and mammals that have a single Kiss1 gene and a single Kiss1r gene, multiple Kiss ligand and receptor genes are found in nonmammalian vertebrates. Their functional relationship, however, is poorly understood. Here, we report that the duplicated zebrafish *kiss1r* genes have evolved a distinct gene expression pattern, different ligand selectivity, and novel nuclear isoforms. While a single *kiss1ra* mRNA was detected exclusively in the brain, 5 *kiss1rb* transcripts were found in many peripheral tissues. Functional assays showed that *kiss1ra* encodes a receptor activated by both Kiss1 and Kiss2, while *kiss1rb* encodes a receptor that has a preference for Kiss1. The four alternatively spliced *kiss1rb* mRNAs encoded 4 truncated isoforms, denoted *kiss1rb*-derived protein (KRBDP)1–4. When their subcellular localization was examined, KRBDP3 and KRBDP4 were found in the nucleus in cultured mammalian cells and in zebrafish embryos. One-hybrid transcription activation assays revealed that KRBDP3, but not KRBDP4, possesses ligand-independent transactivation activity. These findings highlight how the duplication of Kiss1r genes may facilitate their adaptation of specialized functions. The discovery of a nuclear Kiss1r isoform raises the possibility of novel function of Kiss1r in the nucleus.—Onuma, T. A., Duan, C. Duplicated Kiss1 receptor genes in zebrafish: distinct gene expression patterns, different ligand selectivity, and a novel nuclear isoform with transactivating activity. *FASEB J.* 26, 2941–2950 (2012). www.fasebj.org**

Key Words: alternative splicing · nuclear localization

The kisspeptin (Kiss1) and Kiss1 receptor (Kiss1r) pathway plays a central role in the neuroendocrine control of reproduction in vertebrates (1–3). Clinical and animal studies have shown that the Kiss1-Kiss1r signaling regulates the hypothalamic-pituitary-gonadal (HPG) axis and puberty. Loss-of-function mutations in the human *KISS1* and/or *KISS1R* genes have been linked to hypothalamic hypogonadism (4, 5). Gain-of-function mutations in these genes are implicated in gonadotropin-releasing hormone (GnRH)-dependent precocious puberty (6–8). Knockout of the *Kiss1* gene (5, 9) or the *Kiss1r* gene (10, 11) leads to hypogonadism and infertility in the mouse model. Likewise, ablation of kisspeptin neurons in adult mice results in the loss of the estrous cycle and infertility (12). Furthermore, administration of Kiss1 increases GnRH secretion and advances puberty onset (13–16). Administration of Kiss1r antagonist delays onset of puberty (17). These studies have underscored the central importance of the Kiss1-Kiss1r pathway in reproduction in mammals.

It is now understood that the major components of the HPG axis, including GnRH, GnRH receptors, and gonadotropins, are conserved from jawless fishes to humans (18, 19). Recent studies suggest that the Kiss1-Kiss1r system is also conserved and functional in many nonmammalian vertebrates (20, 21). In contrast to the presence of a single Kiss1 gene and a single Kiss1r gene in humans and mice, however, multiple Kiss ligand and receptor genes have been identified in nonmammalian species (3, 20, 21). For instance, zebrafish have 2 Kiss ligand genes (*kiss1* and *kiss*2) and 2 Kiss1r genes (referred to as *kiss1ra/GPR54-2* and *kiss1rb/GPR54-1*)

Abbreviations: CHO, Chinese hamster ovary; DAPI, 4′,6-diamidino-2-phenylindole; DBD, DNA-binding domain; dpf, days postfertilization; GnRH, gonadotropin-releasing hormone; GPCR, G-protein coupled receptor; HEK, human embryonic kidney; hpf, hours postfertilization; HPG, hypothalamic-pituitary-gonadal; KRBDP, *kiss1rb*-derived protein; Luc, luciferase; ORF, open reading frame; PTC, premature termination codon; SRE, serum-responsive element; TA, transactivation; TMD, transmembrane domain

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(22–25). The multiple kiss ligand and receptor genes are thought to be a result of an additional round of genome-wide duplication that has occurred in some teleost and/or amphibian lineages (3, 20, 21). Gene duplication is considered to be a major driving force for reshaping genes and their roles in physiology (26). The retention of two functional Kiss ligand and Kiss1r genes has provided a unique opportunity to gain insights into how the duplicated Kiss1-Kiss1r system may have adapted specialized functions.

In this study, we tested the hypothesis that the duplicated zebrafish *kiss1r* genes have evolved distinct gene expression patterns and functions. Our results suggest that zebrafish *kiss1ra* and *kiss1rb* genes exhibit distinct gene expression patterns and encode receptors with different ligand selectivity. Furthermore, we have identified several novel alternatively spliced *kiss1rb* transcripts. Unexpectedly, one of these transcripts encodes a nuclear isoform that has strong transactivation (TA) activity.

MATERIALS AND METHODS

Materials

All chemicals and reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA) unless stated otherwise. Restriction enzymes were purchased from Promega (Madison, WI, USA) or New England Biolabs (Ipswich, MA, USA). *Taq*DNA polymerase and Expand High Fidelity PCR system were purchased from New England Biolabs and Roche (Basel, Switzerland), respectively. The mouse M2 anti-FLAG antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Cy3-conjugated second antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Superscript III reverse transcriptase, oligonucleotide primers, and cell culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Decapeptides encoding the active core of zebrafish Kiss1 (Z1-10, YNLNSFGLRY-NH_2) and Kiss2 (Z2-10, FNYNPFGLRY-NH_2) were synthesized by the University of Michigan Protein Core Facility. They were dissolved and diluted in 0.1% trifluoroacetic anhydrate (Sigma-Aldrich).

Animals

Wild-type zebrafish (*Danio rerio*) were maintained on a 14/10-h light-dark cycle at 28°C and fed twice daily. Males and females were maintained in separate tanks until the day of natural crossing. Fertilized eggs were raised following standard methods. All experiments were conducted in accordance with guidelines approved by the University of Michigan Committee on the Use and Care of Animals.

Identification and cloning of multiple *kissr* genes and cDNAs

To clone open reading frames (ORFs) of zebrafish *kissr* genes for functional analysis, RT-PCR was carried out with the Expand High Fidelity PCR system and primers shown in Supplemental Table S1 and RNA prepared from adult zebrafish brain. The PCR products were purified, cloned into the pGEM-T Easy vector (Promega), and sequenced at the University of Michigan DNA Sequencing Core Facility. In addition to the previously reported Kiss1ra and Kiss1rb cDNAs (22, 24, 27), several additional Kiss1rb variants were obtained. On the basis of sequence comparison with the genome sequence (http://www.genome.ucsc.edu/cgi-bin/hgBlat), they were likely products of alternative splicing. Their deduced amino acid sequences and GenBank accession numbers are shown in Supplemental Table S2.

RT-PCR analysis

Total RNA was extracted from embryos, larvae, and various adult tissues using TRIzol reagent (Invitrogen). Total RNA (1 μg) was transcribed into single-stranded cDNA using oligo dT primer and Superscript III reverse transcriptase. RT-PCR was performed using *Taq*DNA polymerase. The PCR was carried out at 94°C for 2 min, followed by 38 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min. The primers were designed to specifically cover the deletion and/or insertion regions of each Kiss1r isoform (Supplemental Table S2 and Supplemental Fig. S1).

Plasmid construction

To study their role in mediating Kiss signaling, cDNAs encoding various zebrafish Kiss1r isoforms were amplified by PCR and subcloned into the pcDNA3.1(+) expression vector (Invitrogen) using the *Bam*HI and *Xho*I sites. For the subcellular localization study, cDNAs encoding various Kiss1r ORFs (with the stop codon deleted) were subcloned into the pCS2+/EGFP expression vector. To generate a Flag-tagged Kiss1r fusion protein construct, cDNA encoding *kiss1rb*-derived protein 3 (KRBDP3) ORF was subcloned into the pCMV-Tag1 expression vector, as reported previously (28). To generate various Gal4-DNA-binding domain (DBD) and KRBDP fusion protein constructs for the 1-hybrid assay, DNA fragments encoding various KRBDP fragments were generated by PCR using KOD DNA polymerase (Toyobo, Osaka, Japan) and subcloned into the pBind vector (Promega) to fuse KRBDP in-frame to the C terminus of Gal4-DBD. Deletion mutants were generated by PCR using primers shown in Supplemental Table S1, and subcloned into the pBIND vector. All of these plasmids were verified by sequencing.

Cell culture

Human embryonic kidney (HEK) 293 cells, Chinese hamster ovary (CHO)-K1 cells, and African green monkey SV40-transformed kidney fibroblast (COS-7) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The GT1-7 cells, a hypothalamic GnRH1 neuron-derived cell line (29), were kindly provided by Dr. Pamela Mellon, (University of California—San Diego, La Jolla, CA, USA). The cells were cultured in DMEM or DEME/F12 medium in the presence of 10% FBS, penicillin, and streptomycin in a humidified-air atmosphere incubator containing 5% CO_2. Zebrafish embryonic (ZF4) cells, obtained from ATCC, were grown as reported previously (30).

Receptor function assay

COS-7, CHO-K1, and HEK293 cells, placed in 24-well plates, were transfected with various Kiss1r constructs using Lipofectamine (Invitrogen). For each transfection, 460 ng pcDNA3.1(+)-Kiss1r, 200 ng serum-responsive element–luciferase (SRE-Luc) reporter vector, and 40 ng pRenilla-Luc vector DNA were used for transfection. The SRE-Luc vector

(which has an SRE upstream of the firefly luciferase gene; ref. 31) was kindly provided by Dr. Jessica Schwartz (University of Michigan). Immediately after transfection, cells were serum-starved for 24 h and treated with various concentrations of Z1-10 or Z2-10 peptide for 6 h. The treated cells were washed with 1× PBS and harvested; then, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was normalized by *Renilla* luciferase activity.

Subcellular localization

COS-7 cells and GT1-7 cells were transiently transfected with various pCS2+-Kiss1r:EGFP constructs using Lipofectamine. At 24 h after the transfection, cells were washed with 1× PBS, fixed with 4% paraformaldehyde, and stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The stained cells were washed, mounted, and photographed under a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

Immunocytochemistry

COS-7 cells were transfected with the empty pCMV-Tag1, pCMV:KRBDP3-FLAG construct, or pCMV:IGFBP5-FLAG (28). At 24 h after the transfection, cells were washed with 1× PBS, fixed with 4% paraformaldehyde, and blocked with 20% sheep serum plus 0.1% Tween 20 for 1 h at room temperature. They were then incubated with a mouse M2 anti-FLAG antibody in the blocking buffer at 4°C overnight. After washing, the cells were incubated with Cy3-conjugated anti-mouse IgG for 2 h at room temperature and counterstained with DAPI. The stained cells were washed, mounted, and photographed under a Nikon Eclipse E600 fluorescence microscope.

Microinjection experiment

To determine whether KRBDP3-EGFP is localized in the nucleus *in vivo*, capped mRNA synthesis was carried out using a mMessag mMachine kit (Ambion, Austin, TX, USA) and linearized plasmid DNA as a template. KRBDP3-EGFP mRNA (1 nl) was microinjected into zebrafish embryos (500 pg/embryo) at the 1- to 2-cell stage. Wild-type and GFP mRNA-injected embryos were used as controls. For nuclear visualization, 12-hour postfertilization (hpf) embryos were incubated in embryo-rearing medium containing Hoechst 33342 (2 μg/ml; Invitrogen) for 12 h. The living embryos were dechorionated, deyolked, placed on glass slides, covered by coverslips, and photographed under a Nikon Eclipse E600 fluorescence microscope. To confirm successful protein expression, the embryos were homogenized and subjected to 10% SDS-PAGE followed by Western immunoblot using an anti-GFP antibody (Torrey Pines Biolabs, East Orange, NJ, USA).

One-hybrid transcription activation assay

One-hybrid assay was used to determine whether KRBDP3 and KRBDP4 have any TA activity. For this, Lipofectamine 2000 and FuGene 6 (Roche) were used for transfection in HEK293 and ZF4 cells, respectively. At 24 h after transfection, cells were washed, lysed, and subjected to luciferase assay using the Dual-Luciferase Reporter Assay System. The GAL4: IGFBP-5N construct was used as a positive control (34). To confirm successful expression of GAL4 fusion proteins, cell lysates were separated by 12.5% SDS-PAGE, followed by Western immunoblot using an anti-GAL4 antibody (RK5C1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-tubulin antibody (Sigma-Aldrich). The total protein level in the cell lysates was measured with the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The luciferase activity was normalized by *Renilla* luciferase activity (28, 34) unless otherwise mentioned.

Statistics

Data are shown as means ± SE. Statistical significance among experimental groups was determined by 1-way ANOVA, followed by the Tukey's multiple comparison test. Significance was accepted at $P < 0.05$. The agonist concentrations that induce half-maximum response (EC_{50}) were measured using the Origin 8.6 software (OriginLab, Northampton, MA, USA).

RESULTS

Zebrafish *kiss1ra* and *kiss1rb* genes exhibit different modes of alternative splicing and distinct expression patterns

Recent studies have shown that there are two *kiss1r* genes in zebrafish (22, 24, 27). To obtain their full-length cDNA for functional studies, RT-PCR experiments were carried out. As a result, a single *kiss1ra* transcript and 5 *kiss1rb* transcripts were obtained. The *kiss1ra* transcript is identical to the previously reported sequence, and it encodes a protein with 7 transmembrane domains (TMDs; **Fig. 1*A***). In addition to the previously reported full-length *kiss1rb* transcript, 4 novel transcripts, likely the result of alternative splicing, were identified. They are named KRBDP1–4, respectively (Fig. 1*B*). The *KRBDP1* mRNA lacks the exon 2 sequence, while the *KRBDP2* mRNA lacks sequences encoded by exon 3 and exon 4. The *KRBDP3* mRNA is generated by a partial retention of intron 1 and a partial deletion of exon 1, while the *KRBDP4* mRNA has partial intron 1 sequence (Fig. 1*B*). These alternative spliced transcripts cause frame shifts and/or premature termination codons (PTCs) in the protein coding region (Fig. 1*B*). For instance, *KRBDP3* lacks all TMDs because of the presence of a PTC in the first TMD. *KRBDP1* and *KRBDP4* only have one TMD due to a PTC after the first TMD (Fig. 1*B*). These results suggest that *kiss1ra* and *kiss1rb* undergo very different modes of post-transcriptional regulation.

Next, RT-PCR assays were developed and used to detect the multiple *kiss1r* transcripts simultaneously (Supplemental Fig. S1). In adult fish, *kiss1ra* mRNA was detected in the brain exclusively (**Fig. 2*A***). In comparison, *kiss1rb* mRNA was detected in the brain, eye, fin, and liver (Fig. 2*A*). *KRBDP1* mRNA had a similar tissue distribution pattern (Fig. 2*A*). *KRBDP2* mRNA was detected in all tissues except the fin. The *KRBDP3* transcript was detected in all adult tissues examined. The *KRBDP4* mRNA, on the other hand, was only detected in the muscle. During development, the *kiss1ra* mRNA became detectable as early as 1 day postfertilization (dpf). Its levels increased markedly at 3 dpf and remained high thereafter (Fig. 2*B*). In juvenile

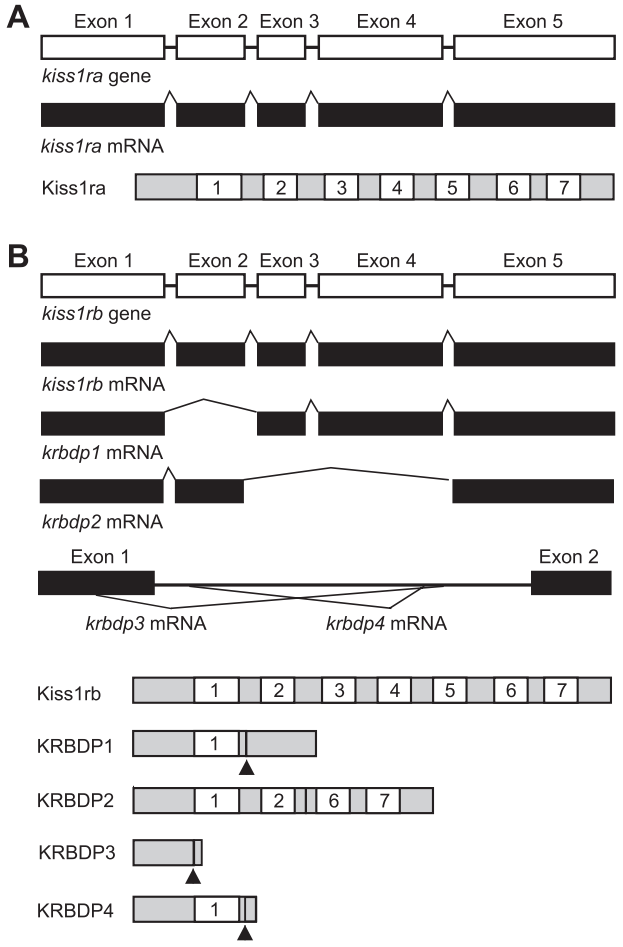

Figure 1. Structure of the zebrafish *kiss1ra* and *kiss1rb*, their mRNAs, and proteins. *A*) Schematic illustration of the *kiss1ra* gene (top), its mRNA (middle), and protein (bottom). Open boxes represent the 7 TMDs. *B*) Schematic illustration of the *kiss1rb* gene (top), its multiple mRNAs (middle), and proteins (bottom). Open boxes represent TMDs; arrowheads represent premature termination codons (PTCs).

and young adult stages, only the head was sampled because of the brain-specific expression of *kiss1ra*. Higher levels of *kiss1ra* mRNA were found at 2 wk, and they gradually decreased (Fig. 2*B*). In comparison, the *krbdp* mRNAs were expressed at very low levels until 7 dfp (Fig. 2*B*). The *kiss1rb* mRNA became easily detectable at 3, 4, and 7 dpf and showed increases to 14 dpf. Of note, *KRBDP1* and *KRBDP3* mRNAs were most abundant at 7 dpf, and their levels decreased thereafter (Fig. 2*B*). These three mRNAs are expressed in the head region in juveniles and young fish (Fig. 2*B*). In contrast, *KRBDP2* was not detectable in the head region until 2 mo. These results suggest that zebrafish *kiss1ra* and *kiss1rb* exhibit distinct gene expression patterns.

Zebrafish *kiss1ra* and *kiss1rb* encode 2 functional Kiss receptors with different ligand selectivity

To determine whether these *kiss1r* transcripts encode functional receptors, their cDNA was subcloned into the pcDNA3.1(+) expression vector and transfected into COS-7 cells. SRE-Luc reporter construct was used to monitor ligand response because Kiss1r signals through phospholipase C-protein kinase C, and the MAPK signaling cascades (32). The transfected cells were stimulated with Z1-10 and Z2-10, two synthetic peptides corresponding to the 10 core amino acids of zebrafish Kiss1 and Kiss2, respectively. As shown in **Fig. 3*A*, *B***, both Z1-10 and Z2-10 caused concentration-dependent increases in the reporter gene activity in COS-7 cells transfected with pcDNA3.1(+)-Kiss1ra. The EC_{50} values were 21.4 and 12.9 nM, respectively. In the pcDNA3.1(+)-Kiss1rb transfected-cells, Z1-10 caused a concentration-dependent increase in the SRE-Luc activity (Fig. 3*A*) with an EC_{50} value of 118.7 nM, whereas Z2-10 had little effect (Fig. 3*B*). Similar results were obtained with CHO-K1 cells (Fig. 3*C*, *D*) and HEK293 cells (Fig. 3*E*, *F*), although the overall EC_{50} values were somewhat higher in these two cell types, probably due to different transfection efficiencies. Not only did Z1-10 have a higher affinity for the Kiss1ra than Kiss1rb, the magnitude of its response was also greater in all the three cell types. This was particularly evident in HEK293 cells. While the Kiss1ra-transfected cells showed a 3-fold increase in SRE-Luc activity in response

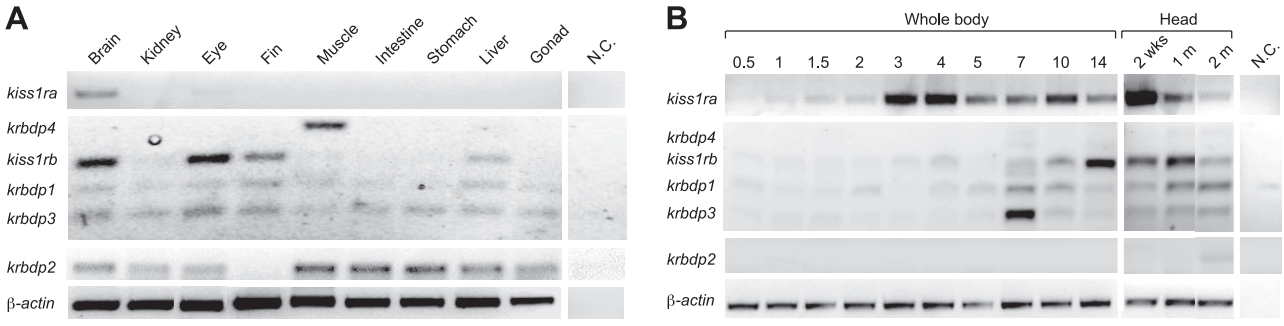

Figure 2. Distinct gene expression patterns of zebrafish *kiss1ra* and *kiss1rb*. *A)* Tissue distribution of *kiss1ra*, *kiss1rb*, *KRBFP1*, *KRBFP2*, *KRBFP3*, and *KRBFP4* mRNAs in the indicated adult zebrafish tissues. Distilled water was used as negative control (N.C.). Primers were designed to detect multiple mRNA transcripts; see validation in Supplemental Fig. S1. Similar results were obtained from 3 male and 3 female fish. No gender difference was observed. *B*) Temporal expression patterns. RNA was isolated from whole-body embryo, larvae, and juveniles at 0.5–14 days postfertilization. For fish older than 2 wk, heads were used for RNA isolation.

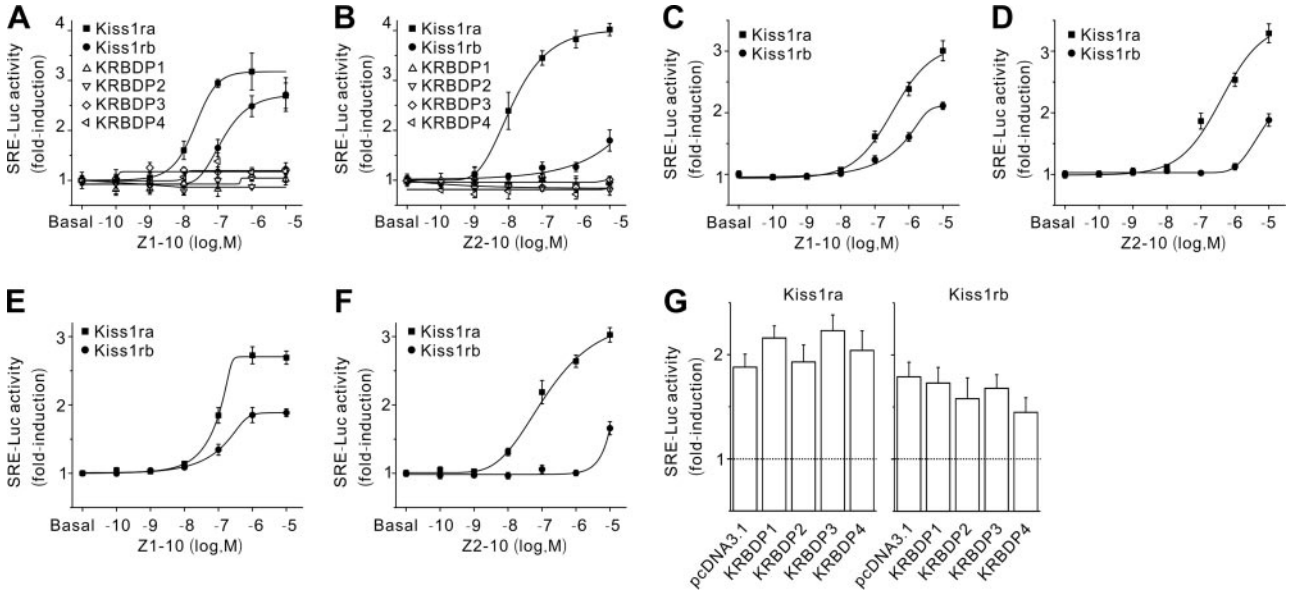

Figure 3. Different ligand selectivity of Kiss1ra and Kiss1rb. *A–F*) COS-7 cells (*A*, *B*), CHO-K1 cells (*C*, *D*), and HEK293 cells (*E*, *F*) were transfected with the indicated pcDNA3.1(+)-Kiss1r plasmid together with the SRE-Luc reporter plasmid. At 24 h after transfection, the cells were treated with Z1-10 peptide (*A*, *C*, *E*) or Z2-10 peptide (*B*, *D*, *F*) at the indicated concentrations for 6 h. Reporter gene activity was determined and expressed as fold increase over the no treatment control group. Values are expressed as means ± SE of 3 independent experiments, each performed in triplicate. *G*) Expression of alternatively spliced Kiss1rb isoforms does not affect the full-length receptor-mediated signaling. COS-7 cells were transfected with 100 ng pcDNA3.1(+)-Kiss1ra (left panel) or pcDNA3.1(+)-kiss1rb-1 DNA (right panel), 200 ng SRE-Luc reporter DNA, and 360 ng DNA encoding the indicated KRBDPs. They were treated with Z1-10 (100 nM for Kiss1ra and 1 μM for Kissrb-expressing cells) for 6 h, and SRE-Luc luciferase activity was determined. Values are expressed as means ± SE (n=3). Broken line indicates no-Z1-10 basal level.

to the Z1-10 treatment, the Kiss1rb-transfected cells had a <2-fold increase (Fig. 3*E*). This result suggests that zebrafish *kiss1ra* encodes a functional receptor that can be activated by both Kiss1 and Kiss2, while *kiss1rb* encodes a specific Kiss1 receptor.

We also examined the possible roles of KRBDP1–4 in mediating zebrafish Kiss1 or Kiss2. For this, COS-7 cells were transfected with plasmids encoding KRBDP1–4. These cells did not respond to either Z1-10 or Z2-10 (Fig. 3*A*, *B*), suggesting that these *kiss1rb*-derived isoforms do not mediate Kiss signaling. It is well known that many G-protein-coupled receptors (GPCRs) undergo alternative splicing, generating isoforms with different pharmacological, signaling, and regulatory properties and even isoforms that can alter or abolish the functions of their full-length counterparts (33). To test whether any of these truncated Kiss1rb isoforms can affect the functions of Kiss1ra and/or Kiss1rb, cotransfection experiments were performed. Coexpression of KRBDP1–4 with either Kiss1ra or Kiss1rb did not affect their signaling activity in response to Z1-10 (Fig. 3*G*).

Zebrafish KRBDP3, but not other isoforms, is localized in the nucleus and has strong TA activity

Next, the subcellular localization of these *kiss1rb* variants was investigated. For this, Kiss1r DNA was subcloned into the pCS2-EGFP vector and transfected into COS-7 cells, and the resulting EGFP signal was examined in living cells. The Kiss1ra-EGFP signal and Kiss1rb-EGFP signal were absent from the nucleus and observed in the Golgi apparatus (in the shape of a crescent moon outside of the nucleus) and on the cell surface (**Fig. 4*A***). Likewise, the KRBDP1-EGFP and KRBDP2-EGFP signals were also absent in the nucleus. In contrast, KRBDP3-EGFP signal was observed in the nucleus. The KRBDP4-EGFP signal was also detected in the perinuclear region at a relatively low level (Fig. 4*A*). The nuclear localization of KRBDP3 was also observed in GT1-7 cells (Fig. 4*B*), a mouse GnRH neuron-derived cell line expressing abundant levels of the endogenous Kiss1r.

Next, we tested whether this nuclear localization occurs *in vivo*. For this, capped mRNA encoding KRBDP3-EGFP or EGFP was injected into zebrafish embryos, and the EGFP signal was examined. As shown in Fig. 4*C*, the KRBDP3-EGFP signal was observed in the nucleus of the embryonic cells. In comparison, the EGFP signal was seen both in the cytoplasm and the nucleus (Fig. 4*C*). To ascertain the successful expression of the fusion protein, the injected embryos were subjected to Western blotting. As shown in Fig. 4*D*, both EGFP and KRBDP3-EGFP were successfully expressed.

To further examine the nuclear localization of KRBDP3 using an independent approach, we generated a KRBDP3-FLAG construct and introduced it to COS-7 cells by transient transfection. The subcellular localization of KRBDP3-FLAG was examined by immu-

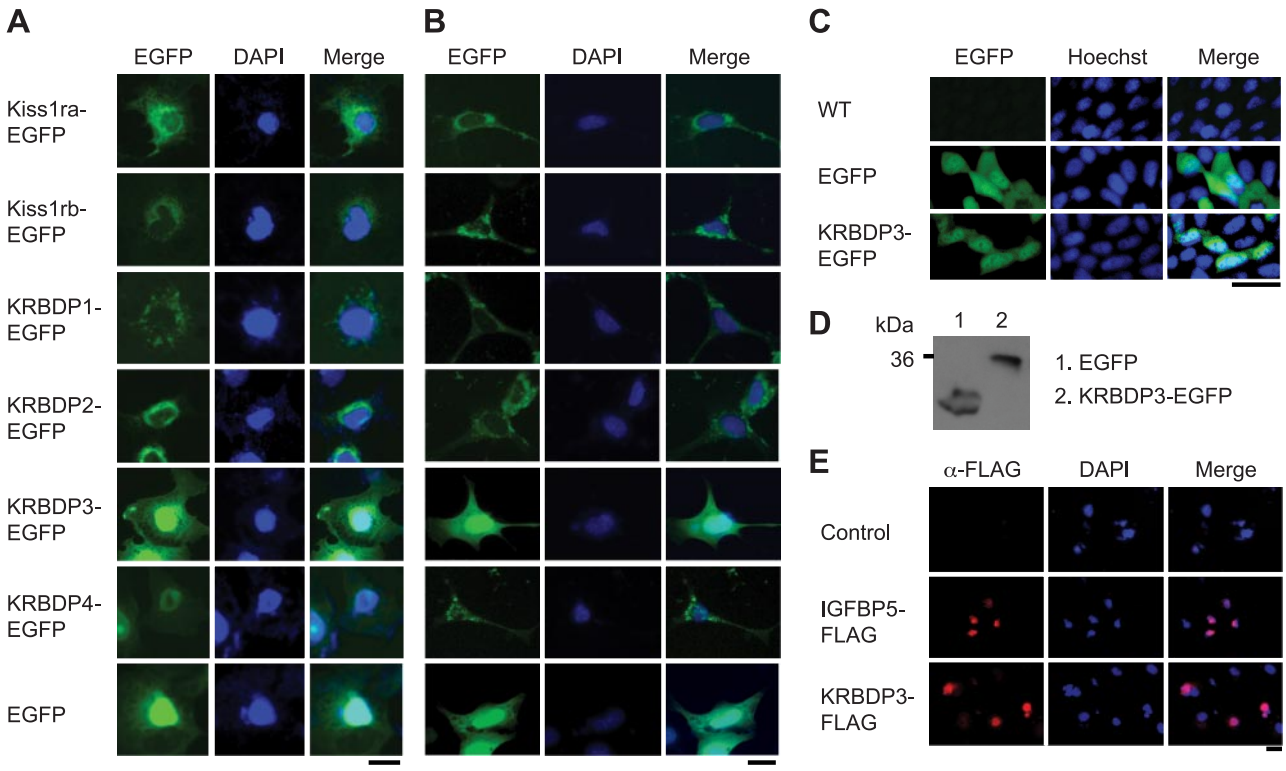

Figure 4. Subcellular localization of Kiss1rs and KRBDPs. *A, B*) COS-7 cells (*A*) or GT1-7 cells (*B*) were transfected with the indicated pCS2+-EGFP plasmid. At 36 h after transfection, cells were counterstained with DAPI, and GFP signal was detected. *C*) Nuclear localization of KRBDP3-EGFP in zebrafish embryos. Wild-type zebrafish embryos were injected with mRNA encoding KRBDP3-EGFP or EGFP. GPF signal was detected at 24 hpf. Nuclei were stained with Hoechst. *D*) Western blotting of embryos described in *C*. *E*) COS-7 cells were transected with the indicated FLAG-tagged plasmid. At 24 h after transfection, cells were subjected to immunocytochemistry using an anti-FLAG antibody and counterstained with DAPI. Scale bars = 20 μm. Right panels show merged images.

nocytochemical staining. Cells transfected with the empty vector or the pCMV:IGFBP-5-FLAG plasmid (28) were used as negative and positive controls, respectively. As shown in Fig. 4*E*, KRBDP3-FLAG signal was detected exclusively in the nucleus in successfully transfected cells. This signal was authentic because it was abolished by the elimination of either the anti-FLAG antibody or the secondary antibody. As reported previously (30), FLAG-IGFBP-5 signal was found exclusively in the nucleus, while no signal was detected in the empty vector-transfected cells (Fig. 4*E*).

The nuclear localization of KRBDP3 raised the intriguing possibility that this protein may be involved in transcriptional regulation directly or indirectly. The C terminus of KRBDP3 has several proline residues (Supplemental Table S2). A proline-rich sequence is a typical feature of TA domains. To determine whether KRBDP3 has any TA activity, its ORF DNAs were fused to Gal4-DBD and transfected into HEK293 cells together with a Gal4 reporter gene. The Gal4-DBD-IGFBP-5N plasmid was utilized as positive control because it has a strong TA activity and is readily available (28, 34). KRBDP4 was also tested for comparison of TA activity. As shown in **Fig. 5*A***, Gal4-DBD-KRBDP3 caused a 10-fold, significant increase in Gal4-dependent reporter gene expression. This activity was comparable with that of the IGFBP-5 N domain. KRBDP4, on the other hand, did not have such an effect. We next examined the TA activity of KRBDP3 in ZF4 cells, a cell line derived from zebrafish embryos. Again, KRBDP3 and IGFBP5N had significant TA activity, while KRBDP4 had little activity in these zebrafish cells (Fig. 5*B*). To map the region in KRBDP3 that is critical for its TA activity, three fragments covering residues 17–47, 31–47, and 1–30 of KRBDP3 were generated, and their TA activities were tested (Fig. 5*C*). Deletion of the first 16 aa (residues 17–47) resulted in a modest but statistically significant reduction in TA activity. Deletion of the first 30 aa (residues 31–47) completely abolished the TA activity of KRBDP3. Deletion of the C-terminal 17 aa (residues 1–30) resulted in a >80% reduction in TA activity. We also tested the effects of kisspeptin treatment on the TA activity. The addition of 1 μM Z1-10 and Z2-10 did not affect the KRBDP3-induced reporter gene expression (Fig. 5*D*), indicating that TA activity of KRBDP3 is likely kiss independent.

When the expression levels of these fusion proteins were examined, we found that they were expressed (Fig. 5*E*). However, the levels of Gal4-DBD-KRBDP4 were markedly lower than Gal4-DBD-KRBDP3 (Fig. 5*E*). To rule out the possibility that the lack of TA activity of Gal4-DBD-KRBDP4 is due to its low expression and/or increased degradation, we titrated the plasmid DNA concentration to make the KRBDP3 and

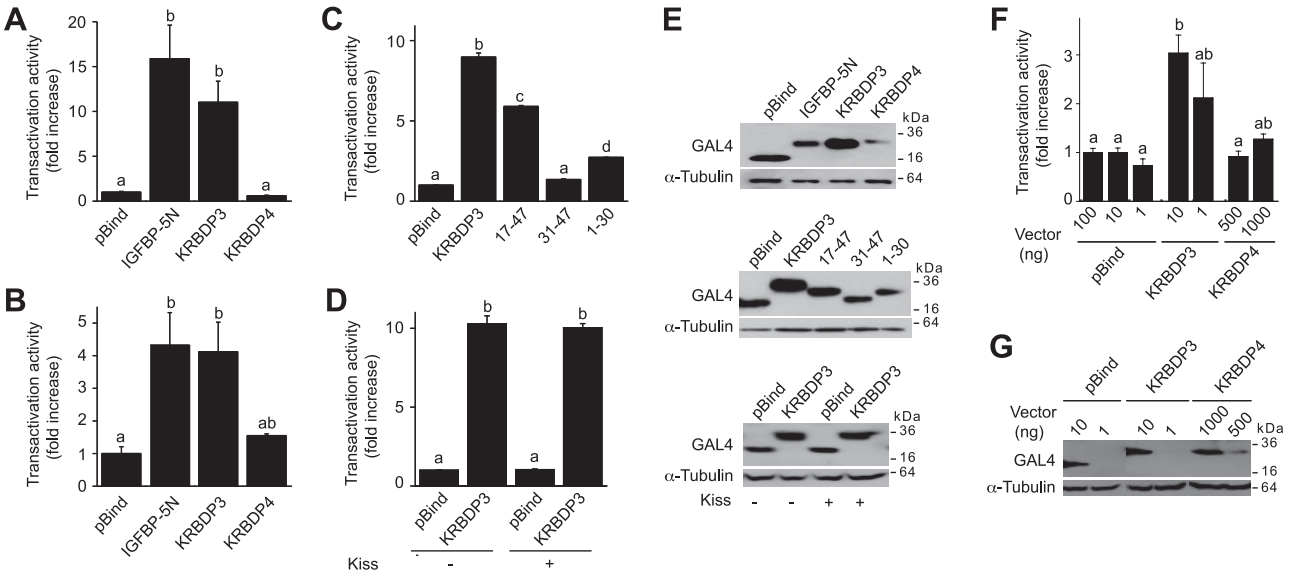

Figure 5. KRBDP3 has TA activity. *A*, *B*) pBind, pBind-KRBDP3, or pBind-KRBDP4 DNA (500 ng/well) was transfected into human HEK293 cells (*A*) and zebrafish (ZF) cells (*B*) together with 500 ng Gal4 reporter plasmid DNA. TA activity was determined and expressed as a fold increase over the pBind control group. Groups labeled with different letters are significantly different from each other ($P<0.005$). *C*) TA activity of various truncated forms of KRBDP3. DNA fragments covering aa 17–47, 31–47, and 1–30 of KRBDP3 were fused to Gal4DBD, and their activities were tested as described in *A*. *D*) Kisspeptin treatment does not affect the TA activity of KRBDP3. pBind or pBind-KRBDP3 vectors were transfected and treated with 1 μM Z1-10 and Z2-10 for 8 h, and their activities were determined as described in *A*. *E*) Western blot analysis results. Expression of the indicated Gal4-fusion proteins was analyzed by immunoblotting using an anti-Gal4 antibody and an anti-tubulin antibody. Note the reduced KRBDP4 expression levels. *F*) HEK293 cells were transfected with pBind, pBind-KRBDP3, or pBind- KRBDP4 DNA at the indicated concentrations. Transcription activity was measured and expressed as fold increase over the pBind control group. Gal4 reporter activity was normalized by total protein levels. *Renilla* luciferase activity could not be used because different doses of expression vector DNA used. Groups labeled with different letters are significantly different from each other ($P<0.005$). *G*) Western blot analysis results of samples tested in *F*. Values are expressed as means ± SE of 2 (*A–C*, *F*) or 3 (*D*) separate experiments, each performed in triplicate.

KRBDP4 levels comparable. As shown in Fig. 5*F*, transfection of cells with KRBDP3 caused significant increases in Gal4-dependent reporter gene expression at the doses of 10 and even 1 ng/well DNA (Fig. 5*G*). In contrast, KRBDP4 had no TA activity at either 1000 or 500 ng/well. Western blot analysis results showed that the two fusion proteins levels were comparable in the Gal4-DBD-KRBDP3 (10 ng/well) group and the Gal4-DBD-KRBDP4 (1000 ng/well) group. These results suggest the lack of TA activity of Gal4-DBD-KRBDP4 is not due to its low expression and/or increased degradation.

DISCUSSION

In this study, we have provided evidence suggesting that the duplicated zebrafish *kiss1r* genes have diverged in several major ways. *1*) The two zebrafish *kiss1r* genes have evolved distinct gene expression patterns. While *kiss1ra* is exclusively expressed in the brain, *kiss1rb* is expressed in many non-neural tissues. The two genes also showed different expression patterns during early development. *2*) The two genes encode distinct GPCRs with different ligand selectivity. While Kiss1ra signaling can be activated by both Z1-10 and Z2-10, Kiss1rb has a preference for Z1-10. *3*) Zebrafish *kiss1ra* and *kiss1rb* undergo different modes of post-transcriptional regulation. In contrast to a single *kiss1ra* transcript, there are five alternatively spliced transcripts in the *kiss1rb* gene. These alternatively spliced mRNAs encode truncated isoforms that are localized in different cellular compartments and may confer novel biological actions.

Recent studies suggest that multiple Kiss1r genes are present in teleosts and amphibians, likely a result of the third round of genome-wide duplication that occurred in some teleost and/or amphibian lineages (3, 20, 21). In this study, we show that the two zebrafish Kiss1r genes have evolved diverged gene expression patterns. During early development, the *kiss1ra* gene started to express much earlier than the *kiss1rb* gene. In the adult stage, the *kiss1ra* mRNA was only detected in the brain, while the *kiss1rb* mRNAs were found in the brain, as well as other many tissues. In mammals, kisspeptin has peripheral functions, as well as central reproductive function. For instance, kisspeptin regulates adrenal steroidogenesis, promotes adipocyte differentiation, kidney morphogenesis, and insulin secretion (35–38). In this study, we showed that *kiss1rb* transcripts are expressed in several peripheral tissues, such as eye, fin, and liver. Functional assay revealed that the *kiss1rb* encoded a functional receptor and mediates Kiss1-dependent signaling. These results suggest that the Kiss1-Kiss1rb signaling may be functional in these pe-

ripheral tissues. Future studies will be needed to elucidate the peripheral function of the Kiss1-Kiss1rb signaling system.

In addition to two *kiss1r* genes, the zebrafish genome also contains two distinct *kiss* ligand genes (23, 24). However, the functional relationship between the duplicated Kiss ligand and receptors is not clear nor is their relative importance in regulating the HPG axis. In this study, we demonstrated that both Z1-10 and Z2-10 can activate Kiss1ra-mediated signaling in a concentration-dependent manner, albeit with different potencies. In the case of Kiss1rb, Z1-10 stimulated its signaling, while Z2-10 was not effective even at μM concentrations. Therefore, Kiss1rb appears to preferentially interact with Kiss1 and may function as a specific Kiss1 receptor. It should be pointed out that our receptor assays only used Z1-10 and Z2-10. Recent studies suggest that the receptor binding and signal activity of a kisspeptin peptide can be influenced by post-translational modifications. Lee *et al.* (24), for example, tested the effects of Z1-p15 (a pyroglutated Kiss1 peptide consisting of 15 aa) and Z1-15 (the nonpyroglutated 15-aa Kiss 1 peptide), and found that Z1-p15 was 50 to 8 times more potent than Z1–15 for Kiss1ra and Kiss1rb, respectively. Nevertheless, our findings are in good agreement with previous studies. Biran *et al.* (22) examined the effect of Z1-10 in activating Kiss1ra and Kiss1rb-mediated SRE-Luc activity and showed that Z1-10 had a greater potency in activating Kiss1ra than Kiss1rb. These researchers, however, did not investigate Kiss2 signaling. Lee *et al.* (24) reported that Z2–12 had greater potency in activating zebrafish Kiss1ra than Kiss1rb. The effect of Z1–10 was not tested in that study. Collectively, these receptor assay results indicate that the action of Kiss2 may be primarily mediated by Kiss1ra, while the Kiss1 signal may be mediated by both Kiss1ra and Kiss1rb.

Servili *et al.* (25) recently reported that Kiss2 neurons, but not Kiss1 neurons, project fibers to the GnRH3 neurons in the hypothalamic regions in zebrafish. These researchers also reported that Kiss1ra, but not Kiss1rb, is expressed in the preoptic area and hypothalamus (25). The zebrafish GnRH3 neurons are the counterparts of the mammalian hypophysiotropic GnRH1 neurons and are critical in regulating the HPG axis (39, 40). Therefore, it is likely that the Kiss2/Kiss1ra pathway plays a critical role in regulating the HPG axis in zebrafish. This hypothesis is also consistent with the finding that Z2-10 had higher potency than Z1-10 in increasing FSHβ and LHβ gene expression in zebrafish (23) and in stimulating LH release in sea bass (41).

In this study, we have identified 4 novel *kiss1rb* splicing variants, *i.e.*, *KRBDP1–4*, in addition to the previously reported *kiss1rb* mRNA (22, 24). These four transcripts encode various truncated forms of Kiss1r. Our functional assays showed that all these truncated forms can be translated, but none was capable of mediating the cellular response to kisspeptins. This is perhaps not surprising, because the structural domains between TMDs are essential for generating the ligand-binding pocket of GPCRs (42). In 7-transmembrane GPCRs, truncated variants often interfere with full-length receptors (33). For instance, truncated forms of the LH receptor, GnRH receptor, and GHRH receptor misroute their full-length partners and inhibit their responses to ligands (43–45). Our coexpression experiments, however, suggest that none of the KRBDPs interfere with Kiss1-stimulation on Kiss1ra and Kiss1rb. It has been reported that alternative splicing controls the pre-mRNA pool of full-length receptors (33). For instance, alternative splice variants of CRH receptors show differential expression in myometrium during pregnancy and labor (46) and in human skin on environmental stimuli (47, 48). Our data showed that all of *kissrb* variant mRNAs are expressed in various tissues. The relative abundance of full-length Kiss1rb promRNA pool might be controlled by unexplored physiological conditions.

A surprising and novel finding made in this study is that the *KRBDP3* mRNA encodes a nuclear isoform that has TA activity. When the subcellular localization was examined in COS-7 cells, the KRBDP3-EGFP signals were observed in the nucleus. This nuclear localization is unique to KRBDP3, because the other four isoforms are not detected in the nucleus. Likewise, the FLAG-tagged version of KRBDP3 was also found in the nucleus. COS-7 cells do not express Kiss1r. We also used GT1-7 cells, which are more physiologically relevant because these cells express abundant endogenous Kiss1r (1–3). Similar results were obtained in the two different cell types. Another independent line of evidence is that the KRBDP3-EGFP signal was localized in the nucleus in zebrafish embryos. Our functional assays suggest that KRBDP3, but not KRBDP4, possesses TA activity. To our knowledge, this is the first study demonstrating that an alternative spliced isoform of a GPCR is located in the nucleus and has TA activity. The TA activity is authentic because the magnitude of TA activity of KRBDP3 was comparable with that of IGFBP-5N, which has been shown to have an evident TA activity (28, 34); the TA activity was observed in both zebrafish cells and mammalian cells; the TA activity was dramatically reduced or abolished by deletions of middle or C-terminal sequences of KRBDP3; and the TA activity was specific to KRBDP3. KRBDP4, when expressed at comparable levels, had no such activity. It is also noted that treatment with Z1-10 and Z2-10 did not alter the TA activity of KRBPD3, suggesting that its nuclear action may be kisspeptin independent. Future studies are needed to elucidate the functional roles of KRBDP3 in the nucleus and to identify its possible partners and targets in the nucleus. FJ

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