Discovery of growth hormone-releasing hormones and receptors in non-mammalian vertebrates

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Abbreviations: GH, growth hormone; GHRH, growth hormone-releasing hormone; GHRH-R, GHRH-receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1-R, PACAP receptor; PHI, peptide histidine isoleucine; PRP, PACAP-related peptide; PRP-R, PACAP-related peptide receptor; VIP, vasoactive intestinal polypeptide

Abstract

In mammals, growth hormone-releasing hormone (GHRH) is the most important neuroendocrine factor that stimulates the release of growth hormone (GH) from the anterior pituitary. In non-mammalian vertebrates, however, the previously named GHRH-like peptides were unable to demonstrate robust GH-releasing activities. In this report, we provide evidence, for the first time, that these GHRH-like peptides are homologues of mammalian PACAP-related peptides (PRP). Instead, novel GHRH peptides encoded in cDNAs isolated from goldfish, zebrafish and African clawed frog were identified. Moreover, receptors specific for these GHRHs were characterized from goldfish and zebrafish. These GHRHs and GHRH-Rs are phylogenetically and structurally more similar to their mammalian counterparts than the previously named GHRH-like peptides and GHRH-like receptors. Information regarding their chromosomal locations and organization of neighboring genes confirmed that they share the same origins as the mammalian genes. Functionally, the goldfish GHRH dose-dependently activates cAMP production in receptor-transfected CHO cells as well as GH release from goldfish pituitary cells. Tissue distribution studies showed that the goldfish GHRH is expressed almost exclusively in the brain, while the goldfish GHRH-R is actively expressed in brain and pituitary. Taken together, these results provide evidence for a novel GHRH-GHRH-R axis in non-mammalian vertebrates. Based on these data, a comprehensive evolutionary scheme for GHRH, PRP-PACAP, PHI-VIP genes in relation to 3 rounds of genome duplication early on in vertebrate evolution is proposed. The newly discovered GHRHs, also found in flounder, *Fugu*, medaka, stickleback, *Tetraodon* and rainbow trout, provide new research directions regarding the neuroendocrine control of growth in vertebrates.

Introduction

Growth hormone-releasing hormone (GHRH), also known as growth hormone-releasing factor, was initially isolated from pancreatic tumors causing acromegaly (1, 2), and hypothalamic human GHRH was shown to be identical to the one isolated from the pancreas tumor (3). Thereafter, the sequences of GHRHs were determined in various vertebrate species and in protochordates (4). In mammals, GHRH is mainly expressed and released from the arcuate nucleus of the hypothalamus (5). The primary function of GHRH is to stimulate GH synthesis and secretion from anterior pituitary somatotrophs via specific interaction with its receptor, GHRH-R (5). In addtion, GHRH activates cell proliferation, differentiation and growth of somatotrophs (6-8). There are many other reported activities of GHRH such as modulation of appetite and feeding behavior, regulation of sleeping (9-10), control of jejunal motility (11), and increase of leptin levels in modest obesity (12).

In mammals, GHRH and pituitary adenylate cyclase-activating polypeptide (PACAP) are encoded by separate genes: GHRH is encoded with a C-peptide with no known function, whereas, PACAP and PACAP-related peptide (PRP) are present in the same transcript (13-14). In non-mammalian vertebrates and protochordates, GHRH and PACAP were believed to be encoded by the same gene and hence processed from the same transcript and prepropolypeptide (15). It was suggested that mammalian GHRH was evolved as a consequence of a gene duplication event of the PACAP gene which occurred just before the divergence that gave rise to the mammalian lineage (3). Prior to this gene duplication event, PACAP was the true physiological regulator for GH release while its function was progressively taken over by the GHRH-like peptides encoded with PACAP after the emergence of tetrapods (16). The GHRH-like peptide was later evolved to PRP in mammals, and the second copy of the ancestral GHRH-like/PACAP gene, after gene duplication, became the physiological GHRH in mammals (3). This

hypothetical evolutionary scheme of GHRH and PACAP genes previously proposed could successfully accommodate and explain most of the information available. However, by data mining of the genomic sequences from several vertebrates, genomic sequences encoding for putative GHRHs and GHRH-Rs from amphibian (*Xenopus laevis*) and teleost (zebrafish) were found. The corresponding proteins share a higher degree of sequence identity with mammalian GHRH and GHRH-R, when compared with the previously identified GHRH-like peptides and receptors. In this report, by analyzing the phylogeny, chromosomal location, and function of these ligands and receptors, a new evolutionary scheme for GHRH, PRP-PACAP and PHI-VIP genes in vertebrates is proposed. More importantly, the discovery of novel mammalian GHRH homologues by *in silico* analysis in other fish provides new research directions regarding the neuroendocrine control of growth in vertebrates.

Results

Predicted Amino Acid Sequences of Fish and Amphibian GHRHs. Recently, several non-mammalian vertebrate genome databases of avian [*Gallus gallus* (chicken)], amphibian [*Xenopus tropicalis*. (Africa clawed frog)] and fish [*Danio rerio* (Zebrafish); *Takifugu rubripes* (*Fugu*); *Tetraodon nigroviridis* (Pufferfish)] species were completely or partially released. Using these valuable resources, we performed bioinformatics analyses to look for novel GHRHs and GHRH-Rs in amphibian and fish. Putative genes for GHRH were predicted from *Xenopus tropicalis*, goldfish (gfGHRH), and zebrafish (zfGHRH), and with the help of these sequences, we successfully cloned their full-length cDNAs. (supplemental data S1 to S3). By aligning all known vertebrate GHRH precursor sequences (S4), it was found that only the N-terminal region (1-27) of GHRHs is conserved. Human shares 81.5% and 74.1% sequence identity with goldfish/zebrafish and *Xenopus laevis* GHRHs, respectively (S11A). Within the first 7 amino acids, there is only one amino acid substitution in goat (position 1), human (position 1), mouse (position 1) and *Xenopus* (position 2). These observations clearly show that a strong selective pressure has acted to preserve the GHRH sequence in evolution, indicating that this peptide plays important functions from fish to mammals. It should be noted that within the first 27 amino acids, the previously named GHRH-like peptides share much lower level of sequence identity with these fish and mammalian GHRHs (51.9% - 59.3% for gfPRPsalmon, and 37% - 44.4% for gfPRPcatfish, S5). Instead, these GHRH-like peptides are structurally homologous to mammalian PRPs (Fig. 1A).

Cloning of GHRH-Rs from Zebrafish and Goldfish. Putative GHRH-R cDNAs were cloned from zebrafish and goldfish (zfGHRH-R and gfGHRH-R) (see S6 and S7). The amino acid sequences of these GHRH-R receptors share 78.4% identity among

themselves, and 51.1% and 49.7%, respectively, with the human GHRH-R (S8). Kyte-Doolittle hydrophobicity plots of these receptors indicated the presence of seven hydrophobic transmembrane regions that are conserved in all class IIB receptors (S9). In addition, the eight cysteine residues in the N-termini of fish and human GHRH-Rs, that presumably structurally maintain the ligand binding pocket (17), are identical. These receptors also contain the basic motifs in the third endoloop, indicating that they can also couple to the cAMP pathway, similar to other members in the same PACAP/glucagon receptor family (18).

A comparison of the fish GHRH-R with other class IIB receptors (S11B) clearly shows that fish GHRH-Rs, sharing 64.8-78.4% sequence identity, are distinct from the previously identified GHRH-like receptors (PRP-Rs). The sequence identity between goldfish GHRH-R and PRP-R is 39.7%, which is not different (36.4-38.3%) from other goldfish receptors in the same gene family, including PAC1-R, VPAC1-R and PHI-R. The sequence identity shared by goldfish, avian and mammalian GHRH-Rs is 48.9-51.1%, which is significantly higher than any other receptors in the same class IIB family, whether in goldfish or in human.

Phylogenetic Analysis of the Novel GHRH and GHRH-Rs. Based on the novel GHRH sequences, a revised phylogenetic tree for GHRH, GHRH-like and PRP peptides was constructed using PACAP as the out-group (Fig. 1A). The newly identified GHRH peptides from fish and amphibian were grouped together with all other previously cloned or predicted GHRH sequences from mammals to fish. Interestingly, the previously named GHRH-like peptides were phylogenetically closer to mammalian PRPs and resembled to form a distinct branch together. Within the GHRH grouping, GHRHs clustered together according to their class with the exception of rodents. Within all PRPs, avian, amphibian and fish PRPsalmon-like (formerly

GHRHsalmon-like) clustered to form a sub-branch, whereas, the fish PRPcatfish-like peptides formed a different group. Apparently, there are two forms of PRPs in fish, but only the salmon-like PRPs are observed in avian and amphibian species. Catfish-like PRP, therefore, is uniquely present in fish, and hence must have evolved by gene duplication after the split that gave rise to tetrapod and ray-finned fish.

Similarly, the newly found gfGHRH-R and zfGHRH-R, and the predicted GHRH-R from chicken and *Fugu* branched together with mammalian GHRH-Rs, whereas the fish PRP-Rs (formerly GHRH-like receptor) formed a separate branch (Fig. 1B). These data confirmed that, together with GHRHs, receptors that are structurally closely related to mammalian GHRH-Rs also exist in other classes. It is interestingly to note that PRP-R might have been lost in mammals (see below). As the function of PRP in non-mammalian vertebrates is not known, the implications of losing PRP-R in mammals is unclear.

Chromosomal Synteny of GHRH and GHRH-R Genes. According to the latest genome assembly versions, the locations, gene structures and organizations of neighboring genes of GHRHs and GHRH-Rs in Zebrafish (zebrafish assembly version 4, Zv4) and *Xenopus* (*Xenopus tropicalis* 4.1) were determined (S10). All exon-intron splice junctions agree with the canonical GT/AG rule. Both fish and amphibian GHRH genes have 4 exons and the mature peptides are located in exon 3. In mammals, GHRH genes also have 4 exons, whereas the mature peptides are found in exon 2 instead of exon 3. Also, exon 2 and its encoded sequences in frog and goldfish are different and are unique in their own species (S4). This information indicated that, although there were frequent exon rearrangements, the mature peptide-encoding exon, in terms of sequence and structure, have been relatively well preserved during evolution. Finally, the GHRH genes are also structurally distinct from PRP-PACAP genes (S10), in which

the three exons encoding cryptic peptide, PRP and PACAP are arranged in tandem. Thus, the organizations of GHRH and PRP-PACAP genes also indicate that they have different origins in evolution.

Fig. 2 summarizes the genomic locations of GHRH and GHRH-R in various vertebrates. For the GHRH genes, the nearest neighboring genes of GHRH are RPN2 and MANBAL in human and chicken. In fact, the RPN2 genes are found also in similar locations in all species analyzed except zebrafish (Fig. 2A). Other than RPN2, EPB41L1, C20orf4, DLGAP4, MYL9, and CTNNBL1 are also closely linked to the GHRH gene loci in human and frog. In *Fugu* and *Xenopus*, BPI and EPB41L1 genes are both linked to GHRH while in *Fugu* and zebrafish, the CDK5RAP1 gene is found close to the GHRH locus. These kinds of similarities in chromosomal syntenic linkage were not observed when comparing the GHRH and PRP-PACAP genes in vertebrate genomes, again confirming the idea that two different genes encoding for GHRH and PACAP exist from fish to mammal.

Regarding the receptor, the GHRH-R and PAC1-R gene loci are in close proximity in human, chicken, zebrafish and *Fugu* (Fig. 2B), indicating that they are homologues in different species. Interestingly, we found that the PRP-R locus is also linked to PAC1-R in chicken. Despite our efforts, no PRP-R gene could be found in mammalian genomes (human, chimpanzee, mouse, rat and rabbit), and hence it is likely that the PRP-R gene was lost after the divergence that gave rise to the mammalian lineage.

Comparison of Fish GHRH and PRP in Activating GHRH-R and PRP-R. To confirm the functional identity of fish GHRH and its receptor, zfGHRH-R-transfected CHO cells were exposed to 100 nM of the various related peptides (Fig. 3A). Both fish and frog (1-27) GHRHs were able to stimulate cAMP accumulation above background levels (no peptide or pcDNA3.1-transfected cells). Graded concentrations of

fishGHRH and xGHRH induced dose-dependent stimulation of zfGHRH-R with EC_{50} values of 3.7 x 10^{-7} and 8.4 x 10^{-7} M, respectively, the fish peptide being 2.3 folds more efficacious than xGHRH (Fig. 3B). In contrast, gfPRPsalmon-like and gfPRPcatfish-like did not induce cAMP production in zfGHRH-R-transfected cells. Interestingly, in gfPRP-R-transfected cells, gfPRPsalmon-like, fishGHRH, and xGHRH could all stimulate intracellular cAMP production dose-dependently with EC_{50} values of: gfPRPsalmon-like, 5.8 x 10^{-9}M > fishGHRH, 1.8 x 10^{-7}M > xGHRH, 4.8 x 10^{-7} M (Fig. 3C).

To test if fish GHRH and gfPRPsalmon-like can exert a regulatory role at the pituitary level to modulate GH secretion, goldfish pituitary cells were challenged with graded concentrations of goldfish GHRH and PRP. A 4-hr incubation of cells with goldfish GHRH induced a dose-related increase in GH release, the minimum effective dose being 10 nM (Fig. 3D) whereas gfPRPsalmon-like, at concentrations up to 1 μM was totally inactive (Fig. 3E).

Tissue Distribution of GHRH and GHRH-R in Goldfish and *Xenopus laevis*. The physiological relevance of GHRH and its cognate receptor in goldfish was tested by measuring their transcript levels using real-time PCR (Fig. 4). GHRH mRNA was detected mostly in the brain while GHRH-R mRNA was found in both brain and pituitary. The expression patterns of these genes in the brain-pituitary axis further support the functional role of the novel GHRH acting to stimulate GH release from the anterior pituitary.

Discussion

The Newly Identified GHRHs, but not GHRH-Like Peptides, are Mammalian GHRH Homologues. The primordial gene for the PACAP/VIP/glucagon gene family

arose more than 650 million years ago and, through exon duplication and insertion, gene duplication, point mutation and exon loss, the family of peptides has developed into the forms that are now recognized. Based on sequence comparison, phylogenetic study and chromosomal locations of GHRH and GHRH-R in vertebrates, we here show that the previously named GHRH-like peptides are homologues of mammalian PRPs, and hence should be renamed as PRP from now on. Moreover, the tissue distribution of these PRPs in the fish brain is different when compared to mammalian GHRHs (19). Functionally, PRPs were unable to stimulate zfGHRH-R, as well as incapable of activating GH release from fish pituitary as shown in this and previous studies (20-22).

More importantly, the present report demonstrates the presence of authentic GHRH peptides in non-mammalian vertebrates. In addition to goldfish and zebrafish, several ray-finned fish GHRHs were identified in medaka, strickland, *Fugu*, *Tetraodon*, flounder, and rainbow trout (see S11) by *in silico* analysis. The mature GHRH sequences are almost identical in these eight fish species, with only one substitution in rainbow trout and medaka GHRHs at position 25 (S) and 26 (I), respectively. Moreover, the peptide cleavage sites (R and GKR) are also conserved. This information clearly suggests an important physiological function of GHRH in such diversified fish species, and shows that the newly discovered GHRHs are homologues of mammalian GHRHs. In parallel with the evolution of GHRHs, novel GHRH-Rs that are homologous to their mammalian counterparts in structure, function, chromosomal localization and tissue distribution are also found in fish. We have therefore shown that, unlike what was hypothesized in previous reports (15-16) both GHRH and its receptor genes actually existed before the split for tetrapods and ray-finned fish.

Another interesting observation is the existence of PRP-specific receptors from fish (23-25) to chicken, suggesting that PRP potentially is a physiological regulator in these species. However, despite our efforts, we were unable to find the PRP-R gene in

any of the mammalian genome databases (totally 12), including the almost completed human and mouse genomes, indicating that PRP-R (but not PRP) was lost in the mammalian lineage. A possible explanation is that PRP-R was lost in a chromosomal translocation event. As shown in Fig. 3B, in chicken, GHRH-R, PAC1-R, PRP-R and VPAC1-R genes are clustered in the same chromosomal region, while, in mammals, VPAC1-R is translocated to a different chromosome and the DNA sequences between VPAC1-R and PAC1-R containing the PRP-R, somehow disappeared.

Implications of the Newly Discovered GHRHs on our Understanding of the Evolution of GHRH and PRP-PACAP Genes. The "one-to-four" rule is the most widely accepted and prevalent model to explain the evolution of many vertebrate genes. This model is based on the "two round genome duplication" hypothesis (26). According to this 2R hypothesis, a first genome duplication occurred as early as in deuterostomes and this was followed by a second round of genome duplication, that took place before the origin of gnathostomes, resulting in having 4 copies of the genome. Based on this hypothesis and our findings, we propose a scheme for the evolution of GHRH and PRP-PACAP genes in vertebrates (Fig. 5). In the beginning, the ancestral chordates possessed one copy of the PACAP-like gene which encoded a single peptide, and by exon duplication, the PRP-PACAP ancestral gene was formed. As a result of two rounds of genome duplication (1R and 2R), the ancestral gene produced 4 paralogous genes, but only three of them, PRP-PACAP, PHI-VIP and GHRH genes, persisted in later lineages. One of the duplicated copies could have been lost via a global process called diploidization (27-29). Although there are two highly conserved PRP-PACAP genes in fish and tunicates, they are produced neither by 1R nor 2R, and this will be discussed later.

The earliest PRP-PACAP genes were identified in protochordates (Chelyosoma productum, 15), in which two PRP-PACAP genes with little differences in gene structure were found. Protochordates are invertebrates that split from vertebrates before the two rounds of genome duplication. Therefore, if these two PRP-PACAP genes existed before 1R and 2R, there should be 8 PRP-PACAP paralogues in vertebrates. Also, the tunicate PRP-PACAP genes share very high levels of similarity among themselves in terms of gene structure and peptide sequence. Hence, these duplicated copies of PRP-PACAP genes are the result of a more recent gene duplication event that occurred after the initial divergence. Interestingly, in the phylogenetic tree, the tunicate PRP-like peptide belongs neither to GHRH nor to PRP groupings. The N-termini of these peptides are similar to PACAP, whereas the C-termini share higher similarity with PRP. Therefore, it is likely that these tunicate PRP-like peptides are evolved from the same ancestral sequences of PRP and/or PACAP.

Based on our model, the evolution of GHRH, PRP-PACAP and PHI-VIP genes could be explained using the concept of Duplication-Degeneration-Complementation (DDC) (30-31). After the first round of genome duplication (1R), the ancestral PRP-PACAP gene duplicated into two and each of them independently mutated. One of the genes had mutations mainly in the PRP-like region, thus giving rise to the PRP-PACAP gene with the function of the ancestral gene mostly preserved in the PACAP domain. Together with PRP's changes, a duplicated PACAP receptor evolved to interact specifically with PRP. Although we have no information regarding the function of PRP from fish to birds, the presence of a specific receptor that is expressed in a tissue-specific manner strongly suggests that this peptide exerts some physiological role. As the primary function was secured by the conservation of PACAP, in the other copy, PRP is free to mutate to GHRH while PACAP sequences were lost by exon deletion, producing the GHRH gene in vertebrates. After 2R, the duplicated gene copy

for PRP-PACAP was free to mutate and hence to acquire new functions to become PHI-VIP, and consistently, PHI and VIP are similar to PRP and PACAP, respectively. Until now, we have not been able to find a second GHRH homologue in vertebrates, and we thus propose that the duplicated GHRH gene may have been deleted or lost in the process of genome rediploidization.

The final question is what is the origin of the two forms of PRP, salmon-like and catfish-like, and correspondingly two PRP-PACAP genes, in fish? We named these PRPcatfish-like and salmon-like as there are clearly two branches in the phylogenetic tree in which the avian and amphibian PRPs are more similar to fish PRPsalmon-like. Based on previous reports and sequence analysis, we found that, in fact, all bony fish possess a PRPsalmon-like gene, supporting the idea that PRPsalmon-like is the ancestral form while PRPcatfish-like existed before the tetrapod/fish split, a duplicated copy arising from a more recent duplication event that occurred after the appearance of the teleost lineage (32). This is confirmed by the presence of duplicated PHI-VIP and glucagon genes in the *Fugu* and zebrafish genomes, as well as two genes for PAC1-R, VPAC1-R and VPAC2-R in fish (33). There is further evidence supporting the hypothesis of a third round of genome duplication; for example, zebrafish possesses seven HOX clusters on seven different chromosomes, instead of four clusters found in mammals (34). Also, there are many duplicated segments in the zebrafish genome where similar duplication are not found in the mammalian genome. This information suggests that some of the genome was duplicated in an ancient teleost. Based on this, we hypothesize that catfish-like and salmon-like PRP encoding genes were produced from the actinopterygian-specific genome duplication at around 300-450MYA.

Materials and Methods

Animals and Peptides. Zebrafish and goldfish were purchased from local fish markets, and *Xenopus laevis* was bought from Xenopusone Inc. Mi. Peptides including *X. laevis* GHRH (xGHRH), zebrafish or goldfish GHRH (fish GHRH), goldfish PRPsalmon-like (previously gfGHRHsalmon-like, 24), and goldfish PRPcatfish-like (previously gfGHRHcatfish-like, 24) were synthesized by The Rockefeller University.

Data Mining and Phylogenetic Analysis. By searching genome databases (Ensembl genome browse and NCBI database) of chicken (*Gallus gallus*), *Xenopus* (*Xenopus tropicalis*), zebrafish (*Danio rerio*) and *Fugu* (*Fugu rubripes*), putative GHRH and GHRH-R sequences were found. These sequences were used for phylogenetic analyses by MEGA3.0 to produce neighbor-joining (NJ) trees with Dayhoff matrix (35). Predicted protein sequences were then aligned by ClustalW. Sequence-specific or degenerate primers were designed accordingly for cDNA cloning by PCR amplifications.

Molecular Cloning of GHRHs (*Xenopus laevis*, Zebrafish and Goldfish) and GHRH-Rs (Zebrafish and Goldfish). Total RNAs from brain (zebrafish and goldfish) and pituitary (*Xenopus laevis*) were extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Rapid Amplification of cDNA Ends (5' and 3' RACE) was performed using the 5' and 3' RACE amplification kits (Invitrogen). Full length cDNA clones encompassing the 5' to 3' untranslated regions were produced by PCR using specific primers and confirmed by DNA sequencing. Full-length GHRH-R cDNAs were subcloned into pcDNA3.1+ (Invitrogen) for functional expression. Primers used in this study are listed in S12.

Tissue Distribution of GHRH and GHRH-R in Goldfish. Goldfish GHRH and GHRH-R transcript levels in various tissues were measured by real-time RT-PCR. First strand cDNAs from various tissues of sexually matured goldfish were prepared using the oligo-dT primer (Invitrogen). GHRH and GHRH-R levels were determined using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) together with specific primers (sequences listed in S13). Fluorescence signals were monitored in real time by the iCycler iQ system (Bio-Rad, Hercules, CA). The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence reaches 10-fold standard deviation of the baseline (from cycle 2 to 10). The ratio change in target gene relative to the β-actin control gene was determined by the $2^{-\Delta\Delta Ct}$ method (36).

Functional Studies of Fish GHRH-R and PRP-R. For functional studies, zfGHRH-R cDNA in pcDNA3.1 (Invitrogen) was permanently transfected into Chinese Hamster Ovary (CHO) cells (ATCC, Manassas, VA) using the Genejuice reagent (Novagen, Darmstadt) and followed by G418 selection (500 μg/ml) for 3 weeks. Several clones of receptor-transfected CHO cells were produced by dilution into 96-well plates. Following RT-PCR to monitor the expression levels of individual clones, colony with the highest expression was expanded and used for cAMP assays. A gfPRP-R-transfected (previously gfGHRH-R, 25) permanent CHO cell-line was also used for comparison in functional expression studies.

To measure cAMP production upon ligand activation, 2 days prior to stimulation, 2.5×10^5 CHO-zfGHRH-R or CHO-gfPRP-R cells were seeded onto 6-well plates (Costar, CA). The assay was performed essentially as described earlier (37) using the Correlate-EIA Immunoassay Kits (Assay Design, Mi).

Measurement of GH Release from Goldfish Pituitary Cells. Goldfish in late stages

of sexual regression were used for the preparation of pituitary cell cultures. Fish were sacrificed by spinosectomy after anesthesia in 0.05% tricane methanesulphonate (Syndel, Vancouver, BC, Canada). Pituitaries were excised, diced into 0.6 mm fragments, and dispersed using the trypsin/DNA II digestion method (38) with minor modifications (39). Pituitary cells were cultured in 24-well cluster plates at a density of 0.25×10^6 cells/well. After overnight incubation, cells were stimulated for 4 hr and culture medium was harvested for GH measurement using a radioimmunoassay previously validated for goldfish GH (40).

Data Analysis. Data from real time PCR are shown as the means ± SEM of duplicated assays in at least three independent experiments. All data were analyzed by one-way ANOVA followed by a Dunnett's test using PRISM (version 3.0, GraphPad Software Inc., San Diego, CA).

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Figure Legends

Fig. 1. Phylogenetic analysis of GHRH, PRP and GHRH-like peptide (1-27), GHRH-Rs and PRP-Rs. Predicted peptide and receptor sequences are marked with asterisks. (A) PACAP sequences were used as an out-group. (B) Novel receptors identified in this report are underlined.

Fig. 2. Chromosomal locations of GHRH and GHRH-R in various vertebrate species. Genes adjacent to GHRH and GHRH-R in different genomes are shown. The genes are named according to their annotation in the human genome. GHRH and GHRH-R genes are boxed

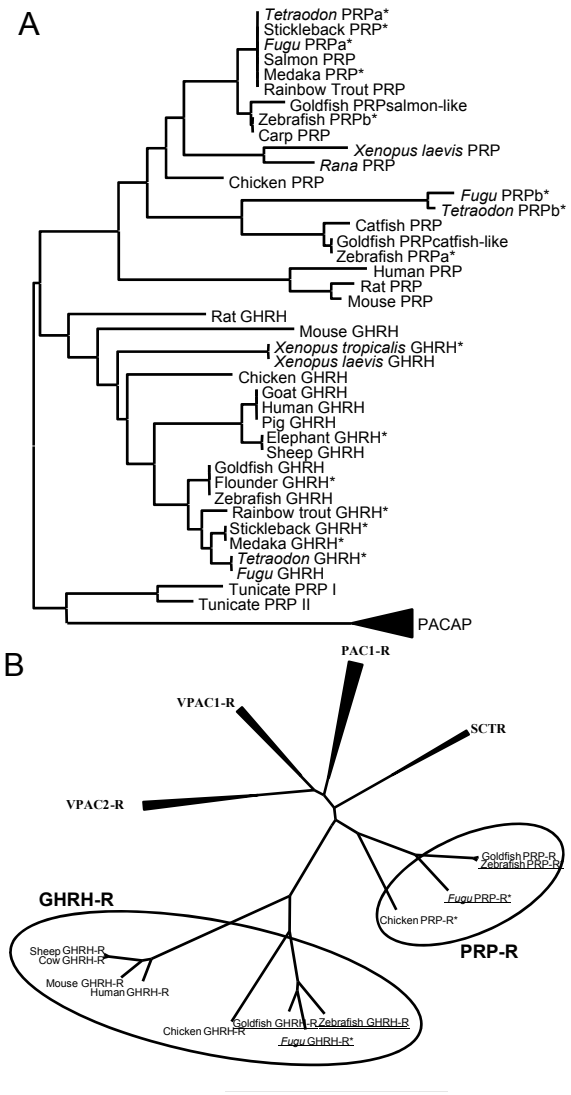
Fig. 3. Functional assays. (A) Intracellular cAMP accumulation in CHO-zfGHRH-R cells stimulated with 100 nM peptides: fish GHRH, *Xenopus* GHRH, goldfish PRPsalmon–like, goldfish PRPcatfish-like, goldfish PHI, goldfish glucagon, zebrafish GIP, human PACAP-27 and human PACAP-38; (B,C) Effect of graded concentrations (from 10^{-11} to 10^{-5} M) of various GHRHs and gfPRPs on cAMP accumulation in CHO-zfGHRH-R cells (B) and CHO-gfPRP-R cells (C). Values represent means ± S.E from 3 independent experiments each in duplicates. Effect of graded concentrations (from 10^{-11} to 10^{-6} M) of (D) fishGHRH and (E) gfPRPsalmon-like on GH release from cultured goldfish pituitary cells. Cells were incubated for 4 hrs with fishGHRH (D, n=12) or gfPRPsalmon-like (E, n=4). Values represent means ± S.E.M. from at least two experiments performed in duplicate.

Fig. 4. Relative abundance of (A) GHRH and (B) GHRH-R transcripts in different tissues in goldfish. A relative abundance of 1 was set arbitrarily for both genes in the

brain. Data are from at least 3 experiments performed in duplicate. Values are expressed as means ± SEM.

Fig. 5. A proposed evolutionary scheme of GHRH, PRP-PACAP, PHI-VIP genes with respect to several rounds of genome duplication. Labeling of peptides and gene organizations are shown in the legend. The genome duplication events are highlighted by light blue boxes. Unknown or unclear paths are marked with question marks. Time for divergence in MYA was taken from (41).

Figure 1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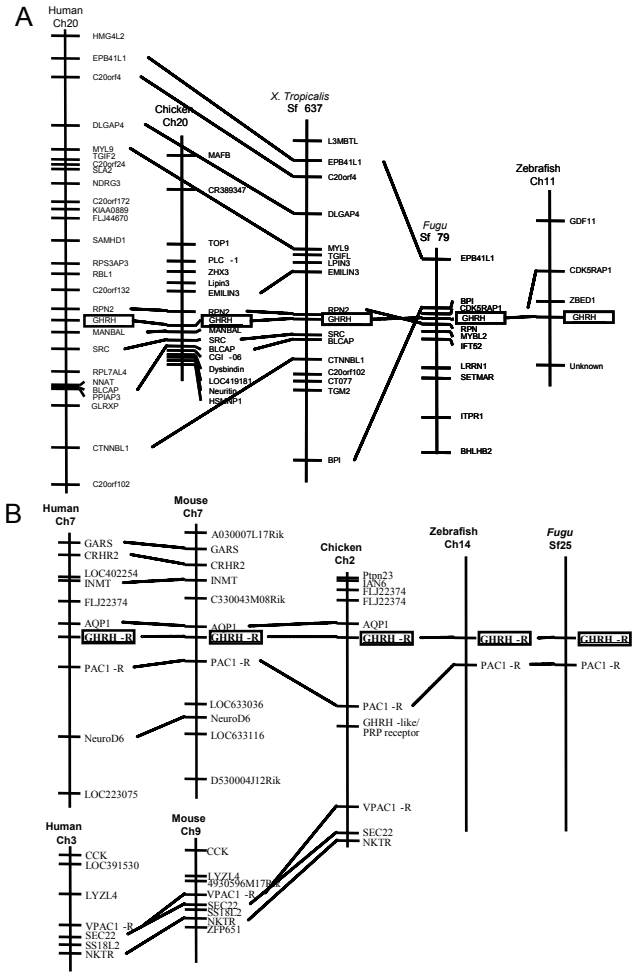
Stickleback PRP*
Fugu PRPa*
Salmon PRP
Medaka PRP*
Rainbow Trout PRP
Goldfish PRPsalmon-like
Zebrafish PRPb*
Carp PRP
Xenopus laevis PRP
Rana PRP
Chicken PRP
Fugu PRPb*
Tetraodon PRPb*
Catfish PRP
Goldfish PRPcatfish-like
Zebrafish PRPa*
Human PRP
Rat PRP
Mouse PRP
Rat GHRH
Mouse GHRH
Xenopus tropicalis GHRH*
Xenopus laevis GHRH
Chicken GHRH
Goat GHRH
Human GHRH
Pig GHRH
Elephant GHRH*
Sheep GHRH
Goldfish GHRH
Flounder GHRH*
Zebrafish GHRH
Rainbow trout GHRH*
Stickleback GHRH*
Medaka GHRH*
Tetraodon GHRH*
Fugu GHRH
Tunicate PRP I
Tunicate PRP II
PACAP
B
PAC1-R
VPAC1-R
SCTR
VPAC2-R
Goldfish PRP-R
Zebrafish PRP-R
Fugu PRP-R*
Chicken PRP-R*
PRP-R
GHRH-R
Sheep GHRH-R
Cow GHRH-R
Mouse GHRH-R
Human GHRH-R
Chicken GHRH-R
Goldfish GHRH-R
Zebrafish GHRH-R
Fugu GHRH-R*

Figure 2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Human Ch20
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EPB41L1
C20orf4
DLGAP4
MYL9
TGIF2
C20orf24
SLA2
NDRG3
C20orf172
KIAA0889
FLJ44670
SAMHD1
RPS3AP3
RBL1
C20orf132
RPN2
GHRH
MANBAL
SRC
RPL7AL4
NNAT
BLCAP
PPIAP3
GLRXP
CTNNBL1
C20orf102
Chicken Ch20
MAFB
CR389347
TOP1
PLC -1
ZHX3
Lipin3
EMILIN3
RPN2
GHRH
MANBAL
SRC
BLCAP
CGI -06
Dysbindin
LOC419181
Neuritin
HSMNP1
X. Tropicalis Sf 637
L3MBTL
EPB41L1
C20orf4
DLGAP4
MYL9
TGIF1
LPIN3
EMILIN3
RPN2
GHRH
SRC
BLCAP
CTNNBL1
C20orf102
CT077
TGM2
BPI
Fugu Sf 79
EPB41L1
BPI
CDK5RAP1
GHRH
RPN
MYBL2
IFT52
LRRN1
SETMAR
ITPR1
BHLHB2
Zebrafish Ch11
GDF11
CDK5RAP1
ZBED1
GHRH
Unknown
B
Human Ch7
GARS
CRHR2
LOC402254
INMT
FLJ22374
AQP1
GHRH -R
PAC1 -R
NeuroD6
LOC223075
Mouse Ch7
A030007L17Rik
GARS
CRHR2
INMT
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AQP1
GHRH -R
PAC1 -R
LOC633036
NeuroD6
LOC633116
D530004J12Rik
Chicken Ch2
Ptpn23
FLJ22374
AQP1
GHRH -R
PAC1 -R
GHRH -like/ PRP receptor
VPAC1 -R
SEC22
NKTR
Zebrafish Ch14
GHRH -R
PAC1 -R
Fugu Sf25
GHRH -R
PAC1 -R
Human Ch3
CCK
LOC391530
LYZL4
VPAC1 -R
SEC22
SS1RL2
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SEC22
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NKTR
ZFP651

Figure 3

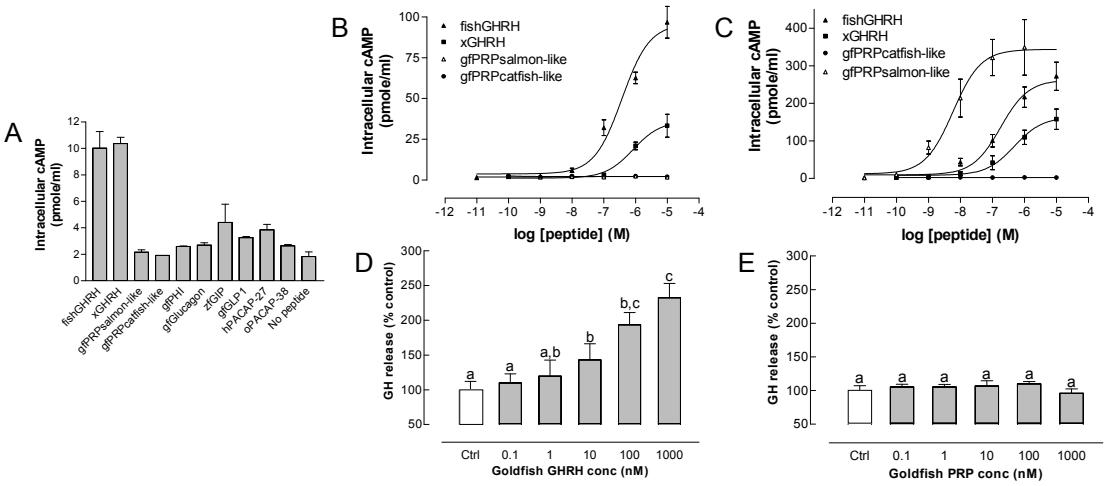

A
Intracellular cAMP (pmole/ml)
fishGHRH
xGHRH
gfPRPsalmon-like
gfPRPcatfish-like
gfPHI
gfGlucagon
zfGIP
gfGLP1
hPACAP-27
oPACAP-38
No peptide
B
fishGHRH
xGHRH
gfPRPsalmon-like
gfPRPcatfish-like
Intracellular cAMP (pmole/ml)
log [peptide] (M)
C
fishGHRH
xGHRH
gfPRPcatfish-like
gfPRPsalmon-like
Intracellular cAMP (pmole/ml)
log [peptide] (M)
D
GH release (% control)
Ctrl
0.1
1
10
100
1000
Goldfish GHRH conc (nM)
E
GH release (% control)
Ctrl
0.1
1
10
100
1000
Goldfish PRP conc (nM)

Figure 4

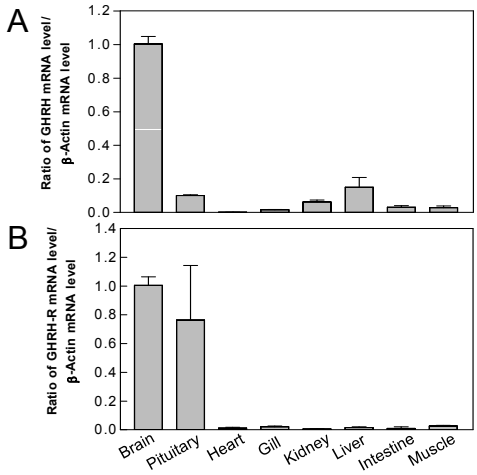
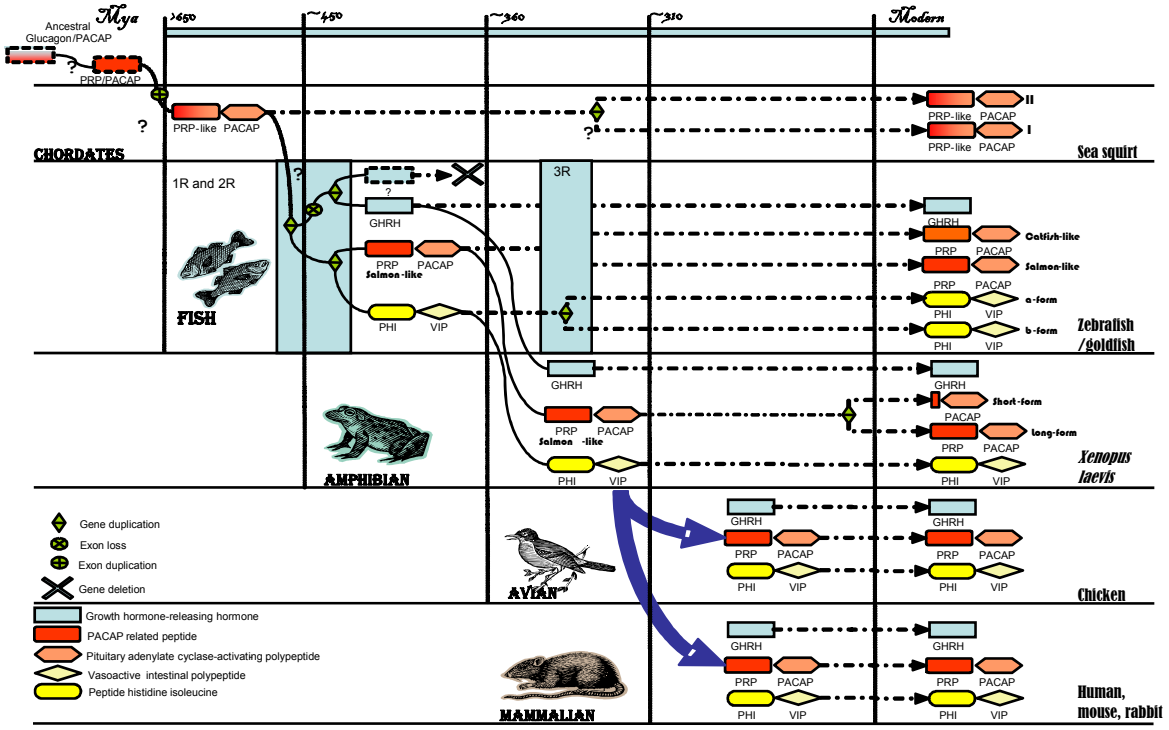

A
Ratio of GHRH mRNA level/ β-Actin mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B
Ratio of GHRH-R mRNA level/ β-Actin mRNA level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Brain
Pituitary
Heart
Gill
Kidney
Liver
Intestine
Muscle

Figure 5


Mya
>650
~450
~360
~310
Modern
Ancestral
Glucagon/PACAP
PRP/PACAP
PRP-like PACAP
CHORDATES
Sea squirt
1R and 2R
3R
FISH
GHRH
PRP PACAP
salmon-like
PHI VIP
Catfish-like
salmon-like
a-form
b-form
Zebrafish
/goldfish
AMPHIBIAN
PRP PACAP
salmon -like
short-form
long-form
Xenopus
laevis
AVIAN
Chicken
MAMMALIAN
Human,
mouse, rabbit
Gene duplication
Exon loss
Exon duplication
Gene deletion
Growth hormone-releasing hormone
PACAP related peptide
Pituitary adenylate cyclase-activating polypeptide
Vasoactive intestinal polypeptide
Peptide histidine isoleucine

Supplemental data

Figure S1

Zebrafish GHRH

```
  1   actacaatca ccaacagtag aggctgggaa taaaatcaaa tcctcagtga
 51   gagaaagatc ttgtttccgt cacatttgtt acaaacctgg accagaaatc
            M   Q  V  G   V  I  Q  R   T  A  L   L  V  L
101   tttgatcATG CAAGTGGGAG TAATTCAGAG AACTGCCTTG CTAGTGCTAT
      C  C  L  L   L  S  A   S  S  S   P  V  Y  P   A  L  K
151   GCTGCTTGTT ACTATCAGCA TCATCTTCCC CAGTCTACCC AGCACTGAAG
       F  G  R  H  A  D  A   I  F  T   N  S  Y   R  K  V  L
201   TTTGGAAGAC ATGCTGATGC CATCTTTACC AACAGCTACA GAAAAGTTCT
        G  Q  I   S  A  R   K  F  L  Q   T  V  M   G  K  R
251   TGGTCAAATA TCTGCCAGAA AATTTCTTCA AACTGTTATG GGAAAAAGAC
      L  G  P  E   T  E  S   N  V  K   R  Q  S  S   M  Y  G
301   TTGGACCAGA AACTGAAAGC AATGTAAAGC GTCAGTCAAG TATGTATGGA
       N  T  Y   K  Q  D  V   D  M  N   V  I  E   S  E  Q  S
351   AACACCTACA AGCAAGATGT GGACATGAAT GTCATTGAGA GTGAACAAAG
        Y  R  D   P  Q  K   F  K  F  A   L  I  M   H  *
401   TTACAGAGAC CCACAAAAAT TTAAGTTTGC ATTAATTATG CACTGAatta
451   aaatgtaaag tgcatataca ttatgaatat ttaaaaggga actattgcat
501   ttgctgtatt tcatattgga taattaaaac tcacccatgt tataatc
```

Figure S1 cDNA and predicted amino acid sequences of zfGHRH. The numbers on the left are the positions of the nucleotide sequences. The UTR sequences are in lower case. The coding sequences are in upper case. The mature peptide sequence is highlighted in bold letter. Potential peptide cleavage sites are in open boxes. Stop codon is represented by an asterisks.The zfGHRH cDNA encompasses a 107-bp 5' UTR, a 339-bp open reading frame (ORF) encoding a 113-amino acid protein, and a 101-bp 3' UTR. The predicted prepro-zfGHRH has a putative 23-amino acid signal peptide and potential peptide processing sites R and GKR at position 34 and 62-64, respectively, suggesting the 27-amino acid zfGHRH is located between 35 to 61.

Figure S2

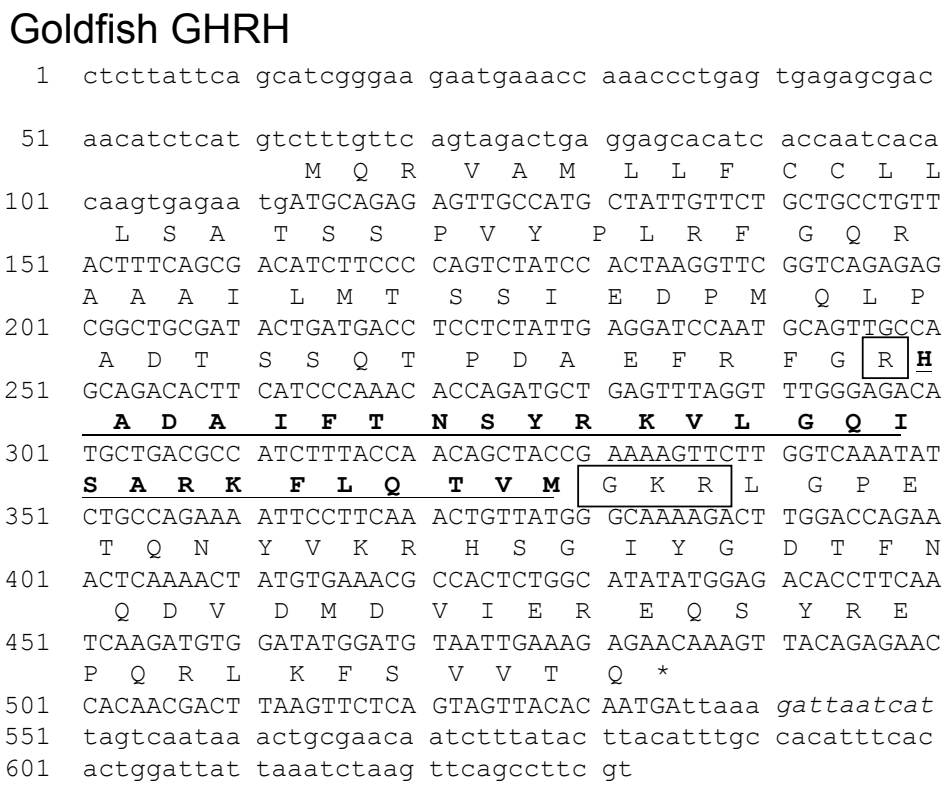


Figure S2 cDNA and predicted amino acid sequences of gfGHRH. The numbers on the left are the positions of the nucleotide sequences. The UTR sequences are in lower case. The coding sequences are in upper case. The mature peptide sequence is highlighted in bold letter. Potential peptide cleavage sites are in open boxes. Stop codon is represented by an asterisks. The sizes of the 5'UTR, ORF and 3'UTR are 112, 420, and 97-bps, respectively, and, within the ORF, the predicted GHRH sequences between the putative cleavage sites (R and GKR, position 62 and 90-92) are identical to those of zfGHRH. As both fish GHRHs are followed immediately by GKR, they are likely α-amidated at the C-termini.

Figure S3

X. laevis GHRH

```
  1 aatttcgcag accctccggc tgtaaggatt ctacagcttt gggaagcacg
 51 ccattgtaga ggaagaagtt cagcaggact gactacacag tttttactgc
                M  R  R   T  L  C   L  L  L  L   H  F  A
101 aaaaaaagaa ggATGCGCAG AACACTTTGC CTGTTGCTAC TCCATTTTGC
     L  C  V   Q  C  Y   I  F  H  P   K  Y  S   S  Y  Q
151 ATTATGTGTG CAGTGTTACA TCTTTCATCC AAAATACAGT TCTTACCAGA
    T  P  G  D   F  N  I   E  T  I   E  P  L  Q   S  Q  D
201 CTCCAGGCGA CTTTAATATC GAGACAATTG AGCCCTTGCA AAGCCAAGAC
     W  S  S   L  D  E  K   K  E  Y   V  R  G   L  S  E  N
251 TGGTCATCTT TGGATGAGAA AAAGGAATAT GTCAGGGGAC TGTCAGAAAA
      R  V  E   R  H  V  D   A  I  F   T  N  T   Y  R  K
301 CAGGGTGGAA CGGCATGTGG ATGCCATATT CACTAACACA TACAGAAAAT
    F  L  G  Q   I  S  A   R  R  Y   L  Q  N  M   M  G  K
351 TCCTGGGGCA GATTTCAGCC AGGAGGTACC TGCAGAACAT GATGGGGAAA
    T  L  G  Q   D  T  Q   K  K  A   D  P  E   D  G  V  L
401 ACCCTGGGGC AGGATACCCA GAAAAAGGCA GATCCTGAAG ATGGTGTGCT
     G  E  E   V  I  T   L  L  S  D   S  G  I   P  D  W
451 GGGGGAAGAA GTGATCACCC TACTGAGTGA CAGTGGGATT CCAGATTGGA
    R  A  E  E   H  R  E   T  R  L   *
501 GGGCAGAAGA GCACCGTGAG ACCCGGCTAT AAactagaag cctacaggat
551 ccgtcatcaa atgttcagcc taatgcgtta attccgtgcc aacaaaatgg
601 acagcaatgg caacatacat gatagagcat tggtcattgt gatttctgag
651 ttggtaataa aatataacat tatggggcaa aaaaaactag gtcaagtttt
701 atgccaattc cctgttgaga aaaatcactt tttgcatcta atcaagaagc
751 agttgtacag aggtatttca taaaataaaa agttttagtg
```

Figure S3 cDNA and predicted amino acid sequences of xGHRH. The numbers on the left are the positions of the nucleotide sequences. The UTR sequences are in lower case. The coding sequences are in upper case. The mature peptide sequence is highlighted in bold letter. Potential peptide cleavage sites are in open boxes. Stop codon is represented by an asterisks. *Xenopus laevis* GHRH cDNA is 790 bps in length with a 112-bp 5'UTR, a 420-bp ORF and a 258-bp 3' UTR (supplemental data S3). Within the 140-amino acid xGHRH precursor protein, the N-terminal processing site (R at position 67) is conserved while the C-terminal site (GKT at position 95-97 or KK at position 104-105) is unclear. The mature xGHRH peptide may be 27- or 28-amino acid long, depending whether G at 95 is processed to the C-terminal amide, or 36-amino acid long (68 to 103, processing site KK at position 104-105).

Figure S4

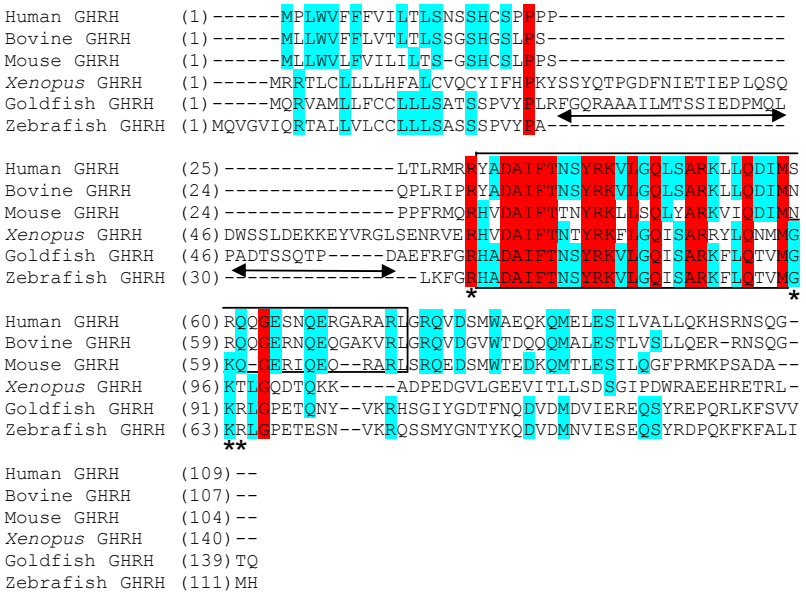


Figure S4 Alignment of GHRH precursor proteins. The alignment was generated by using the default settings of the VectorNTI 10 (Invitrogen) with AlignX program. Identical and conserved residues are highlighted in red and blue, respectively. Putative GHRH peptides are in the open box. Regions found only in *Xenopus* and goldfish are underlined by arrows.

Figure S5

Species	Position	Sequences (1-27)	%Identity	Ref
GHRH				
Goldfish	Unknow	HADAIFTNSYRKVLGQISARKFLQTVM	100	--
Zebrafish	Ch11	HADAIFTNSYRKVLGQISARKFLQTVM	100	--
Fugu	Sf 701	HADAIFTNSYRKVLGQISARKILQTIM	92.6	*
Tetraodon	Ch11	HADAIFTNSYRKVLGQISARKILQTIM	92.6	*
X. laevis	Unknow	HVDAIFTNTYRKFLGQISARRYLQNMM	74.1	--
X. tropicalis	Sf 637	HVDAIFTNTYRKFLGQISARRYLQNMI	70.4	*
Chicken	Ch20	HADAIFTDNYRKFLGQISARKFLQTII	81.5	*
Rat	Ch3	HADAIFTSSYRRILGQLYARKLLHEIM	63.0	27
Mouse	Ch2	HVDAIFTTNYRKLLSQLYARKVIQDIM	59.3	28
Goat	Unknow	YADAIFTNSYRKVLGQLSARKLLQDIM	81.5	29
Human	Ch20	YADAIFTNSYRKVLGQLSARKLLQDIM	81.5	30
PRP				
Goldfish-catfish	Unknow	HADGLLDRALRDILVQLSARKYLHSLM	44.4	51
Goldfish-salmon	Unknow	HADGMFNKAYRKALGQLSAFHYLHTLM	59.3	51
Chicken	2	HADGIFSKAYRKLLGQLSARKYLHSLM	66.7	31
X. laevis	Sf420	HADELLNKVYRNVLGHLSARKYLHTLM	40.7	32
Mouse	17	VAHEILNEAYRKVLDQLSARKYLQSVV	55.6	33
Human	18	VAHGILNEAYRKVLDQLSAGKHLQSLA	46.4	34

Figure S5 Percentage similarity and identity of gfGHRH with other GHRHs. Chromosomal locations of GHRHs in species not yet determined are labeled as unknown. In the alignment, conserved and identical residues for GHRH and PRP are highlighted in blue and red, respectively. Residues that are conserved in PRP are labeled in green. The % identity was calculated by comparing with gfGHRH. Sf, scaffold number; Ch, chromosome number; --, sequences isolated in this report; *, sequences predicted from the genome database.

Figure S6

Zebrafish GHRH Receptor

```
                                      M  L  P  C  A  R  G  L  F  W  L  L  T  C  L  T
   1  gcgctgtgga ctcgactgga gagATGCTGC CGTGTGCGCG TGGACTTTTC TGGCTGCTGA CATGTCTCAC
        T  V  L  C  S  L  H  P  E  C  E  Y  I  F  Q  L  A  R  D  E  Q  R  C
  71  AACTGTGTTG TGTAGTTTGC ATCCCGAGTG TGAATATATC TTTCAGCTGG CGAGAGATGA ACAGCGGTGT
        L  R  E  I  T  D  L  G  N  L  S  S  S  S  G  C  L  S  V  W  D  S  V
 141  CTGCGAGAGA TCACAGATCT GGGGAACCTC AGCAGCTCCT CAGGTTGTCT TTCAGTATGG GATTCAGTGG
        V  C  W  P  S  A  V  V  G  E  T  V  Q  S  P  C  P  A  V  F  S  L  F  E
 211  TCTGCTGGCC CAGTGCTGTG GTGGGTGAAA CTGTCCAGTC TCCCTGTCCT GCTGTTTTCT CTCTCTTCGA
        N  N  T  E  G  V  V  S  R  N  C  T  V  T  G  W  S  R  P  F  P  P  Y
 281  GAACAACACA GAAGGTGTAG TGAGTCGAAA CTGCACAGTC ACAGGCTGGT CCCGACCGTT TCCTCCTTAT
        H  V  A  C  S  A  E  D  N  I  P  E  E  S  Y  F  A  T  V  K  L  I  Y
 351  CATGTGGCCT GCAGCGCTGA GGACAATATC CCAGAGGAGT CGTATTTTGC CACTGTGAAG CTGATCTACA
        T  V  G  Y  G  A  S  L  L  S  L  S  V  A  V  L  I  L  L  L  F  R  R  L
 421  CTGTGGGCTA TGGAGCATCT CTGCTCTCGC TCTCTGTTGC TGTGCTAATA CTGCTGCTCT TCAGGCGACT
        H  C  T  R  N  Y  I  H  M  Q  L  F  F  T  F  I  L  K  S  V  A  V  F
 491  GCACTGCACT CGAAACTATA TCCACATGCA GCTGTTTTTC ACCTTCATCC TGAAATCTGT GGCCGTGTTC
        I  K  D  V  T  L  F  S  S  D  D  T  D  H  C  S  L  S  T  V  A  C  K
 561  ATCAAAGATG TGACGCTGTT CTCCAGCGAC GACACAGATC ACTGCTCTCT GTCTACTGTG GCCTGTAAAA
        T  A  V  V  F  C  H  Y  C  V  M  S  N  F  F  W  L  L  V  E  A  V  Y  L
 631  CAGCGGTGGT GTTTTGTCAC TATTGCGTCA TGTCTAATTT CTTCTGGTTG CTGGTTGAAG CTGTGTATCT
        N  S  L  L  V  S  V  F  L  R  R  R  R  C  L  W  A  F  A  L  L  G  W
 701  GAACTCTCTG CTGGTGTCCG TGTTTCTGCG CAGACGCCGC TGTCTGTGGG CTTTTGCTCT GTTGGGCTGG
        G  V  P  L  V  C  I  V  L  W  I  F  S  R  L  Y  F  E  D  T  E  C  W
 771  GGTGTTCCAC TGGTCTGCAT TGTCCTTTGG ATCTTCTCCA GACTGTATTT TGAAGACACT GAATGCTGGG
        D  I  N  E  D  S  P  Y  W  W  I  I  K  G  P  I  V  I  S  I  A  V  N  F
 841  ACATAAATGA AGATTCTCCT TATTGGTGGA TCATAAAGGG CCCCATTGTA ATATCTATTG CGGTGAACTT
        L  L  F  L  N  I  I  R  I  L  M  Q  K  L  N  P  R  L  I  Q  F  N  N
 911  TCTTCTCTTC TTGAACATTA TCAGGATTCT GATGCAGAAA CTGAACCCTC GCTTGATCCA GTTCAACAAT
        S  D  Q  Y  R  R  L  T  K  S  T  L  L  L  I  P  L  F  G  T  H  Y  M
 981  TCAGACCAGT ACAGGCGACT GACCAAGTCC ACACTCCTGC TCATCCCTCT CTTCGGCACT CATTACATGA
        I  F  S  F  L  P  D  Y  F  N  T  G  L  R  L  C  I  E  L  C  L  G  S  F
1051  TCTTCAGCTT TCTCCCAGAC TACTTCAACA CGGGCTTGCG GCTCTGCATT GAGCTGTGTC TGGGCTCCTT
        Q  G  L  I  V  A  I  L  Y  C  F  L  N  Q  E  V  Q  N  E  I  R  L  R
1121  CCAGGGCCTG ATTGTGGCAA TTCTCTACTG TTTCCTGAAC CAGGAGGTTC AGAATGAGAT ACGGTTACGA
        W  L  R  Y  Q  E  G  S  Y  A  V  V  P  V  G  V  K  G  S  Q  M  D  T
1191  TGGCTTCGGT ATCAAGAAGG AAGTTATGCT GTCGTCCCCG TCGGAGTGAA AGGAAGTCAG ATGGACACTC
        P  V  H  R  Q  T  R  R  N  S  I  K  H  F  T  V  C  I  L  F  C  S  F  Y
1261  CAGTCCACCG TCAAACCAGA AGAAATTCAA TCAAACATTT TACCGTATGC ATATTGTTTT GCTCTTTTTA
        M  T  T  I  G  L  K  G  T  *
1331  TATGACAACA ATTGGCTTAA AGGGGACCTA Atatgcacat ttttacaaga tctaaaataa gtctctggta
1401  tccctaaagt gcgcatgtga agtttcagct caaaatacca cacaaatgat gttttataac ttattaaaac
1471  ggatcccttt aagctttgat tctaattgtg gcattttggt gactgtcgct ttaaattca
```

Figure S6 cDNA and predicted amino acid sequences of zebrafish GHRH-R. The seven transmembrane domains are underlined. The RLTK motif is boxed. The conserved cystine residues at the N-terminal are circled. The putative signal peptide is in bold letter. The stop codon is represented by an asterisk. The N-glycosylation site is marked in a black box.

Figure S7

Goldfish GHRH Receptor

```
                                M  T  G  D  M  R  S  W  A  R  G  I  L  W  L  S  S  F
1   agcgcgcgct gtggacATGA CTGGAGACAT GCGCTCGTGG GCTCGCGGGA TCCTGTGGTT GTCATCTTTT
    T  T  V  L  S  S  L  H  P  E  C  E  Y  I  F  Q  L  A  R  D  E  Q  R
71  ACAACTGTGT TGTCTAGTTT GCATCCTGAG TGTGAGTATA TCTTTCAGCT GGCAAGAGAT GAGCAGCGGT
    C  L  R  E  I  T  D  L  G  N  L  S  S  S  S  G  C  L  P  V  W  D  A  V
141 GTCTCAGAGA GATCACAGAT CTTGGAAACC TCAGCAGCTC TTCAGGTTGT CTGCCAGTAT GGGATGCTGT
    V  C  W  P  R  A  A  V  G  E  T  I  H  L  L  C  P  A  V  F  S  L  F
211 AGTGTGTTGG CCCAGAGCTG CAGTGGGTGA GACGATCCAC TTGCTGTGTC CTGCTGTTTT CTCTCTCTTC
    K  N  N  T  G  I  V  S  R  N  C  T  A  G  G  W  S  H  P  F  P  P  Y
281 AAGAACAACA CAGGTATAGT GAGTCGTAAC TGCACGGCTG GCGGCTGGTC ACATCCGTTC CCTCCGTACC
    H  E  A  C  I  V  E  D  E  I  P  E  E  S  Y  F  F  T  V  K  L  I  Y  T
351 ACGAGGCCTG CATTGTTGAG GACGAAATCC CAGAGGAGTC GTATTTCTTC ACTGTGAAAC TGATCTACAC
    V  G  Y  G  A  S  L  L  S  L  S  V  A  V  A  I  L  M  L  F  R  R  L
421 TGTTGGATAC GGAGCATCAC TGCTGTCGCT CTCTGTTGCC GTGGCAATAC TGATGCTCTT CAGGCGATTA
    H  C  A  R  N  Y  I  H  M  Q  L  F  F  T  F  I  L  K  S  V  A  V  F
491 CACTGCGCTC GAAACTATAT CCACATGCAG CTGTTTTTCA CCTTCATCCT GAAATCGGTG GCTGTGTTTA
    I  K  D  A  T  L  F  S  S  D  D  T  D  H  C  S  L  S  R  T  A  C  K  A
561 TCAAAGATGC TACGCTGTTC TCCAGTGACG ACACAGATCA CTGCTCTCTC TCCAGAACGG CCTGTAAGGC
    A  V  V  F  C  H  Y  C  I  M  T  N  F  F  W  L  L  V  E  A  V  Y  L
631 AGCGGTGGTA TTTTGTCATT ACTGTATCAT GACTAATTTC TTCTGGTTGC TGGTTGAAGC CGTGTATCTG
    N  S  L  L  V  S  D  F  P  R  S  R  R  C  L  W  T  F  A  L  L  G  W
701 AACTCTCTGC TGGTCTCAGA CTTCCCGCGC AGCCGCCGCT GTCTCTGGAC TTTCGCTCTG CTGGGCTGGG
    G  F  P  L  V  C  I  V  L  W  I  C  S  R  L  Y  F  E  D  T  E  C  W  N
771 GCTTTCCACT GGTTTGCATT GTCCTCTGGA TCTGTTCCAG ACTGTATTTT GAAGACACAG AGTGCTGGAA
    I  N  E  D  S  P  Y  W  W  I  I  K  G  P  I  V  V  S  I  G  M  N  F
841 CATAAATGAA GACTCTCCTT ATTGGTGGAT CATCAAGGGT CCCATTGTAG TTTCCATAGG GATGAACTTC
    L  L  F  M  N  I  I  R  I  L  V  Q  K  L  N  P  R  L  I  Q  F  N  N
911 CTTCTCTTCA TGAACATTAT CAGGATTCTG GTGCAGAAAC TGAACCCTCG CTTGATCCAA TTCAACAATT
    S  A  Q  Y  R  R  L  T  K  S  T  L  L  L  I  P  L  F  G  T  H  Y  M  V
981 CAGCTCAGTA CAGGCGACTG ACCAAGTCCA CCCTCCTCCT CATCCCTCTC TTCGGCACTC ATTACATGGT
    F  N  F  L  P  D  Y  F  N  V  S  L  R  L  C  I  E  L  C  L  G  S  F
1051 CTTCAACTTC CTCCCAGACT ACTTCAACGT GAGCCTGCGG CTTTGCATCG AGCTGTGTCT GGGCTCCTTC
    Q  G  L  I  V  A  V  L  Y  C  F  L  N  Q  E  V  Q  K  E  I  R  L  R
1121 CAGGGCCTCA TTGTGGCAGT TCTCTACTGT TTCCTGAACC AGGAGGTTCA GAAAGAAATA CGCTTACGTT
    W  M  R  Y  Q  E  G  S  Y  I  V  V  P  V  G  A  K  G  S  Q  M  D  T  P
1191 GGATGCGATA TCAAGAGGGG AGTTACATTG TCGTCCCTGT TGGAGCCAAA GGAAGTCAAA TGGACACTCC
    F  *
1261 ATTCTAGagt cattaaaatt caccggtgcc tctaatatac agacctcaac acctccagaa accatgaaca
1331 tctgaatcca acagttttat ttaatacaga ttgtattgct cagtcgatat gacaacaaat ggcttaaaca
1401 gccagaatat acagtatttg ggctgcagta tatctgtgaa ttaacacact taacagagtt cattagagag
1471 catggtaaaa atgtgcaatt catcagaaca aaactgctgc ttttgggggt ttcttggtgg gtttaattaa
1541 agtatgtaca tatatagaga aaagcctatc aaaatatact actgctgccc ttcaaattat ttattacttt
1611 aaggggnaat gttaaactga gatatgacag ttttgaatgt gtatgtattt agctgtatgt atgtacatgt
1681 ttacagtatt aacattggta aagagaaata ttaagatttt tgagtgagat aggcctacag gtaaccaggc
1751 aagttatgtt ttatatgttt attctgccaa atttgttact tcacttcgat tggcaaaaca gtggtttgat
1821 atttccttat catttcacaa gttgctttgt gtttttaact gtatttatat tttgctgaaa cgcctgttat
1891 aggctgttta atcaatgtca agttccctta gtgtgacaac acgcctgcaa tttgctcatc atatgttcct
1961 gtatctttcc ccctgtcata tacatgttca aaatcatatt tgtagcaaag acccactctc aaaatgacta
2031 ttttgagcta aaggagtgta acaactggcc ctggtttctc ctttctgctg cttgattctt ttatttttaa
2101 tacttaaaaa tgacattagt att
```

Figure S7 cDNA and predicted amino acid sequences of goldfish GHRH-R. The seven transmembrane domains are underlined. The RLTK motif is boxed. The conserved cystine residues at the N-terminal are circled. The putative signal peptide is in bold letter. The stop codon is represented by an asterisk. The N-glycosylation site is marked in a black box.

Figure S8

Species	%Identity	References
GHRH-R		
Goldfish	100	--
Zebrafish	78.4	--
Fugu	64.8	*
Chicken	48.9	53,35,36
Cow	49.7	37
Mouse	50.3	38
Sheep	50.7	37
Human	51.1	39
Other goldfish receptors		
PAC1-R	38.3	40
VPAC1-R	37.7	41
PHI-R	36.4	42
PRP-R	39.7	52
Other human Class II receptors		
GHRH-R	51.1	39
PAC1-R	35.8	43
VPAC1-R	42.1	44
VPAC2-R	43.6	45
SCT-R	40.1	46
GIP-R	29.9	47
GLP1-R	31.6	48
GLP-2R	25.2	49
Glu-R	28.8	50

Figure S8 Percentage similarity and identity of gfGHRH-R with other GHRH receptors, other goldfish receptors in the same gene family, and other class IIB receptors in human.

Figure S9

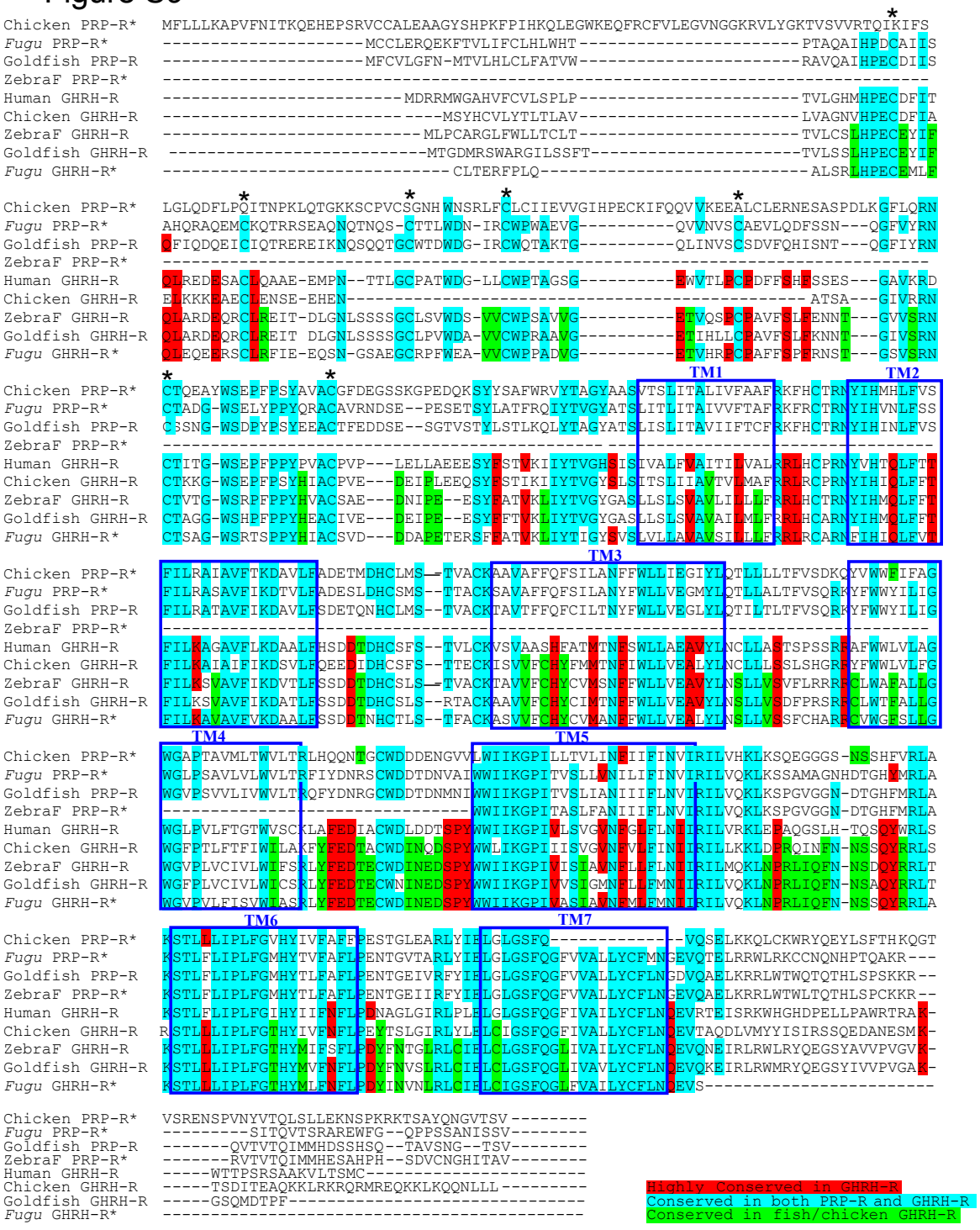


Figure S9. Amino acid alignment of GHRH-Rs and PRP-Rs. The alignment was generated by using the default settings of the VectorNTI 10 (Invitrogen) with AlignX program. Conserved residues for both GHRH-R and PRP-R are highlighted in blue. Residues that are conserved only among GHRH-Rs are in red. Residues conserved in fish and chicken GHRH-Rs are in green. Putative transmembrane domains (TM1 to TM7) are in open blue boxes. *, conserved cysteine residues.

Figure S10

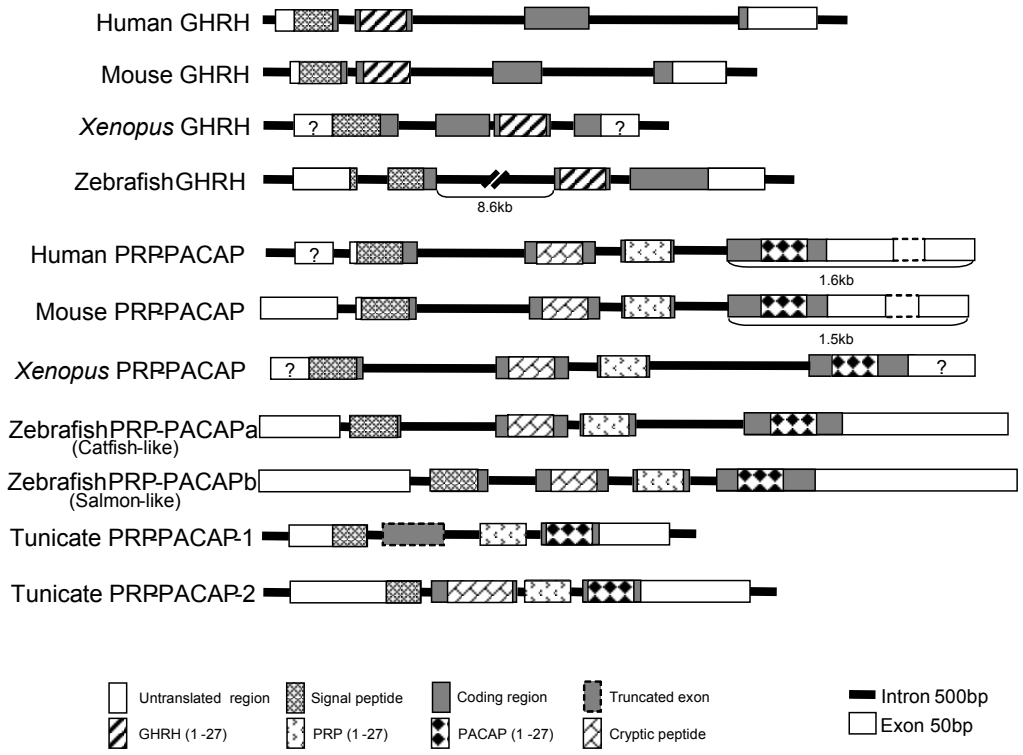


Figure S10 Diagrammatic representation of GHRH and PRP-PACAP gene structures in various vertebrates. The boxes represent exons. The solid lines represent introns. Different domains of the precursors are shown in the legend.

Figure S11

GHRH				
Species	Accession number (Nucleotide)	Accession number (Protein)	Database	Remarks/ reference
Human (*Homo sapiens*)	NM_021081	NP_066567	GenBank (NCBI)	1
Mouse (*Mus musculus*)	NM_010285	NP_034415	GenBank (NCBI)	2
Rat (*Rattus norvegicus*)	NM_031577	NP_113765	GenBank (NCBI)	3
Pig (*Sus scrofa*)	None	P01287	GenBank (NCBI)	4
Goat (*Capra hircus*)	None	P63293	GenBank (NCBI)	5
Elephant	Predicted	Predicted	Genome (ENsembl)	
Sheep (Ovis aries)	None	P07217	GenBank (NCBI)	5
Chicken (*Gallus gallus*)	Predicted	Predicted	Genome (NCBI)	Ch20
Xenopus Tropicalis	Predicted	Predicted	Genome (Ensembl)	Sf38
Xenopus Laevis	DQ991245	ABJ55979	Genome (NCBI)	From this report
Flounder	CX283280	Predicted from unidentified EST	GenBank (NCBI)	Unidentified EST
Fugu	Predicted	Predicted	Genome (Ensembl)	Sf79
Goldfish	DQ991243	ABJ55977	Genome (NCBI)	From this report
Medaka	Predicted	Predicted	Genome (UTGB)	Sf70
Rainbow trout	BX870571	Predicted from unidentified EST	GenBank (NCBI)	Unidentified EST
Stickleback	Predicted	Predicted	Genome (Ensembl)	GpXVII
Tetraodon	Predicted	Predicted	Genome (Ensembl)	Ch11
Zebrafish	DQ991246	ABJ55980	Genome (NCBI)	From this report

Figure S11

PRP/PACAP

Species	Accession number (Nucleotide)	Accession number (Protein)	Database	Remarks/ reference
Human (*Homo sapiens*)	NM_001117	NP_001108	GenBank (NCBI)	6
Mouse (*Mus musculus*)	NM_009625	NP_033755	GenBank (NCBI)	7
Rat (*Rattus norvegicus*)	NM_016989	NP_058685	GenBank (NCBI)	8
Rana ridibunda	AF221633	AAF74571	GenBank (NCBI)	9
Chicken (*Gallus gallus*)	AY956323	AAX56089	GenBank (NCBI)	10
Xenopus Laevis	AF187877	AAD56956	GenBank (NCBI)	11
Carp (*Cyprinus carpio*)	None	P42692	GenBank (NCBI)	12
Salmon (*Oncorhynchus nerka*)	X73233	P41585	GenBank (NCBI)	13
Goldfish a			Personal Comm.	
Goldfish b			Personal Comm.	
Fugu a	Predicted	Predicted	Genome (Ensembl)	Sf223
Fugu b	Predicted	Predicted	Genome (Ensembl)	Sf80
Medaka	Predicted	Predicted	Genome (UTGB)	Sf652
Stickleback a	Predicted	Predicted	Genome (Ensembl)	GpXXI
Stickleback b	Predicted	Predicted	Genome (Ensembl)	GpIII
Tetraodon a	Predicted	Predicted	Genome (Ensembl)	Ch6
Tetraodon b	Predicted	Predicted	Genome (Ensembl)	Unknown
Catfish (*Clarias macrocephalus*)	X79078	P48144	GenBank (NCBI)	14
Zebrafish a	AF329730	AAG59830	GenBank (NCBI)	15
Zebrafish b	NM_152885	NP_690841	GenBank (NCBI)	16
Rainbow trout	AF343976	AAK28557	GenBank (NCBI)	25
Tunicate I	Reference	Reference	Reference	26
Tunicate II	Reference	Reference	Reference	26

Figure S11

GHRH-R

Species	Accession number (Nucleotide)	Accession number (Protein)	Database	Remarks/ reference
Human (*Homo sapiens*)	NM_000823	NP_000814	GenBank (NCBI)	17
Mouse (*Mus musculus*)	NM_001003685	NP_001003685	GenBank (NCBI)	18
Sheep (*Ovis aries*)	NM_001009454	NP_001009454	GenBank (NCBI)	19
Cow	NM_181020	NP_851363	GenBank (NCBI)	19
Chicken (*Gallus gallus*)	NM_001037834	ABB84385	GenBank (NCBI)	10, 20, 21
Goldfish	DQ991244	ABJ55978	Genome (NCBI)	From this report
Fugu	Predicted	Predicted	Genome (Ensembl)	Sf25
Zebrafish	DQ991247	ABJ55981	Genome (NCBI)	From this report

PRP-R

Species	Accession number (Nucleotide)	Accession number (Protein)	Database	Remarks/ reference
Chicken	XM_425958	XP_425958	GenBank (NCBI)	A
Goldfish	AF048819	AAC15698	GenBank (NCBI)	23
Fugu	AJ296145	CAC82589	GenBank (NCBI)	22
Zebrafish	AY738797	AAW65133	GenBank (NCBI)	24

Predicted – Sequence is predicted from genome database
None – No sequence is available
Ch – Chromosome Number
Sf – Scaffold Number
A – Automatic prediction from genome project

Figure S12

Primer sequences used in PCR cloning of GHRH and GHRH-R cDNAs

Amplification	Primer Name	Primer Sequence (5′ to 3′)
Goldfish GHRH		
3′ RACE	FishGHRH-F1	GACTCGAGCAYGCYGATGCCATCTTTAC
	FishGHRH-F2	GACTCGAGCTKGGTCAAATRTCWGCCAG
5′ RACE	FishGHRH-R1	GAAAGCTTTGTAGCTGTTKGTAAAGATG
	FishGHRH-R2	GAAAGCTTCAYTGWAGAAWYTTYCTGGC
Full length	gfGHRH-F4	CAGCTCGAGGAATGAAACCAAACCCTGAG
	gfGHRH-R3	CAGAAGCTTGTTCGCAGTTTATTGACT
Zebrafish GHRH		
3′ RACE	FishGHRH-F1	GACTCGAGCAYGCYGATGCCATCTTTAC
	FishGHRH-F2	GACTCGAGCTKGGTCAAATRTCWGCCAG
5′ RACE	FishGHRH-R1	GAAAGCTTTGTAGCTGTTKGTAAAGATG
	FishGHRH-R2	GAAAGCTTCAYTGWAGAAWYTTYCTGGC
Full length	zfGHRH-F4	CAGCTCGAGAACTGCCTTGCTAGTGCT
	zfGHRH-R3	CAGAAGCTTCAGCAAATGCAATAGTTCC
Xenopus GHRH		
PARTIAL	xGHRH-F1	ATGCGAAGAACACTTTGC
	xGHRH-F2	ATGTGGATGCCATATTCA
	xGHRH-R1	TCATAGCCGTGTTCCACG
3′ RACE	xGHRH-F3	TTCCTGGGGCAGATTTCAGC
	xGHRH-F2	ATGTGGATGCCATATTCA
5′ RACE	xGHRH-R2	TCTTCTGCCCTCCAATCTGG
	xGHRH-R3	GCTGAAATCTGCCCCAGGAA
Full length	xGHRH-FLF1	CCCCAAGCTTGGGAAGCACGCCATTGTAG
	xGHRH-FLR1	CGGAATTCCATTGCTGTCCATTTTGTTG
Goldfish GHRH-R		
3′ RACE	Fish-GHRH-R-mF3	CAGCTCGAGGARWWKMTSTTTCAGCTGG
	Fish GHRH-R-mF1	TTCGGCACTCATTACATG
5′ RACE	gf-GHRH-R-mR3	ATGATCCACCAATAAG
	gf-GHRH-R-mR5	GGCTTCAACCAGCAAC
5′ RACE	gf-GHRH-R-mR4	CAGCTCGAGATCCAGAGGACAATGC
	gf-GHRH-R-mR7	CTGTGTTGTTCTTGAAGAGA
Partial clone	Fish-GHRH-R-mF2	CAGCTCGAGCCGYCSTACGAGGAAGC
	gf-mGHRH-R-R2	CAGAAGCTTCCAACAGGGACGACAATG
	gf-mGHRH-R-F1	CAGCTCGAGGCCTCATTGTGGCAGTTC
	gf-mGHRH-R-F6	CAGAAGCTTAATACTGATGCTCTTC
Full length	gf-mR-Start-F	CAGCTCGAGTCGCGCGCGACCTG
	gf-mR-Stop-R	CAGAAGCTTAGAGGCACCGGTGAAT
Zebrafish GHRH-R		
3′ RACE	Fish-GHRH-R-mF3	CAGCTCGAGGARWWKMTSTTTCAGCTGG
	Fish-GHRH-R-mF1	TTCGGCACTCATTACATG
5′ RACE	Fish-GHRH-R-R2	CAGAAGCTTAAMCCCTGGAAMGAGCCCAG
	Fish-GHRH-R-mR1	CAGAAGCTTTCTGGGYCTGCCACMTCC
5′ RACE	zf-mGHRH-R-R4	CAGAAGCTTGTAACCGTATCTCATTC
	Fish-mGHRH-R-R5	CAGAAGCTTGCACTTTAGGGATACCAGAG
5′ RACE	Fish-mGHRH-R-R5	CAGAAGCTTGCACTTTAGGGATACCAGAG
	zf-mGHRH-R-220R	ACCCACCACAGCACTGG
Partial clone I	zf-mGHRH-R-R5	CAGAAGCTTGCACTTTAGGGATACCAGAG
	Fish-GHRH-R-F7	CAGCTCGAGACASWGRRTGCTGGGAC
Partial clone II	Fish-GHRH-R-R2	CAGAAGCTTAAMCCCTGGAAMGAGCCCAG
	Fish-GHRH-R-F8	CAGCTCGAGTTYGCCACKGTGAAGCTG
Full length	zf-mGHRH-R-F4	CAGCTCGAGTGTGGACTCGACTGG
	zf-mGHRH-R-R5	CAGAAGCTTGCACTTTAGGGATACCAGAG
Others		
5′ & 3′ RACE	AUAP	GGCCACGCGTCGACTAGTAC
3′ RACE	AP	GGCCACGCGTCGACTAGTAC$(T)_{17}$
5′ RACE	AnP	$(CUA)_4$GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG

Figure S13

PCR primers for real-time analysis of goldfish GHRH and GHRH-R

Amplification	Primer Name	Primer Sequence (5′ to 3′)
GHRH	gfGHRH-F5	ATCTTTACCAACAGCTAC
	gfGHRH-R2	TCATCTTGATTGAAGGTG
GHRH-R	gf-GHRH-R-Q1	TTCAACAATTCAGCTCAGTA
	gf-GHRH-R-Q1R	TAGTCTGGGAGGAAGTTGAA

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