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The orphan G protein-coupled receptor 25 (GPR25) is activated by Apelin and Apela in non-mammalian vertebrates

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ABSTRACT

G protein-coupled receptor 25 (GPR25) is an orphan G protein-coupled receptor in vertebrates, that has been implicated to be associated with autoimmune diseases and regulate blood pressure in humans. However, the endogenous ligand of GPR25 remains unknown in vertebrates. Here, we reported that in non-mammalian vertebrates (zebrafish, spotted gars, and pigeons), GPR25 could be activated by Apelin and Apela peptides, which are also the two endogenous ligands of vertebrate Apelin receptor (APLNR). Using the pGL3-CRE-luciferase reporter assay and confocal microscopy, we first demonstrated that like APLNR, zebrafish GPR25 expressing in HEK293 cells could be effectively activated by zebrafish Apelin and Apela peptides, leading to the inhibition of forskolin-stimulated cAMP production and receptor internalization. Like zebrafish GPR25, pigeon and spotted gar GPR25 could also be activated by Apelin and Apela, and their activation could inhibit forskolin-induced cAMP accumulation. Interestingly, unlike zebrafish (/spotted gar/pigeon) GPR25, human GPR25 could not be activated by Apelin and Apela under the same experimental conditions. RNA-seq analysis further revealed that *GPR25* is expressed in a variety of tissues, including the testes and intestine of zebrafish/spotted gars/humans, implying the potential roles of GPR25 signaling in many physiological processes in vertebrates. Taken together, our data not only provides the first proof that the orphan receptor GPR25 possesses two potential ligands 'Apelin and Apela' and its activation decreases intracellular cAMP levels in non-mammalian vertebrates, but also facilitates to unravel the physiological roles of GPR25 signaling in vertebrates.

1. Introduction

Up to date, nearly 87 class A G protein-coupled receptors (GPCRs) are still classified as orphan GPCRs and their ligands have not yet been identified [1]. The G protein–coupled receptor 25 (GPR25) is an example of orphan receptors in the class A GPCR family. GPR25 was first cloned in 1997 and mapped on human chromosome 1 [2]. GPR25 is expressed in human memory T-cells and NK-cells and identified as a primary causal gene associated with autoimmune diseases revealed by *cis*-eQTL mapping based on a genome-wide association study (GWAS) [3]. Moreover, GPR25 is reported to be associated with arterial stiffness, and presumably, it may be capable of binding an unknown ligand to regulate blood pressure (BP) [4].

Despite the progress on understanding the potential roles of GPR25, the identity of its endogenous ligand remains unknown. Within the class A GPCR family, GPR25 shares a relatively high degree of amino acid sequence identity (29–34%) with vertebrate Apelin receptor (APLNR) [5]. It is clear that APLNR is activated by two distinct peptides, named Apelin and Apela (also known as Elabela and Toddler), which are encoded by two separate genes (*APELIN* and *APELA*), in humans [6], rats [7] and zebrafish [8,9].

Apelin is a peptide of 36 or 13 amino acids (designated as Apelin-36 and Apelin-13 respectively) and identified as the first endogenous ligand of APLNR [10]. Both Apelin and APLNR are reported to be widely expressed in the central nervous system (CNS) and peripheral tissues and involved in the regulation of many physiological/pathological processes, including the cardiovascular functions, angiogenesis, water balance and stress-induced disorders in vertebrates [11–13]. Apela is an alternative endogenous ligand of APLNR which was identified in zebrafish and mammals

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just recently. Apela is a peptide of 36 (Apela-36) or 22 (Apela-22) amino acids. Despite the low degree of amino acid sequence identity (25%) shared between Apelin and Apela peptides [14], both peptides can equipotently activate APLNR, which is functionally coupled to Gi protein and its activation decreases intracellular cAMP levels. In zebrafish, Apela is reported to be essential for cell movement during gastrulation and heart development [8,9]. In mammals, Apela is highly expressed in the kidneys of humans [6] and rats [7] and plays important roles in angiogenesis [6], fluid homeostasis [7], placental development [15] and cardiovascular development [16].

The structural similarity shared between APLNR and GPR25 led us to hypothesize that like APLNR, GPR25 could be activated by Apelin and/or Apela in vertebrates. To test this hypothesis, in this study, we cloned GPR25 from several representative vertebrate species, including zebrafish, pigeons, spotted gars and humans, and tested whether this receptor could be activated by Apelin and Apela *in vitro*. Our data, for the first time, demonstrated that the orphan receptor GPR25 can be activated by Apelin and Apela in non-mammalian vertebrates, and its activation can decrease intracellular cAMP levels. Our findings will help to unravel the physiological roles of GPR25 signaling in vertebrates.

2. Materials and methods

2.1. Chemicals and peptides

Chicken Apelin-36, Apela-32 (without a disulfide bond) and zebrafish Apelin-36, Apela-36 and Apela-22 (without a disulfide bond) peptides were synthesized (>95% purity) by GL Biochem (Shanghai, China) and their structures were verified by mass spectrometry. Dual-luciferase reporter assay system was purchased from Promega (Madison, Wisconsin). Primers used in this study were listed in Supplementary Table 1. All other experimental reagents were purchased from Sigma (Sigma-Aldrich, China).

2.2. Animal experiments

Zebrafish, pigeons and spotted gars purchased from local suppliers were sacrificed and various tissues were collected. All these animal experiments were conducted in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China. The experimental protocol used in this study was approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University.

2.3. Cloning of zebrafish, spotted gar, pigeon and human GPR25 and construction of expression plasmids

Genomic DNA was extracted from human embryonic kidney 293 (HEK293) cells or from heart tissues of zebrafish, pigeon and spotted gar using a genome DNA extraction kit (Tiangen Biotech, China) following the manufacturer's instructions. According to the predicted (or reported) cDNA sequences of zebrafish (XM_021479994), pigeon (XP_005510493), spotted gar (XM_015342099), and human (NM_005298) *GPR25* deposited in the GenBank, gene-specific primers were designed to obtain the complete open reading frame (ORF) of GPR25 from these species. The ORF of GPR25 was amplified with the high-fidelity KOD DNA polymerase (Toyobo, Japan) using genomic DNA as the template (note: the coding regions of *GPR25* in these species are intronless). Then, PCR products were cloned into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA) and sequenced by Beijing Genome Institute (BGI).

Using the same approach, the expression plasmids encoding zebrafish APLNR (also named APLNRa, NM_001075105) and human APLNR (NM_005161) were also constructed in this study. For internalization assays, three pcDNA3.1 (+) expression plasmids encoding zebrafish GPR25-EGFP, human GPR25-EGFP and APLNR-EGFP were constructed by tagging EGFP to the C-terminus of GPR25 or APLNR.

2.4. Functional characterization of GPR25 in cultured HEK293 cells

According to the method established in our previous studies [17,18], the functionality of various cloned receptors was examined in HEK293 cells by a system of co-transfected pGL3-CRE-luciferase reporter construct and receptor expression plasmids. In brief, HEK293 cells transiently expressing GPR25/APLNR were treated with Apelin/Apela peptide for 6 h in the presence of forskolin (5 μM). Then, receptor-inhibited cAMP levels were monitored by a pGL3-CRE-Luciferase reporter assay [17,18].

2.5. Internalization assay by confocal microscopy

HEK293 cells cultured on six-well plates were transfected with 1 μg/well expression plasmid encoding zebrafish/human GPR25-EGFP (or human APLNR-EGFP). Then, cells were subcultured on poly-D-lysine coated glass coverslips in 24-well plates for 24 h. Cells were treated with serum-free DMEM medium or ligands (10 μM Apelin or Apela) for 30 min, and then fixed with 4% paraformaldehyde for 10 min. After fixation, cells were washed 3 times in cold PBS, and a subsequent 1-min incubation with 2 μg/ml DAPI in PBS was used to stain the nuclei. EGFP signals were examined by a Leica TCS SP5 II confocal microscope (Leica, Germany).

2.6. Statistical analysis

The luciferase activities in each treatment group were expressed as percentage compared with the control group. The data were analyzed by nonlinear regression followed by one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA). To validate the results, all these experiments were repeated at least 3 times.

3. Results

3.1. Cloning of GPR25 from zebrafish, pigeons and spotted gars

To identify the potential ligands for the orphan receptor GPR25 in vertebrates, we first cloned the coding region of *GPR25* from several representative vertebrate species, including zebrafish (MG732937), spotted gars (MG732939) and pigeons (MG732938) (Supplementary Figs. 1–3). The coding region of zebrafish, spotted gar and pigeon *GPR25* are predicted to encode a receptor of 377, 376 and 371 amino acids respectively, which are similar in length to human GPR25 (361 amino acids, NM_005298). Amino acid sequence alignment revealed that zebrafish GPR25 shows high amino acid identity with that of spotted gars (52%), pigeons (38%), coelacanth (45%) and humans (39%), and the highest identity was noted in the seven transmembrane domains (TM1-7). In addition, the conserved D/ERY motif known for G protein-coupling and two cysteine residues for a disulfide bond formation are also present in GPR25 of all vertebrate species examined (Fig. 1).

Interestingly, we also found that vertebrate GPR25 shares some degree of amino acid sequence identity with zebrafish/human APLNR (34%/29%) (Fig. 1).

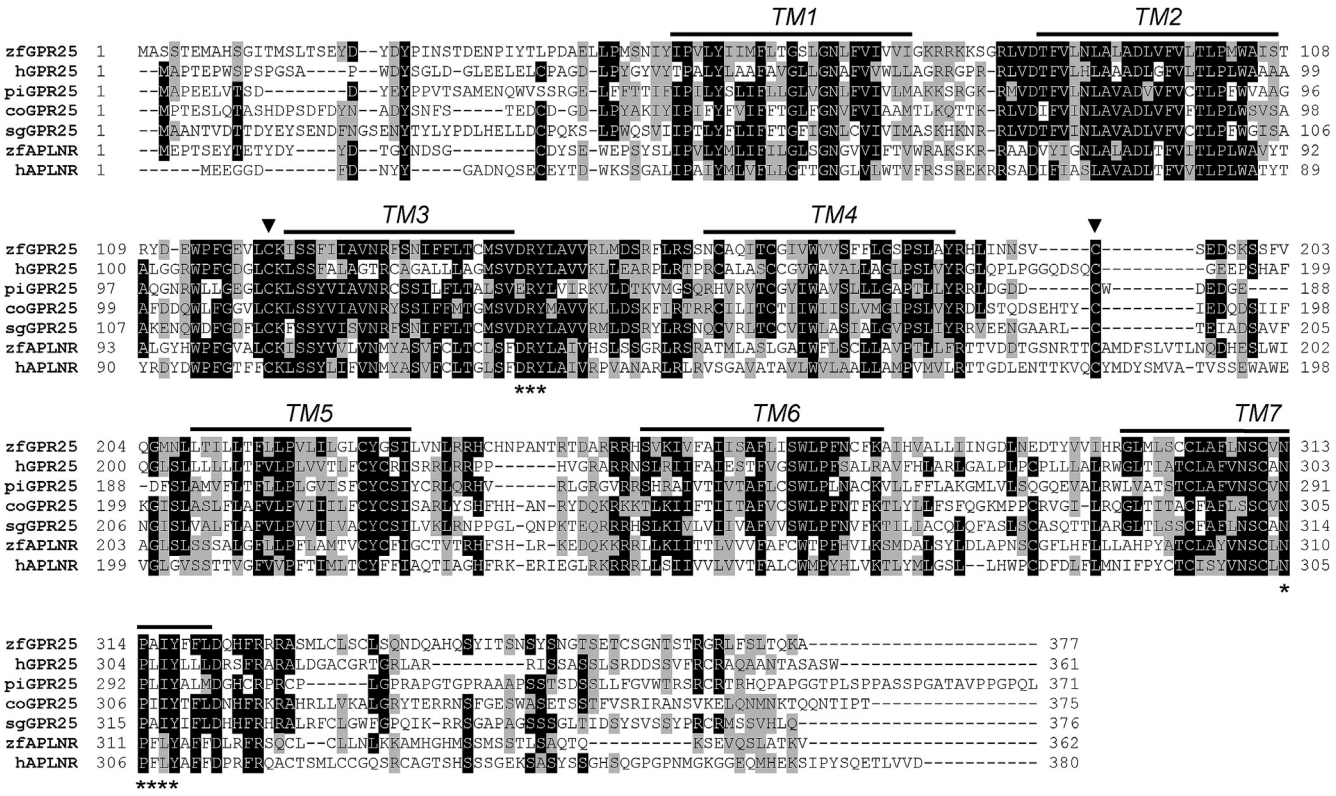

Fig. 1. Alignment of zebrafish GPR25 (zfGPR25) with that of human GPR25 (hGPR25), pigeon GPR25 (piGPR25), coelacanth GPR25 (coGPR25, XP_005988535), spotted gar GPR25 (sgGPR25), zebrafish APLNR (zfAPLNR, NM_001075105) and human APLNR (hAPLNR, NM_005161). Identical residues are shaded in black, and similar residues are shaded in gray. Asterisks indicate the 'D/ERY motif' and 'NPxxY motif'. The two cysteine residues used for disulfide bond formation are marked by arrowheads. Horizontal lines mark the seven transmembrane domains (TM1-7).

3.2. Functional analysis of GPR25 in zebrafish, pigeons, spotted gars and humans

To test whether like APLNR, GPR25 could be activated by Apelin and Apela peptides, zebrafish, spotted gar, pigeon and human GPR25 were transiently expressed in HEK293 cells and treated by zebrafish or chicken Apelin and Apela peptides (Fig. 2A). The receptor-inhibited cAMP levels were then monitored by a pGL3-CRE-luciferase reporter system established in our laboratory [17].

As shown in Fig. 2B, zebrafish Apelin-36, Apela-36 and Apela-22 could inhibit forskolin (5 μM)-induced luciferase activity of HEK293 cells expressing zebrafish GPR25 dose-dependently, and their EC_{50} values are: 0.39 ± 0.14 μM, 0.32 ± 0.09 μM and 0.52 ± 0.16 μM, respectively. Likewise, zebrafish GPR25 could also be activated by chicken Apelin-36 (EC_{50}: 0.66 ± 0.16 μM) and Apela-32 (EC_{50}: 0.74 ± 0.17 μM) (Fig. 2C). As a positive control, zebrafish APLNR could be potently activated by zebrafish Apelin-36 (EC_{50}: 1.14 ± 0.78 nM), Apela-36 (EC_{50}: 1.41 ± 0.36 nM) and Apela-22 (EC_{50}: 1.87 ± 0.68 nM) (Fig. 2D) [8,9]. All these findings clearly demonstrated that like APLNR, GPR25 is a functional receptor coupled to Gi-cAMP signaling pathway and can be activated by Apelin and Apela in zebrafish, despite a relatively high concentration of each peptide is required for its activation.

Similarly, we also demonstrated that both chicken (and/or zebrafish) Apelin and Apela could activate pigeon or spotted gar GPR25 expressed in HEK293 cells (Fig. 3A-D), with Apelin being slightly more potent than Apela. Strikingly, human GPR25 expressed in HEK293 cells showed no response to chicken Apelin/Apela treatment (Fig. 3E), though both peptides could potently activate human APLNR under the same condition (EC_{50} of Apelin-36: 0.64 ± 0.07 nM; Apela-32: 0.64 nM ± 0.07) (Fig. 3F). These findings further support the hypothesis that like APLNR, GPR25 can be activated by Apelin and Apela in non-mammalian vertebrates.

3.3. GPR25 internalization induced by Apelin and Apela

To investigate whether like APLNR, GPR25 could be induced to internalize following agonist treatment, HEK293 cells expressing zebrafish GPR25-EGFP were treated by 10 μM of zebrafish Apelin-36 or Apela-36 for 30 min, and the receptor internalization was examined by confocal microscopy. As shown in Fig. 4A, the strong signal of zebrafish GPR25-EGFP was mainly localized at the cell membrane in the absence of peptide. However, when treated by Apelin-36/Apela-36, GPR25-EGFP signal was rapidly internalized to form large and hollow intracellular vesicles, as shown in Fig. 4B and C. As a positive control, human APLNR could also internalize after Apelin and Apela treatment and formed the large and hollow intracellular vesicles in the cytoplasm (Fig. 4H and I). These findings further indicate that like APLNR, zebrafish GPR25 could be activated by both Apelin and Apela, thus resulting in agonist-induced receptor internalization (Fig. 4).

Interestingly, unlike zebrafish GPR25-EGFP or human APLNR-EGFP, human GPR25-EGFP appears unable to internalize with peptide treatment (Fig. 4), although its signal could be observed at the cell membrane and cytoplasm (surrounding nuclei) in the absence of peptide (Fig. 4D). This finding suggests that human GPR25 may not be activated by Apelin/Apela *in vitro*, as shown in our functional assays (Fig. 3E).

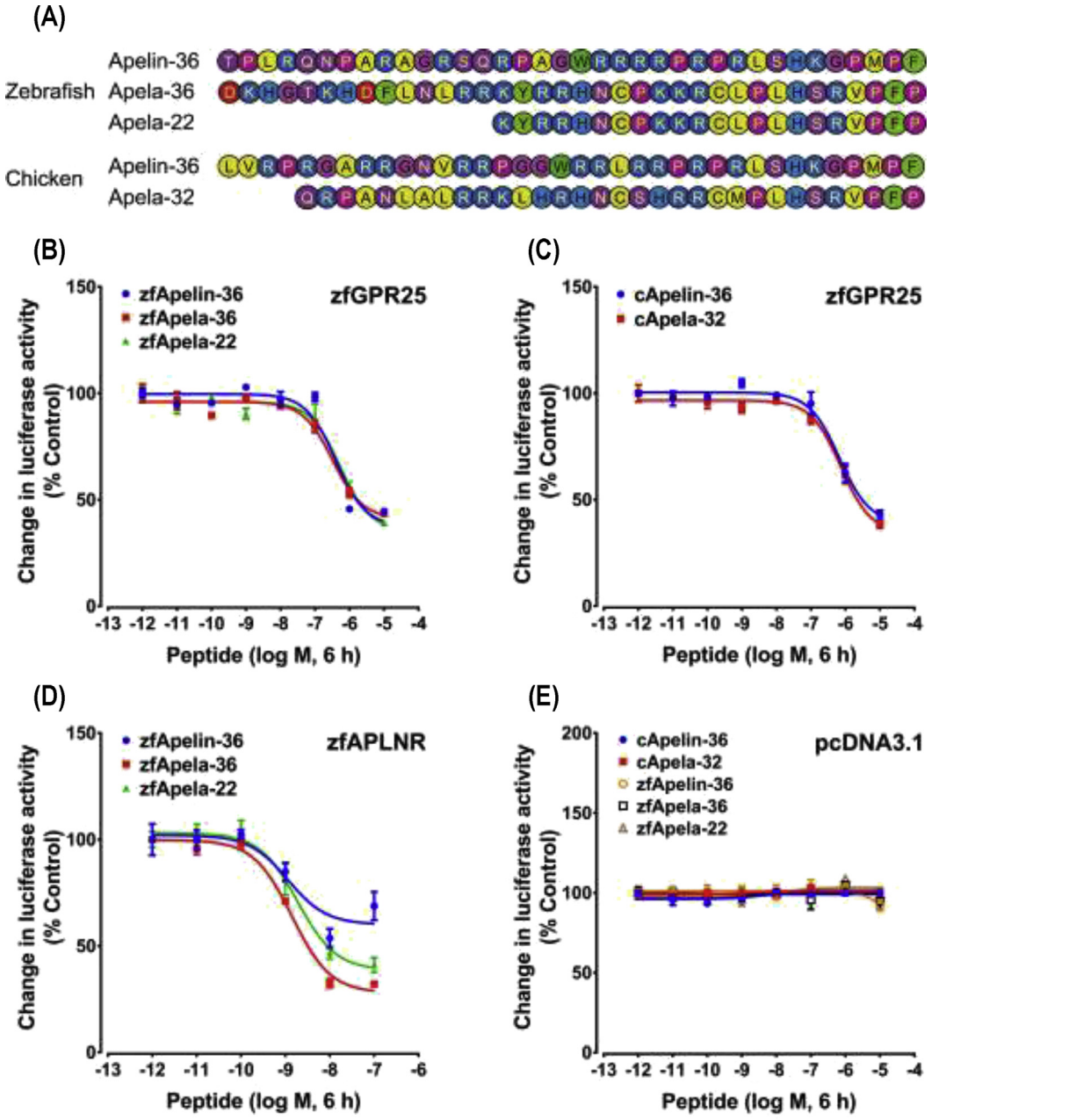

Fig. 2. Functional analysis of zebrafish GPR25. **(A)** Amino acid sequences of zebrafish (zfApelin-36, zfApela-36 and zfApela-22) and chicken (cApelin-36, cApela-32) peptides used (note: chicken Apelin/Apela peptides were deduced from their cDNA sequences, KX017222/KX017223). (**B, C**) Effects of zebrafish Apelin-36/Apela-36/Apela-22 (**B**) or chicken Apelin-36/Apela-32 (**C**) on forskolin (5 μM)-stimulated luciferase activity in HEK293 cells expressing zebrafish GPR25 (zfGPR25), monitored by a pGL3-CRE-luciferase reporter system. (**D**) Effects of zebrafish Apelin-36/Apela-36/Apela-22 on forskolin-stimulated luciferase activity in HEK293 cells expressing zebrafish APLNR (zfAPLNR). (**E**) No effect on forskolin-stimulated luciferase activity was noted when HEK293 cells transfected with an empty pcDNA3.1 (+) vector were treated with Apelin/Apela peptides. Each data point represents mean ± SEM of three replicates ($N = 3$).

4. Discussion

In this study, the orphan receptor *GPR25* was cloned from several representative vertebrate species including zebrafish. Although the overall amino acid sequence identity among these species is not high (38–52%), our synteny analysis clearly indicates that the cloned zebrafish, spotted gar and pigeon *GPR25* are orthologous to human *GPR25* (Supplementary Fig. 4). In addition, we noted that the seven transmembrane domains of vertebrate GPR25 share higher sequence identities with those of human GPR25, ranging from 48% to 53%. Like other members of class A GPCR, the conserved D/ERY motif for G protein-coupling and NPxxY motif are also observed in these cloned GPR25. All these conserved structural features imply that GPR25 may be a functional receptor in vertebrates. Moreover, we noted that vertebrate GPR25 shows a relatively high degree of amino acid sequence identity with that of zebrafish APLNR (34%) and human APLNR (29%), particularly in their transmembrane domains. This finding implies that GPR25 may have a close evolutionary relationship with APLNR. This speculation was supported by the phylogenetic analysis shown in Supplementary Fig. 5, in which vertebrate GPR25 shows a much closer evolutionary relationship to vertebrate APLNR, than to other

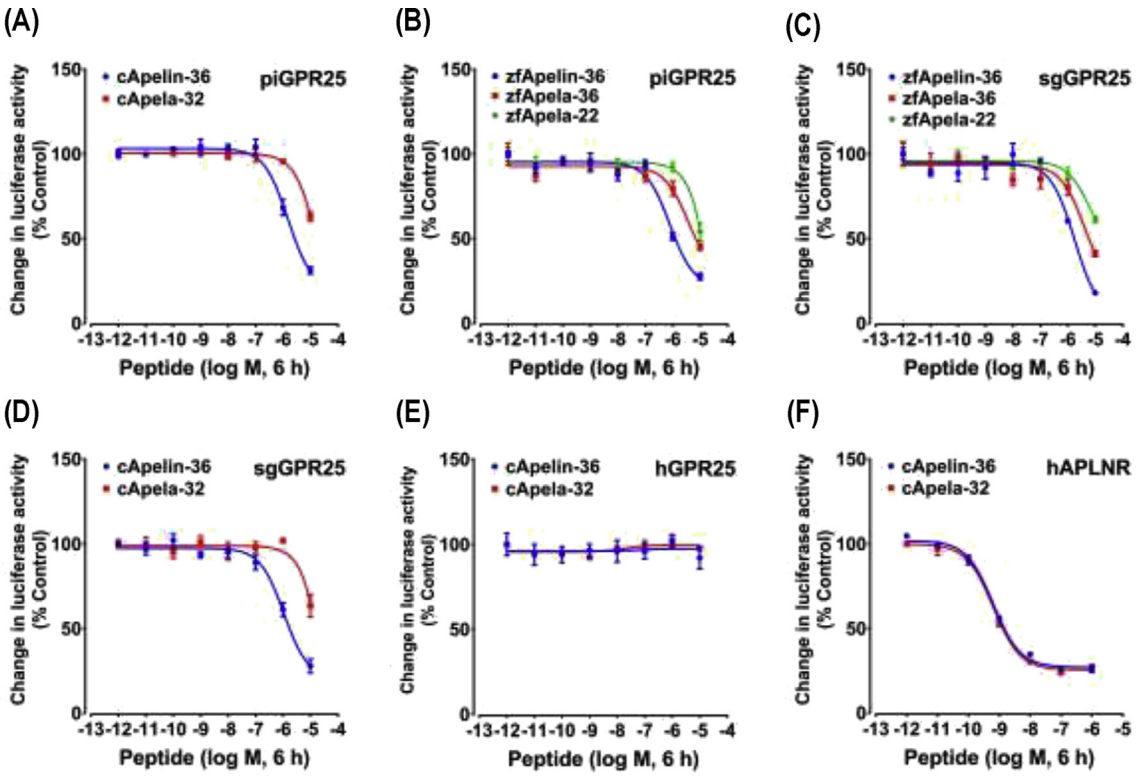

Fig. 3. Functional analysis of GPR25 in pigeons, spotted gars and humans. (**A, B**) Effects of chicken Apelin/Apela (**A**) or zebrafish Apelin/Apela (**B**) on forskolin (5 μM)-stimulated luciferase activity of HEK293 cells expressing pigeon GPR25 (piGPR25). (**C, D**) Effects of zebrafish Apelin/Apela (**C**) or chicken Apelin/Apela (**D**) on forskolin-stimulated luciferase activity of HEK293 cells expressing spotted gar GPR25 (sgGPR25). (**E**) Effects of chicken Apelin/Apela on forskolin-stimulated luciferase activity of HEK293 cells expressing human GPR25 (hGPR25). (**F**) Effects of chicken Apelin/Apela on forskolin-stimulated luciferase activity of HEK293 cells expressing human APLNR (hAPLNR). Each data point represents mean ± SEM of three replicates ($N = 3$).

members of class A GPCR family, such as angiotensin II receptor type 1/2 (AGTR1/2).

The structural similarity, together with the close phylogenetic relationship, between GPR25 and APLNR led us to hypothesize that GPR25 and APLNR may share functional similarity in vertebrates, and GPR25 can also be activated by the two known endogenous ligands of APLNR: Apelin and Apela. In agreement with this hypothesis, we proved that like zebrafish/human APLNR, zebrafish GPR25 could be activated by both Apelin and Apela, and receptor activation could inhibit forskolin-stimulated cAMP production, monitored by a pGL3-CRE-luciferase reporter system [17,18]. Our finding, for the first time, indicates that zebrafish GPR25 possesses two potential ligands 'Apelin and Apela' and is functionally coupled to Gi protein(s), whose activation inhibits adenylate cyclase (AC) activity, similar to APLNR. Like zebrafish GPR25, pigeon and spotted gar GPR25 could also be activated by Apelin and Apela (Fig. 3A-D). Strikingly, we found that chicken Apelin-36 and Apela-32 seem unable to activate human GPR25 (Fig. 3E), though both peptides could activate human APLNR potently (Fig. 3F). Since confocal microscopy indicates human GPR25 could localize to the cell membrane (Fig. 4D), the inability of Apelin/Apela in activating human GPR25 is likely due to their extremely low (or no) binding affinity to GPR25 *in vitro*. This is partially congruent with the finding in a recent study, in which the recombinant alkaline phosphatase-conjugated Apela cannot bind to rat GPR25 *in vitro* [7]. Taken together, our data suggest that like APLNR, GPR25 could be activated by Apelin and Apela in vertebrates, or, at the very least, in non-mammalian vertebrates.

Ligand-induced receptor endocytosis is a common feature of many GPCRs in response to ligand stimulation [19]. In this study, we found that zebrafish GPR25 is localized at the cell surface. Nevertheless, human GPR25 and APLNR could also show some degree of intracellular retention (Fig. 4), as suggested in previous studies reporting intracellular retention of human APLNR expressed in HEK293 cells [6,20]. Like human APLNR (Fig. 4) [6,20], zebrafish GPR25 is rapidly internalized and forms the large, hollow vesicles upon Apelin and Apela stimulation (Fig. 4), whereas human GPR25 seems unable to undergo internalization. This finding further supports the notion that both Apelin and Apela could activate GPR25 in non-mammalian vertebrates.

The activation of GPR25 by Apelin and Apela in non-mammalian vertebrates, and not in mammals, suggests that mammalian GPR25 may have undergone a dramatic structural change after the divergence of birds and mammals, thus leading to its functional difference between non-mammalian and mammalian species (Figs. 2–4). Similar to the findings in the present study, another orphan receptor Bombesin receptor subtype-3 (BRS3) was demonstrated to be potently activated by gastrin-releasing peptide (GRP) and neuromedin B (NMB) in non-mammalian vertebrates (chickens and spotted gars), but not in mammals (mice) in our recent study [21]. These studies from our laboratory may provide an alternative key approach to de-orphanize other orphan GPCRs from an evolutionary perspective in future. In this study, we also noted that activation of GPR25 in zebrafish, spotted gars and pigeons requires high concentrations of Apelin or Apela (≥100 nM), therefore, we still cannot exclude the possibility that other potential 'high-affinity' ligand(s) for GPR25 may exist in vertebrates.

The functional similarity between GPR25 and APLNR, ie both can be activated by Apelin and Apela and are coupled to Gi-cAMP signaling pathway (Figs. 2–4), strongly suggests that GPR25

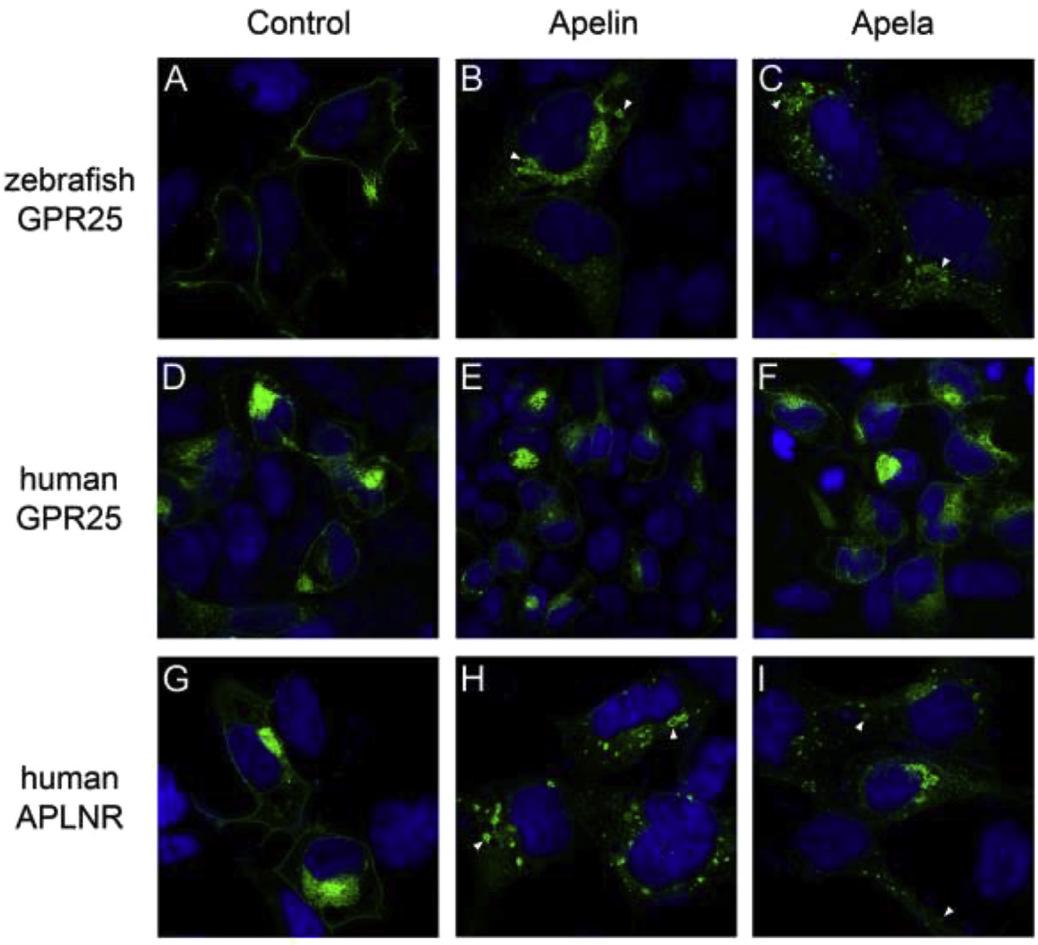

Fig. 4. Confocal microscopy showing the internalization of GPR25/APLNR. (**A-C**) Zebrafish Apelin-36 (**B**) and Apela-36 (**C**) could induce the internalization of zebrafish GPR25-EGFP expressed in HEK293 cells, while in the absence of ligand (**A**: control), GPR25-EGFP was mainly localized at cell membrane. (**D-F**) Chicken Apelin (**E**)/Apela (**F**) seems unable to induce the internalization of human GPR25-EGFP. (**G-I**) Human APLNR expressed at cell membrane (**G**: control) was rapidly internalized when treated by chicken Apelin-36 (**H**) or Apela-32 (**I**). Arrowheads mark the internalized GPR25/APLNR signals in the large, hollow vesicles.

signaling may play important physiological and pathological roles in vertebrates more or less similar to those of APLNR signaling, such as regulation of blood pressure [4,11]. Despite the failure of Apelin and Apela in activating human GPR25 in *vitro*, the activation of GPR25 by Apelin or Apela *in vivo* should not be ruled out, as this may be possible in the presence of some unknown accessory proteins *in vivo*. In addition, there has been increasing evidence showing that many GPCRs possess constitutive activity in the absence of their endogenous ligands, such as melanocortin receptor 4 (MC4R) in the absence of α-melanocyte-stimulating hormone (α-MSH) [22]. Therefore, there is also a possibility that GPR25 may possess constitutive activity to regulate physiological/pathological processes, such as blood pressure and arterial stiffness in humans [4]. It is reported that APLNR is capable of forming hetero-dimers with other GPCRs, such as kappa opioid receptor [23], thus, we should not exclude the possibility that GPR25 may hetero-dimerize with other GPCR(s) (*e.g.* APLNR) to exert its functions. Our future studies on these issues will help to define the functionality of vertebrate GPR25 more precisely.

To understand the physiological roles of GPR25 in vertebrates, RNA-Seq data was used to analyze the distribution of *GPR25* in zebrafish, spotted gars and humans (Supplementary Fig. 6). *GPR25* mRNA was detected to be widely expressed in most tissues examined, including the brain, heart, gills, kidneys, bones, intestines and testes of zebrafish and spotted gars. Although zebrafish *APLNR* was previously reported to be expressed in abundance during embryonic development [8,9], *GPR25* mRNA was hardly detected in zebrafish embryos. It implies that unlike APLNR, GPR25 signaling may not play an active role in zebrafish embryogenesis [8]. Like zebrafish and spotted gar *GPR25,* human *GPR25* mRNA is also expressed in a variety of tissues, including the testes and intestines [24]. The wide tissue distribution of *GPR25* across vertebrate species hints that GPR25 may play a wide range of roles in vertebrates, which is worthy of further investigation.

In summary, this study represents the first to report that the orphan receptor GPR25 can be activated by Apelin and Apela in non-mammalian vertebrates, and its activation can decrease intracellular cAMP levels. Our data suggests that GPR25 possesses two potential ligands 'Apelin and Apela' and is coupled to Gi-cAMP signaling pathway. These findings imply that GPR25 signaling may act in a way more or less similar to APLNR signaling in vertebrates. Although many questions regarding GPR25 have yet been answered, our study undeniably sets up a critical starting point to explore the physiological roles of GPR25 signaling in vertebrates, including zebrafish and humans.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.04.229.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.04.229.

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Supplementary Materials

Title:

The orphan G protein-coupled receptor 25 (GPR25) is activated by Apelin and Apela in non-mammalian vertebrates

Authors:

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```
   1 ATGGCAAGCAGCACAGAGATGGCACACTCTGGAATTACAATGAGCTTGACAAGTGAATATGACTATGACTATCCAATTAATTCCACGGAC  90
   1  M  A  S  S  T  E  M  A  H  S  G  I  T  M  S  L  T  S  E  Y  D  Y  D  Y  P  I  N  S  T  D  30

  91 GAGAATCCAATCTACACCCTTCCCGACGCTGAACTGCTGCCCATGTCTAACATCTACATTCCTGTGCTGTACATCATTATGTTCCTCACC 180
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 181 GGCTCTTTGGGAAACCTGTTTGTGATTGTGGTCATTGGAAAGCGGCGCAAGAAAAGTGGACGCCTGGTAGACACCTTCGTGCTGAACCTG 270
  61  G  S  L  G  N  L  F  V  I  V  V  I  G  K  R  R  K  K  S  G  R  L  V  D  T  F  V  L  N  L  90

 271 GCACTAGCCGACCTCGTGTTTGTTCTCACGCTGCCAATGTGGGCAATTTCGACCCGTTATGACGAGTGGCCCTTTGGTGAGGTGTTATGC 360
  91  A  L  A  D  L  V  F  V  L  T  L  P  M  W  A  I  S  T  R  Y  D  E  W  P  F  G  E  V  L  C  120

 361 AAGATTAGCAGCTTTATCATTGCGGTTAACCGCTTTTCCAACATCTTCTTCCTCACCTGCATGAGCGTGGACCGCTACCTGGCAGTGGTG 450
 121  K  I  S  S  F  I  I  A  V  N  R  F  S  N  I  F  F  L  T  C  M  S  V  D  R  Y  L  A  V  V  150

 451 CGCCTAATGGACTCTCGGTTTCTCCGCAGCAGCAATTGTGCACAAATTACCTGTGGCATCGTGTGGGTGGTTTCCTTCTTTCTAGGATCT 540
 151  R  L  M  D  S  R  F  L  R  S  S  N  C  A  Q  I  T  C  G  I  V  W  V  V  S  F  F  L  G  S  180

 541 CCATCTCTGGCGTACCGTCACTTGATCAACAATTCGGTGTGCTCTGAAGATTCCAAATCCTCTTTTGTTCAAGGAATGAATCTTCTGACT 630
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 631 ATATTGCTAACCTTCTTGCTCCCGGTACTCATTCTAGGCCTCTGTTATGGGTCGATTTTGGTCAACCTGCGACGTCACTGTCACAATCCT 720
 211  I  L  L  T  F  L  L  P  V  L  I  L  G  L  C  Y  G  S  I  L  V  N  L  R  R  H  C  H  N  P  240

 721 GCCAACACACGCACTGATGCCAGACGAAGGCATTCGGTTAAGATTGTGTTTGCTATCATTTCAGCCTTTCTGATCTCCTGGCTTCCATTC 810
 241  A  N  T  R  T  D  A  R  R  R  H  S  V  K  I  V  F  A  I  I  S  A  F  L  I  S  W  L  P  F  270

 811 AACTGCTTCAAGGCCATCCATGTTGCGTTACTGATCATCAATGGAGATCTTAATGAGGACACCTATGTTGTCATACATAGAGGACTCATG 900
 271  N  C  F  K  A  I  H  V  A  L  L  I  I  N  G  D  L  N  E  D  T  Y  V  V  I  H  R  G  L  M  300

 901 CTCTCCTGCTGTCTGGCATTTCTCAACAGCTGCGTCAATCCGGCTATCTACTTCTTTCTCGATCAACATTTCAGACGCAGAGCTTCCATG 990
 301  L  S  C  C  L  A  F  L  N  S  C  V  N  P  A  I  Y  F  F  L  D  Q  H  F  R  R  R  A  S  M  330

 991 TTGTGTCTGAGCTGTCTGAGTCAAAATGACCAAGCACACCAGAGTTACATCACCTCGAATTCTTACTCCAACGGCACATCTGAGACCTGC 1080
 331  L  C  L  S  C  L  S  Q  N  D  Q  A  H  Q  S  Y  I  T  S  N  S  Y  S  N  G  T  S  E  T  C  360

1081 TCTGGAAATACATCAACCCGCGGGCGGCTTTTCTCACTTACTCAAAAGGCATGA 1134
 361  S  G  N  T  S  T  R  G  R  L  F  S  L  T  Q  K  A  *  378
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Supplementary Fig. 1 The nucleotide and deduced amino acid sequence of zebrafish *GPR25* (accession no. MG732937).

```
   1 ATGGCGGCGAATACCGTGGACACGACGGACTACGAGTACTCTGAAAATGACTTCAACGGAAGCGAGAACTACACATACCTGTATCCTGAC  90
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  91 TTGCATGAGTTATTGGATTGTCCTCAGAAATCCTTGCCCTGGCAGTCCGTCATTATACCTACCCTCTACTTTCTCATCTTCTTCACTGGC 180
  31  L  H  E  L  L  D  C  P  Q  K  S  L  P  W  Q  S  V  I  I  P  T  L  Y  F  L  I  F  F  T  G  60

 181 TTCATTGGGAATCTGTGCGTCATCGTCATCATGGCCTCCAAGCACAAGAACAGGAGGCTGGTGGACACCTTTGTCATTAACCTGGCCGTG 270
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 271 GCGGACTTGGTCTTCGTGTGCACTTTACCGTTCTGGGGGGATTTCGGCGGCGAAGGAAAACCAATGGGACTTCGGCGATTTCCTCTGCAAG 360
  91  A  D  L  V  F  V  C  T  L  P  F  W  G  I  S  A  A  K  E  N  Q  W  D  F  G  D  F  L  C  K  120

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 451 ATGCTGGACTCCAGATACCTTCGCAGCAACCAATGCGTACGCTTGACCTGTTGCGTCATCTGGTTGGCGTCCATCGCGCTAGGAGTGCCC 540
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 541 TCTCTTATCTACCGGAGGGTGGAGGAAAATGGCGCGGCCCGTTTGTGCACCGAAATCGCCGACTCAGCTGTTTTCAACGGGATCAGCCTT 630
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 901 ACCCTGTCCTCTTGCTTTGCCTTCCTAAACAGCTGCGCTAACCCCGCCATTTACATCTTCCTGGACCACCACTTCAGGCATCGCGCTCTG 990
 301  T  L  S  S  C  F  A  F  L  N  S  C  A  N  P  A  I  Y  I  F  L  D  H  H  F  R  H  R  A  L  330

 991 CGCTTCTGTCTCGGCTGGTTCGGCCCACAGATCAAGCGGCGGAGTGGAGCTCCAGCCGGGTCCTCTTCCGGGCTCACCATTGACAGTTAC 1080
 331  R  F  C  L  G  W  F  G  P  Q  I  K  R  R  S  G  A  P  A  G  S  S  S  G  L  T  I  D  S  Y  360

1081 TCGGTGTCCAGTTACCCTCGCTGCCGCATGTCTTCAGTTCACTTGCAGTGA 1131
 361  S  V  S  S  Y  P  R  C  R  M  S  S  V  H  L  Q  *  377
```

Supplementary Fig. 2 The nucleotide and deduced amino acid sequence of spotted gar *GPR25* (accession no. MG732939).

```
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 181 ATGGCCAAGAAGAGCAGGGGCAAGAGGATGGTGGACACGTTCGTGCTGAACCTGGCGGTGGCCGATGTCGTGTTTGTCTGCACGTTGCCC 270
  61  M  A  K  K  S  R  G  K  R  M  V  D  T  F  V  L  N  L  A  V  A  D  V  V  F  V  C  T  L  P  90

 271 TTCTGGGTGGCAGCGGGGGCGCAAGGGAACCGCTGGCTGCTCGGTGAAGGGCTCTGCAAGCTCAGCAGCTACGTCATCGCCGTCAACCGC 360
  91  F  W  V  A  A  G  A  Q  G  N  R  W  L  L  G  E  G  L  C  K  L  S  S  Y  V  I  A  V  N  R  120

 361 TGCTCCAGCATCCTCTTCCTCACCGCCCTCAGCGTGGAGCGCTACCTGGTCATCAGGAAGGTGCTGGACACCAAGGTGATGGGTTCCCAG 450
 121  C  S  S  I  L  F  L  T  A  L  S  V  E  R  Y  L  V  I  R  K  V  L  D  T  K  V  M  G  S  Q  150

 451 AGACACGTGCGCGTCACCTGCGGCGTCATCTGGGCCGTGTCCCTCCTCCTGGGCGCCCCCACCCTGCTGTACCGGCGGCTGGACGGGGAC 540
 151  R  H  V  R  V  T  C  G  V  I  W  A  V  S  L  L  L  G  A  P  T  L  L  Y  R  R  L  D  G  D  180

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 631 TGCTCCATCTACTGCCGGCTCCAGCGCCACGTCCGCCTGGGCAGGGGTGTCCGACGGTCCCACCGCGCCATCGTCACCATCGTCACCGCC 720
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 721 TTCCTCTGCTCCTGGCTGCCCCTGAACGCCTGCAAGGTGCTGCTCTTCTTCCTCGCCAAGGGGATGCTGGTCCTGTCTCAGGGCCAGGAG 810
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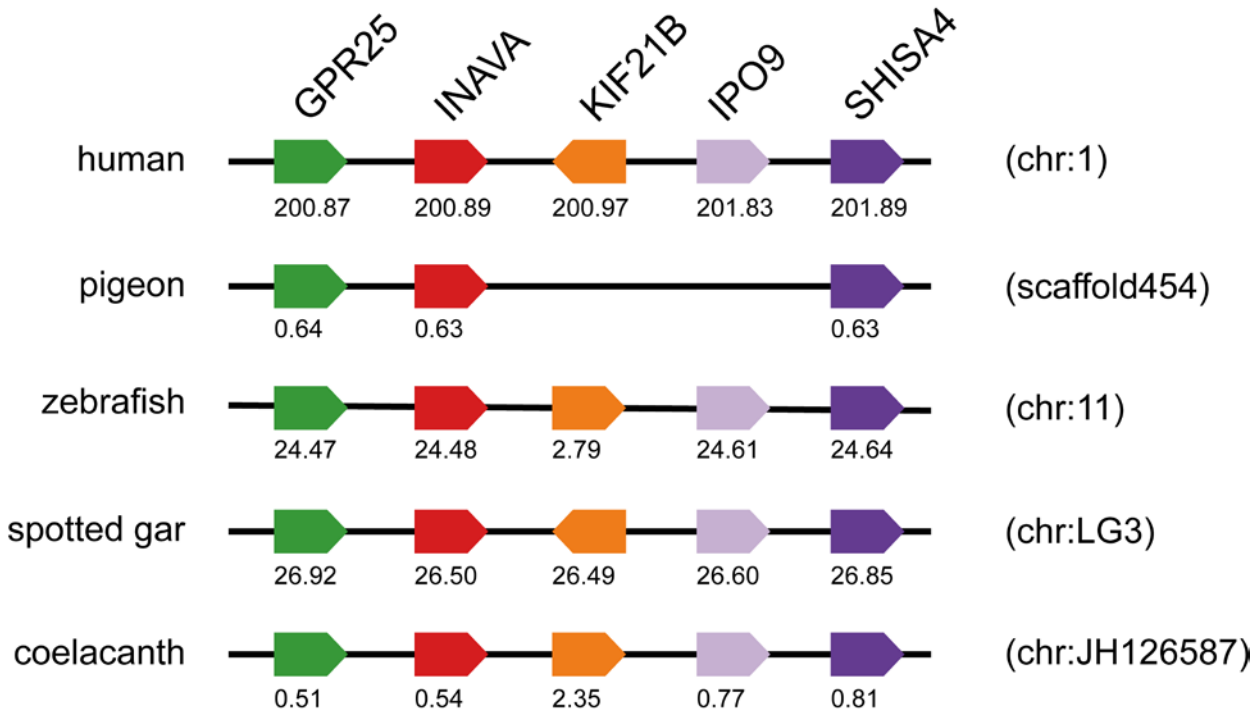
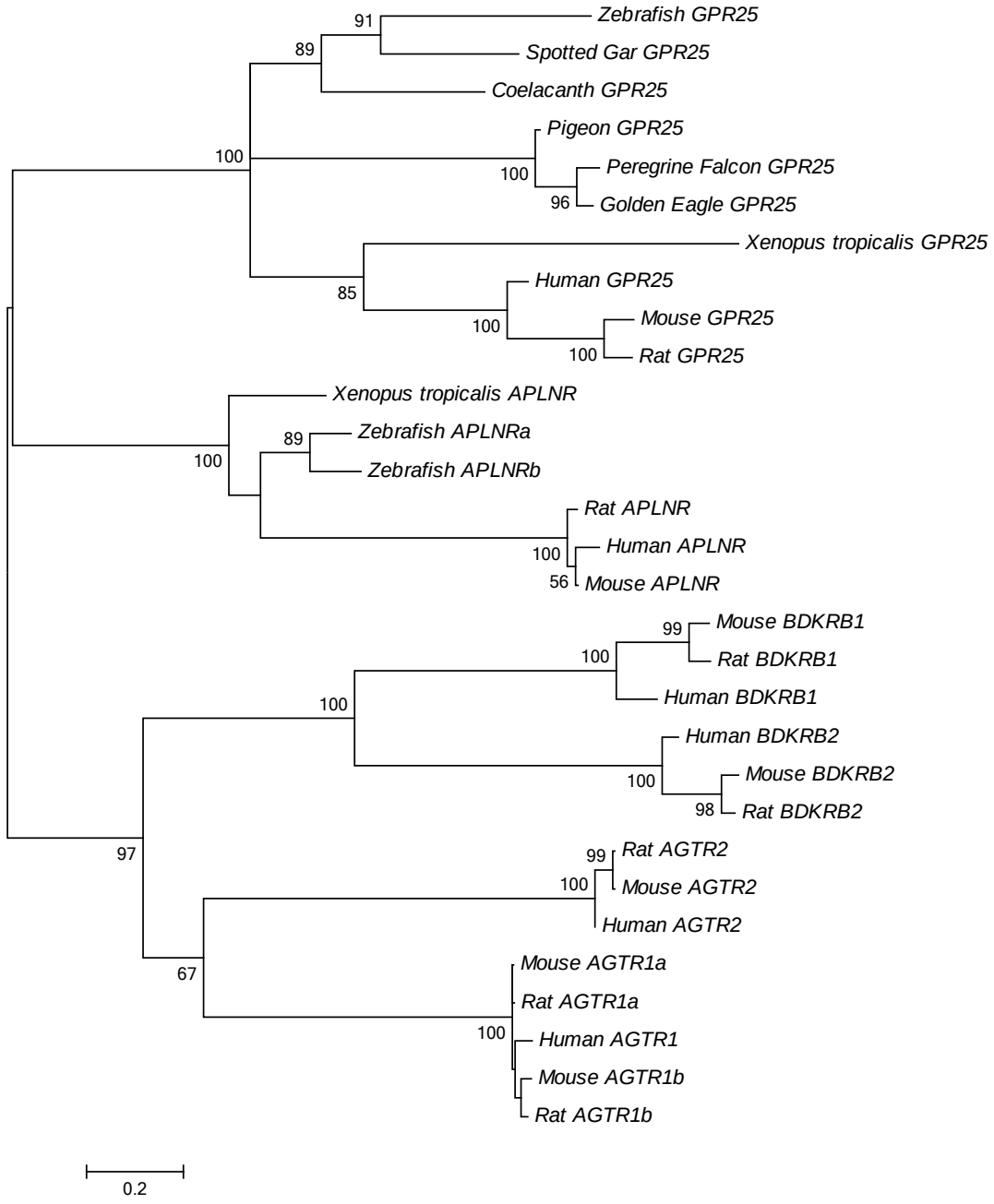
 991 CTCTTCGGTGTCTGGACCCGCTCCCGGTGCAGGACCCGGCACCAGCCGGCACCGGGGGGCACCCCGCTGAGTCCCCCCGCCTCGTCCCCA 1080
 331  L  F  G  V  W  T  R  S  R  C  R  T  R  H  Q  P  A  P  G  G  T  P  L  S  P  P  A  S  S  P  360

1081 GGTGCCACCGCTGTCCCCCCTGGTCCCCAGCTGTGA 1116
 361  G  A  T  A  V  P  P  G  P  Q  L  *  372
```

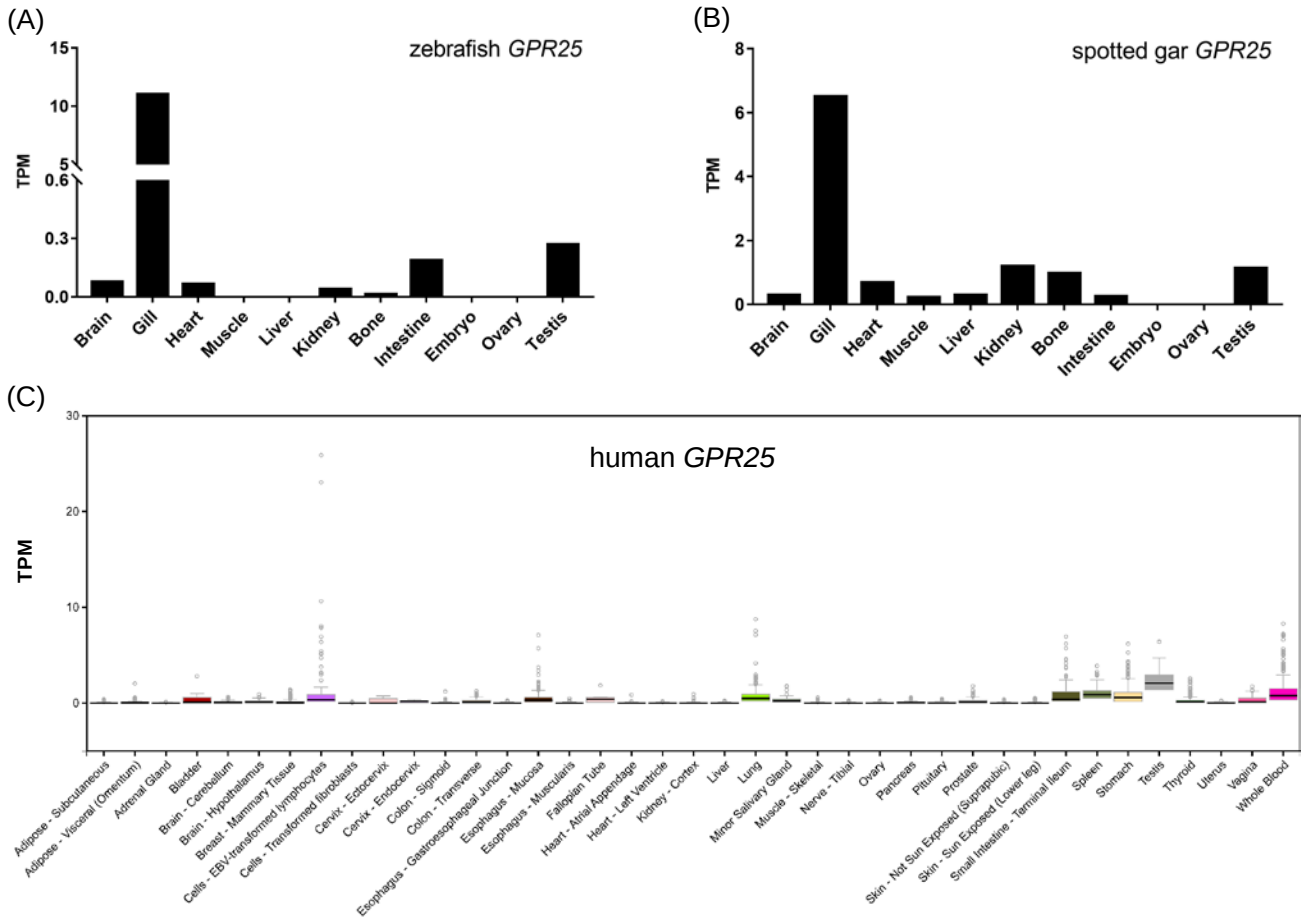
Supplementary Fig. 3 The nucleotide and deduced amino acid sequence of pigeon *GPR25* (accession no. MG732938).



Supplementary Fig. 4. Synteny analysis of *GPR25* in vertebrates. The orphan receptor GPR25 is localized in a syntenic region conserved in all vertebrate species examined including humans, pigeons, zebrafish, spotter gars and coelacanths. The orthologous gene was marked in the matching colors. The number below each gene indicates the gene location (Megabyte, Mb) in corresponding chromosome (chr) or scaffold shown in brackets on the right.



Supplementary Fig. 5 Phylogenetic analysis of GPR25 among vertebrates**.** Phylogenetic analysis was performed using the amino acid sequences containing GPR25, APLNR, AGTR1, AGTR2, BDKRB1 and BDKRB2 from vertebrate species, including human and other vertebrate species indicated. Phylogenetic tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model in MEGA7 [1]. Numbers near branch points indicate the bootstrap values.



Supplementary Fig. 6 Expression of *GPR25* in zebrafish, spotted gars and humans. **(A, B)** The tissue distribution of *GPR25* mRNA transcript was examined in zebrafish (**A**) and spotted gars (**B**) using RNA-Seq data (Accessions: SRP044781 and SRP044782) [2], including 11 different tissues: brain, gill, heart, muscle, liver, kidneys, bone, intestines, embryos, ovaries and testes. The quantification of reads was performed with Salmon v0.8.2 [3] against the Ensembl database. (**C**) The tissue distribution of human *GPR25* mRNA transcripts was examined by analyzing the RNA-Seq data obtained from the GTEx Consortium (https://www.gtexportal.org/home/gene/GPR25) [4]. The transcripts per million (TPM) values were used to estimate the abundance of *GPR25* mRNA transcript.

Supplementary Table 1 Primers used in this study [a]

Gene/construct name	Sense/ antisense	Primer sequence (5'-3')	Size (bp)
[b]*Primers for construction of the expression plasmids*			
Zebrafish *gpr25*	Sense	CGGGGTACCGCACAGATGGCAAGCAGCAC	1187
	Antisense	CGGGGTACCAGAAGCGAAAGTTGGCAGTC	
Human *GPR25*	Sense	CGGGGTACCAGGCCATGGCCCCCACAGAG	1321
	Antisense	CGGGGTACCGGAACAGCTAGTCACAGGAG	
Pigeon *GPR25*	Sense	CCGGAATTCGCCCCCATGGCTCCCGAGGAG	1202
	Antisense	CCGGAATTCTCAAACCGGGTTCCTGTTACC	
Spotted Gar *gpr25*	Sense	CCGGAATTCGCGACATGGCGGCGAATACC	1149
	Antisense	CCGGAATTCGAGAGTAGGTCCTTCACTGC	
Zebrafish *aplnra*	Sense	CCGGAATTCCAAATGGAGCCAACGTC	1242
	Antisense	CCGGAATTCATACTCGCATCCACTCATC	
Human *APLNR*	Sense	CCGGAATTCAGCATGGAGGAAGGTGGTG	1148
	Antisense	CCGGAATTCCTAGTCAACCACAAGGGTC	
Zebrafish Gpr25-EGFP	Sense	GTGTGGTGGAATTCGCCACCATGGCAAGCAGCACAGAGAT	1171
	Antisense	TCCTCGCCCTTGCTCACCATTGCCTTTTGAGTAAGTGAGA	
Human GPR25-EGFP	Sense	TAGTCCAGTGTGGTGGAATTCGCCACCATGGCCCCCACAG	1128
	Antisense	CTCGCCCTTGCTCACCATCCAGGAGGCCGAGGCAGTGT	
Human APLNR-EGFP	Sense	GTGTGGTGGAATTCGCCACCATGGAGGAAGGTGGTGAT	1180
	Antisense	TCCTCGCCCTTGCTCACCATGTCAACCACAAGGGTCTCCT	
pcDNA3.1-EGFP	Sense	ATGGTGAGCAAGGGCGAGGA	6159
	Antisense	GGTGGCAATTCCACCACACT	

[a] All primers were synthesized by Tsingke (Beijing, China).

[b] Restriction sites added at the 5'-end of the primers are underlined.

References in supplementary materials

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