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Evolution of the vertebrate *pth2* (*tip39*) gene family and the regulation of PTH type-2 Receptor (*pth2r*) and its endogenous ligand *pth2* by Hedgehog signaling in zebrafish development

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Abstract

In mammals, parathyroid hormone (PTH), secreted by parathyroid glands, increases calcium levels in the blood from reservoirs in bone. While mammals have two PTH receptor genes, *PTH1R* and *PTH2R*, zebrafish has three, *pth1r*, *pth2r* and *pth3r*. PTH can activate all three zebrafish Pthrs while PTH2 (*alias* tuberoinfundibular peptide 39, TIP39) preferentially activates zebrafish and mammalian PTH2Rs. We know little about the roles of the PTH2/PTH2R system in the development of any animal. To determine the roles of PTH2 and PTH2R during vertebrate development, we evaluated their expression patterns in developing zebrafish, observed their phylogenetic and conserved synteny relationships with humans, and described the genomic organization of *pth2*, *pth2r*, and *pth2r* splice variants. Expression studies showed that *pth2* is expressed in cells adjacent to the ventral part of the posterior tuberculum in the diencephalon, whereas *pth2r* is robustly expressed throughout the CNS. Otic vesicles express both *pth2* and *pth2r*, but heart expresses only *pth2*. Analysis of mutants showed that Hedgehog (*Hh*) signaling regulates the expression of *pth2* transcripts more than that of nearby *gnrh2*-expressing cells. Genomic analysis showed that a lizard, chicken, and zebra finch lack a *PTH2* gene, which is associated with an inversion breakpoint. Likewise, chickens lack PTH2R, while humans lack PTH3R, a case of reciprocally missing ohnologs (paralogs derived from a genome duplication). The considerable evolutionary conservation in genomic structure, synteny relationships, and expression of zebrafish *pth2* and *pth2r* provides a foundation for exploring the endocrine roles of this system in developing vertebrate embryos.

Keywords

pth; pth receptor; pth2 evolution; pth2 development; pthr gene duplication

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Introduction

PTH (parathyroid hormone), PTH2, and PTHLH (PTH like hormone, alias PTHRP, parathyroid hormone related protein) are members of a small gene family (Papasani et al. 2004). Although PTH is an endocrine hormone that regulates serum calcium, PTHLH regulates patterning of chondrogenic and odontogenic tissues in mammals (Miao et al. 2002; Schipani & Provot 2003). Zebrafish has two co-orthologs of *Pth* (Gensure et al. 2004; Hogan et al. 2005) that appear to have originated during genome duplication at the base of teleost radiation (Amores et al. 2004; Hoegg 2004; Jaillon et al. 2004; Postlethwait et al. 1999; Postlethwait et al. 1998; Taylor 2003). In humans, PTH provides an important therapy for osteoporosis (Swarthout et al. 2002) and deregulation of PTHLH is responsible for most instances of humoral hypercalcemia of malignancy (HHM, high calcium levels in the blood associated with breast, lung, and myeloma cancers) (Guerreiro et al. 2007; Mangin et al. 1988). Despite the importance of PTH and PTHLH for human health and disease, the functions of PTH2 are not well understood in any species.

The expression patterns of PTH gene family members are distinct. In mammals, PTH is expressed primarily in the parathyroid glands with lower levels detected in the hypothalamus and pituitary (Fraser et al. 1991; Fraser et al. 1990; Harvey & Hayer 1993) and thymus (Günther et al. 2000; Postlethwait et al. 1999; Tucci et al. 1996). In contrast, PTHLH is expressed in many mammalian cell types, including cartilage, bone, mammary glands, teeth, skin, pancreatic islets and smooth muscles in the cardiovascular system, and is widely expressed in neurons of cerebral cortex, hippocampus, and cerebellum (Broadus & Nissenson 2006; Merendino et al. 1986; Weaver et al. 1995; Weir et al. 1990; Wysolmerski & Stewart 1998). Whereas *PTH2* is expressed in the subparafascicular area and in the medial paralemniscal nucleus of the CNS in 3-day-old macaque, nothing is known about its expression in human brain (Bago et al. 2009). Rat CNS expresses *Pth2* in posterior ventral thalamic areas, medial paralemniscal nucleus, and in dorsal and dorsolateral hypothalamus (Dobolyi et al. 2003a; Dobolyi et al. 2003b), as in other mammals, suggesting roles substantially different from those other PTH paralogs play in skeletal development and maintenance. Zebrafish, on the other hand, has duplicate orthologs of the human *PTH* gene (Gensure et al. 2004) called *pth1a* and *pth1b* that are expressed along the lateral line before neuromast migration and in the neuromasts, as well as in the ventral neural tube (Hogan et al. 2005). Our previous study showed generalized expression of *pth2* in the forebrain-midbrain boundary and heart in two day old embryos (Papasani et al. 2004). Here we report the genomic structure of zebrafish *pth2* and the results of conserved synteny investigations among zebrafish, human, chicken and lizard chromosomes showing that *PTH2* was lost in the lineage leading to lizards and birds. In addition, we provide a detailed analysis of *pth2* expression in zebrafish embryos and its regulation by *shh*.

PTH, PTH2, and PTHLH interact with the G-protein coupled receptors, PTH1R, PTH2R, and PTH3R (Rubin & Jüppner 1999a; Rubin & Jüppner 1999b). PTH and PTHLH bind and activate PTH1R nearly equivalently (Gardella & Jüppner 2001). Although PTH partially activates PTH2R (Mannstadt et al. 1999; Usdin et al. 1999b), PTH2 is likely the endogenous PTH2R ligand (Hoare, 2000; Hoare et al. 2000a; John et al. 2002). Functional *in vitro* studies show that zebrafish Pth3r expressed in COS-7 cells binds Pthlh and Pth and shows preferential activation by Pthlh (Rubin & Jüppner 1999a). We previously observed *pth2r* expression throughout the developing zebrafish brain at 48 and 72h (Papasani et al. 2004) and here provide detailed expression profiles over time. We describe the conserved genomic structure of *pth2r*; it's conserved syntenies between among zebrafish, human, and chicken chromosomes showing that loss of chicken *PTH2R* was associated with chromosome breakpoints. In addition, we isolated a novel splice variant (SV#19) of the original gene

(*pth2r*). Our aim was to obtain detailed information regarding the genomic structure of *pth2r* that would illuminate our understanding of the human *PTH2*/*PTH2R* system.

Materials and Methods

Zebrafish

AB wild type zebrafish and *smo* and *syu* mutants were obtained from the Oregon Fish Facility. Embryos were incubated at 28 C (Kimmel et al. 1995). Embryos used for *in situ* hybridization on whole mounts and cryo sections were treated with 0.003% 1-phenyl-2-thiourea before 24 hpf (hours post fertilization) to inhibit pigmentation. All protocols approved by local IACUC committees.

RNA extraction and RT-PCR

Each RT-PCR used 22 whole embryos. Embryos were homogenized in Tri Reagent (Sigma-Aldrich); at least two independent total RNA preps were extracted following the manufacturer's protocol and treated with DNase I (Roche). After determining RNA concentration and quality by spectrophotometer and agarose gel electrophoresis, cDNA was synthesized using 5.0 μg total RNA (25 μl total reaction volume as previously described (Rubin & Jüppner 1999a; Rubin et al. 1999; Shoemaker et al. 2006) with oligo (dT) primers using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Gene-specific primers (Table 1; Supplemental Data) were used to perform PCR as described (Papasani et al. 2004). To control for genomic DNA amplification, all RT-PCRs used DNase-treated RNA and the resulting amplicons crossed multiple introns. The amplicons were compared (Blast and Aligned) to gDNA and no gDNA contamination was observed (Supplemental Figure 1).

Rapid amplification of cDNA ends (RACE), and DNA sequencing

Splice variants were isolated by 5'RACE as described (Rubin et al. 1999). In short, total zebrafish RNA was obtained using the microRNA isolation kit following the manufacturer's guidelines (Promega, Madison, WI). To identify the 5'end of the cDNA encoding PTH2, approximately 1.0 μg of DNAse-treated total RNA from zebrafish was reverse transcribed using Omniscript II reverse transcriptase (Qiagen, Hilden, Germany) and a gene-specific reverse primer (zPTH2-3ut#1) Table 1. One tenth of the RT-PCR product was used for an initial PCR consisting of reverse zPTH2-3ut#2, forward zPTH2-5ut#2, and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), with the following reaction profile: initial denaturation at 94C for 3 min, and 35 cycles with denaturation at 94C for 1 min, annealing at 54 C for 1 min, polymerization at 72C for 2 min, and final extension at 72C for 10 min. A nested PCR using 2 μl of the initial PCR product was performed using reverse zPTH2-3ut#2 and forward zPTH2-5ut#3 following the same reaction profile. The 5'RACE amplicons were electrophoresed through a 2% agarose gel containing ethidium bromide, purified, ligated to pGEM-Teasy (Promega) and named zPTH2-5'RACE/pGEMT (Rubin et al. 1999), and used to transform *E. coli* TOP10 cells (Invitrogen). Bacterial colonies were screened by PCR using gene-specific primers. At least two independent plasmids containing pth2 cDNAs were purified by miniprep (Invitrogen), and sequenced in duplicate according to manufacturer's protocols (ABI, Perkin-Elmer Corp, Foster City, CA). Orientations were determined after resequencing cDNA amplicons and confirmed using zebrafish ENSEMBL (www.ENSEMBL.org).

Genomic analysis for pth2 and pth2r

To investigate conserved syntenies between zebrafish *pth2/pth2r* and human *PTH2/PTH2R*, we used the Synteny Database (Catchen et al. 2009)

(http://teleost.cs.uoregon.edu/synteny_db/). In Figure 1A, along the bottom of the dot plot the gray dots represent genes in order along zebrafish (*Danio rerio*, Dre) chromosome 17 (Dre17), which contains *pth2*. The plot places a cross on the chromosome appropriate for the location of each zebrafish gene's human ortholog, so the horizontal gene order corresponds to the zebrafish chromosome. Open circles show positions of *pth2*, the human (*Homo sapiens*, Hsa) *PTH2* (Hsa19), and its paralogs, *PTH* (Hsa11) and *PTHLH* (Hsa12). For phylogenetic analysis, (Fig. 5A) sequences were aligned by Multiple Sequence Comparison by Log-Expectation (MUSCLE, http://www.ebi.ac.uk/Tools/muscle/index.html) and subjected to maximum likelihood analysis (http://atgc.lirmm.fr/phyml/) (Guindon & Gascuel 2003; Guindon et al. 2005).

In situ hybridization

At least two independent whole mount *in situ* hybridizations were performed using 20–30 embryos for each complementary RNA (cRNA) probe to ensure reproducibility. In addition, *in situ* hybridizations for control (sense RNA) and experimental embryos were conducted in parallel to minimize variances between days. The synthesis of cRNA probes followed published protocols: *pth2* (Papasani et al. 2004); *gnrh2* (Gopinath et al. 2004); *gh1* (Herzog et al. 2004); *vmhc* and *myl7* (Yelon et al. 1999). To synthesize the *pth2* cRNA probe, *pth2*/pGEMT was linearized with *Mfe1* and transcribed with Sp6 polymerase using the digoxingenin (DIG) RNA labeling kit following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). The *pth2* probe corresponded to bases 256 to 786 of accession number AY306196 (Suppl Fig. 1). To synthesize the *pth2r* cRNA probe, *pth2r*/pCRII was linearized using *BamHI* and transcribed with Sp6 polymerase using the DIG RNA labeling kit as described above. The *pth2r* probe corresponded to bases 940 to 2429 of accession number NM_131377. Sense probes (control) were utilized to observe non-specific expression and compared to their previously verified cRNA expression patterns (*pth2* (Papasani et al. 2004); *gnrh2* (Gopinath et al. 2004); *gh1* (Herzog et al. 2003; Herzog et al. 2004); *vmhc* and *myl7* (Yelon et al. 1999)).

Embryos were cryosectioned and used for *in situ* hybridization as described (Rodriguez-Mari et al. 2005). For *pth2* and *gnrh2* double expression, two day old embryos were treated with Proteinase K (10 μg/ml) for 20 mins and fixed in 4% paraformaldehyde/phosphate buffer saline (PBS) for 20 mins at room temperature (RT). Subsequently embryos were washed in PBT (PBS plus 0.1% Tween 20) and incubated at 65 °C ovenight with equal amounts of *pth2* and *gnrh2* probe in 50% formamide buffer solution. After a series of washes, embryos were treated in blocking solution for 2 hrs at RT. Hybridization was detected by alkaline phosphatase-conjugated anti-DIG antibody and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) following manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Both the experimental and the control reactions were stopped at the same time by washing them with PBT.

Results

Conserved syntenies for pth2

Conserved syntenies provide evidence for the conservation of genome regions across evolutionary history. The conserved syntenies for *pth2* (Fig. 1A) show that none of the human (Hsa) chromosomes that contain *PTH* paralogs (Hsa11, *PTH*; Hsa12, *PTHLH*; Hsa19, *PTH2*, see Fig. 1F) had extensive conserved synteny with *Danio rerio* linkage group 17 (Dre17), the location of *pth2*. Two genes immediately to the left and three immediately to the right of *pth2* have orthologs widely separated on Hsa14, and as the dot plot shows, many other genes on Dre17 have orthologs on Hsa14; but Hsa14 has no *PTH*-related gene. Thus, human and zebrafish *pth2* genes do not show conserved syntenies.

At least three hypotheses can account for these results. First, the location of the zebrafish gene may be incorrect due to an error in genome assembly. To test this possibility, we compared the position of *pth2* in zebrafish to that in the well-assembled genome of stickleback (*Gasterosteus aculeatus*, Gac). Results showed that genes near zebrafish *pth2* had orthologs near stickleback *pth2* (Fig. 1B). The agreement of these two genomes makes the incorrect-assembly mechanism unlikely. A second hypothesis is that chromosome rearrangements in the fish and/or tetrapod lineages destroyed any conserved synteny that might have originally existed. To test this mechanism, we compared human chromosome 19 (Hsa19), which contains *PTH2*, to the stickleback genome. Results showed that stickleback has two clear copies of most regions of Hsa19 (boxed in Fig. 1C), but that stickleback orthologs of the region around human *PTH2* are distributed over several stickleback chromosomes, especially linkage groups II, V, VIII, XI, and XX (Fig. 1C). These results suggest that substantial chromosome rearrangements occurring in one or both lineages after the stickleback and human lineages diverged. Third, zebrafish *pth2* may not be orthologous to human *PTH2*. To test this possibility, we constructed a maximum likelihood tree of the whole gene family. Results showed that zebrafish and pufferfish Pth2 clustered with human and mouse PTH2 (Fig. 1D). We conclude that *pth2* and *PTH2* are orthologous genes.

Our searches of the chicken genomic and EST databases failed to identify a gene closely related to *PTH2*, and this loss was confirmed by analysis of the zebra finch genome, suggesting that the *PTH2* gene was lost in the bird lineage. To explore this point further, we searched ENSEMBL for chicken orthologs of genes neighboring *PTH2*, and discovered that nearly all neighbors within ten genes of *PTH2* were missing from both chicken and zebra finch. In contrast, orthologs of most *PTH2* neighbors – but not *PTH2* itself – were present in the anole lizard (*Anolis carolinensis*), which, like birds and crocodiles, is a diapsid, an animal with two temporal fenestra on each side of the skull (mammals are synapsids). To identify a mechanism for the loss of *Pth2* from the lizard-dinosaur-bird lineage, we compared human and lizard genome databases. Results showed that, like birds, lizard lacks *PTH2*, but has many nearby neighbors (Fig. 1E). A local inversion with a breakpoint between the two neighbors of the human *PTH2* gene distinguishes the lizard and human regions. This result could happen if an inversion breakpoint destroyed *PTH2* in diapsids. Further chromosome rearrangements may have contributed to the loss of additional neighboring genes from the bird lineage. We conclude that lizards and birds lack an ortholog of *PTH2* due to a chromosome breakage event.

Finally, examination of paralogous chromosomes in the human genome show that PTH, PTH2, and PTHLH occupy paralogous chromosome segments in Hsa11, Hsa19, and Hsa12, respectively (Fig. 1F). These chromosome segments, along with a portion of Hsa1, most likely (see Dehal and Boore, 2005), are paralogous chromosome segments (paralogons) arising from the R1 and R2 rounds of early vertebrate genome duplication. We conclude that *PTH*, *PTH2*, and *PTHLH* are ohnologs arising in the R1 and R2 genome duplication events and that the fourth ohnolog went missing from bony vertebrates (Postlethwait, 2007; Wolfe, 2000).

Expression of pth2

We evaluated expression of *pth2* by whole mount *in situ* hybridization and RT-PCR in various stages of development using *beta-actin* as control: cleavage (0.75–2 hour post fertilization, h), blastula (2.25–4.66 h), segmentation (10.33–22h) and pharyngula (24–42h) until the hatching period (48–72h). Compared to control sense probe (no hybridization signal, data not shown), *in situ* hybridization using cRNA probes showed *pth2* transcript expression during cleavage (1.75h) and blastula stages (4h) (Fig. 2. A–D). During segmentation (19–22h), *pth2* was expressed in forebrain, midbrain, hindbrain and in cells lining brain ventricles (Fig. 2. E, F). During the pharyngula stage (26h), *pth2* transcript was

observed in midbrain and otic vesicles (Fig. 2. G, H). Expression of *pth2* in otic vesicles became more prominent at 36h (Fig. 2. I, J). In the hatching period (48 –72h), expression of *pth2* in brain became restricted to the paired domains near forebrain-midbrain boundary that became more intense over time (Fig. 2. K, L, M, N). These paired domains lie adjacent to the ventral part of posterior tuberculum (Mueller and Wullimann, 2003). Bilateral *pth2*-expressing domains lie beneath dorsal thalamus anterior to preoptic region (Fig. 2. O, P). Zebrafish *gnrh2* has been reported to be expressed near the *pth2* domains we describe here (Gopinath et al. 2004; Kuo 2005). To evaluate how close the expression of *pth2* and *gnrh2* are to each other spatially, we double-labeled zebrafish embryos for *pth2* and *gnrh2* expression. Double labels showed that the *pth2* expression domain was anterio-ventral to *gnrh2* expressing cells (Supplemental Figure 2). Thus, *pth2* and *gnrh2* transcripts are expressed in two distinct but nearby paired domains. RT-PCR supports the conclusion from the whole mount *in situ* study that *pth2* mRNA was present at all stages tested (Supplemental Figure 3). We conclude that *pth2* transcript is present in embryos long before the midblastula transition, the stage at which zygotic genes are first expressed and thus *pth2* is expressed very early in zygotes (messenger-RNA that is synthesized during oogenesis and deposited in the cytoplasm of the cells in the egg).

Factors that regulate the development of PTH2-expressing cells have been incompletely investigated. Because *shha* and *pth2* are expressed within several cell diameters of each other (Papasani et al. 2004), we hypothesized that *hedgehog* signaling might direct the development of *pth2*-expressing cells, consistent with the regulation of *nk2.2*-expressing cells several cell diameters distant from *shh*-expressing cells (Barth & Wilson 1995). To test this hypothesis, we evaluated expression of *pth2* and *gnrh2* (a gene expressed by the hypothalamus) in animals lacking either *shha* activity (*syu*; *sonic-you* mutants (Schauerte et al. 1998) or all hedgehog signaling (*smo*; *slow-muscle-omitted* mutants (Varga et al. 2001). Compared to wild types (Fig. 3. A, B), *syu* mutant embryos had fewer cells expressing *pth2* and fewer cells expressing *gnrh2* (Fig. 3. C, D). This result shows that *shh* signaling is essential for the development of *pth2*- and *gnrh2* expression, but is not required for the specification of at least some *pth2*-and *gnrh2*-expressing cells. In contrast, removal of all Hedgehog signaling by mutation of *smo*, which encodes the receptor for Shh and other Hedgehog proteins (Varga et al. 2001), dramatically reduced the development of *pth2*-transcript expression but merely diminished the number of *gnrh2*-transcript expression (Fig. 3. E, F).

Because Pth2 can regulate the hypothalamo-pituitary axis in rats (Wang et al. 2002; Ward et al. 2001), we performed single and double-label experiments to examine *pth2*- and *gh1*-expressing cells (Herzog et al. 2003). Results showed that *gh1*-expressing cells of the anterior pituitary (Supplemental Fig. 4. C, D) occupied a single medial cell group located ventral and posterior to the paired *pth2* domains (Supplemental Fig. 4. A, B, E, F). We conclude that, if Pth2 regulates Gh1 secretion in zebrafish, as suggested in rat, then it likely does so indirectly, possibly by regulating the hypothalamo-pituitary axis. Further studies are necessary to fully understand the mechanism.

The *pth2* gene was expressed not only in the CNS and in the developing otic vesicles but also in the zebrafish heart (Papasani et al. 2004). To better understand the role of *pth2* in the developing heart, we marked various chambers using myosin light polypeptide 7 (*myl7*, alias *cmlc2*), which is expressed throughout the ventricular and atrial portions of the heart tube (Supplemental Fig. 5. C, D) and ventricle-specific myosin heavy chain gene *vmhc* (Supplemental Fig. 5. E, F) (Yelon 2001). We observed diffuse expression of *pth2* throughout the atrial and ventricular regions of the developing heart tube at 48h (Supplemental Fig. 5. A, B). We conclude that *pth2* expression is not confined to a single portion of the heart tube at the stages examined.

Genomic structure of pth2r

The Pth2 ligand acts by binding and activating the Pth2r (John et al. 2002; Papasani et al. 2004). To understand the evolutionary origin and biological roles of Pth2r in zebrafish, we first studied its genomic structure. We used BLAST searches of the ENSEMBL zebrafish Zv8 genomic database (Rubin et al. 1999) (http://pre.ENSEMBL.org/Danio_rerio/) to identify contigs with sequence identity to our *pth2r* cDNA (NM_131377). Contig CU459122.18 contains exons EL2, M5, M6/7 and M7 and T along with the corresponding introns (Fig. 4A), contig BX001055.11 contains exons S, E1, E3, G, M1, M2, M3 and M4, and contig CU862080.5_01118 contains exons M4, EL2, M5 and M6/7.

The organization of *pth2r* was deduced from our cDNA (Rubin et al. 1999) and by designing *pth2r* exonic primers to determine intron-exon borders and intron lengths on genomic DNA. Our deduced *pth2r* gene consists of 15 exons (including the splice variant SV#19 and exon U, Fig. 4A). By comparing cDNA to gDNA, we validated the intron-exon borders of the 15 exons (from exon S through T) and sizes of many introns. A comparison of zebrafish *pth2r* to human *PTH2R* (transcript ID ENST00000413482) (Fig. 4A) showed that human *PTH2R* has 14 exons (from exon U through T) similar to that of zebrafish *pth2r* (excluding the splice variant SV#19).

pth2r splice variants

We previously isolated two *pth2r* transcripts, one of 2429 bp (which we call pth2r-predominant form) and one with a 5′ splice variant pth2r(43) of 2378 bp that lacked 17 amino acids in the amino-terminal extracellular domain (Rubin et al. 1999). In studies reported here, we confirmed the original two forms, pth2r-predominant and the 5′ splice variant pth2r(43), but further identified two additional splice variants by multiple and independent 5′ RACE experiments using adult zebrafish total RNA with *pth2r*-specific primers. The first new *pth2r* splice variant lacked exon S and began at exon E1 (pth2r -No-S, Fig. 4B). The second new *pth2r* splice variant is pth2r-SV#19 and is encoded by a novel signal peptide SV#19 (accession GU002363) (Fig. 4B). A search of the zebrafish genome at ENSEMBL identified significant sequence identity corresponding to the pth2r-SV#19 cDNA sequence on contig BX001055.11.

To define exons encoding our 5′ RACE products (predominant-pth2r, pth2r(43), pth2r No S and pth2rSV#19), we compared structures to *pth2r* gDNA. We found that Exon S for *pth2r* gDNA has an intron acceptor site (agTT) that is 14 nucleotides 5′ to the initiator ATG, which is found in both the predominant-pth2r form and in pth2r(43) (Fig. 4C). In the predominant-pth2r form, an intron donor site (AGca) is 164 nucleotides 3′ to the initiator ATG, while an intron donor site (GCgt) observed in pth2r(43) is 112 nucleotides 3′ of the ATG (Fig. 4C). Thus, pth2r(43) is shorter than predominant-pth2r at the 3′ end while the rest of the nucleotides remain constant in exon S for both of them. The newly discovered pth2r-SV#19 transcript has consensus intron donor and acceptor sites (AGgt and agAT, respectively) (Fig. 4. D). The novel splice variant pth2r-SV#19 we found here either lacks a mammalian equivalent or its mammalian equivalent is yet to be identified.

Phylogenetic analysis of Pthr genes

To help understand the relationships and histories of vertebrate *Pthr* genes, we conducted a phylogenetic analysis (Fig. 5A). Results confirmed that vertebrates have three Pthr genes (Rubin et al. 1999). *Pth1r* is present in tetrapods, birds, an amphibian and teleosts, and tree topology matches accepted species relationships (Fig. 5A). The zebrafish *pth2r* gene (Rubin et al. 1999) falls in the *PTH2R* clade with strong bootstrap support along with the *pth2r* of other teleosts (Fig. 5A). Furthermore, while mammals and an amphibian have a clear *PTH2R* ortholog, reciprocal best amino acid identity matches by BLAST (basic local

alignment search tool) (Altschul et al. 1997) searches of two sequenced bird genomes (chicken and zebra finch) failed to identify any *Pth2r* ortholog. This suggests that *Pth2r* was present in the last common ancestor of all vertebrates but was lost from the bird lineage after it diverged from the mammalian lineage. Reciprocal best BLAST analyses revealed a single clear ortholog of *PTH3R* in the genomes of two birds, an amphibian, and several teleosts (Fig. 5A), but none in mammalian genomes. We conclude that the *Pth3r* gene was present in the last common ancestor of all vertebrates but was lost from mammalian genomes after they diverged from bird genomes. Thus, the ancestral functions of the *Pthr* gene family must be partitioned differently in mammals and other vertebrates (Fig. 5F).

Zebrafish pth2r shares conserved syntenies with human PTH2R

The hypothesis that zebrafish *pth2r* is an ortholog of human *PTH2R* predicts that the two genes should reside in orthologous chromosome segments. To test this property, we investigated conserved syntenies using the Synteny Database (Catchen et al. 2009). Results showed that zebrafish *pth2r* has neighbors that have human orthologs residing near *PTH2R* on human Chromosome 2 (Hsa2) (Fig. 5B). We conclude that *pth2r* has conserved synteny with the human genome, consistent with orthology.

To investigate the genomic basis for the loss of *Pth2r* from birds, we compared human and chicken genomes (Fig. 5C). Results showed that the 2Mb segment orthologous to the human *PTH2R* neighborhood (Fig. 5 C1, 2) extends over three different regions of chicken chromosome 7 (Fig. 5 C3, 4). The close linkage of *Pth2r* with *Idh1*, *Plekhm3* and *Fzd8* is shared by human and zebrafish and is hence ancestral (Fig. 5B), but these regions are widely separated on chicken chromosome 7. The parsimonious explanation is that a transposition event disturbed the region between *PIP5K3* and *MAP2* in the avian lineage and that this breakage event may have led to the loss of the avian *Pth2r* gene.

Reciprocally, *Pth3r* was lost from the mammalian genome (Fig. 5A). In chicken, *Pth3r* is located between *Myl4* and *Ddx42* (Fig. 5 D1, 2), but the human orthologs of *MYL4* and *DDX42* are located far apart on Hsa17 (Fig. 5 D3). This arrangement would be predicted if a chromosome transposition/inversion event disrupted the *Pth3r* gene in a mammalian ancestor and separated genes that were ancestral neighbors.

Two rounds of whole genome duplication occurred in an ancestor to all extant vertebrates (Dehal & Boore 2005; Garcia-Fernandez & Holland 1994; Holland et al. 1994; Spring, 1997). We wondered whether the *PTHR* gene family originated in these events. We used the Synteny Database (Catchen et al. 2009) to examine the distribution of human paralogs surrounding *PTH2R* (Fig. 5E). Results showed that Hsa2, 3, 7, 10, 12, and 17 had large numbers of paralogs of Hsa2 genes. Coupled with the conserved synteny analysis of Figure 5D, the results suggest that *PTH3R* `should have' been located on Hsa17 if it had not gone missing (Fig. 5F).

Expression of pth2r

To compare gene expression patterns of Pth2 and its receptor Pth2r, we evaluated *pth2r* (pth2r-predominant form) distribution in space and time by whole mount *in situ* hybridization and its expression by RT-PCR. Compared to control sense probe (no hybridization signal, data not shown), *in situ* hybridization using cRNA probes showed *pth2r* transcript during cleavage before the mid-blastula transition (1.75–2h), indicating that *pth2r* is very early expressed (Fig. 6. A, B, C, D). Nuclei at 2h appeared to have accumulated *pth2r* transcript (Fig. 6. C, D, enlarged and cell-specific nuclei are indicated with arrowhead) and many cells in the blastula (4h) showed *pth2r* expression (Fig. 6. E, F). During early segmentation at 12h, *pth2r* transcript was expressed throughout the developing

CNS (Fig. 6. G) and at 15 and 18h, *pth2r* transcript accumulated in brain, eye and notocord (Fig. 6. H, I). At 22h, *pth2r* was expressed in forebrain, midbrain and hindbrain (Fig. 6J). At 28h, we found strong expression in the epiphysis (Fig. 6K). At 36h, we observed prominent expression in the otic vesicles and pharyngeal arches (Fig. 6L). At 48h, *pth2r* was robustly expressed in the forebrain, midbrain, hindbrain, retina and pharyngeal arches (Fig. 6 M, N) and also in the otic vesicles (Fig 6. O, P). At 72h, *pth2r* expression became more restricted to the midbrain-hindbrain region (Fig. 6. Q, R). RT-PCR experiments supported the whole mount *in situ* study that *pth2r* mRNA was present at all stages tested, suggesting early onset and continued presence of the transcript (Supplemental Figure 1).

These results show that *pth2r* is widely and robustly expressed throughout the CNS in a pattern much broader than *pth2* at the same stages. The very early onset of ligand and receptor expression suggests their involvement in early development. Ligand (Fig. 2E) and receptor (Fig. 6J) genes were both expressed in the forebrain, midbrain and hindbrain in segmentation stages, but later, expression of the ligand became more restricted. Although we detected expression of the receptor in the retina (Fig. 6. I, K, L, M), we didn't detect it in the heart where the ligand gene was expressed. We also observed the expression of the receptor gene in the otic vesicles. We found that the ligand and receptor are both expressed in otic vesicles, but the highest concentration of ligand expression (Fig 2. G, H, I, J) is not at the same location as that of receptor (Fig 6 L, O, P). Nevertheless, the expression of *pth2* and *pth2r* mRNA in the ear at the same time suggests a role in otic development, although further functional studies are required to confirm this proposed interaction.

Discussion

Different vertebrates have different subsets of PTH ohnologs

Our analysis of paralogons in the human genome show that *PTH*, *PTH2*, and *PTHLH* are ohnologs, paralogs derived from the R1 and R2 rounds of whole genome duplication that occurred at the base of the vertebrate radiation (Dehal & Boore 2005). Zebrafish *pth2* and human *PTH2* are highly likely to be orthologous genes (Papasani et al. 2004) despite the lack of conserved syntenies, which probably happened by chromosome arrangements that stirred the *PTH2* or *pth2* neighborhoods with respect to each other in 450 million years since the last common ancestor of human and zebrafish and thereby abolished any evidence of conserved synteny. Examination of bird and lizard genomes showed that the diaspid lineage lacks an orthologue of *PTH2*, which was neatly deleted from the lizard genome and which was part of a larger chromosome segment that is deleted in the bird genome. The significance of this finding is that birds and lizards perform the combined roles of *PTH*, *PTH2*, and *PTHLH* solely by the use of *PTH* and *PTHLH*. Whether birds and lizards apportion the mammalian roles of *PTH2* between their *PTH* and *PTHLH* genes, or whether they lack the gene-specific roles of *PTH2* is a question for future research. In any event, this finding has significance because it means that different lineages of vertebrates have different PTH-family genes and hence variations in PTH-family gene functions.

Zebrafish *pth2* has overlapping expression patterns with the mammalian *PTH2* gene

Expression studies showed that in zebrafish, *pth2* was expressed at very early stages of development, and then showed wide-spread expression in zygotes that gradually become constrained to the heart and otic vesicles and to the forebrain-midbrain boundary close to *gnrh2* expressing cells; suggesting roles in early brain (Blind et al. 2003; Wortmann et al. 2003) and heart development (Yelon 2001; Yelon et al. 2002; Dobolyi et al. 2003).

Factors that regulate the development of PTH2-expressing cells have been incompletely investigated. Our results show that knockdown of Hedgehog signaling substantially reduces

the number of cells expressing *pth2* but has less effect on the number of cells expressing *gnrh2*. Knockdown of all Hedgehog signaling by mutation of the receptor was more severe than removal of Shha alone, suggesting that the expression of Indian hedgehog genes (*ihha* and *ihhb*), which are expressed in the branchial arches (Avaron et al. 2006) or less likely *shhb* (Ekker et al. 1995), may influence the development of *pth2*-expressing cells. We further conclude that the development of *pth2*-expressing cells is more sensitive to Hedgehog signaling than is the development of *gnrh2*-expressing cells. These results are consistent with the finding that Hh signaling regulates development of the diencephalon and hypothalamus (Mathieu et al. 2002; Scholpp et al. 2006). Because desert hedgehog (*dhh*) is not expressed in zebrafish until approximately 6dpf (Avaron et al. 2006), it is unlikely to be involved in signaling relevant to *pth2*-expressing cells. Previous studies showed that intracerebroventricular administration of *Pth2* in rat brain increases GRF (growth hormone releasing factor) and decreases pulsatility of growth hormone (GH) release; thus, Pth2 can control the hypothalamo-pituitary axis (Wang et al. 2002; Ward et al. 2001). Our Pth2 and Gh1 expression study suggests that if Pth2 regulates Gh1 secretion in zebrafish as it does in rats, then control is likely indirect by regulating the hypothalamo-pituitary axis.

Comparison of *Pth2* expression among various vertebrates provides clues to the evolution of its developmental roles. Pth2-positive neurons are widely expressed in two distinct brain regions in both mice and rats (Dobolyi et al. 2003a; Faber et al. 2007), including the subparafascicular area (Wang et al. 2006) and the medial paralemniscal nucleus at the midbrain-pons junction (Varga et al. 2008). PTH2-expressing neurons were also present in the subparafasicular area and in the medial paralemniscal nucleus in 3-day-old male macaque brain (Bago et al. 2009). *PTH2* mRNA was found abundantly in the human central nervous system, trachea, fetal liver, and, to a lesser degree, in human heart and kidney (Hansen et al. 2002). We observed zebrafish *pth2* expressed adjacent to the ventral posterior tuberculum at 48h and throughout the CNS at younger stages. Thus *pth2* is expressed in overlapping subsets of brain regions in zebrafish, rodents and humans. We conclude that the *Pth2* expression domain in the ventral forebrain plays an ancient phylogenetically conserved role in vertebrate development or physiological function.

Zebrafish and mammals share additional *pth2* expression domains. As in zebrafish, the human heart and rat aorta express *Pth2* (Eichinger et al. 2002; Hansen et al. 2002). We also observed *pth2* mRNA in zebrafish otic vesicles at 48h, consistent with the hypothesis that *pth2* is involved in otic vesicle development or auditory functioning (Dobolyi et al. 2003a). The correspondence of gene expression patterns suggests that *PTH2* has specific broadly shared developmental and/or physiological roles in the brain, heart and otic vesicles among different species and thus reflects ancestral functions present at least at the origin of bony fishes 450 million years ago.

pth2r gene structure

Comparative genomics of the zebrafish *pth2r* and the human *PTH2R* genes showed similar exon structure, but identified two novel splice variants. The zebrafish splice variant that lacked exon S and starts with exon E1 is likely nonfunctional like the corresponding human *PTH1R* transcript (Joun et al. 1997). For the splice variant pth2r-SV#19, residues located downstream of the signal peptide are in-frame and code for the predominant-pth2r transcript previously described (Rubin et al. 1999). Phylogenetic and conserved synteny analysis showed that Pth2r was lost from the bird lineage after it diverged from the mammalian lineage and that *Pth3r* was lost from mammalian genomes after they diverged from bird genomes. Our analysis supports the conclusion that the last common ancestor of teleosts and tetrapods had three *Pthr* genes, probably arising in two rounds of whole genome duplication in a stem vertebrate (R1 and R2), and that *Pth3r* went missing in the mammalian lineage after it diverged from the bird lineage, while *Pth2r* was deleted from the bird lineage after it

diverged from the mammalian lineage. Because no extant organisms that have been shown to have a fourth *pthr*, we conclude that *pth4r* was lost shortly after 2R, consistent with the finding that loss of a paralog after gene duplication is the most common fate of a pair of gene duplicates (Haldane 1933; Nei & Roychoudhury 1973; Bailey et al. 1978; Watterson 1983). Our genomic analysis shows that, as with paralogs for *PTH*-gene family ligands, paralogs for the *PTHR* receptors are different in different classes of vertebrates. The important implication, again, is that different lineages of vertebrates may accomplish different functions with these different gene sets, or that, *in toto*, they accomplish the same functions but that these functions are spread out over different individual genes.

Analysis of zebrafish *pth2r* expression patterns revealed a pattern generally similar to that previously reported in mouse and primate brains in the hypothalamus, cerebellum, and cerebral cortex (Bago et al. 2009; Faber et al. 2007). Pth2r has also been detected throughout the cardiovascular system, including vascular endothelium and smooth muscle of rat (Usdin et al. 1999), but we were unable to detect *pth2r* expression in the zebrafish embryonic heart. Additionally, we detected *pth2r* in the ear in a different location to that of *pth2*, which is nevertheless consistent with the action of a diffusible ligand. *In situ* hybridization experiments, of course, do not detect protein; thus to address whether Pth2 is transported necessitates the use of a Pth2 antibody.

Conclusions

This study revealed genomic and functional similarities and differences among vertebrate lineages for the PTH2-PTH2R ligand-receptor system. We identified an additional and novel signal peptide pth2r-SV#19. The identification of additional signal exons may facilitate future studies on the number and location of promoters for PTH2R. We also established that both the ligand and the receptor are expressed in otic vesicles and are thus positioned to be involved in auditory development. Moreover, the expression of the ligand throughout the heart suggests its possible involvement in heart development. Future knockout studies are essential to test these hypotheses. Additionally, we found that Hedgehog signaling regulates the development of Pth2 ligand-producing cells but has less effect on *gnrh2* expressing cells. This information provides a foundation essential for future functional analyses of this ligand-receptor complex in zebrafish.

Of broader importance, our conserved synteny and phylogenetic studies showed that the three vertebrate *PTH*-family and *PTHR*-family genes likely arose in two rounds of whole genome duplication at the base of the vertebrate radiation; that the *PTH2* ligand is present in mammals and fish but absent from the sequenced genomes of lizards and birds; and that the *PTH2R* receptor is absent from birds, reciprocally, that the *PTH3R* receptor is absent from mammals, and finally that zebrafish has copies of all three genes. We conclude that the variation in gene content across vertebrate classes provides ample leeway for variations in functions of the genes that constitute this ligand-receptor system in each vertebrate lineage. The variation among animal genomes shown here is particularly important in two cases; first, when suggesting functions for a human gene of medical importance from investigations on a teleost fish or bird model, we must be certain we are comparing orthologs. Second, when trying to infer the evolutionary origin of endocrine systems, such as the parathyroid gland, if we do not compare orthlogous genes from different taxa, then we may make inappropriate inferences. Thus, our data provide a foundation for further investigation of the biological roles of the Pth2-Pth2r complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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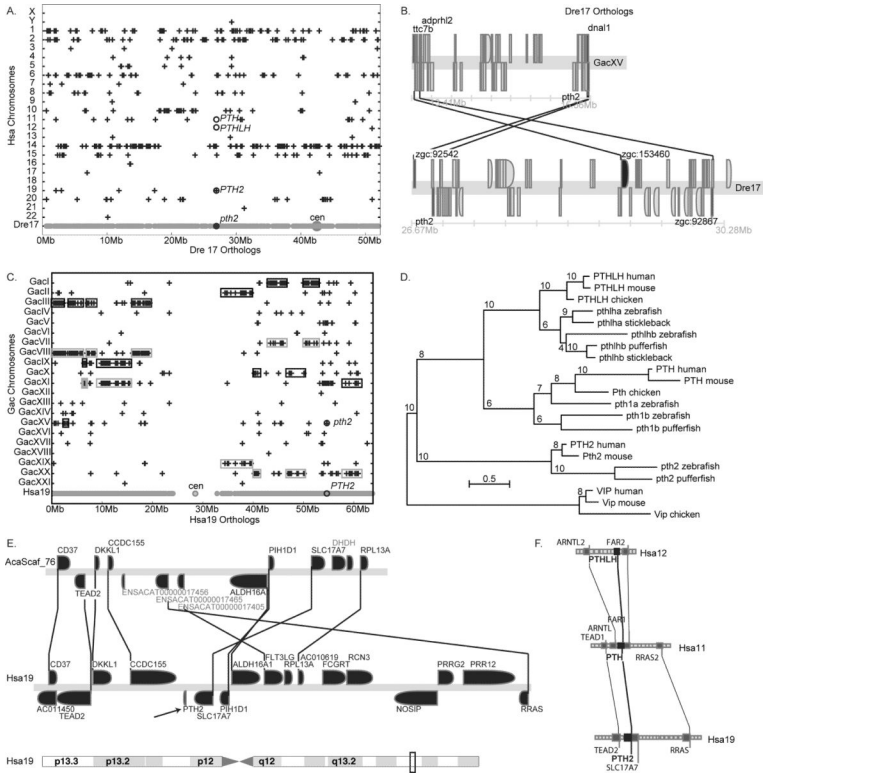

Figure 1. Conserved synteny analysis for *pth2*

(A) Dot plot showing zebrafish chromosome Dre17 across the bottom with the position of *pth2* and the centromere (cen) marked. Directly above each gene on Dre17 (gray dots), the figure plots a cross on the human chromosome that carries the ortholog of each zebrafish gene. Human chromosomes containing *PTH2*, *PTH*, and *PTHLH* are indicated with circles. The plot shows little conservation of synteny between Dre17 and Hsa19, the site of *PTH2*, but substantial conservation of syntenies with Hsa14. (B) Conserved syntenies around zebrafish *pth2* with the stickleback region on linkage group XV showing shared pairs of orthologs (connecting lines). (C) Dot plot comparing Hsa19 to stickleback chromosomes. Most regions of Hsa19 have two clear regions of conserved synteny with stickleback, which are surrounded by pairs of rectangles, paralogons resulting from the teleost genome duplication event. The exception is the region around *pth2*, which has no clear pair of co-orthologous chromosome segments, suggesting substantial chromosome rearrangement in the fish lineage with respect to the human lineage. (D) Maximum likelihood tree. Bootstrap numbers are of ten replicates. (E) Conserved syntenies for the human region around *PTH2* compared to the anole lizard *Anolis carolinensis*. (F) Examination of paralogous chromosomes in the human genome show that PTH, PTH2, and PTHLH occupy paralogous chromosome segments. The top row shows the lizard scaffold_76 and its orthologous region in human in the next line down. An arrow indicates the position of *PTH2* and an arrowhead indicates the site of the inversion breakpoint. The location of the human chromosome segment on Hsa19 is indicated on the idiogram below. Abbreviations and Accession numbers are in Supplemental Data.

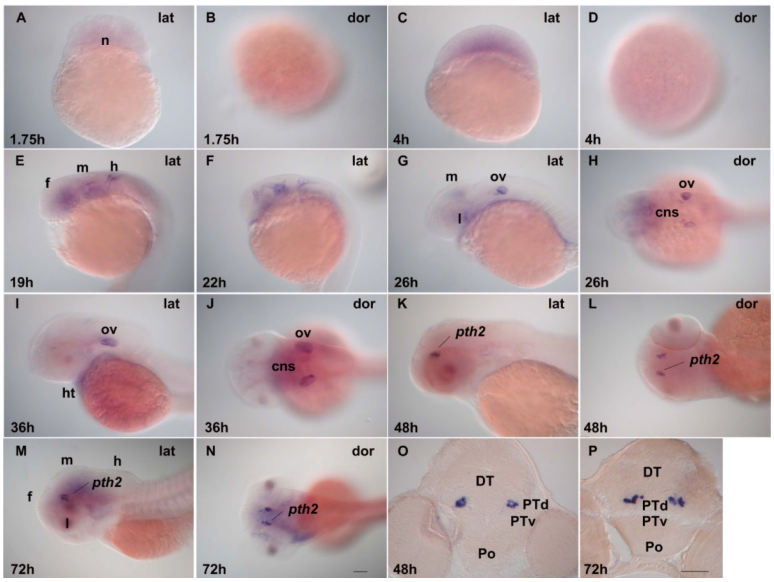

Figure 2. Developmental expression of zebrafish *pth2* by whole-mount *in situ* hybridization

(A–N) cleavage through hatching. (O, P) Cross sections of 48h (M) and 72h (N) embryos. Abbreviations: cns, central nervous system; d, dorsal; DT, dorsal thalamus; f, forebrain; h, hindbrain; l, lens; lat, lateral; m, midbrain; ov, otic vesicles; Po, preoptic region; PTd, dorsal part of posterior tuberculum; PTv, ventral part of posterior tuberculum, ap, animal pole. Scale bar is 50 μM (A–N and O–P). Images O and P are 2× the magnification of all other panels (A through N).

Fig. 3

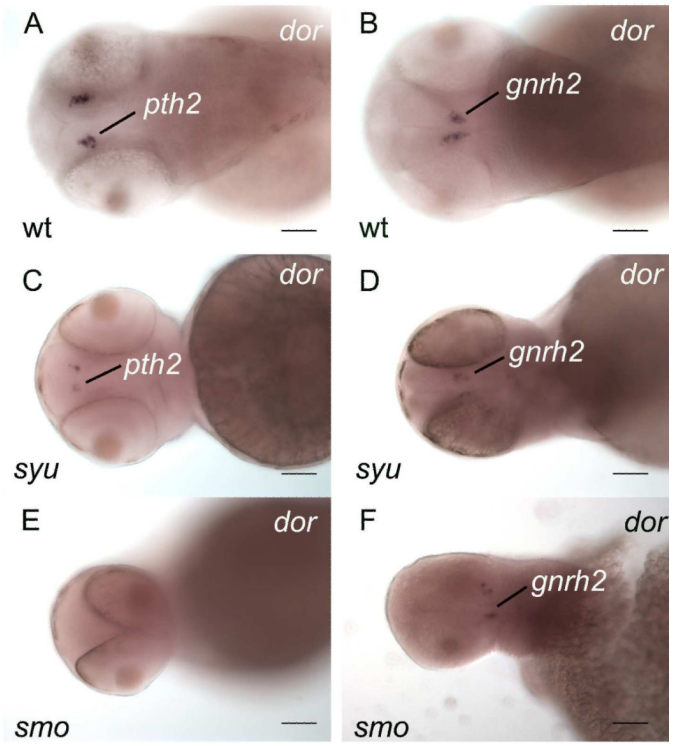

Figure 3. Localization of *pth2* and *gnrh2* assessed by whole-mount *in situ* hybridization double labeling

Whole mount *in situ* hybridization to detect expression of *pth2* and *gnrh2* in 48h zebrafish embryos. Expression of *pth2* mRNA was detected on both sides of the midline, slightly anterior to the *gnrh2* expression domain. The lateral (lat) and dorsal (dor) views display the gap between the two clusters of *pth2*- and *gnrh2-expressing cells* (A–F). (G) Higher magnification view of the preparation shown in F, confirming that *pth2*-expressing cells are anterior-ventral to the cells expressing *gnrh2*. Forebrain (f), midbrain (m) and eye (e). Scale bar is 50 μM.

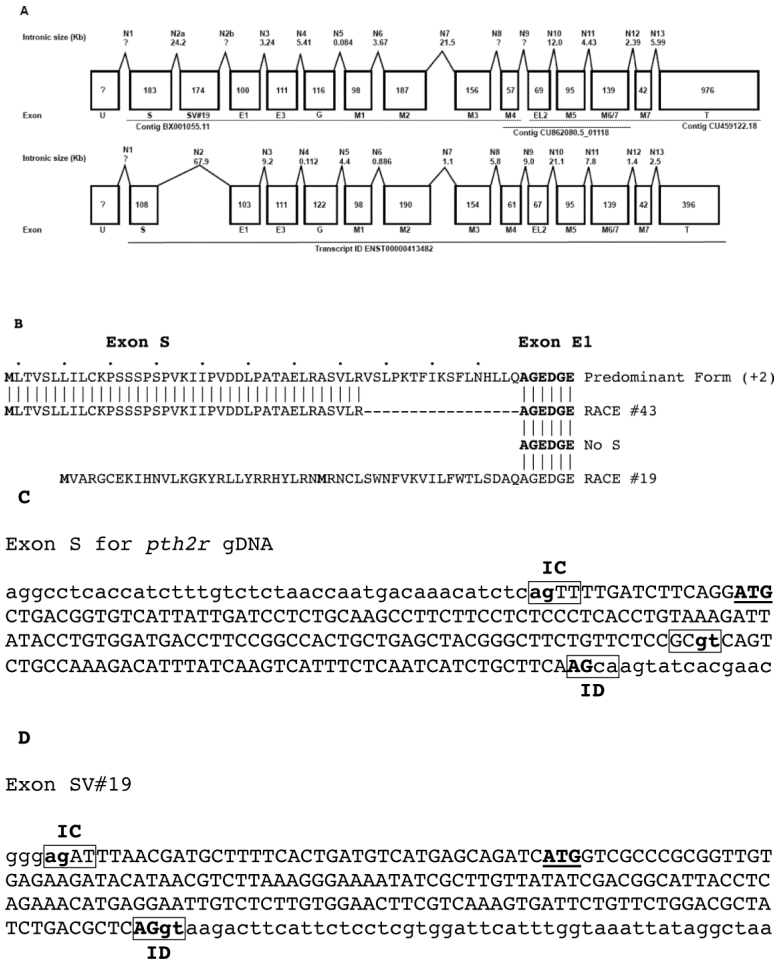

Figure 4. Consensus structure of the genomic sequence encoding the zebrafish *pth2r* and 5'-splice variants

(A) Zebrafish *pth2r* genomic structure (top panel) compared to human *PTH2R* (transcript ID ENST00000413482, bottom panel). Boxes show exon sizes in base pairs (bp) with their identifications directly below. The intronic sequences (N1, N2a, N2b……N13) with respective sizes are indicated between their flanking exonic boxes. N? indicates an intron of unknown size. Bars indicate contigs containing the respective exons. (B) Multiple and independent 5'-RACE experiments using adult total zebrafish RNA produced four different amplicons encoding exon S. Amino acids were aligned at Exon E1 (in bold) and subsequently aligned and extended 5' (Exon S). In addition to the predominant amplicon (predominant pth2r) and pth2r(43) (shorter by 17 residues than the predominant *pth2r*) (Hoare et al. 2000a; Papasani et al. 2004; Rubin and Jüppner, 1999a; Rubin and Jüppner, 1999b; Rubin et al. 1999), we identified two additional transcripts that indicated alternate splicing (Joun et al. 1997). (C) The nucleotide sequence of *pth2r* genomic DNA encoding exon S for both the predominat pth2r and pth2r(43) and (D) a novel signal peptide arising from exon SV#19, respectively. The pth2r-SV#19 transcript has a conserved intron donor (ID) and intron acceptor (IC) recognition sequence. The initial methionine ATG is bold and underlined. Coding nucleotides are in uppercase and untranslated introns are in lowercase font. Splice donor and acceptor sites are in boxes.

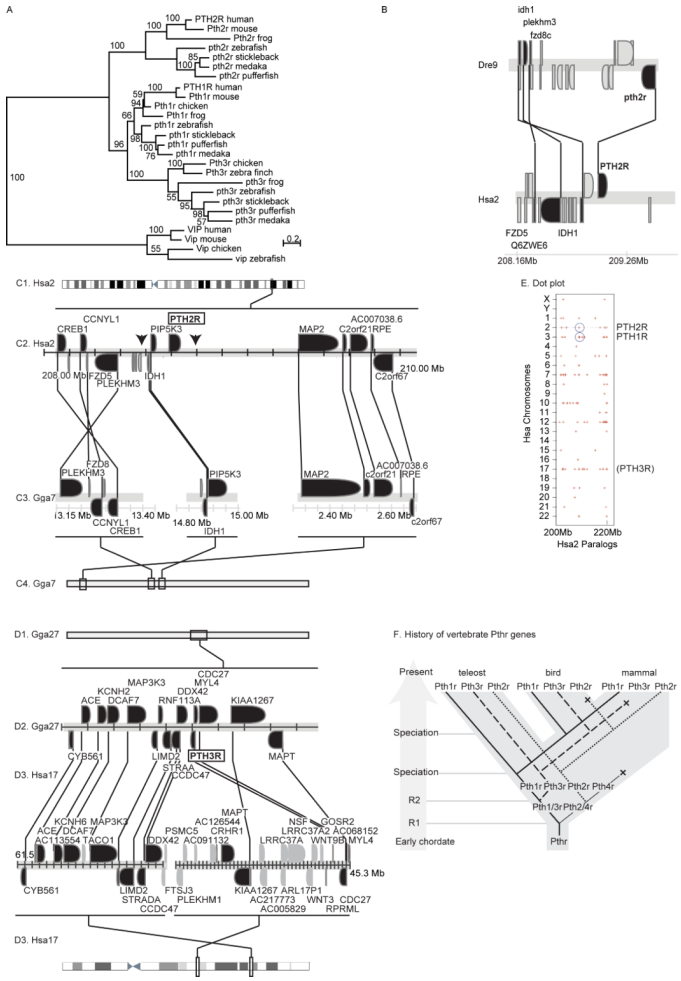

Figure 5. Phylogenetic and conserved synteny analysis for PTH receptors

(A) Maximum likelihood tree of Pthr amino acid sequences rooted on the related sequences of VIP. Numbers on branches are bootstrap values of 100 iterations. Results show that teleost *pth2r* genes are orthologs of human *PTH2R*; that *Pth1r* is present in teleosts, birds, and mammals, and that *Pth3r* is present in teleosts, an amphibian, and birds but is missing from mammals. (B) Conserved syntenies of human and zebrafish *PTH2R* and *pth2r* genes. The portion of zebrafish chromosome Dre9 containing *pth2r* shows conserved syntenies with human chromosome 2 (Hsa2) near *PTH2R*. Lines connect orthologs between zebrafish and human. (C). Conserved syntenies suggest a mechanism for the loss of *Pth2r* from the avian lineage. C1. Idiogram of human chromosome 2 (from Ensembl). C2. Expansion of the region boxed in part C1, showing genes transcribed left to right on the top, and in the reverse orientation on the bottom. C3. The three regions of chicken chromosome 7 (Gga7) that are orthologous to the human chromosome segment containing *PTH2R*. C4. Chicken chromosome 7 with the regions shown in detail in part C marked with boxes. The human *PTH2R* gene lies near a chromosome transposition break points (arrowheads). D. Conserved syntenies suggest a mechanism for the loss of *Pth3r* from the human lineage. D1. Chicken chromosome 27 with the boxed area blown up in D2. D3. Two portions of human chromosome 17 that contain the chicken orthologs of the region surrounding *Pth3r*. D4. The position of the two human chromosome segments on Hsa17 that are orthologous to the single region centered on *Pth3r* in the chicken genome. E. A dot plot showing paralogs of genes surrounding *PTH2R* on Hsa2. The location of *PTH2R* and its paralog *PTH1R* are

marked by circles and the presumed location of the missing *PTH3R* gene on Hsa17 is indicated in parentheses. F. A history of the Pthr family. The most parsimonious explanation from evidence from phylogenetic and conserved synteny analysis is that *Pth1r* (solid line), *Pth2r* (dotted line), and *Pth3r* (dashed line) arose in the R1 and R2 rounds of vertebrate genome duplication and that the fourth expected gene, Pth4r (thin line) was lost shortly thereafter (X). After the speciation event separating teleost and tetrapod lineages, both lineages initially had genes for Pth1r, Pth2r, and Pth3r, but after the speciation event separating bird and mammalian lineages, *Pth3r* was lost in the mammalian lineage and *Pth2r* was lost in the bird lineage (X's). The investigation of gene functions in this gene family has the potential to show how ancestral gene functions evolve and partition after gene duplication and lineage-specific gene loss. Abbreviations and Accession numbers are in Supplemental Data.

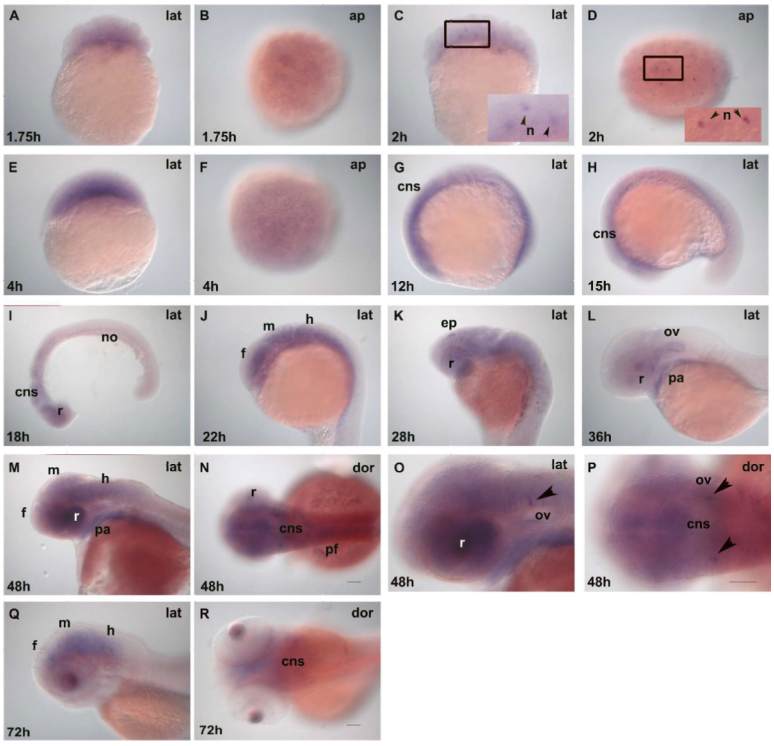

Figure 6. Developmental expression of zebrafish *pth2r*(pth2r predominant form)

Tissue-specific expression of zebrafish *pth2r* mRNA by whole-mount *in situ* hybridization using *pth2r* probe. Lateral (lat) and dorsal (dor) images of *pth2r* expression from cleavage through hatching periods (A–P) showed the expression of *pth2r* transcript in cns (central nervous system), ep (epiphysis), f (forebrain), h (hindbrain), m (midbrain), ov (otic vesicle), n (nucleus, blown up and indicated with arrowhead), no (notochord), pa (pharyngeal arches), pf (pectoral fin) and r (retina). O and P are the higher magnification view of the preparation shown in M and N confirming that *pth2r*-expressing cells are there in the developing otic vesicles (indicated with arrowhead). ap indicates animal pole. Scale bar is 50 μM (A–N, O–P, and Q–R). Images O and P are 2× the magnification of all other panels.

Table 1

Primer sequences used for PCR amplification (A–C) and RT-PCR (D–G).

	Gene	Primer sequences (5′–3′	Amplicon Size (bp)
A	*pth2r*	5′-TGGGGTGTTCCTGCCGTCTTTGT - For 5′-TTATCTGACTGATCACCACATCGT - Rev	825
B	*pth2*	5′-TGACCCTCATGGCATCGGCATTT - For 5′-TTGTCTCTCCATTGCTGTCAACA - Rev	367
C	β-actin	5′-ATGGACTCTGGTGATGGTGTGA - For 5′-TTGAAGGTGGTCTCGTGGATA - Rev	383
D	zPTH2-3ut#1	5'-TTTTCCCTATACCATACTTTATA - Rev	
E	zPTH2-3ut#2	5'-TTACAATTACTTTGAATTAACTAC - Rev	
F	zPTH2-5ut#2	5'-GAGTGTTAGAGAGAAACTCTG - For	
G	zPTH2-5ut#3	5'-CATGGACGATTTGCGAATTAG - For	

For = Forward Primer

Rev = Reverse Primer