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Duplicated zebrafish co-orthologs of parathyroid hormone-related peptide (PTHrP, Pthlh) play different roles in craniofacial skeletogenesis

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Abstract

In mammals, parathyroid hormone-related peptide (PTHrP, alias PTH-like hormone (Pthlh)) acts as a paracrine hormone that regulates the patterning of cartilage, bone, teeth, pancreas, and thymus. Beyond mammals, however, little is known about the molecular genetic mechanisms by which Pthlh regulates early development. To evaluate conserved pathways of craniofacial skeletogenesis, we isolated two *Pthlh* co-orthologs from the zebrafish (*Danio rerio*) and investigated their structural, phylogenetic, and syntenic relationships, expression, and function. Results showed that *pthlh* duplicates originated in the teleost genome duplication. Zebrafish *pthlha* and *pthlhb* were maternally expressed and showed overlapping and distinct zygotic expression patterns during skeletal development that mirrored mammalian expression domains. To explore the regulation of duplicated *pthlh* genes, we studied their expression patterns in mutants and found that both *sox9a* and *sox9b* are upstream of *pthlha* in arch and fin bud cartilages, but only *sox9b* is upstream of *pthlha* in the pancreas. Morpholino antisense knockdown showed that *pthlha* regulates both *sox9a* and *sox9b* in the pharyngeal arches but not in the brain or otic vesicles and that *pthlhb* does not regulate either *sox9* gene, which is likely related to its highly degraded nuclear localization signal. Knockdown of *pthlha* but not *pthlhb* caused *runx2b* overexpression in craniofacial cartilages and premature bone mineralization. We conclude that in normal cartilage development, *sox9* upregulates *pthlh*, which downregulates *runx2*, and that the duplicated nature of all three of these genes in zebrafish creates a network of regulation by different co-orthologs in different tissues.

Introduction

Parathyroid hormone (PTH) acts as the main hypercalcemic hormone while PTH-related protein (PTHrP, official human symbol PTHLH (PTH-like hormone) and referred to as Pthlh in this manuscript), is essential for embryonic development, differentiation, and tissue patterning (Philbrick *et al*. 1996). Unregulated paracrine secretion of Pthlh is associated with a type of tumor that results in elevated blood calcium levels, a condition called humoral

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The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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hypercalcemia of malignancy (HHM), while regulated secretion of Pthlh during mouse embryogenesis is essential for the developmental patterning of cartilage, bone, teeth, CNS, pancreas, and other tissues (Karperien *et al*. 1996, Philbrick *et al*. 1996, Clemens *et al*. 2001).

Pthlh structure is highly conserved. For example, human, chicken, and rodent Pthlh are 98% identical within the first 111 residues, while the average protein is only 85% identical compared with chickens and humans (Lagerström *et al*. 2006). Alternative splicing of human *PTHLH* transcripts produces three PTHLH variants that differ in their carboxy termini (Clemens *et al*. 2001, Sellers *et al*. 2004). Splice variants share a 'prepro' segment including the signal sequence, followed by 139 shared amino acids (Philbrick *et al*. 1996). The sequence of Pthlh, including the amino-terminus (N-terminus), mid-region, and nuclear localization signal (NLS) with its associated RNA-binding region, is remarkably conserved among species, suggesting functional conservation (Henderson *et al*. 1995, Wu *et al*. 1996; Fig. 1). Posttranslational processing produces forms with distinct physiological functions that work through unique yet poorly characterized receptors (Orloff *et al*. 1996, Valin *et al*. 2001) to regulate cartilage differentiation and apoptosis, chondrocyte metabolism, and matrix synthesis and to inhibit bone resorption (Clemens *et al*. 2001).

Mice with a disrupted *Pthlh* gene have a lethal chondrodysplasia characterized by abnormal growth plates with fewer resting and proliferative chondrocytes, premature endochondral and perichondral ossification, and death shortly after birth due to breathing complications. Mice chondrocytes overexpressing Pthlh display delayed chondrocyte differentiation in contrast to the premature chondrocyte differentiation syndrome caused by *Pthlh* knockdown (Karaplis *et al*. 1994). As a result, surviving mutant pups are born with a cartilaginous endochondral skeleton. After birth, long bones in mutants slowly ossify from the outside in but retain a cartilaginous center, which may be due to high concentrations of Pthlh in the center of the cartilaginous bone mold (Weir *et al*. 1996). These results show that Pthlh plays an essential role in the development of the endochondral skeleton by modulating the timing and extent of chondrocyte differentiation and being tightly regulated by several factors such as Indian hedgehog (Ihh) and Sox9.

Pthlh acts through a reciprocal interaction with Ihh, at least in rodents (Karaplis *et al*. 1994, Vortkamp *et al*. 1996). Although *ihh* genes are expressed in zebrafish skeletogenesis (Avaron *et al*. 2006), the *pthlh/ihh* mechanism remains poorly understood in fish skeletogenesis (Provot & Schipani 2005). Similarly, although zebrafish *sox9* is required for chondrogenesis, a *sox9/pthlh* interaction is unknown (Yan *et al*. 2002, 2005).

Thus, because little is known about the roles of Pthlh in nonmammalian vertebrates, we isolated zebrafish *pthlh* genes to evaluate two hypotheses: 1) zebrafish *pthlh* is functionally conserved compared with human PTHLH and 2) similar to human *PTHLH*, zebrafish *pthlh* is expressed and tightly regulated during development. We found two co-orthologs of human *PTHLH*, as with several other genes that regulate craniofacial skeletogenesis, including *bmp2*, *runx2*, *sox9*, and *ihh*, all of which arose during a genome duplication event in the teleost lineage, the teleost genome duplication (TGD; Amores *et al*. 1998, Postlethwait *et al*. 2000, Jaillon *et al*. 2004, Taylor & Raes 2004, Nakatani *et al*. 2007). Duplicated genes derived from this genome duplication often partitioned ancient subfunctions between the co-orthologs (Force *et al*. 1999, Postlethwait 2007), which may help facilitate dissection of these pathways, including the regulatory mechanisms governing reciprocal Pthlh signaling between chondrogenic and osteogenic developmental programs (Yan *et al*. 2005, Flores *et al*. 2006). We show here that Sox9 upregulates Pthlh, which downregulates Runx2 during craniofacial and odontogenic development.

Materials and Methods

Identification of gDNA regions encoding putative zebrafish pthlh sequences

A cDNA encoding a partial sequence of *pthlh* from the puffer fish *Sphoeroides nephelus* (accession DQ023267) was used as a probe to search the zebrafish genome website (http://134.174.23.160/HumanblastZebrafish/) for homologous sequences. Two sequences, *pthlha* (NM_001024627) on chromosome 4 and *pthlhb* (NM_001043324) on chromosome 25, were identified by BLAST analyses that showed the greatest sequence similarity to puffer fish and human *PTHLH* genes (*PTHLH*, accession M57293 and M32740).

Zebrafish (WT strain AB) were reared, anesthetized, and killed according to established IACUC-approved protocols (Westerfield 2000). We use the ZFIN guide for gene and protein nomenclature (http://zfin.org/zf_info/nomen.html). For example, genes for zebrafish, human, and mouse are designated, respectively, *pthlha*, *PTHLH*, and *Pthlh* and proteins Pthlh, PTHLH, and Pthlh. Although the official symbol is PTHLH, some of the aliases include PTHrP, HHM, Pthlp, PTH-L, and PLP (Abbink & Flik 2007, Pinheiro *et al.* 2010).

Isolation of zebrafish pthlh cDNAs

For *pthlha*, 5′-RACE was performed on an adult zebrafish head cDNA library constructed in λZAP (Stratagene) using platinum Taq DNA polymerase (Invitrogen) with the primers SK (5′-CCGCTCTAGAACTAGTGGATC) and reverse (REV) *pthlha*-1 (5′-CCTCCGGATGCCCTTCATCATCAT). Nested PCR was performed with SK and Rev *pthlha*-2 (5′-GATTTGTGGGTCTCCTGCTGCGG). Amplicons were gel purified, then ligated to pGEM-Teasy (Promega), and called *pthlha*-5′-RACE/pGEMT. 3′-RACE was performed with platinum Taq DNA polymerase, T7 primer (5′-TAATACGACTCACTATAGG), and primer forward(FOR)*pthlha*(5′-ACCCACGCTCAGCTGATGCAT). Nested PCR was performed with T7 and primer For *pthlha*-3 (5′-GATAAAGGCCGAACGCTGCAG). Amplicons were gel purified, then ligated to pGEMT, and called *pthlha*-3′-RACE/pGEMT. Zebrafish *pthlha*-5′-RACE/pGEMTand *pthlha*-3′-RACE/pGEMT were used to transform *Escherichia coli* T0P 10 cells (Invitrogen). Plasmids containing *pthlha* 5′-RACE and 3′-RACE cDNAs in pGEMTwere sequenced according to manufacturer protocols (ABI, Perkin-Elmer Corp., Foster City, CA, USA).

For *pthlhb*, total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and Rev *pthlhb*-1 (5′-CTTTTATCACTCTCTCATGTGCAT). To isolate the cDNA encoding the 5′-nucleotides of *pthlhb*, a 1:100 dilution of the reverse transcriptase reaction was amplified with Rev *pthlhb*-3 (5′-CATCTTACTCTTCCTCTTCATG) and For *pthlhb*-5 (5′-ATGTTAAGGCACTGGGGCTT). A nested amplification used the following primers: Rev *pthlhb*-4 (5′-TGGCTGCTCTTCATATGTCAG) and For *pthlhb*-5. To identify the 3′-RACE cDNAs encoding *pthlhb*, total zebrafish RNA was reverse transcribed using Superscript II reverse transcriptase and an oligo-dT anchor primer (Bhattacharya *et al.* 2011). The first strand was then amplified by PCR using the primer For *pthlhb*-1 (5′-GTCACGCTCAGATGATGCAC) and AUAP primer (5′-GGCCACGCGTCGACTAGTAC; Invitrogen). A nested amplification used the following primers: For *pthlhb*-2 (5′-ACAGGAGCCGCTCTCTGCAT) and an adapter AUAP primer. 3′-RACE cDNAs encoding *pthlhb* were isolated, ligated to pGEM-Teasy, and sequenced as described earlier.

Determination of intron/exon boundaries, sequence alignments, and phylogenetic analyses

The intron/exon structure of the zebrafish *pthlh* genes was determined using (http://www.searchlauncher.bcm.tmc.edu/seq-search/gene-search.html), location of RNA splice sites was determined using (http://www.fruitfly.org/seq_tools/splice.html), and signal sequence was predicted using (http://www.cbs.dtu.dk/services/SignalP/). Sequence alignments and phylogenetic analyses were performed as described (Guindon *et al.* 2005, Bhattacharya *et al.* 2011).

Zebrafish sox9a⁻ and sox9b⁻ mutants

sox9 single mutants (*sox9a*⁻ and *sox9b*⁻) and double mutant (*sox9a*⁻/*sox9b*⁻; Yan *et al.* 2002, 2005) were used to evaluate the expression of *pthlha* and *pthlhb* during development.

Zebrafish pthlh antisense morpholino oligonucleotide injection

Antisense morpholino oligonucleotides (MOs) were obtained (http://www.gene-tools.com) and injected in one- to two-cell zebrafish embryos as described (Draper *et al.* 2001). The zebrafish *pthlha* splice-blocking MO (e2i2 (exon-2, intron-2) 5′-CACAGACACTTACATTCGGCTGCTG, which blocks the exon-2 donor site, Fig. 6A) or the *pthlhb* splice-blocking MO (i2e3 (intron-2, exon-3) 5′-CTGAACGCCGTCTGGAACATAGACA, which blocks the exon-3 acceptor site, Fig. 6B; 1.0–2.0 nl of a 3.0 mg/ml MO stock), was injected into about 500 embryos each session for at least three independent sessions to assess phenotypes compared to noninjected embryos. Controls were of two types: 1) the injection of control MO (TTTTTGCTCATACAGGTGGCCTGAG, which represents the sense MO for zebrafish *egr2b*, NM_183341) and which gave no effect, and 2) the co-injection of *pthlha* or *pthlhb* MO along with *tp53* (NM_131327) MO (GCGCCATTGCTTTGCAAGA-ATTG) at 2.0 mg/ml, which follows the recommended procedure to ensure that MO-induced phenotypes are not caused by nonspecific cell death due to MO toxicity (Robu *et al.* 2007, Eisen & Smith 2008). MO-injected embryos were allowed to develop to 24, 48, and 72 h post-fertilization (hpf) before whole-mount *in situ* hybridization, examining at least 50 animals per time point (Bhattacharya *et al.* 2011). To evaluate disruption of *pthlha* or *pthlhb* mRNA, RT-PCR was performed on noninjected controls and pooled MO-injected embryos using either *pthlha* primers (For+120, 5′-AACT-TTGAGGAGACTGGCGCT; Rev−745, 5′-AACTCCTGCAGTGATCGAGCT; Fig. 6A) or *pthlhb* primers (For-in, 5′-CGCTGTGGTGCCCCCTGATAA; Rev-R, 5′-CTTTTATCACTCTCTCATGTGCA; Fig. 6B).

Whole-mount *in situ* hybridization and alcian blue/alizarin red staining

Zebrafish *pthlha* and *pthlhb* cRNA probes were produced by linearizing *pthlha*-5′-RACE/pGEMT with NotI and *pthlhb*-5′-RACE/pGEMT with NcoI and then transcribing the cDNA using the DIG RNA labeling kit (Roche Applied Science) using T7 RNA polymerase for *pthlha* and Sp6 for *pthlhb* probes. The whole-mount *in situ* hybridization on zebrafish embryos was performed as described previously (Yan *et al.* 2002, Bhattacharya *et al.* 2011). For cellular resolution, cryosections were subjected to *in situ* hybridization as described (Rodriguez-Mari *et al.* 2005). Alcian blue and alizarin red staining of cartilage and bone were performed as described (Walker & Kimmel 2007).

Results

Identification of cDNAs encoding zebrafish pthlha and pthlhb

To identify zebrafish *pthlh* genes, we used the *pthlh* cDNA sequence of *S. nephelus* to search the zebrafish genome database. Two putative zebrafish gDNA regions were identified that showed sequence similarity to the human *PTHLH* transcript, which we call *pthlha* and *pthlhb*. To determine whether these putative genes are expressed, gene-specific primers for zebrafish *pthlha* and *pthlhb* were designed to amplify products by RT-PCR using adult zebrafish total RNA. Based upon partial *pthlha* and *pthlhb* cDNA sequences, 5′- and 3′-RACE reactions were performed to obtain the full-length cDNAs (accession: *pthlha*, DQ022615 and Ensembl, ENSDARG00000031737; *pthlhb*, DQ022616 and Ensembl, ENSDARG00000071070).

Genomic regions with sequence identity to the cDNA sequences were analyzed to predict intron–exon boundaries. The organization of the zebrafish *pthlha* and *pthlhb* genes is simpler than that of the human *PTHLH* gene due to the lack of exons encoding additional C-terminal variants. In general, the gene structures for *pthlha* and *pthlhb* are organized similar to *PTHLH* in which the 5′-UTR is encoded on exon-1, exon-2 encodes the prepro sequence (presequence includes −40 through −7 for Pthlha and −32 through −7 for Pthlhb, and part of the prosequence, including −6 through −3), and exon-3 encodes the rest of the prosequence (−2 and −1) and the mature peptide (ENSDARG 00000031737 and ENSDARG00000071070). More specifically, Pthlha encompasses three exons where exon-1 encodes the 5′-UTR, exon-2 encodes the transcription start site and N-terminus, and exon-3 encodes the mid-region, NLS and RNA-binding region, and C-terminus regions of Pthlh (ENSDARG00000031737). Although Pthlha exon-1 encoded 219 nucleotides of 5′-UTR, the longest transcript encoding Pthlhb that we isolated lacked exon-1, contained exon-2 encoding 18 nucleotides of 5′-UTR as well as the transcription start site and N-terminus, and exon-3 encoding a conserved mid-region but a poorly conserved NLS and C-terminus regions (ENSDARG00000071070).

Structural analysis of zebrafish Pthlh polypeptides

Similar to PTHLH, each zebrafish Pthlh protein contained a predicted signal sequence (Supplementary Table 1, see section on supplementary data given at the end of this article) and dibasic cleavage site (either RR or KR) immediately preceding the first amino acid residue of the predicted mature peptide (Fig. 1A). The predicted mature Pthlha and Pthlhb polypeptide hormones are 158 and 130 amino acids long respectively (Fig. 1A). Tblastx pairwise alignments showed that Pthlha is more similar to PTHLH (54% identity) than Pthlhb (35%), while the two zebrafish Pthlh proteins showed 57% identity to each other (Fig. 1A). Regions that showed the greatest similarity to PTHLH and conservation with other Pthlh ligands include the Pthlh(1–34) N-terminus of the mature peptide, while the mid-region (Wu *et al.* 1996) and NLS/RNA-binding domain (Henderson *et al.* 1995, Nguyen & Karaplis 1998) showed less conservation. Zebrafish Pthlh showed several N-terminal residue changes (A1S, H5A/V, and F23W) that are similar to residues in human PTH (hPTH), suggesting that Pthlh may function similar to hPTH for calcium regulation, while the Pthlhb mid-region showed the least conservation with a gap of 49 residues and a significantly degraded NLS sequence (Gardella & Jüppner 2000), which may suggest a lack of conservation for nuclear functions.

Phylogenetic relationships among PTH, Pthlh, and PTH2 peptides

A key question is the origin of the zebrafish *pthlh* gene duplicates. Using PhyML (Guindon *et al.* 2005), we analyzed phylogenetic relationships of Pthlh, PTH, and PTH2 from teleosts (zebrafish, stickleback, and puffer fish) and tetrapods, using VIP, which represents the next

most closely related gene clade, as outgroup (Bhattacharya *et al*. 2011). While the Pthlh clade had highly significant bootstrap support (ten of ten replicates, Fig. 1B), the PTH clade was strong but not quite as robust (six of ten replicates). The Pthlh and PTH clades clearly grouped significantly as sister groups (eight of ten replicates), with the PTH2 (alias TIP39) clade grouping basal to the PTH-Pthlh supergroup, using VIP as outgroup. Within each Pthlh, PTH, and PTH2 clade, sequences followed the accepted phylogenetic relationships of the species themselves, from basal to derived. Teleost Pthlha and Pthlhb sequences fell as duplicates arising after the divergence of the teleost and tetrapod lineages, consistent with the interpretation that they are co-orthologs of human *PTHLH*, having arisen in the TGD event.

Genomic origin of the Pthlh family

The phylogenetic analysis confirmed that Pthlh, PTH, and PTH2 form a tight gene family. To further understand the historical relationships of these genes, we used our automated engine for the analysis of conserved syntenies beginning with PTH2 (Catchen *et al*. 2009). For every gene in a 6 Mb region centered on *PTH2* in human chromosome 19 (Hsa19), the engine searched for paralogs on other human chromosomes and placed a dot on the chromosome above the *Hsa19* gene. Open circles in Fig. 1C locate *PTH2* on Hsa19 and its paralogs *PTH* and *PTHLH* on Hsa11 and Hsa12 respectively. Results showed that genes neighboring PTH2 have paralogs on many human chromosomes, but Hsa11 and Hsa12 are the major paralogons for the region of Hsa19 containing PTH2; these two regions have paralogs of 27 and 23 *Hsa19* genes respectively (Fig. 1C). Hsa7 and Hsa1 have 14 and 15 paralogs each and fewer other chromosomes. These results would be expected if an original chromosome segment containing a single *PTH/PTHLH* gene experienced two rounds of duplication to make four chromosome segments, three of which today are parts of human chromosomes 11, 12, and 19 containing *PTHLH*, *PTH*, and *PTH2*, respectively, and the fourth is part of either Hsa1 or Hsa7, but this fourth segment has lost its *PTHLH*-related gene, the most common fate of duplicated genes. We conclude that the vertebrate *PTHLH*, *PTH*, and *PTH2* genes arose in the two rounds of genome duplication that occurred at the base of the vertebrate radiation (Garcia-Fernandez & Holland 1994, Dehal & Boore 2005).

Syntenic relationships of zebrafish *pthlh* genes

Inferring a gene's origins requires the investigation of its conserved syntenies. Fig. 2A, B, C, D and E compares genomic regions surrounding the two zebrafish *pthlh* genes to the human genome, while Fig. 2F and G compares, reciprocally, the genomic region surrounding the human *PTHLH* gene to the two zebrafish chromosomes. Zebrafish *pthlha* and *pthlhb* reside at the left ends of linkage groups (LGs) 4 and 25 respectively (Fig. 2A and E). Figure 2B shows an enlargement of the marked region on LG25 in Fig. 2A. Four genes immediately flanking *pthlhb* (Fig. 2B) have human orthologs, as judged by best reciprocal BLAST analysis (Wall *et al*. 2003), on Hsa12 (Fig. 2C), and orthologs of four genes immediately to the left of *pthlha* (Fig. 2D) are also located on Hsa12 (Fig. 2C). Reciprocally, of the 20 annotated genes surrounding *PTHLH* on Hsa12 (Fig. 2G, an enlargement of the bar indicates the position of *PTHLH* in Fig. 2C), all except one has orthologs on zebrafish LG4 or LG25 (Fig. 2F and H). The most parsimonious explanation for these data is that the last common ancestor of zebrafish and humans had a single chromosome region with this gene set, that the chromosome segment duplicated in the zebrafish lineage, and that the two duplicated regions evolved into the current genomic arrangements by gene loss and inversions. Because *pthlha* and *pthlhb* are parts of this duplicated chromosome region, we conclude that they are co-orthologs of the human *PTHLH* gene arising in the TGD event.

Early developmental expression of zebrafish Pthlh co-orthologs

Key insights into a gene's functions come from its pattern of expression. RT-PCR of one cell, 5, 10, 24, 48, and 72 hpf embryos detected transcripts of *pthlha* and *pthlhb* at all stages (Supplementary Fig. 1, see section on supplementary data given at the end of this article). These results showed that *pthlha* and *pthlhb* are maternally expressed but at levels that are not clearly detected by *in situ* hybridization (Fig. 3A and B). At 12, 24, and 48 hpf, both *pthlha* and *pthlhb* showed expression in the region of presumptive branchial arch-4 (Fig. 3C, D, E, F, G and H). At 72 hpf, *pthlha* was expressed in otic vesicles (ov), superoptic cartilages (sopc), and pancreas (pan) (Fig. 3I); in tooth-forming cells (tee; Philbrick *et al*. (1998)); and in scattered cells of the retina similar to the distribution of amacrine cells (eye (e); Fig. 3K; Yokoi *et al*. 2009; Fig. 3M). These expression domains are similar to those of *Pthlh* in developing mouse embryos (Lee *et al*. 1995, Philbrick *et al*. 1996). Surprisingly, zebrafish embryos did not appear to express *pthlh* genes in keratinocytes of the skin (Wysolmerski *et al*. 1994) or scales or fins (Trivett *et al*. 1999). At 72 hpf, *pthlhb* was expressed in a triangular patch of 20–30 mesenchymal cells of unknown identity near the presumptive thymus (Figs 3J, L and 4H) and in spinal neuromasts (Fig. 3N), where it may be associated with the formation of the caudal neurosecretory system (urophysis; Ingleton *et al*. 2002). The ceratohyal and opercular provided useful elements for position due to their location and size (Albertson *et al*. 2005).

Pthlh co-ortholog expression during tissue patterning and ossification

In situ hybridization studies on cryosections revealed the expression pattern of *pthlha* and *pthlhb* in the developing craniofacial skeleton in single-cell detail. In histological sections, *pthlha* expression appeared robustly throughout the brain (Fig. 4A, 53 hpf), the cartilage core of branchial arches 1–4 (Fig. 4C, 3 dpf), pancreatic endocrine cells (Fig. 4E, 3 dpf; Shor *et al*. 2006), retinal amacrine cells within the inner nuclear layer and ganglionic layer of the retina (Fig. 4I, 6 dpf; Yokoi *et al*. 2009), teeth (Fig. 4K, 6 dpf), spinal cord (Fig. 4M, 6 dpf), and in cartilages, including the perichondrium and in hypertrophic chondrocytes, for example, the ceratohyal (Fig. 4G, 6 dpf). In contrast to *pthlha*, expression of *pthlhb* was not generally associated with the development of brain, spinal cord, pancreas, or teeth but showed weak expression in the eye (Fig. 4B, 53 hpf), hypertrophic chondrocytes of the hyosymplectic and opercular (Fig. 4D and F, 4 dpf), and in the proximity of the thymus (Fig. 4H, 4 dpf; *rag1*-labeled cRNA probe, control).

The region around the pharynx gives rise to not only elements of the craniofacial skeleton but also to the thymus, thyroid, and in terrestrial vertebrates to parathyroid glands, so we asked whether the pharyngeal *pthlhb* domain included any of these organs. The *pthlhb* expression domain in the pharyngeal arch region is unlikely to be the thyroid because in fish embryos the thyroid develops as an unpaired structure in the midline (Rohr & Concha 2000, Alt *et al*. 2006) rather than as a paired organ in the arches like the *pthlhb* expression domain (Figs. 3J and L, 4H).

To ask whether the pharyngeal domain of *pthlhb*-expressing cells is related to the thymus, we compared the expression pattern of *pthlhb* to that of the thymus marker *rag1* (Willett *et al*. 1997). Results showed that the *rag1* domain was more ventrally and centrally located than the *pthlhb* domain (Figs. 4F, H and 5E, I, M, Q). Sections showed that, while the *rag1* domain was in the endoderm (Fig. 4H), the *pthlhb* domain was expressed at the posterior border of the hyosymplectic and opercular (Fig. 4D and F) ruling out the possibility that *pthlhb* expression labels thymus cells.

Fish lack anatomically distinct parathyroid glands, which is a major difference between the teleost and tetrapod endocrine systems (Wendelaar Bonga & Pang 1991). Furthermore, in

zebrafish, *pth1* and *pth2* are not expressed in cells likely to correspond to a gland equivalent to a pharyngeal-derived parathyroid but are expressed in early sites of skeletal calcification (Hanaoka *et al*. 2004, Hogan *et al*. 2004). Thus, although the *pthlhb*-positive domain is approximately in the position expected for parathyroid gland cells, it is unlikely to represent the parathyroid.

Regulatory interactions of Sox9 and Pthlh

Because zebrafish *sox9* genes are essential for pharyngeal arch development (Yan *et al*. 2005), we wondered whether this transcription factor is necessary for the development of *pthlh*-expressing cells of the arches. Zebrafish has two *Sox9* co-orthologs, *sox9a* and *sox9b*, that are derived from the TGD and are involved in different aspects of arch development (Chiang *et al*. 2001, Yan *et al*. 2005). To discern whether zebrafish *sox9a* or *sox9b* regulates *pthlh*-expressing cells, we evaluated *pthlh* gene expression in single mutants lacking function of one or the other *sox9* co-ortholog and in double mutants. Results showed that *pthlha* expression was diminished in the pharyngeal arches, sopc, and pectoral fin buds of *sox9a* mutants (Fig. 5A and B). We conclude that *sox9a* regulates *pthlha* expression in many craniofacial cartilages and in the pectoral fin (*sox9a→pthlha* in chondrocytes). This is consistent with the known role of *sox9a* in the development of osteochondroprogenitor cells, including the cartilaginous pharyngeal skeleton (Akiyama *et al*. 2005, Yan *et al*. 2005). By contrast, the expression of *pthlha* in the pancreas was unaffected (Fig. 5B). In *sox9b* mutants (Fig. 5C), *pthlha* expression was also reduced in the arches and, in addition, was reduced in the pancreas, a known target of *Sox9* action (Seymour *et al*. 2008). We conclude that *sox9b* rather than *sox9a* regulates pancreatic expression of *pthlha* (*sox9b→pthlha* in pancreas). In double mutants, most *pthlha* expression was eliminated (Fig. 5D). Thus, we conclude that *pthlha* is downstream of *sox9a* in some tissues and downstream of *sox9b* in other tissues.

Sox9 activity is also necessary for embryonic expression of *pthlhb*: the expression of *pthlhb* disappeared in *sox9a* but not in *sox9b* mutants (Fig. 5F, J, G and K respectively). We conclude that *sox9a* but not *sox9b* function is required for expression of the *pthlhb* domain in the pharyngeal arches and/or for the development of *pthlhb-expressing* cell types (*sox9a→pthlhb*). The thymus, as defined by *rag1* expression, was controlled by *sox9b* but not *sox9a* (Fig. 5O, S, N and R respectively). The finding that *sox9a* regulates the *pthlhb*-positive cells but that *sox9b* regulates the *rag1*-expressing cells (Fig. 5M, N, Q and R) is consistent with the interpretation that the *pthlhb*-expressing cells are a different cell type from the *rag1*-expressing cells (Fig. 5E, I, G and K). Overall, these results show that *sox9a* stimulates expression of *pthlha* and *pthlhb* (*sox9a→pthlha* and *pthlhb*) in chondrocytes.

The *sox9* mutants help to rule out possible identities of the small triangular patch of *pthlhb*-expressing cells. In *sox9a* mutants, the opercular bone develops normally (Yan *et al*. 2005) but the triangular patch of *pthlhb*-expressing cells is gone. By contrast, in *sox9b* mutants, the opercular is greatly reduced but the *pthlhb* domain is normal. This shows that *pthlhb* is not expressed in the opercular.

Evaluation of Pthlh knockdown phenotypes

To understand the roles of *pthlha* and *pthlhb* in development, we knocked down activity using antisense MOs directed against splice donor and acceptor sites. To assess the efficacy of knockdown, we isolated total RNA from control or morpholino-injected embryos and performed RT-PCR using genomic DNA as control. The *pthlha* splice-blocking MOs were targeted to the splice donor site of intron-2 (MO-p1e2i2) and the splice acceptor site of intron-2 (MO-p1i2e3), which should delete exon-2 and thus remove the signal sequence and hence block secretion. To check whether the MOs did indeed delete exon-2, we amplified cDNA using primers in exon-1 and exon-3 (Fig. 6A). For *pthlha*, MO injection blocked the

excision of intron-2 and caused the intron-1 donor site to splice directly to the intron-2 acceptor site, thereby deleting exon-2 (Fig. 6A, left panel). Because the quantity of the exon-2-deleted transcript was greater than the quantity of normal transcript, we concluded that knockdown of *pthlha* was substantial. For *pthlhb*, splice-blocking MOs were designed for the splice acceptor site of intron-1 (MO-p2i1e2) and the splice acceptor site of intron-2 (MO-p2e2i2). Efficacy of knockdown was checked using primers in intron-2 and in the 3′-UTR (Fig. 6B). RT-PCR analysis showed no amplification from RNA extracted from normal controls, as expected if the mature mRNA lacks intron-2, the site of one of the primers. By contrast, results showed the substantial amplification of a band of 586 bp from RNA isolated from MO-treated animals, as expected from an aberrant transcript that had failed to be spliced (Fig. 6B, left panel). We conclude that these MOs knocked down normal splicing of *pthlhb*, but they did not cause message degradation because whole-mount *in situ* hybridization experiments on *pthlha* and *pthlhb* MO-injected embryos at 24, 48, and 72 hpf showed that neither the *pthlha* nor the *pthlhb* MO altered the level of *pthlha* and *pthlhb* transcripts (Supplementary Fig. 2, see section on supplementary data given at the end of this article), supporting the finding of altered transcript by RT-PCR (Fig. 6A and B).

To examine the role of zebrafish *pthlh* co-orthologs during skeletal development, we studied their epistatic relationships with the skeletal regulatory genes *sox9* and *runx2b*. MOs were injected into one-cell embryos and developing embryos were evaluated for the expression of *sox9a*, *sox9b*, and *runx2b* (Fig. 6C, D, E, F, G, H, I, J and K). Control animals injected with a standard control MO gave no phenotype different from uninjected embryos. To guard against phenotypes caused by nonspecific effects involving cell death, animals were injected simultaneously with *pthlh* MOs and a MO directed against *p53* (Robu *et al*. 2007, Eisen & Smith 2008). The phenotype of double knockdown animals was the same as the *pthlh* single knockdowns, so we conclude that the *pthlh* MOs do not cause off-target effects mediated by p53-related cell death. In normally developing 2 dpf embryos, *pthlha* and *sox9a* expression overlaps in branchial arches and otic vesicles (Figs. 3G, I and 4C, G) while *sox9a* is expressed in the forebrain, dorsal hindbrain, branchial arches, otic vesicles, pectoral girdle, and limb buds (Fig. 6C), and *sox9b* is expressed in the tectum, retina, dorsal hindbrain, otic vesicle, branchial arches, and pectoral fin bud (Fig. 6D), as described previously (Chiang *et al*. 2001, Yan *et al*. 2005).

At 3 dpf, *pthlha* knockdown led to embryos that had slightly altered *sox9a* expression in the branchial arches, otic vesicle, and pectoral fin bud (Fig. 6F) and significantly reduced *sox9b* expression in the tectum, retina, dorsal hindbrain, branchial arches, and pectoral fin bud (Fig. 6G). Knockdown of *pthlhb* led to 2 dpf embryos with substantially reduced *sox9a* expression (most notably in pharyngeal arch 1 and 2, Fig. 6I) and *sox9b* expression (retina and branchial arches, Fig. 6J). We conclude that *pthlha* and *pthlhb* play a significant role in the regulation of *sox9a* and *sox9b* at 3 dpf during craniofacial skeleton development.

Sox9 is a major regulator of chondrogenesis while *Runx2* is a major regulator of tooth and bone development (D'Souza *et al*. 1999, Kim *et al*. 1999), and in zebrafish, *runx2b* is expressed in bone-forming elements (Flores *et al*. 2006). Morpholino knockdown of *pthlha* produced animals that overexpressed *runx2b* in skeletal elements throughout the craniofacial region (Fig. 6H). We conclude that *pthlha* inhibits or delays *runx2b* (*pthlha* –|| *runx2b*). Similarly, morpholino knockdown of *pthlhb* produced animals with altered craniofacial morphology and overexpression of *runx2b* in skeletal elements and teeth (Fig. 6K). Both endochondral (palatoquadrate, ceratohyal, ceratobranchials-1 to -4, and especially the tooth-bearing ceratobranchial-5) and dermal bones (opercle and parasphenoid, Mabee *et al*. (2000)) showed enhanced expression of *runx2b*. We conclude that *pthlha* action normally inhibits *runx2b* expression in both endochondral and dermal bones and that MO knockdown of primarily *pthlha* (and to an extent *pthlhb*, Fig. 6K) relieves that inhibition.

To understand the role of *pthlha* and *pthlhb* during cartilage and bone development, we stained *pthlha*- and *pthlhb*-knockdown animals with alcian blue for cartilage and alizarin red for mineralized bone. Results showed that 6 dpf *pthlha* knockdown animals (Fig. 7B) had significantly more alizarin bone staining than controls in the bone collar of the ceratohyal, palatoquadrate, first ceratobranchial, and hyomandibular (Fig. 7A and B, arrows). We conclude that *pthlha* action inhibits or delays bone mineralization (*pthlha* –|| bone mineralization). Knockdown of *pthlhb* (Fig. 7C) caused under- and deformed development of the ceratobranchials and ceratohyals with minimal additional alizarin staining on the palatoquadrate and hyomandibular as controls (Fig. 7A). Double knockdown of *pthlha* and *pthlhb* (Fig. 7D) caused substantial deformity along with more alizarin staining than controls. These results are comparable to the murine *Pthlh* knockout mutants showing premature bone formation (Karaplis *et al*. 1994). Thus, the finding that *pthlha* knockdown led to premature bone deposition is consistent with the hypothesis that the function of *Pthlh* is conserved between zebrafish and mammals and is necessary for chondrogenesis.

Discussion

The zebrafish genome contains two co-orthologs of the human *PTHLH* gene with *pthlha* showing more similarity than *pthlhb* to human *PTHLH* (Fig. 1A). In mammals, PTHLH is associated with chondrogenesis and development of the pancreas, the craniofacial skeleton, and other tissues (Philbrick *et al*. 1996). One current hypothesis is that in teleosts, Pthlh is an endocrine hormone that regulates serum calcium homeostasis and osmoregulation. This hypothesis is supported because teleost serum shows high plasma Pthlh levels in heterologous RIAs (Abbink & Flik 2007) while Pthlh levels are typically low in mammalian plasma. Our hypothesis is that, because Pthlh is expressed during early development, zebrafish Pthlh may have a dual role as a paracrine hormone that is necessary for chondrogenesis and osteogenesis, similar to mammals, and as a circulating hormone for serum calcium homeostasis and osmoregulation.

The partitioning of ancestral subfunctions appears to have occurred in Pthlh co-orthologs (Force *et al*. 1999, Postlethwait 2007). For example, the NLS of human PTHLH appears to be more strongly conserved in Pthlha than in Pthlhb (Henderson *et al*. 1995, Nguyen & Karaplis 1998). Furthermore, Pthlha showed considerable conservation in the N-terminus, mid-region, NLS/RNA-binding region, and C-terminus compared with Pthlhb (Miao *et al*. 2008, Toribio *et al*. 2010). This situation might be expected under the hypothesis that Pthlha has a conserved functional role in the development of bone and other tissues compared with Pthlhb.

Our phylogenetic analysis showed that proteins encoded by human *PTHLH*, *PTH*, and *PTH2* form a clade with the relationship ((Pthlh, PTH) PTH2). Analyses of conserved syntenies show that the chromosomal paralogons in which these genes are embedded are as expected if they arose in two rounds of whole genome duplication that occurred at the base of the vertebrate radiation (Dehal & Boore 2005). The fourth predicted paralog has been lost, which is the most common fate of one member of a pair of duplicated genes, but paralogon analysis suggests that it would likely have been in Hsa1 or Hsa7 (Fig. 1B and C). We conclude that the pre-duplication ancestor to all vertebrates had a single gene that may have had many of the functions of these three genes today, but that after the vertebrate genome duplications, these genes diversified in function.

Zebrafish have two copies of many human genes that regulate chondrogenesis and craniofacial patterning (for example, *RUNX2*, *SOX9*, *IHH*, *BMP2*, and *MSX2*; Ekker *et al*. 1997, Martinez-Barbera *et al*. 1997, Yan *et al*. 2005, Avaron *et al*. 2006, Flores *et al*. 2006), and now, as we describe here, *PTHLH*. These paralogs arose in the TGD (R3) that occurred

at the base of the teleost radiation (Amores *et al*. 1998, Postlethwait *et al*. 2000, Jaillon *et al*. 2004, Taylor & Raes 2004, Nakatani *et al*. 2007). The TGD produced a pair of zebrafish PTH co-orthologs (Gensure *et al*. 2004) and a pair of Pthlh co-orthologs that today reside in duplicated chromosome regions.

To evaluate the developmental roles of Pthlha and Pthlhb during chondrogenic and osteogenic developmental programs, we characterized their amino acid composition and conservation, their expression in wild-type and *sox9*-deficient embryos (Yan *et al*. 2005, Flores *et al*. 2006), and their functions by analysis of knockdown animals.

Zebrafish *Pthlh* co-orthologs were expressed in the developing teeth, pancreas, spinal cord, bone, and cartilage, similar to that of their mammalian orthologs (Clemens *et al*. 2001), demonstrating a conservation for 450 million years. In the 300 million years since the TGD, *pthlha* and *pthlhb* evolved significantly different embryonic expression patterns. The specialization of *pthlhb* is especially notable because its embryonic expression is restricted to chondrocytes at the posterior border of the hyosymplectic and opercular (Fig. 4D and F), while *pthlha* is expressed in many elements of the pharyngeal skeleton including the ceratobranchials, ceratohyal, and teeth (Figs 3I, M and 4C, G, K). These results show that the *pthlh* paralog that maintained the most ancestral expression domains (*pthlha*) is also the one that preserved the most ancestral protein coding domains.

In tetrapods, Ihh secreted by maturing chondrocytes stimulates surrounding perichondrial osteoblasts to secrete Pthlh (Inada *et al*. 1999). Pthlh diffuses back to prehypertrophic chondrocytes, where, by reciprocal signaling, it binds a receptor (Pth1r or Pth3r), thereby blocking further chondrocyte maturation. Chondroblasts thus continue to proliferate and do not develop to the Ihh-secreting stage, thereby completing a hypothesized negative feedback loop (Vortkamp *et al*. 1996). Pthlh may slow chondrocyte maturation by inhibiting *Runx2* expression via Creb1 (Iwamoto *et al*. 2003, Li *et al*. 2004). Although the process is not yet fully understood, our data show that in *pthlha* knockdown zebrafish embryos, *runx2b* transcript is upregulated, which is consistent with the hypothesis, and that *pthlha* knockdown leads to premature alizarin staining in the ceratohyal bone collar, suggesting that Pthlha in zebrafish is necessary to slow chondrocyte maturation. Combined, our results suggests that the role of *pthlha* in zebrafish chondrogenic and osteogenic pathways conserves the role of *Pthlh* in mammals.

Although previous studies observed *Sox9* expression during retinogenesis (Poché *et al*. 2008, Yokoi *et al*. 2009), the robust expression of *Pthlh* in the retina is a novel finding. Furthermore, *Pthlhb* expression during retinogenesis was significantly reduced in zebrafish *sox9b* mutants, which suggests that retinal expression of Pthlh may be under the control of *Sox9* (Fig. 7F, G, I and J). The recent finding that Sox9 is important for development of the retina in teleosts and tetrapods suggests the hypothesis that Sox9 may exert its effect in eye development at least in part via Pthlh (Poché *et al*. 2008, Yokoi *et al*. 2009). Because zebrafish Pthlh co-orthologs showed discrete tissue-specific expression, it is likely that additional novel Pthlh functions may be observed in zebrafish.

The data clearly suggest that *pthlh* is necessary during the development of neural crest cell-derived craniofacial endochondrogenesis (Figs. 6 and 7). Furthermore, zebrafish *Pthlh* knockdown animals showed a reduction in *osx* expression in the neurocranium, pharyngeal arches, and pectoral girdle similar to that in humans with campomelic dysplasia (Supplementary Fig. 3, see section on supplementary data given at the end of this article; Yan *et al*. 2002). The regulation of *sox9* by Pthlh showed considerable conservation between zebrafish and mammals. The increased expression of *runx2b* in knockdown Pthlha animals (Fig. 7) suggests that Pthlh may have a direct role in downregulating *runx2b* expression. Our

data are consistent with the hypothesis that Pthlh decreases the expression of *runx2*, which then retards bone mineralization. In the *pthlha* knockdown animals, *runx2a* is upregulated, which leads to premature bone mineralization.

In summary, these studies identified the anciently duplicated teleost orthologs of mammalian Pthlh. Functional studies using mutants and morpholino knockdowns showed that zebrafish *pthlh* duplicates responded differently to their upstream regulators *sox9a* and *sox9b* and a downstream target *runx2b* (Supplementary Fig. 4, see section on supplementary data given at the end of this article). These studies lay the groundwork for teasing apart the further functional roles of Pthlh in vertebrate development. Future studies to evaluate the developmental pathways associated with Pthlha and Pthlhb proteins may yield information that would be useful to understand the general rules governing the evolution of duplicated genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abbink W, Flik G. Minireview: parathyroid hormone-related protein in teleost fish. General and Comparative Endocrinology. 2007; 152:243–251.10.1016/j.ygcen.2006.11.010 [PubMed: 17188690]

Akiyama H, Kim J-E, Nakashima K, Balmes G, Iwai N, Deng J, Zhang Z, Martin J, Behringer R, Nakamura T, et al. Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. PNAS. 2005; 102:14665–14670.10.1073/pnas.0504750102 [PubMed: 16203988]

Albertson R, Payne-Fereira T, Postlethwait J, Yelick P. Zebrafish acvr2a and acvr2b exhibit distinct roles in craniofacial development. Developmental Dynamics. 2005; 233:1405–1418.10.1002/dvdy.20480 [PubMed: 15977175]

Alt B, Reibe S, Feitosa N, Elsalini O, Wendl T, Rohr K. Analysis of origin and growth of the thyroid gland in zebrafish. Developmental Dynamics. 2006; 235:1872–1883.10.1002/dvdy.20831 [PubMed: 16680726]

Amores A, Force A, Yan Y-L, Joly L, Amemiya C, Fritz A, Ho R, Langeland J, Prince V, Wang Y-K, et al. Zebrafish *hox* clusters and vertebrate genome evolution. Science. 1998; 282:1711–1714.10.1126/science.282.5394.1711 [PubMed: 9831563]

Avaron F, Hoffman L, Guay D, Akimenko M. Characterization of two new zebrafish members of the hedgehog family: atypical expression of a zebrafish *Indian hedgehog* gene in skeletal elements of both endochondral and dermal origins. Developmental Dynamics. 2006; 235:478–489.10.1002/dvdy.20619 [PubMed: 16292774]

Bhattacharya P, Yan Y-L, Postlethwait J, Ruibn D. 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. Journal of Endocrinology. 2011; 211:187–200.10.1530/JOE-10-0439 [PubMed: 21880859]

Catchen J, Conery J, Postlethwait J. Automated identification of conserved synteny after whole-genome duplication. Genome Research. 2009; 19:1497–1505.10.1101/gr.090480.108 [PubMed: 19465509]

Chiang EF-L, Pai C-I, Wyatt M, Yan Y-L, Postlethwait J, Chung B-C. Two Sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. Developmental Biology. 2001; 231:149–163.10.1006/dbio.2000.0129 [PubMed: 11180959]

Clemens T, Cormier S, Eichinger A, Endlich K, Fiaschi-Taesch N, Fischer E, Friedman P, Karaplis A, Massfelder T, Rossert J, et al. Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. British Journal of Pharmacology. 2001; 134:1113–1136.10.1038/sj.bjp.0704378 [PubMed: 11704631]

Danks J, Ho P, Notini A, Katsis F, Hoffmann P, Kemp B, Martin T, Zajac J. Identification of a parathyroid hormone in the fish *Fugu rubripes*. Journal of Bone and Mineral Research. 2003; 18:1326–1331.10.1359/jbmr.2003.18.7.1326 [PubMed: 12854844]

Dehal P, Boore J. Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biology. 2005; 3:e314.10.1371/journal.pbio.0030314 [PubMed: 16128622]

Draper B, Morcos P, Kimmel C. Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. Genesis. 2001; 50:154–156.10.1002/gene.1053 [PubMed: 11477696]

D'Souza R, Aberg T, Gaikwad J, Cavender A, Owen M, Karsenty G, Thesleff I. Cbfa1 is required for epithelial–mesenchyme interactions regulating tooth development in mice. Development. 1999; 126:2911–2920. [PubMed: 10357935]

Eisen J, Smith J. Controlling morpholino experiments: don't stop making sense. Development. 2008; 135:1735–1743.10.1242/dev.001115 [PubMed: 18403413]

Ekker M, Akimenko M, Allende M, Smith R, Drouin G, Langille R, Weinberg E, Westerfield M. Relationships among msx gene structure and function in zebrafish and other vertebrates. Molecular Biology and Evolution. 1997; 14:1008–1022.10.1093/oxfordjournals.molbev.a025707 [PubMed: 9335141]

Flanagan JA, Power DM, Bendell LA, Guerreiro PM, Fuentes J, Clark MS, Canario AVM, Danks JA, Brown BL, Ingleton PM. Cloning of the cDNA for the Sea Bream (*Sparus aurata*) parathyroid hormone-related protein. General and Comparative Endocrinology. 2000; 118:373–382.10.1006/gcen.2000.7481 [PubMed: 10843788]

Flores M, Lam E, Crosier P, Crosier K. A hierarchy of runx transcription factors modulate the onset of chondrogenesis in craniofacial endochondral bones in zebrafish. Developmental Dynamics. 2006; 235:3166–3176.10.1002/dvdy.20957 [PubMed: 17013873]

Force A, Lynch M, Pickett FB, Amores A, Yan Y-L, Postlethwait J. Preservation of duplicate genes by complementary, degenerative mutations. Genetics. 1999; 151:1531–1545. [PubMed: 10101175]

Garcia-Fernandez J, Holland P. Archetypal organization of the amphioxus *Hox* gene cluster. Nature. 1994; 370:563–566.10.1038/370563a0 [PubMed: 7914353]

Gardella T, Jüppner H. Interaction of PTH and PTHrP with their receptors. Reviews in Endocrine & Metabolic Disorders. 2000; 1:317–329.10.1023/A:1026522619828 [PubMed: 11706746]

Gensure R, Ponugoti B, Gunes Y, Papasani M, Lanske B, Bastepe M, Rubin D, Jüppner H. Identification and characterization of two parathyroid hormone-like molecules in zebrafish. Endocrinology. 2004; 145:1634–1639.10.1210/en.2003-0964 [PubMed: 14684608]

Guindon S, Lethiec F, Duroux P, Gascuel O. PHYML Online – a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Research. 2005; 33:W557–W559.10.1093/nar/gki352 [PubMed: 15980534]

Hanaoka R, Ohmori Y, Uyemura K, Hosoya T, Hotta Y, Shirao T, Okamoto H. Zebrafish *gcmb* is required for pharyngeal cartilage formation. Mechanisms of Development. 2004; 121:1235–1247.10.1016/j.mod.2004.05.011 [PubMed: 15327784]

Henderson J, Amizuka N, Warshawsky H, Biasotto D, Lanske B, Goltzman D, Karaplis A. Nucleolar localization of parathryoid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. Molecular and Cellular Biology. 1995; 15:4064–4075. [PubMed: 7623802]

Hogan B, Hunter M, Oates A, Crowhurst M, Hall N, Heath J, Prince V, Lieschke G. Zebrafish *gcm2* is required for gill filament budding from pharyngeal ectoderm. Developmental Biology. 2004; 276:508–522.10.1016/j.ydbio.2004.09.018 [PubMed: 15581882]

Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, et al. Maturational disturbance of chondrocytes in *Cbfa1*-deficient mice. Developmental Dynamics. 1999; 214:279–290.10.1002/(SICI)1097-0177(199904)214:4<279::AID-AJA1>3.0.CO;2-W [PubMed: 10213384]

Ingleton P, Bendell L, Flanagan J, Teitsma C, Malment R. Calcium-sensing receptor and parathyroid hormone-related protein in the caudal neurosecretory system of the flounder (*Platichthys flesus*). Journal of Anatomy. 2002; 200:487–497.10.1046/j.1469-7580.2002.00036.x [PubMed: 12090395]

Iwamoto M, Kitagaki J, Tamamura Y, Gentili C, Koyama E, Enomoto H, Komori T, Pacifici M, Enomoto-Iwamoto M. Runx2 expression and action in chondrocytes are regulated by retinoid signaling and parathyroid hormone-related peptide (PTHrP). Osteoarthritis and Cartilage. 2003; 11:6–15.10.1053/joca.2002.0860 [PubMed: 12505482]

Jaillon O, Aury J, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A. Genome duplication in the teleost fish *Tetradon nigroviridis* reveals the early vertebrate proto-karyotype. Nature. 2004; 431:946–957.10.1038/nature03025 [PubMed: 15496914]

Karaplis AC, Luz A, Glowacki J, Bronson R, Tybulewicz V, Kronenberg HM, Mulligan RC. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes and Development. 1994; 8:277–289.10.1101/gad.8.3.277 [PubMed: 8314082]

Karperien MC, Lanser P, de Laat SW, Boonstra J, Defize LHK. Parathyroid hormone related peptide mRNA expression during murine postimplantation development: evidence for involvement in multiple differentiation processes. International Journal of Developmental Biology. 1996; 40:599–608. [PubMed: 8840192]

Kim I, Otto F, Zabel B, Mundlos S. Regulation of chondrocyte differentiation by Cbfa1. Mechanisms of Development. 1999; 80:159–170.10.1016/S0925-4773(98)00210-X [PubMed: 10072783]

Lagerström M, Hellström A, Gloriam D, Larsson T, Schiöth H, Fredriksson R. The G protein-coupled receptor subset of the chicken genome. PLoS Computational Biology. 2006; 2:e54.10.1371/journal.pcbi.0020054 [PubMed: 16741557]

Lee K, Deeds JD, Segre GV. Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acid during fetal development of rats. Endocrinology. 1995; 136:453–463.10.1210/en.136.2.453 [PubMed: 7835276]

Li T-F, Dong Y, Ionescu A, Rosier R, Zuscik M, Schwarz E, O'Keefe R, Drissi H. Parathyroid hormone-related peptide (PTHrP) inhibits Runx2 expression through the PKA signaling pathway. Experimental Cell Research. 2004; 299:128–136.10.1016/j.yexcr.2004.05.025 [PubMed: 15302580]

Mabee P, Olmstead K, Cubbage C. An experimental study of intraspecific variation, developmental timing, and heterochrony in fishes. Evolution. 2000; 54:2091–2106. [PubMed: 11209785]

Martinez-Barbera J, Toresson H, Rocha SD, Krauss S. Cloning and expression of three members of the zebrafish Bmp family: Bmp2a, Bmp2b and Bmp4. Gene. 1997; 198:53–59.10.1016/S0378-1119(97)00292-8 [PubMed: 9370264]

Miao D, Su H, He B, Gao J, Xia Q, Zhu M, Gu Z, Goltzman D, Karaplis A. Severe growth retardation and early lethality in mice lacking the nuclear localization sequence and C-terminus of PTH-related protein. PNAS. 2008; 105:20309–20314.10.1073/pnas.0805690105 [PubMed: 19091948]

Nakatani Y, Takeda H, Kohara Y, Morishita S. Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. Genome Research. 2007; 17:1254–1265.10.1101/gr.6316407 [PubMed: 17652425]

Nguyen M, Karaplis A. The nucleus: a target site for parathyroid hormone-related peptide (PTHrP) action. Journal of Cellular Biochemistry. 1998; 70:193–199.10.1002/(SICI)1097-4644(19980801)70:2<193::AID-JCB5>3.0.CO;2-J [PubMed: 9671225]

Orloff JJ, Ganz MB, Nathanson H, Moyer MS, Kats Y, Mitnick M, Behal A, Gasalla-Herraiz J, Isales CM. A midregion parathyroid hormone-related peptide mobilizes cytosolic calcium and stimulates formation of inositol trisphosphate in a squamous carcinoma cell line. Endocrinology. 1996; 137:5376–5385.10.1210/en.137.12.5376 [PubMed: 8940360]

Philbrick WM, Wysolmerski JJ, Galbraith S, Holt E, Orloff JJ, Yang KH, Vasavada RC, Weir EC, Broadus AE, Stewart AF. Defining the roles of parathyroid hormone-related protein in normal physiology. Physiological Reviews. 1996; 76:127–173. [PubMed: 8592727]

Philbrick WM, Dreyer BE, Nakchbandi IA, Karaplis AC. Parathyroid hormone-related protein is required for tooth eruption. PNAS. 1998; 95:11846–11851.10.1073/pnas.95.20.11846 [PubMed: 9751753]

Pinheiro P, Cardoso J, Gomes A, Fuentes J, Power D, Canario A. Gene structure, transcripts and calciotropic effects of the PTH family of peptides in *Xenopus* and chicken. BMC Evolutionary Biology. 2010; 10:373.10.1186/1471-2148-10-373 [PubMed: 21122104]

Poché R, Furuta Y, Chaboisser M, Schedl A, Behringer R. Sox9 is expressed in mouse multipotent retinal progenitor cells and functions in Müller glial cell development. Journal of Comparative Neurology. 2008; 510:237–250.10.1002/cne.21746 [PubMed: 18626943]

Postlethwait J. The zebrafish genome in context: ohnologs gone missing. Journal of Experimental Zoology. 2007; 308B:563–577.10.1002/jez.b.21137 [PubMed: 17068775]

Postlethwait J, Woods I, Ngo-Hazelett P, Yan Y-L, Kelly P, Chu F, Huang H, Hill-Force A, Talbot W. Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Research. 2000; 10:1890–1902.10.1101/gr.164800 [PubMed: 11116085]

Provot S, Schipani E. Molecular mechanisms of endochondral bone development. Biochemical and Biophysical Research Communications. 2005; 328:658–665.10.1016/j.bbrc.2004.11.068 [PubMed: 15694399]

Robu M, Larson J, Nasevicius A, Beiraghi S, Brenner C, Farber S, Ekker S. p53 activation by knockdown technologies. PLoS Genetics. 2007; 3:e78 0787–0801.10.1371/journal.pgen.0030078 [PubMed: 17530925]

Rodriguez-Mari A, Yan Y, Bremiller R, Wilson C, Canestro C, Postlethwait J. Characterization and expression pattern of zebrafish anti-Müllerian hormone (Amh) relative to sox9a, sox9b, and cyp19a1a, during gonad development. Gene Expression Patterns. 2005; 5:655–667.10.1016/j.modgep.2005.02.008 [PubMed: 15939378]

Rohr K, Concha M. Expression of *nk2.1a* during early development of the thyroid gland in zebrafish. Mechanisms of Development. 2000; 95:267–270.10.1016/S0925-4773(00)00345-2 [PubMed: 10906475]

Rotllant J, Liu D, Yan Y-L, Postlethwait J, Westerfield M, Du S-J. Sparc (osteonectin) functions in morphogenesis of the pharyngeal skeleton and inner ear. Matrix Biology. 2008; 27:561–572.10.1016/j.matbio.2008.03.001 [PubMed: 18430553]

Sellers R, Luchin A, Richard V, Brena R, Lima D, Rosol T. Alternative splicing of parathyroid hormone-related protein mRNA: expression and stability. Journal of Molecular Endocrinology. 2004; 33:227–241.10.1677/jme.0.0330227 [PubMed: 15291755]

Seymour P, Freude K, Dubois C, Shih H, Patel N, Sander M. A dosage-dependent requirement for Sox9 in pancreatic endocrine cell formation. Developmental Biology. 2008; 323:19–30.10.1016/j.ydbio.2008.07.034 [PubMed: 18723011]

Shor R, Halabe A, Aberbuh E, Matas Z, Fux A, Boaz M, Wainstein J. PTHrP and insulin levels following oral glucose and calcium administration. European Journal of Internal Medicine. 2006; 17:408–411.10.1016/j.ejim.2006.02.019 [PubMed: 16962947]

Taylor J, Raes J. Duplication and divergence: the evolution of new genes and old ideas. Annual Review of Genetics. 2004; 38:615–643.10.1146/annurev.genet.38.072902.092831

Toribio R, Brown H, Novince C, Marlow B, Hernon K, Lanigan L, Hildreth B, Werbeck J, Shu S, Lorch G, et al. The mid-region, nuclear localization sequence, and C terminus of PTHrP regulate

skeletal development, hematopoiesis, and survival in mice. FASEB Journal. 2010; 24:1947–1957.10.1096/fj.09-147033 [PubMed: 20145205]

Trivett M, Officer R, Clement J, Walker T, Joss J, Ingleton P, Martin T, Danks J. Parathyroid hormone-related protein (PTHrP) in cartilaginous and bony fishes. Journal of Experimental Zoology. 1999; 284:541–548.10.1002/(SICI)1097-010X(19991001)284:5<541::AID-JEZ10>3.0.CO;2-3 [PubMed: 10469992]

Valin A, Guillen C, Esbrit P. C-terminal parathyroid hormone-related protein (PTHrP) (107–139) stimulates intracellular Ca^{2+} through a receptor different from the type 1 PTH/PTHrP receptor in osteoblastic osteosarcoma UMR 106 cells. Endocrinology. 2001; 142:2752–2759.10.1210/en.142.7.2752 [PubMed: 11415993]

Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science. 1996; 273:613–622.10.1126/science.273.5275.613 [PubMed: 8662546]

Walker M, Kimmel C. A two-color acid-free cartilage and bone stain for zebrafish larvae. Biotechnic & Histochemistry. 2007; 82:23–28.10.1080/10520290701333558 [PubMed: 17510811]

Wall D, Fraser H, Hirsh A. Detecting putative orthologs. Bioinformatics. 2003; 19:1710–1711.10.1093/bioinformatics/btg213 [PubMed: 15593400]

Weir EC, Philbrick WM, Amling M, Neff LA, Baron R, Broadus AE. Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes skeletal dysplasia and delayed endochondral bone formation. PNAS. 1996; 93:10240–10245.10.1073/pnas.93.19.10240 [PubMed: 8816783]

Wendelaar Bonga SE, Pang P. Control of calcium regulating hormones in the vertebrates: parathyroid hormone, calcitonin, prolactin, and stanniocalcin. International Review of Cytology. 1991; 128:139–213. [PubMed: 1917377]

Westerfield, M. The Zebrafish Book: a Guide for the Laboratory Use of Zebrafish (Danio rerio). Eugene, Oregon: Univeristy of Oregon Press; 2000.

Willett C, Zapata A, Hopkins N, Steiner L. Expression of zebrafish rag genes during early development identifies the thymus. Developmental Biology. 1997; 182:331–341.10.1006/dbio.1996.8446 [PubMed: 9070331]

Wu TL, Vasavada RC, Yang K, Massfelder T, Ganz M, Abbas SK, Care AD, Stewart AF. Structural and physiological characterization of the mid-region secretory species of parathyroid hormone-related protein. Journal of Biological Chemistry. 1996; 271:24371–24381.10.1074/jbc.271.40.24371 [PubMed: 8798692]

Wysolmerski JJ, Broadus AE, Zhou J, Fuchs E, Milstone LM, Philbrick WM. Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. PNAS. 1994; 91:1133–1137.10.1073/pnas.91.3.1133 [PubMed: 7508121]

Yan Y-L, Miller C, Nissen R, Singer A, Liu D, Kirn A, Draper B, Willoughby J, Morcos P, Amsterdam A, et al. A zebrafish *sox9* gene required for cartilage morphogenesis. Development. 2002; 129:5065–5079. [PubMed: 12397114]

Yan Y-L, Willoughby J, Liu D, Crump J, Wilson C, Miller C, Singer A, Kimmel C, Westerfield M, Postlethwait J, et al. A pair of sox: distinct and overlapping functions of zebrafish *sox9* co-orthologs in craniofacial and pectoral fin development. Development. 2005; 132:1069–1083.10.1242/dev.01674 [PubMed: 15689370]

Yokoi H, Yan Y-L, Miller M, BreMiller R, Catchen J, Johnson E, Postlethwait J. Expression profiling of zebrafish sox9 mutants reveals that Sox9 is required for retinal differentiation. Developmental Biology. 2009; 329:1–15.10.1016/j.ydbio.2009.01.002 [PubMed: 19210963]

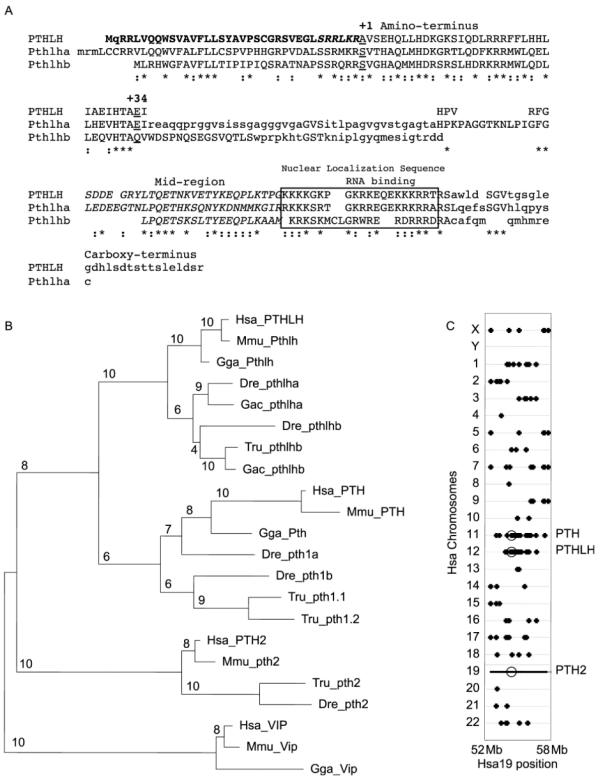

Figure 1.

Phylogenetic and conserved synteny analysis. (A) Human (M57293 and M32740) and zebrafish Pthlha (DQ022615) and Pthlhb (DQ022616) amino acid sequences aligned (upper case) as described (Bhattacharya *et al.* 2011). The first residue of human PTHLH is designated as +1 (underlined). The signal sequence and 'pro' peptides (residues to the left of +1) are bold. The Pthlh sequence alignments revealed a 41 amino acid gap of low sequence homology plus conserved regions including the N-terminus (1–34; 34th residue is underlined), nuclear localization sequence (boxed) containing the RNA-binding domain, and mid-region (uppercase italics). Because the zebrafish genome contains two Pthlh orthologs that differ in their conservation in these regions, they are useful natural variants to evaluate the roles different domains play during chondrogenic and osteogenic development. *Identical residues: conservative substitutions (Flanagan *et al.* 2000). (B) Phylogenetic analysis by maximum likelihood, maximum parsimony, and neighbor joining methods agreed in topology (maximum likelihood shown using JTT substitution model, four substitution rate categories, http://atgc.lirmm.fr/phyml/(Guindon *et al.* 2005). Bootstrap values are from ten replicates. Analyses suggest that PTH and Pthlh are sister groups and that PTH2 (alias TIP39) is the sister group to this super-clade. PTH, Pthlh, and PTH2 thus form a superfamily, whereas secretin falls as a sister group to this larger superfamily. (C) Dotplots mapping genes on Hsa19 from 52 to 58 Mb displayed along the horizontal axis to their orthologs on the other human chromosomes displayed as a dot on the proper chromosome along the vertical axis directly above each *Hsa19* gene. Circles indicate the locations of *PTH2* on Hsa19 and its paralogs *PTH* and *PTHLH* on human chromosomes Hsa11 and Hsa12 respectively. The PTH2 chromosome segment has paralogs mainly on Hsa11 and Hsa12. *Tetrapods*: chicken (*Gallus gallus*), Gga_Pth_M36522; human, Hsa_PTH_NM_000315; mouse, Mmu_Pth_NM_020623, Gga_Pthlh_X52131, Hsa_PTHLH_J03580, Mmu_Pthlh_M60056, Hsa_PTH2_NP_848544, Mmu_PTH2_NP_444486, Gga_Vip_AAA87896, Mmu_ Vip_BAB31301,

VIP_Hsa_NP_003372; *Fish*: zebrafish (*Danio rerio*), Dre_PTH1a_NM_212950, Dre_PTH1b_NM_212949, fugu Tru_pth1.1 (Danks *et al*. 2003), Tru_pth1.2_AAQ73561; Tru_pthlhb_AJ249391; stickleback (*Gasterosteus aculeatus*), Gac_pthlhb_ENSGACG00000004317, Gac_pthlha_ENSGACT00000000765, pthlha_Dre_NP_001019798, pthlhb_Dre_NP_001036789, Tru_pth2_T004305 Scaffold_4305, Dre_pth2_NP_991140.

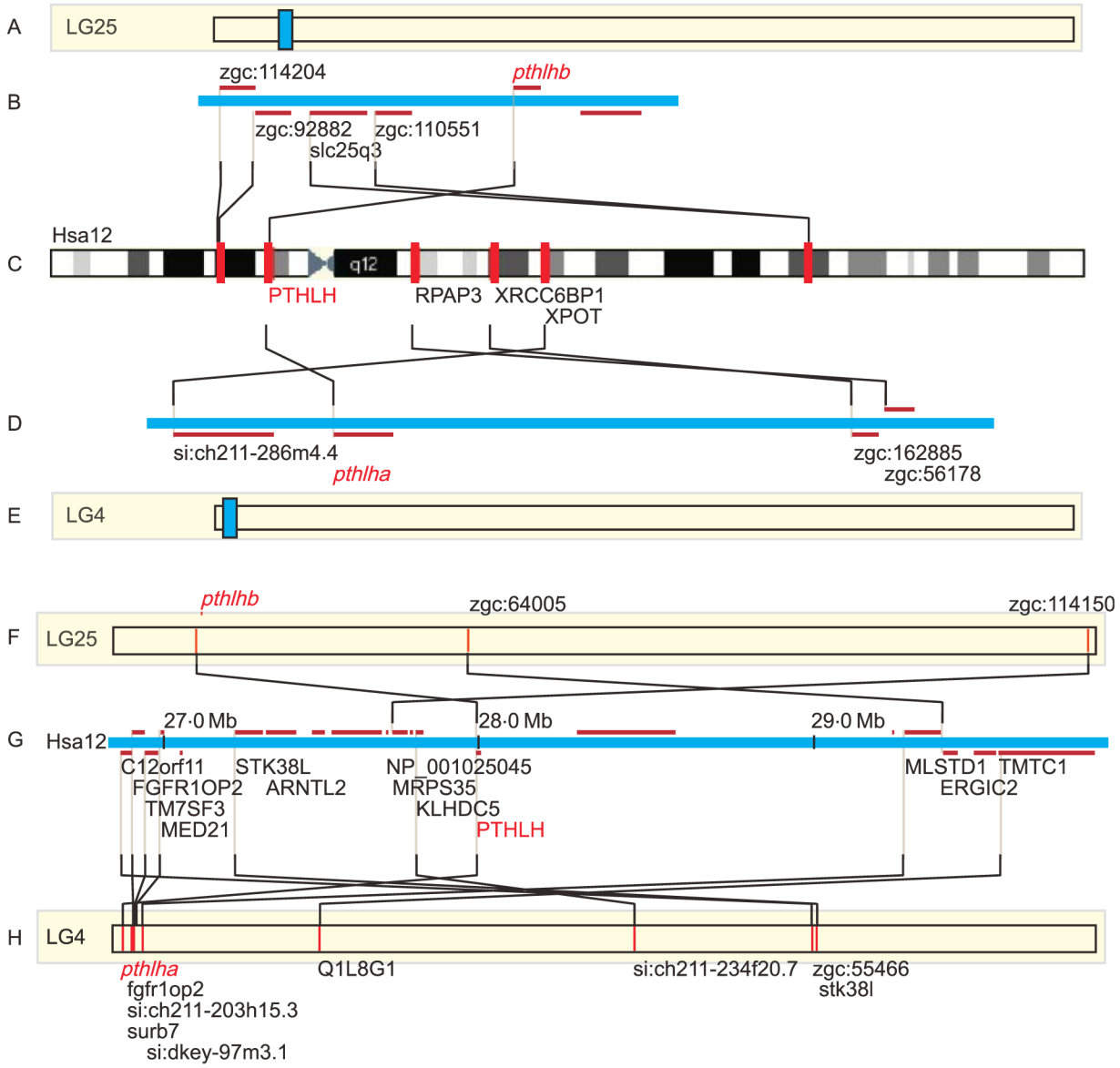

Figure 2.

Conserved syntenies for zebrafish *pthlha* and *pthlhb* genes. (A, E, F and H) Represent entire zebrafish chromosomes (Zv7). (B and D) Show all annotated genes (red horizontal lines) in the regions of the zebrafish chromosomes indicated in blue in A and E. (C) Shows an ideogram of human chromosome 12, with the locations of human orthologs of zebrafish genes shown as red bars. Lines link orthologs between the two species. (G) Shows the region of human chromosome 12p11.2 with all annotated genes indicated in red horizontal lines. Annotated sequences for which no zebrafish ortholog is called are in gray. Black lines link human genes to zebrafish orthologs. The ortholog of *ARNTL2* is on LG18.

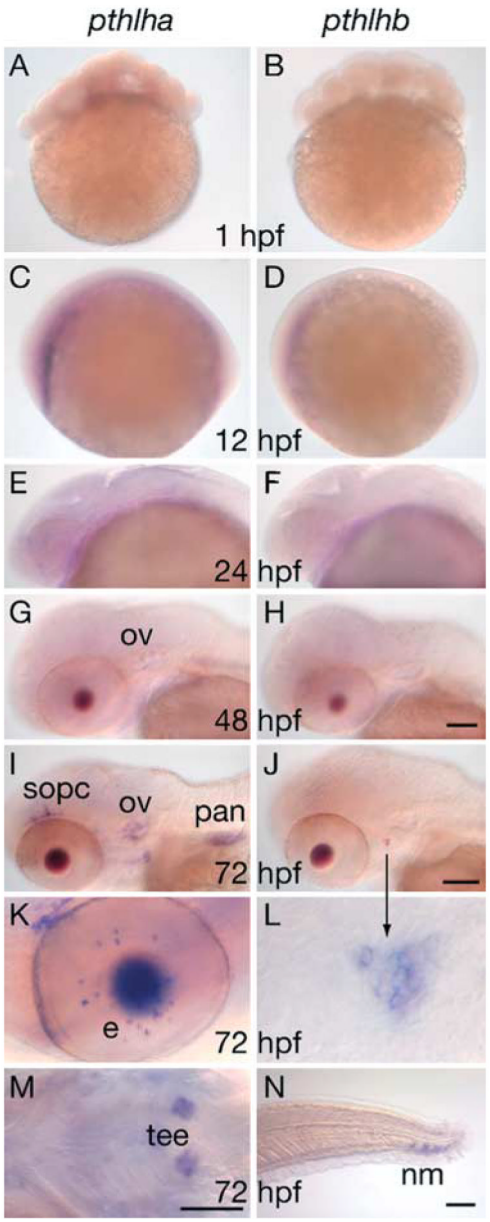

Figure 3.

Tissue-specific expression of zebrafish *pthlh* co-orthologs. Expression of *pthlha* and *pthlhb* assessed by whole-mount *in situ* hybridization (Pthlha panels A, C, E, G, I, K and M; Pthlhb panels B, D, F, H, J, L and N) assessed at 1, 12, 24, 48, and 72 hpf. Panels A and B show very low level of expression at the embryo–yolk interface for both *pthlh* mRNAs. The *pthlha* gene was expressed in the otic vesicle (ov, panels G and I); super optic cartilages (sopc), pancreas (pan, panel I), retina (e, panel K), and teeth (tee, panel M). The *pthlhb* gene was expressed in the pharyngeal arch region (panels J and L, arrow) and urophysis (neuromasts) of the tail (nm, panel N). Panels A, B, C, D, E, F, G, H, I and J, lateral view, anterior toward the left. All scale bars, 100 μm.

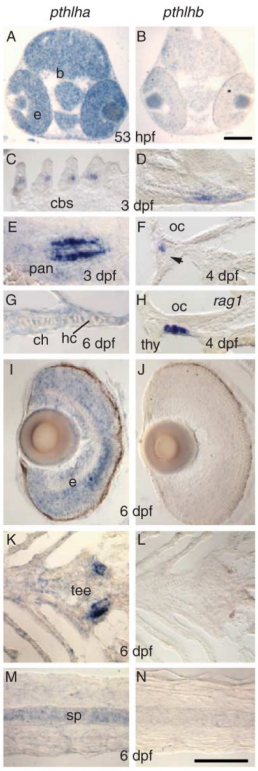

Figure 4.

Histological analysis of tissue-specific expression of zebrafish *pthlh* co-orthologs. Expression of *pthlha* and *pthlhb* transcripts in sections of zebrafish embryos (*pthlha* panels A, C, E, G, I, K and M; *pthlhb* panels B, D, F, H, J, L and N) at 53 hpf, 3, 4, and 6 dpf. Expression of *pthlha* was robust in the brain (b) and retina (e, A), ceratobranchial arch cartilages (cbs, C), pancreas endocrine cells (pan, E), hypertrophic chondrocytes (hc) of the ceratohyal cartilage (ch, G), eye (e, A, I), teeth (tee, K), and spinalcord (sp, M). Expression of *pthlhb* was slight in the eye (retina, B), chondrocytes at the posterior border of the hyosymplectic and opercular (Fig. 4D and F respectively), and the thymus region (thm, H; *rag1*-labeled cRNA probe, control); oc, opercular chondrocytes (F and H). All scale bars, 100 μm.

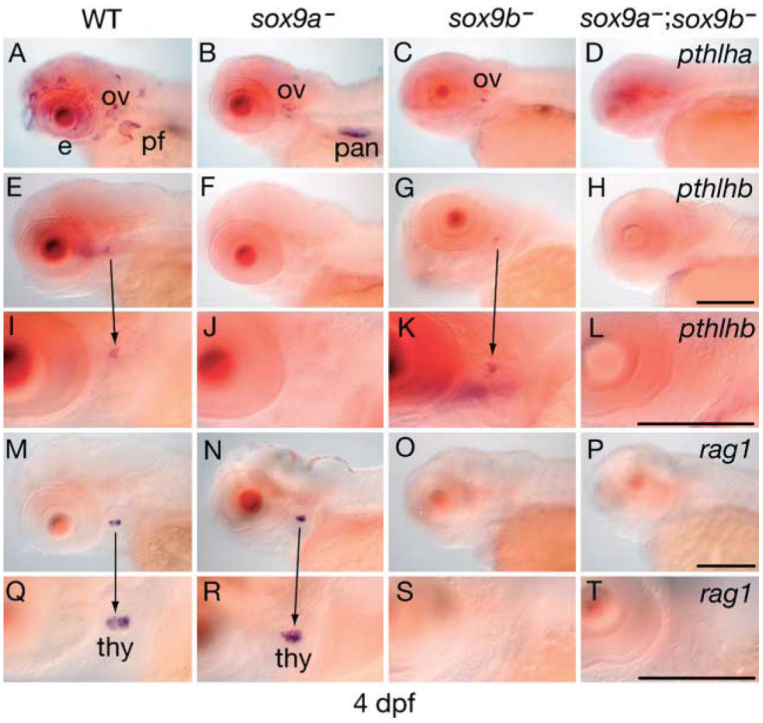

Figure 5.

pthlh expression in *sox9* mutants. Expression of *pthlha*, *pthlhb*, and *rag1* in WT, *sox9a*⁻ and *sox9b*⁻ single mutants, and *sox9a*⁻/*sox9b*⁻ double mutants in 4 dpf embryos. *pthlha* expression (A) was diminished in the pharyngeal arches, superoptic cartilages, and pectoral fin buds of *sox9a* (B) and *sox9b* mutants (C). In double mutants, most *pthlha* expression was eliminated (D). We conclude that *sox9a* regulates *pthlha* expression in many craniofacial cartilages and in the pectoral fin (*sox9a*→*pthlha* in chondrocytes) and that *pthlha* is downstream of *sox9a* in some tissues and downstream of *sox9b* in other tissues. *pthlhb* (and not *pthlha*) showed expression in the a distinctive domain about 20 cells in the pharyngeal arch region (E, I and G, K). *pthlhb* expression in that pharyngeal arch region is regulated by *sox9a* (F, J) while *rag1* expression in thymus is regulated by *sox9b* (O, S). The arrows show the hybridized region in a more amplified image below. Thus for example, the triangular region on panel E is shown in panel I at a greater magnification. Similarly the *rag1* expression in panel M is shown in panel Q at a greater magnification. e, retina; ov, otic vesicle; pan, pancreas; pf, pectoral fin; and thm, thymus. Anterior towards the left. Scale bars, 100 μm.

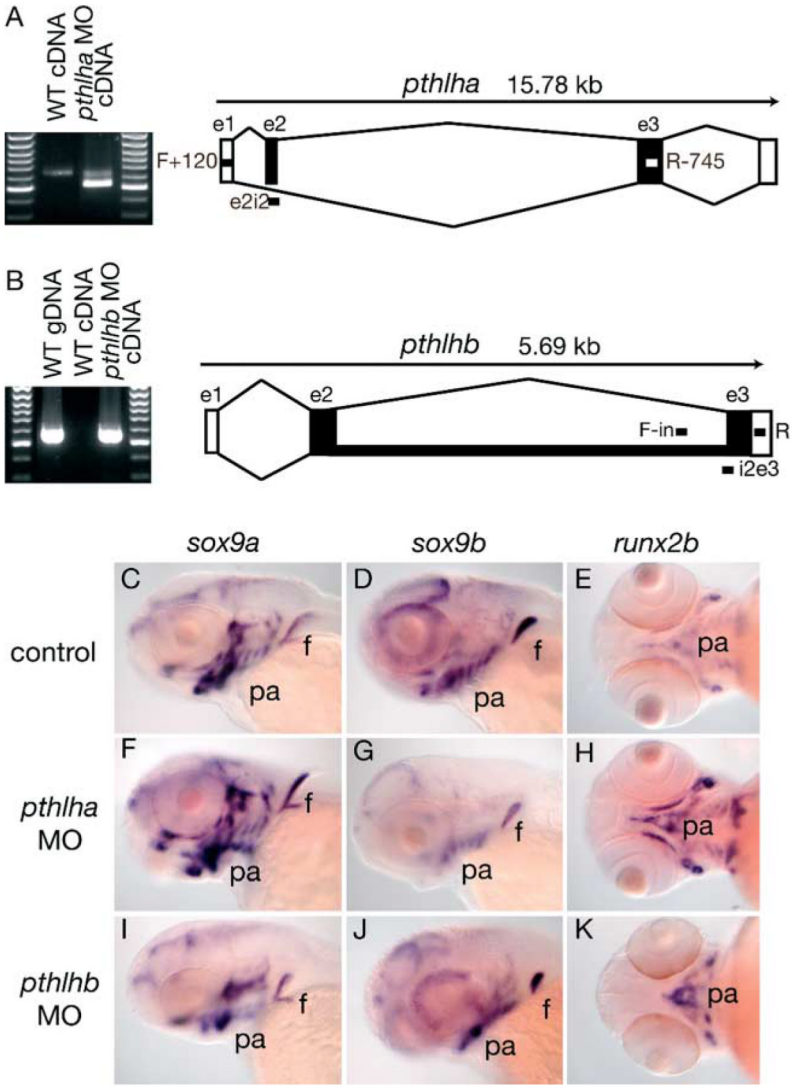

Figure 6.

Morpholino knockdown of *pthlha* and *pthlhb*. (A) *pthlha*; left, *pthlha* amplicon size difference between cDNAs isolated from WT and MO-treated embryos, right, schematic indicating exons 1–3 (boxes), introns (lines), primers (forward, F+120 and reverse, R−745), and splice-blocking MO (e2i2). Compared with WT, the *pthlha* morphant showed a significantly decreased amplicon size due to the elimination of exon-2 from the pre-mRNA. (B) *pthlhb*; left, *pthlhb* amplicon size difference between WT and MO-treated embryos. Right, schematic indicating the location of exons 1–3, primers (forward, F-in and reverse, R) and splice-blocking MO (i2e3). Compared with WT gDNA, the *pthlhb* morphant showed a similar amplicon size indicating the lack of intron excision in the mRNA whereas WT cDNA yielded no amplicon. (C, D, E, F, G, H, I, J and K) Regulation of *sox9* expression in *pthlha* knockdown embryos (C, F and I) and *pthlhb* knockdown embryos (D, G and J) at 3 dpf queried by *in situ* hybridization for *sox9a*, *sox9b*, and *runx2b* as indicated in the figure. C, D, F, G, I and J, lateral views. E, H and K, ventral views with anterior to the left. f, pectoral fin; pa, pharyngeal arches.

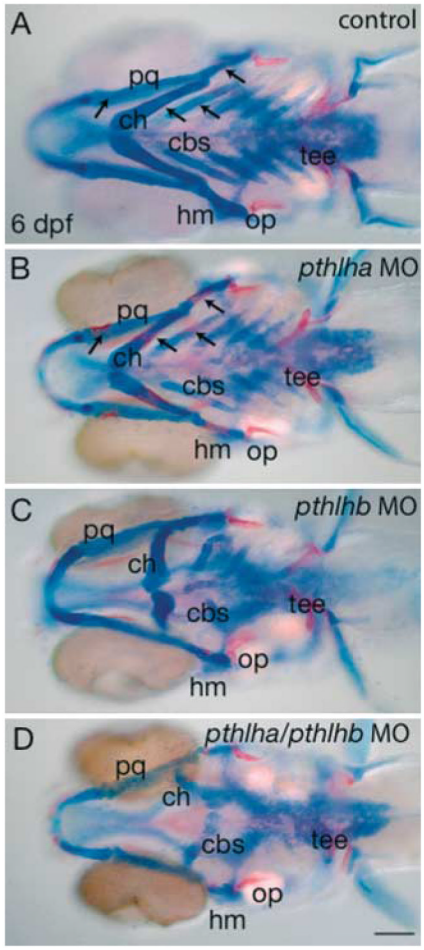

Figure 7.

Regulation of pharyngeal arch development by *Pthlh* co-orthologs at 6 dpf. 6 Dpf fish stained with alcian blue and alizarin red. *pthlha* MO-treated embryos (B) showed significantly more premature alizarin red staining (bone formation, arrows) compared with noninjected controls (A). *pthlhb* MO-treated embryos (C) showed deformed pharyngeal skeletons compared with noninjected controls (A) and *pthlha* knockdown (B). Double knockdown of *pthlha* and *pthlhb* (D) showed severe abnormality in the pharyngeal skeleton. cbs, ceratobranchials; ch, ceratohyal; hm, hyomandibular; op, opercular; pq, palatoquadrate; tee, teeth. Scale bar is 100 μm.