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Expression of the G protein gamma T1 subunit during zebrafish development

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Abstract

Here, we report the identification and expression analysis of the zebrafish G protein gamma T1 subunit gene (*gngT1*) during development. Similar to its human and mouse homologs, we confirm zebrafish *gngT1* is expressed in the developing retina, where its transcription overlaps with the photoreceptor cell-specific marker, *rhodopsin* (*rho*). Surprisingly, we also show zebrafish *gngT1* is expressed in the dorsal diencephalon, where its transcription overlaps with the pineal specific markers, *arylalkylamine N-acetyltransferase-2* (*annat-2*) and *extra-ocular rhodopsin* (*exorh*). Analysis of the proximal promoter sequence of the zebrafish *gngT1* gene identifies several conserved binding sites for the cone-rod homeobox/orthodenticle (Crx/Otx) homeodomain family of transcription factors. Using a morpholino antisense approach in zebrafish, we show that targeted knockdown of *otx5* potently suppresses *gngT1* expression in the pineal gland, whereas knockdown of *crx* markedly reduces *gngT1* expression in the retina. Taken together, these data indicate that pineal- and retinal-specific expression of the *gngT1* gene are controlled by different transcription factors and exogenous signals.

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Keywords

Zebrafish; G protein; *gngT1*; Signal transduction; Rhodopsin; Exo-rhodopsin; Pineal gland; Retina; Photoreceptor cells

1. Results and discussion

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are an evolutionarily conserved group of proteins that transduce signals from a plethora of cell surface receptors responding to a wide variety of hormones, neurotransmitters and sensory stimuli. Structurally, the G proteins are composed of equimolar amounts of α , β , γ subunits. Functionally, their unique signaling roles are generally ascribed to their α subtypes for which 16 genes have been identified. However, this assumption is being called into question with the discovery of a similar number and heterogeneity of γ subtypes for which 12 genes have been identified in the mouse and human genomes (Downes et al., 1999; Hurowitz et al., 2000; Robishaw and Berlot, 2004). Although the recent analyses of gene targeted mice lacking certain γ subunit genes have begun to highlight important functional differences (Schwindinger et al., 2003; Schwindinger et al., 2004), the vast majority of the γ subunit genes do not have assigned signaling roles more than a decade after their discovery (Robishaw and Berlot, 2004).

To better understand the functions of distinct G protein γ subtypes, we have begun studies to characterize the γ subunit genes expressed in zebrafish (Leung et al., 2006). In contrast to humans and mice, zebrafish offers a genetically tractable system to study the ontogeny of this important class of multi-subunit G proteins (Cheng et al., 2003). In this regard, the combined use of both *in situ* hybridization and reverse genetic techniques provides a powerful approach for the functional analysis of large multi-gene families expressed in this system (Leung et al., 2006). In this paper, we focus on the identification and expression analysis of the *gngT1* gene in zebrafish. Since this gene has not been previously identified outside mammals (Nordström et al., 2004), information on the spatial and temporal expression of the *gngT1* gene will provide a framework for future analyses of its functions in the context of the whole organism, such as zebrafish.

1.1 Zebrafish cDNA isolation and sequence analysis

The human *gngT1* sequence (Scherer et al., 1996) was used to search for related sequences in the zebrafish databases. As a result of this analysis, several EST fragments were identified. Using primers corresponding to these sequences (**NM199967**, **BI879345** and **BI846907**), RT-PCR was used to produce a complete zebrafish cDNA that was subjected to automated DNA sequence analysis. Translation of the open reading frame predicts a 73 amino acid polypeptide with high homology to several γ subunit genes. Based on their primary structures, the γ subunit genes have been divided into five subclasses, with each subclass showing less than 50% amino acid identity to members of other subclasses (Ray et al., 1995). Phylogenetic analysis (Fig. 1A) indicates that the zebrafish cDNA is most closely related to members of subclass I that exhibit a number of unique structural and biochemical properties that set them apart from the other subclasses (Balcueva et al., 2000). For instance, a characteristic feature of this subclass is the presence of a CAAX motif (where C=cysteine; A=aliphatic; and X=serine, threonine, or cysteine) that directs the addition of a farnesyl moiety to these proteins, thereby accounting for the unusual ability of this subclass to reversibly associate with the plasma membrane where signaling occurs (Ray et al., 1995; Balcueva et al., 2000). Notably, the newly identified zebrafish cDNA shares this motif (Fig. 1B).

Subclass I includes the mammalian *gngT1*, *gngT2*, and *gngI1* subtypes (Balcueva et al., 2000). Of these, only the *gngT2* subtype has been identified outside mammals (Thisse et al.,

2004; Nordström et al., 2004). Sequence comparisons indicate the newly identified zebrafish cDNA is clearly distinct from the zebrafish *gngT2*, with the predicted protein showing only 50.0% amino acid identity (Fig. 1B). Further comparisons reveal the zebrafish cDNA is most similar to the human *gngT1* and *gngI1*, with the predicted proteins showing 66.2 and 61.6% amino acid identity, respectively. Of the differences, seven residues are shared with the human $G\gamma_{T1}$ protein (purple residues in Fig. 1B) and three residues with the human $G\gamma_{I1}$ protein (yellow residues). Because of its close resemblance to both the human $G\gamma_{T1}$ and $G\gamma_{I1}$ subtypes, we cannot conclusively identify the zebrafish cDNA as one or the other subtype based on sequence analysis alone. Therefore, we examined the expression pattern of the zebrafish cDNA to provide a more definitive identification.

1.2. Tissue distribution of zebrafish mRNA

The mammalian *gngT1* is exclusively expressed in the eye, where it functions in phototransduction (Kisselev and Gautam 1993; Arshavsky et al., 2002), whereas the *gngI1* transcript is highly expressed in the heart, lung, and skeletal muscle, where its signaling role remains elusive (Balcueva et al., 2000). To determine the tissue distribution of the newly identified zebrafish cDNA, we first performed RT-PCR analysis on a panel of adult zebrafish tissues. The zebrafish transcript shows strong expression in the eye and weak expression in the brain and testis (Fig. 2). Notably, the zebrafish transcript is not detected in the heart where high expression of the mammalian *gngI1* transcript is observed (Balcueva et al., 2000). Taken together, the high sequence homology and the abundant expression in the eye identify the zebrafish cDNA as the homolog of the mammalian *gngT1* subunit gene. (By convention (<http://zfish.uoregon.edu/index.html>), zebrafish homologs of mammalian genes are given the same name and abbreviation; hence, the designation as zebrafish *gngT1*).

1.3. Developmental expression of zebrafish *gngT1*

To analyze the temporal and spatial expression pattern of zebrafish *gngT1*, we performed whole mount *in situ* hybridization on embryos at different developmental stages. At 24 hours post-fertilization (hpf), a lateral view of the embryo shows the *gngT1* mRNA is expressed at a single site in the roof of the brain (Fig. 3A). At the same time point, a dorsal view of the head region shows the expression of the *gngT1* gene starts from two narrow domains along the midline of the dorsal diencephalon that later merge to form the presumptive pineal gland (Fig. 3B). Between 36 and 48 hpf, the level of *gngT1* expression increases but remains restricted to this region (Fig. 3A-B). Between 48 and 60 hpf, the expression of *gngT1* extends to the photoreceptor layer of the eye (Fig. 3A-B).

This is the first study to show that the *gngT1* transcript is expressed in a restricted region in the brain as well as the eye. To more precisely identify its localization in the brain, two-color *in situ* hybridization experiments were performed on 44 hpf embryos, using a digoxigenin-labeled probe corresponding to the *gngT1* gene and a fluorescein-labeled probe corresponding to the *aanat-2* gene. In zebrafish, the protein encoded by the *aanat-2* gene regulates melatonin production in the pineal gland, and therefore, its expression serves as a useful marker for this tissue (Falcon et al., 2003). As evidenced by co-localization of these two probes, the *gngT1* mRNA is expressed in the developing pineal gland in zebrafish (Fig. 3C).

1.4 Expression of *gngT1* in pineal- and retinal-specific photoreceptor pathways

As the major source of circulating melatonin, the pineal gland has profound effects on the diurnal behavior and seasonal reproduction of many vertebrates (Cahill, 1996). In lower vertebrates, light acts on the pineal photoreceptor (exo-rhodopsin) to synchronize melatonin synthesis with the environmental light conditions (Cahill, 1996; Mano, et al., 1999). Comparison of the zebrafish *exorh* and *gngT1* genes reveals that both genes share a similar temporal and spatial expression pattern in the developing pineal gland (Fig. 4A). Like

gngT1, the expression of *exorh* is observed in the pineal gland between 24 and 72 hpf (Fig. 4A). However, unlike *gngT1*, the expression of *exorh* is not detected in the eye even by 72 hpf. Although the exo-rhodopsin receptor has been reported in the pineal gland previously (Mano, et al., 1999), the G protein subunits acting downstream of this receptor have not been identified. Two-color *in situ* hybridization studies on 48 hpf embryos confirms co-localization of the *gngT1* and *exorh* transcripts in the developing pineal gland (Fig. 4B), thereby providing the first line of evidence that the products of these two genes may work together in the same signaling pathway to control melatonin production in this tissue. This is especially noteworthy since expression of *gngT1* in the pineal gland has not been reported in any species (Nordström et al., 2004), although other phototransducing components have been localized there (Bönigk et al., 1996; Kasahara et al., 2000).

In higher vertebrates, light acts on retinal photoreceptors (rhodopsin and melanopsin), which project to the suprachiasmatic nucleus to control melatonin production in the pineal gland (Pandi-Perumel et al., 2006). Comparison of zebrafish *rho* and *gngT1* transcripts reveals that these two genes share a similar expression pattern in the retina. At 60 hpf, the expression of *rho* and *gngT1* is readily detected in the ventral patch of the retina (Fig. 5A), the site at which the earliest differentiating neurons begin to give rise to the first photoreceptor cells (Larison and BreMiller, 1990; Robinson et al., 1995; Raymond et al., 1995; Schmitt et al., 1999). Between 72 and 84 hpf, the expression of both genes extends medially and then dorsally in a pattern that closely mimics rod cell differentiation (Hargrave and McDowell, 1992; Burrill and Easter, 1995; Raymond et al., 1995; Schmitt and Dowling, 1996) (Fig. 5A). Two-color *in situ* hybridization studies on 72 hpf embryos confirms that *gngT1* and *rho* are co-expressed in the photoreceptor layer of zebrafish retina (Fig. 5B). These data support earlier studies showing the G protein γ_{T1} subunit acts downstream of the rhodopsin receptor in the retina to produce a visual image in the cortex (Kisselev and Gautam 1993; Arshavsky et al., 2002). In addition, these results suggest that the G protein γ_{T1} subunit might act downstream of the exo-rhodopsin receptor in the pineal gland to regulate melatonin production (Cahill, 1996; Mano, et al., 1999).

1.5. Factors controlling tissue-specific expression of zebrafish *gngT1*

Virtually nothing is known regarding transcriptional regulation of the G protein *gngT1* gene in any species. To begin to identify the factors controlling zebrafish *gngT1* expression, we identified several possible transcription factor binding sites in the 5'-flanking region of the zebrafish *gngT1* gene. Of particular interest are five copies of the TAATC sequence (Fig. 6 highlighted in green). First identified in the *rho* and *exorh* genes (Chen et al., 1997; Furukawa et al., 1997; Livesey and Cepko, 2001; Asaoka et al., 2002), this consensus sequence is recognized by the Crx/Otx family of transcription factors. In mammals, Crx regulates retinal-specific expression of several phototransduction genes, including those encoding rhodopsin, arrestin, phosducin, G protein α_{T1} subunit, cGMP phosphodiesterase, and Crx itself (Livesey et al., 2000; Zhu and Craft, 2000; Gamse et al., 2001; Pittler et al., 2004). In zebrafish, Otx5 controls pineal-specific expression of several circadian clock genes, including those encoding *aanat-2*, the interphotoreceptor retinoid-binding protein, and *Reverb α* (Gamse et al., 2001; Appelbaum et al., 2005).

Both Crx and Otx5 bind to the same consensus sequence, suggesting that one or both of these transcription factors may be involved in regulating the expression of *gngT1*. To identify the relative roles of these two transcription factors, a morpholino antisense oligonucleotide (morpholino) approach was used to examine the expression of *gngT1* in embryos injected with a morpholino against *otx5* or *crx* (Gamse et al., 2001). The *in situ* hybridization results show that the *otx5* morpholino markedly reduces *gngT1* expression in the pineal gland (Fig. 7A) even though the same concentration of morpholino has little or no effect on *gngT1* expression

in the retina (Fig. 7B). Moreover, the degree of suppression is similar at 48 and 72 hpf (Fig. 7A), indicating that *otx5* knockdown causes a loss rather than a delay of *gngTl* expression in the pineal gland. To confirm that the *otx5* morpholino does not cause a non-specific delay in the ontogeny of the pineal gland that could account for this phenotype, we monitored the development of the pineal gland in transgenic fish expressing GFP under control of the *annat-2* promoter (Gothilf et. al, 2002). The finding that the control and morpholino-treated embryos express similar levels of fluorescence suggests that *otx5* knockdown does not adversely affect the development of the pineal gland even though the same dose of morpholino potently inhibits the expression of *gngTl* in this tissue (Compare Fig. 7A and 7C).

In contrast to the *otx5* morpholino, the *crx* morpholino markedly inhibits *gngTl* expression in the retina but has no apparent effect on its expression in the pineal gland. (Fig. 8A-B). To demonstrate that the *crx* morpholino does not cause a non-specific delay in the ontogeny of the retina that could account for this phenotype, we prepared transverse plastic sections through the retinas of control and morpholino-treated embryos. The results show that the *crx* morpholino, at the low dose used in this study, has no discernable effect on retinal differentiation even though the same dose of morpholino markedly reduces the expression of *gngTl* in this tissue (Compare Fig. 8B-C).

Collectively, our results (Fig. 7-8) demonstrate that Otx5 is more important for regulating *gngTl* expression in the pineal gland, whereas Crx is more important for regulating *gngTl* expression in the retina. Since both transcription factors are expressed in these two tissues (Gamse et al., 2001), the basis for their differential regulation is not clear. We are currently exploring the possibility that Crx and Otx5 function in concert with other factors that may be differentially expressed between these two tissues.

1.6. Conclusions

Based on both sequence and expression analyses, we conclude that the newly identified zebrafish cDNA is orthologous to human *gngTl*. Although not previously identified outside mammals (Nordström et al., 2004), our most recent analysis shows that *gngTl* is present in many teleost (zebrafish, *Takifugu*, medaka, Atlantic salmon and sockeye salmon), amphibian (*Xenopus*), and avian (chicken) species. Although showing 63% to 81% amino acid identity across this range of species, this level of homology for the G γ T1 protein is considerably lower than that observed for other G γ subtypes that typically show >90% amino acid identity from fish to man (Leung et al., 2006). This lower level of homology may reflect the role of *gngTl* in a photoreceptor signaling pathway that has adapted to different environmental conditions.

In mammals, the *gngTl* gene is positioned in tandem with the *gng11* gene on human chromosome 7, where the two genes are thought to have arisen as the result of gene duplication. Since neither gene has previously been identified outside mammals (Nordström et al., 2004), it has not been possible to say when this gene duplication took place. Our analysis of the syntenic region of the zebrafish chromosome 19 coupled with an exhaustive search of the available zebrafish EST databases failed to identify the presence of the *gng11* gene in zebrafish. Collectively, these results suggest that teleosts contain only the *gngTl* gene and that gene duplication giving rise to the *gng11* gene occurred later. Following gene duplication in mammals, the expression of *gngTl* appears to have become restricted to the retina, whereas expression of *gng11* appears to have emerged in the pineal gland (www.ncbi.nlm.nih.gov/projects/geo/).

Cell-specific factors controlling retinal- and pineal-specific expression of the *gngTl* gene have not been determined in any species. Here, we identify Crx and Otx5 as possible transcription factors. Since both factors are co-expressed in these tissues, it is likely that Otx5 and Crx are necessary, but are not sufficient to account for the differential expression of *gngTl*. In this

regard, a pineal expression-promoting element (PIPE) with the sequence TGACCCCAATCT has been identified in the promoter region of *exorh* (Asaoka et al., 2002). We identified a sequence with partial homology to PIPE in the 5' flanking region of *gngT1* (TGTCCCC; Fig. 6 highlighted in purple). In addition, we identified a second sequence with partial homology to PIPE in the first intron of *gngT1* (TGACCCCAAACC; data not shown). In future experiments, we will determine whether either of these sequence elements are critical for regulation of *gngT1* expression in the pineal gland.

2. Experimental procedures

2.1 Fish Stocks

Danio rerio (Florida wildtype strain; 'That Fish Place', Lancaster, Pennsylvania; and Longfin strain, Scientific Hatcheries, Huntington Beach, California) were maintained at 28.5°C on a 14:10 light-dark (LD) cycle. Embryos were staged according to Westerfield, 1994.

2.2 Cloning of zebrafish *gngT1*

RNA was isolated from 30-hpf embryos and 6-month old adults using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized by reverse transcription reaction, and was used as a template to amplify a portion of the zebrafish *gngT1* gene by polymerase chain reaction, using the primer pair: *gngT1*-f1 5'-GACCTCATCGTAAGCAGCAAA-3' (forward, from -22 to -2 bp) and *gngT1*-r1 5'-GGGATTTTCTGTCCACTTTAGC-3' (reverse, from 218 to 239 bp). A nested primer pair: *gngT1*-f2 5'-ATCGATATGCCGATCATAGATGTAGAAA-3' (forward, from 1 to 22 bp) and *gngT1*-r2 5'-CTCGAGTTAGCAAATGACACATCCACCTT-3' (reverse, from 200 to 217 bp) was used to confirm the PCR product.

2.3 RT-PCR

To determine tissue distribution, RT-PCR analysis was performed on total RNAs isolated from various adult tissues, using the ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA) with the primer pair *gngT1*-f1 and *gngT1*-r1. The PCR amplification reaction was carried out for 30 or 34 cycles under the following conditions: 1 min for denaturation at 94°C; 45 seconds for annealing at 58°C; and 1 min for elongation at 72°C.

2.4 Whole mount in situ hybridization

The zebrafish *gngT1* construct was generated by subcloning the PCR product described above into the ClaI/XhoI sites of the pBluscript SK⁺ vector. The digoxigenin (Dig) RNA labeling Kit (Roche Applied Science, Indianapolis, IN) was used to generate the labeled *gngT1* probe. A portion of the first-strand cDNA described above was used as the template to PCR amplify a portion of the zebrafish *exorh* gene (GenBank accession number NM131212), using the primer pair: *exorh*-f1 5'-TGCCG ATC GTCCAC TTCTC (forward, from -23 to -5) and *exorh*-r15'-CTGATTGGCTGA TGGCAGC (reverse, from 1081 to 1099); or the zebrafish *rho* gene (GenBank accession number NM131084), using the primer pair: *rho*-f1 5'-ATGAACGGTACAGAGGGACCG-3' (forward, from 1 to 21) and *rho*-r1 5'-CGCCGGAGACACGGAGCTGGA-3' (reverse, from 1042 to 1062). The zebrafish *exorh* and *rho* constructs were generated by subcloning the PCR products described above into the pCRII-TOPO dual promoter vector (Invitrogen, Carlsbad, CA). The plasmid for making the *aanat-2* probe was provided by Dr. David C. Klein. Either the Digoxigenin (Dig) or the Fluorescein (Flu) RNA labeling Kits (Roche Applied Science, Indianapolis, IN) were then used to prepare the labeled *exorh*, *rho*, and *aanat-2* probes.

For whole mount *in situ* hybridization, embryos were treated with 30mg/L 1-phenyl-2-thiourea (PTU) to prevent pigment formation. The staged embryos were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and more than 30 embryos were analyzed for each observation. Whole-mount one- or two-color *in situ* hybridizations were performed essentially as described by Thisse et al, 1993, Hauptmann, 2001, and Long and Rebagliati, 2002. The immunodetection was carried out using anti-digoxigenin alkaline phosphate-conjugated Fab fragment (1:3000 dilution) or anti-fluorescein alkaline phosphate-conjugated Fab fragment (1:1000 dilution) (Roche Applied Science, Indianapolis, IN). 5-bromo-4-chloro-3-indohyl phosphate (BCIP) and 4-nitro blue tetrazolium NBT (Roche Applied Science, Indianapolis, IN) were used for single-color *in situ* hybridization. Fast Red (Sigma-Aldrich, St. Louis, MO) and NBT/BCIP were used for two-color *in situ* hybridization. The images were viewed using a MZFL3 stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a DEI-750D CE digital video camera (Optronics, Golenta, CA); were captured using ImagePro+ version 4.1 software (Media Cybernetics, Silver Spring, MD); and were processed using Adobe Photoshop (Adobe Systems Incorporated).

2.5 Antisense morpholino oligonucleotides

Two morpholino oligonucleotides targeting *otx5* (MO-*otx5*; 5'-CATGACTAACTCTCTCTCTCTCTC-3') and *crx* (MO-*crx*; 5'-ATGTAGGACATCATTCTTGGGACGG-3') were designed according to Games et al, 2001 and were synthesized by Gene-tools (Pholomath, OR). Approximately 1nL of different concentration of MO was injected into 1 -to 2-cell stage zebrafish embryos. Control zebrafish were injected with Danieau's buffer (1×: 58mM NaCl; 0.7mM KCl; 0.4 mM MgSO₄; 0.6mM Ca(NO₃)₂ and 5.0 mM HEPES, pH7.6).

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Fig. 1.

Phylogenetic analysis and sequence alignment of zebrafish *gngT1*. (A) The GenBank accession numbers for human G protein γ subunits are: GyT1 (NP 068774); GyT2 (NP 113686); Gy2 (NP 444292); Gy3 (NP 036334); Gy4 (NP 004476); Gy5 (NP 005265); Gy7 (NP 005136); Gy8 (NP 150283); Gy10 (NP 001017998); Gy11 (NP 004117); Gy12 (NP 061329); Gy13 (NP 057625). The GenBank accession numbers for zebrafish γ subunits are: GyT1 (NP 956261); and GyT2 (AAH59612). Phylogenetic tree prediction was performed using the TREE TOP program (<http://www.Genebee.msu.su/genebee.html>). (B) Multiple sequence alignment was performed using the MAP program (<http://searchlauncher.bcm.tmc.edu/multi-align.html>). Identical residues shared by all sequences are shown in green; residues identical to human GyT1 are shown in purple; and residues identical to human Gy11 are shown in yellow. The identity values (%) are shown at the end of the sequences. The CAAX motif is marked with a black box.

Fig. 2.

Tissue distribution of *gngT1*. RT-PCR analysis was performed on various tissues from 6-month old zebrafish. PCR amplification was carried out for the indicated number of cycles for *gngT1* and β -actin. The latter one was used as a loading control.

Fig. 3.

Expression pattern of *gngT1* during zebrafish development. (A) Lateral view and (B) dorsal view of whole mount *in situ* hybridization of *gngT1* in the zebrafish embryos from 24 to 60 hpf. (C) Two-color *in situ* hybridization using *aanat-2* and *gngT1* probes at night (44 hpf). Fast red staining for *aanat-2* (red) and NBT/BCIP for *gngT1* (blue). Flu, fluorescein in red; Dig, digoxigenin in blue. Arrow points to the pineal and arrowheads point to the eyes.

Fig. 4. Comparison of the *gngT1* and *exorh* expression in the pineal gland. (A) Lateral view of the *gngT1* and *exorh* transcripts at 24, 48 and 72 hpf. (B) Two-color *in situ* hybridization using *exorh* and *gngT1* probes at 48 hpf. Fast red staining for *exorh* (red) and NBT/BCIP for *gngT1* (blue). *exorh*, extra-ocular rhodopsin; Flu, fluorescein; Dig, digoxigenin.

Fig. 5.

Comparison of the *gngT1* and *rho* expression in the retina. (A) Lateral view of the *gngT1* and *rhodopsin* transcripts at 60, 72 and 84 hpf. (B) Two-color *in situ* hybridization using *gngT1* and *rhodopsin* probes in the retinal photoreceptor layer at 72 hpf. Fast red staining for *rho* (red) and NBT/BCIP for *gngT1* (blue). *rho*, *rhodopsin*; Flu, fluorescein; Dig, digoxigenin. Scale bar equals 50 μ m.

-1260 TAGTCCCTTTT **TAATC**TGGGGTTGCCACAGCAGAATAAACTACTAATTTATCCAGCATATGTTTTACGT
 -1190 AGCAGATGCCCTTCCAGCTG **TAATC**CATCACTGGGAAACACCCATAGACTCCTATTACACACATAAACT
 -1120 ATTATACACACTATATATAAGACATAATATATAACCAACCAATAAAAGTAGAATTATCA **TAATC**ATTAT
 -1050 TATTTTATAATAAATACGACAAATTAACAATATATACCATAAAATACTATAATATGTCTAAACATAATA
 -980 TAATAAAATATAAATATACTAAATATAATTATTATTGTAATTAAATATCTTCTATAAATGTGACAACGA
 -910 TAATAGTAGCATATAATAAAATTATTATTTTGTGATAAATATATTAATAATATTAAATAAATAAATGAT
 -840 GATACATAATATTGTAGACTGATTACAATCCAGTACTAAAAATAAATTAATAACAAAGCCAAAAAATAA
 -770 AATAAAACAAATTTAAAAATAAATCATTTTAAAGTTTAAATTTGGTTTAAAAATTATTTATGTAGAAATA
 -700 AACGAATACAATTTAAATTTAAACTTTTTTAAATAAAAGAAACCTTTTCAAGTTGATGAACCTAATTTT
 -630 ATATACATTATTTAACAATAAAATAAAATACAGAAATTAATGAGTAAATAAATAATATTTTATAATAT
 -560 AATATAATATGATATAATATATGTTTAAATGTAGCGCATATATAACATTTTGTGAAAGAACACATGTCA
 -490 TATCTTGTATGATCATGTATTATCTAATGTGACAGGTTAAGGGTAACATGGATTAGCATGCTCATCACAG
 -420 TAGCCTGTAATACTTTCTAT **ATG** **CCCC**TTGACCTACATTGCAATCCATCGCTCATTAGCAGAAAACCTTTC
 -350 ATGCCAATTTGATTATCTTTAGGATATAATTGAGTTGTACAAGTATGCATTTTCATTGCGCAATGTATTT
 -280 **TAATC**CACAATAATAGTTAACCACAAAGAAAAAGAAGACTATAGAAAGGTAAATAAGAGTACTTGT
 -210 TTAATGAGATTAAGAGATTGCAATGTTTATGCAGATTACTTTATAAACCGGATTGAGAGAGATGTGCAGA
 -140 ATCTCTGTCCATTTTCCAGAGTGGAGACCTCATCGTAAGCAGCAGTAAGTTTGCTTATTTCCAGTATATT
 -70 GAATATCAAAATA **TAATC**CATTTCATTCAACACAACATGTAAATGATATGATTGTTTGATTTCATGCAGAAA
ATGCCG

Fig. 6.

The genomic sequence of the proximal promoter region of the zebrafish *gngT1*. The putative Crx/Otx5 binding sites are in green. The sequence motif with partial homology to PIPE is in purple and the start codon ATG is in bold. Zebrafish EST NM 199967, BI706840 were used to blast NCBI zebrafish genomic sequence. Underlined sequence is corresponding to the 5' UTR of the zebrafish *gng T1* transcript. The GenBank accession number for this genomic sequence is [BX323007](#).

Fig. 7.

Regulation of the zebrafish *gngT1* expression by *otx5* in the pineal gland. The embryos were injected with MO-*otx5* and the transcripts were detected by Dig labeled *gngT1* probe. (A) Dorsal view of *gngT1* expression in the pineal gland. (B) Lateral view of *gngT1* expression in the pineal and the retina at 72 hpf. (C) Expression of the GFP in the pineal gland at 72 hpf using the transgenic zebrafish line TG[*aanat-2*:GFP].

Fig. 8. Regulation of zebrafish *gngT1* expression by *crx* in the retina. The embryos were injected with MO-*crx* and the transcripts were detected by Dig labeled *gngT1* probe. (A) Dorsal view of *gngT1* expression in the pineal gland at 48 hpf. (B) Lateral view of *gngT1* expression in the pineal gland and the retina at 72 hpf. (C) Plastic section of zebrafish eyes at 72 hpf.