Zebrafish aversive taste co-receptor is expressed in both chemo- and mechanosensory cells and plays a role in lateral line development

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Fishes rely on both chemical and tactile senses to orient themselves to avoid predators, and to detect and taste food. This is likely achieved by highly coordinated reception of signals by mechano- and chemosensory receptors in fish. A small co-receptor from zebrafish, receptor activity modifying protein (RAMP)-like triterpene glycoside receptor (RL-TGR), was previously found to be involved in recognition of triterpene glycosides, a family of naturally occurring compounds that act as chemical defenses in various prey species. However, its localization, function, and how it impacts sensory organ development in vivo is not known. Here we show that RL-TGR is expressed in zebrafish in both i) apical microvilli of the chemosensory cells of taste buds including the epithelium of lips and olfactory epithelium, and ii) mechanosensory cells of neuromasts belonging to the lateral line system. Loss-of-function analyses of RL-TGR resulted in significantly decreased number of neuromasts in the posterior lateral line system and decreased body length, suggesting that RL-TGR is involved in deposition and migration of the neuromasts. Collectively, these results provide the first in vivo genetic evidence of sensory cell-specific expression of this unusual co-receptor and reveal its additional role in the lateral line development in zebrafish.

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zebrafish G-protein coupled receptor (GPCR), transduces a signal in the presence of triterpene glycoside ligands. The location and distribution of RL-TGR are important factors in understanding the role of this receptor in organism-environment interactions, and are expected to affect the kinds of ligand(s) RL-TGR can recognize and the message being transduced. The ligands could be cues from the external environment as is the case for chemical compounds in food or they could be secreted by cells as in the case of intereukins, growth factors, etc.5,6.

Given its proposed function in chemoreception of prey chemical defenses, RL-TGR expression is expected to be localized in chemosensory tissues, namely olfactory epithelium and taste buds in the mouth. The taste buds allow fish to discriminate between palatable and noxious food items, and are found throughout the oropharyngeal epithelium, on the branchial arches, and on the head and other regions of the body in some species.7 In this study, we sought to first determine the location and distribution pattern of RL-TGR in zebrafish in order to dissect the molecular basis of its cellular function in vivo. Further, we performed morpholino-mediated knockdown of RL-TGR to reveal its physiological effects on early development of zebrafish.

Results

Expression pattern of RL-TGR in zebrafish. Reverse transcriptase quantitative PCR (RT-qPCR) analysis in zebrafish whole embryos showed that rltgr starts to be expressed at 8 hours post fertilization (hpf) with a major increase at 3 days post fertilization (dpf) onwards (Fig. 1a). Using whole-mount in situ hybridization (WISH) labeling, spatiotemporal rltgr expression was detected from 8 hpf to 19 dpf (Fig. S1). Predominantly, rltgr expression was detected in the head, pharyngeal region, and intestine (7 dpf embryo) (Fig. 1b,b'). Its expression was also detected in the neurmasts of the posterior lateral line system (Fig. 1b). From 7 dpf onwards, rltgr was detected by WISH in the pharyngeal region, intestine, and neurmasts, a pattern observed until at least 19 dpf (Fig. S1). To analyze the expression of RL-TGR protein in zebrafish embryos, we performed whole-mount immunofluorescence experiments. To this end, we generated a rabbit polyclonal antibody against a peptide containing the 18 amino acid residues (KYGEDALKVTVKLKVHKE) from the C-terminal portion of the predicted transmembrane domain of RL-TGR, which showed immunoreactivity with the recombinant RL-TGR protein expressed in HEK 293 cells (Fig. S2). We further evaluated the specificity of the anti-RL-TGR antibody by immunostaining the cichlid (Maylandia zebra) embryos with anti-RL-TGR antibody (Fig. S3). Because the M. zebra genome (GenBank assembly accession: GCA_000238955) sequence does not contain the rltgr gene, it serves as an ideal negative control to evaluate the specificity of our custom generated anti-RL-TGR antibody. As expected, there was no immunoreactivity in cichlid embryos at both 3 and 7 dpf with anti-RL-TGR antibody confirming its specificity with RL-TGR protein (Fig. S3). However, we were unable to obtain specific reactivity via western blot using this antibody.

In zebrafish, RL-TGR-positive cells were detected in the epithelium of the upper and lower lips (Fig. 1c,c'), as well as in neurmasts of both the cephalic (Fig. 1d,d') and posterior (Fig. 1e,e') lateral line system, as evidenced by immunostaining of the 7 dpf embryos with anti-RL-TGR antibody. Consistent with the gene expression findings, RL-TGR-positive cells were detected throughout the embryos at 3–18 hpf (Fig. S4a–c). In 1 dpf embryos, RL-TGR-positive cells were detected in the nasal sensory epithelium of the olfactory pit (Fig. S4d). In 2 dpf embryos, immunofluorescence was detected both in the olfactory pit and in the brain (Fig. S4e,f). In 3 to 7 dpf embryos, RL-TGR immunostaining signal occurred in the epithelium of upper and lower lips, olfactory pit, and neurmasts (Fig. S4g–l). The number and size of anti-RL-TGR-labeled neurmasts in the anterior and posterior lateral line were increased in 7 dpf larvae (Fig. S4k,l). These RL-TGR protein expression patterns are in agreement with the rltgr gene expression patterns in different sensory tissues (Fig. 1b–b') and point to a function of RL-TGR protein in the zebrafish sensory system during early development.

In adult zebrafish, tissue-specific expression of the rltgr gene evaluated using RT-qPCR corroborated observations with 5–19 dpf stage larvae (Fig. S1), whereby rltgr is expressed in the intestine and mouth (both upper and lower jaw including lips) (Fig. 1f–f'). In adults, rltgr expression was also detected in muscle which could be due to the presence of neurmasts on the body surface. We validated our expression results by analyzing the expression of rltgr in different tissue-specific zebrafish transcriptomes from publicly available RNASeq data (Fig. 1g). Consistent with the tissue-specific expression, rltgr is highly expressed in the intestine and head. However, insignificant numbers of reads were detected via this approach from the olfactory epithelium (OE) and muscle (Fig. 1g).

RL-TGR is expressed in zebrafish taste buds, lips, and olfactory epithelium. The taste buds with open receptor areas appear in the larval zebrafish mouth and oropharyngeal cavity around 4–5 dpf, when they start feeding. Therefore, we examined the upper and lower lips in 7 dpf embryos and used calretinin, a calcium binding protein, as a taste bud marker. RL-TGR-positive cells were localized in the epithelial layer of both upper and lower lips (Fig. 2a) and colocalized with calretinin in the basal cells and microvilli (tip or receptor area) of the taste buds in the upper and lower lips (Fig. 2b). RL-TGR-positive cells were also located in the nasal sensory epithelium of the olfactory pit (Fig. 2c) and colocalized with calretinin in the sensory epithelium region where both are expressed in the olfactory receptor neurons of the olfactory pit. Using acetylated tubulin as a marker for ciliated cells, we found that RL-TGR-positive cells were located in the ciliated cells of the olfactory epithelium (Fig. 2d).

RL-TGR is expressed in zebrafish mechanosensory neurmasts. Neurmasts of the lateral line system sense hydrodynamic signals of water currents and help fish to orient, avoid predators, and detect food.8 Immunostaining with RL-TGR antibody revealed that RL-TGR is expressed in neurmasts of the lateral line system. RL-TGR-positive cells were arranged in a typical rosette pattern, indicating expression of RL-TGR in hair cells, support cells, and mantle cells of the neurmast (Fig. 3a). Further, colocalization of RL-TGR and acetylated tubulin confirmed the localization of RL-TGR in both kinocilia and cell body of the hair cells (Fig. 3b).
Additionally, colocalization of RL-TGR with Prospero-related homeobox gene 1 (Prox1), a transcription factor expressed in the lateral line primordium and used as a marker for neuromasts, confirmed the presence of RL-TGR in the stereocilia of the hair cells and in progenitor cells (mantle or supporting) surrounding neuromasts. The colocalized expression of RL-TGR and parvalbumin in the hair cells further confirmed the strong expression of RL-TGR in the hair cells of the neuromast (Fig. 3c). The expression of RL-TGR in the lateral line system suggests its potential signaling role in the development of neuromasts and/or their stereotypical distribution and function.
Loss of RL-TGR activity affects lateral line development. In order to investigate the role of RL-TGR in embryonic development, we performed knockdown experiments by injecting translation-blocking antisense rltgr morpholino oligonucleotides (rltgr MO1) in 1- to 2-cell stage embryos. We could not generate splicing-blocking morpholino oligonucleotides because rltgr is coded within a single exon. Compared to control MO-injected embryos (control embryos), rltgr MO1-injected embryos (morphants) exhibited a significant decrease in the immunostaining fluorescence intensity using the anti-RL-TGR antibody, demonstrating the MO’s protein translation blocking efficiency (Fig. S5). At both 3 and 5 dpf, control embryos did not exhibit any significant morphological defects (Fig. S6). In contrast, the morphants exhibited dose-dependent phenotypes characterized by a shortened anterior–posterior axis (body length), bent trunks, and curved tails (Fig. S6a). The observed rltgr MO1 dose-dependent phenotype revealed significant differences in the RL-TGR morphants compared to control-MO injected individuals at 3 and 5 dpf (P < 0.0001) (Fig. S6b). A significant decrease in the body length (at 3 dpf, P < 0.01; 5 dpf, P < 0.0001) and number of neuromasts (at 5 dpf, P < 0.0001) were observed in an rltgr MO1 dose-dependent manner (Fig. S7a,b). Injection of rltgr MO1 caused a decrease in the number of deposited neuromasts in the posterior lateral line, which was efficiently rescued by rltgr mRNA (Fig. 4a,b). Moreover, the body length was also restored upon co-injection with rltgr mRNA (Fig. 4a,c). It should be noted that the morpholino oligonucleotide (rltgr MO1) was designed to target the region upstream of start codon and that the mRNA used in the rescue experiment contained ORF of rltgr and not the morpholino target site, in order to ensure that there was no titration effect leading to false positive rescue results. It should also be noted that less severe phenotypes (short and curved body) were used for statistical purposes because it was difficult to assess the number of neuromasts in the more severe phenotypes with deformed posterior structures; this bias made the contrast more conservative. RL-TGR expression analysis in rltgr morphants revealed that RL-TGR-positive cells were confined to hair cells in the neuromasts (Fig. 4d). There was also a decrease in the expression of the neuromast marker Prox1 in rltgr morphants (Figs S5 and 4d).
Loss of RL-TGR activity has little effect on taste bud development. Although RL-TGR activity is required for the lateral line development, it did not seem to affect the development of taste buds. At both 3 and 5 dpf, a majority of the morphants showed unaltered morphology of the mouth and structure of the taste buds (Fig. 5a,b), whereas a small population of morphants with severe body phenotypes exhibited deformity in the mouth with either no or underdeveloped taste buds. In both cases, the expression of RL-TGR was almost absent in the lip epithelium and tip of the taste buds, while the expression of calretinin was not affected in the lip epithelium and taste buds (Fig. 5).

Validation of translation-blocking morpholino knockdown of rltgr. In order to confirm that the observed phenotypes of RL-TGR knockdown zebrafish juveniles were due to specific interaction between the morpholino (rltgr MO1) and the target sequence (rltgr), a second, non-overlapping, translation-blocking morpholino rltgr MO2 was designed and tested. We observed the same effect as with rltgr MO1 (Fig. 6). Both rltgr MO1 and MO2 morphants exhibited phenotypes characterized by a shortened anterior–posterior axis (body length), bent trunks, and curved tails with decreased number of lateral neuromasts (Fig. 6a). Yet, the head neuromasts developed normally in uninjected individuals as well as morphants (using either MO1 or MO2) suggesting that knockdown of rltgr affected the migration of posterior lateral line neuromast primordium only. At 3 dpf, a significant dose-dependent decrease in the body length and number of neuromasts was observed in embryos injected with rltgr MO1 or rltgr MO2 (Fig. 6b,c). Another potential side effect of morpholino treatment could be toxicity to cells which may have affected elongation of the body axis. To rule out the general development delay...
in morpholino-injected larvae arising from morpholino toxicity, we performed alcian blue cartilage staining at 5 dpf. We observed that cartilage development was not affected in both control and morpholino injected embryos (Fig. 6d). This suggests that the observed reduction in body size was not due to developmental delay.

Discussion

**RL-TGR expression in both chemo- and mechanosensory cells.** The observation that RL-TGR is expressed in chemosensory tissues *in vivo* such as lip epithelium, taste buds, and olfactory epithelium (Fig. 2) supports its role as a chemoreceptor which was previously indicated by heterologous expression in *Xenopus* oocytes. Given that RL-TGR functions in conjunction with a GPCR, it is unlikely that RL-TGR senses both chemical and tactile signals since mechanical stimuli may not be expected to be transduced via GPCRs due to their relatively slow signaling cascades. However, the lateral line system, typically implicated in mechanosensation, has also been reported to play a role in feeding, particularly in prey and food detection. Even though each sensory system is sufficient by itself to accomplish behavioral objectives, fishes can modulate their responses using different sensory systems that might complement each other's function to achieve complex behavior. Feeding behavior in fishes is stimulated by integration of multiple types of cues providing information about prey suitability, prey location, and other aspects of the foraging environment, all dependent upon functioning visual, chemo- and mechanosensory systems. Further, spatiotemporal expression profile of *rltgr* (WISH) (Fig. S1, 1b), qPCR and RNASeq data (Fig. 8g) revealed that it is also strongly expressed in the intestine. The expression of *rltgr* in zebrafish gut is consistent with multiple findings of taste receptors expressed in the guts of mammals and insects. These
receptors may function as a second line of defense against potentially noxious compounds as a part of enteric nervous system, supporting the hypothesis that RL-TGR functions in aversive taste reception.

**Possible roles of RL-TGR in the taste buds and olfactory epithelium.** Identified previously as a co-receptor for sensing chemicals, RL-TGR expression in the apical microvilli of taste buds protruding from the epithelium (open taste receptor area) (Fig. 2) justifies its role in sensing chemical compounds. In the apical microvilli of taste buds, its expression colocalizes with calretinin, a calcium binding protein that regulates intracellular Ca²⁺ homeostasis. Calretinin has been found to be widely distributed in the central and peripheral nervous system as well as in chemosensory cells such as taste buds and olfactory epithelium. RL-TGR is expressed in the labial epithelial cells embedding the taste buds (Fig. 2). It was observed that most calretinin-positive cells do not express RL-TGR in the taste buds (Fig. 2b) which could be due to the difference in the origin of the cells. Calretinin-positive cells in the taste buds may have an endodermal origin, whereas RL-TGR positive basal epithelial cells arise from the ectoderm.

Since the cells of taste buds have a limited life span, they are maintained by continuous proliferation of epithelial progenitor and stem cells. In mice, basal keratinocytes originating from the epithelial bilayer serve as progenitor populations that supply new cells to taste buds. Even though the specific role of RL-TGR in the basal epithelial region of the taste buds is presently unclear, the expression pattern suggests that it may be involved in signaling in the progenitor or stem cell populations during taste bud development and/or regeneration. However, RL-TGR signaling may not be indispensable as the RL-TGR loss-of-function phenotype (less severe individuals with shortened bodies) had normally developed taste buds and lip epithelia (Fig. 5). Nevertheless, we did find mouth deformity with less or underdeveloped labial taste buds in morphants displaying the more severe phenotype (data not shown).

It has been reported that olfactory organs in fishes are formed much earlier in development than taste buds or gustatory organs. Specifically, the receptors that bind odorants start to develop within 24 to 30 hpf (before hatching). RL-TGR co-receptor expression is consistent with the development pattern of chemosensory organs including both olfaction and taste. RL-TGR expression was first detected in olfactory epithelium at 1 dpf and then at 2 dpf (Fig. S4d,e). RL-TGR expression was found in the lip epithelium from 3 dpf onwards, consistent with prior observations that zebrafish taste buds develop starting at 3 dpf. This order of onset of RL-TGR expression in parallel with different sensory modalities suggests one possibility that RL-TGR may help zebrafish larvae detect chemosensory cues via its expression in olfactory epithelium, and later helps larvae evaluate the quality of food through expression in taste buds when fish are actually able to feed.

Figure 5. RL-TGR has a minimal effect on taste bud development. (a) Confocal images of the mouths of control embryos and rltgr morphants (dorsal view, anterior to the top) at 3 dpf stained with RL-TGR (green) and the taste bud marker calretinin (red). (b) Confocal images of mouth of control embryos and rltgr morphants (dorsal view, anterior to the top) at 5 dpf stained with RL-TGR (green) and taste bud marker calretinin (red). n > 10; scale bars are shown in the images.
RL-TGR is involved in lateral line development. The function of a co-receptor is expected to depend on the kinds of ligand(s) it binds and the stimulus that is being transduced. The ligands can be from the external environment as is the case for chemical compounds in food or they can be ligands secreted by the cells such as interleukins and growth factors. In the latter case, co-receptors have been reported to be involved in chemical signaling during cell proliferation, migration, and embryonic development. The zebrafish phenotype observed upon knockdown of RL-TGR, in which deposition of neuromasts was impaired (Fig. 4), points towards a likely role of RL-TGR in neuromasts. RL-TGR, a chemoreceptor, may sense a chemotactic gradient necessary for lateral line primordium migration. The primordium is a group of migrating epithelial cells that differentiates into neuromasts which requires coordination of diverse cellular behaviors via chemical signaling. RL-TGR expression was observed in the primordium of the lateral line (data not shown). Recently, GPCR-mediated signaling was discovered in the context of lateral line development, whereby G-proteins induce primordium migration through chemokine receptors, Cxcr4. Given that RL-TGR functions in conjunction with a yet-unidentified zebrafish GPCR, RL-TGR may be participating in pathways controlled and regulated by GPCRs at different levels to promote migration of the lateral line primordium. Therefore, disruption in this activity by RL-TGR knockdown might have led to impaired migration and decreased number of deposited neuromasts (Figs 4 and 6). However, we cannot rule out the possibility that the lateral line phenotype and decreased body length could be indirect effects of RL-TGR knockdown impacting signaling factors, such as fibroblast growth factors or cysteine-rich glycoproteins (Wnt), important for lateral line development.

In a previous study, the role of RL-TGR in chemoreception was identified using a heterologous system, whereas the location, distribution, and function of RL-TGR in sensory organs in vivo are established for the first time in the current study. Herein we report that RL-TGR is expressed in sensory specific cells of both chemo- and
mechanosensory organs. More importantly, a new role for RL-TGR is revealed in the development of lateral line system, which is crucial in fish for food detection and predator avoidance. Most likely, RL-TGR is one of the first components in the signaling cascade; therefore, its inactivity might affect the entire signaling pathway directing the early developmental processes. Further studies are required to identify the endogenous GPCR with which RL-TGR interacts in various tissues and the downstream molecules that are stimulated during the signaling.

**Methods**

**Ethics statement.** This study was approved by the Institutional Animal Care and Use Committee at Georgia Institute of Technology (A14039). All animal work was performed according to procedures approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology.

**Zebrafish strains.** Adult fish and embryos (AB/Tuebingen - wildtype) were raised and maintained under standard laboratory conditions. Breeding wild type strains were maintained at 28°C on a 14 h light/10 h dark cycle. Embryos were collected by natural spawning, staged according to Kimmel and colleagues, and raised at 28°C in fish water (Instant Ocean, 0.1% methylene blue) in Petri dishes. We report the embryonic ages in hours post fertilization (hpf) and days post fertilization (dpf).

**In situ hybridization and immunohistochemistry.** Whole-mount in situ hybridization (WISH), was carried out as previously described on embryos fixed overnight in 4% paraformaldehyde/phosphate buffered saline (PBS), then rinsed with PBS-Tween (0.1% Tween-20), dehydrated in 100% methanol and stored at –20°C until processed for WISH. rltgr antisense riboprobes were previously in vitro labelled with modified nucleotides (digoxigenin, Roche). The template for the antisense RNA probe was amplified from embryonic cDNA with the following primers: forward: 5′-CCAGGATCCCTAGGTGTTTTTTATGTAATCGGAC-3′; reverse: 5′-GCGGCCGCTTTCAAACAGTCTGTGATCG-3′. Immunohistochemistry on whole-mount zebrafish embryos was performed as previously described using the following antibodies: rabbit anti-RL-TGR (1:200; custom synthesized, Covance), goat anti-calretinin (1:100; Millipore Sigma), goat anti-Prox1 (1:20; R&D Systems), mouse anti-acetylated tubulin (1:1000; Sigma), mouse anti-parvalbumin (1:200; Millipore Sigma) and fluorescently conjugated Alexa antibodies (1:200; Molecular Probes). For communostaining with Prox1 or calretinin or acetylated tubulin or parvalbumin, embryos were incubated with all primary antibodies and finally with secondary antibodies. Embryos were either mounted in Vectashield with DAPI (Vector Laboratories) and imaged on a Zeiss LSM 700 A or B confocal microscope or embedded in 1% agarose and imaged on a Zeiss Lightsheet Z.1 microscope. The neuromasts were assessed by staining the embryos with SYTOX green dye (Thermo Fisher Scientific) and observed under Zeiss epifluorescence microscope. SYTOX green stains the nuclei of neuromast epithelium (ERR375744-47, ERR375748-49), muscle (SRR1609753-55, SRR891510, ERR145653) and intestine.
Briefly, we mapped all reads per time-point independently back to the zebrafish genome (GRCz10; Ensembl) with CLC Genomics Workbench (version 8.5.1) and reads counts mapped to rltgr level of mapped the reads from heads and intestines to both genic and intergenic regions. For comparison, the expression level of rltgr in a tissue was defined by the number of uniquely mapped reads in rltgr divided by one thousandth of the whole exon length of rltgr, and then was normalized by dividing by one millionth of the total number of valid reads in the respective samples.

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**Author Contributions**

N.M. designed and performed experiments, analyzed the data, and wrote the manuscript. J.X. collected zebrafish embryos and performed all injections. Z.B. and B.I. evaluated the specificity of anti-RLTGR antibodies. J.X., C.H.S., and J.K. also designed the experiments and analyzed the data. N.A.M. and B.I. contributed additional trouble-shooting and data interpretation. N.A.M., C.H.S., and J.K. contributed towards reagents and equipments. All authors contributed towards review of the final manuscript.

**Additional Information**

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**Competing Interests:** The authors declare that they have no competing interests.

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