Running title

Medaka orthologue for tissue-type transglutaminase

Title

Biochemical characterization of the medaka (*Oryzias latipes*) orthologue for mammalian tissue-type transglutaminase (TG2)

Author names

[1]Yuki Takada ¶ , [1]Yuko Watanabe ¶ , [1]Kazuho Okuya, [1]Hideki Tatsukawa, [2]Hisashi Hashimoto, and [1]Kiyotaka Hitomi

Affiliations

1 Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya 464-8601

2. Bioscience Biotechnology Center Nagoya University, Nagoya 464-8601

Corresponding author: Kiyotaka Hitomi, Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, 464-8601, Japan. Tel: +81 52 747 6807; Fax: +81 52 747 6810; e-mail: hitomi@ps.nagoya-u.ac.jp

¶ These two authors contribute equally.

Abbreviations:

bio-Cd, 5-(biotinamido)pentylamine; BSA, bovine serum albumin; DTT, dithiothreitol; IPTG, β-D-1-thiogalactopyranoside; PBS, Phosphate–buffered saline; TBS, Tris–buffered saline; TGase, transglutaminase.

Abstract

Transglutaminase is an enzyme family responsible for post-translational modification such as protein cross-linking and the attachment of primary amine and/or deamidation of glutamine-residue in proteins. Medaka (*Oryzias latipes*), a recently established model fish, has similar functional proteins to those characterized in mammals. Previously, we found the apparent orthologues that correspond to human transglutaminases in medaka. In this study, regarding the medaka orthologue of human tissue-type transglutaminase (OlTGT), recombinant protein was expressed in an active form in bacteria cultured at low temperature. Using the recombinant protein, we biochemically characterized the enzymatic activity and also obtained a monoclonal antibody that specifically recognized OlTGT. Immunochemical analysis revealed that OlTGT was not expressed ubiquitously, unlike its mammalian orthologue, but in primarily limited tissues such as the eye, brain, spinal cord, and gas-gland.

Keywords: transglutaminase; *Oryzias latipes*; calcium

Transglutaminases (TGases) are a family of Ca^{2+}-dependent enzymes that catalyze protein cross-linking reactions resulting in functional and structural modification in a variety of biological processes [1-3]. In mammals, the TGase family consists of eight members (TG1-TG7 and Factor XIII (FXIII)) as isozymes that are characterized by their various distribution and regulatory mechanism of activity. Among the family members, FXIII is involved in blood coagulation by polymerizing of fibrin [4]. TG1, TG3 and TG5 have functional roles in skin barrier formation by cross-linking of various structural proteins in differentiating keratinocytes [5-6]. TG2, major isozyme, is ubiquitously expressed in mammals and has multiple roles, such as in the stabilization of extracellular matrix proteins, inactivation of transcription factors, and modification of signal transduction molecules [7-8]. Furthermore, in the case of TG2, non-catalytic functions have been found, such as GTP binding proteins and interacting proteins for scaffolding. Several related diseases affected by aberrant regulation of TG2 enzymatic activity have been suggested, such as celiac disease, cancer, and neurodegenerative diseases. However, the physiological functions of TG2 have yet to be clarified.

Possible orthologues for TG2 are also found in a wide variety of species including both vertebrates and invertebrates [1]. In animals other than mammals, TG2 primarily appears to be required for recovery from injury or barrier formation. However, in mammals and the other animals, the physiological significance of TG2 and its relevance to diseases are still valuable issues to be addressed. Considering the essential and various roles of TG2, unknown functions of this protein will expand upon the related phenomena of this isozyme.

Medaka, a model fish recently used in parallel to zebrafish (*Danio rerio*), has several advantages for investigating the functions of genes of interest [9-

[11]. Taking advantages of feasible maintenance, high fertility, and short generation period, several kinds of gene-modified fishes have been established and used for drug screening in research areas, including pharmaceutical investigations [12, 13]. We recently discovered the orthologues of TGases in medaka, which consists of seven genes: OlTGK1, OlTGK2, OlTGK3 (for hTG1), OlTGB (for hFXIII), OlTGT (for hTG2), and OlTGF and OlTGO that do not correspond with specific mammalian TGase [14]. In our previous study, biochemical characterization of the OlTGK1 gene product indicate that this enzyme has cross-linking activity and was expectedly localized to the skin epidermis and spine bone. However, for the other TG isozymes in medaka, investigations are ongoing to determine their expression pattern and biochemical data. In the present study, for OlTGT, we carried out these experiments using recombinant proteins and the obtained monoclonal antibody which is essential for expression analyses of isozymes. Since TG2 is a major isozyme and has multiple functions in mammals, we believe the investigation of OlTGT, as medaka orthologue, including its biochemical properties and expression pattern will contribute to improved understanding of the physiological significance.

Materials and methods

Animal experiments

For use of rat and medaka in a series of experiments, animal care and experiments were carried out according to the Regulations for Animal Experiments in Nagoya University.

Expression and purification of recombinant OlTGT

The plasmid vector harboring OlTGT was obtained from NBRP (National

105 BioResourses Project of Japan, Okazaki, Japan), as clone ID: olsp62a16. The
106 cDNA sequence was analyzed and the data was deposited to DDBJ
107 (LC068826).
108 The purification of recombinant OlTGT was carried out as previously
109 reported [14]. Briefly, using the plasmid vector DNA (olsp62a16) as a template,
110 the possible full-length OlTGT cDNA was amplified by PCR with specific
111 primers, to attach the SmaI and XhoI recognition sites at 5' and 3',
112 respectively (forward; 5'-atcccggggctcaatctgtggacattgaac-3, reverse; 5'-
113 atctcgagttactttccaatgatgacgtttc-3'). The amplified cDNA was confirmed its
114 sequence and the SmaI-XhoI fragment was inserted into the expression
115 vector, pET24dHis which includes the initiation codon and enables to
116 express the protein attached with hexahistidine at the N-terminus. *E. coli.*
117 BL21(DE3)LysS was transformed with the constructed plasmid vector and
118 the transformant was grown in L-broth. Induction of recombinant OlTGT
119 expression was carried out by a standard method using isopropyl β-D-1-
120 thiogalactopyranoside (IPTG).
121 The harvested bacteria were lysed in lysis buffer (10 mM Tris-Cl, pH 8.0,
122 10 mM NaCl, 1 mM β-mercaptoethanol, phehylmethylsulfonyl fluoride, and
123 benzamidine) following sonication. The supernatant obtained by
124 centrifugation was applied to TALON (Clontech-TAKARA, Kyoto Japan)
125 and then eluted by elution buffer (wash buffer containing 100 mM imidazole).
126 Then the eluted proteins were subjected to size separation gel
127 chromatography (Superdex-200 increase; GE Healthcare Bio-Sciences AB
128 Uppsala, Sweden).
129
130 *Measurement of in vitro activity of transglutaminase*
131 The enzymatic activity was measured by incorporation of 5-

(biotinamido)pentylamine (bio-Cd, Pierce, IL, USA) into β-casein fixed on the 96 well microtiterplates in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 15 mM $CaCl_2$, 1 mM dithiothreitol. After incubation, microtiter well was washed with TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.1% tween-20. After adding strpetavidin-peroxidase (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and washing, the amounts of the reaction product was measured by color development of the peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB, Sigma-Aldrich, St. Louis, MO).

TG2 enzymatic activity was reported to be inhibited by GTP [15]. To examine the inhibitory effect on the enzymatic activity by GTP, the recombinant proteins for human TG2 (Zedira, Darmstadt, Germany) and OlTGT were used for the enzymatic reaction. GTP solution (Sigma-Aldrich), dissolved in 0.1 M Tris-Cl (pH. 8.0) , was contained at the indicated concetrations in the reaction as descrived above.

Establishment of rat hybridoma secreting monoclonal antibody against OlTGT

The purified recombinant proteins for OlTGT was used for immunization to Wister rat as antigen with Freund complete (once) or incomplete (twice) adjuvant in two months at the footpad. The lymphocytes from the immunized rat was prepared and fused with proliferating myeloma (Sp2/0-Ag14) using polyethylenglycol 4000 by a standard method. The hybridomas were selected in HAT medium, and then the supernatants of the grown colonies were analyzed for the production of target antibody by ELISA method. The hybridomas were amplified, cloned, and then grown for production of the antibody against OlTGT (T9D). From the supernatant from cultured cells in

159 the serum-free medium (Hybridoma-SFM, Gibco, Lifetechnologies)
160 monoclonal antibody was affinity-purified using Protein-A immobilized gel
161 by a standard method.
162
163 *Immunoblotting and immunostaining*
164 The paraffin section of medaka (cab, adult fish) was prepared by standard
165 method after fixation of Davidson solution (acetic acid, formalin and
166 ethanol) for 7 days. The 10 μm section on the slide glass was prepared and
167 de-paraffin was carried out using xylene and ethanol. To the section that was
168 blocked by blocking solution (Vectastain blocking solution including rabbit
169 normal serum, Vector Laboratories; Burlingame, CA, USA), the monoclonal
170 antibody (T9D) diluted with 0.1 % BSA-containing PBS was added and then
171 incubated at 4ºC overnight. After washing the section with the wash buffer,
172 in order to amplify the immunoreaction, biotin-labeled secondary antibody
173 was reacted. Then, followed by adding avidin and biotin-peroxidase, color
174 development was performed using 3, 3'-diaminobenzidine (DAB) staining.
175 Rat immunoglobulin G was used for negative control. The section was
176 observed under the fluorescence microscope (BZ-9000; Keyence, Osaka,
177 Japan).
178
179 **Results**
180 *Deduced primary structure of OlTGT*
181 Construction of the phylogenic tree including OlTGT and all the human
182 isozymes let us confirm the cDNA sequence as the orthologue to hTG2
183 (supplemental figure 1 and ref. 14). Then, sequence alignment of OTGT with
184 human TG2 is shown in the Fig. 1. Based on the homology with human TG2,
185 this orthologue consists of 677 putative amino acid residues with overall

186 homology to that of TG2, suggesting that OlTGT contains also four domains:
187 β-sandwich, catalytic core, β-barrels 1 and 2. In addition to the catalytic triad
188 such as Asn, His, and Cys (the active-site residue), the surrounding amino
189 acid residues around the active site (Cys) required for substrate recognition
190 and essential Trp were also preserved in the OlTGT sequence. Moreover,
191 possible calcium binding sites were observed at certain residues with a
192 similar pattern to human TG2. As for GTP binding region, however, there
193 were less homologous sequences, suggesting that OlTGT plays a role only
194 in transamidation activity.
195
196 *Production and biochemical characterization of recombinant OlTGT in*
197 *bacteria*
198 For expression of the recombinant protein, we compared the pattern in two
199 different temperatures for cultivation at 37°C or 25°C. It has previously been
200 reported that OlTGT was harvested as the soluble fraction as expected size
201 of 75 kDa when incubated at the lower temperature (Fig. 2A) [14]. Under this
202 culture condition, the protein expressed in *E. coli* was successfully purified
203 to homogeneity by metal affinity-purification, followed by size exclusion
204 chromatography (Fig. 2B). The amounts of purified protein enabled us to
205 perform biochemical characterization studies and to also obtain monoclonal
206 antibodies as an antigen.
207 For evaluation of the enzymatic activity, biotin-labeled pentylamine (bio-
208 Cd), as a glutamine-acceptor substrate, was incorporated into the microtiter-
209 well coated β-casein, as a glutamine- donor substrate. Depending on the
210 concentration of this glutamine-acceptor substrate, incorporation of bio-Cd
211 into β-casein was increased in the presence of OlTGT (Fig. 3A). This
212 reaction is also time-dependent (Fig. 3B) and the co-incubation of EDTA in

both reactions abrogated the incorporation indicating the enzyme reaction is calcium ion-dependent.

In mammalian TG2, the enzymatic activity is regulated by GTP. Then, we examined its inhibitory effect on the enzymatic activities using recombinant proteins for both hTG2 and OlTGT. As shown in Fig. 3C, whereas the activity of hTG2 was inhibited by the presence of GTP in a concentration-dependent manner (1-100 μM), OlTGT did not show any inhibitory regulation. This result is consistent with the fact that there was no reported GTP-interacting sites in the primary sequence of OlTGT.

Immunochemical analyses of OlTGT

A hybridoma that secreted a monoclonal antibody against OlTGT was established using the purified recombinant protein as the antigen. Upon analysis, since confirmation of the cross-reactivity against other medaka TGase isozymes is important, immunoblotting was carried out using each recombinant protein prior to immunohistochemical experiments (supplemental figure 2). The antibody recognized and mainly reacted to OlTGT but slightly cross-reacted with OlTGK1.

Regarding the immunohistochemical analysis, expression was unexpectedly not ubiquitous as reported in mammals. Higher level of the expression was apparently observed at the site of the eye, brain, spinal cord, and gas-gland as shown in Fig. 4A. In Fig. 4B, the enlarged pictures indicate that in the eye, brain and spinal cord, staining was specified around the retina, optic tectum and myelin sheath, respectively. Upon immunostaining, we also confirmed less cross-reactivity using the serial section for OlTGK1 antibody reaction that gave no signal (supplemental figures 3 and 4).

Discussion

Calcium ion-dependent cross-linking reactions catalyzed by TGase are characteristic to vertebrate TGases but also observed in a wide range of the invertebrates TGases. So far, TGases with mammalian-like structures have also been characterized in various organisms including horseshoe crab, *Drosophila*, grasshopper, and slime mold [1)]. Among eight human TG isozymes, since tissue-type TGase (TG2) is ubiquitously expressed in and plays multiple roles, a lot of studies on the biochemical properties and physiological functions of this isozyme have been conducted including on other animals. Fish is evolutionally the lowest vertebrate but they maintain several physiological functions of those preserved in mammals. As a well-known model fish, zebrafish has been used in studies of various biological phenomena including TGase [16-17)]. In addition, *in situ* expression pattern of one orthologue of TGase in medaka (identical to OlTGB; orthologue for mammalian FXIII) was reported during embryogenesis [18)]. However, in these studies, investigations at the protein and histological analyses on this isozyme (tissue-type TG; TG2) as TGase family have not been included.

In this fish model, medaka, we very recently identified all the orthologues of human TGases some of which apparently corresponded with the eight mammalian isozymes in medaka genes [14)]. OlTGT (T means tissue-type), obviously the orthologue for mammalian TG2, has a total deduced molecular mass of 75 kDa, which is similar to human TG2 obtained from the results of recombinant proteins (Fig. 2). Although we did not confirm with the other possible translation initiation site in the 5' flanking region, it would be

greatly possible our examined cDNA sequence encoded open reading frame because of high homology with human TG2 sequence. As shown in Fig. 1, when compared to human TG2, residues essential for the calcium-dependent cross-linking reaction were mostly conserved, which is consistent with the biochemical data.

To characterize the biochemical properties of OlTGT, we produced recombinant protein in bacteria, which was successfully expressed at the lower temperature probably due to sterically stabilized form. These experiments demonstrated that the enzyme was sterically reproducible and active during bacterial expression. Using this sterically stabilized protein as an antigen, we obtained biochemical data and a monoclonal antibody used for the tissue distribution analysis. As expected, the purified recombinant protein for OlTGT showed the apparent enzymatic activity in a calcium-ion dependent manner. Only the difference in the enzymatic property is that OlTGT was not affected by GTP, which inhibits the enzymatic activity of transamidation of mammalian TG2 [1-3, 15]. This result was expected because there is less homologous on the GTP-binding region between human TG2 and OlTGT (Fig. 1) This tendency is also found in the case of frog TG2 orthologue (*Xenopus laevies*; NP_001085410). It is interesting that medaka and frog orthologues for TG2 do not have such a GTP-binding region from the aspect of molecular evolution, but its physiological significance is currently unknown.

The immunohistochemical analysis indicated the data that OTGT is expressed in limited tissues: eye, brain, spinal cord, and gas-gland. It is

notable that OlTGT is expressed not ubiquitously, which is different property from mammalian TG2.

In the eye, the expressed areas are concentrated around the retina, which was observed in another fish (goldfish) where TG activity is essential for recovery from injury [19]. The precise significance of mammalian TG2 in brain remains unknown, however, evidence suggests that it may be involved in IL-2 cross-linking and overexpression may lead some cytotoxic factors to oligodendrocyte [20]. As for spinal cord, spontaneous recovery from injury was investigated in adult zebrafish [21]. OlTGT may be involved in the event since TG2 contribute to recovery of tissue injury. Although the expressions of gas-gland is first finding in the fish, identification of substrates might give a clue to know the physiological significance.

In conclusion, we characterized OlTGT, an orthologue for mammalian TG2 involved in various biological processes, both biochemically and immunohistochemically. Limited expression rather than ubiquitous distribution was observed in the eye, brain, spinal cord and gas-gland in this organisms, which has a possibility novel functions rather than mammals. In addition to analysis on substrates, the functional significance of this expression will be clarified by tissue-specific deletion, which is possible by using a genome editing system. Research is ongoing in these area.

Author Contribution

The first (Y. T.) and the second (Y. W.) authors performed the experiments with equal contribution. The other authors (K.O., H.T., H.H.) support the experiments and data analyses. The last author (K. H.) organized the

planning and completion of the manuscript.

Acknowledgment

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393 Figure legend

394 Fig. 1. Sequence alignment of OlTGT and human tissue-type
395 transglutaminase (TG2).

396 Amino acid sequences from medaka (OlTGT) and human (hTG2) are
397 aligned. The identical residues are boxed. The closed triangles and rectangle
398 represent the catalytic triad (C268, H325, and D348 in OlTGT sequence) and
399 essential residue (W241) for activity, respectively. The open circles and
400 boxes represent the calcium binding domain and required region for the GTP
401 interaction of human TG2, respectively.

402

403 Fig. 2. Expression and purification of recombinant OlTGT

404 The supernatant and precipitate fractions from *E. coli* expressing the
405 recombinant OlTGT cultured in the presence or absence of IPTG at the 37°C
406 or 25°C were subjected to 7.5% SDS-PAGE. For subjected samples, sup and
407 ppt indicate the supernatant and the precipitated fractions in the cellular
408 lysate (A). Recombinant protein was purified from the lysates by TALON
409 affinity chromatography and size exclusion chromatography. Each step of
410 the purification was analyzed by SDS-PAGE. The molecular mass marker
411 was paralleled (B). The arrowhead indicates the OlTGT protein.

412

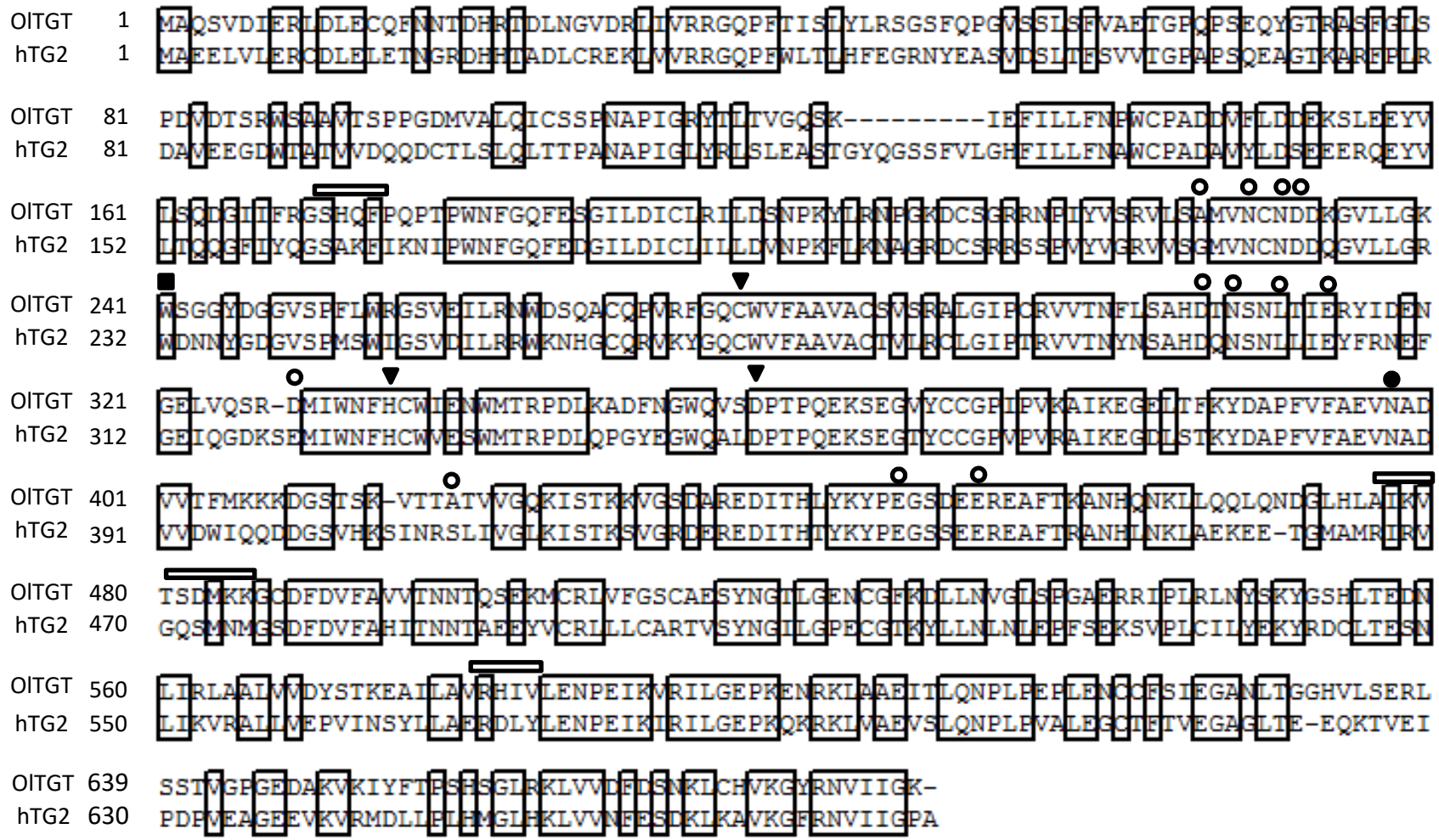
413 Fig. 3. Enzymatic activity of recombinant OlTGT

414 The purified protein was analyzed for its enzymatic activity by measuring
415 the incorporation of bio-Cd into β-casein. In the 100 μL of the reaction
416 mixture, 1 μg of recombinant OlTGT was included along with 15 mM $CaCl_2$
417 and bio-Cd (A: 0-800 μM; B: 500 μM). Incorporation of bio-Cd at various
418 concentrations of bio-Cd in the reaction mixture (A) and indicated times (B)

were measured in the presence (closed circle) or absence (closed rhombus) of 30 mM EDTA. (C) In the co-presence of the indicated final concentrations of GTP, the enzymatic activities of hTG2 and OlTGT were measured using 500 ng of recombinant proteins in the reaction mixture (15 min). The open and closed boxes indicate the activities of hTG2 and OlTGT, respectively. The inhibitory ratio was calculated based on the activity in the absence of GTP. In all the experiments, data represent means ± SD of triplicate samples.

Fig. 4. Immunohistochemical analysis on whole medaka section

From the fixed and paraffin-embedded medaka, tissue sections were prepared. The serial sections were subjected to hematoxylin and eosin (HE) staining (upper), and immunoreaction using the monoclonal antibody T9D (middle) as well as the same amounts of rat immunoglobulin G (lower). The immunoreaction was carried out and developed for a whole section. The scale bar indicates 2 mm (A). The images indicated by arrows are enlarged for eye, brain, spinal cord, and gas-gland (B). The scale bars for the former three tissues and gas-gland are 100 and 500 μm, respectively.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hTG2 1
OlTGT 81
hTG2 81
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hTG2 391
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OlTGT 560
hTG2 550
OlTGT 639
hTG2 630

Fig. 1

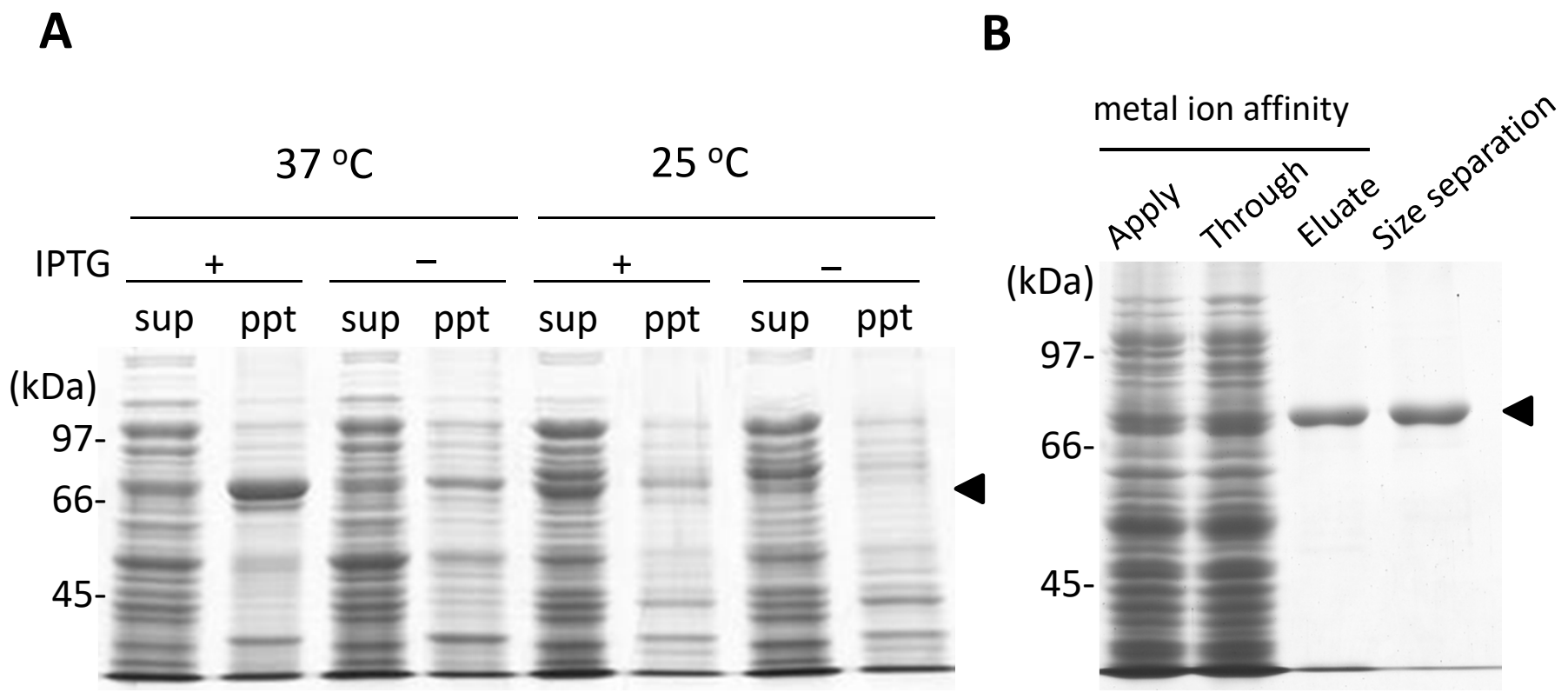
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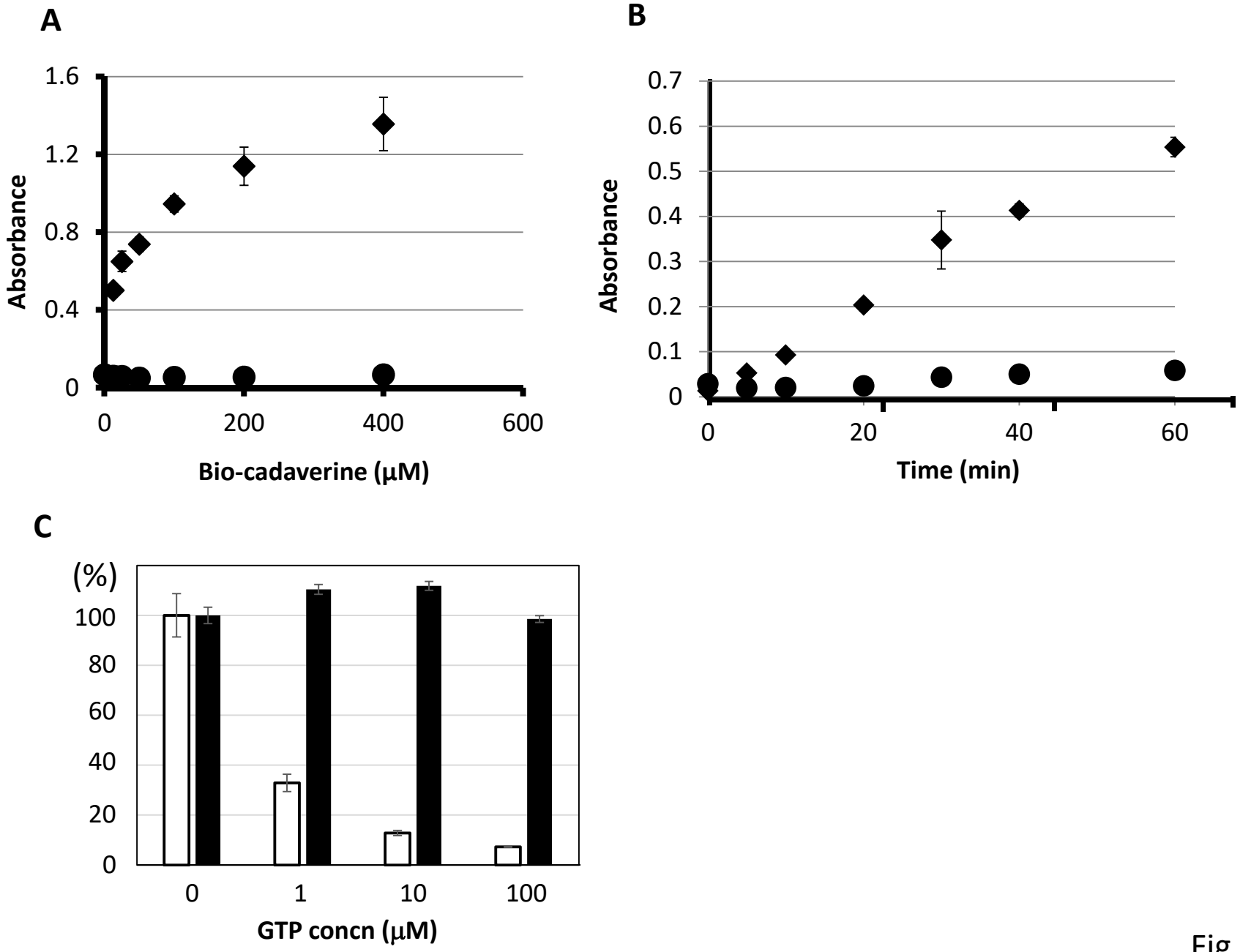
Fig. 2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0.4
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Absorbance
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Fig. 3

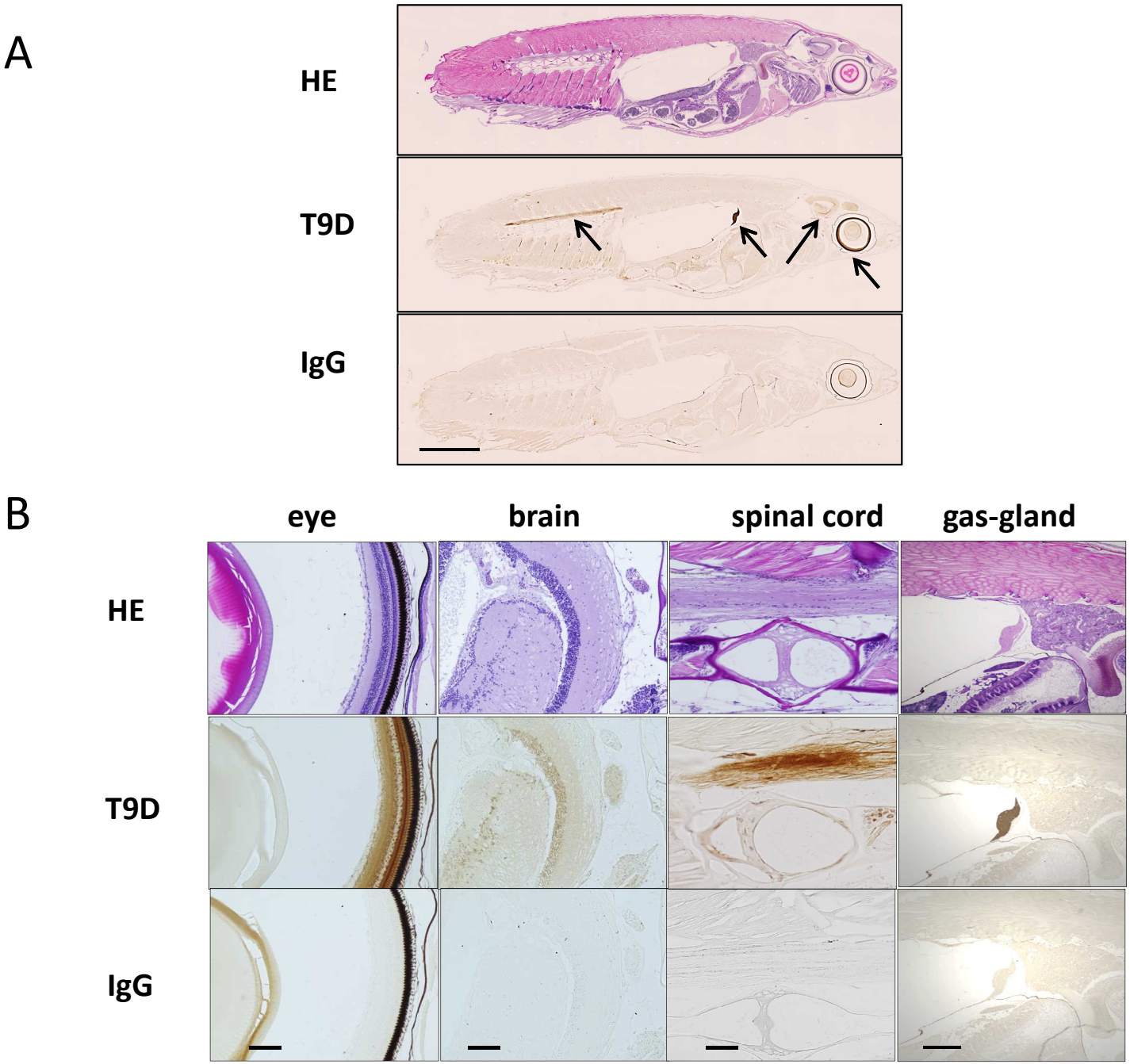
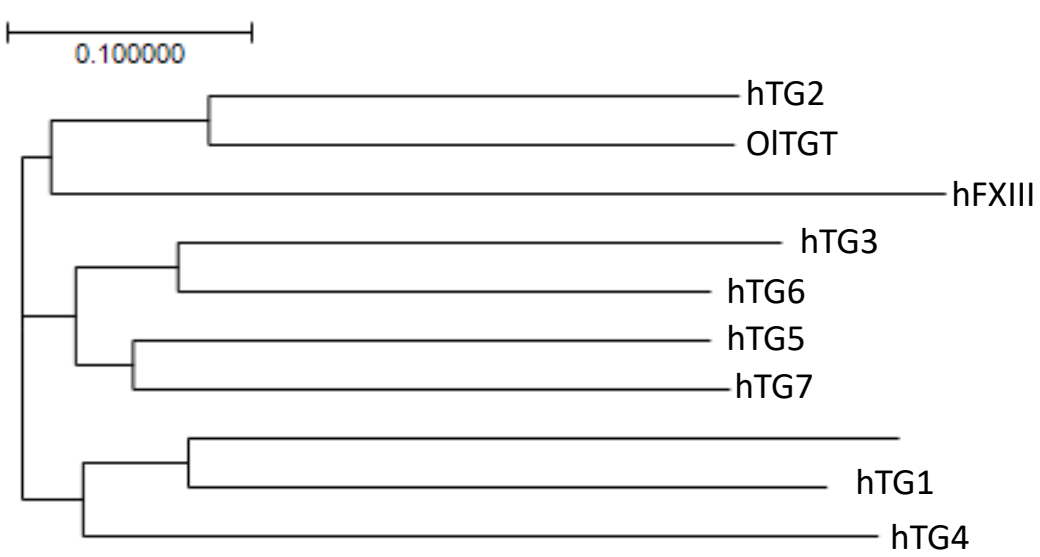
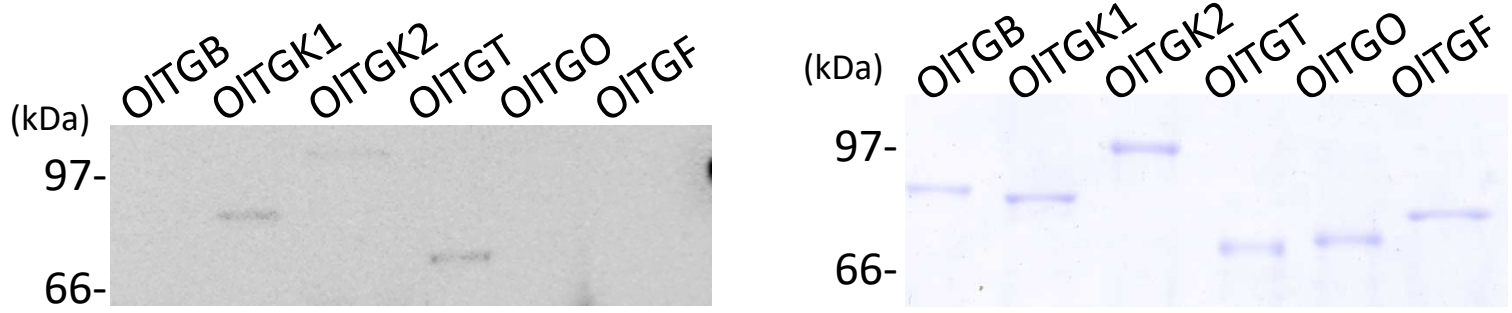


Fig. 4



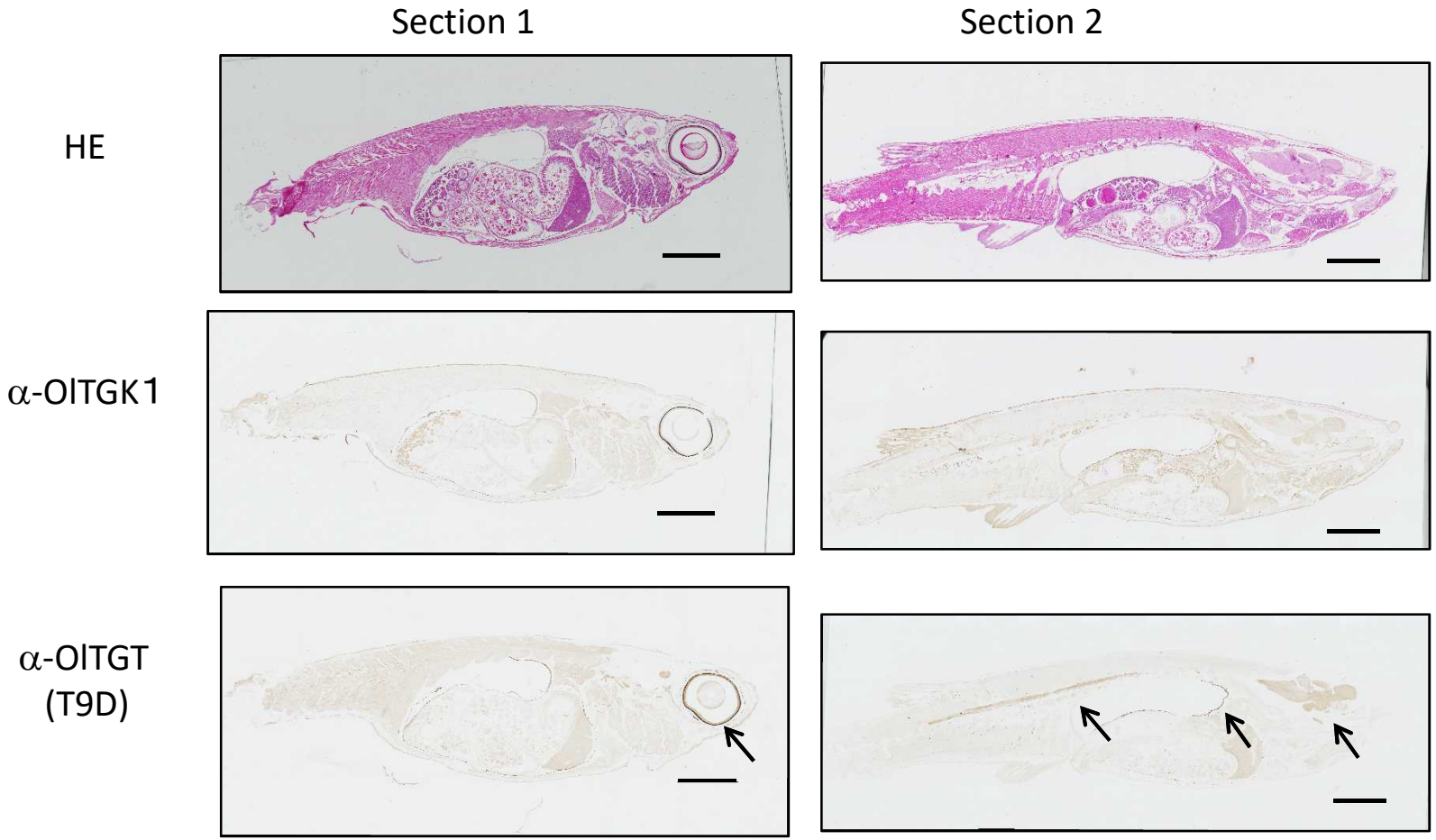
Supplemental Fig. 1

The phylogenic tree was depicted (1000 boostrap trials, Neighbor-Joining Method plot) based on the deduced primary sequences of human FXIII (NP_000120), TG1 (NP_000350), TG2 (NP_004604), TG3 (NP_003236), TG4 (NP_003232), TG5 (NP_963925), TG6 (NP_945345), TG7 (NP_443187), and OlTGT (LC068826).



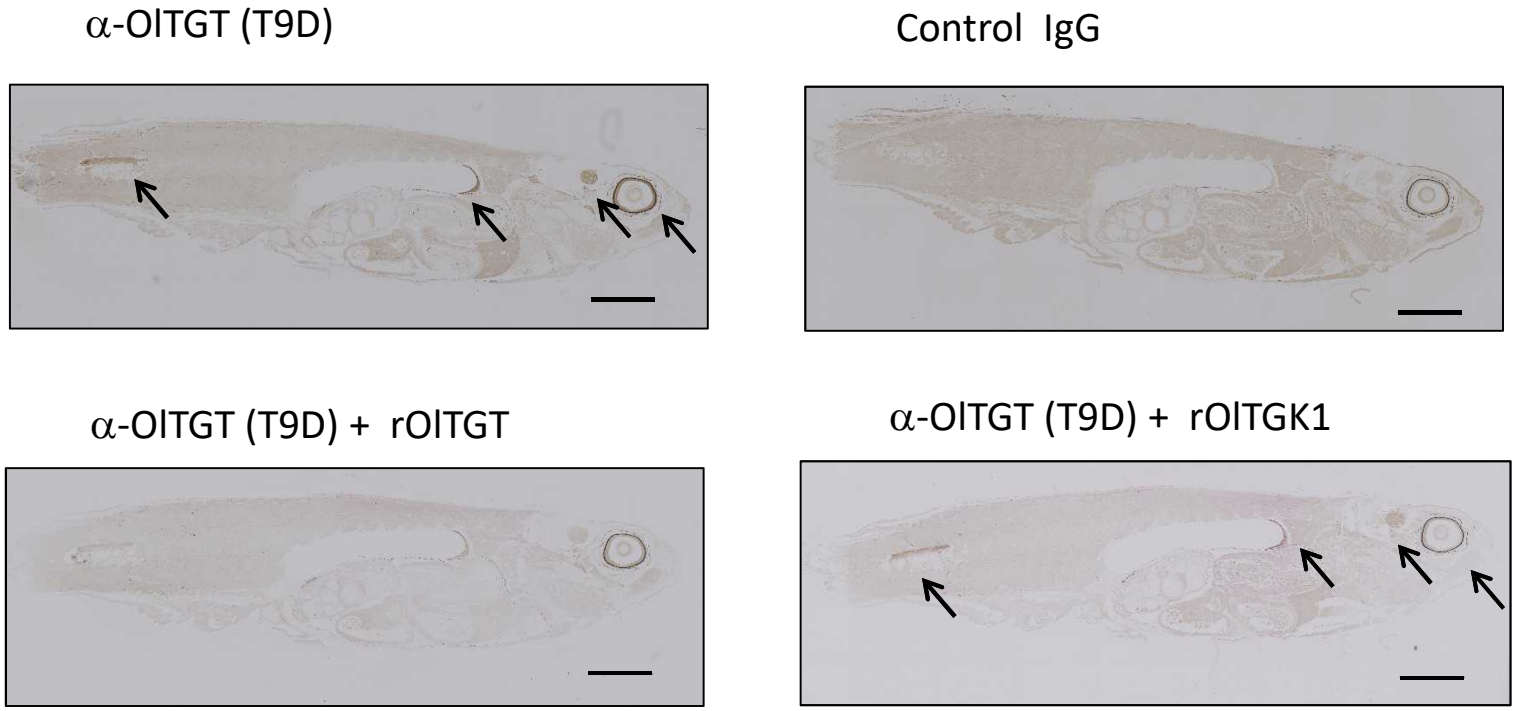
Supplemental Fig. 2

Immunoblotting using T9D analyzed on 10 ng of the recombinant proteins for OlTGB, OlTGK1, OlTGK2, OlTGT, OlTGO and OlTGF (left). CBB-staining (100 ng) is also shown. Immunoblotting was carried out as a standard method as described previously (14).



Supplemental Fig. 3

Immunostaining for serial sections of two distinct areas of whole medaka body using anti-antibodies for either OlTGK1 (α-OlTGK1) or OlTGT (α-OlTGT, T9D). The arrows indicate the specific signals that were not cross-reacted by anti-OlTGK1 reactions. HE staining was paralleled. The scale bar shows 2 mm.



Supplemental Fig. 4

Comparative immunostaining using pre-absorbed antibodies by two antigens. Monoclonal antibody solution was pre-incubated for 30 min at room temperature with excess molar (1:20) of antigens, the purified recombinant proteins for OlTGT and OlTGK1, and then reacted with the serial whole sections as in the cases of Figs 4. and S3. The specific signals (arrows) were obtained in the case of anti-OlTGT antibody (α-OlTGT), not in the case of rat immunoglobulin G (control IgG). The specific signals (arrows) were decreased with antibody that was pre-absorbed with recombinant OlTGT (OlTGT+rOlTGT), but not in the case of recombinant OlTGK1 (α-OlTGT + rOlTGK1). The scale bar shows 2 mm.