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# Expression analysis of *thg1l* during *Xenopus laevis* development

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**ABSTRACT The tRNA-histidine guanylyltransferase 1-like (*THG1L*), also known as induced in high glucose-1 (*IHG-1*), encodes for an essential mitochondria-associated protein highly conserved throughout evolution, that catalyses the 3'–5' addition of a guanine to the 5'-end of tRNA-histidine (tRNA$^{His}$). Previous data indicated that THG1L plays a crucial role in the regulation of mitochondrial biogenesis and dynamics, in ATP production, and is critically involved in the modulation of apoptosis, cell-cycle progression and survival, as well as in cellular stress responses and redox homeostasis. Dysregulations of THG1L expression play a central role in various pathologies, including nephropathies, and neurodevelopmental disorders often characterized by developmental delay and cerebellar ataxia. Despite the essential role of THG1L, little is known about its expression during vertebrate development. Herein, we examined the detailed spatio-temporal expression of this gene in the developing *Xenopus laevis*. Our results show that *thg1l* is maternally inherited and its temporal expression suggests a role during the earliest stages of embryogenesis. Spatially, *thg1l* mRNA localizes in the ectoderm and marginal zone mesoderm during early stages of development. Then, at tadpole stages, *thg1l* transcripts mostly localise in neural crests and their derivatives, somites, developing kidney and central nervous system, therefore largely coinciding with territories displaying intense energy metabolism during organogenesis in *Xenopus*.**

**KEYWORDS:** Post-transcriptional modifications, tRNA, mitochondria, Spinocerebellar Ataxia

## Introduction

Post-transcriptional modifications of tRNAs are highly evolutionary conserved mechanisms required for accurate translation. These modifications play a number of different roles, such as regulating the decay of both pre-tRNA and mature tRNA, controlling specificity and accuracy of mRNA decoding, and modulating the efficiency and specificity of tRNA aminoacylation (Phizicky and Hopper, 2023). Several data have shown that impairments of tRNA post-transcriptional modification machinery have a central role in various pathological conditions, such as neurological disorders and cancer, and also affect embryonic development (Balke *et al.*, 2015; Blaze and Akbarian, 2022). The tRNA-histidine guanylyltransferase 1-like (THG1L), also known as "induced in high glucose-1" (IHG-1), encodes for a highly evolutionarily conserved essential protein associated with the inner mitochondrial membrane (Bhreathnach *et al.*, 2017; Hickey *et al.*, 2011) that catalyses the 3'–5' addition of a guanine to the 5'-end of tRNA-histidine (tRNA$^{His}$) (Murphy *et al.*, 2013). With few exceptions, the unusual 5'-end consisting of an extra guanylate residue called G–1 in the acceptor stem is required for tRNA$^{His}$ aminoacylation by histidyl-tRNA synthetases, both in prokaryotes and in eukaryotes (Rosen *et al.*, 2006). Although its essential role has been underlined, *THG1L* is still a little-studied gene, whose molecular pathways and involvement in physiological and pathological conditions are far from being elucidated.

To date, previous studies have described THG1L as a key regulator of mitochondrial function that, by stabilizing the transcriptional cofactor peroxisome proliferator–activated receptor-γ coactivator 1α (PGC-1α), increases the mitochondrial respiratory capacity, ATP production, biogenesis and fusion, therefore influencing cell survival in conditions of oxidative stress (Edvardson *et al.*, 2016; Hickey *et al.*, 2011; Hickey *et al.*, 2014). Moreover, *in vitro* experiments on HeLa cells have suggested that IHG-1 may have a role in the regulation of cell cycle progression, cell morphology, and proliferation (Guo *et al.*, 2004). In the same line, *in silico* analysis have reported that THG1L functionally interacts with various key mediators involved in apoptosis, proliferation, and also with regulators of cellular stress response and redox homeostasis (Bhreathnach *et al.*, 2017). Previ-

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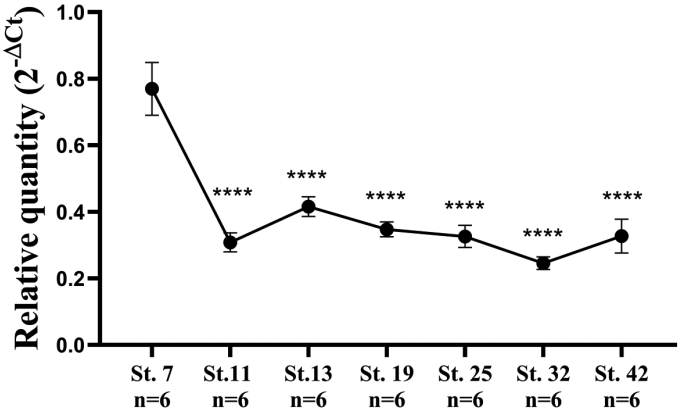


**Fig. 1. RT-qPCR analysis of *thg1l* expression during development.** cDNAs were synthesized from embryos and larvae at different developmental stages (St., indicated at the bottom of the panel) and amplified using specific primers for *thg1l*, as well as *sub1.S*, *slc35b1.L* and *ppp1ca.L*, used for normalization. As technical control, reverse transcriptase was omitted. Asterisks (*) indicate statistically significant difference between each stage and St. 7. Data are expressed as mean ± standard error of the mean; n, number of samples analysed. ANOVA followed by Tukey post-hoc test: ****$p < 0.0001$.

ous works have indicated that THG1L expression dysregulations have a central role in the pathogenesis of tubulointerstitial fibrosis promoted by the major pro-fibrotic key mediator, the transforming growth factor 1-β (TGF1-β), in both diabetic nephropathy in humans and in experimental renal fibrosis (Bhreathnach *et al.*, 2017; Corcoran *et al.*, 2013; Murphy *et al.*, 2008). Although far to be elucidated, it has been suggested that THG1L plays a critical role during development. In particular, specific *THG1L* variants have been identified in patients showing various degrees of developmental delay and cerebellar ataxia, sometimes associated with microcephaly, cerebellar hypoplasia, dysarthria, pyramidal signs and epileptic encephalopathy, as well as, in rare cases, other multisystem abnormal manifestations (Edvardson *et al.*, 2016; Han *et al.*, 2023; Rabin *et al.*, 2021; Shaheen *et al.*, 2019; Walker *et al.*, 2019). Indications of a potential involvement of *thg1l* in central nervous system (CNS) development have been also provided by microarray analyses in developing *Xenopus laevis* embryos, showing that *thg1l* is among the genes coherently regulated by the paired-like homeodomain transcription factor Rax/Rx1 (Giudetti *et al.*, 2014), a well-known critical factor for vertebrate retina specification and morphogenesis, which also plays a key role in brain development (Terada *et al.*, 2006). Finally, considering that dysregulations of mitochondrial function have an active role in the pathogenesis of several diseases, including neurodegenerative, cardiac, skeletal muscle disorders (Chen *et al.*, 2023; Schulz and Schluter, 2023), as well as in embryo development (Bruna de Lima *et al.*, 2023; May-Panloup *et al.*, 2021), clearly emerges the importance of research aimed at increasing knowledge about this gene. To our knowledge, the expression pattern of THG1L during embryogenesis is a topic that has been poorly investigated. As *Xenopus laevis* is a well-known experimental model in biomedical research and development, we have performed a detailed spatio-temporal expression analysis of *thg1l* on developing *Xenopus*, with the aim to increase the knowledge on this tRNA post-transcriptional modifier and provide a foundation for further studies on its functional role.

## Results and Discussion

### Temporal expression of *thg1l*

To analyze the temporal expression pattern of *thg1l* during embryonic development, we performed real-time reverse transcription-polymerase chain reaction (RT-qPCR) assay on cDNAs obtained from *Xenopus* embryos at different developmental stages. Our results show that this gene is expressed during embryonic life, in line with some evidence provided by *in situ* hybridization (ISH) in zebrafish (Thisse and Thisse, 2004), as well as by microarray in *Xenopus laevis* (Giudetti *et al.*, 2014). In detail, our temporal expression analysis (Fig. 1) shows that *thg1l* transcripts are already detectable at blastula 64-cell stage (stage, St. 7), and therefore before mid-blastula transition (St. 8 according to Newport and Kirschner (1982)), indicating the presence of maternal transcripts, as also suggested by previous transcriptomic data (Session *et al.*, 2016; Yanai *et al.*, 2011), and a potential role for *thg1l* during the earliest phases of embryonic development. In the same line, maternal expression of *thg1l* has been reported in zebrafish (Thisse and Thisse, 2004). Maternal transcripts of other highly conserved tRNA post-transcriptional modifiers have been reported in *Xenopus* and zebrafish as, for instance, GTP-binding protein 3 (*gtpbp3*) (Chen *et al.*, 2016), tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*trmu*) (Zhang *et al.*, 2018), tRNA nucleotidyl transferase 1 (*trnt1*) (DeLuca *et al.*, 2016) and tRNA (guanine(37)-N1)-methyltransferase 5 (*trmt5*) (White *et al.*, 2017). Interestingly, in line with data on THG1L, defects in these genes are responsible for rare syndromes characterized by severe pathological traits in humans, such as varying degrees of brain defects, developmental delay, intellectual disability, seizure, dysarthria and ataxia (Argente-Escrig *et al.*, 2022; Chakraborty *et al.*, 2014; Reinhart *et al.*, 1993; Sasarman *et al.*, 2015; Wiseman *et al.*, 2013; Zhang *et al.*, 2023). Successively, *thg1l* expression levels in *Xenopus laevis* are quickly reduced, probably due to the consumption of maternal mRNA, reaching the lowest expression level by gastrula stage (St. 11), and then remaining almost unchanged until St. 42 tadpole. A similar temporal expression profile was previously reported, by RNA-sequencing analysis, in the Xenbase database for *thg1l* (Fisher *et al*., 2023) and also for other tRNA modifiers such as *trnt1*, *trmu*, and *trmt5* (Session *et al*., 2016; Yanai *et al*., 2011), indicating that specific post-transcriptional processing of tRNAs, including the 3'–5' addition of a guanine to the 5'-end of tRNA-histidine ($tRNA^{His}$), is regulated in a time-dependent manner, and may have a fundamental role especially in the initial phases of vertebrate embryogenesis. As THG1L influences mitochondrial activity (Edvardson *et al.*, 2016; Hickey *et al.*, 2011; Hickey *et al.*, 2014), this possibility is in line with the fact that the function of mitochondria is finely modulated during development, playing a pivotal role during the earliest stages, as previously reported in various experimental models, including *Xenopus laevis* (Han *et al.*, 2018; Klymkowsky, 2011).

### Spatial expression of *thg1l*

To determine the spatial expression of *thg1l* we performed ISH throughout development. During the earliest blastula stages (St. 7), before mid-blastula transition, maternal *thg1l* transcripts localise in the animal hemisphere (Fig. 2 A,B,C,F). In particular, the hybridization signal appears mostly localised in the basal portion of animal blastomeres at this developmental stage (Fig. 2F) suggesting the possibility that the asymmetric localization of *thg1l* mRNA at early

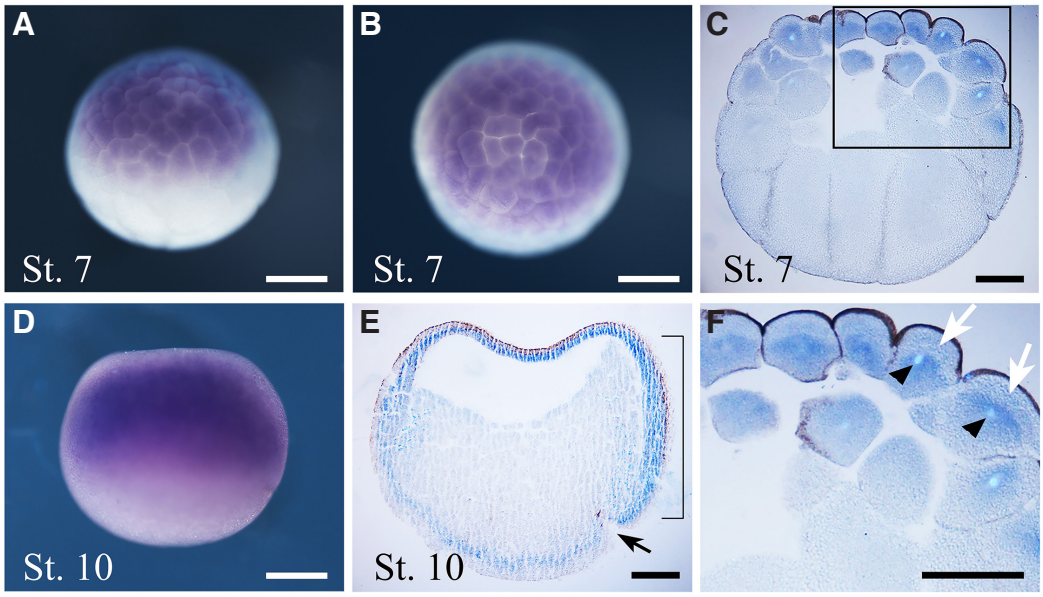


**Fig. 2. *In situ* hybridisation analysis of *thg1l* during segmentation and gastrulation.** Embryo developmental stages (St.) are indicated at the bottom left corner of each panel. **(A,D)** Lateral view, animal pole to the top and vegetal pole to the bottom, and **(B)** animal view, of whole-mount stained embryos. **(C,E)** Sagittal sections from whole-mount embryos, animal pole to the top and vegetal pole to the bottom. Black arrow in **(E)** points to the dorsal lip of the blastopore. **(F)** Magnified view of the boxed region indicated in **(C)**, showing the intracellular localization of *thg1l* transcripts in animal blastomeres. White arrows show absence of the transcript in the apical portion of cytoplasm, whereas black arrowheads indicate Hoechst-stained nuclei. Brackets in **(F)** indicate the dorsal involuting mesoderm. Scale bars: 200 μm in **(A,B,D)**; 150 μm in **(C,E,F)**.

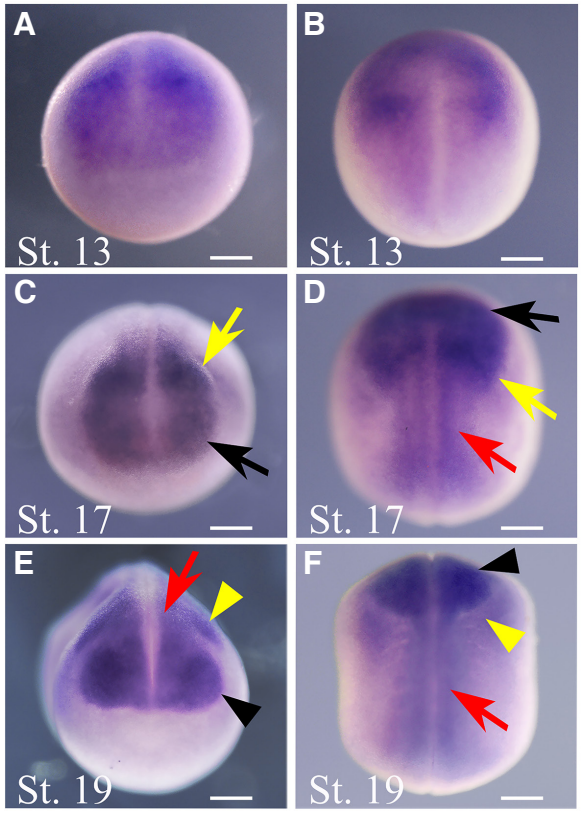


**Fig. 3. *In situ* hybridisation analysis of *thg1l* during neurulation.** Embryo developmental stages (St.) are indicated at the bottom left corner of each panel. **(A,C,E)** Frontal view, dorsal to the top, and **(B,D,F)** dorsal view, frontal to the top, of whole-mount hybridized embryos. Black arrows indicate the anterior neural plate. Yellow arrows point to the neural crests. Red arrows indicate the neural tube. Black arrowheads point to the optic vesicles. Yellow arrowheads indicate migrating neural crest cells. Scale bar: 250 μm.

developmental stages may be involved in cell-fate determination as reported for various mRNA asymmetrically distributed in the cells (Skamagki *et al.*, 2013). At early gastrula St. 10 (Fig. 2 D,E), *thg1l* is expressed in the ectoderm and in the presumptive mesoderm of the marginal zone. At early neurula stage (St.13, Fig. 3A,B), *thg1l* signal localizes in anterior regions of the neural plate and, although at significantly lower level, also in the posterior neural plate. This pattern becomes more demarcated at mid-neurula (St. 17, Fig. 3 C,D) when the expression of *thg1l* increases in the most anterior region of the neural plate and in territories of neural crests, while additional lower signal continues to be detectable in the in the posterior neural plate. Successively, after neural tube closure at late-neurula (St. 19, Fig. 3 E,F), *thg1l* hybridization signal is observed in the neural tube, in migrating neural crest cells, and in optic vesicles.

At early tailbud (St. 22-23, Fig. 4A), *thg1l* transcripts mostly localise in eye vesicles and in migrating neural crest material, with a weak signal also detectable in the prosencephalon; therefore, all structures originating from the territories where *thg1l* is expressed at neurula stage. Additionally, a weak expression is present in somites and in the posterior region of the trunk, including the posterior wall.

By St. 28 (Fig. 4B), *thg1l* is clearly recognizable in the prosencephalon, rostral region of the spinal cord, branchial arches, developing eye, otic vesicles, pronephros, somites in the posterior half of the trunk, and in the growing tail. As shown in Fig. 4C, a similar expression pattern is mostly maintained at later tailbud stage (St.33/34), when a hybridization signal becomes observable in the midbrain-hindbrain boundary, and a weak signal is also distinguishable in cranial placodes, including olfactory epibranchial and lateral line placode, mesencephalon and rhombencephalon. Additionally, histological sections at this stage (Fig. 4 D-H) show a clear signal in lens, whereas a faint signal is observed in the retina, Rathke's pouch and posterior region of the notochord, suggesting that *thg1l* expression level in these regions is likely to be weak. Suggestive of a possible influence of *thg1l* on mitochondrial activ-

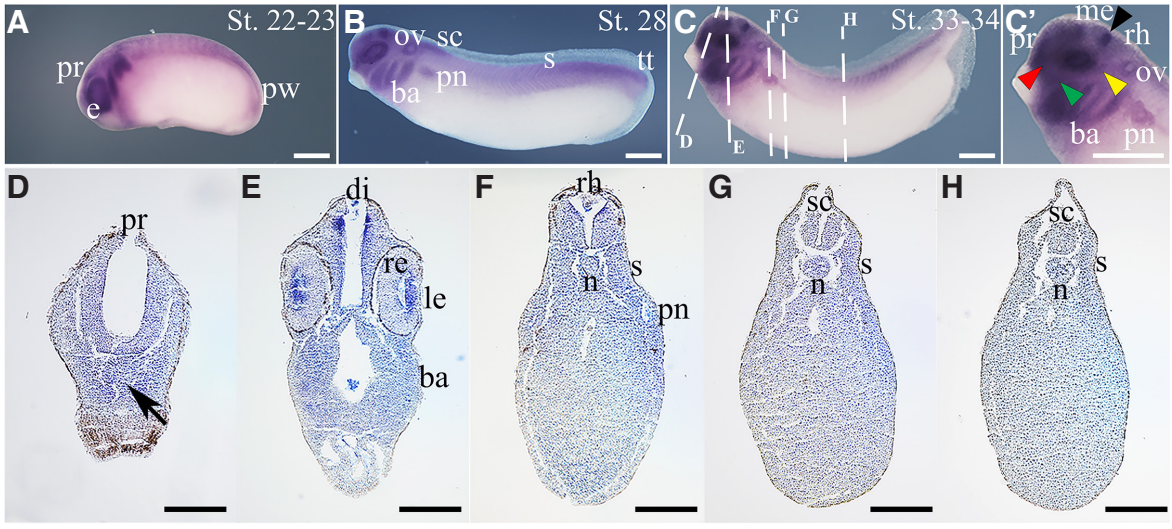


**Fig. 4. *In situ* hybridisation analysis of *thg1l* in tailbud embryos.** Embryo developmental stages (St.) are indicated at the top right corner of each panel. **(A,B,C)** Lateral views of whole-mount hybridized embryos, anterior to the left; **(C')** magnified view of **(C)**. Dashed lines in **(C)** indicate the section planes shown in **(D-H)**. **(D-H)** Transverse sections from whole-mount St. 33-34 embryos, dorsal to the top. Black arrow in **(D)** points to the Rathke's pouch. Black arrowhead in **(C')** indicates the midbrain-hindbrain boundary; yellow arrowhead indicates lateral line placode; green arrowhead indicates epibranchial placode, and red arrowhead indicates olfactory placode. Scale bars: 500 µm in **(A-C, C')**; 250 µm in **(D-H)**. ba, branchial arches; di, diencephalon; e, eye; le, lens; me, mesencephalon; n, notochord; ov, otic vesicle; pn, pronephros; pr, prosencephalon; pw, posterior wall; re, retina; rh, rhombencephalon; s, somites; sc, spinal cord; tt, tail tip.

ity also during embryogenesis, a previous work in *Xenopus laevis* reported that the nervous system, eye, skeletal muscle and kidney tissues (territories in which *thg1l* is clearly expressed), are sites with high energy requirements during organogenesis. In addition, the same authors have shown that the spatial expression pattern of GRIM-19, a newly identified subunit of the Mitochondrial Respiratory Chain (MRC) complex I, which plays crucial roles in basic biological processes through the production of cellular energy, generation of reactive oxygen species, and initiation of apoptosis, is largely similar to the one we report for *thg1l* (Chen *et al.*, 2007).

As shown in (Fig. 5), in the head region of St. 42 tadpoles, *thg1l* transcripts localise in the olfactory pits and Jacobson's organ, telencephalon, hypothalamus, mesencephalon and rhombencephalon (Fig. 5 A-C). In addition, the hybridization signal is clearly observed in the velar plate, parachordal cartilages, cranial muscles including mandibular muscles, as well as a weak expression is localized in the infrarostral cartilage (Fig. 5 B,C). Moreover, in the eye region, at the time when the retina is mature, *thg1l* transcripts mostly localize in the lens, inner plexiform layer and external ocular muscles, although a weak signal is diffused in all retinal layers (Fig. 5 B,B').

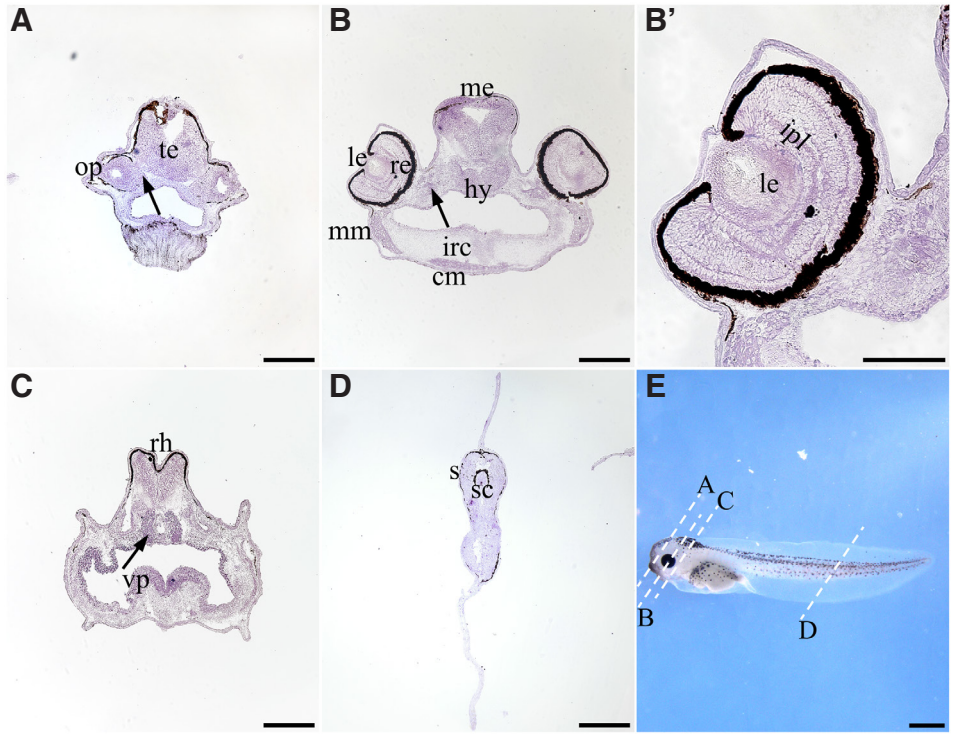


**Fig. 5. *In situ* hybridisation analysis of *thg1l* in stage 42 larvae. (A,B,B',C,D)** Transverse cryosections, dorsal to the top. **(E)** Lateral views of the larvae, anterior to the left. Dashed lines in **(E)** indicate the section planes shown in **(A, B, B',C,D)**. Black arrows indicate the Jacobson's organ in **(A)**, external ocular muscles in **(B)**, and parachordal cartilages in **(C)**; **(B')** high magnification of the eye represented in **(B)**. Scale bars, 200 µm in **(A,B,C,D)**; 1 mm in **(E)** and 100 µm in **(B')**. cm, cranial muscles; hy, hypothalamus; ipl, inner plexiform layer; irc, infrarostral cartilage; le, lens; me, mesencephalon; mm, mandibular muscles; op, olfactory pit; te, telencephalon; re, retina; rh, rhombencephalon; s, somites; sc, spinal cord; vp, velar plate.

Finally, in the trunk-caudal region, *thg1l* expression was observed in the spinal cord and somites (Fig. 5D).

In conclusion, our study elucidates the previously undescribed spatio-temporal expression pattern of *thg1l* in *Xenopus laevis*. The dynamic expression of the tRNA-histidine guanylyltransferase mRNA, together with the activity of its encoded protein, suggest an important role for *thg1l* during the development of various organs and tissues. Moreover, the differential regulation of *thg1l* in different cells during development is necessary for proper tissues morphogenesis and function.

## Materials and Methods

### Total RNA extraction and RT-qPCR analysis

*Xenopus laevis* embryos were generated and staged in accordance with published works (Messina *et al.*, 2015; Newport and Kirschner, 1982). For each biological sample, total RNA was extracted from a pool of six-ten embryos, using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Phenol-chloroform extraction was performed to isolate RNA that successively was precipitated and purified using RNAeasy Plus MiniKit (Qiagen, Venlo, The Netherlands), in line with manufacturer's instructions. DeNovix™ (Wilmington, DE, USA) spectrophotometer was used to measure concentration and purity of total RNA, while agarose-gel electrophoresis was performed to evaluate RNA integrity. According to manufacturer's instructions, first-strand cDNA was synthesised using QuantiTect Reverse Transcription Kit (Qiagen).

Relative measurements of mRNA expression levels were performed by RT-qPCR using the SYBR Green method (SensiMix SYBR kit, Bioline, London, UK) on a QuantStudio 3 system (ThermoFisher), following the manufacturer's protocol. Transcript levels of *thg1l* were normalised on the expression of three reference genes (*sub1.S*, *slc35b1.L* and *ppp1ca.L*), in line with previous works (Martini *et al.*, 2023; Mughal *et al.*, 2018; Vandesompele *et al.*, 2002), and calculated as $2^{-(\Delta CT)}$. Specific primer sequences were designed using the Primer-BLAST tool in the NCBI browser. Specificity and efficiency of primer-pairs were also evaluated before RT-qPCR analysis. Primer-pairs showing 100% efficiency were chosen. Additionally, to exclude genomic DNA contamination, reverse transcriptase obtained without reverse transcriptase was used as control. Primers sets used in this work are shown below, along with the sizes of their respective expected amplification products:

*thg1L*: Fw 5'- CTATCTGCCTGGCCTTTGGG -3',
Rv 5'- GTGGGTCATAAACTTGCTCGC -3' (98 bp);
*sub1.S*: Fw 5'-GCAGGAGAAATGAAGCCAGG-3',
Rv 5'-CCGACATCTGCTCCTTCAGT-3' (79 bp);
*slc35b1.L*: Fw 5'-CGCATTTCCAAACAGGCTCC-3',
Rv-5'-CAAGAAGTCCCAGAGCTCGC-3' (107 bp);
*ppp1ca.L*: Fw 5'-ACGAGTCTCTCATGTGCTCC-3',
Rv-5'-CAGAGCTGGGAGGGGTCATT-3' (140 bp).

In addition to the canonical *thg1l* gene sequence (NM_001095128.1), four predicted transcript variants are reported (XM_041585200.1, XM_018251143.2, XM_041585201.1, XM_041585202.1). *thg1l* forward and reverse primer anneal to exon 3 and to the junction between exon 3 and 4 of the canonical gene, respectively. Both regions bound by forward and reverse primers are conserved also in all predicted transcript variants.

RT-qPCR data were expressed as Mean ± standard error of the mean (SEM) from n=6 samples for each embryonic stage in three independent experiments. Statistical analysis was performed using the software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) and, after verification of the normal distribution, ANOVA followed by post-hoc Tukey's Multiple comparison test was used. Differences were considered significant when $p < 0.05$.

### *In situ* hybridization

*thg1l* antisense RNA probe was transcribed *in vitro* from the cDNA clone IMAGE:4970193 (GenBank Acc. n. BC085067.1), by plasmid linearization with SalI followed by T7 RNA polymerase transcription. To evaluate the strength of the hybridization signal compared to potential background staining, a *thg1l* sense probe was transcribed after plasmid linearization with XhoI, followed by transcription with SP6 RNA polymerase, and then assessed (Supplementary Fig. S1). Whole-mount ISH has was performed as previously described (Giannaccini *et al.*, 2013), and BM Purple substrate (Roche, Indianapolis, IN, USA) was used for signal detection. Images of whole-mount embryos were acquired by a Nikon SMZ18 stereomicroscope (Nikon Corporation, Minato, TO, Japan) connected to a Nikon DS-Ri3 digital camera and equipped with the software NIS-Elements AR 5.11.03. In specific cases, whole mount hybridized embryos were sectioned using a microtome to obtain 20 µm-thick sections from paraffin-embedded whole-mount preparations. Sections were collected onto slides and successively mounted with Eukitt (O. Kindler, Freiburg, Germany) or Aqua-Polymount (cat. No. 18606, Polysciences Inc., Warrington, PA, USA). St. 42 tadpoles were fixed for 1 h in 4% paraformaldehyde at room temperature, cryoprotected in 25% sucrose in phosphate buffer saline overnight, sectioned using a cryostat (12 µm-thick sections) and finally collected onto polarised slides. In accordance with published methods (D'Autilia *et al.*, 2010), sections were subjected to ISH and then mounted with Aqua-Polymount. Images from hybridized sections were acquired by a Nikon Eclipse Ti microscope, connected to a Nikon DS-Ri3 digital camera and equipped with the software NIS-Elements AR 5.11.03. To prepare all image panels shown in this work, the software Adobe Photoshop CS 8.0.1 was used.

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