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Expression Pattern of Xtshz1 and Xtshz3 During Xenopus Early Eye Formation

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Expression Pattern of *Xtshz1* and *Xtshz3* During *Xenopus* Early Eye Formation

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Abstract

The *Drosophila* gene *teashirt* (*tsh*) codes for a transcription factor that is part of a genetic network specifying eye identity. Three vertebrate *tsh* homologs have been identified (*tshz1-3*). All three mouse *tshz* genes can induce ectopic eyes in *tsh* loss-of-function flies. It is not yet known if any of the three *tshz* genes are required for vertebrate eye formation. This study was conducted to determine if any of the three *Xenopus laevis tshz* genes is expressed in the eye field or eye primordia during development. Although *tshz1* mRNA was expressed in the neuroectoderm, spinal cord, branchial arches, and olfactory placode, transcripts were never detected in either the eye field or eye primordia at any of the stages tested. In contrast to *tshz1*, *tshz3* mRNA was detected in the eye primordia as early as stage 22 with strongest expression at stage 24. In addition, *tshz3* was also detected in the neuroectoderm, spinal cord, branchial arches, neural fold, and hindbrain. Expression of *tshz3* in the eye primordia suggests its possible involvement in vertebrate early eye formation.

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Introduction

The systematic expression of transcription factors leads to the specification and ultimate differentiation of cells in developing embryos. Transcription factors within the tsh-related Zn-finger *tsh* family regulate both insect and vertebrate development. *Drosophila teashirt tsh* is a single gene expressed during fly embryo and adult development (Fasano, et al. 1991). Research has shown that this gene is vital to the successful development of *Drosophila* due to its role in trunk patterning (Mathies, et al. 1994), midgut morphogenesis (Roder, et al. 1992), and development of adult appendages (Abu-Shaar and Mann. 1998; Erkner, et al. 1999). In *tsh* loss-of-function mutants, a labial head segment arises in place of the most anterior trunk segment (Fasano, et al. 1991; Roder, et al. 1992). *tsh* is also crucial for *Drosophila* eye development (Pan and Rubin. 1998; Bessa, et al. 2002). It has been suggested that the Tsh protein is required to prevent the untimely expression of downstream transcription factors involved in development of the eye-antennal imaginal disc (Bessa, et al. 2002).

Three *tsh*-like genes (*tshz1, tshz2, tshz3)* have been identified in mouse. Similar to *tsh*, all three murine *tshz* genes participate in trunk, gut, and limb development (Caubit, et al. 2000). Tsh amino acid sequence conservation is very low 35% between *Drosophila* and mouse Tsh proteins (Manfroid, et al. 2004). Despite this variation, research has shown that the murine and *Drosophila tsh* genes perform similar functions (Fasano, et al. 1991; Caubit, et al. 2000; Manfroid, et al. 2004). All three *tshz* genes can be used to avoid developmental defects in *tsh* loss-of-function *Drosophila* mutants. In addition, target proteins and genes of *Drosophila tsh* that are required for eye development can interact with mouse Tshz proteins (Manfroid, et al. 2004).

Three *tsh*-like genes (*Xtszh1, Xtshz2*, and *Xtshz3)* also exist in *Xenopus laevis*. Previous research has determined that *Xtshz1* regulates brain and cranial neural crest development (Koebernick, et al. 2006), while *Xtshz3* plays a role in dorsal determination by enhancing canonical Wnt signaling (Onai et al., 2007). There has been no published report on the function of *Xtshz2.* It is possible that one or more *Xtshz* is a functional ortholog of *tsh*, but further research is needed to confirm and understand the potential role of each *Xtshz* during early eye formation.

The objective of this study was to determine the expression patterns of each of the three *Xtshz* genes during *X. laevis* early eye formation. Specifically, we were interested in determining if any of the *tshz* genes are expressed in the eye field or eye primordial. Expression patterns of *tshz1* and *tshz3* were obtained through *in situ* hybridization. *tshz1* mRNA was not detected by *in situ* hybridization during early eye formation. In contrast, *tshz3* mRNA was detected in the eye primordia as early as stage 22, with strongest expression at stage 24. Expression in the eye primordia suggests that *tshz3* is possibly involved in early eye formation. The data obtained through this study will contribute to the larger goal of determining if any of the *Xenopus tshz* genes are functionally similar to the *tsh* gene necessary for *Drosophila* eye development.

Methods

Animals

Fertilized eggs were obtained from *Xenopus laevis* injected to induce egg laying with 500 U of human chorionic gonadotropin (Sigma-Aldrich Company, USA). Embryos

were dejellied as described previously (Zuber, et al. 2003) and staged according to Nieuwkoop and Faber (1994).

In situ hybridization

pGEMTxTsh1ORF1 (a gift from Pieler lab, Germany) and pCS2-xTsh3 (a gift from Sasai Laboratory, Japan) were transformed into DH5-α competent cells. Transformed cells were cultured according to the Qiagen Plasmid Midi Kit and linearized by restriction digest accordingly (table 1).

Table 1. Enzymes used in restriction digest for probe synthesis.

	Antisense	**Sense**
Xtshz1	Not1	Nco1
Xtshz3	BamH1	Not1

Linearized DNA was cleaned through phenol chloroform extraction and precipitated with ethanol. Digoxigenin (DIG)-labeled antisense and sense RNA probes were generated as described in Ambian (Austin, TX) however, probes were carbonate treated. For all *Xtshz* probes, t7 and sp6 RNA polymerase plus were used for antisense and sense, respectively. As a positive control, Rx antisense probes were synthesized in the same manner using Nco1 and sp6 RNA Polymerase Plus.

Whole-mount in situ hybridization was performed on *Xenopus laevis* embryos stages 9, 12.5, 15, 18, 20, 22, and 24 using previously described procedures (Zuber, et al. 2003; Viczian, et al. 2006).

Results

Primers were designed to amplify specific regions of each *Xtshz* gene for both probe synthesis and PCR time course of gene expression. PCR products for *Xtshz1* and *Xtshz3* were cloned into the pGEMT-EZ vector and transformed into DH5-α competent cells, however, products were never sequenced or used in probe synthesis due to arrival of clones requested from other laboratories. Data for the PCR time course of gene expression was not obtained, due to contamination problems.

The *rax* gene was used as a positive control to identify the location of the eye field and eye primordia at each of the developmental stages tested. *rax* mRNA was first detected at stage 12.5 using *in situ* hybridization (Figure 2 A-C). In addition to continuous detection in the eye field and eye primordia (Figure 2-7 A-C), *rax* was detected in the ventral forebrain and the pineal gland at stage 24 (Figure 8 A-C).

tshz1 mRNA was first detected using *in situ* hybridization at stage 9 in the animal cap (Figure 1, D-F) and was detected only in the neuroectoderm (Figure 2- 4 D-F) until stage 18. At stage 20, *tshz1* was detected in the branchial arches and the neural folds (Figure 5 D-F) until expression expanded to include the spinal cord and olfactory placode in stage 20-24 (Figure 6-7 D-F). *tshz1* was also detected in the pronephros in stage 24 (Figure 7 D-F). *tshz1* transcripts were never detected using *in situ* hybridization in either the eye field or eye primordia at any of the stages tested. The various stained presumptive tissues were identified through previous research (Onai, et al. 2007).

tshz2 was not successfully amplified using the primers we created and has not been previously published therefore *in situ* hybridization could not be conducted.

tshz3 mRNA was first detected using *in situ* hybridization at stage 15 (Figure 3 G-I) where it was found in the neuroectoderm and the branchial arches until stage 18 (Figure 4 G-I). At stage 20, expression was located in the neural fold, the branchial arches and the hindbrain (Figure 5 G-I). Expression expanded to include the eye primordia lightly in stage 22 and strongly in stage 24 (Figure 6-7 G-I). The various stained presumptive tissues were identified through previous research (Koebernick, et al. 2006). Expression in the eye primordia suggests that *tshz3* is possibly involved in early eye formation.

Discussion

Previous research has identified three vertebrate homologs of the *tsh* gene crucial for *Drosophila* eye development (Pan and Rubin. 1998; Bessa, et al. 2002). Manfroid determined that the three mouse *tsh* homologs can all induce ectopic eye formation in *tsh* loss-of-function flies. This study was conducted in order to begin determining if any *Xenopus tshz* genes are functional homologs to the *Drosophila tsh* gene. Although this study did determine the *in situ* hybridization expression pattern of *Xenopus tshz1* and *tshz3,* additional work will be necessary to determine which, if any, of the three *tshz* genes are required for normal eye formation.

For this study, we began by designing primers to amplify specific regions of mRNA expressed by each of the three *Xenopus tshz* genes. These primers were designed for two purposes. First, they were needed to conduct a PCR time course of gene expression. Since PCR is a much more sensitive technique than *in situ* hybridization, this time course is necessary to determine if any of the *tshz* genes are

expressed in the eye field or eye primordia at levels undetected by *in situ* hybridization. Unfortunately, contamination of primers and non-specific *Xtshz2* primers prevented the PCR time course of gene expression from being completed. New primers must be created in order to complete this time course of gene expression. The primers designed in this experiment were also necessary for *in situ* hybridization probe synthesis. These primers were never used for this purpose due to arrival of *tshz1* and *tshz3* clones from other laboratories.

Information regarding the function of *tshz1* and *tshz3* has already been published (Koebernick, et al. 2006; Onai, et al. 2007) and clones from these laboratories were requested for our *in situ* hybridization. Since *tshz2* has never been published, clones were not available. This prevented us from determining the expression pattern of *tshz2.* Future work will be needed to design *tshz2* primers to amplify a region to be used as an *in situ* probe.

Using the *tshz1* and *tshz3* clones requested, as well as the positive control *rax*, probes were synthesized and in situ hybridization was performed. *In situ* hybridization revealed that *tshz1* and *tshz3* mRNA was present in the *Xenopus* embryo during early eye field formation. Using Onai's previous *tshz1* work a guide, we determined that *tshz1* mRNA was expressed in the neuroectoderm, spinal cord, branchial arches, and olfactory placode. In addition, using Koebernick's previous *tshz3* work as a guide, we determined that *tshz3* mRNA was expressed in the neuroectoderm, spinal cord, branchial arches, neural fold, and hindbrain. In contrast to *tshz1*, *tshz3* mRNA was also detected in the eye primordia as early as stage 22 with strongest expression at stage 24. This suggests possible involvement of *tshz* in early eye formation. Additional work

consisting of *in situ* hybridizations of sectioned embryos and double *in situ* hybridization experiments will be necessary to identify the presumptive cell types that each gene is expressed in. Once the expression patterns for all *tshz* genes are determined, knock-down and over expression studies will be used to determine if any of these genes are required for early eye formation in *Xenopus*.

In conclusion, we were able to determine that *tshz1* and *tshz3* are both expressed in *Xenopus* during early eye formation. Using *in situ* hybridization, we determined that *tshz3* is expressed in the eye primordia, suggesting possible involvement in vertebrate eye formation. Future research is needed to determine the function of all three *Xenopus tshz* genes as it relates to eye development. Obtaining this information will provide researchers with a piece of the puzzle in understanding vertebrate eye formation.

Acknowledgements

I would like to thank both the Pieler and Sasai laboratories for their generous gift of *tshz1* and *tshz3* plasmids. Thank you also to the members of the Zuber and Viczian laboratories for their guidance and suggestions throughout this project.

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Appendix

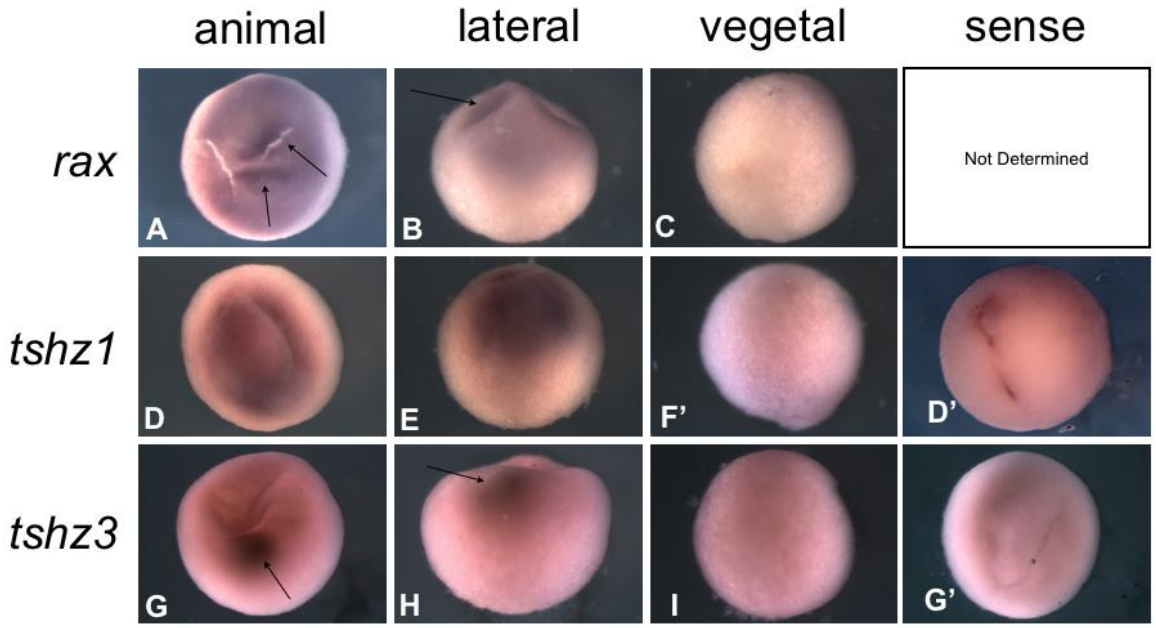

Figure 1. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 9 *Xenopus laevis* embryos. A-C) No specific staining was detected with *rax* probes. Arrow points to non-specific staining due to embryonic damage. (**D-F)** *tshz1* is detected in the animal pole. **G-I)** No specific staining was detected with *tshz3* probes. Arrows point to non-specific staining due to embryonic damage. No staining was detected with either *tshz1* (**D'**) or *tshz3* (**G'**) sense probes.

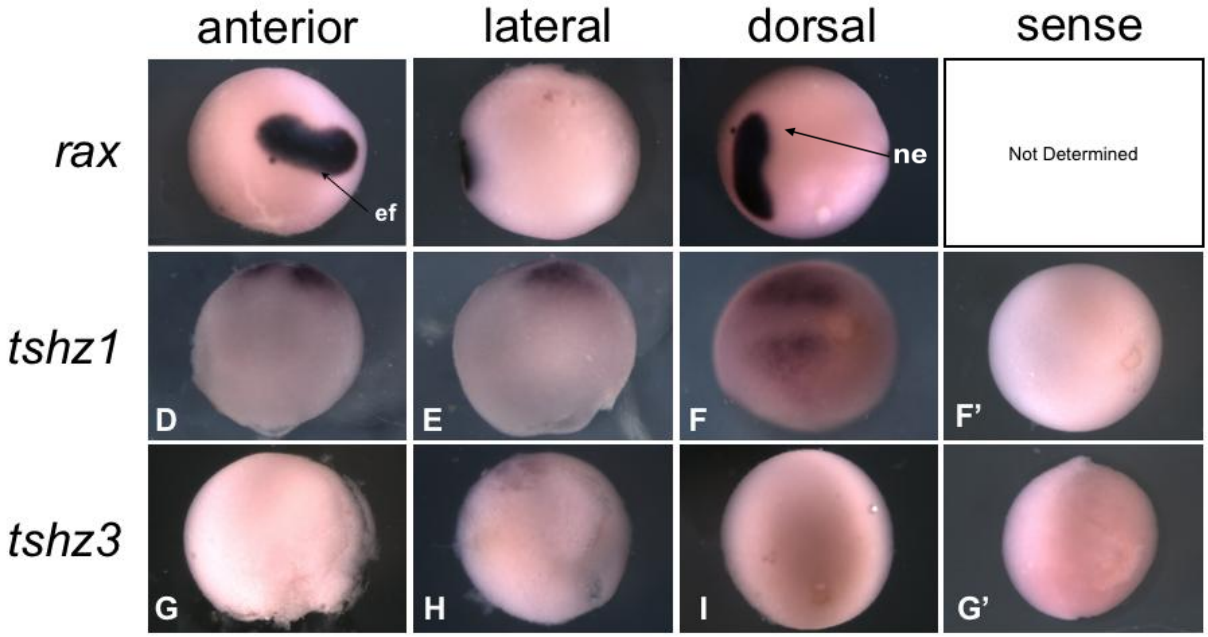

Figure 2. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 12.5 *Xenopus laevis* embryos. A-C) *rax* expression is detected in the eye field (ef). **D-F)** *tshz1* is detected in the neuroectoderm. **G-I)** No specific staining was detected with *tshz3* probes. No staining was detected with either *tshz1* (**F'**) or *tshz3* (**G'**) sense probes. Anterior is to the left in lateral and dorsal views.

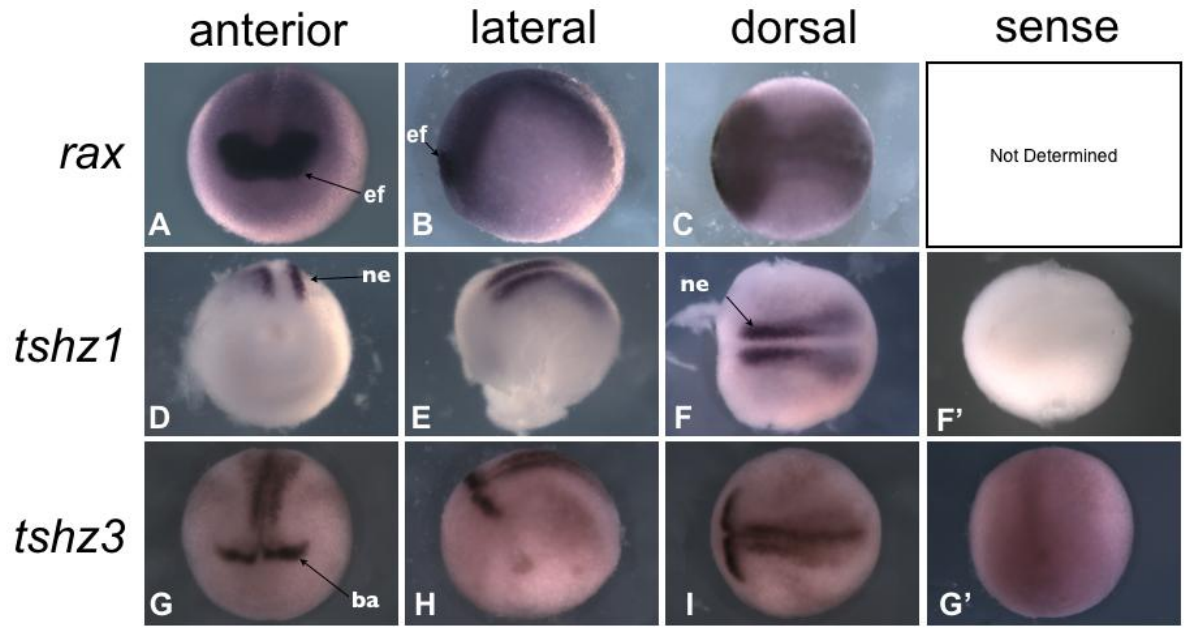

Figure 3. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 15 *Xenopus laevis* embryos. A-C) *rax* expression is detected in the eye field (ef). **D-F)** *tshz1* is detected in the neuroectoderm (ne). **G-I)** *tshz3* is expressed in the neuroectoderm, and branchial arches (ba). No staining was detected with either *tshz1* (**F'**) or *tshz3* (**G'**) sense probes. Anterior is to the left in lateral and dorsal views.

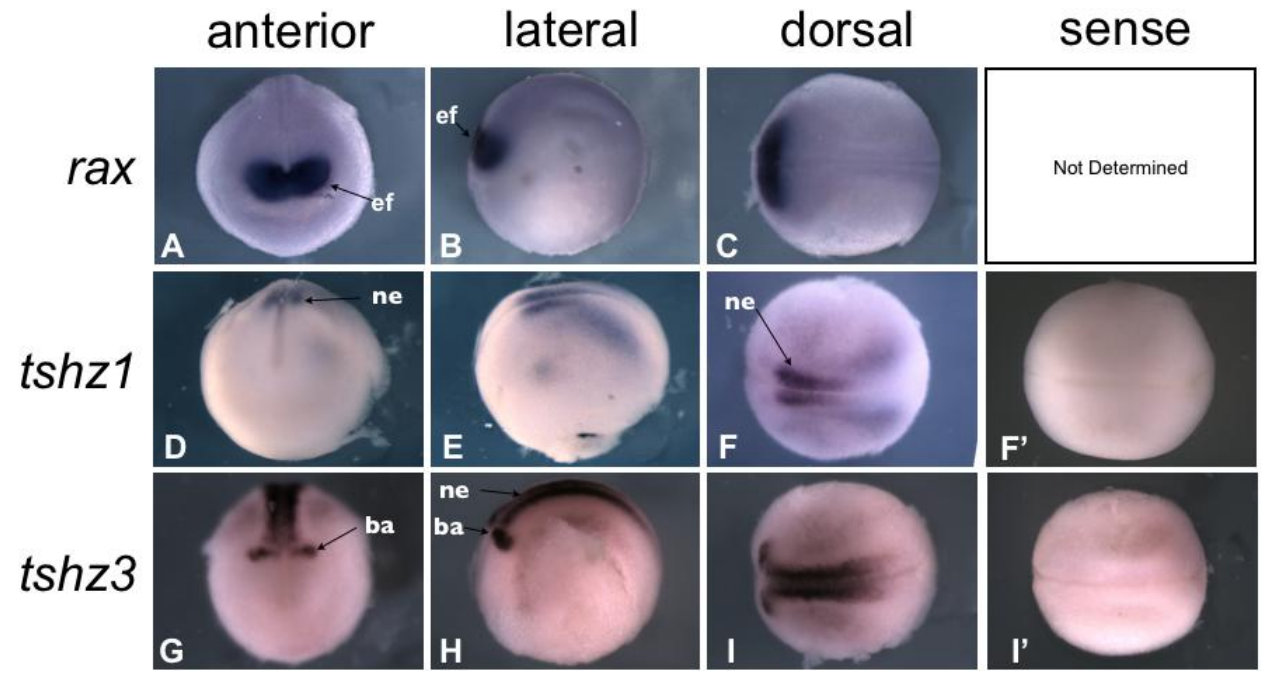

Figure 4. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 18 *Xenopus laevis* embryos. A-C) *rax* expression is detected in the eye field (ef). **D-F)** *tshz1* is detected in the neuroectoderm (ne). **G-I)** *tshz3* is expressed in the neuroectoderm, and presumptive branchial arches (ba). No staining was detected with either *tshz1* (**F'**) or *tshz3* (**I'**) sense probes. Anterior is to the left in lateral and dorsal views.

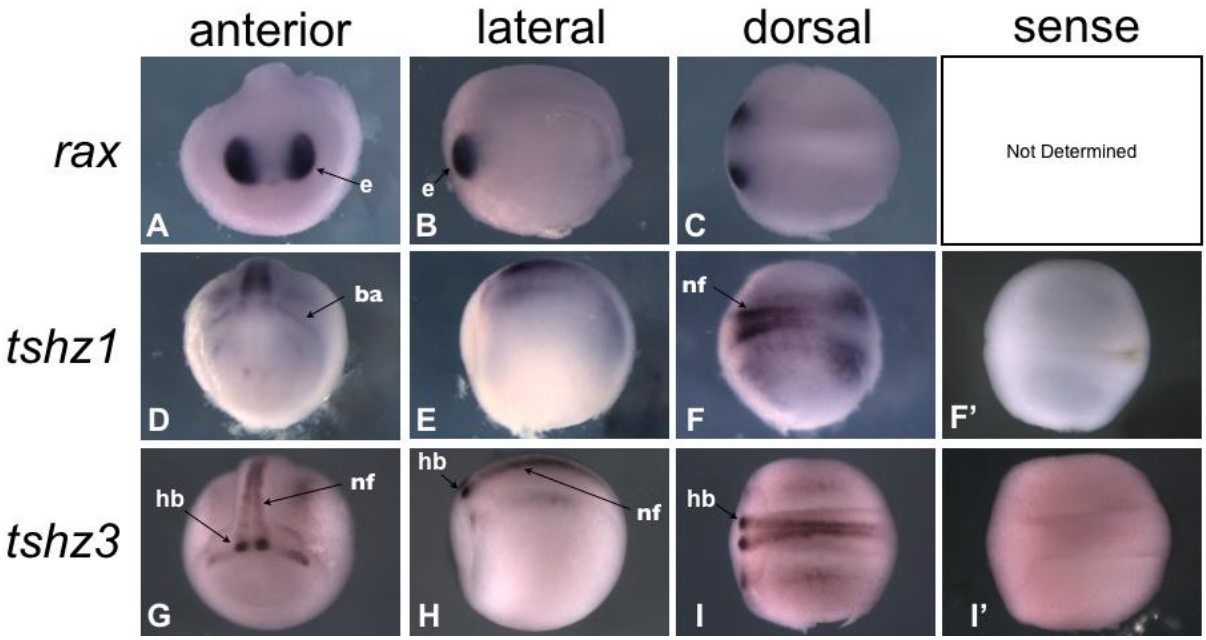

Figure 5. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 20 *Xenopus laevis* embryos. A-C) *rax* expression is detected in the eyes (e). **D-F)** *tshz1* is detected in the neural fold (nf) and branchial arches (ba). **G-I)** *tshz3* is expressed in the hindbrain (hb), neural fold, and branchial arches. No staining was detected with either *tshz1* (**F'**) or *tshz3* (**I'**) sense probes. Anterior is to the left in lateral and dorsal views.

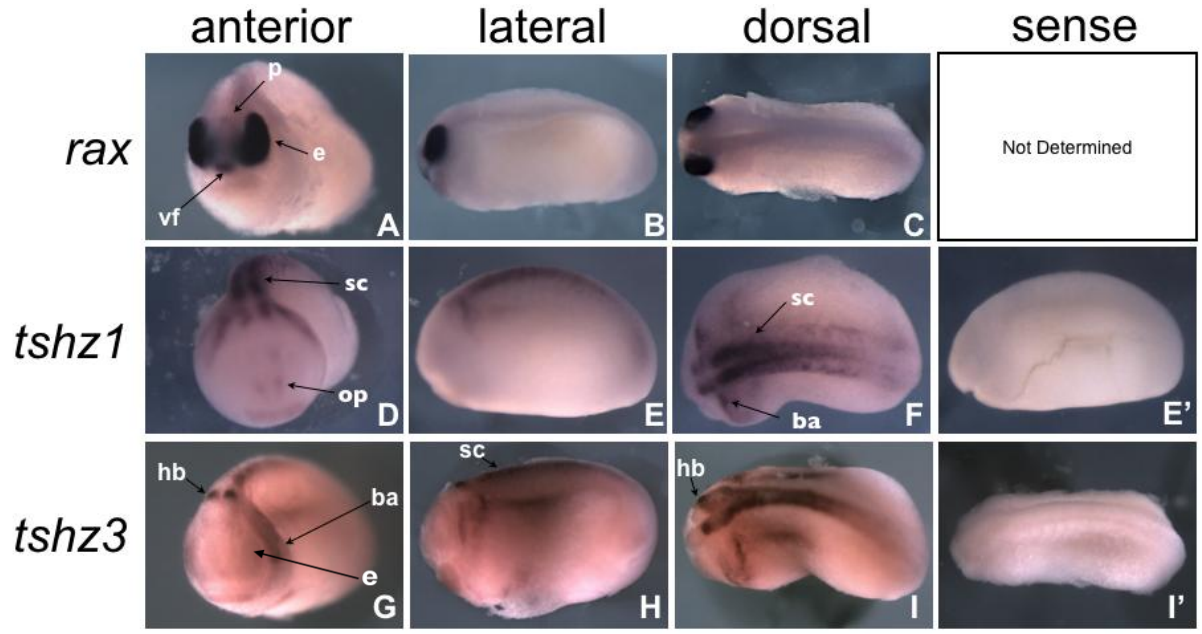

Figure 6. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 22 *Xenopus laevis* embryos. A-C) *rax* expression is detected in the eye primordia (e), pineal gland (p) and ventral forebrain (vf). **D-F)** *tshz1* is detected in the olfactory placode (op), spinal cord (sc) and branchial arches (ba). **G-I)** *tshz3* is expressed in the hindbrain (hb), spinal cord, eye primordia and branchial arches. No staining was detected with either *tshz1* (**E'**) or *tshz3* (**I'**) sense probes. Anterior is to the left in lateral and dorsal views.

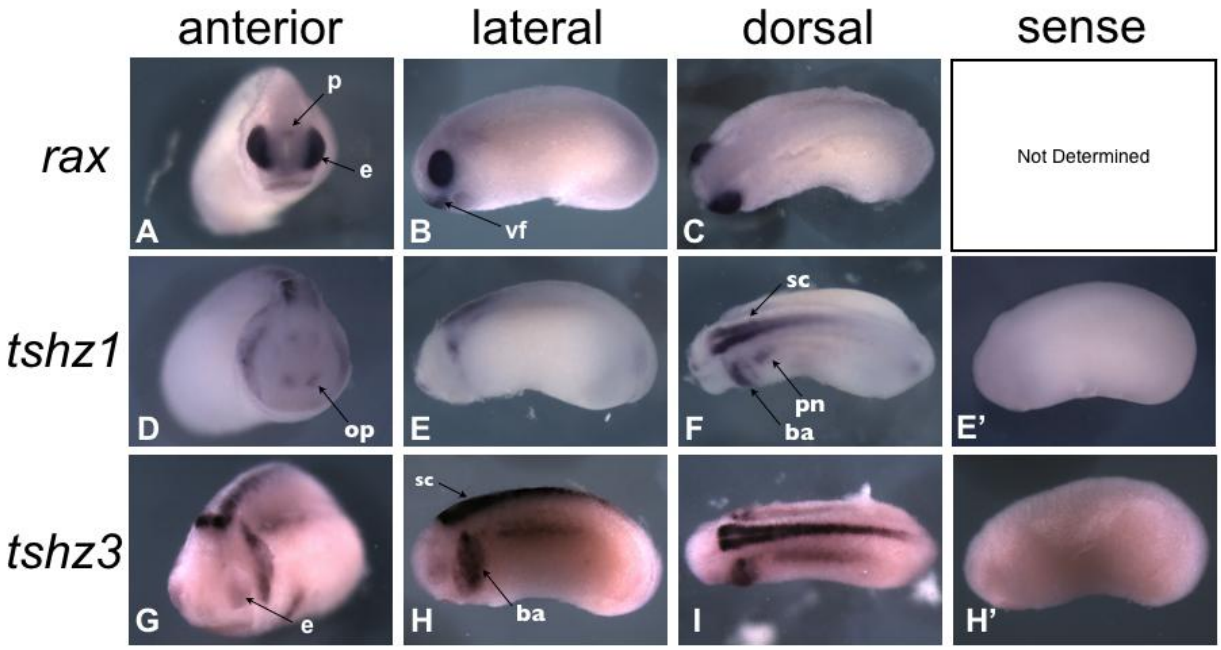

Figure 7. In situ expression patterns of *rax*, *tshz1*, and *tshz3* in stage 24 *Xenopus laevis* embryos. A-C) *rax* expression was detected in the eye primordia (e), pineal gland (p) and ventral forebrain (vf). **D-F)** *tshz1* is detected in the olfactory placode (op), spinal cord (sc), pronephros (pn), and branchial arches (ba). **G-I)** *tshz3* is expressed in the eye primordia, spinal cord, and branchial arches. No staining was detected with either *tshz1* (**E'**) or *tshz3* (**H'**) sense probes. Anterior is to the left in lateral and dorsal views.