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Sijia Wang 04/15/2014

Functional Analysis of Endothelin Receptor Signaling in Zebrafish Enteric Nervous System Development

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Abstract

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Endothelin-3 (EDN3) and its obligate receptor endothelin receptor B (EDNRB) are thought to play an essential role in enteric nervous system (ENS) development by regulating the proliferation and differentiation of enteric neural crest cells and their precursors. Patients with pediatric condition Hirschsprung disease (HSCR), a congenital ENS disorder, have been identified with a missense point mutation in the human EDNRB gene. To confirm the evolutionary conservation for the requirement of Endothelin-3 signaling in ENS development, we have identified and cloned two EDNRB orthologues in zebrafish. Bioinformatics analysis has revealed that the second copy of EDNRB gene has arisen because of the hypothesized genome duplication that occurred in teleosts after they diverged from the other vertebrate lineages. Subsequently, antisense digoxigenin-labeled probes were generated to characterize the expression pattern of EDNRB orthologues during embryogenesis. We found that both EDNRB1a and EDNRB1b orthologues are expressed in the neural crest precursors as these cells begin to enter the gastrointestinal tract and migrate caudally along its length. These results support our hypothesis that there is a conserved function of EDNRB orthologues in neural crest migration and ENS development in zebrafish model system.

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Introduction

The vertebrate enteric nervous system (ENS) plays a crucial role in controlling gut motility and regulating gastrointestinal function (Furness and Costa, 1987). It is principally derived from a population of embryonic stem cells called the neural crest cells (NCCs), which arise at the border between neural ectoderm and non-neural ectoderm as the neural plate begins to fold and form the neural tube (Gershon el al., 1993). When the neural tube closes, these neural crest cells (NCCs) become specified to produce a diverse cell lineages including but not limited to melanocytes, jaw cartilage elements and enteric neural crest-derived cells (ENCCs) (Gershon el al., 1993). Subsequently these ENCCs migrate to and colonize the whole length of the gastrointestinal tract, giving rise to an integrated network of neurons and glia that constitute the enteric nervous system (ENS) (Newgreen and Young, 2002). Carefully regulated proliferation, differentiation and migration of enteric precursors is necessary for normal ENS development. In humans, the impairment in neural crest cell proliferation and migration leads to the intestinal motility disorder Hirschsprung disease (HSCR) (Kapur, 1999). HSCR is characterized by the aganglionosis of the distal colon that results in congenital intestinal obstruction with an incidence of 1/5000 among newborns (Kapur, 1999). Multiple mutant loci are associated with this complex genetic disorder. Significantly, with respect to this study, a missense point mutation in the gene encoding endothelin receptor B (EDNRB) has been identified in patients with hereditary Hirschsprung disease (HSCR) (Baynash et al., 1994).

Endothelin receptor B (EDNRB) is a member of the family of G protein-coupled heptahelical receptors with seven transmembrane domains (Pla and Laura, 2003). Three sub-types of EDNR (A,B,C) have been identified, each of which preferentially binds to different endothelin (EDN) ligands (Arai et al., 1990). Endothelins constitute a family of 21 amino-acid vasoactive peptides, of which there are three members: EDN1, EDN2, and EDN3 (Rubanyi and Polokoff, 1994). EDNRB has similar affinities for all three ligands. Only EDNRA and EDNRB are found in mammals (Pla and Laura, 2003). Both receptors initiate several intracellular signal transduction events via heterotrimeric G proteins, leading to a variety of biological actions.

Previous studies have shown that the specific signal conveyed by EDN3 and EDNRB plays a significance role in the normal development of epidermal melanocytes and enteric ganglion neurons in mice and humans. In cell culture, EDN3 has been discovered to inhibit NCC differentiation and delay Schwann cell maturation (Hearn et al., 1998). Thus the EDNRB signaling pathway is required to maintain the NCCs in an undifferentiated state to ensure they migrate to the final destination in the gut before full differentiation. Significantly "knockout" mice that lack either the gene encoding EDN3 or EDNRB exhibit a recessive phenotype of aganglionic megacolon and white-spotted coat color, and eventually die (Baynash et al., 1994; Hosoda et al., 1994). Critically, defects in the EDN3 and EDNRB genes can result in a similar congenital aganglionosis in humans (Puffenberger et al., 1994). Therefore, the function of EDN3 and EDNRB is directly linked to Hirschsprung's disease.

In this study, we sought to investigate the role of EDN3 and EDNRB in neural crest cell's proliferation and differentiation in the zebrafish model system. Previous studies in zebrafish have identified a zebrafish null mutant in one of the zebrafish EDNRB orthologues. Surprisingly this mutant is viable and does not have a defect in its ENS (Parichy et al., 2000). Recently we have identified a second EDNRB orthologue whose function may explain why no phenotype was observed in the previously identified zebrafish EDNRB mutant *rose*. To determine if the role of EDNRB in ENS development has been conserved evolutionarily, we have cloned both these orthologues and have performed whole-mount in situ hybridization at various developmental stages to exam the temporal and spatial expression of both genes. We also designed reagents to block their function (morpholino anti-sense oligonucleotides). We hypothesized that during embryogenesis, endothelin signaling pathway involving all zebrafish EDN3 and EDNRB orthologues is required for normal development of zebrafish ENS. This result would be consistent with previous studies that have shown a conserved function in ENS development for orthologues of other identified HSCR genes in zebrafish (Shepherd et al., 2004). The overlapping expression patterns of EDNRB orthologues suggest these orthologues may have redundant function in zebrafish ENS development, which would explain the lack of an ENS phenotype in the *rose* EDNRB1a mutant. This data is consistent with our hypothesis that EDRNB signaling is responsible for regulating the proliferation and differentiation of enteric neural crest cells and their precursors in all vertebrate species.

Material and Methods

Bioinformatics analysis

A BLAST search was performed first to identify the EDNRB orthologues in zebrafish. FASTA software was then used to analyze the percent of identity between zebrafish EDNRB genes and those of mouse and human. Genome structure analysis was completed using GenBank and the Zebrafish Genome browser. CLUSTALW sequence alignment program was used to carry out the alignment of amino acid sequences of the zebrafish, mouse and human orthologues. Drawtree and Drawprogram were used to conduct a phylogenetic analysis.

PCR cloning and sequencing

To clone the cDNA of both EDNRB1a and EDNRB1b orthologues, degenerative reverse transcriptase PCR was firstly performed to isolate and amplify the 5' and 3' overlapping segments of the open reading frame. RACE (rapid amplification of cDNA ends) was then used to amplify 5' and 3' ends of this transcript. RACE cDNA was isolated from 72hpf embryos using a Smart RACE cDNA Amplification Kit (Clonetech). The PCR products were subcloned and sequenced to assemble full-length EDNRB1a and EDNRB1b cDNAs. To confirm the nucleotide sequences, we compared sequences of PCR-amplified cDNA with existing database sequence.

Whole-mount in situ hybridization

Methods for riboprobe synthesis and in situ hybridization of embryos have been previously described (Thisse et al., 1993). Digoxigenin-labeled riboprobes were prepared from templates linearized with *Xho*I using Sp6 RNA polymerase for both EDNRB1a and EDNRB1b. Hybridizations were carried out at 65°C, followed by stringency washes at the same temperatures.

EDNRB1a and EDNRB1b antisense oligo injections

The 25-mer morpholino antisense oligonucleotides were synthesized by Gene Tools, LLC (http://www.gene- tools.com). Both translational blocking and splice blocking morpholinos were designed. Translational blocking morpholinos prevent a targeted transcript from being transcribed by binding to the 5' untranslated region and the translational start site preventing ribosomes from assembling at the translational start site (Bill et al., 2009). The translational MOs were designed with the following sequences to target the translational start site:

EDNRB1a morpholino 5'-GGAAACGCATGACTATTTAACAGTC-3'

EDNRB1b morpholino 5'-TCAGAAGAGTGATGTTTCCCAGCAT-3'

Splice blocking morpholinos bind to the splice-junctions at exon intron boundaries thus preventing pre-mRNA processing (Bill et al., 2009). Exon 4/exon 5 junctions in the zebrafish EDNRB orthologues were chosen as targets for splice-site MOs with the following base composition:

EDNRB1a morpholino 5’-CTCATTCAGTAATACTCTGACCTGT-3’

EDNRB1b morpholino 5’-ACGAGGTGTAGAAACGCACCTGTTT-3’,

The morpholinos will be injected into one-cell stage embryos using a gas-driven microinjection apparatus.. We will inject morpholinos to the orthologues singly or together into wild type embryos. Alternatively we will inject the morpholinos blocking EDNRB1b orthologue function only into the *rose* EDNRB1a mutant embryos.

Results

Evolutionary conservation of EDNRB1a and EDNRB1b

Using sequence data from GenBank and the Zebrafish Genome browser (https://www.ncbi.nlm.nih.gov/genbank/; http://www.ensembl.org/Danio_rerio/Info/Index) for both EDNRB orthologues, we performed a comparison of gene features. Both EDNRB1a and EDNRB1b are comprised of seven exons. Sequence comparison demonstrates substantial conservation of nucleotide sequences (Fig.1). The similar genome organization that we observe suggests that these two orthologues arose due to the probable genome duplication event that occurred in teleosts after they diverged from the other vertebrate lineages (Postlethwait et al., 2000). The nucleotide sequences of zebrafish EDNRB1a and EDNRB1b were also compared with published sequence of mammalian EDNRB orthologues (Fig.2). This analysis reveals significant similarities in both exon arrangement and sequences, showing strong evolutionary conservation of EDNRB gene structure (Fig.2). Unsurprisingly there is also strong cross species conservation in the alignment of amino acid sequences of zebrafish EDNRB orthologues with those from other vertebrate species. All EDNRB orthologues share highly conserved transmembrane domains that are characteristic of EDNRB proteins, implying the zebrafish orthologues may potentially have the same function as the mammalian orthologues (Fig.3).

The two zebrafish EDNRB orthologues likely occurred due to a genome duplication event

Syntenic anlaysis of the gene arrangement surrounding the two zebrafish EDNRB orthologues show that in addition to the duplicated EDNRB genes, in the zebrafish genome there are also two copies of several other genes in the loci of the two EDNRB orthologues, for example klf12 and nlgn4 (Fig.4). This finding further supports our hypothesis that the two EDNRB orthologues arose due to a genome duplication event. Moreover, though the order of the genes is changed due to probable genetic rearrangements that occurred after the genome duplication event, it appears that the zebrafish EDNRB orthologue located on chromosome 9 is syntenically more similar to the mammalian EDNRB genes (Fig.4). This finding leads us to hypothesize that EDNRB1b orthologue may be the ancestral gene despite its later discovery, while EDNRB1a is the duplicated gene. Phylogram of the EDNRB gene family further demonstrates that EDNRBs from teleost lineage are more closely related to each other than to their mammalian orthologues (Fig.5). Interestingly, besides zebrafish, several ancient teleosts like cod also contain two orthologues encoding EDNRB in their genome (Fig.5). This phenomenon underscores the significance of genome duplication that occurred in teleosts during the evolution of this branch of the vertebrate lineage. It has been suggested that, through the massive genome rearrangements followed by several rounds of whole-genome duplication, the early vertebrates were able to expand their gene repertoire (Braasch et al., 2009). Furthermore, the duplicated genes clearly subsequently evolved independently from each other and the EDNRB1b orthologues appear more homologous to the mouse and human orthologues (Fig.5).

Expression of EDNRB orthlogues in the neural crest cells

DIG-labeled antisense riboprobes were synthesized for both zebrafish EDNRB orthologues (Fig.6). We performed whole-mount in situ hybridization to determine the specific expression patterns and spatial locations of these genes during embryogenesis (Fig.7).

Spatial and temporal expression patterns of EDNRB1a in zebrafish neural crest cells are similar to previous published expression patterns ((Parichy et al., 2000; Lister et al., 2006; Arduinito et al., 2008; Lopes et al., 2008). In the early stages of zebrafish embryogenesis, EDNRB1a is expressed widely through out the anterior head (Fig.7). No EDNRB1a cells are present in the ventral edge of the trunk, where the intestine is located. At later ages, when the ENS is developing, EDNRB1a expressing cells are found in the gut and appear to be migrating along the ventromedial edge of the trunk (Fig.7), which is consistent with the ENCC intestinal migratory pathway.

Comparatively, the expression of EDNRB1b orthologue is more abundant in the anterior region, where neural crest precursors originate (Fig.7). Between 48 and 72h of development, embryos exhibit accumulation of EDNRB1b expressed cells at the midbrain-hindbrain boundary, which later migrate towards the anterior gut (Fig.7). These expression patterns are consistent with a potential role for both orthologues in ENCC migration and proliferation. The overlapping EDNRB orthologues' expression in the anterior head region indicates that EDNRB1a and EDNRB1b may be functional redundant in the early stages of zebrafish embryogenesis, despite EDNRB1a's more prominent role in regulating ENCC migration.

Discussion

We cloned and sequenced two orthologues of EDNRB in zebrafish. Our results are consistent with our hypothesis that the endothelin pathway does play a conserved critical role in the development of the enteric nervous system in zebrafish. Specifically we believe the endothelin receptor B is required for the proliferation and migration of enteric neural crest-derived cells. Bioinformatics analysis has revealed that two EDNRB orthologues in the zebrafish genome have arisen because of the hypothesized genome duplication that occurred in teleosts after they diverged from the other vertebrate lineages (Postlethwait et al., 2000). The overlapping EDNRB orthologues' expression patterns in zebrafish in the ENCC migratory pathway support our hypothesis that EDNRB plays a crucial role in the early precursor migration along the intestine. However we still need to confirm that the EDNRB orthologues are specifically expressed in the ENCC. It is possible that the expression patterns we observe might be related to the previously shown developmental role for endothelin signaling in the development of other neural crest-derived cell types, such as melanocytes. This will require double label experiments in which the markers of zebrafish ENCC and ENS neurons are co-localized with the expression of the EDNRB genes.

If both EDNRB orthologues are expressed in ENCCs, the presence of the second EDNRB orthologue in ENCCs could explain why no ENS phenotype was observed in the previously identified zebrafish EDNRB mutant *rose* (Parichy et al., 2000). This lack of phenotype in the *rose* mutant is likely to have resulted from the functional redundancy of the

two zebrafish orthologues similar to that seen for the GFRapha1 orthologues in zebrafish (Shepherd et al., 2004).

To further support our hypothesis for a conserved functional role for the zebrafish EDNRB orthologues in ENS development, we plan to undertake morpholino injections into zebrafish embryos to determine the gene knock down phenotypes in the ENS. Furthermore to test if there is functional redundancy of the orthologues, we will need to inject morpholinos singly or together into wild type embryos. Alternatively we will inject the morpholinos blocking EDNRB1b orthologue function only into *rose* mutant embryos.

In conclusion, our results are consistent with previous studies in other vertebrate species that EDNRB signaling pathway is required for normal ENS development. It remains to be tested whether zebrafish EDNRB orthologues are functionally redundant. The high homology between the zebrafish orthologues of EDNRB and those of other species, including human, supports our hypothesis that there has been evolutionary conservation in function. In the long term these findings will potentially have implications for our understanding of the Hirschsprung disease and further support the use of zebrafish as a model system to study the genetic basis of HSCR given the apparent strong evolution conservation of various signaling pathways in vertebrate ENS development.

References

Arai, H., Hori, S., Aramori, I., Ohkubo, H., & Nakanishi, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature, 348*(6303), 730-732.

Arduini, B. L., Gallagher, G. R., & Henion, P. D. (2008). Zebrafish endzone regulates neural crest-derived chromatophore differentiation and morphology.*PloS one*, *3*(7).

Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E., & Yanagisawa, M. (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell*, *79*(7), 1277-1285.

Bill, B. R., Petzold, A. M., Clark, K. J., Schimmenti, L. A., & Ekker, S. C. (2009). A primer for morpholino use in zebrafish. *Zebrafish*, *6*(1), 69-77.

Braasch, I., Volff, J. N., & Schartl, M. (2009). The endothelin system: evolution of vertebrate-specific ligand–receptor interactions by three rounds of genome duplication. *Molecular biology and evolution*, *26*(4), 783-799.

Furness, J. B., & Costa, M. (1987). *The enteric nervous system*. Edinburgh etc.: Churchill Livingstone.

Gershon, M. D., Chalazonitis, A., & Rothman, T. P. (1993). From neural crest to bowel: development of the enteric nervous system. *Journal of neurobiology*, *24*(2), 199-214.

Hearn, C. J., Murphy, M., & Newgreen, D. (1998). GDNF and ET-3 Differentially Modulate the Numbers of Avian Enteric Neural Crest Cells and Enteric Neurons *in Vitro*. *Developmental biology*, *197*(1), 93-105.

Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A., & Yanagisawa, M. (1994). Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell*, *79*(7), 1267-1276.

Kapur, R. P. (1999). Hirschsprung disease and other enteric dysganglionoses. *Critical reviews in clinical laboratory sciences*, *36*(3), 225-273.

Lister, J. A., Cooper, C., Nguyen, K., Modrell, M., Grant, K., & Raible, D. W. (2006). Zebrafish Foxd3 is required for development of a subset of neural crest derivatives. *Developmental biology*, *290*(1), 92-104.

Lopes, S. S., Yang, X., Müller, J., Carney, T. J., McAdow, A. R., Rauch, G. J., Jacoby, A.S., Hurst, L.D., Delfino-Machin, M., Haffter, P., Geisler, R., Johnson, S.L., Ward, N., & Kelsh, R. N. (2008). Leukocyte tyrosine kinase functions in pigment cell development. *PLoS genetics*, *4*(3)

Newgreen, D., & Young, H. M. (2002). Enteric nervous system: development and developmental disturbances—part 2. *Pediatric and Developmental Pathology*, *5*(4), 329-349.

Parichy, D. M., Mellgren, E. M., Rawls, J. F., Lopes, S. S., Kelsh, R. N., & Johnson, S. L. (2000). Mutational Analysis of Endothelin Receptor b1 (*rose*) during Neural Crest and Pigment Pattern Development in the Zebrafish *Danio rerio*. *Developmental biology*, *227*(2), 294-306.

Pla, P., & Larue, L. (2003). Involvement of endothelin receptors in normal and pathological development of neural crest cells. *International Journal of Developmental Biology*, *47*(5), 315-326.

Postlethwait, J. H., Woods, I. G., Ngo-Hazelett, P., Yan, Y. L., Kelly, P. D., Chu, F., Huang, H., Hill-Force, A., & Talbot, W. S. (2000). Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome research*, *10*(12), 1890-1902.

Puffenberger, E. G., Kauffman, E. R., Bolk, S., Matise, T. C., Washington, S. S., Angrist, M., Weissenbach J., Garver, K.L., Mascari, M., Ladda, R., Slaugenhaupt, S.A., & Chakravarti, A. (1994). Identity-by-descent and association mapping of a recessive gene for Hirschsprung disease on human chromosome 13q22. *Human Molecular Genetics*, *3*(8), 1217-1225.

Rubanyi, G. M., & Polokoff, M. A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacological reviews*, *46*(3), 325-415.

Shepherd, I. T., Pietsch, J., Elworthy, S., Kelsh, R. N., & Raible, D. W. (2004). Roles for GFRα1 receptors in zebrafish enteric nervous system development. *Development*, *131*(1), 241-249.

Thisse, C., Thisse, B., Schilling, T. F., & Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development*, *119*(4), 1203-1215.

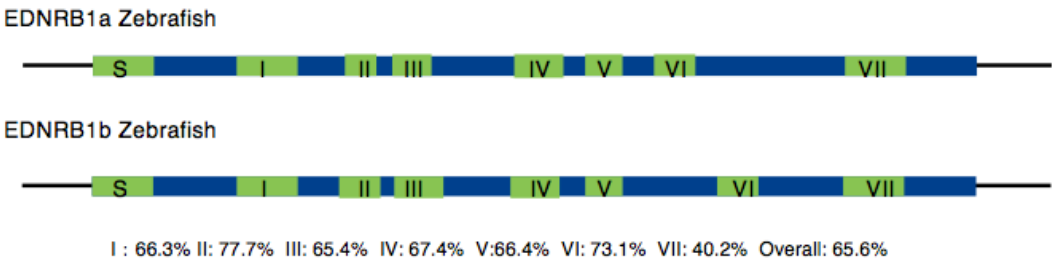
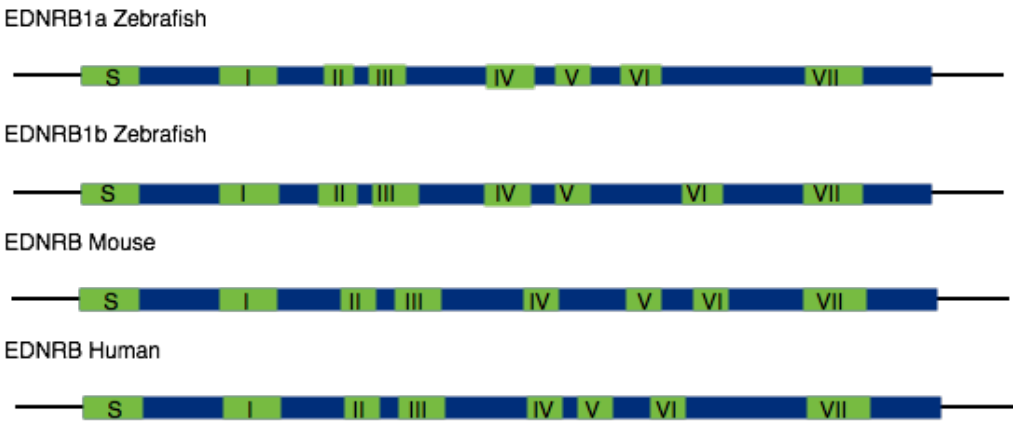


Fig.1 Gene comparison between EDNRB1a and EDNRB1b orthologues. The substantial sequence similarities between the two orthologues suggest they arose from a genome duplication event. Shown is a schematic of the exon and intron arrangement of the EDNRB1a and EDNRB1b genes. Green indicates predicted transcriptional start site and exons; while dark blue represents the introns. The percent identity for each exon sequence is shown below the schematic.



Comparison		I	II	III	IV	V	VI	VII	Overall
EDNRB1a Zebrafish	EDNRB Mouse	69.9%	71.7%	68.8%	76.7%	70.1%	74.8%	59.3%	70.0%
	EDNRB Human	68.3%	74.0%	68.3%	77.3%	73.1%	76.4%	60.7%	70.9%
EDNRB1b Zebrafish	EDNRB Mouse	69.2%	75.9%	63.7%	68.6%	66.4%	77.4%	50.4%	68.1%
	EDNRB Human	69.7%	72.6%	65.2%	64.8%	70.1%	77.2%	44.4%	68.4%

Fig.2 EDNRB homolog analysis between different species. Shown are the arrangement of the exons and introns of EDNRB orthologues in zebrafish, mouse and human. Green represents the predicted transcriptional start site and exons while dark blue represents intronic regions. The percent of sequence conservation for each exon between each of the zebrafish EDNRB orthrologues and that of mouse and human is listed in the table.

Fig. 3 Alignment of the amino acid sequences of zebrafish EDNRB1a, and EDNRB1b with those of mouse and human EDNRB. Alignment was carried out using the CLUSTALW sequence alignment program. Identical amino acids are marked by * and dashes represent gaps in the alignment. Conserved transmembrane domains are colored in blue.

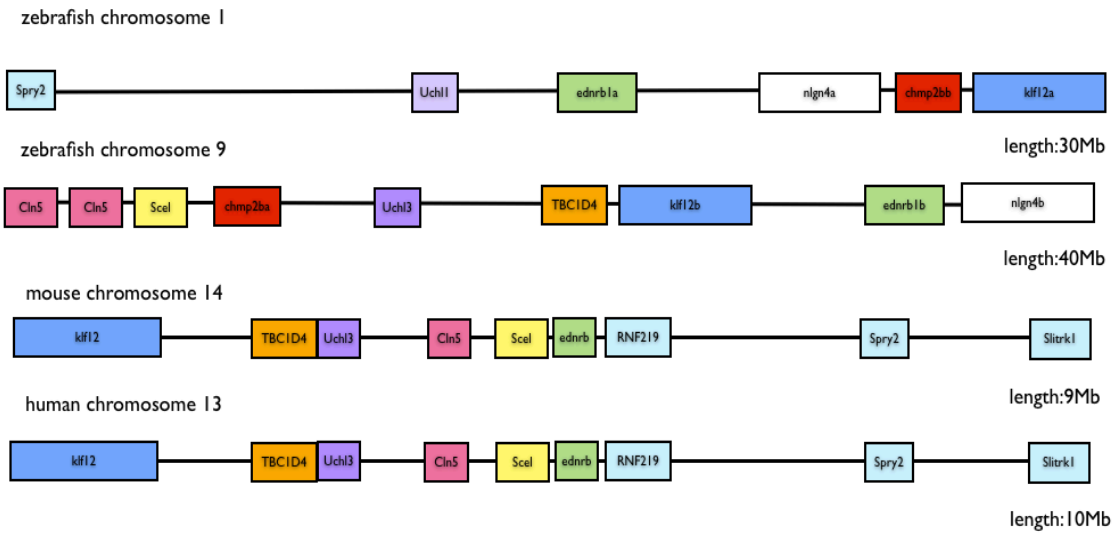


Fig. 4 Syntenic analysis of the two zebrafish EDNRB orthologues and mammalian EDNRB genes. The same genes are colored the same. The length of each syntenic region aligned is shown. Despite the changed gene order, due to probable genetic rearrangement post the genome duplication event, the gene order in the chromosome surrounding the duplicated EDNRB genes appears highly conserved.

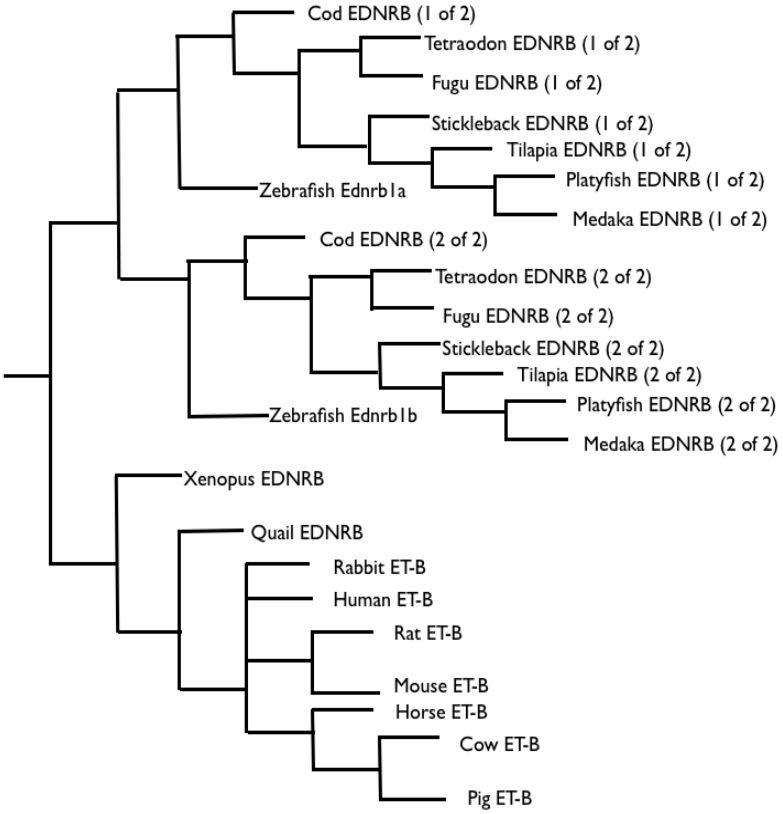


Fig. 5 Rooted phylogram of the EDNRB orthologue family. The tree was generated from the sequence alignment made by CLUSTRALW program. It shows the relationship between the two zebrafish ortholgues and those of other species.

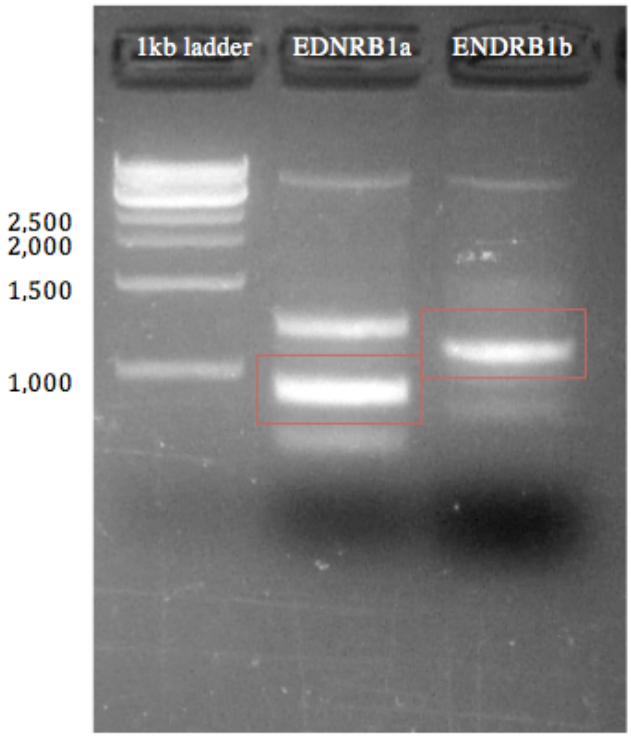


Fig. 6 Gel image of the DIG-labeled riboprobes prepared from templates linearized with *Xho*I using Sp6 RNA polymerase. Correct sized bands are shown in red box.

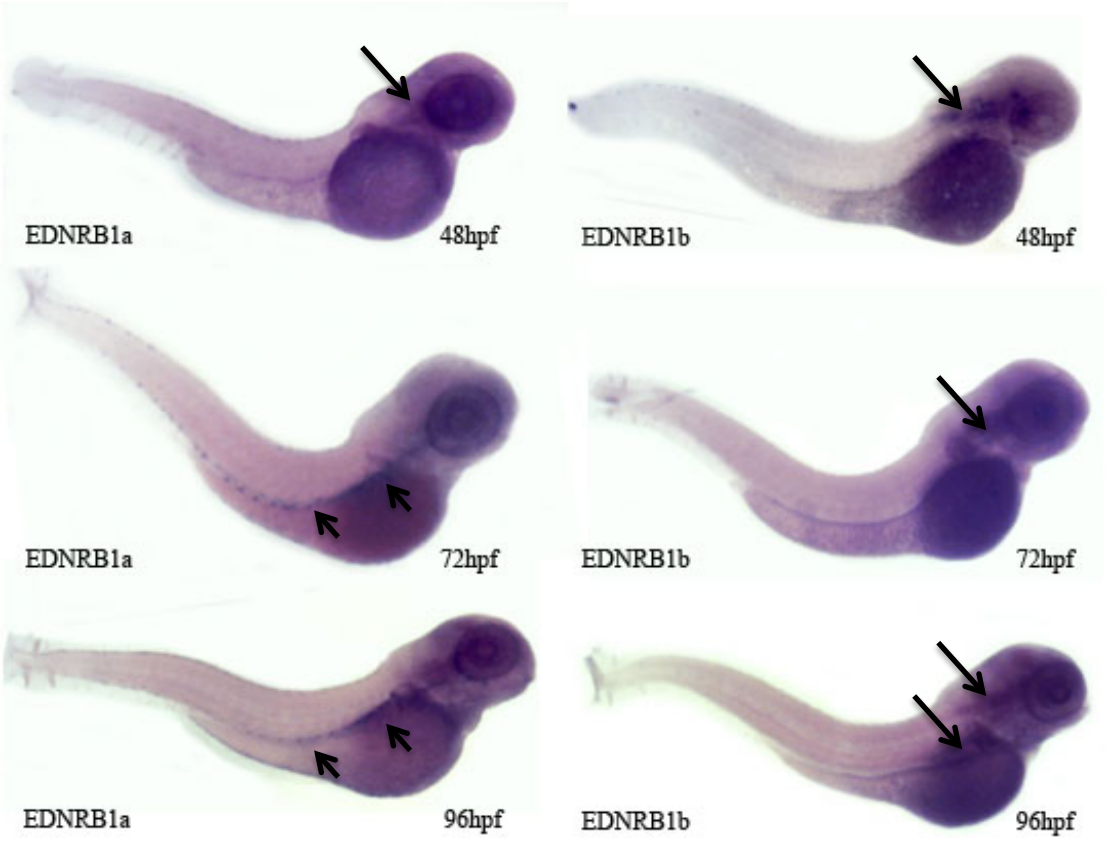


Fig. 7 EDNRB1a and EDNRB1b spatial and temporal expression. Embryos with ages of 48 hpf, 72 hpf and 96 hpf were hybridized with riboprobes for EDNRB1a and EDNRB1b. In situ hybridization studies show EDNRB1a and EDNRB1b are expressed throughout these stages of embryogenesis. Arrows indicate gene expression in the anterior head region and in gut tube.