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## Expression patterns of alternative transcripts of the zebrafish *olfactomedin 1* genes

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### Abstract

Olfactomedin 1 (Olfm1) is a founding member of the family of olfactomedin domain-containing proteins. It is a secreted protein that performs different roles in different species. Although the molecular mechanisms of Olfm1 action are not known, its possible roles include the regulation of neural crest cell production, neuronal differentiation and ischemic neuronal death in adult. Two zebrafish *olfm1* gene (*olfm1a* and *olfm1b*) located on chromosomes 5 and 21 were identified in zebrafish genome. Four different transcripts are produced from each *olfm1* gene. The distribution of these transcripts in the course of zebrafish early development was studied by *in situ* hybridization and quantitative RT-PCR. Different variants of *olfm1* mRNA were present mainly in neurogenic tissues and demonstrated overlapping expression patterns.

### Keywords

olfactomedin; zebrafish; development; *in situ* hybridization; motor neurons; Rohon-Beard neurons; trigeminal ganglia; neural crest

## 1. Results and discussion

Olfactomedin 1 (Olfm1), also known as noelin (Barembaum et al., 2000; Moreno and Bronner-Fraser, 2001) and pancortin (Nagano et al., 1998), is a highly conserved secreted glycoprotein that was originally isolated from the bullfrog olfactory neuroepithelium (Snyder et al., 1991). The C-terminal olfactomedin domain was subsequently identified in a family of proteins consisting of at least 13 members in mammals (Zeng et al., 2005). Some family members, such as latrophilins and gliomedin, are membrane-bound proteins with the olfactomedin domain in the extracellular N-terminal region, while the intracellular C-terminal domain of these proteins is essential for the transduction of extracellular signals to intracellular signaling pathways (Volynski et al., 2004; Eshed et al., 2005). Other family members are secreted glycoproteins that may play important roles in normal development and diseases, although molecular mechanisms of their action are still not clear. Available data suggest that Olfm1 may have differing functions in different vertebrates. Chicken Olfm1 is involved in the regulation of the production of neural crest cells by the neural tube (Barembaum et al., 2000), while *Xenopus* Olfm1 promotes neuronal differentiation (Moreno and Bronner-Fraser, 2001; Moreno and Bronner-Fraser, 2005). Recent data suggest that mouse Olfm1 interacts with WAVE1 and Bcl-xL and forms a mitochondria-associated protein complex that mediates ischemic neuronal

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death in adult, but non embryonic, cerebral cortical neurons (Cheng et al., 2007). Olfactomedin domain in *Olfm1* may be essential for interaction with other proteins (Torrado et al., 2002), including receptors and extracellular matrix proteins (Eshed et al., 2005; Liu et al., 2006).

Four structurally different mRNA, named *AMY*, *BMZ*, *AMZ*, and *BMZ*, are produced from the *Olfm1* gene. They share a common central region (M) and have two different transcription start sites (A or B), and two different 3'-regions (Y and Z) produced by alternative splicing (Danielson et al., 1994). *AMY* and *BMZ* variants encode a shorter form of *Olfm1* that lacks the olfactomedin domain, while *AMZ* and *BMZ* variants encode proteins containing the olfactomedin domain.

Expression of the *Olfm1* gene is somewhat different in several studied species. In chicken, *Olfm1* is expressed in the closing neural tube and later becomes restricted to the dorsal neural folds and migrating neural crest (Barembaum et al., 2000). In *Xenopus*, *Olfm1* mRNA was detected mainly in postmitotic neurogenic tissues in the developing central and peripheral nervous systems, first appearing after neural tube closure (Moreno and Bronner-Fraser, 2001; Moreno and Bronner-Fraser, 2002; Moreno and Bronner-Fraser, 2005). In mice, *AMZ* and *BMZ* variants of *Olfm1* mRNA were detected in the early neural plate and ectoderm. Later they become detected in brain, some cranial placodes, cranial neural crest and pharyngeal endoderm. As development progresses, these mRNAs were detected in marginal zone cells of the neural tube, cranial ganglia and dorsal root ganglia (Moreno and Bronner-Fraser, 2005). In adult rats, only *BMZ* and *BMZ* *Olfm1* mRNAs were abundant in the cerebral cortex including the hippocampus and the olfactory bulbs, while the levels of *AMY* and *AMZ* variants was very low (Nagano et al., 1998).

Zebrafish represents an excellent experimental system to study gene expression and function in early development. In this paper, we have identified two zebrafish *olfm1* genes and characterized the expression patterns of different splice forms. Zebrafish *olfm1* genes demonstrate conservation in sequence and in gene structure to other vertebrates showing the overlapping expression patterns more closely resembling the expression pattern of *Xenopus Olfm1*.

### 1.1. Nomenclature and sequence analysis of zebrafish *olfm1* genes

Four genes encoding proteins highly homologous to mouse *Olfm1* protein were identified in the zebrafish genome. These genes were located on chromosomes 5, 14, 21 and 24. Proteins encoded by the genes located on chromosomes 5 and 21 showed 84% identity to mouse *Olfm1* (Nagano et al., 1998) and lower identity to *Olfm2* (53–54% identity) and *Olfm3* (60% identity). Proteins encoded by genes located on chromosomes 14 and 24 showed the highest identity to mammalian *Olfm2* (74%) and *Olfm3/optimed* (83%) (Torrado et al., 2002), respectively, while their identity with *Olfm1* was about 60%. Since a significant part of the zebrafish genome has been duplicated during evolution, we concluded that the chromosomes 5 and 21 genes are orthologs of mouse *Olfm1*. The zebrafish *olfm1* genes located on chromosomes 5 and 21 will be called *olfm1a* and *olfm1b*, respectively. *Olfm1a* and *Olfm1b* are 88% identical at the protein level.

Similar to mouse *Olfm1*, four different transcripts were predicted for both *olfm1a* and *olfm1b* genes, giving at least eight different *olfm1* transcripts in zebrafish (Fig. 1). The existence of these transcripts was supported by several lines of evidence. First, multiple clones were identified in the zebrafish EST database that contained exons 1 or 1' followed by exons 2 and 3 and 4' for both *olfm1a* and *olfm1b* genes. Second, full-length cDNA of the *olfm1b* gene containing exons 1, 2–6 was amplified and cloned from 72 hpf zebrafish embryos. Finally, exon 6 of the *olfm1a* gene and exon 4 of both *olfm1* genes were amplified by RT-PCR from different developmental stages.

Fig. 2 compares the deduced amino acid sequences from these transcripts to the sequences of the mouse AMZ and BMZ Olfm1 forms. It is interesting to note that when Olfm1 sequences belonging to different vertebrate species are compared, the M part, which is retained in all Olfm1 isoforms, is as conserved as the C-terminal Z part containing the olfactomedin domain.

## 1.2. Expression pattern of the *olfm1a* and *olfm1b* genes during embryonic development of zebrafish

Since there are no sequences that would be unique to each transcript, we have studied expression patterns of groups of transcripts from different promoters. Each group consisted of long and short forms of the transcripts from the same promoter. These groups will be referred to as *TR1* (*AMZ* and *AMY* transcripts of the *olfm1a* gene), *TR2* (*BMZ* and *BMZ* transcripts from the *olfm1a* gene), *TR3* (*AMZ* and *AMY* transcripts from the *olfm1b* gene) and *TR4* (*BMZ* and *BMZ* transcripts from the *olfm1b* gene) through the paper (see Fig. 1).

The spatial expression patterns of each group of transcripts was obtained by whole-mount *in situ* hybridization using the DIG-labeled antisense RNA probes specific for the 5'-end of each group. Different transcripts of the *olfm1* genes showed complex overlapping patterns of expression as summarized in Table 1. In brief, for both *olfm1a* and *olfm1b* genes, transcripts having exon 1' as the first exon (*TR2* and *TR4* groups) were detected first starting from 16 hpf (Fig. 3A). *TR2* transcripts were present in the developing eye, trigeminal placodes and the ventral neural tube, while *TR4* transcripts were strongly expressed in the trigeminal placodes. The levels of *TR2* and *TR4* expression increased in these areas between 16 and 24 hpf, and additional sites of expression were detected in the epibranchial placodes/ganglia, midbrain and telencephalon. Sectioning of the stained embryos demonstrated that in the eye *TR2* transcripts were present mainly in the dorsal-posterior retina (Fig. 3O–P). In the neural tube, *TR2* transcripts were present in motor neuron-containing outer layers of the ventral neural tube (Wilson and Maden, 2005). *TR4* transcripts were instead found in the dorsal neural tube, in the sensory Rohon-Beard neurons (Wilson and Maden, 2005) (Fig. 3K). This is confirmed by immunostaining of *TR2 in situ* hybridization samples with islet antibodies, which stain motor and sensory neurons and ganglia (Korz et al., 1993; Appel et al., 1995). *TR2* and islet showed overlapping expression pattern in trigeminal ganglia (Fig. 3Q) and ventral neural tube (not shown), although in the neural tube *TR2* stained a broader population of neurons compared with islet. At later developmental stages (35 and 55 hpf), expression of both *TR2* and *TR4* was significantly reduced in the neural tube (Fig. 3R,T), and was detected in telencephalon, ventral diencephalon, and dorsolateral and epibranchial placode/ganglia (Fig. 3R, T, V, X). In addition, *TR4* was also expressed in the optic tectum. This is similar to mouse brain development, where high levels of Olfm1 are detected in the adult but not in the embryonic cortex (Cheng et al., 2007).

*TR1* and *TR3* had similar expression patterns but later onset than *TR2* and *TR4*. At 16 hpf, there was no obvious expression of *TR1* (not shown) and only very weak expression of *TR3* (Fig. 3B). At 24 hpf, expression of *TR3* was detected in the developing kidney (Fig. 3J). At an advanced developmental stage (55 hpf), levels of *TR1* and *TR3* expression were comparable with those of *TR2* and *TR4*. At this stage, *TR1* demonstrated more limited expression pattern compared to *TR2*. For example, no *TR1* signals were detected in the olfactory placode (Fig. 3Q, U).

The expression patterns of the *olfm1* genes during early embryonic development (10–31 hpf) were analyzed by Q-PCR. When oligonucleotide primer sets specific to the 5'-ends of different *olfm1* mRNA variants were used, *TR1* and *TR2* messages, transcribed from the *olfm1a* gene, were first detected at 12 hpf, while *TR3* and *TR4* messages, transcribed from the *olfm1b* gene, were first detected at 20 hpf and 14 hpf, respectively (data not shown). The abundance of all transcripts dramatically increased with development until the last analyzed stage (31 hpf). To

estimate relative amounts of the long (*AMZ* and *BMZ*) and short (*AMY* and *BMY*) forms of *olfm1a* and *olfm1b* mRNAs, forward primer located in exon 3 and reverse primers located in exon 4' or 4 were used in Q-PCR. These experiments demonstrated that the activation of the *olfm1a* gene transcription in early development was mainly due to the accumulation of the long forms which were the predominant forms, comparing to the shorter forms, which did not show a significant increase in the first 24 hpf. On the other hand, both longer and shorter forms transcribed from the *olfm1b* gene showed strong increase from 18 hpf (not shown).

### 1.3. Expression pattern of BAC DNA encoding *olfm1b* gene

To evaluate the size of the promoter/enhancer region of the zebrafish *olfm1* gene, BAC DNA encoding the full-length *olfm1b* gene including 8 kb of the 5'-flanking and 85 kb of the 3'-flanking sequences was injected into zebrafish embryos. This DNA showed a mosaic expression as judged by *in situ* hybridization with probes specific for *TR3* or *TR4* transcripts. The levels of mRNA transcribed from the injected BAC DNA was significantly higher than from endogenous *olfm1b* gene and this mRNA was detected after 3 h of staining after *in situ* hybridization, while 30 h were normally needed to visualize the expression of the endogenous gene, so that we could distinguish the expression of ectopically introduced *olfm1b* gene from the endogenous expression. Both *TR3* and *TR4* transcripts from BAC DNA were detected in the same cells (Rohon-Beard neurons, trigeminal placode/ganglia and the telencephalon) as the expression of endogenous gene in 24 hpf embryos, with *TR4* producing stronger and more numerous hybridization signals as compared with *TR3* (Fig. 4 and Table 2). *TR3* and *TR4* transcripts were also detected in the hindbrain of few embryos, although we could not distinguish whether they were present in the neural tube or epibranchial placodes. We concluded that most of the promoter-enhancer sequences for the *TR4* transcript are present in BAC DNA, while some of the promoter/enhancer sequences for the *TR3* transcript may be located outside of the 8 kb 5-upstream sequence.

In summary, different splice variants of the *olfm1* genes demonstrated overlapping but not identical expression patterns and were expressed mainly in neural tissues in the course of early development. These expression patterns were more similar to the pattern of expression of the *Xenopus Olfm1* gene than to the chicken *Olfm1* gene (Barembaum et al., 2000; Moreno and Bronner-Fraser, 2001; Moreno and Bronner-Fraser, 2002). Similar to *Olfm1* gene expression in *Xenopus*, the zebrafish *olfm1* genes are expressed mainly after formation of neural crest which in the head region occurs at 12 hpf (Schilling and Kimmel, 1994). The activation of the *olfm1* genes expression takes place after the beginning of neural crest migration that starts around 15–16 hpf in the anterior region of the embryos (Schilling and Kimmel, 1994). Zebrafish *olfm1* genes are most actively expressed in the neurogenic tissues of the developing central and peripheral nervous systems. Further studies are in progress to elucidate the role of the *Olfm1* in neural development by protein knockdown using morpholinos and overexpression of the *olfm1* genes.

## 2. Experimental procedures

### 2.1. Husbandry of fishes

Wild-type zebrafish were maintained as described by Westerfield (2000). Embryos were produced by natural matings. All experiments using animals were approved by the NEI Animal Use and Care Committee.

### 2.2 Identification of the zebrafish *olfm1* genes

The mouse *Olfm1* protein sequence ([BAA28765](#)) was used for a blast search (<http://www.ncbi.nlm.nih.gov/BLAST/>) of the zebrafish genome database (<http://www.ensembl.org/Multi/blastview>). Zebrafish EST IMAGE clones were purchased from Open Biosystems.

Clones 7274404 and 7256463 correspond to two transcripts from two different promoters of the zebrafish *olfm1a* gene, while clones 7904578 and 7429384 correspond to two transcripts from two different promoters of the zebrafish *olfm1b* gene (see Results and discussion). BAC DNA (CH211-11G18) containing the zebrafish *olfm1b* gene and 8 kb and 85 kb of 5'- and 3'-flanking sequences was obtained from BACPAC Resources.

### 2.3. *In situ* RNA hybridization

The 5'-probes specific to each of the cDNA clones (see above) were amplified by PCR and cloned into the pcDNA3 vector (Invitrogen). Each of these probes recognizes two different transcripts having different 3'-ends as a result of alternative splicing. They will be called *TR1*, *TR2*, *TR3* and *TR4* through the text (see Results and discussion for more details). The following primers were used to prepare TR1-4 probes (the sizes of the amplicons are shown in the parenthesis):

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<i>TR1</i> (FW):	5'-ATAAGCTTAGCGATGATGCCTACTGGA-3' and
<i>TR1</i> (RV):	5'-ATAAGCTTGGGTGAGTTCGGTACCA-3' (331 bp)
<i>TR2</i> (FW):	5'-ATAAGCTTCGAGTGGAGAGAGAAGAC-3' and
<i>TR2</i> (RV):	5'-ATAAGCTTGCCAGTGCCTGTCCGGCA-3' (387 bp)
<i>TR3</i> (FW):	5'-ATAAGCTTGGAGAGACACACGGGAGAAA-3' and
<i>TR3</i> (RV):	5'-ATAAGCTTGCCTGAGTTCCTGCGCCA-3' (327 bp)
<i>TR4</i> (FW):	5'-ATAAGCTTCTAAAGGGCGTGAACGCACA-3' and
<i>TR4</i> (RV):	5'-ATAAGCTTCTATACTCCGGTCCGGAT-3' (440 bp).

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Digoxigenin-labeled cRNA probes were synthesized by *in vitro* transcription of the corresponding linearized plasmids using SP6-, T7- or T3-polymerase and DIG-RNA-labeling mixture (Roche). Whole mount *in situ* hybridization was performed as described previously (Toyama et al., 1995). After the visualization of *in situ* hybridization signals, some embryos were embedded in OCT and 10 µm frozen sections were prepared and counterstained with DAPI (1 µg/ml). The mounted sections were observed under an Axoplan2 microscope (Zeiss).

### 2.4. Quantitative RT-PCR (Q-PCR)

Ten embryos from each developmental stage were dechorionated and homogenized in 500 µl of Trizol (Invitrogen) using Polytron (Kinematica, Inc.) at the maximum power for 1 minute. Total RNA was isolated from the lysates following the manufacturer's protocol. RNA was dissolved in distilled water at concentrations of 0.1–0.3 µg/µl. Q-PCR was performed using 0.5 µg of total RNA as described previously (Hemish et al., 2003). Briefly, RNA was converted to cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems) and then dispensed into a 96-well PCR plate. Forward and reverse primers for a target gene and SYBR Green PCR Master Mix (Applied Biosystems) were added into each wells and PCR reaction was performed and monitored using a 7900HT Real Time Thermocycler (Applied Biosystems). *Elongation factor 1α* (*EF1α*) was amplified simultaneously for normalization after confirmation that its expression was not dramatically changed during developmental stages under study. Primers used for the Q-PCR were as follows:

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<i>TR1</i> (FW):	5'-ACCTCATTCTGCCGCTGAATT-3'
<i>TR1</i> (RV):	5'-CTGTCCGGCTCGATGCTT-3'
<i>TR2</i> (FW):	5'-CGATGATCACCAACTGGATGTC-3'
<i>TR2</i> (RV):	5'-GCCACGCTTAATTGGTGCTA-3'
<i>TR3</i> (FW):	5'-TCTCTGTCTATCCGTTGCTGA-3'
<i>TR3</i> (RV):	5'-GTGCTCACTCCTCGCTCTCC-3'
<i>TR4</i> (FW):	5'-GGTGCTCAGCACCATGGC-3'
<i>TR4</i> (RV):	5'-GCGGTCAGTTTGGTGGTGTT-3'
<i>Olfm1a</i> Y form (FW):	5'-GCTGCAGAGACTGAAGAACAAGTT-3'
<i>Olfm1a</i> Y form (RV):	5'-AACTGAATGATGCGATCAGTTGAC-3'
<i>Olfm1a</i> Z form (FW):	5'-CCACAACAGGGTGTCTAATCTTGA-3'
<i>Olfm1a</i> Z form (RV):	5'-AGTCAGCTTCCCACAGGTAAT-3'
<i>Olfm1b</i> Y form (FW):	5'-GAGAGCCAGAGGCCAGAAGAA-3'
<i>Olfm1b</i> Y form (RV):	5'-CGTGACATAAACAGCGTACTGA-3'

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<i>Olfm1b</i> Z form (FW):	5'-TGCAGAATCTGACTGCGAGTCT-3'
<i>Olfm1b</i> Z form (RV):	5'-GGAGTGCAGGTCATCATAGTCATAA-3'
<i>ef1a</i> (FW):	5'-CAGTGCTGGATTGCCACACT-3'
<i>ef1a</i> (RV):	5'-CCAGAACGACGGTCGATCTT-3'

## 2.5. Immunohistochemistry

Anti-Islet1 antibodies 39.4D5 and 40.2D6 were purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Embryos were first used for *in situ* hybridization with *TR2* RNA probe, then fixed with 4% paraformaldehyde, and incubated in the blocking buffer (1% bovine serum albumin, 0.1% Triton X-100, 0.05% dimethylsulfoxide and 2% goat serum in PBS) at 4°C for one hour. The blocking buffer was replaced with the anti-Islet antibody (1:500) diluted in the blocking buffer, and the incubation was continued at 4°C overnight. After washing three times with PBS-T (0.1% Triton X-100 in PBS) for 2 h, embryos were stained using an ABC labeling kit (Vector) according to the manufacturer's instructions. Embryos were embedded in OCT and 10 µm frozen sections were prepared. Images were collected using Zeiss Axoplan2 microscope (Zeiss).

## 2.6. BAC DNA injections

BAC DNA (30 pg) containing full length *olfm1b* gene as well as 8 kb of the 5'-flanking and 85 kb of the 3'-flanking sequences was injected into 1 cell stage embryos. Whole mount *in situ* hybridization with *TR3*- or *TR4*-specific probes was performed 24 h after injection as described above.

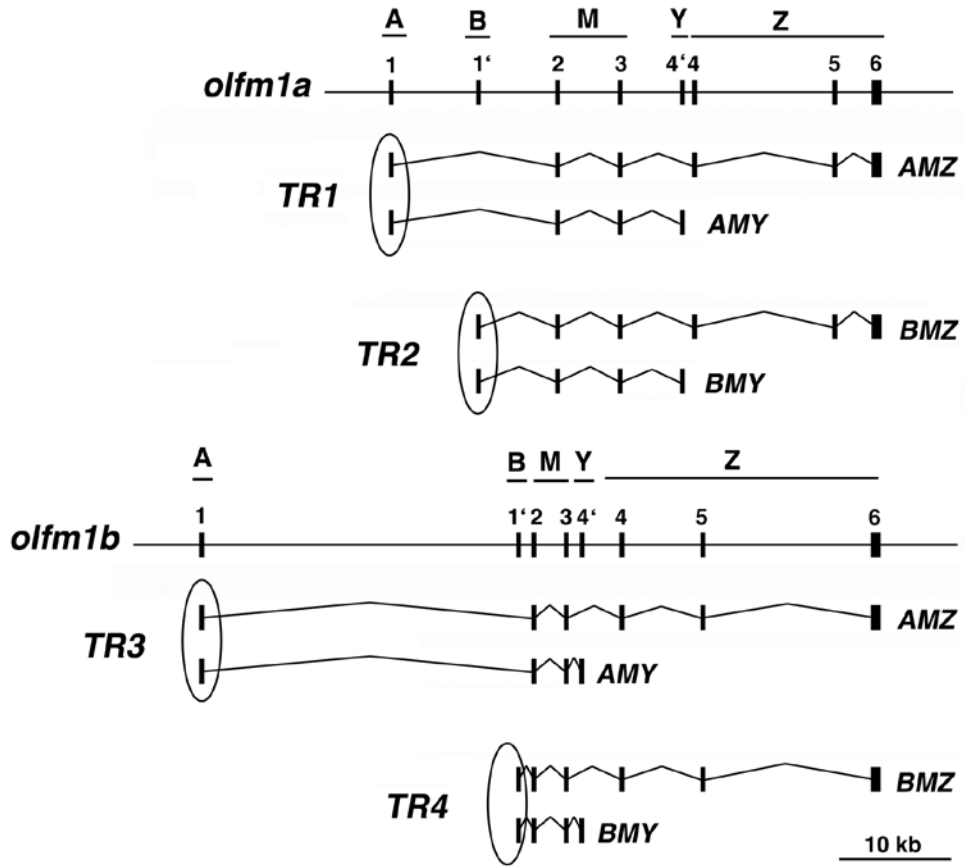
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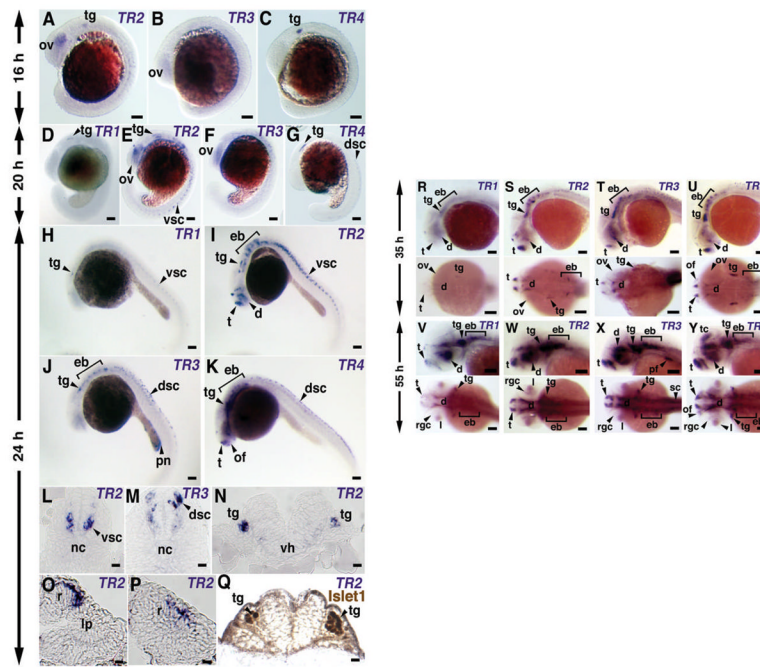


**Fig. 1.** Exon-intron organization of the zebrafish *olfm1* genes and the structure of corresponding transcripts. A, B, M, Y and Z correspond to different regions of *olfm1* mRNAs previously identified in *Olfm1* mRNAs of other vertebrates. Exons 1 (region A) or 1' (region B) and 4' (region Y) or 4 (part of region Z) are not used in the same transcripts. Transcripts having the same 5'-exons and transcribed from the same promoter were unified in the groups (TR1-4). Circles over exon 1 or 1' indicate the position of probes used for *in situ* hybridization.



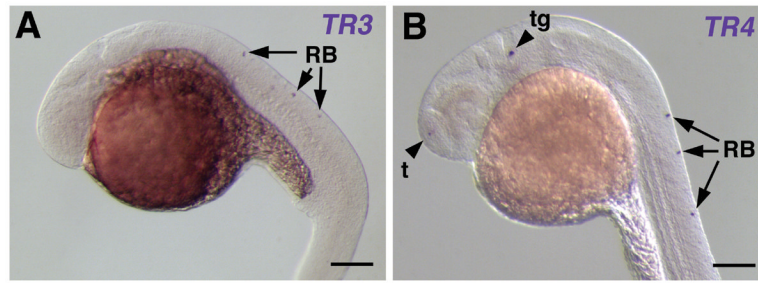
	<b>A</b>	
Olfm1a AMZ specific	MQPANKLLSLTLVLMGTELQ	22
Olfm1b AMZ specific	--RVH-----IV-----	22
mOlfm1 AMZ specific	---R-----LV-LV-----	22
	<b>B</b>	
Olfm1a BMZ specific	MSVPLLLKIGVVLSTMAMITNWMSQTLPSLVGLNSTKLSVAPPVPPDRSTG	50
Olfm1b BMZ specific	-----T---TA-QG-Y----IG	50
mOlfm1 BMZ specific	-----T-R-A-SG-TL-----	50
	<b>M</b>	
Olfm1a common	VLPANPEESWQVYSSAQDSEGRVCVTVVAPQQTMCSDARTKQLRQLLEKVVQNMSTQIQN	110
Olfm1b common	-----V	110
mOlfm1 common	---T-----I-----S---EV	110
	<b>I</b>	
Olfm1a common	LDQRTQKDLQYVQRMEVQLRGLTEKFKQVEESHKQNIKAKYKAIKAKMEELRPMIPVLEE	170
Olfm1b common	-----VK--D-----R---N-----A---L-----	170
mOlfm1 common	--R--R-----EK--N-MK-----HL-R-F-----D---L-----	170
	<b>Z</b>	
Olfm1a common	YKADAKLVMQFKKEVKNLTSVLSLQEEIGAFDYEELHNRVSNLEERLRACMQKLAGKGL	230
Olfm1b common	-----R-Q-----Q---AS-GL-Q-M---Y--DD--S--VS-----	230
mOlfm1 common	-----L-----Q-----N-----Y--D--QS-----	230
	<b>F</b>	
Olfm1a common	TGISDAITIKTSGSRYGSMWTDPLAPDGDTRVWYMDGYHNNRFRVREYKSMADFMYSDFNT	290
Olfm1b common	-----F-----E-----D-----TS---	290
mOlfm1 common	-----PV-V-----F-----E--N-----V---NT---	290
	<b>M</b>	
Olfm1a common	SHRLPHWPGTGQVVYNGSIYFNKFSNTIIKPFDFKTATISKSGQLDYAGFNRRYHYSWG	350
Olfm1b common	-----M-----S-M---R--N---S-T--A--	350
mOlfm1 common	-----HI--R--L--E--L-TRS-----Y--M--A--	350
	<b>H</b>	
Olfm1a common	GHSIDLMLVDEGGLWAIYATNQAGNIVISKLNPATLQIVKSYTTHPKRSAGEAFMCG	410
Olfm1b common	-----V-----H-I--HIIQ-W-----R-----S-----	410
mOlfm1 common	-----N---V-----D-VS---LQTWN-SY-----I---	410
	<b>T</b>	
Olfm1a common	TLVVTNGYSGGTKVYYAFHTNSSTYEYIDIPLQNKYSHISMLDYNPRDRALYAWNNGHQV	470
Olfm1b common	-----Y-----V-----	470
mOlfm1 common	-----H--YQ--A-----PF-----K-----T	470
	<b>L</b>	
Olfm1a common	LYNVTLFHVIRSEEL	485
Olfm1b common	-----Q-	485
mOlfm1 common	-----D--	485

**Fig. 2.** Comparison of the zebrafish *Olfm1* sequences with that of mouse *Olfm1*. The “-“ symbols in *Olfm1b* and mouse *Olfm1* mark amino acids that are identical to those in *Olfm1a*. A, B, M and Z denote parts encoded by different regions of *olfm1* mRNAs (see Fig. 1). Exon 4' encodes only one amino acid (Glycine) and is not shown in the Fig. Numbering for the M and Z parts is shown for the BMZ form.



**Fig. 3.**

Expression patterns of *TR1-4* in developing zebrafish embryos. Antisense RNA probes for whole-mount *in situ* hybridization were specific for each group and prepared as described in Material and Methods. (A–C) 16 hpf embryos. (D–G) 20 hpf embryos. (H–P) 24 hpf embryos. L, M and N show 10 μm frozen sections at the trunk level (L and M) and at the level of hindbrain (N). *TR2* and *TR3* mRNAs are expressed in the different part of neural tube (L and M). *TR2* expression was also observed in trigeminal ganglia (N). O and P show 10 μm frozen sections at the eye level. The strong *TR2* signal was detected in dorsal (O) and posterior (P) retina. (Q) 24 hpf embryos first hybridized to *TR2* probe and then stained with *islet1* antibodies. Frozen section at the hindbrain level is shown. Trigeminal ganglia (tg) express both *TR2* (N) and *islet1* (Q). (R–U) 35 hpf embryos. *TR1* showed weak signal while all other transcripts are strongly expressed in telencephalon, ventral diencephalons and dorsolateral and epibranchial placodes/ganglia. *TR3* has a stronger expression at the preoptic commissure than other transcripts and *TR4* is expressed in the olfactory placode. (V–Y) 55 hpf embryos. All transcripts strongly expressed in telencephalon, diencephalons and dorsolateral and epibranchial placodes/ganglia. *TR4* has specific expressions in the optic tectum (arrowhead). Abbreviations: d – diencephalon; dsc – dorsal spinal cord; eb – epibranchial placode/ganglia; l – lens; lp – lens placode; nc – notochord; of – olfactory placode; ov - optic vesicle; pn – pronephros; r – retina; rgc – retinal ganglion cells; sc – spinal cord; t – telencephalon; tc – tectum; tg – trigeminal placode/ganglia; vh– ventral hypothalamus; vsc – ventral spinal cord. A-C Scale Bar, 0.1 mm. D, E Scale bar, 0.01 mm. F, G Scale bar, 0.1 mm.



**Fig. 4.**

Expression pattern of the *olfm1b* gene from ectopically injected BAC DNA. One cell stage embryos were injected with 30 pg of *olfm1b* BAC DNA and analyzed by *in situ* hybridization for *TR3* and *TR4* expression. The signal of *TR3* was only observed in the dorsal spinal cord (A, arrowheads), while *TR4* expression was detected mainly in the telencephalon (t), trigeminal ganglia (tg) and Rohon-Beard neurons (RB) (B). Scale bar, 0.1 mm.

Table 1

Summary of *TR1-TR4* expression pattern

	Telencephalon	Diencephalon	Hindbrain	Optic vesicle	Olfactory placode	Spinal cord	Trigeminal ganglia	Epibranchial placode	Pronephros
TR1	-	-	-	-	-	-	-	-	-
TR2	-	-	-	+	-	-	+	-	-
TR3	-	-	-	-	-	-	-	-	-
TR4	-	-	-	-	-	-	++	-	-
TR1	-	-	-	-	-	++	+	-	-
TR2	-	-	-	++	-	++	++	++	-
TR3	-	+	-	+	-	++	-	+	++
TR4	-	-	-	-	-	+	++	-	-
TR1	+	-	-	-	-	+	+	-	-
TR2	++	-	-	++	-	++	++	++	-
TR3	-	+	-	+	-	++	++	+	++
TR4	++	-	-	++	-	++	-	-	-
TR1	+	+	-	-	-	-	++	++	-
TR2	+++	+	++	++	-	+	+	+	-
TR3	++	++	++	++	-	++	++	++	-
TR4	+++	+	-	+	++	+	+++	+++	-
TR1	+	+	++	++	-	-	+	+	-
TR2	+++	+++	+++	+	-	++	+++	+++	-
TR3	+++	+++	+++	+	-	++	+++	+++	-
TR4	+++	+++	+++	++	++	+	+++	+	-

- indicates the absence of hybridization signals, while +, ++, and +++ indicate weak, medium and strong hybridization signals, respectively

**Table 2**  
Distribution *TR3* and *TR4* expression in 24 hpf embryos injected with *olfm1b* BAC DNA

Transcripts	% embryos			
	Telencephalon	Trigeminal ganglia	Epibranchial placode/ neural tube	Rohon- Beard neurons
<i>TR3</i>	0	38	25	75
<i>TR4</i>	63	88	38	50

8 embryos were analyzed for both *TR3* and *TR4* probes.