Int. J. Dev. Biol. 54: 905-911 (2010)
doi: 10.1387/ijdb.092882ql

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Expression of *protocadherin-19* in the nervous system of the embryonic zebrafish

QIN LIU*,[1], YUN CHEN[1], FUMITAKA KUBOTA[2], JEAN J. PAN[1] and TOHRU MURAKAMI[3]

[1]Department of Biology, University of Akron, Akron, Ohio, USA, [2]Gunma University Hospital, Maebashi, Gunma, Japan and [3]Department of Anatomy, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

ABSTRACT We have analyzed the expression pattern of protocadherin-19, a member of the δ2-protocadherins, in the nervous system of developing zebrafish using *in situ* hybridization methods. mRNA encoding *protocadherin-19 (Pcdh19)* began to be expressed at about 12 hours post fertilization (hpf) showing a segmental expression pattern in the anterior 1/3 of the neural keel, with strong expression in the presumptive forebrain, cerebellum/rhombomere 1 and rhombomere 4. *Pcdh19* expression in the posterior neural keel was continuous and confined to the midline region. By 24 hpf, *Pcdh19* was expressed widely in the brain and spinal cord, with higher expression levels in the ventral telencephalon, dorsal and central thalamus, optic tectum, central tegmentum, cerebellum and dorsolateral regions of the hindbrain. As development proceeded, *Pcdh19* expression domains became restricted to the dorsal and/or lateral regions of the central nervous system, and *Pcdh19* expression was not detected in the spinal cord of two- and three-day old embryos. *Pcdh19* was also expressed by the eye primordium, developing retina, lens and otic vesicle. Similar to its expression in the brain, *Pcdh19* expression in the eye and ear was also spatially and temporally regulated.

KEY WORDS: *development, cell adhesion molecule, central nervous system, retina, otic vesicle*

Introduction

Cell adhesion molecules cadherins play important roles in tissue and organ development, function and maintenance of adult structures (Yagi and Takeichi 2000; Gumbiner 2005; van Roy and Berx 2008). So far more than 100 cadherins have been identified, and they are grouped into several subfamilies, including classical cadherins, protocadherins, desmosomal cadherins and flamingo cadherins (Nollet *et al*. 2000). The protocadherins (Pcdhs) subfamily contain more members than any other cadherin subfamilies, and Pcdhs are divided into several groups, such as clustered Pcdhs: α-, β-, and γ-Pcdhs, and non-clustered δ-Pcdhs (Suzuki 1996; Frank and Kemler 2002; Noonan *et al*. 2004; Wu 2005). The δ-Pcdhs are further divided into δ1-Pcdhs and δ2-Pcdhs based mainly on presence of several conserved motifs in the cytoplasmic domains, with the δ1-Pcdhs (e.g. Pcdh1, 7 and 9) having three conserved motifs CM1-CM3, whereas the δ2-Pcdhs (e.g. Pcdh10, 18 and 19) containing only two (CM1 and CM2) of the motifs (Redies *et al*. 2005; Vanhalst *et al*. 2005).

Results and Discussion

Zebrafish Pcdh19

Zebrafish *Pcdh19* encodes a protein with a putative hydrophobic signal sequence, extracellular region containing six repeats, transmembrane and cytoplasmic domains (Fig. 1). Phylogenetic analysis (Fig. 2) and alignment of several Pcdhs sequences (Fig. 3) show that the zebrafish Pcdh19 is most similar to human and mouse Pcdh19 at the amino acid level, less similar to Pcdh18, another δ2-Pcdh, and even less similar to Pcdh1, a δ1-Pcdh (Figs. 2 and 3 A,B). Like other δ2-Pcdhs, the zebrafish Pcdh19 contains only two conserved motifs, CM1 and CM2, in the cytoplasmic domain (Fig. 3A). The zebrafish CM1 is identical to human and mouse CM1, while the zebrafish CM2 has a high degree of identity (88.2%) with the human and mouse CM2 (Fig. 3A). As in other

Abbreviations used in this paper: hpf, hours post fertilization; *Pcdh19*, protocadherin-19 gene or mRNA; Pcdh19, protocadherin-19 protein; Pcdhs, protocadherins;.

***Address correspondence to:** Dr. Qin Liu. Department of Biology, University of Akron, 185 East Mill Street, Akron, Ohio 44325, U.S.A. Fax: +1-330-972-8445. e-mail: qliu@uakron.edu

Accepted: 24 July 2009. *Final author-corrected PDF published online:* 14 August 2009. *Associate Editor:* Makoto Asashima.

ISSN: Online 1696-3547, Print 0214-6282

```
Sig
MHSKDMDFVQMFVCFLLCWTGVDAVFNLK                                          29
EC1
YTVEEELRAGTKIANVTADAKVAGFALGNRQPYLRVISNSEPRWVNLSPAGLLITKQKID
RDAVCRQTPKCFISLEVMSNSMEICVIKIEIIDVNDNAPRF                             130
EC2
PTNHIDIEISENAAPGTRFPLEGASDPDSGSNGIQTYTITPNDIFGLEIKTRGDGSKIAE
LVVEKTLDRETQSRYTFELTAEDGGDPPKSGTVQLNIKVIDSNDNNPVF                     239
EC3
DEPVYTVNVLENSPINTLVIDLNATDPDEGTNGEVVYSFINFVSNLTKQMFKIDPKTGVI
TVNGVLDHEELHIHEIDVQAKDLGPNSIPAHCKVIVNVIDINDNAPEI                      347
EC4
KLLSENSEMVEVSENAPLGYVIALVRVSDNDSGANGKVQCRLQGNVPFRLNEFESFSTLL
VDGRLDREQRDMYNLTILAEDSGYPPLRSSKSFAVKVTDENDNPPYF                       454
EC5
TKPHYQAMVLENNVPGAFLLAVSARDPDLGMNGTVSYEIIKSEVRGMSVESYVTVNSNGE
IYGVRAFNHEDTRTFEFKVSAKDGGDPPLTSNATVRIVVLDVNDNTPVM                     563
EC6
TTPPLVNGTAEVSIPKNAGVGYLVTQIKADDYDEGENGRLTYSISEGDMAYFEIDQINGE
VRTTKTFGENAKPSYQITVVAHDHGQTSLSASAYIVIYLSPDLNAQEQIGPVN                 676
TM
LSLIFIIALGSIAVILFVTMIFVAV                                              701
Cyto
KCKRDNKEIRTYNCRVAEYSYGNQKKSSKKKKLSKNDIRLVPRDVEETDKMNVVSCSSLT
SSLNYFDYHQQTLPLGCRRSESTFLNVENQNSRNAAPNHGYHHTFTGQGPQQPDLIINGM
PLPETENYSIDSSYVNSRAHLIKSTSTFKDMEGNSLKDSGHEESDQTDSEHDVQRGHYAD         CM1
TAVNDVLNMTVPSNNSQIPDQDQSEGFHCQDECRILGHSDRCWMPRVPIPARAKSPEHGR         CM2
NVIALSIEATTVDVPHYEDCGTTKRTFATFGKDGPDEDRAEQRGRRQTAEPAVCSPKTNG
AVREAGNGREAVSPITSPVHLKSPQSKAPSTYNTLKCRDAERIANHSLLRQPEGKDSEPA
MREINTLLQDGRDKESPGSKRLKDIVL                                           1088
```

Fig. 1. Deduced amino acid sequence of zebrafish *Pcdh19*. *The putative hydrophobic signal sequence (Sig), and the conserved cytoplamic motifs 1 and 2 (CM1 and CM2, respectively) are underlined. Other abbreviations: cyto, cytoplasmic domain; EC1-EC6, extracellular domains 1-6; TM, transmembrane domain.*

Pcdh domains, the zebrafish CM1 and CM2 are more similar to those of Pcdh18 (δ2) than Pcdh1 (δ1) (data not shown). Zebrafish Pcdh19 has two isoforms, which are identical except that isoform 1 is slightly shorter (missing amino acids 755-826 in the cytoplasmic domain, Fig. 1) than isoform 2. The human PCDH19 also has two variants but they differ by only one amino acid, with variant 1 containing an extra serine at amino acid 847 in the protein.

Pcdh19 expression

Compared to our extensive knowledge of classical cadherins (e.g. cadherin-1 and cadherin-2, also known as E- and N-cadherins, respectively) expression and function, little is known about Pcdh19 expression and function in developing vertebrates, and to the best of our knowledge, there is no published report on Pcdh19 expression in nonmammalian vertebrates.

Using reverse transcriptase-polymerase chain reaction (RT-PCR) and whole mount *in situ* hybridization methods, we analyzed expression of *Pcdh19* in embryonic zebrafish from 6 hours post fertilization (hpf) to 72 hpf. RT-PCR experiments showed that both *Pcdh19* isoforms were expressed by embryos of 10 hpf to 72 hpf, with isoform 2 expression slightly stronger than isoform 1 in 18-22 hpf and 72 hpf embryos (Fig. 4A). cRNA probes designed to detect both *pcdh19* isoforms were used to perform whole mount *in situ* hybridization. There was no *Pcdh19* expression found in young embryos of 6 hpf (Fig. 4B) and 9 hpf (data not shown). At 12-13 hpf, *Pcdh19* expression was observed in the neural keel. In the anterior neural keel, *Pcdh19* expressing domains in the presumptive forebrain and hindbrain were separated by regions with little or no *Pcdh19* expression (Fig. 4 C,D). The eye primordia also contained *Pcdh19* (Fig. 4D). To determine the relative positions of the *Pcdh19* expression domains in the presumptive hindbrain, we performed double-labeling experiments using digoxigenin-labeled *Pcdh19* cRNA probes coupled with fluorescein-labeled *pax2a* (labeling the boundary of the mid- and hindbrains, Krauss *et al.* 1991) or *krox20* (labeling the rhombomeres 3 and 5, Oxtoby and Jowett 1993). The first *Pcdh19* expression domain (indicated by an arrow) in the presumptive hindbrain was located immediately posterior to the boundary between the mid- and hindbrains (Fig. 4F), while the second *Pcdh19* expression domain (indicated by an arrowhead) in the presumptive hindbrain was located between the rhombomeres 3 and 5 labeled by the *krox20* probe (Fig. 4 G,H). Therefore, the first and second *Pcdh19* expression domains in the presumptive hindbrain were likely situated in the presumptive cerebellum/rhombomere 1 and rhombomere 4, respectively. *Pcdh19* expression in the posterior neural keel was continuous, but appeared to be restricted to regions along the midline (indicated by two arrows in Fig. 4E).

At 18 hpf, *Pcdh19* was expressed in both the brain and spinal cord (Fig. 5 A-D), with obvious regional differences in expression levels, judged by staining intensities, in the fore- and midbrains (Fig. 5B). The ventral telencephalon, ventral diencephalon and tegmentum showed stronger *Pcdh19* expression than the remaining regions of the fore- and midbrains. Similar to *Pcdh19* expression in the younger embryos (Fig. 4), reduced *Pcdh19* expression was found between the cerebellum and optic tectum (arrowhead in Fig. 5B). No regional difference in expression levels was detected in the hindbrain (Fig. 5C), except there was a narrow region, between the cerebellum and the remaining hindbrain, with reduced *Pcdh19* expression (arrow in Fig. 5 B,C). This region was likely derived from the region between the first *Pcdh19* expression domain and rhombomere 3 in the presumptive hindbrain of younger embryos (Fig. 4 G,H). In the spinal cord, *Pcdh19* expression appeared to be stronger in the floor plate region (Fig. 5D). In addition to the

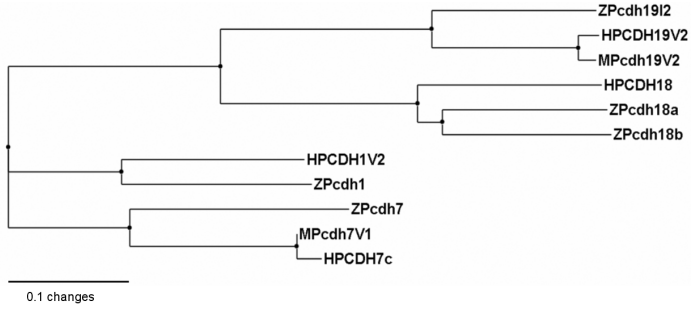
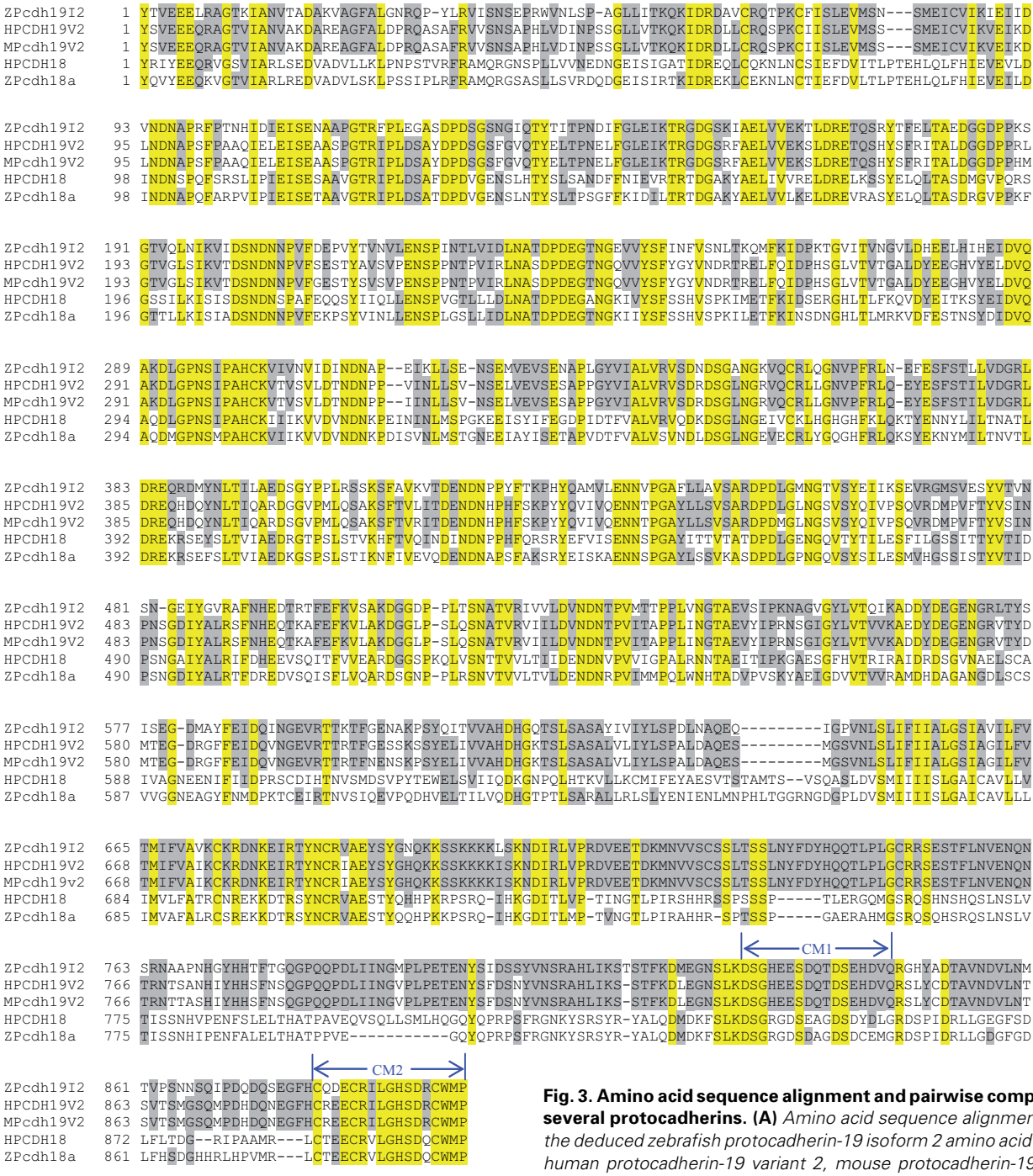


Fig. 2. Unrooted phylogram resulting from neighbor-joining distance analysis of EC1 through the carboxy-terminal protein sequence alignment. *Abbreviations: human protocadherin-1 variant 2, HPCDH1V2; human protocadherin-7, variant c, HPCDH7c; human protocadherin-18, HPCDH18; human protocadherin-19 variant 2, HPCDH19V2; mouse protocadherin-7 variant 1, MPcdh7V1; mouse protocadherin-19 variant 2, MPcdh19V2; zebrafish protocadherin-1, ZPcdh1; zebrafish protocadherin-7, ZPcdh7; zebrafish protocadherin-18a, ZPcdh18a; zebrafish protocadherin-18b, ZPcdh18b; zebrafish protocadherin-19 isoform 2, ZPcdh19l2.*

A



B

	ZPcdh19I2	HPCDH19V2	MPcdh19V2	HPCDH18	MPcdh18	ZPcdh18a	HPCDHV2	ZPcdh1
ZPcdh19I2		71.28%	71.76%	34.20%	34.29%	35.84%	22.75%	22.05%
HPCDH19V2			96.46%	33.99%	32.80%	35.56%	22.46%	22.29%
MPcdh19V2				33.57%	32.95%	35.78%	22.79%	23.30%
HPCDH18					93.12%	66.84%	22.06%	22.95%
MPcdh18						66.10%	22.31%	23.60%
ZPcdh18a							23.71%	23.41%
HPCDH1V2								68.64%
ZPcdh1								

Fig. 3. Amino acid sequence alignment and pairwise comparisons of several protocadherins. (A) *Amino acid sequence alignment between the deduced zebrafish protocadherin-19 isoform 2 amino acid sequence, human protocadherin-19 variant 2, mouse protocadherin-19 variant 2, and two δ2–protocadherins: human protocadherin-18 and zebrafish protocadherin-18a. The alignment shows sequences between published sequences from the EC1 to the end of the conserved cytoplasmic motif 2. Sequences highlighted by yellow boxes indicate residues that are common to all five sequences, and sequences highlighted by gray boxes indicate amino acids that are identical to at least three of the sequences. The conserved cytoplasmic motifs CM1 and CM2 are indicated.* **(B)** *Sequence identity percentages for pairwise comparisons between the above five δ2-protocadherin sequences, a mouse protocadherin-18 sequence (MPcdh18), and two δ1-protocadherin sequences: human protocadherin-1 variant 2 and zebrafish protocadherin-1. Diagonal shaded boxes indicate sequence comparisons between the same sequences, and therefore, represent 100% identity. Sequence comparisons were performed using ClustalW2. Other abbreviations are the same as in Fig. 2.*

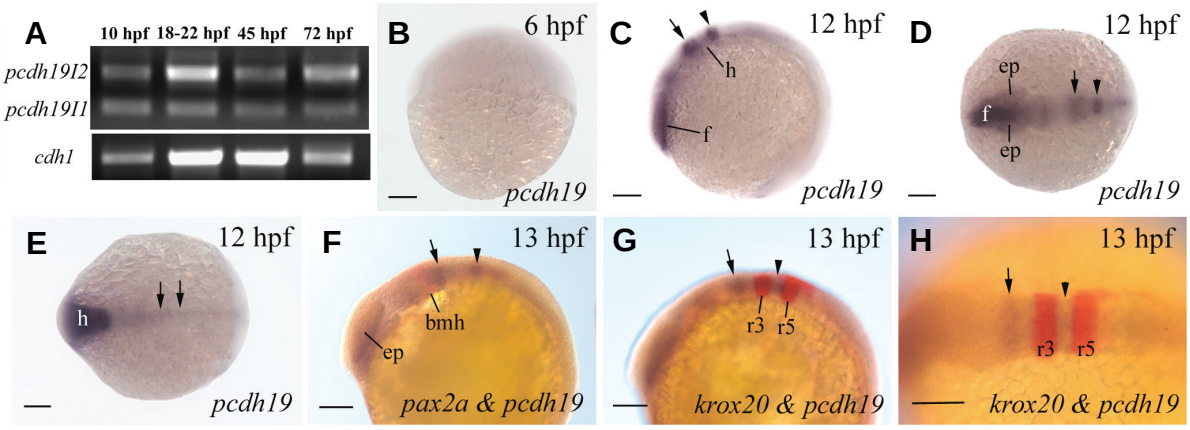


Fig. 4. *Pcdh19* expression in 6-13 hpf zebrafish embryos. (A) *RT-PCR analysis of* Pcdh19 *isoforms 1 and 2 expression in embryonic zebrafish using total RNAs. RT-PCR for* cdh1 *was performed as loading control. The remaining panels show whole mount embryos labeled with* Pcdh19 *cRNA probes* **(B-E)**, Pcdh19 *and* pax2a *cRNA probes* **(F)**, *or* Pcdh19 *and* krox20 *cRNA probes* **(G,H)**. **(B,C)** *Lateral views of the entire embryos (head towards the lower left corner for C).* **(D,E)** *Dorsal views (anterior to the left) of the entire embryos.* **(F,G)** *Lateral views of the anterior half of the embryos (anterior to the left and dorsal up), while* **(H)** *is a dorsal view of the presumptive hindbrain region of an embryo (anterior to the left). The arrow and arrowhead in (C, D, F, G and H) point to the first and second* Pcdh19 *expression domains, respectively, in the presumptive hindbrain. The two arrows in panel E indicate* Pcdh19 *expression in the middle neural keel. Abbreviations: bmh, boundary of the mid- and hindbrains; ep, eye premordium; f, presumptive forebrain; h, presumptive hindbrain; r3 and r5, rhombomeres 3 and 5, respectively. Scale bars, 100 μm.*

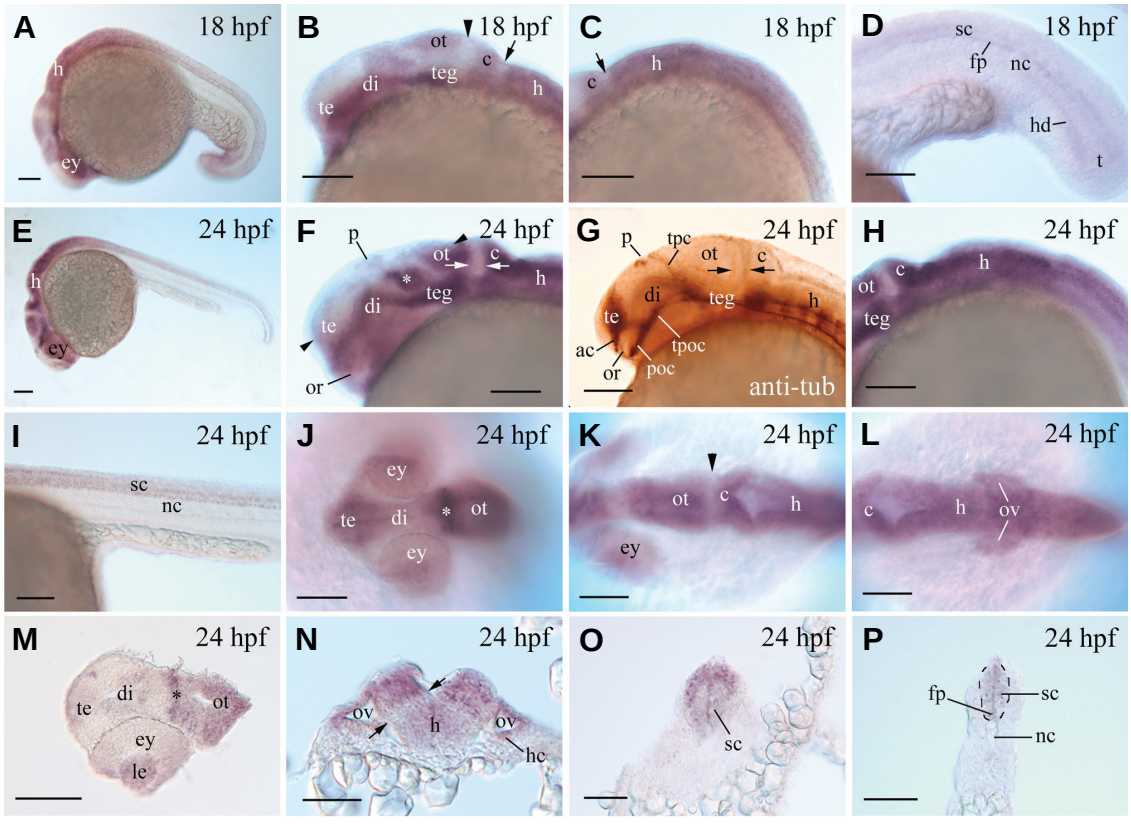


Fig. 5. *Pcdh19* expression in 18 and 24 hpf zebrafish embryos. (A-F) *and* **(H-L)** *Whole mount embryos labeled with* Pcdh19 *cRNA.* **(G)** *is the head region of a whole mount embryo labeled with the anti-acytelated tubulin antibody.* **(A,E)** *Lateral views of the entire embryos (head towards the lower left corner).* **(B,F)** *Lateral views of the fore-and midbrains, while* **(C,H)** *are lateral views of the hindbrain (anterior to the left, dorsal up).* **(J,K,L)** *Dorsal views of the head region (anterior to the left).* **(D,I)** *Lateral views of the posterior half of the body and mid-trunk region, respectively (anterior to the left). The arrowhead in* **(B,K)**, *and the opposing arrows in* **(F,G)** *indicate the same region with reduced* Pcdh19 *expression, located between the posterior border of the optic tectum and boundary of the mid-hindbrains. The arrow in* **(B,C)** *point to the same area with reduced* Pcdh19 *expression, located immediately posterior to the cerebellum. The asterisk in* **(F,J,M)** *points to the same* Pcdh19 *expressing area in the dorsal thalamus.* **(M-P)** *Sections from whole mount embryos processed for* Pcdh19 in situ *hybridization. The apposing arrowheads in (F) indicate the plane of section for the image in (M) (anterior to the left), while* **(N-P)** *are cross sections (dorsal up) at the otic vesicle (ov), anterior-trunk, and mid-trunk regions, respectively. The opposing arrows in (N) indicate a band of tissue with reduced* Pcdh19 *staining. Other abbreviations: ac, anterior commissure; c, cerebellum; di, diencephalon; fp, floor plate of the spinal cord; h, hindbrain; hc, hair cells; hd, hypochord; le, lens; nc, notochord; or, optic recess; ot, optic tectum; p, pineal organ; poc, postoptic commissure; sc, spinal cord; te, telencephalon; teg, tegmentum; tpc, tract of posterior commissure; tpoc, tract of postoptic commissure. The remaining abbreviations are the same as in Fig. 4. Scale bars, 100 μm for (A-M), and 50 μm for (N-P).*

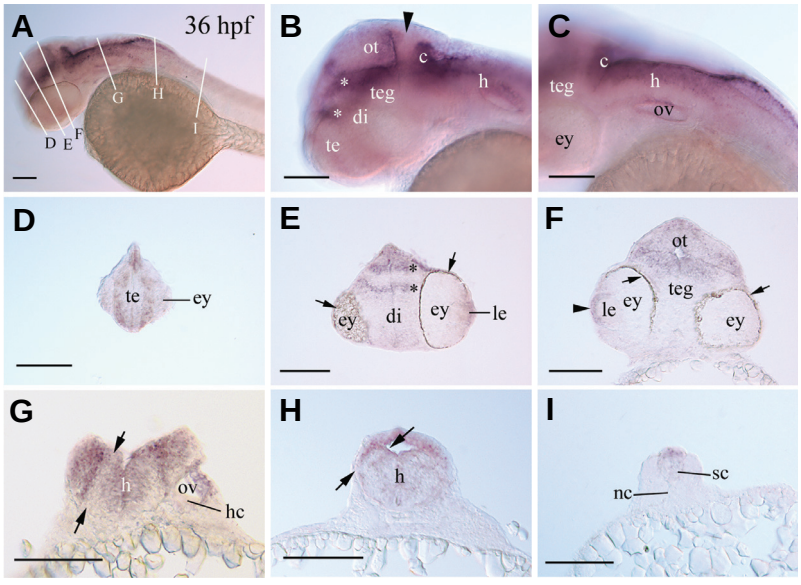


Fig. 6. *Pcdh19* expression in 36 hpf embryos. (A,B,C) *Lateral views of whole mount embryos showing the anterior 2/3 of the fish, the anterior head, and posterior head regions, respectively (anterior to the left and dorsal up).* **(D-I)** *Cross sections (dorsal up) from whole mount embryos processed for* Pcdh19 in situ *hybridization. Levels of the sections are shown in (A). The arrowhead in (B) points to the region with reduced* Pcdh19 *expression (also see Fig. 5F). Arrows in* **(E,F)** *indicate pigmented epithelium. The arrowhead in (F) points to* Pcdh19 *expression in the epithelial layer of the lens. Asterisks in (B,E) indicate the same thalamic regions with stronger* Pcdh19 *expression. The opposing arrows in (G,H) indicate the band of tissue with reduced* Pcdh19 *expression (also see Fig. 5N). Abbreviations are the same as in Fig. 5. Scale bars, 100 μm.*

nervous tissue, *Pcdh19* was also seen in the hypochord below the notochord near the tail (Fig. 5D). Embryos of 24 hpf showed somewhat similar *Pcdh19* expression patterns in the fore- and midbrains as 18 hpf embryos, except that the staining was stronger in the dorsal thalamus (ventroanteral to the optic tectum, and adjacent to the tract of the posterior commissure, indicated by an asterisk in Fig. 5F) and optic tectum (Fig. 5F) than 18 hpf embryos (Fig. 5B). In the telencephalon, the *Pcdh19* expressing domain was located anterodorsal to the optic recess (Fig. 5 F,G). The stronger labeled regions in the telencephalon, central thalamus and tegmentum appeared to form a continuous thick bank viewing laterally (Fig. 5F). The stripe of tissue between the posterior border of the optic tectum and the boundary of the mid- and hindbrains continued to show much reduced staining (Fig. 5 F,G, indicated by two opposing arrows). In the hindbrain, *Pcdh19* expression was continuous from the cerebellum to the spinal cord, with stronger expression levels detected in the cerebellum and dorsolateral hindbrain (Fig. 5 H,N). A band of tissue between the dorsolateral and the ventromedial hindbrain showed reduced staining (indicated by two opposing arrows in Fig. 5N). In the anterior spinal cord, its dorsal 1/3 area was also more strongly labeled than the ventral spinal cord (Fig. 5O), while in the mid-trunk and tail regions of the spinal cord, regional differences in the staining was not detected and the floor plate region continued to express *Pcdh19* (Fig. 5P). *Pcdh19* was also expressed by the retina, lens (Fig. 5 J,M), and otic vesicle (5L and N). The lens and peripheral retina (future retinal marginal zones) were more strongly labeled than the central retina (Fig. 5M). Epithelial cells in the lateral otic vesicle and the hair cells were strongly labeled (Fig. 5N).

Pcdh19 expression was reduced and regional differences in expression levels became more pronounced in some regions of the CNS in 36 hpf embryos (Fig. 6). Stronger *Pcdh19* staining continued to be observed in the dorsal and central thalamus (Fig. 6 B,E,F), the cerebellum (Fig. 6 B,C), and the dorsolateral regions of the hindbrain (Fig. 6 B,C,G,H). In the telecephalon, stronger *Pcdh19* expression was found in the lateral portion (Fig. 6D). In the diencephalon, the strongly labeled *Pcdh19* expression domains in the dorsal and central thalamus

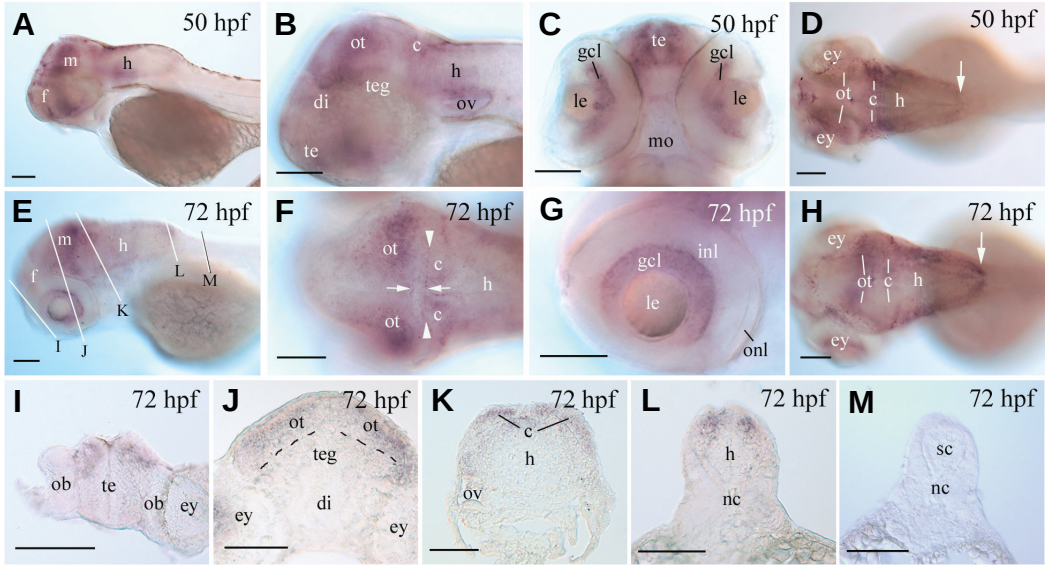


Fig. 7. *Pcdh19* expression in older embryos. Pcdh19 *expression in 50 hpf* **(A-D)** *and 72 hpf (the remaining panels) embryos.* **(A-H)** *Whole mount embryos showing lateral views (anterior to the left and dorsal up) of the anterior fish* **(A,E)**, *head* **(B)** *and eye* **(G)**. **(C)** *Ventral view of the head (dorsal up), while* **(D,F,H)** *are dorsal views (anterior to the left). The arrow in* **(D,H)** *points to the posterior border of* Pcdh19 *expression domain in the hindbrain, while the arrowheads in* **(F)** *indicate the boundary of the mid-hindbrains. The apposing arrows in (F) indicate the area between the posterior border of the optic tectum and boundary of the mid-hindbrains with reduced* Pcdh19 *expression.* **(I-M)** *Cross sections (dorsal up) with their section levels indicated in (E). The dashed lines in* **(J)** *indicate the boundary between the optic tectum and tegmentum. Abbreviations: f, forebrain; gcl, retinal ganglion cell layer; inl, inner nuclear layer; m, midbrain; onl, outer nuclear layer. Other abbreviations are as in Fig. 4. Scale bars, 100 μm.*

had the appearance of two horizontal bands in cross sections (Fig. 6E, indicated by two asterisks). In the midbrain, *Pcdh19* expression was confined mainly to the optic tectum and dorsal tegmentum (Fig. 6F). Again, the region between the posterior border of the optic tectum and the boundaries of mid- and hindbrains (arrowhead in Fig. 6B), and the region between the dorsolateral and the ventromedial hindbrain (opposing arrows in Fig. 6 G,H) had less *Pcdh19* expression than their neighboring areas. The dorsal spinal cord contained higher *Pcdh19* expressing cells than the ventral spinal cord even in the mid-trunk region (Fig. 6I). *Pcdh19* expression in the retina and lens was reduced compared to 24 hpf embryos, except in the retinal marginal zones and the lens epithelial layer (Fig. 6 E,F). Strong *Pcdh19* expression continued in the lateral epithelial cells of the otic vesicle, but its expression was reduced in the hair cells (Fig. 6G).

Generally speaking, *Pcdh19* expression in 50 hpf embryos was similar to those of 72 hpf (Fig. 7). Similar to the younger embryos, stronger *Pcdh19* staining was seen in the dorsal thalamus and dorsal tegmentum (Fig. 7B), optic tectum (Fig. 7 B,F,J), cerebellum (Fig. 7 B,K), and dorsolateral hindbrain (Fig. 7 D,H,K), but *Pcdh19* expression in the central thalamus, tegmentum and hindbrain was reduced compared to 24 and 36 hpf embryos. At 50 hpf, *Pcdh19* expression in the telencephalon (Fig. 7C) was mainly in the lateral regions (similar to 36 hpf embryos, Fig. 6D), but stronger expression became restricted to the dorsal telencephalon by 72 hpf (Fig. 7I). The region between the posterior border of the optic tectum and the boundary of the mid-hindbrains continued to show reduced staining (Fig. 7F), while *Pcdh19* expression in the ventromedial hindbrain was greatly reduced (Fig. 7K) compared to younger embryos (Figs. 5N and 6G). *Pcdh19* expression in the spinal cord was no longer detectable at both 50 hpf (Fig. 7D, data not shown for spinal cord sections) and 72 hpf (Fig. 7 H,M). In the eye, *Pcdh19* expression became mainly confined to the retinal ganglion cell layer, while there was no *Pcdh19* expression in the lens (Fig. 7 C,G). *Pcdh19* expression in the otic vesicle became further reduced (Fig. 7K).

Pcdh19 expression in the embryonic zebrafish is somewhat similar to that in embryonic mice (Gaitan and Bouchard, 2006). In both species, early *Pcdh19* expression is mainly confined to the nervous system. *Pcdh19* is found in the telencephalon and diencephalon, in the spinal cord, in the developing retinal ganglion cell layer and lens. In adult rat brain, *Pcdh19* was expressed in specific regions of the telencephalon, diencephalon, midbrain and hindbrain (Kim *et al*. 2007). In addition to the neural tissue, *Pcdh19* is also expressed by several other tissues in human (RT-PCR analysis, Wolverton and Lalande 2001) and mouse (Gaitan and Bouchard 2006), including the heart and kidney. It remains to be determined if *Pcdh19* is expressed by these tissues in larval and adult zebrafish.

Materials and Methods

Zebrafish embryos were obtained from in house breeding, and maintained as described in the Zebrafish Book (Westerfield 2005). Embryos for whole mount *in situ* hybridization were raised in PTU (1-phenyl-2-thiourea, 0.003%) at 28.5°C, staged in hours post fertilization, and fixed in phosphate buffered 4% paraformaldehyde.

Cloning of zebrafish Pcdh19

A TBlastn search using human PCDH19 protein sequence as a query resulted in a zebrafish genomic DNA sequence CR318607. A zebrafish *Pcdh19* cDNA fragment was amplified by PCR from a zebrafish embryonic cDNA Uni-ZAP XR library (discontinued; Stratagene, La Jolla, CA) using primers (forward primer 0, 5'-CGTTAGTCATAGACCTGAACGCCACTGACC -3, reverse primer 0, 5'-TTACTACCAAGCCACGATGACAGTCTGAGC -3') flanking a predicted zebrafish *Pcdh19* coding region. The PCR product was cloned in the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA). Sequences of the clones indicated 2 isoforms, *Pcdh19* isoforms 1 and 2 (GenBank, accession number: AB 362378 and AB 362379, respectively, F. Kubota and T. Murakami). Analysis of the deduced amino acid sequences of these isoforms revealed that they lack the N-terminal 1/3 (from signal sequence to about half of EC3) of the proteins compared to other protocadherins. A BLAST search using the *Pcdh19* isoform 2 sequence resulted in a zebrafish cDNA clone sequence (wu:fc83e05, GenBank accession number: BC129243, Strausberg *et al*.) that contains all the incomplete *Pcdh19* isoform 2 sequence, plus most of the missing N-terminal sequence. Compared to other protocadherins including the mammalian Pcdh19 proteins, this zebrafish sequence (zebrafish protocadherin-19 isoform 2) appeared to be missing some of the signal sequence in the 5' region. Using primers designed according to genomic sequence of wu:fc83e05 (GenBank accession number: NW 001884478) and mRNA sequence of the wu:fc83e05 (forward primer 1, 5'-CGCGTGAAGACAGACATCAA-3', forward primer 2, 5'-TTCCAAGGACATGGATTTCG-3', 58 and 25 nucleotides, respectively, 5' to the starting codon of the published wu:fc83e05 sequence; reverse primer 1, 5'-AGTAGACCACCTCGCCATTG-3', corresponding to the nucleotides 748 to 767 of the wu:fc8305), and total RNAs from 24-70 hpf whole zebrafish embryos, we performed RT-PCR and obtained the missing signal sequence (Fig. 1).

RT-PCR analysis of Pcdh19 expression in developing zebrafish

RT-PCR analysis of *Pcdh19* isoforms temporal expression profiles was performed using *Pcdh19* specific primers (forward primer 3, 5'-GCCTTGGGCTCTATTGCAGTCA-3'; reverse primer 2, 5'-AGCATAGTGCCCTCTCTGGA-3', corresponding to nucleotides 1284-1305 and 1855-1874, respectively, of zebrafish *Pcdh19* isoform 2, GenBank accession number AB 362379). These primers amplified bands of 377 bp and 590 bp for *Pcdh19* isoforms 1 and 2, respectively (Fig. 4A). Zebrafish *cdh1* transcripts (Liu *et al*. 2007) were used as the control for the RT-PCR experiments because *cdh1* was shown to be strongly expressed by young zebrafish embryos (Babb *et al*. 2005).

In situ *hybridization and immunocytochemistry*

For obtaining a *Pcdh19* DNA fragment (corresponding to the nucleotides 43-985 of the incomplete zebrafish *Pcdh19* isoforms 1 and 2) as a template for synthesizing cRNA probes, zebrafish *Pcdh19* specific primers (forward primer 4, 5'-CAATGGCGAGGTGGTCTACT-3'; reverse primer 3, 5'-CAACTCCAGCGTTTTTAGGG-3'), and total RNA isolated from 20-50 hpf whole zebrafish embryos were used for RT-PCR. This cDNA fragment was cloned into the pCRII-TOPO vector (Invitrogen), and was verified by restriction mapping and a PCR experiment using a pair of *Pcdh19* specific primers that were internal to the previous set of primers (forward primer 5, 5'-GCCCGAAATCAAACTGTTGT-3'; reverse primer 4, 5'-GCACCTCCGATTTGATGATT-3'). This experiment produced a cDNA fragment corresponding to the nucleotides 266-729 of the incomplete zebrafish *Pcdh19* isoforms 1 and 2). The larger *Pcdh19* cDNA fragment in the pCRII-TOPO vector was used as a template for the synthesis of digoxigenin-labeled zebrafish *Pcdh19* RNA sense or antisense probes for *in situ* hybridization. Both *Pcdh19* isoforms contained this cDNA fragment. cDNAs used to generate the fluorescein-labeled antisense *pax2a* and *krox20* were provided by Drs. Pamela Raymond (University of Michigan) and Lisa Maves (Fred Hutchinson Cancer Research Center), respectively. Detailed procedures for the cRNA probe synthesis and whole mount *in situ* hybridization were described previously (Liu *et al*. 1999; Liu *et al*. 2007; Kubota *et al*. 2008). There was no

staining in zebrafish embryos from 24-50 hpf using the sense *Pcdh19* probes (data not shown).

Anti-acetylated tubulin antibody (Sigma, St Louis, MO) was used at 1:3000. The secondary antibody (used at 1:250) was biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA). Whole-mount immunocytochemistry was carried out according to protocols described in the Zebrafish Book (Westerfield 2005).

Acknowledgements

This work was supported by grants from NIH EY13879 to Q. L., Grants-in-Aid awarded to T.M. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT), and the Initiative for Attractive Education in Graduate Schools awarded to Gunma University from MEXT. We thank Drs. Pamela Raymond (University of Michigan, Ann Arbor, MI) and Lisa Maves (Fred Hutchinson Cancer Research Center, Seattle, WA) for the pax2a *and* krox20 *containing plasmids, respectively.*

References

BABB S.G., BARNETT J., DOEDENS A.L., COBB N., LIU Q., SORKIN B.C., YELICK P.C., RAYMOND P.A. and MARRS J.A. (2001). Zebrafish E-cadherin: expression during early embryogenesis and regulation during brain development. *Dev Dyn* 221: 231-237.

FRANK M. and KEMLER R. (2002). Protocadherins. *Curr Opin Cell Biol* 14: 557-562.

GAITAN Y. and BOUCHARD M. (2006). Expression of the δ–protocadherin gene *Pcdh19* in the developing mouse embryo. *Gene Expr Patterns* 6: 893-899.

GUMBINER B.M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* 6: 622-634.

KIM S.-Y, SUN CHUNG H, SUN W. and KIM H. (2007). Spatiotemporal expression pattern of non-clustered protocadherin family members in the developing rat brain. *Neurosci* 147: 996-1021.

KRAUSS S, JOHANSEN T, KORZH V, and FJOSE A. (1991). Expression of the zebrfish paired box gene pax[zf-b] during early neurogenesis. *Development* 113: 1193-1206.

KUBOTA F, MURAKAMI T, TAJIKA Y, and YORIFUJI H. (2008). Expression of protocadherin-18 in the CNS and pharyngeal arches of zebrafish embryos. *Int J Dev Biol* 52: 397-405.

LIU Q, SANBORN K.L, COBB N, RAYMOND P.A, and MARRS J.A. (1999). R-cadherin expression in the developing and adult zebrafish visual system. *J Comp Neurol* 410: 303-319.

LIU B, DUFF R.J, LONDRAVILLE R.L, MARRS J.A, and LIU Q. (2007). Cloning and expression analysis of cadherin7 in the central nervous system of the embryonic zebrafish. *Gene Expr Patterns* 7: 15-22.

NOONAN J.P, GRIMWOOD J, SCHMUTZ J, DICKSON M, and MYERS R.M. (2004). Gene conversion and the evolution of protocadherin gene cluster diversity. *Genome Res* 14: 354-366.

NOLLET F, KOOLS P, and VAN ROY F. (2000). Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol* 299: 551-572.

OXTOBY L, and JOWETT T. (1993). Cloning of the zebrafish *krox-20* (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res* 21: 1087-1095.

REDIES C, VANHALST K, and VAN ROY F. (2005). δ–Protocadherins: unique structures and functions. *Cell Mol Life Sci* 62: 2840-2852.

SUZUKI S.T. (1996). Protocadherins and diversity of the cadherin superfamily. *J Cell Sci* 109: 2609-2611.

VAN ROY F, and BERX G (2008). The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci* 65: 3756-3788.

VANHALST K, KOOLS P, STATES K, VAN ROY F. and REDIES C. (2005). δ-Protocadherins: a gene family expressed differentially in the mouse brain. *Cell Mol Life Sci* 62: 1247-1259.

WESTERFIELD M. (2005). *The zebrafish Book*. Eugene, OR: University of Oregon Press.

WOLVERTON T, and LALANDE M. (2001). Identification and characterization of three members of a novel subclass of protocadherins. *Genomics* 76: 66-72.

WU Q. (2005). Comparative genomics and diversifying selection of the clustered vertebrate genes. *Genetics* 169: 2179-2188.

YAGI T. and TAKEICHI M. (2000). Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev* 14: 1169-1180.