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Expression of *protocadherin-9* and *protocadherin-17* in the nervous system of the embryonic zebrafish

Qin Liu^{*,1}, Yun Chen¹, Jean J. Pan¹, and Tohru Murakami²

¹Department of Biology, University of Akron, Akron, Ohio 44325

²Department of Anatomy, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Abstract

In this study we analyzed expression patterns of two δ -protocadherins, protocadherin-9 and protocadherin-17, in the developing zebrafish using in situ hybridization and RT-PCR methods. Both protocadherins were mainly detected in the embryonic central nervous system, but each showed a distinct expression pattern. Protocadherin-9 message (*Pcdh9*) was expressed after 10 hours post fertilization (hpf). It was found mainly in small clusters of cells in the anteroventral forebrain and ventrolateral hindbrain, and scattered cells throughout the spinal cord of young embryos (24 hpf). *Pcdh9* expression in the hindbrain was segmental, reflecting a neuromeric organization, which became more evident at 34 hpf. As development proceeded, *Pcdh9* expression increased throughout the brain, while its expression in the spinal cord was greatly reduced. *Pcdh9* was also found in the developing retina and statoacoustic ganglion. Protocadherin-17 message (*Pcdh17*) expression began much earlier (1.5–2 hpf) than *Pcdh9*. Similar to *Pcdh9* expression, *Pcdh17* expression was found mainly in the anteroventral forebrain at 24 hpf, but its expression in the hindbrain and spinal cord, confined mainly to lateroventral regions of the hindbrain and anterior spinal cord, was more restricted than *Pcdh9*. As development proceeded, *Pcdh17* expression was increased both in the brain and spinal cord: detected throughout the brain of two- and three-day old embryos, strongly expressed in the retina and in lateral regions of spinal cord in two-day old embryos. Its expression in the retina and spinal cord was reduced in three-day old embryos. Our results showed that expression of these two *protocadherins* was both spatially and temporally regulated.

Keywords

development; cell adhesion molecules; brain; retina; spinal cord

1. Results and Discussion

Cadherins are Ca^{++} -dependent cell adhesion molecules involved in tissue and organ development, function and maintenance of adult structures (Yagi and Takeichi, 2000; Gumbiner, 2005; van Roy and Berx, 2008). Cadherin superfamily constitutes more than 100 members that are grouped into several subfamilies, including classical cadherins,

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*To whom correspondence should be addressed. Phone: 330-972-7558; Fax: 330-972-8445; qliu@uakron.edu.

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protocadherins, desmosomal cadherins and flamingo cadherins (Nollet et al., 2000). There are about 80 members in the protocadherins (Pcdhs) subfamily, making them the largest subgroup of cadherins. Compared to classic cadherins, cell adhesion mediated by homophilic protocadherin interactions is generally weaker, but pcdhs may be more involved in intracellular signaling (e.g. via protein phosphatase-1 α) than strong cell-cell adhesion (Frank and Kemler, 2002; Redies et al., 2005). Based mainly on gene organization, Pcdhs are divided into clustered Pcdhs represented by 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 can be further divided into δ 1-Pcdhs (e.g. Pcdh1, 7 and 9) and δ 2-Pcdhs (e.g. Pcdh10, 17 and 18) (Redies et al., 2005; Vanhalst et al., 2005), based on the number of homologous repeats in their extracellular domains (δ 1-Pcdhs have 7, while δ 2-Pcdhs have 6) and the number of conserved motifs in their cytoplasmic domains (δ 1-Pcdhs have 3, while δ 2-Pcdhs have 2). Pcdhs expression patterns have mainly been examined in mammals, and there is little information on Pcdhs expression in nonmammals.

1.1 Zebrafish Pcdh9 and Pcdh17

Zebrafish *Pcdh9* and *Pcdh17* cDNA sequences were recently published in GenBank (accession numbers [XM_001343475](#), [XM_684743](#), respectively). To obtain cDNA fragments serving as templates for synthesizing in situ hybridization *Pcdh9* and *Pcdh17* cRNA probes, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from 22–50 hpf zebrafish embryos and zebrafish *Pcdh9* or *Pcdh17* specific primers (see Section 2).

Alignments of several Pcdhs sequences (Fig. 1 and Fig 2) show that at the amino acid level the zebrafish Pcdh9 and Pcdh17 are most similar to human and mouse Pcdh9 and Pcdh17, respectively. Each Pcdh is also less similar to other zebrafish members of their respective subgroup (e.g. zebrafish Pcdh9 vs. Pcdh1, both are δ 1-Pcdhs), and even less similar to members of the other subgroup (e.g. Pcdh9 vs. Pcdh18). In both human and mouse, alternative splicing was found in several δ -Pcdhs including PCDH9/Pcdh9 (Vanhalst et al., 2005). The zebrafish Pcdh9 is most similar to the short transcript of the human and mouse PCDH9/Pcdh9, lacking any of the conserved motifs in the cytoplasmic domain that are present in standard transcripts for PCDH9/Pcdh9 and most other δ -Pcdhs (Vanhalst et al., 2005). It is unknown whether there are alternative zebrafish Pcdh9 transcripts. The zebrafish Pcdh17 is similar to Pcdh17 in other species with two conserved motifs in the cytoplasmic domain, and alternative transcripts have not been reported for zebrafish and other species.

1.2. Pcdh9 Expression

Using RT-PCR and whole mount in situ hybridization methods, we analyzed expression of *Pcdh9* in embryonic zebrafish from 1.5–2 hours post fertilization (hpf) to 72 hpf. RT-PCR experiments revealed that *Pcdh9* transcripts were absent from young embryos (1.5–10 hpf), but strongly expressed in older embryos (18–50 hpf, Fig. 3). By 24 hpf when major brain regions become readily distinguishable (Kimmel, 1993), whole mount in situ hybridization experiments showed that *Pcdh9* expression was detected in the forebrain, hindbrain and spinal cord (Fig. 4). In the telencephalon, *Pcdh9* was observed in the anteroventral region. There were three clusters of cells in the anteroventral diencephalon posterior to the optic recess (Fig. 4A and B). These *Pcdh9* expressing clusters were located in lateral regions of the anteroventral diencephalon (Fig. 4I and J). In the epithalamus, there were two small clusters of cells, adjacent to the pineal gland, expressing *Pcdh9* (Fig. 4B). In the hindbrain, six small clusters of *Pcdh9* expressing cells were found in the lateroventral regions (Fig. 4C). These clusters appeared to be arranged in a segmental pattern. The first cluster was located ventral to the cerebellum, while the fourth cluster was located ventral to the statoacoustic ganglion that also contained *Pcdh9* (Fig. 4C and K). In the spinal cord, cells expressing *Pcdh9* were found scattered throughout the structure (Fig. 4L). By 34 hpf there are more differentiating neurons in the CNS

and the major axonal tracts become more prominent. *Pcdh9* expression in 34 hpf embryos also increased compared to 24 hpf embryos (Fig. 5). In the telencephalon, *Pcdh9* expressing cells were found mainly in lateral regions adjacent to the olfactory placode (Fig. 5B and I). In the diencephalon, *Pcdh9* expression was observed in the dorsal and ventral thalamus, in the anterior hypothalamus posterior to the optic recess (Fig. 5B). *Pcdh9* expression was also detected in the lateral tegmentum (Fig. 5B and J) and an area immediately anteroventral to the optic tectum (pretectal region, indicated by an asterisk in Fig. 5B). The segmental expression of *Pcdh9* in the hindbrain became more evident (Fig. 5C and D). Except for the first cluster, which was faintly labeled, the other five *Pcdh9* expressing clusters became larger, especially the fourth cluster above the statoacoustic ganglion, than in the younger embryos. This segmental expression pattern is similar to cadherin-4 expression in the embryonic hindbrain (Liu et al., 2001), reflecting a neuromeric organization of the zebrafish hindbrain (Kimmel, 1993). *Pcdh9* expression in the posterior hindbrain and spinal cord was not segmental, confined mainly to cells in lateral regions (Fig. 5L). The statoacoustic ganglion continued expressing *Pcdh9* (Fig. 5C and D).

Pcdh9 expression in the brain became further increased in 2 and 3 day-old embryos (Fig. 6). In the forebrain, *Pcdh9* continued to be expressed in the telencephalon, dorsal and ventral thalamus, and anterior hypothalamus (Fig. 6B and J). In the midbrain, in addition to the tegmentum, the optic tectum also expressed *Pcdh9* (Fig. 6B, C, J and K). *Pcdh9* expression in the pretectal area (asterisk in Fig. 6B and J) became stronger than at 34 hpf (Fig. 5B). The region between the posterior border of the optic tectum and the boundaries of mid- and hindbrains (indicated by apposing arrows in Fig. 6B, C, J and K) had little or no *Pcdh9* expression compared to its neighboring areas. The reduced *Pcdh9* expression in this region was similar to *protocadherin-19* expression in embryonic zebrafish (Liu et al., 2009). In the hindbrain, *Pcdh9* was expressed widely, and the segmental expression pattern seen in younger embryos (24 and 34 hpf) was no longer observed. The cerebellum was also *Pcdh9* positive (Fig. 6B, C, J and K). *Pcdh9* expression in the spinal cord had become much reduced (Fig. 6A and I). *Pcdh9* was not detected in the retina of younger embryos (Fig. 5K). In 50 hpf embryos, *Pcdh9* expressing cells were seen in the retinal ganglion cell layer in the ventroanterior region of the retina (Fig. 6D) where differentiation of retinal cells is initiated (Hu and Easter, 1999). By 72 hpf, *Pcdh9* expression had expanded to the entire retinal ganglion cell layer (Fig. 6L). The statoacoustic ganglion continued expressing *Pcdh9* (Fig. 6B, C, G, and J).

1.3. *Pcdh17* expression

Unlike *Pcdh9* expression, *Pcdh17* transcripts were detected in all stages examined, including 1.5–2 hpf embryos (Fig. 3). *Pcdh17* expression increased as development proceeded (Fig. 3). Whole mount in situ hybridization experiments revealed that at 24 hpf, *Pcdh17* expression was observed in the forebrain, hindbrain and spinal cord (Fig. 4E–G, M–P). In the forebrain, *Pcdh17* expressing cells were mainly located in the ventral telencephalon and ventrolateral diencephalon (Fig. 4F and M), which was similar to *Pcdh9* expression pattern at this stage, except that *Pcdh17* expressing cells in the ventrolateral diencephalon (Fig. 4F and M) were not organized into compact clusters as the *Pcdh9* expressing cells in this region (Fig. 4B, I and J). In the hindbrain, *Pcdh17* expressing cells were found in the lateroventral hindbrain (Fig. 4G and O). The same expression pattern was also seen in the anterior spinal cord (Fig. 4N–P), but *Pcdh17* became undetectable in the mid-trunk and tail regions of the spinal cord (Fig. 4P). *Pcdh17* was also expressed in the ventroanterior region of the developing retina (Fig. 4H) where the earliest differentiating retinal neurons are found a few hours later (28 hpf, Hu and Easter, 1999).

Pcdh17 expression was greatly increased in the brain of 34 hpf embryos (Fig. 5). *Pcdh17* expressing cells were found throughout the fore- and midbrains (Fig. 5E and F). In the

forebrain, higher expression levels (judging from staining intensity) were observed in the lateroventral telencephalon (Fig. 5M), lateral regions of the dorsal and ventral thalamus, and anterior hypothalamus immediately posterior to the optic recess (Fig. 5F and N). In the midbrain, *Pcdh17* expression was detected in both the optic tectum and tegmentum (Fig. 5F). Like the younger embryos, *Pcdh17* expression continued in the lateroventral regions of the hindbrain and spinal cord (Fig. 5G, H and P). Unlike the younger embryos, *Pcdh17* expressing cells were also detected in more medial regions of the ventral hindbrain, forming two columns of cells lateral to the midline of the hindbrain (Fig. 5H). *Pcdh17* was also expressed throughout the developing retina (Fig. 5O) and statoacoustic ganglion (Fig. 5H). Compared to *Pcdh9* expression in the embryonic brain at this stage, *Pcdh17* expression was less restricted (e.g. *Pcdh9* expression was not detected in the optic tectum and retina, Fig. 5B and K).

Pcdh17 continued to be expressed widely in the brain of older embryos (50 and 72 hpf, Fig. 6), and its expression levels, judging from staining intensity, were increased in the dorsal and ventral thalamus, anterior hypothalamus and tegmentum (Fig. 6F). *Pcdh17* was expressed throughout the hindbrain, with reduced expression along the midline, except in an area between the otic vesicle (Fig. 6F, G, N and O). *Pcdh17* expression in the spinal cord was greatly increased, although mainly confined to the lateral regions (Fig. 6E and insert). *Pcdh17* continued to be widely expressed by the retina, with much reduced expression in the newly developed outer nuclear layer where differentiating photoreceptors are located (Fig. 6H). Generally speaking, *Pcdh17* expression was similar between 50 hpf and 72 hpf embryos (Fig. 6F, G, N and O), except that in the older embryos *Pcdh17* expression was reduced in the spinal cord (Fig. 6E and M), and its expression in the retina became mainly restricted to the outer portion of the inner nuclear layer where horizontal cells are located (Fig. 6P).

In 24 hpf embryos, both *Pcdh9* and *Pcdh17* expressing cells in the telencephalon and diencephalon appeared to be located within regions (e.g. dorsorostral cluster and ventrorostral cluster in the telencephalon and diencephalon, respectively) where the earliest differentiating neurons (called primary neurons by Kimmel, 1993) are located (Ross et al., 1992). *Pcdh9* expressing cells in the hindbrain at these stages seemed to be a subset of the primary neurons in the hindbrain (Ross et al., 1992; Kimmel, 1993) because both types of cells exhibit neuromeric organization, and *Pcdh9* expressing clusters were smaller than the primary neuron clusters. As development proceeded, there were more differentiating neurons throughout the brain, with differentiating neuronal clusters become bigger and by 48 hpf, almost the entire brain was labeled with neuronal differentiating markers (e.g. HNK-1, Ross et al., 1992). Concomitantly, expression of both *Pcdh9* and *Pcdh17* increased as development proceeded, and their expression domains expanded to almost the entire brain in 2–3 day-old embryos. Moreover, regions with reduced *Pcdh9* expression (e.g. between the posterior border of the optic tectum and the boundaries of mid- and hindbrains), and reduced *Pcdh9* and *Pcdh17* expression (e.g. along the midline of both the brain and spinal cord) coincide with regions with high levels of cell proliferation (Wullmann and Knipp, 2000). Taken together, it appears that both *Pcdh9* and *Pcdh17* are expressed by differentiating and/or postmitotic neurons.

Although both *Pcdhs* were widely distributed in the brain of 2–3 day old embryos, their expression patterns continued to be distinct. *Pcdh9* expression in the optic tectum was stronger than *Pcdh17*. Reduced *Pcdh9* expression in the region between the posterior border of the optic tectum and the boundaries of mid- and hindbrains of 50 hpf embryos was more prominent compared to *Pcdh17*. *Pcdh9* expression in the retina was confined to the retinal ganglion cell layer (Fig. 6D and L), whereas *Pcdh17* expression was widely spread (Fig. 6H).

Expression of *Pcdh9* was analyzed in humans (Strehl et al., 1998), mouse (Vanhalst et al., 2005), and rat (Kim et al., 2007). Northern blot analysis in human tissues revealed that *PCDH9* was mainly expressed by the fetal brain tissue, and strongly by tissues isolated from

various regions of the adult brain including the cerebral cortex, occipital cortex and medulla. Other brain regions (e.g. thalamus and spinal cord) expressed low to moderate levels of *PCDH9* (Strehl et al., 1998). In embryonic mouse, *Pcdh9* was strongly expressed by the pretectal area, occipital cortex, anteroventral hypothalamus, and presumptive retinal ganglion cell layer (Vanhalst et al., 2005). A similar *Pcdh9* expression pattern was found in the developing rat brain (Kim et al., 2007). *Pcdh9* expression in the developing zebrafish is similar to these species in that it is mainly expressed in the nervous tissue: it is observed in similar regions of the brain such as the telecephalon (cerebrum), pretectal area, anteroventral hypothalamus, optic tectum (occipital cortex), and hindbrain (medulla). A segmental hindbrain expression of *Pcdh9* was not described in the other species, perhaps partially due to limited stages examined, and tissue sections, instead of whole mount embryos, were analyzed.

Much less is known about *Pcdh17* expression in the vertebrate central nervous system (Redies et al., 2005; Kim et al., 2007). In developing rat brain (postnatal day 3), *Pcdh17* was detected in the cerebral cortex, in the ventral thalamus, throughout the hypothalamus, and in the cerebellum (Kim et al., 2007). This expression pattern is somewhat similar to *Pcdh17* expression in the developing zebrafish brain. Moreover, in both the rat and zebrafish, there appears to be an anterior-posterior gradient in *Pcdh17* expression in the brain, with anterior brain regions (e.g. anteroventral forebrain) contained more *Pcdh17* than posterior regions (e.g. hindbrain, see Fig. 4F, Fig 5F and G).

2. Experimental procedures

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%) treated fish tank water at 28.5°C, anesthetized in 0.02% MS-222 and fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline.

To obtain a zebrafish *Pcdh9* cDNA fragment as a template for synthesizing in situ hybridization cRNA probes, RT-PCR was performed using zebrafish *Pcdh9* specific primers (forward primer 1, 5'-GCGAGTGCTCCTTTGAAATG-3'; reverse primer 1, 5'-CGGATTGTCGTTCTCATCCT-3') and total RNA from zebrafish 20–50 hpf embryos. The resulting DNA fragment, corresponding to nucleotides 317–1396 of the published zebrafish *Pcdh9* sequence (GenBank accession number: [XM_001343475](#)), was cloned into the pCRII-TOPO vector (Invitrogen), and was verified by restriction enzymes digestion and a PCR experiment, using a pair of zebrafish *Pcdh9* specific primers that were internal to the last set of primers (forward primer 2, 5'-CCGCTTTGCTATTCCTTCTG-3'; reverse primer 2, 5'-TCACAGTACCGTTGGTTGGA-3'). For obtaining a *Pcdh17* DNA fragment (corresponding to the nucleotides 678–1525 of the zebrafish *Pcdh17*, GenBank accession number: [XM_684743](#)) as a template for synthesizing in situ hybridization cRNA probes, zebrafish *Pcdh17* specific primers (forward primer 3, 5'-CTGTGTTTGAACAGCCCTCA-3'; reverse primer 3, 5'-TTGCACCATCAGTGGGTTTA-3'), and total RNA isolated from 45–72 hpf 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 *Pcdh17* specific primers that were internal to the last set of primers (forward primer 4, 5'-GAAGGGATTAATGGGCAGGT-3'; reverse primer 4, 5'-CCAGAACAGAACCCAGGTATTC-3').

RT-PCR analysis of *Pcdh9* and *Pcdh17* temporal expression profiles was performed using *Pcdh9* primers (forward primer 2 and reverse primer 2, see above), *Pcdh17* primers (forward primer 4 and reverse primer 4, see above), and zebrafish *cdh1* specific primers (forward primer: 5'-TGTCAGAGTTGAGCGTGTCC-3'; reverse primer: 5'-TTGAAAACCCACCCTTGTC-3', GenBank Accession No. AF364811) bracketing a

similar cadherin region (EC1 to EC3). *cdh1* transcripts were used as the control for the RT-PCR experiments because *cdh1* was shown to be expressed by young zebrafish embryos (Babb et al., 2001).

Detailed procedures for digoxigenin-labeled cRNA probe synthesis and whole mount in situ hybridization were described previously (Liu et al., 1999; Kubota et al., 2008). There was no staining in zebrafish embryos from 24–50 hpf using the sense *Pcdh9* or *Pcdh17* probes (data not shown).

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Figure1a

HPCDH9 1 YTIREEEPENVPIGNIPKDLNISHINAATGTSASLVYRLVSKAGDAPLVKVSSTGEIFTTSNRIDREKLCAGASYAEENECEFFLEVVI---LPNDFFRLLIKIK
 MPcdh9 1 YTIREEEPENVPIGNIPKDLNISHINAATGTSASLVYRLVSKAGDAPLVKVSSTGEIFTTSNRIDREKLCAGASYAEENECEFFLEVVI---LPNDFFRLLIKIK
 ZPcdh9 1 YPIREEEQENVLIGNIPKDLNISHMNSATGATSNLVYRLVSKAGDTPLLRVMSNTGEIFTTSNRIDREKLCPGPSFED-SECSFELEVVI---LPNDYFRLLIKIK
 ZPcdh1 1 YQVTEEQPPNLTIGSLASDQGLPDT-----GHLYKLEVGA---PYLRVDGKTGDIYTTENSIDRESLKDCRNLFGDPCELEFEVSIITDLMKGTGPRLLIEGR

HPCDH9 103 IIVKDTNDNAPMFSPVINISIPENTLINSRFPISATDPDTGFNGVQHYELLNG---QSVFGLDIVETPEGEKWPQLIVQQNLDRQKDTYVMKIKVEDGGTPO
 MPcdh9 103 IIVKDTNDNAPMFSPVINISIPENTLINSRFPISATDPDTGFNGVQHYELLNG---QSVFGLDIVETPEGEKWPQLIVQQNLDRQKDTYVMKIKVEDGGTPO
 ZPcdh9 102 IIVKDTNDNAPMFSPVINISIPENTLINSRFAIPSATDPDTGPNVHKYELLNG---QSAFGLDIVETPEGEKWPQLIVQQNLDRQKDTYVMKIKVEDGGNPO
 ZPcdh1 95 IEILDENDNTPOESSPVLTLSIPENTHVGALEAIPVATDRDSGSGNIADYALTTPCATNLFSIQVAQDSD-EKLPQLIVLGNLDRELDRSDLDLNIKVVDDGSGEP

HPCDH9 205 KSSTAILQVTVSDVNDNRVPFKEGQVEVHIPENAPVGTSVIQLHATDADIGSNAEIRYIFGAQVAPATKRLFALNNTTGLITVQRSLDREETAIHKVTVLASDG-
 MPcdh9 205 KSSTAILQVTVSDVNDNRVPFKEGQIEVHIPENAPVGTSVIQLHATDADIGSNAEIRYIFGAQVAPATKRLFALNNTTGLITVQRSLDREETAIHKVTVLASDG-
 ZPcdh9 204 KSSTAILQVTVSDVNDNRVPFKEGQIEVHIPENAPVGTSVIQLHATDADIGSNAEIRYIFGAQVAPATKRLFALNNTTGLITVQRSLDREETAIHKVTVLASDG-
 ZPcdh1 200 RQSSALIRITITDANDNVKFEERSHYEGELPENSVPVGHSLQVQKANDSDMGPNGEVYSLHQPSSEVMR-LLSIDRNTGIIIFVKGSVDREEISILKFFVQAKDNG

HPCDH9 309 -SSTPARATVTINVTDVNDNPPNIDLR--YIISPINGTVYLSEKDPVNTKIALITVSDKDDTVNGKVICFIEREVPPHKLKAVYDN-----CYLLETSSLLDYEG
 MPcdh9 309 -SSTPARATVTINVTDVNDNPPNIDLR--YIISPINGTVYLSEKDPVNTKIALITVSDKDDTVNGKVICFIEREVPPHKLKAVYDN-----CYLLETSSLLDYEG
 ZPcdh9 308 -SSSPARATITINVTDVNDNAPNIDLR--YIISPNGTVMLSEKDPINTKIALITVSDKDDTVNGKVICFIERKDVPPHKLKAVYDN-----CYLLETSSALLDYEG
 ZPcdh1 304 PQSSKSKALVTITVKDQNDNAPSIRIRGIGLVTHEDGVANISEMDPVGTAVALVEVSDRDEGENAVVTCVVAGDVPPQLRSASETGNDKRKRYFLQTTTLLDYER

HPCDH9 405 TKEFSFKIVASDSGKPSLNQTALVRVKLEDENDNPPIFNQPVIELSVSENNRRGLYLTTSATDEDSGKNADIVYQLG---PNASFDDLDRKTGVLTAASRVFDRE
 MPcdh9 405 TKEFSFKIVASDSGKPSLNQTALVRVKLEDENDNPPIFNQPVIELSVSENNRRGLYLTTSATDEDSGKNADIVYQLG---PNASFDDLDRKTGVLTAASRVFNRE
 ZPcdh9 404 TKEYIFKIVASDSGKPSLNQTALVRVKLEDENDNPPIFTQPVIELAVMENNLRDMFLTTISATDEDSGRNAEIVYQLG---PNASFDDLDRKTGVLTAASRVFDRE
 ZPcdh1 409 IKEYRIEIVAVDSGNPALSSTNSLKVQVTDMDNNAFVESPTLYEVEFFEEENQPDCKVLVDVATDADSGTNAELTYSITGGSVPEGLFEINPNTGEVVRVMSQLDRE

HPCDH9 507 EQERFIFTVTARDNGTPPLQSQAIVITVLDENDNSPKFTHNHQFFVSENLPKYSTVGVIITVTDADAGENKAVTSLILNDNENFVLDPSYSGVIKSNVSFDREQQ
 MPcdh9 507 EQERFIFTVTARDNGTPPLQSQAIVITVLDENDNSPKFTHNHQFFVSENLPKYSTVGVIITVTDADAGENKAVTSLILNDNENFVLDPSYSGVIKSNVSFDREQQ
 ZPcdh9 506 EQERFLFTVTARDNGTRALQSQAIVITVLDENDNSPKFTHNHQFFVSENLPKYSTVGVIITVTDADAGENAVALSLILNDNENFVLDPSYSGVIKSNVSFDREQQ
 ZPcdh1 514 QLDHYQELVAAAKGVESLRGTATVVVKVLDNRNDNPKFEMNGYSFVSQENMPLSPVGMVTVNDHDEGENARVQLSVEFDNGKFMIONGTYTILSTISFDREKE

HPCDH9 612 SSYTFDVKATDGGQPPRSSSTAKVTINVMVDNDNSPVVISPPSNTSFKLVPLSAIPGSVVAEVFAVDVDTGMNAELKYTIVSGNNKGLFRIDPVTGNITLEEKPAP
 MPcdh9 612 SSYTFDVKATDGGQPPRSSSTAKVTINVMVDNDNSPVVISPPSNTSFKLVPLSAIPGSVVAEVFAVDIDTGMNAELKYTIVSGNNKGLFRIDPVTGNITLEEKPAP
 ZPcdh9 611 SSYTFDVRADNGSPPCSSAAKVITINVIDVNDNTPIVIYPPSNTSFKLVPLSAIPGSVVAEVFAVDGDTGMNAELKYTIVSGNVRSLFRIIDPVTGNITLEEKPTI
 ZPcdh1 619 STYSFRLKAVDGGDPPRSSYVGITINVLDENDNAPYVTKP-ANSSYKFLDPRTSBETHVESVEAEDIDIGLNSLDFNIVGGNPHGLFRI-SEGEITLAKETFTPS

HPCDH9 726 TDVGLHRLVNVNISDLGYPKSLHTLVLVFLYVNDTAGNASYIYDLIRRTMETPLDRNIGDSSQPYQNEDEY--
 MPcdh9 726 TDVGLHRLVNVNISDLGYPKALHTLVLVFLYVNDTAGNTSYIYDLIRRTMETPLDRNIGDSSQPYQNEDEY--
 ZPcdh9 725 ADIGLHRLVNVNISDLGYPKSLHTLVLVFLVNDTVGNSSYIHDLIRRTMETPLDRNIGESSETYQNVND--
 ZPcdh1 721 KHIGLHRLVVKVKDKGTP-PRQTTALVHIYVNETIGNVSHVEGLVGHSLYTPLEMDTAGDPDYSRDPHSNI

Figure1b

HPCDH17 1 YSVPEEQGAGTVIGNIGRDARLQGLPPAERGGG-GRSKSGSYRVLENSAPHLLDVADSGLLYTKQRIDRESLCRHNACQLSLEVFANDKE---ICMIKVEI
 MPcdh17 1 YSVPEEQGAGTVIGNIGKDARLQGLPPAERGGSGSGRSKSGSYRVLENSAPHLLDVADSGLLYTKQRIDRESLCRHNACQLSLEVFANDKE---ICMIKVEI
 ZPcdh17 1 YSIPEEKIQG-VIGNIAKDAELEGE-----QKKSNFRVLENSAPHLLDVDPESGLLYTKQRIDRETLCRONSKQLSMEVFANDKE---ICMIKVEI
 ZPcdh18a 1 YQVYEEQKVGTVIARLREDVADVLSKLP-----SIPILRFAMQRGSASLLSVRDQGEISIRTKIDREKLCEKRNLCNCTIEFDVLTLPTEHLQLFHEVEI

HPCDH17 101 QDINDNAPSFSSDQIEMDISENAAPGTRFPLTSAHDDPDAGENGLRTYLLTRDDHGLFGLDVKSRGDGTKFPELVIQKALDREQQNHHTLVLTALDGGEPPTSAT
 MPcdh17 102 QDINDNAPSFPSDQIEMDISENAAPGTRFPLTSAHDDPDAGENGLRTYLLTRDDHGLFGLDVKSRGDGTKFPELVIQKALDRELQNHHTLVLTALDGGEPPTSAT
 ZPcdh17 91 QDINDNAPSFPSQIDIDISENAAPGTRFPLAAAYDPDTKENGKTYQITRDDYISFSLDVKSRGDGTKFPELVVQSRSLDREERSHHTLIIITATDGGEPKSGT
 ZPcdh18a 97 LDINDNAPQFARPVLPPIEISETAAGVTRIPLDSATDPDVGENSLNTYSLT--PSGFEKIDILTRTDGAKYAEVLVVKELDREVRASYELQLTASDRGVPEKFGT

HPCDH17 205 VQINVKVIDSNDNSPVFEAPSYLVPELPEAPLGTTVVIDLNATDADEGPNGEVLYSFSSYVPDRVRELFSDPKTGLIRVKGNLDYEENGMLIEDVQARDLGPNP
 MPcdh17 206 VQINVKVIDSNDNSPVFEAPSYLVPELPEAPLGTTVVIDLNATDADEGPNGEVLYSFSSYVPDRVRELFSDPKTGLIRVKGNLDYEENGMLIEDVQARDLGPNP
 ZPcdh17 195 MQINVKVIDSNDNSPVFEQPSYVVEIPENAPLGTTVVIDLNATDSDEINGQVYTSFSCYVPDRIKELFSDPRTGVTKIQGKIDFEENPIIIEDVQAKDQGNP
 ZPcdh18a 199 TLLKISIASSNDNNPVFEKPSYVINLENSPLGSLLDLNATDPDEGTNGKIIYSFSSHSVPKILETEKINSNDNGHILMRKVDFTSTNSYIDVQADMGPN

HPCDH17 309 IPAHCKVTVKLIDRNDNAPSIGFVSVRQG-----ALSEAAPPGTVIALVRVTDKDSGKNGQLQCRVLGGGTGGGGGLGGPGGSVPFKLEENYDNFYTVVTDRE
 MPcdh17 310 IPAHCKVTVKLIDRNDNAPSIGFVSVRQG-----ALSEAAPPGTVIALVRVTDKDSGKNGQLQCRVLGGGTGGG--LGGPG-SVPFKLEENYDNFYTVVTDRE
 ZPcdh17 299 IPGHCKVTVKVLDNRNDNAPSIGFVAVRQG-----AVSEAAATPGTVIALVRVTDKDSGRNGQLQCRILG-----NVPFKLEENYDNFYTVVTDRE
 ZPcdh18a 303 MPAHCKVVIKVVVDVNDKPDLSVNLMTSTNEEYAIYSETAEVDTFVALVSVNLDLSGLNGEVECRLYG-----QGHRELQKSYEKNYMILTNVT

HPCDH17 408 LDRETQDEYNVTIVARDGGSPPLNSTKSFVKILDENDNPPRFTKGLYVLQVHENNIPGEYLGSVLAQDPDLGQNGTVSYSLPSSHIGDVSITYTVSVNPTNGA
 MPcdh17 406 LDRETQDDYNVTIVARDGGSPPLNSTKSFVKILDENDNPPRFTKGLYVLQVHENNIPGEYLGSVLAQDPDLGQNGTVSYSLPSSHIGDVSITYTVSVNPTNGA
 ZPcdh17 383 LDREVKDEYNITIVAKDNGNPPLNSTKSFVKILDENDNAPRFTKMYLLQVPENNIPGEYLGSVLAHDPDLGQNGTVSYSLPSPNSVEESITTYVNIKPTDGA
 ZPcdh18a 392 LDREKRSEFSTIVIAEDKGSPLSTIKNFIVEVQDENDNAPSFASKRYEISKAENNSPGAYLSSVKA SDPDLGPNGQVSYSLPESMVHGSSISTYVVTIDPSNGD

HPCDH17 512 IYALRSFNFEQTKAFEFKVLAKDSGAPAHLESNATVRVTVLVDNDNAPVIVLPTLQNDTAEQLQVPRNAGLGYLSTVRALDSDFGESGRLYEIVDGNDDHLFE
 MPcdh17 510 IYALRSFNFEQTKAFEFKVLAKDSGAPAHLESNATVRVTVLVDNDNAPVIVLPTLQNDTAEQLQVPRNAGLGYLSTVRALDSDFGESGRLYEIVDGNDDHLFE
 ZPcdh17 487 IYALRSFNFEQTKYEDFKVQAKDSGNP-HLESNTTVRVSVLDVNDNIPVIVLPLQNDTAEIHVPRNVGVGHITTVRAVDNDFGESGRLYEISDGNNEHLFE
 ZPcdh18a 496 IYALRTFDREDVQSISELVQARDSGNP-PLRSNVTVVLTVLENDNRPVIMMQLWNHTADVVPVSKYAEIGDVTTVVRAMDHADAGANGDLSCSVVGNEAGYFN

HPCDH17 616 IDPSSGEIRTLHPFWEEDVTPVVELVVKVTDHGKPTLSAVAKLIIRSVSGSLPEGVPRVNGEQHH---WDMS
 MPcdh17 614 IDPSSGEIRTLHPFWEEDVTPVVELVVKVTDHGKPTLSAVAKLIIRSVSGSLPEGVPRVNGEQHH---WDMS
 ZPcdh17 590 IDSVTGEVRTAQPLWEEVTPVVELIIVKTDHGKPSLSAMAKLIVKAYSGPLPEGEPRINAEHHN---WDMS
 ZPcdh18a 599 MDEKTCEIRTNVSIQEVQDVELTILVQDHGTPTLSARALLRLSLYENIENLMNHHLTGGRNGDGPLDVS

Figure 1.

Figure 1a. Amino acid sequence alignment between the deduced zebrafish protocadherin-9 amino acid sequence (ZPcdh9), human protocadherin-9 (HPCDH9), mouse protocadherin-9 (MPcdh9), and another $\delta 1$ -protocadherin, zebrafish protocadherin-1 (ZPcdh1). Figure 1b. Amino acid sequence alignment between the deduced zebrafish protocadherin-17 (ZPcdh17), human protocadherin-17 (HPCDH17), mouse protocadherin-17 (MPcdh17), and another $\delta 2$ -protocadherin, zebrafish protocadherin-18a (ZPcdh18a). The alignments show sequences between published sequences from the EC1 to the end of the extracellular domain. Sequences highlighted by yellow boxes indicate residues that are common to all four sequences, and sequences highlighted by gray boxes indicate amino acids that are identical to three of the four sequences.

	ZPcdh9	HPCDH9	MPcdh9	ZPcdh1	ZPcdh17	HPCDH17	MPcdh17	ZPcdh18a
ZPcdh9		86.62%	86.37%	41.24%	31.23%	30.37%	30.28%	29.78%
HPCDH9			98.98%	40.94%	31.23%	31.09%	31.06%	29.78%
MPcdh9				40.94%	31.48%	30.97%	31.73%	29.78%
ZPcdh1					28.80%	29.72%	29.79%	29.04%
ZPcdh17						73.50%	74.16%	44.71%
HPCDH17							98.25%	44.09%
MPcdh17								44.22%
ZPcdh18a								

Figure 2.

Sequence identity percentages for pairwise comparisons between the above eight protocadherin sequences, Diagonal shaded boxes indicate sequence comparisons between the same sequences, and therefore, represent 100% identity. Sequence comparisons were performed using ClustalW2. Abbreviations are the same as in Figure 1.

Figure 3.
RT-PCR analysis of *Pcdh9* and *Pcdh17* expression in embryonic zebrafish using total RNAs.
RT-PCR for *cdh1* was performed as loading control. Numbers represent hours post fertilization.

Figure 4.

Pcdh9 and *Pcdh17* expression in 24 hpf zebrafish embryos. All panels show whole mount embryos labeled with *Pcdh9* cRNA (panels A–D, I–L) or *Pcdh17* cRNA probes (panels E–H, M–P). Panels A and E show lateral views of the anterior half of the embryos (anterior toward the lower left corner, and dorsal to the upper left corner). Panels B, C, F and G show lateral views (anterior to the left and dorsal up) of the head, with panels B and F showing the fore- and midbrains, and panels C and G showing the hindbrain region. Panels D and H show lateral views (anterior to the left and dorsal up) of whole mount eyes. Panels I, J and M show frontal views (dorsal up) of embryo heads with focusing planes at the diencephalon level. Panels K and O are dorsal views of the hindbrain region (anterior to the left). Panels L, N and P are lateral views from the mid-trunk region (anterior to the left and dorsal up). The arrowheads in panels B, F, I and M point to the more anteroventrally located *Pcdh9* or *Pcdh17* expressing cluster, while the arrows in panels B and J point to the more posterodorsally located *Pcdh9* expressing cluster in the anteroventral diencephalon. Asterisks in panels C and K indicate clusters of *Pcdh9* expressing cells in the hindbrain. Arrows in panels G, H, N, O and P point to *Pcdh9* expressing cells. Abbreviations: c, cerebellum; di, diencephalon; ey, eye; h, hindbrain; hd, head; le, lens; nc, notochord; or, optic recess; ot, optic tectum; ov, otic vesicle; p, pineal organ; rpe, retinal pigmented epithelium; sag, statoacoustic ganglion; sc, spinal cord; te, telencephalon; teg, tegmentum, y, yolk.

Figure 5.

Pcdh9 and *Pcdh17* expression in 34 hpf embryos. All panels are from whole mount embryos labeled with *Pcdh9* cRNA (panels A–D, I–L) or *Pcdh17* cRNA (panels E–L, M–P) probes. Panels A and E are lateral views (anterior to the left and dorsal up) of the anterior 2/3 of the embryos. Panels B and F are lateral views (anterior to the left and dorsal up) of the fore- and midbrains, while panels C and G show lateral views of the hindbrain (panel C shows slightly more anterior structures). Panels D, H, L and P are dorsal views (anterior to the left), with panels D, H and L showing mainly the hindbrain, while panel P showing mainly the spinal cord. Panels I and M are frontal views (dorsal up) focusing at the telencephalon level, while panels J and N are frontal views (dorsal up) focusing at the diencephalon and tegmentum levels, respectively. Panels K and O show lateral views (anterior to the left and dorsal up) of the retina. The asterisk in panels B and F indicates the pretectal area. Segmental expression of *Pcdh9* in the hindbrain is indicated by asterisks in panels C and D. Abbreviations: ht, hypothalamus; op, olfactory placode. Other abbreviations are the same as in Figure 4.

Figure 6.

Pcdh9 and *Pcdh17* expression in 50 hpf and 72 hpf embryos. Panels A–D, I–L are from whole mount embryos processed for *Pcdh9* in situ hybridization, while panels E–H, M–P are whole mount embryos labeled with *Pcdh17* cRNA probe. All lateral views have anterior to the left and dorsal up, while dorsal views have anterior to the left. Panels A, E, I and M are lateral views of the anterior half of the embryos showing overall patterns of *Pcdh9* or *Pcdh17* expression. Panels B, F, J and N are lateral views of the head region. Panels C, G, K and O show dorsal views of the mid- and hindbrains. Panels D, H, L and P are lateral views of the retina. The apposing arrows in panels B, C, J and K indicate the area between the posterior border of the optic tectum and boundary of the mid-hindbrains with reduced *Pcdh9* expression. The opposing arrowheads in panel E insert indicate *Pcdh17* expression in the spinal cord. Abbreviations: bmh, boundary of mid- and hindbrains; gcl, retinal ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer. Other abbreviations are the same as in Figure 4 and Figure 5.