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Expression of protocadherin 18 in the CNS and pharyngeal arches of zebrafish embryos

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ABSTRACT Here, we report the results of molecular cloning and expression analyses of a non-clustered protocadherin *(pcdh)*, *pcdh18* in zebrafish embryos. The predicted zebrafish pcdh18 protein shows 65–66% identity and 78–79% homology with its mammalian and *Xenopus* counter-parts. It has a Disabled-1 binding motif in its cytoplasmic domain, which is characteristic of pcdh18. Zebrafish embryos expressed *pcdh18* by the early gastrula stage, 6 h post-fertilization (hpf), in their animal cap but not in the germ ring or the shield. *pcdh18* was expressed in the neural tube and the central nervous system (CNS) from 12 hpf. Some populations of cells in the lateral neural tube and spinal cord of 12–18 hpf embryos expressed *pcdh18*, but expression in these cells disappeared by 24 hpf. The hindbrain of embryos at 24–56 hpf expressed *pcdh18* in cells closely adjacent to the rostral and caudal rhombomeric boundaries in a thread-like pattern running in the dorsoventral direction. The *pcdh18*-positive cells were localized in the ventral part of the hindbrain at 24 hpf and in the dorsal part from 36 hpf. *pcdh18* was also expressed in the telencephalon, diencephalon, tectum, upper rhombic lip, retina and otic vesicle. Expression in the CNS decreased markedly before hatching. Pharyngeal arch primordia, arches, jaws and gills expressed *pcdh18*, and the molecule was also expressed in some endodermal cells in late embryos.

KEY WORDS: *Danio rerio, central nervous system, pharyngeal arch, protocadherin, rhombomere*

Differential adhesion plays a pivotal role in morphogenesis during embryonic development (Steinberg, 1970). The cadherin super-family is thought to provide a major molecular basis for such cell adhesion (for review, Halbleib and Nelson, 2006; Takahashi *et al.*, 2005). Protocadherins (pcdh) are the largest subfamily of cadherins, which have common structural features in the extra-cellular domains, but no extensive similarities in their cytoplasmic domain unlike classic cadherins. Many of *pcdh* form gene clusters analogous to the immunoglobulin clusters. These clustered *pcdh* are more or less differentially expressed in the central nervous system (CNS), and have been suggested to be involved in systematic control of morphogenesis of CNS (Bass *et al.*, 2007, Esumi *et al.*, 2005, Hirayama and Yagi, 2006, Tada *et al.*, 2004). However, recent findings indicate allelic and conbinational gene regulation for clustered *pcdh* (Morishita and Yagi, 2007). This makes non-clustered *pchd* more attractive as a diversely controled adhesion machinary functioning for elaborate morphogenesis.

A few of non-clustered pcdh, namely *pcdh8*, *10*, and *15*, are known to play roles in particular morphogenic events (Kazmierczak *et al.*, 2007, Le Guedard *et al.*, 2007, Murakami *et al.*, 2006, Rhee *et al.*, 2003, Yamamoto *et al.*, 1998), although the biological significance of most non-clustered *pcdh* is unknown. A subclass of non-clustered *pcdh* including *pchd1, 7, 8, 9, 10, 11, 17, 18* and *19* is characterized by a shared and highly conserved cytoplasmic motif of unknown function, CM-2 (Wolverton and Lalande, 2001). In the course of our investigation of this *pcdh* subclass, we cloned zebrafish *pcdh18* and found its interesting expression pattern in developing embryos.

Cloning of pcdh18

Zebrafish *pcdh18* cDNA was amplified by PCR from a cDNA library using primers designed from a zebrafish predicted gene homologous to known *pcdh18* (Supplimentary Fig. S1). The deduced sequence of zebrafish *pcdh18* consists of 1,150 amino

Abbreviations used in this paper: CNS, central nervous system; Dab1, Disabled-1; DNA, deoxyribonucleic acid; dpf, days post-fertilization; hpf, hours post-fertilization; ISH, *in situ* hybridization; *krx-20, krox-20;* pcdh, protocadherin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTU, 1-phenyl-2-thiourea; RNA, ribonucleic acid.

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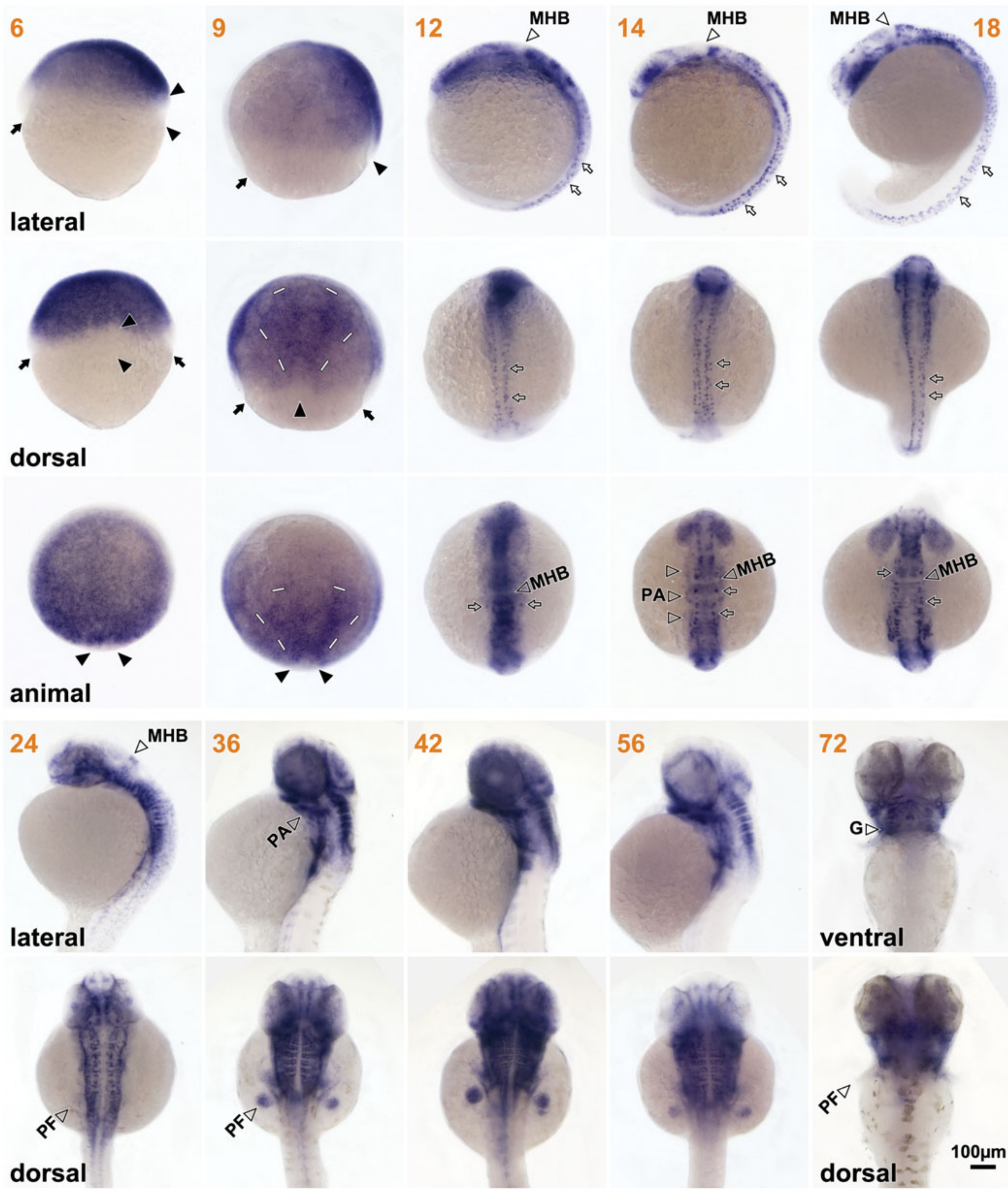

Fig. 1. Overview of *pcdh18* expression in zebrafish embryos. *Panels show extended depth of field photographs (see Experimental Procedures) of embryos stained blue by* in situ *hybridization for* pcdh18 *transcripts. Orange numbers indicate hours post-fertilization. Black arrows indicate the germ ring. Black arrowheads indicate the shield. Thin white bars show the triangular area of expression in 9 hpf embryos. Open arrows indicate punctuate expression in the head and trunk. The animal pole is aligned toward the top in lateral, dorsal, and ventral views. Dorsal is to the right in lateral views and down in animal pole views. Abbreviations: G, gills; MHB, midbrain-hindbrain boundary; PA, pharyngeal arch primordia or arches; PF, pectoral fin.*

acids, and shows conservation of characteristic features of the pcdh family, including a signal sequence, six cadherin domains, a single transmembrane domain, and a cytoplasmic domain (Supplementary Fig. S2). It shows 65–66% identity and 78–79% homology at the amino acid level with known pcdh18 and KIAA1562 proteins of human, mouse, rat, and *Xenopus*. The first five cadherin domains and the transmembrane domain show the highest degrees of identity and homology (Supplementary Fig. S3). Zebrafish pcdh18 has a Disabled-1 (Dab1) binding motif (F/YxNP, or Phe/Tyr-any-Asn-Pro) in its cytoplasmic domain, which is characteristic of pcdh18 of different species (Homayouni *et al.*, 2001) (Supplementary Fig. S2). Dab1 is an adapter protein, which mediates Reelin signals that affect cell migration and positioning in mammalian brain development. Dab1 has been shown to interact with pcdh18 *via* the binding motif. Zebrafish pcdh18 has a CM-2 motif in the cytoplasmic domain.

pcdh18 *expression in early embryos*

The transcript of *pcdh18* was first expressed diffusely in the animal cap of early gastrula embryos at 6 hours post-fertilization (hpf) (Fig. 1, 6 hpf). The expression was somewhat more intense dorsally than ventrally and was excluded from the germ ring and the shield (dorsal organizer). By the mid-gastrula stage or 9 hpf, *pcdh18* became more obviously localized to the dorsal half of the embryos. A roughly triangular pattern of expression appeared in the prospective head region (Fig. 1, 9 hpf), and the head structures continued to express *pcdh18* throughout development (Fig. 1, 9–72 hpf).

By 12 hpf, small punctuate expression appeared in the head and the trunk (Fig. 1, 12–14 hpf). The dots were roughly aligned on two or four parasagittal lines but not in precise symmetry. The punctuate expression began to decrease at 18 hpf and had mostly disappeared by 24 hpf (Fig. 1, 18–24 hpf). Transverse cryostat sections revealed that the dots represented *pcdh18*-expressing cells in the lateral neural tube and spinal cord (Fig. 2, A–C). Based on their location (Bernhardt *et al.*, 1990, Kuwada *et al.*, 1990, Myers *et al.*, 1986), these *pcdh18*-positive cells seemed to correspond to a subset of spinal interneurons, and the dorsolateral cells may be Rohon-Beard neurons. Further studies to determine their identity are currently underway in our laboratory.

Expression in the brain

In 12-hpf embryos, *pcdh18* was expressed diffusely in the rostral region (Fig. 1, 12 hpf). By 14 hpf, the expression became more defined to structures such as eye buds, forebrain, midbrain, hindbrain, and pharyngeal arch primordia (Fig. 1, 14 hpf). In 18-

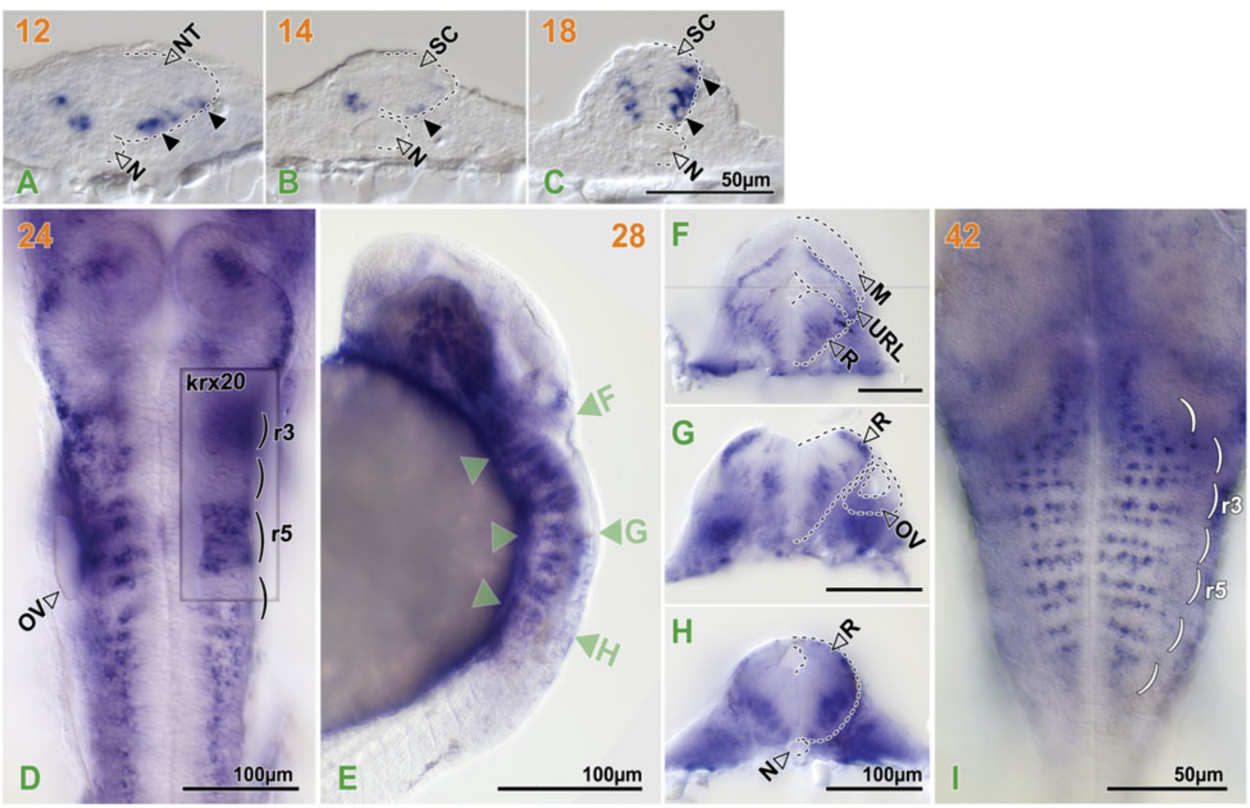

Fig. 2. *pcdh18* expression in the hindbrain and spinal cord. *Orange numbers indicate hours post-fertilization. Black dotted lines outline the structures indicated. Black arrowheads in transverse sections of the trunk of 12–18 hpf embryos* **(A-C)** *(dorsal to top) indicate* pcdh18*-expressing cells in the lateral neural tube and spinal cord.* **(D)** *Dorsal view of a 24 hpf embryo stained for* pchd18 *and* krx-20 *with single-color double* in situ *hybridization. Parentheses indicate rhombomeres. Inset shows a focal plane of an area of* krx-20 *expression which is located ventral to that of* pcdh18 *expression. Green arrowheads in the lateral view of a 28-hpf embryo* **(E)** *indicate focal planes in hand-cut sections* **(F-H)** *(dorsal to top) made from the very same embryo.* **(F)** *A composite picture of two closely adjacent focal planes.* **(I)** *A dorsal view of the hindbrain (rostral to the top) of a 42 hpf embryo with patterns of transverse dotted lines. Abbreviations: M, midbrain; N, notochord; NT, neural tube; OV, otic vesicle; R, rhombencephalon; r3 & 5, rhombomere 3 & 5; SC, spinal cord; URL, upper rhombic lip.*

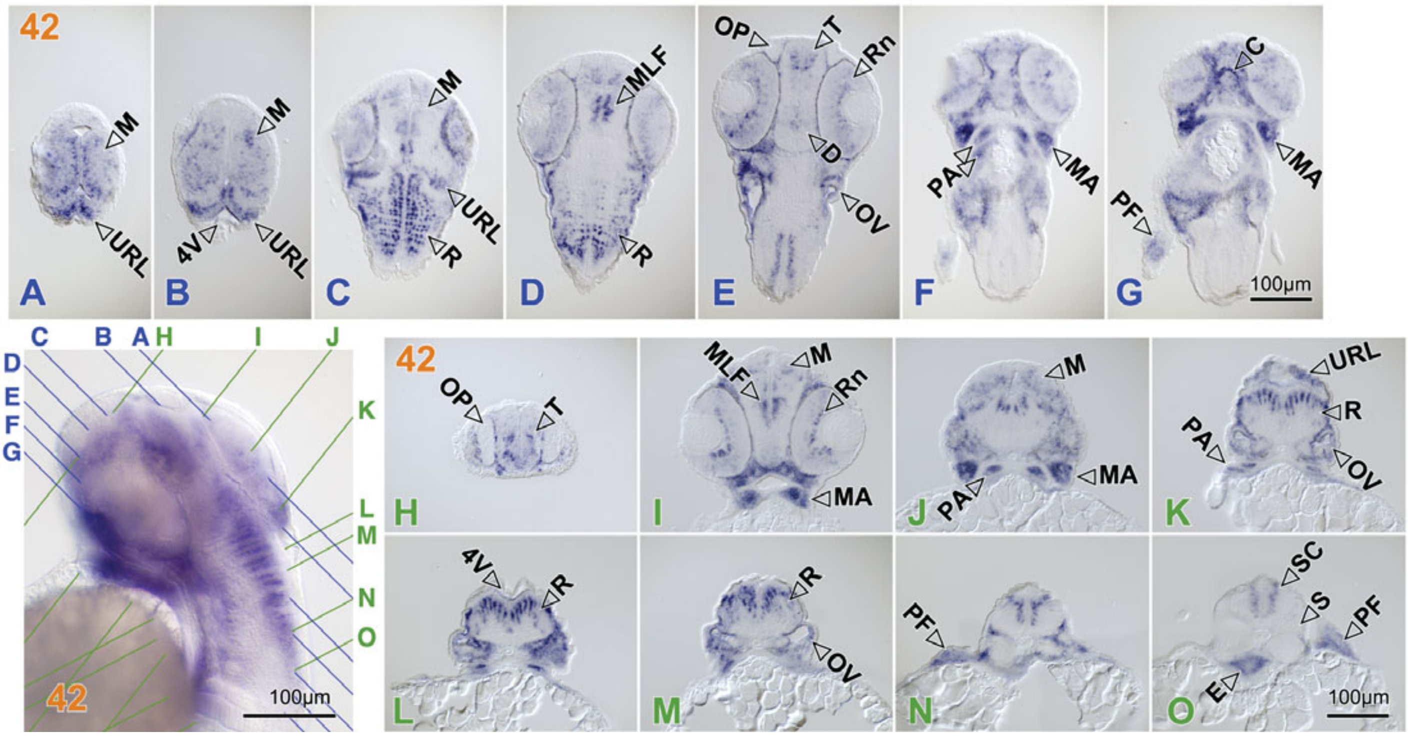

Fig. 3. Expression in the CNS and pharyngeal arches in sections. *Orange numbers indicate hours post-fertilization. Colored lines A–O in the lateral view of a 42-hpf embryo (lower left) indicate planes of serial sections* **(A-G)** *(horizontal sections; rostral to the top) and* **(H-O)** *(transverse sections; dorsal to the top). Abbreviations: 4V, forth ventricle; C, chin; D, diencephalon; E, endoderm; M, midbrain; MLF, medial longitudinal fascicle; OV, otic vesicle; OP, olfactory pit; PA, pharyngeal arches; PF, pectoral fin; R, rhombencephalon; Rn, retina; S, somite; T, telencephalon; URL, upper rhombic lip.*

hpf embryos, *pcdh18* expression continued in the eye buds, otic vesicles, forebrain, and ventral parts of the midbrain through hindbrain (Fig. 1, 18 hpf). There was a gap of expression at the midbrain-hindbrain boundary during the period from 14 to 18 hpf (Fig. 1, 14–18 hpf).

By 24 hpf, transverse lines of expression appeared in dorsal and lateral aspects of the rhombencephalon (Fig. 1, 24–56 hpf). The pattern prompted us to identify the alignment of the lines to rhombomeres in terms of the rhombomere segmentation. Double staining for *pcdh18* and *krox-20* (Oxtoby and Jowett, 1993) transcripts showed that the lines were localized closely adjacent to the rostral and caudal rhombomeric boundaries (Fig. 2, D). Examination of transverse hand-cut sections from a whole-mount stained 28-hpf embryos revealed that the expression was localized in the ventral part of rhombencephalon running in transverse planes from ventrolateral to dorsomedial (Fig. 2, E–H).

During the period from 36 to 56 hpf, *pcdh18* continued to be expressed in the rhombencephalon in transverse lines adjacent to the rhombomere boundaries (Fig. 1, 42–56 hpf; Fig. 2, I). Unlike earlier embryos, *pchd18* was expressed in the dorsal part of the rhombencephalon from 36 hpf (Fig. 1, 36–56 hpf; Fig. 3, K–N). Examination of transverse and coronary sections showed that the staining pattern was actually threads of *pcdh18*-positive cells running dorsoventrally in transverse planes (Fig. 3), similar to the radial glia (Trevarrow *et al.*, 1990). Some signaling and transcription factors such as deltaA (Delta-Notch signaling), rasgef1b (Ras GEF), and beta3.1 (bHLH), have been reported to show similar expression patterns in the hindbrain (Adolf *et al.*, 2004, Epting *et al.*, 2007, Riley *et al.*, 2004). Although functional relationships between these signals and *pchd18* remain to be elucidated, it is interesting to assume that *pcdh18* is involved in rhombomeric boundary formation driven by Delta-Notch signaling.

Analysis of sections also showed that *pcdh18* was expressed in the telencephalon, medial longitudinal fascicle of the diencephalon, tectum, and upper rhombic lip. *pcdh18* was expressed in sensory systems such as the retina and otic vesicles, but not in olfactory pits or lateral lines (Fig. 3, A–O). The expression in the retina was most intense in the innermost ganglion cell layer. The expression in the CNS decreased by 56 hpf (Fig. 1, 56 hpf). The distinct pattern in the hindbrain was almost completely abolished by 72 hpf (Fig. 1, 72 hpf).

Expression in pharyngeal arches and other structures

pcdh18 was expressed in the pharyngeal arch primordia by 14 hpf (Fig. 1, 14 hpf). It was expressed in pharyngeal, maxillary, and mandibular arches in later embryos (Fig. 3, F, G, I, J), and in the branchial and jaw arches at 72 hpf (Fig. 1, 72 hpf, and Supplimentary Fig. S4, A). Cryostat sections of 72 hpf embryos revieled that *pch18* was expressed in the cells encaplulating the branchial cartilages (Supplimentary Fig. S4, A, C). Pectral fin buds expressed *pcdh18* at 24 hpf through 56 hpf (Fig. 1, 24–56 hpf). Some endodermal cells expressed *pcdh18* in late embryos (Fig. 3, O).

Previous studies indicated the involvement of pcdh18 protein and reelin-Dab1 signaling in mammalian odontogenesis (Carroll *et al.*, 2001, Fukasawa *et al.*, 2005, Heymann *et al.*, 2001, Homayouni *et al.*, 2001). Zebrafish has pharyngeal teeth which attach to the 5th branchial arch at 4–6 days post fertilization (dpf). Since zebrafish pcdh18 share Dab1 binding site, we expected to see some increase of *pcdh18* expression in, or related to the teeth. In contrast to the expectations, we could not find tooth-related *pchd18* expression at 72 hpf nor 5 dpf (Supplementary Fig. S4).

Conclusions

We cloned a zebrafish non-clustered pcdh, *pcdh18*, and characterized its embryonic expressions in detail. *pcdh18* was expressed in the neural tube, CNS, eyes, otocysts, pharyngeal arches, and some endodermal cells. The expression in the hindbrain was particularly interesting in terms of the rhombomere segmentation. We are addressing the identities of the cells expressing *pchd18,* and the functional significances of *pcdh18* in the CNS morphogenesis.

The expression pattern of *pcdh18* in zebrafish embryos seemed to be quite different from that in mice; *pcdh18* expression is rather systemic in rodents (Homayouni *et al.*, 2001, Kim *et al.*, 2007), whereas it was limited to the CNS and head structures in zebrafish. Teleost fish including zebrafish often have diversified alleles homologous to a mammalian counterpart supposedly due to ancient whole gene duplications (Stock, 2007, Wittbrodt *et al.*, 1998). Blast searches indicate that there are two alleles (EU267178 and our AB297803) in the zebrafish genome homologous to mammalian *pcdh18*. It is interesting to suppose these *pcdh18* alleles acquired differential rolls in zebrafish development. Nonetheless, further comparative studies on the functional diversity of *pcdh18* in different species are required.

Experimental Procedures

Fish

Zebrafish (AB line) were obtained from the Zebrafish International Resource Center (Eugene, OR; http://zebrafish.org/zirc/). Fish embryos were raised at a standard temperature of 28.5°C (Westerfield, 2000), fixed in 4% paraformaldehyde - PBS at various time points, and stored in methanol at –70°C until use for expression analyses. PTU (1-phenyl-2-thiourea) at 0.003% was used for older embryos to suppress pigmentation.

Cloning of zebrafish pcdh18

Zebrafish *pcdh18* cDNA was amplified by PCR from a zebrafish embryonic cDNA Uni-ZAP XR library (discontinued; Stratagene, La Jolla, CA) using primers 5'-GGA TTT ATC ATG CTT TAA TCT ACA CCG AAC-3' and 5'-GGA TAA TTA GCA TTC ACA AAA CAC AAT ACA TCC-3', designed to flank the coding region of a zebrafish predicted protein similar to pcdh18 (accession: XM_685424). The PCR product was cloned in the *p*CR-Blunt II vector using a ZeroBlunt TOPO PCR Cloning Kit (Invitrogen Japan, Tokyo, Japan). The *pcdh18* cDNA was sequenced using an ABI Prism 3100 (Applied Biosystems Japan, Tokyo, Japan) (accession: AB297803). Pair-wise alignment and multiple alignment analyses were performed using DNA Strider 1.4 (C. Marck, CEA, Cedex, France) and ClustalW (EMBL-EBI, http://www.ebi.ac.uk/clustalw/), respectively.

In situ *hybridization*

RNA probes for *in situ* hybridization (ISH) were synthesized using a DIG RNA Labeling Kit (Roche Diagnostics K.K., Tokyo, Japan). Embryos were stained by whole-mount ISH as described previously (Bellipanni *et al.*, 2000, Kubota *et al.*, 2007, Murakami *et al.*, 2006). Single-color double ISH for *pcdh18* and *krx-20* was done for 24-hpf embryos to identify the alignment of *pchd18* staining to the rombomeres. Two-color double ISH was not successful for us, supposedly due to diminishing expression of *krx-20* at 24 hpf.

Microscopy

Stained whole embryos, embryos with the yolk removed, and hand-cut sections made from stained embryos using rasor blades were used for microscopy. A whole-mount embryo was held stationary in the pit of an agarose plate cast with glass beads. For serial cryostat sections, stained embryos were cryoprotected in 20% sucrose–PBS, immersed in O.C.T. Compound (Sakura Finetek, Tokyo, Japan), and snap-frozen in liquid nigrogen. Nikon AZ-100 and E-1000 compound microscopes were used for microscopic analyses. Photographs were taken using Olympus E-330 and E-3 digital SLR cameras in RAW format and processed with Adobe Lightroom. For extended depth of field pictures of whole-mount specimens, a series of photographs were taken with shifting focus throughout the embryo and processed with the “Extended Depth of Field” plug-in (Forster *et al.*, 2004) (http://bigwww.epfl.ch/demo/edf/) for ImageJ software (http://rsb.info.nih.gov/ij/). Adobe Photoshop was used for adjustments, including color temperature corrections to make the background a neutral gray, automatic black and white level correction, and gamma adjustment to 1.25 for the photographs of whole-mount and hand-cut sections to allow details to be seen clearly in darkly stained areas in prints.

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References

ADOLF, B., BELLIPANNI, G., HUBER, V. and BALLY-CUIF, L. (2004). atoh1.2 and beta3.1 are two new bHLH-encoding genes expressed in selective precursor cells of the zebrafish anterior hindbrain. *Gene Expr Patterns* 5: 35-41.

BASS, T., EBERT, M., HAMMERSCHMIDT, M. and FRANK, M. (2007). Differential expression of four protocadherin alpha and gamma clusters in the developing and adult zebrafish: DrPcdh2gamma but not DrPcdh1gamma is expressed in neuronal precursor cells, ependymal cells and non-neural epithelia. *Dev Genes Evol* 217: 337-51.

BELLIPANNI, G., MURAKAMI, T., DOERRE, O.G., ANDERMANN, P. and WEINBERG, E.S. (2000). Expression of Otx homeodomain proteins induces cell aggregation in developing zebrafish embryos. *Dev Biol* 223: 339-53.

BERNHARDT, R.R., CHITNIS, A.B., LINDAMER, L. and KUWADA, J.Y. (1990). Identification of spinal neurons in the embryonic and larval zebrafish. *J Comp Neurol* 302: 603-16.

CARROLL, P., GAYET, O., FEUILLET, C., KALLENBACH, S., DE BOVIS, B., DUDLEY, K. and ALONSO, S. (2001). Juxtaposition of CNR protocadherins and reelin expression in the developing spinal cord. *Mol Cell Neurosci* 17: 611-23.

EPTING, D., VORWERK, S., HAGEMAN, A. and MEYER, D. (2007). Expression of rasgef1b in zebrafish. *Gene Expr Patterns* 7: 389-95.

ESUMI, S., KAKAZU, N., TAGUCHI, Y., HIRAYAMA, T., SASAKI, A., HIRABAYASHI, T., KOIDE, T., KITSUKAWA, T., HAMADA, S. and YAGI, T. (2005). Monoallelic yet combinatorial expression of variable exons of the protocadherin-alpha gene cluster in single neurons. *Nat Genet* 37: 171-6.

FORSTER, B., VAN DE VILLE, D., BERENT, J., SAGE, D. and UNSER, M. (2004). Complex wavelets for extended depth-of-field: a new method for the fusion of multichannel microscopy images. *Microsc Res Tech* 65: 33-42.

FUKASAWA, K., SAHARA, N., MORIYAMA, K., KUNO, T., FUJII, S., OTOGOTO, J.I., OTA, N., UDAGAWA, N., YAGASAKI, H. and OZAWA, H. (2005). Identification of protocadherin 18-like protein in horse molar cementum. *Journal of the Matsumoto Dental University Society* 31: 160-166.

HALBLEIB, J.M. and NELSON, W.J. (2006). Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* 20: 3199-214.

HEYMANN, R., KALLENBACH, S., ALONSO, S., CARROLL, P. and MITSIADIS, T.A. (2001). Dynamic expression patterns of the new protocadherin families CNRs and Pcdh-gamma during mouse odontogenesis: comparison with reelin expression. *Mech Dev* 106: 181-4.

HIRAYAMA, T. and YAGI, T. (2006). The role and expression of the protocadherin-alpha clusters in the CNS. *Curr Opin Neurobiol* 16: 336-42.

HOMAYOUNI, R., RICE, D.S. and CURRAN, T. (2001). Disabled-1 interacts with a novel developmentally regulated protocadherin. *Biochem Biophys Res Commun* 289: 539-47.

KAZMIERCZAK, P., SAKAGUCHI, H., TOKITA, J., WILSON-KUBALEK, E.M., MILLIGAN, R.A., MULLER, U. and KACHAR, B. (2007). Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. *Nature* 449: 87-91.

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

KUBOTA, F., MURAKAMI, T., MOGI, K. and YORIFUJI, H. (2007). Cadherin-6 is required for zebrafish nephrogenesis during early development. *Int J Dev Biol* 51: 123-9.

KUWADA, J.Y., BERNHARDT, R.R. and NGUYEN, N. (1990). Development of spinal neurons and tracts in the zebrafish embryo. *J Comp Neurol* 302: 617-28.

LE GUEDARD, S., FAUGERE, V., MALCOLM, S., CLAUSTRES, M. and ROUX, A.F. (2007). Large genomic rearrangements within the PCDH15 gene are a significant cause of USH1F syndrome. *Mol Vis* 13: 102-7.

MORISHITA, H. and YAGI, T. (2007). Protocadherin family: diversity, structure, and function. *Curr Opin Cell Biol* 19: 584-92.

MURAKAMI, T., HIJIKATA, T., MATSUKAWA, M., ISHIKAWA, H. and YORIFUJI, H. (2006). Zebrafish protocadherin 10 is involved in paraxial mesoderm development and somitogenesis. *Dev Dyn* 235: 506-14.

MYERS, P.Z., EISEN, J.S. and WESTERFIELD, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. *J Neurosci* 6: 2278-89.

OXTOBY, E. and JOWETT, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res* 21: 1087-95.

RHEE, J., TAKAHASHI, Y., SAGA, Y., WILSON-RAWLS, J. and RAWLS, A. (2003). The protocadherin papc is involved in the organization of the epithelium along the segmental border during mouse somitogenesis. *Dev Biol* 254: 248-61.

RILEY, B.B., CHIANG, M.Y., STORCH, E.M., HECK, R., BUCKLES, G.R. and LEKVEN, A.C. (2004). Rhombomere boundaries are Wnt signaling centers that regulate metameric patterning in the zebrafish hindbrain. *Dev Dyn* 231: 278-91.

STEINBERG, M.S. (1970). Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. *J Exp Zool* 173: 395-433.

STOCK, D.W. (2007). Zebrafish dentition in comparative context. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 308B: 523-549.

TADA, M.N., SENZAKI, K., TAI, Y., MORISHITA, H., TANAKA, Y.Z., MURATA, Y., ISHII, Y., ASAKAWA, S., SHIMIZU, N., SUGINO, H. *et al.* (2004). Genomic organization and transcripts of the zebrafish Protocadherin genes. *Gene* 340: 197-211.

TAKAHASHI, Y. (2005) Common mechanisms for boundary formation in somitogenesis and brain development: shaping the 'chic' chick. *Int. J. Dev. Biol.* 49: 221-230.

TREVARROW, B., MARKS, D.L. and KIMMEL, C.B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* 4: 669-79.

WESTERFIELD, M. (2000). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. University of Oregon Press, Eugene, OR, USA.

WITTBRODT, J., MEYER, A. and SCHARTL, M. (1998). More genes in fish? *Bioessays* 20: 511-515.

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

YAMAMOTO, A., AMACHER, S.L., KIM, S.H., GEISSERT, D., KIMMEL, C.B. and DE ROBERTIS, E.M. (1998). Zebrafish paraxial protocadherin is a downstream target of spadetail involved in morphogenesis of gastrula mesoderm. *Development* 125: 3389-97.

Supplementary Material

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1261 TGACAGTGAC TCGGGTATAA ACGGTGAGGT TGTGTGCCGT CTTCATGGCC ATGGGCATTT
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1381 TGAGAAACGC TCTGAATATA GCTTGACGGT GATCGCCGAA GACCGTGGCA CGCCAAGCCT
1441 ATCCACCATC AAACACTTCA CAGTGCAGGT GCAGGATGAA AATGACAACC CTCCCCGCTT
1501 TGAAAAGAGC CGATATGAAA TCTTCAAGTC TGAGAACAAT TCCCCTGGGG CATACCTGAC
1561 GTCAGTGGTG GCCACTGACC CAGATTTGGG GCCTAATGGA CAGGTGACCT ACTCAATTGT
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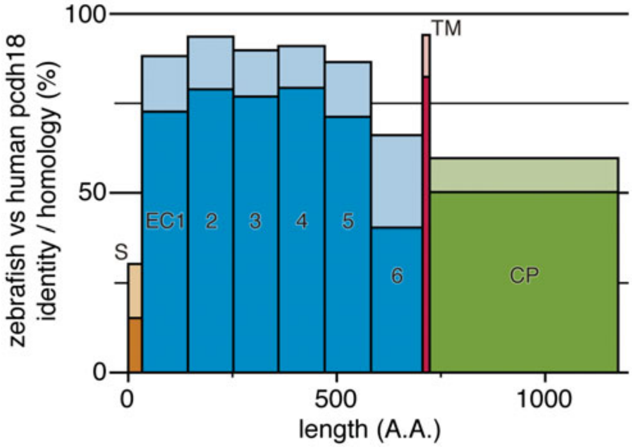
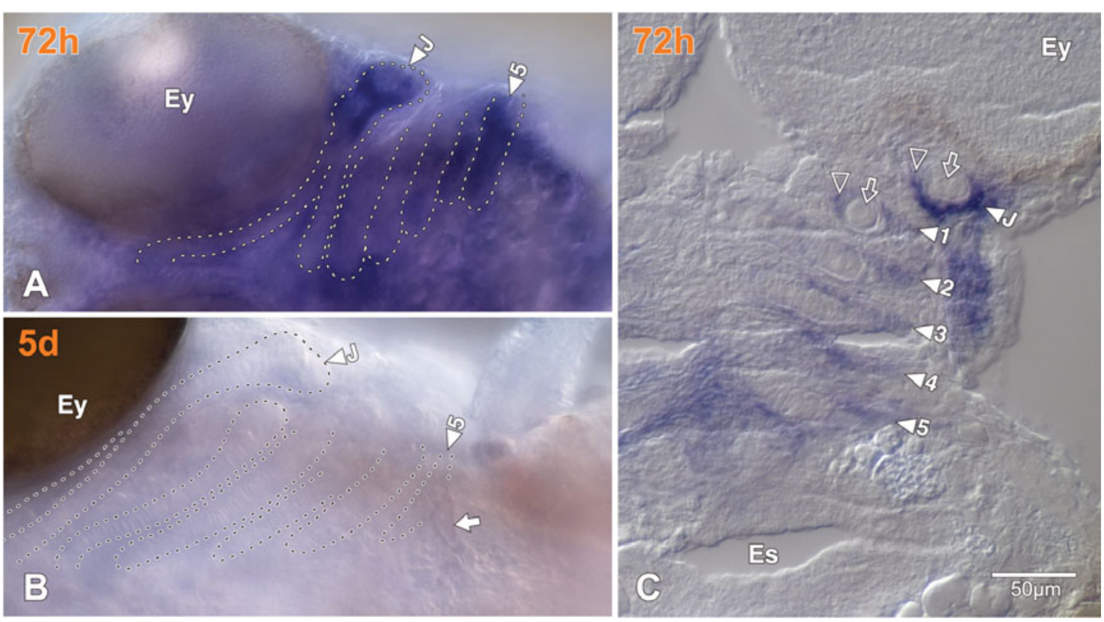
3601 gagaagaaga aaaaaaaaac acagagacct gcaattgtcg ttaaagttgc atttctggat
3661 gtattgtgtt ttgtgaatgc taattatcc
```

Supplementary Figure S1. Sequence of zebrafish *pcdh18* cDNA *(accession number: AB297803). The coding region is shown in green uppercase letters.*

Supplementary Figure S2. Multiple alignment of known pcdh18 proteins. *Multiple alignment of mouse (Accession: NP_569715), rat (EDM15003), human (NP_061908),* Xenopus *(NP_001011150) and zebrafish pcdh18 proteins was performed with ClustalW. S indicates signal sequence; EC1–6, cadherin domains; TM, transmembrane domain; CP, cytoplasmic domain; Green boxes, calcium ion binding sites. The Dab1 binding motif, F/YxNP, is marked with a red box. CM-2 motif conserved among pcdh8, 10, 18, 19 and others is marked with a blue box.*



Supplementary Figure S3. Domain structure and identity/homology of zebrafish pcdh18 protein. *Bars represent the domains of zebrafish pcdh18 protein. Bar widths indicate protein lengths in number of amino acids (A.A.) and bar heights indicate identity (darkly colored) and homology (lightly colored) of the domain with the human counterpart. Orange bar (S), signal sequence; blue (EC1-6), extracellular cadherin domains 1–6; red (TM), transmembrane domain; green (CP), cytoplasmic domain.*



Supplementary Figure S4. *pcdh18* expression in the branchial arches. *Orange numbers indicate embryonic or larval age post-fertilization.* **(A,B)** *Ventral views of embryos at 72 hpf and 5 dpf, respectively, with the yolk removed. Rostral to the left, left to the top. Dotted lines outline the jaw and branchial arches. Closed arrowheads identify the arches.* **(A)** *The 5th arch where the pharyngeal tooth would attach has diffuse but no tooth-specific* pcdh18 *staining at 72 hpf.* **(B)** *A pharyngeal tooth (closed arrow) appeared by 5 dpf when* pcdh18 *expression had diminished from the embryo.* **(C)** *A coronary cryostat section of a stained 72 hpf embryo cut at the level of the arches (the 4th arch is somewhat off the level of the particular section). The cells expressing* pcdh18 *(open arrowheads) surrounds branchial cartilages (open arrows). Abbreviations: 1-5, 1st to 5th branchial arches; Ey, eyes; Es, esophagial lumen, J, jaw arches.*

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