

# Protocadherins

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Protocadherins constitute the largest subgroup within the cadherin family of calcium-dependent cell–cell adhesion molecules. Recent progress in genome sequencing has enabled a refined phylogenetic analysis of protocadherins and led to the discovery of three large protocadherin clusters on human chromosome 5/mouse chromosome 18. Interestingly, many of the circa 70 protocadherins in mammals are highly expressed in the central nervous system. Roles in tissue morphogenesis and formation of neuronal circuits during early vertebrate development have been inferred. In the postnatal brain, protocadherins are possibly involved in the modulation of synaptic transmission and the generation of specific synaptic connections.

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*Current Opinion in Cell Biology* 2002, 14:557–562

0955-0674/02/\$ – see front matter

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*Published online 5 August 2002*

## Abbreviations

AXPC	axial protocadherin
CNR	cadherin-related neuronal receptor
Dab1	Disabled1
EC	cadherin ectodomain
NFPC	neural fold protocadherin
PAPC	paraxial protocadherin

## Introduction

The term ‘protocadherin’ was introduced about a decade ago by Shintaro Suzuki and co-workers [1]. When they used PCR with degenerate primers for extracellular repeats of classical cadherins to identify new members of the cadherin family, they found unexpected PCR fragments coding for cadherin ectodomains (EC) with features strikingly different from those of classical cadherins. These PCR fragments were then isolated from a wide range of vertebrate and invertebrate species, suggesting that these sequences represent an ancient, primordial cadherin motif. This inspired the name ‘protocadherins’ (from the Greek word ‘protos’— the first).

Full-length cloning of two protocadherin cDNAs revealed that their cytoplasmic tails were largely different from each other and from the highly conserved cytoplasmic domain of the classical cadherins. These and other protocadherins examined thereafter [2–7] showed high expression in brain — stimulating protocadherin research from then on. With the recent discovery of three large protocadherin clusters in mammalian genomes [8,9], more than 60 protocadherins are known in mouse and man, thus making them the largest subfamily of the cadherin superfamily. In this

review, we focus on the genomic and molecular features of protocadherins, with emphasis on functional implications in vertebrate development and organization of the central nervous system.

## Classification and genomic organization of protocadherins

Recent progress in genome sequencing of several species enables a refined analysis of cadherin-like molecules. In a phylogenetic tree comparing cadherin EC1 domains [10], a distinct branch comprising the clustered alpha, beta and gamma protocadherins and other protocadherins with six or seven EC domains was segregated as the protocadherin family. In contrast, the seven-pass transmembrane cadherins (Flamingo cadherins) and Fat cadherins, which form their own subfamilies, and other cadherin molecules (for example, DN-cadherin and Dachous of *Drosophila*) which remain in solitary positions, are not included [10]. An overview of the protocadherin family members, following this restrictive phylogenetic definition, and the molecular criteria outlined below is shown in Table 1. The family relationships of three recently discovered protocadherin-like molecules,  $\mu$ -protocadherin, protocadherin 15, and MT-protocadherin, are unclear [11–13]. The  $\mu$ -protocadherin has four EC repeats, as well as mucin-like domains, which may be added by alternative splicing [12]. Protocadherin 15 possesses 11 EC repeats, encoded by multiple exons. Mutations in this protocadherin cause Usher syndrome 1F in humans and similar dysfunctions in the ‘Ames waltzer’ mouse, with deafness as a main phenotype, probably resulting from malformation of sensory hair-cell stereocilia [11,14]. MT-protocadherin, exclusively expressed in the olfactory system of the mouse central nervous system (CNS), contains six EC repeats [13].

Investigations on the genomic structure of protocadherins reveal single large exons coding for multiple ectodomains as a characteristic feature of this subfamily [15]. Typically, this large exon codes for the entire extracellular portion, as well as the transmembrane domain and a short cytoplasmic part, thus giving rise to a complete protocadherin molecule. If additional exons for an extension of the cytoplasmic domain are absent, the corresponding protocadherin is a single-exon gene, as is the case with the beta subfamily of the protocadherin gene cluster [9,16]. Large exons are also found in Fat and Flamingo cadherins, but these code only for some parts of the extracellular domains [15].

Gene clustering, a known feature of many cadherins (for recent review see [17]), is similarly reported for human protocadherins at three chromosomal loci: 5q31, 13q21 and Xq21. [4,8,18,19,20,21]. At 5q31 (mouse chromosome 18c), the  $\alpha$ -,  $\beta$ - and  $\gamma$ -protocadherins are organized in three large, sequential clusters with a total of 52 protocadherin

**Table 1****Overview of protocadherin family members and some of their genomic and molecular features.**

Protocadherin name/synonym	Gene symbol	Locus (human)	Gene structure/ number of exons	Cadherin ectodomains	Splice forms/ domain features
Protocadherin 42 AXPC ( <i>XL</i> )	PCDH1	5q32–33	Single ECE Multiple CPEs	Seven	Variant 1: SEV Variant 2: contains CM2
BH-protocadherin NFPC ( <i>XL</i> )	PCDH7	4p15	Single ECE Multiple CPEs	Seven	Variants a–c, Variant b: SEV Variant c: contains CM2
Arcadlin PAPC ( <i>DR/XL</i> )	PCDH8	13q14.3- q21.1	Single ECE Two CPEs	Six	Exon 1 alternatively spliced Both isoforms contain CM2
Protocadherin 9	PCDH9	13q14.3– q21.1	Single ECE Unknown	Six	Unknown CM2
OL-protocadherin	PCDH10	4q28.3	Single ECE Four CPEs	Six	Variant 1: contains CM2 Variant 2: SEV
PCDHX	PCDH11X	Xq21.3	Large ECE (exon 5) Total of 11 exons	Seven	Variants a–d High similarity to PCDHY
VE-cadherin 2	PCDH12	5q31	Single ECE Three CPEs	Six	Unknown
Protocadherin 15	PCDH15	10q21.1	More than 20 exons	11	Unknown
Protocadherin 68	PCDH17	13q14.3	Unknown	Six	Unknown CM2
Protocadherin 68-like	PCDH18	4q31.1	Single ECE Three CPEs	Six	Unknown; CM2
Protocadherin 19 Kiaa 1313	PCDH19	Xq13.3	Large ECE (exon 1) Five additional exons	Six	Unknown; CM2
PCDHY	PCDH11Y	Yp11.2	Large ECE (exon 5) Total of nine exons	Seven	Variants a–c High similarity to PCDHX
μ-Protocadherin	MUCDHL	11p15.5	17 exons	Four	Variants 1–4; mucin domains Alternatively spliced
MT-protocadherin	Not assigned	10q22.1– q22.3	Unknown	Six	Unknown
α-Protocadherins CNRs	PCDHA@ 15 family members	5q31	Single, variable ECE Three CPEs	Six	Three variants: O (SEV), A and B Splicing in CPE (exon 3)
β-Protocadherins	PCDHB@ 15 family members	5q31	Single exon genes	Six	Unknown
γ-Protocadherins Protocadherin 2 family	PCDHG@ 22 family members	5q31	Single, variable ECE Three CPE	Six	SEV variants Alternative splicing in CPE?

Note that protocadherins typically have six or seven cadherin ectodomains and one large exon (ECE) coding for all, or at least for multiple cadherin ectodomains, respectively, termed 'single ECE' or 'large ECE'. Alternative splicing of exons coding for the cytoplasmic parts (CPE) is observed, generating isoforms with divergent cytoplasmic domains. Moreover, in some protocadherins transcript variants exist that omit the splice donor site on exon 1. Instead the transcript continues and terminates in the 'intronic' sequence. Thus, these transcript variants are derived from a single exon (SEV, single exon variant), which codes for the complete protocadherin protein including ECs, transmembrane domain and a short, unique cytoplasmic tail. Recently, a conserved motif of 17 amino acid residues (CM2) was identified in the cytoplasmic domains of several protocadherins [19]. Owing to alternative splicing, this motif may be restricted to specific transcript variants.

The exact family relationships of MT-protocadherin, protocadherin 15 and μ-protocadherin are unclear. Of note, the cadherin domains of protocadherin 15 and μ-protocadherin are encoded by multiple exons, different from the other protocadherin genes listed in the table. The gene symbols are according to the HUGO Human Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature/>). Information on the gene localization, genomic structure and transcript variants has been obtained from the original publications (see reference list) and was supplemented with recent updates from the public databases of the National Center of Biotechnology Information (NCBI), Bethesda, Maryland at <http://www.ncbi.nlm.nih.gov/> and <http://www.ncbi.nlm.nih.gov/LocusLink/>. *DR*, *Danio rerio*. MUCDHL, mucin and cadherin-like (protein); PCDH, protocadherin; *XL*, *Xenopus laevis*.

genes [8,9,16,22]. In the α- and γ-protocadherin clusters, full-length transcripts are generated by unknown mechanisms from one large 'variable' exon, encoding the individual protocadherin, and from three exons that code for the family-specific, 'constant' cytoplasmic domain. Phylogenetic analysis of the extracellular domains shows that the three families are closely related and indicates that they are likely to have arisen from multiple gene duplications [8,9,16].

Recently, a conserved cytoplasmic motif of 17 amino acid residues was reported in several protocadherins [19] (see Table 1). The motif is not present in all splice variants and its function is unknown. However, it suggests that parts of the cytoplasmic domain of protocadherins are derived from a common ancestral gene.

New insights into the question of whether protocadherins do indeed represent an ancient 'proto'-type of cadherin-like

molecules come from recent analysis of cadherin genes in the fully sequenced genomes of the *Caenorhabditis elegans* and *Drosophila melanogaster* [23••]. Among the 15 and 17 cadherin superfamily members present in the worm and fruit fly, respectively, none could be classified as a direct orthologue of vertebrate protocadherins. Instead, homologies to seven-pass transmembrane cadherins, Fat and Ret-like cadherins and the cytoplasmic domains of classical cadherins were reported. Of note, one cadherin superfamily member with two predicted cadherin ectodomains is conserved from worm to man as an orthologue, demonstrating the reliability of such an analysis. Interestingly, this protein is highly enriched in brain [24].

Taken together, it is tempting to speculate that protocadherins, especially those of the clustered type, arose rather late during evolution, possibly in chordates or early vertebrates, and diverged to great numbers in mammals by gene duplications. Indeed, a zoo-blot analysis of the  $\alpha$ -protocadherin family (cadherin-related neuronal receptors, CNRs) has revealed an increasing number of family members in mammals compared with lower vertebrates [25].

### Adhesion and associated molecules

Several reports indicate that protocadherin ECs mediate weak homophilic adhesion. The design of the adhesion experiments and their interpretation largely follows those made with classical cadherins (i.e. adhesion assays using transfected mouse fibroblast L-cells). Calcium-dependent cell adhesion was observed for  $\mu$ -protocadherin [12]. Similarly, cells transfected with protocadherins 1, 8 (Arcadlin), 10 and 12 (VE-cadherin 2) formed aggregates that typically differed in size and degree of cellular compaction from those formed with classical cadherins, indicating differences in strength of adhesiveness for protocadherins [26–29]. Protocadherin 10 was localized exclusively to homotypic cell–cell contacts in cell culture assays when transfected cells were mixed with cells expressing other protocadherins or R-cadherin. Adhesion was almost absent with the one member of the  $\beta$ -protocadherin family tested [2]. The weak cell adhesion mediated by  $\gamma$ -protocadherin C3 was strongly improved by using a chimeric molecule in which the cytoplasmic domain of E-cadherin replaced the protocadherin intracellular domain [27]. Specific cell aggregation was induced in animal cap assays after injection of axial protocadherin (AXPC) and paraxial protocadherin (PAPC) in *Xenopus* blastomeres [30••,31]. Interestingly, this adhesion was stronger when mutant forms of AXPC or PAPC lacking the cytoplasmic domains were tested, suggesting a negative regulation of adhesion by the cytoplasmic domains of these protocadherins. Similar, neural fold protocadherin (NFPC) resulted in aggregation of cell clones after earlier injection into progenitor cells [6].

So far, only three interaction partners of the cytoplasmic domains of protocadherins have been identified. First, the tyrosine kinase Fyn binds to the constant domain of the

$\alpha$ -protocadherin family (CNRs) and co-immunoprecipitates with these in extracts from brain and transfected cells [5]. Second, the longest splice variant of protocadherin 7 interacts with the alpha isoform of protein phosphatase 1 (PP1) [32]. Finally, very recently the binding of protocadherin 18 to the phosphotyrosine-binding domain of the adaptor protein Disabled 1 (Dab1) was reported [33•]. Again, these protocadherins and their interaction partners are strongly expressed in brain. PP1 has been implicated in synaptic plasticity, and Dab1 is a key molecule in the reelin signaling pathway, which is involved in the correct formation of cortical nerve cell layers.

Interestingly, reelin itself is reported to bind to the EC1 domain of the alpha protocadherins. Antibodies to this region, which contains an RGD motif, inhibited binding of reelin and neuronal aggregation *in vitro*, suggesting that  $\alpha$ -protocadherins act as receptors for reelin [25]. Evidence for the participation of  $\alpha$ -protocadherins in the reelin signaling pathway comes from co-localization studies, which show that  $\alpha$ -protocadherins and Dab1 are expressed adjacent to reelin-positive cells in motoneurons of the spinal cord and during odontogenesis [34,35]. However, a recent study could not confirm the reelin binding of  $\alpha$ -protocadherins [33•]. In conclusion, the exact function of the  $\alpha$ -protocadherins in the reelin receptor complex *in vivo* is unclear at present. It remains a particularly intriguing challenge to understand why such a great diversity of  $\alpha$ -protocadherin receptors is provided for reelin as the sole ligand.

The presence of protocadherins at cellular junctions suggests the anchoring of protocadherins to the cytoskeleton, similar to classical cadherins. For example, VE-cadherin 2 is enriched at endothelial junctions and protocadherin 8 (Arcadlin) and the  $\alpha$ -protocadherins are localized to the synaptic junction [5,28,29]. Moreover, the known participation of protocadherins in cellular movements during fish and frog gastrulation (AXPC/PAPC) or in frog epithelial adhesion (NFPC) indicates cytoskeletal linkage of protocadherins [6,30••,31,36].

### Functional implications in development

The functions of the three protocadherins, NFPC, PAPC and AXPC, have been studied mainly during embryogenesis of *Xenopus* and zebrafish [6,30••,31,36,37••]. NFPC is present in *Xenopus* from early cleavage stages on, and strong expression is found in the developing ectoderm and in the neural folds after gastrulation. Injection of recombinant NFPC promoted ectodermal cell clustering *in vivo*, while NFPC lacking the extracellular domains had a dominant-negative effect, causing large blisters in the inner ectodermal layer after injection. This effect was rescued specifically by the wild-type NFPC or C-cadherin, but not by *Xenopus* E- or N-cadherin, suggesting that NFPC mediates differential adhesiveness required for proper ectodermal differentiation [6].

PAPC, identified in a screen for genes present in the Spemann organizer of *Xenopus* embryos, is expressed during gastrulation and somitogenesis. Somitogenesis is disturbed in zebrafish and *Xenopus* embryos upon expression of secreted forms of dominant-negative PAPC [31,36,37\*\*]. In *Xenopus* presomitic mesoderm PAPC is found in the anterior part of the future somites and is regulated by the transcription factors Thylacine and Mespo, as well as by members of the Notch signaling pathway. Similarly, in zebrafish, PAPC expression is regulated by Mesp proteins and is a downstream target of the T-box gene *spadetail*. Most likely, PAPC mediated cell–cell adhesion is essential for boundary formation and the correct establishment of an anterior–posterior axis in the somites in zebrafish and *Xenopus*. Similar observations were made for AXPC, which is expressed in a pattern complementary to PAPC in the developing notochord [30\*\*,31]. A very recent *Xenopus* study demonstrates that AXPC is critically involved in sorting of pre-notochord cells, since these failed to detach from mesodermal cells in animals injected with mRNA for a secreted dominant-negative AXPC [30\*\*]. These observations suggest that adhesion is the primary function of PAPC, AXPC and NFPC in early development of zebrafish and *Xenopus* and indicate that several different protocadherins may interact in a defined region during tissue morphogenesis. The situation may be more complex in mammals where additional protocadherins or other adhesion molecules could act in a redundant manner. A possible example for this is the lack of any phenotype in mice with null alleles for protocadherin 8, the putative PAPC homologue [38]. Analogous to *Xenopus* PAPC, protocadherin 8 is transiently expressed in the mouse primitive streak, in the newly formed somites and in the paraxial mesoderm derivatives. Strong expression in the CNS, particularly in the midbrain region following embryonic day 8.5, may be related to different molecular functions (see below). However, PAPC might not be a true orthologue of mouse protocadherin 8, since both molecules share only 41% amino acid identity, much less than observed for the homologues of AXPC and NFPC.

### Functions in the central nervous system and in synapse formation

Many members of the cadherin superfamily are expressed in distinct patterns in the developing vertebrate CNS. They are involved in segregation of neuronal precursor populations, axonal outgrowth and synapse formation. These topics have been covered recently by several excellent reviews to which the reader is referred [39–43]. Briefly, the idea that cadherin-family members may provide an adhesive code for the establishment of specific brain structures is based on observations that subpopulations of functionally connected neurons are characterized by the expression of a defined set of cadherins. Moreover, cadherins are localized to the synaptic junctions and may modulate synaptic structure and transmission properties, as was shown recently for N-cadherin [44–47]. Specific molecular determination of distinct neuronal connections

was proposed more than 40 years ago by the chemoaffinity hypothesis of Roger W Sperry [48]. Their (homophilic) adhesion properties and molecular diversity make cadherins prime candidates for such postulated lock-and-key molecules (for a review, see [39]).

Of the non-clustered protocadherins, the most detailed information is available on protocadherin 8 (Arcadlin) and protocadherin 10 (OL-protocadherin) [26,28,38]. Both show defined expression patterns in the developing brain and are localized to synaptic contacts. Protocadherin 8 expression is found following embryonic day 8.5 in midbrain regions, the auditory and visual projections, the limbic system, and, additionally, in the hippocampus. Functionally, Arcadlin expression is regulated by neuronal activity (i.e. enhanced levels of Arcadlin were found after tetanic stimulation of hippocampal neurons). Moreover, application of antibodies to Arcadlin suppressed long-term potentiation of hippocampal granule cells, indicating an important function for Arcadlin in regulating synaptic transmission or synaptic structure, similar to the situation shown for N-cadherin [28,46,47].

Protocadherin 10 is mainly expressed in the olfactory bulb, in most parts of the limbic system and in the cerebellum, where protocadherin 10 marks distinct clusters of Purkinje cells in a pattern of parasagittal bands [26,49\*]. This suggests functions of protocadherin 10 in cerebellar development and in synapse formation of Purkinje cells, similar to observations made for various classical cadherins in earlier cerebellar development [40]. Evidence for expression of the several other protocadherins in the CNS has come from northern blots, cDNA analysis and *in situ* hybridization [1,4,13,19\*,20,21,32,33\*,50]. In contrast,  $\mu$ -protocadherin and protocadherin 12 (VE-cadherin 2) are not detected in the CNS [12,29].

With the discovery of the clustered protocadherins, the number and diversity of cadherins expressed in brain greatly increased. Functions in the generation of synaptic specificity were indicated by the presence of CNRs at the synapse and by differential expression of several protocadherins from the three families in embryonic and postnatal brain [2,5,7,51]. While distinct expression patterns are reported for distinct family members, these are not easily correlated with specific neuronal circuits or particular neuronal populations at present [52,53]. Nonetheless, the distribution may represent recognition patterns of higher order or individual neuronal fingerprints. Moreover, various truncated forms and cytoplasmic splice variants are reported for the  $\alpha$ -protocadherins [22]. They could represent additional functional protocadherin moieties, adding even more degrees of protocadherin diversity. Alternatively, they may be involved in the cellular regulation of the full-length protocadherin adhesion and signaling properties, as is inferred from the experiments with mutated PAPC and NFPC constructs (see above).

## Conclusions and future directions

In summary, genome sequencing revealed protocadherins as the largest subfamily within the cadherin superfamily in mammals. Given the lack of direct protocadherin orthologues in worm and fly, one might speculate about a rather recent evolutionary origin of protocadherins in vertebrates.

From the work discussed here, it seems clear that protocadherins can mediate homophilic adhesion. However, this has a different strength and probably other regulatory mechanisms compared with adhesion of classic cadherins. This could indicate that protocadherins also are prone to form heterophilic interactions and might actually switch their function from an adhesive moiety to a signaling or receptor molecule. Thus, it will be important to establish cell culture models to understand such heterophilic interactions in more detail—for example, those observed between  $\alpha$ -protocadherins and reelin.

The many splice variants found for the cytoplasmic region add to the general diversity of protocadherin tails. This feature might reflect the possible promiscuity of extracellular interactions partners of protocadherins and the resulting multitude of functions. Therefore, it will be essential to identify more molecules that link the cytoplasmic domains to signaling pathways and to the cytoskeleton. Indeed, multiple roles for protocadherins *in vivo* are strongly supported by the reviewed literature: in embryonic stages, protocadherins participate in tissue morphogenesis, whereas in the adult they are involved in differentiation of specific cells (e.g. in the establishment and modulation of synaptic contacts, as has been shown for protocadherin 8).

Genetic engineering in cell culture and mouse models will help to understand how protocadherins function in cellular development and how these versatile tools were evolved to full complexity in vertebrate species.

## Acknowledgements

We would like to thank Kirsten Arndt, Randy Cassada and Ingrid Haas for discussion and their suggestions on the manuscript. With support by the Deutsche Forschungsgemeinschaft SFB 592, to RK.

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