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COMMENTARY

Emerging roles of protocadherins: from self-avoidance to enhancement of motility

Shuichi Hayashi* and Masatoshi Takeichi*

ABSTRACT

Protocadherins are a group of transmembrane proteins belonging to the cadherin superfamily that are subgrouped into 'clustered' and 'non-clustered' protocadherins. Although cadherin superfamily members are known to regulate various forms of cell–cell interactions, including cell–cell adhesion, the functions of protocadherins have long been elusive. Recent studies are, however, uncovering their unique roles. The clustered protocadherins regulate neuronal survival, as well as dendrite self-avoidance. Combinatorial expression of clustered protocadherin isoforms creates a great diversity of adhesive specificity for cells, and this process is likely to underlie the dendritic self-avoidance. Non-clustered protocadherins promote cell motility rather than the stabilization of cell adhesion, unlike the classic cadherins, and mediate dynamic cellular processes, such as growth cone migration. Protocadherin dysfunction in humans is implicated in neurological disorders, such as epilepsy and mental retardation. This Commentary provides an overview of recent findings regarding protocadherin functions, as well as a discussion of the molecular basis underlying these functions.

KEY WORDS: Protocadherin, Cell–cell contact, Self-avoidance, Cell movement, Lamellipodia, WAVE complex

Introduction

Regulation of cell–cell contacts is essential for animal morphogenesis. Cadherins are transmembrane glycoproteins that regulate cell–cell contacts through homophilic or heterophilic interactions between their extracellular regions (Takeichi, 2014). Their extracellular region is subdivided into repetitive 'EC domains' (also called 'EC repeats') (Fig. 1A), and individual EC domains are connected in tandem through a unique set of amino-acid sequences, called the cadherin motif, that function to bind to Ca^{2+} (Tsukasaki et al., 2014). Proteins that contain the cadherin motif comprise the cadherin superfamily (Hirano and Takeichi, 2012). The cadherin superfamily comprises over 100 members in vertebrates (Hulpiau and van Roy, 2009). The 'classic' cadherins, including neuronal (N)-cadherin (also known as Cdh2), are responsible for Ca^{2+}-dependent cell–cell adhesion in animals. The classic cadherins in vertebrates have five EC domains in their extracellular region, and their cytoplasmic regions bind to a specific group of proteins, including p120-catenin and β-catenin (also known as Ctnnd2 and Ctnnb1, respectively) (Takeichi, 2014). β-catenin simultaneously binds to α-catenin (also known as Ctnna1), an actin-binding protein, forming the cadherin–catenin complex, which plays a key role in mechanical adhesions between animal cells. p120-catenin and β-catenin exclusively bind to the classic cadherins and not to other cadherin superfamily members. In fact, the cytoplasmic amino-acid sequences are diversified among the cadherin superfamily members, indicating that their functions are also diversified.

Protocadherins are a subgroup of the cadherin superfamily that have the conserved cadherin motifs (Sano et al., 1993; Suzuki, 1996). Unlike the classic cadherins, protocadherins have more than five EC domains and lack the catenin binding sites in their cytoplasmic region (Fig. 1A). The term 'protocadherin' has been broadly used without clear definition throughout the literature, referring to various members of the cadherin superfamily. In this Commentary, we focus on two specific groups, the 'clustered' and 'non-clustered' protocadherins, which are phylogenetically distinct from other members of the cadherin superfamily (Hulpiau and van Roy, 2009). The clustered protocadherins comprise as many as 53 and 58 members in human and mouse, respectively, accounting for approximately half of the total cadherins in these organisms, whereas only around ten non-clustered protocadherins have been identified (Fig. 1B). Both groups of protocadherins are widely expressed in the nervous system of developing vertebrates, suggesting that they have roles in neural development and function. Clinical and genetic studies suggest that the dysfunction of protocadherins is related to neurological disorders and cancer in humans (Kim et al., 2011; van Roy, 2014). Below, we first review recent progress in the study of clustered and non-clustered protocadherins, before discussing protocadherin-related diseases.

Clustered protocadherins

The clustered protocadherins comprise the protocadherin-α (Pcdhα), protocadherin-β (Pcdhβ) and protocadherin-γ (Pcdhγ) groups. The genes encoding the protocadherins have a characteristic genomic organization, in which the three gene clusters are arranged in tandem on a single chromosome (Wu and Maniatis, 1999) (Fig. 2A). In the mouse, the gene cluster encoding Pcdhα has 14 variable exons and those encoding Pcdhβ and Pcdhγ, 22. Each variable exon encodes an extracellular domain, a transmembrane domain and a variable portion of the cytoplasmic domain. The genes encoding Pcdhα and Pcdhγ, but not those encoding Pcdhβ, also contain three additional constant regions that are located downstream of the variable exons, which encode a shared C-terminus of the cytoplasmic domain. This unique gene organization allows for the production of a large diversity of protocadherin isoforms through an alternative choice of promoters that precede each of the variable exons, as well as through alternative splicing. Details of their gene regulation are reviewed elsewhere (Chen and Maniatis, 2013; Yagi, 2012).

Laboratory for Cell Adhesion and Tissue Patterning, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan.

*Authors for correspondence (hayashi@cdb.riken.jp; takeichi@cdb.riken.jp)

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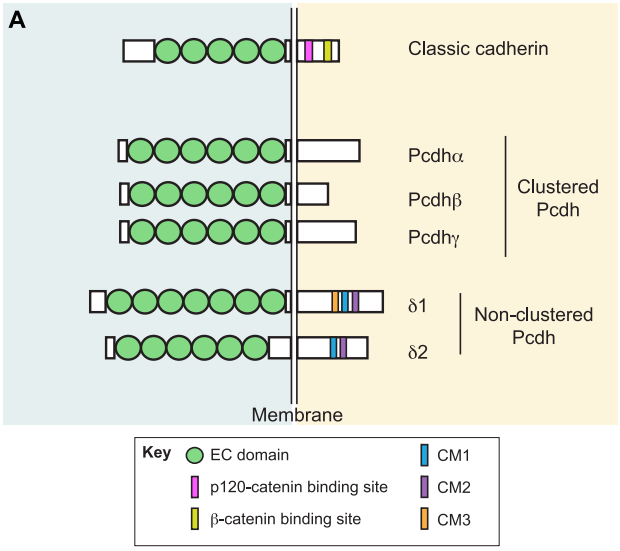

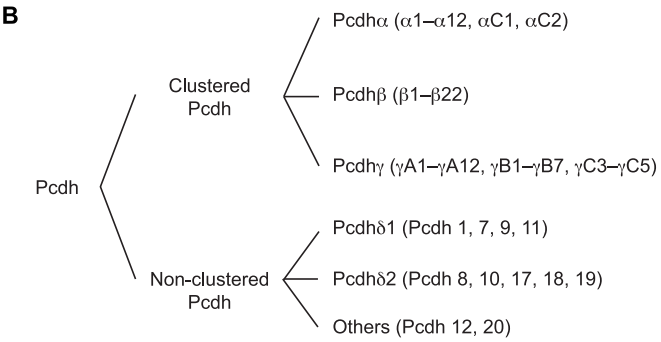

Fig. 1. Structure and classification of the protocadherin family. (A) Structures of clustered and non-clustered protocadherins (Pcdh) in comparison with a classic cadherin. The classic cadherins have five 'EC domains' in the extracellular region, and p120-catenin and β-catenin binding sites in the cytoplasmic region. The clustered protocadherins have six EC domains and no identified motifs in the cytoplasmic region. The δ1 and δ2 groups of the non-clustered protocadherins have seven and six EC domains, respectively, and both groups have conserved motifs, CM1 and CM2, in the cytoplasmic region. The δ1 group has an additional conserved motif, CM3. (B) A phylogenetic tree of protocadherins (Hulpiau and van Roy, 2009). In the mouse clustered protocadherins, the α-group has 14 isoforms, and the β- and γ-groups have 22 each (the isoforms are given in parentheses). The non-clustered protocadherins are grouped as 'δ1', 'δ2' and 'others'; the members of which are shown in parentheses.

Regulation of neurite patterning by clustered protocadherins

Knockout of the Pcdhα gene cluster in mice disturbs the coalescence of homotypic olfactory sensory neuron (OSN) axons into a glomerulus by inducing the formation of ectopic glomeruli (Hasegawa et al., 2008). Based on this observation, the authors of that paper propose that Pcdhα is required to eliminate miswired axons. Another study from the same group showed that the constitutive expression of a single Pcdhα isoform is sufficient to prevent ectopic glomerulus formation (Hasegawa et al., 2012), suggesting that the diversity of extracellular regions is not required for the coalescence of OSN axons.

Deletion of the entire Pcdhγ gene cluster in mice causes them to die shortly after birth. In these mutant mice, loss of neuronal cells and synaptic alterations were observed in the spinal cord and retina (Lefebvre et al., 2008; Wang et al., 2002), suggesting that Pcdhγ is important for neuronal survival. Gene knockout of three Pcdhγ isoforms that are classified as C-type genes (*Pcdhgc3*, *Pcdhgc4* and *Pcdhgc5*) results in phenotypes that are indistinguishable from those obtained when the entire Pcdhγ cluster is deleted (Chen et al., 2012). This suggests that the C-type genes are primarily responsible for neuronal survival. Apart from the regulation of cell survival, it has been shown that the Pcdhγ proteins mediate 'self-avoidance' between neurites in retinal starburst amacrine cells, as well as in cerebellar Purkinje cells – large GABAergic neurons that have highly branched dendrites (Lefebvre et al., 2012) (Fig. 2B). Self-avoidance is a key principle that governs neurite patterning, whereby sister dendrites of the same neuron repel each other. Recently, Slit–Robo signaling, which mediates diverse cell behaviors through the interactions between Slit ligands and Robo receptors, was also found to regulate self-avoidance (Gibson et al., 2014). That study, however, suggested that Pcdhγ and Slit–Robo signals independently mediate dendritic self-avoidance, leaving their functional relationships to be determined in the future. In the cerebral cortex, loss of Pcdhγ genes reduces arborization of the apical dendrites of neurons (Garrett et al., 2012). Furthermore, knockout of the Pcdhα cluster also impairs dendrite arbor formation in hippocampal neurons (Suo et al., 2012). The latter study additionally suggests that Pcdhα proteins, as well as Pcdhγ proteins, regulate dendritic arborization by inhibiting focal adhesion kinase or proline-rich tyrosine kinase 2 (also known as Ptk2b), which is associated with the cytoplasmic regions of these protocadherins (Chen et al., 2009a). It remains to be determined whether the Pcdh-dependent dendritic self-avoidance and dendrite arborization observed in these studies depend on a common or distinct signaling mechanism(s).

Single cell diversification through clustered protocadherins

For proper self-avoidance, neurons must distinguish between self and non-self. In connection with this notion, it has been suggested that clustered protocadherins endow neurons with diverse adhesive labels. Studies on allelic gene expression of the Pcdhα and Pcdhγ gene clusters in Purkinje cells show that, although the variable exons of the C-type protocadherins are constantly expressed from both alleles, random monoallelic and combinatorial expression occurs for the variable exons encoding Pcdhα, PcdhγA and PcdhγB (Esumi et al., 2005; Kaneko et al., 2006). These findings suggest that the various isoforms of protocadherins are stochastically expressed in different combinations in individual neurons.

An *in vitro* study has demonstrated that Pcdhγ isoforms form multimers (probably tetramers) in the cis-orientation and that these multimers show strict homophilic trans-interactions at cell–cell interfaces (Schreiner and Weiner, 2010). Intriguingly, the multimer formation occurs promiscuously among various isoforms, leading the authors of that study to predict that the 22 isoforms of Pcdhγ could form 234,256 distinct adhesive interfaces. A recent study has further found that cells expressing different combinations of Pcdhα, Pcdhβ and Pcdhγ aggregate through the homophilic trans-interactions of the protocadherins that they express; however, these cells fail to co-aggregate with cells that express a different combination of protocadherins, even when the difference is only due to one isoform in the combination (Thu et al., 2014) (Fig. 2C). It is of note that some of the clustered protocadherin isoforms, including the alternative Pcdhα isoforms and PcdhγC4, are unable to induce cell aggregation when individually expressed in cells, but they can do so when coexpressed with other isoforms. This phenomenon has been explained by assuming that these isoforms

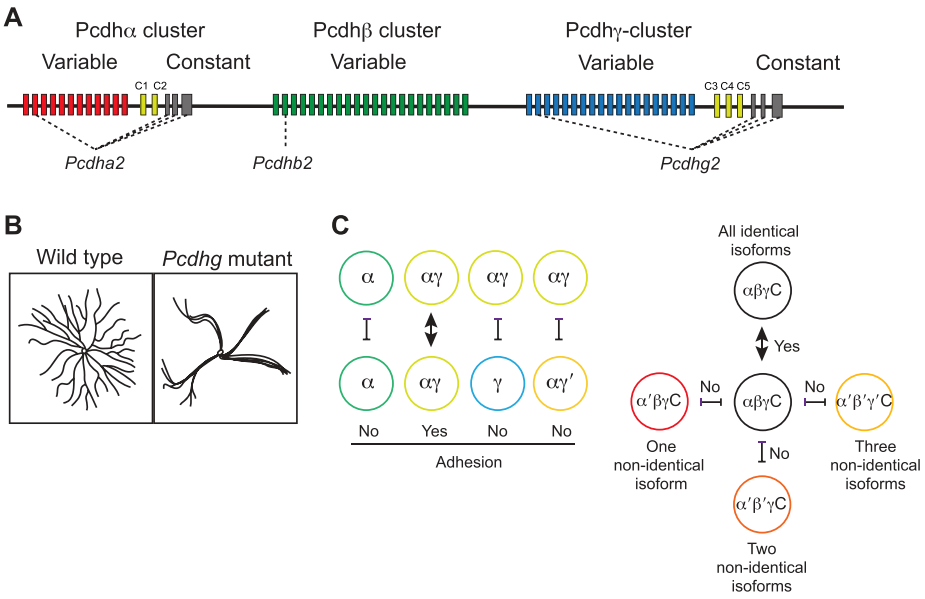

Fig. 2. Generation of unique cell recognition properties through combinatorial expression of clustered protocadherin isoforms. (A) Genomic organization of the clustered protocadherin genes. The generation of *Pcdha2*, *Pcdhb2* and *Pcdhg2* genes is indicated as an example of exon selection that produces various isoforms. (B) Knockout of the Pcdhγ gene cluster in mice results in impaired self-avoidance of dendrites. The diagrams were drawn based on the findings by Lefebvre and colleagues (Lefebvre et al., 2012). (C) Cell–cell adhesion is induced only between cells expressing the same combination of clustered protocadherins (Thu et al., 2014). Expression of a Pcdhα isoform alone does not induce cell–cell adhesion, probably due to its failure to transfer to the plasma membranes, whereas co-expression of Pcdhα and Pcdhγ isoforms induces cell–cell adhesion. Pcdhα- and Pcdhγ-expressing cells adhere to those that express the same combination of isoforms, but not to those expressing either Pcdhγ alone or the same Pcdhα isoform along with another Pcdhγ isoform (shown in the scheme on the left). A cell expressing a combination of Pcdhα, Pcdhβ, Pcdhγ and the C isoforms adheres to a cell with the same combination of isoforms (shown on the right). However, even a single mismatch in Pcdhα, Pcdhβ or Pcdhγ inhibits the adhesion. Mismatches in the C isoforms also inhibit cell adhesion. The diagrams were drawn based on the findings of Thu and colleagues (Thu et al., 2014).

are unable to localize to the plasma membranes independently but that they are rescued by ‘carrier’ isoforms. It remains undetermined whether such interdependency between isoforms also occurs in neurons *in vivo*.

Using these observations, we can speculate that a molecular mechanism exists that regulates the self- and non-self recognition of neurons. In a neuronal population, individual neurons acquire various adhesive specificities based on the combinatorial expression of clustered protocadherin isoforms, resulting in a strong heterogeneity among neurons with regard to the adhesive molecules at their surface. In such systems, neurites should be able to distinguish between those derived from the same or other neurons by detecting differences in protocadherin isoforms that are expressed on the respective surfaces. The trans-interactions of the clustered protocadherins, which can only occur successfully between a pair of neurites of the same origin, could act as a trigger for avoidance signals. The neurites responding to such signals would then repel each other, although intracellular molecular events underlying this putative repelling mechanism remain to be identified. Proteins with adhesion-promoting abilities are not often considered as repelling factors, but such opposing functions could be reconciled as discussed in the Concluding remarks. By contrast, whether the loss of Pcdhα and Pcdhβ genes also affects self-avoidance *in vivo* has not been addressed yet. This point needs to be clarified in the future.

Non-clustered protocadherins

In contrast to the clustered protocadherins, the genes encoding the non-clustered protocadherins are scattered on multiple chromosomes. They are subgrouped into δ1, δ2 and others (Fig. 1B) on the basis of overall homology, number of EC domains and conservation of specific amino-acid motifs (Hulpiau and van Roy, 2009; Redies et al., 2005). The δ1 and δ2 subgroups have seven and six EC domains, respectively. Both the δ1- and δ2-subgroups have conserved cytoplasmic motifs, CM1 and CM2, and δ1-protocadherins have an additional conserved motif, CM3. Recent studies have uncovered unique functions of non-clustered protocadherins, particularly for δ2-subfamily members, as discussed below.

Developmental roles of δ1-protocadherins

Among the δ1 subfamily members, NF-protocadherin (NFPC, *Xenopus* homolog of Pcdh7), which is expressed in the ectoderm of *Xenopus* embryos, is the best studied. Morpholino-mediated depletion of NFPC or expression of its putative dominant-negative forms in embryos disrupts ectoderm layers and impairs neural tube closure (Bradley et al., 1998; Heggem and Bradley, 2003; Rashid et al., 2006). More recent studies have shown that the cytoplasmic domain of NFPC binds to the template-activating factor 1 (TAF1), a histone-binding protein involved in chromatin remodeling, and that depletion of TAF1 mimics that of NFPC, suggesting that NFPC requires TAF1 in order to exert its cellular functions (Heggem and Bradley, 2003; Rashid et al., 2006). The NFPC–TAF1 complex is also expressed in retinal ganglion cells in *Xenopus*, and perturbation of its activity impairs axon elongation and navigation (Leung et al., 2013; Piper et al., 2008). How NFPC functionally couples with such a transcriptional regulator, however, remains to be resolved. Another member of the δ1 subfamily, axial protocadherin (AXPC, *Xenopus* homolog of Pcdh1), is expressed along the axial mesoderm in *Xenopus*

embryos, and its knockdown using morpholinos results in the loss of notochord formation (Yoder and Gumbiner, 2011). Intriguingly, the authors of that study could not detect any alterations in the adhesion-related behavior of cells, such as cell sorting, when AXPC expression was manipulated in embryos. Overall, the cellular and molecular functions of the δ1-protocadherins largely remain mysterious.

δ2-protocadherins in early development

The δ2 subfamily comprises five members – Pcdh8, Pcdh10, Pcdh17, Pcdh18 and Pcdh19 (Table 1). Their functions in early development have been studied in *Xenopus* and zebrafish. In *Xenopus* embryos, paraxial protocadherin (PAPC, *Xenopus* homolog of Pcdh8) is expressed at paraxial mesoderm, in contrast to the expression of AXPC at axial mesoderm (Kim et al., 1998). Earlier studies suggested that PAPC plays a role in cell sorting and convergent extension during *Xenopus* gastrulation (Kim et al., 1998). More recent studies have shown that PAPC controls these morphogenetic events by downregulating the adhesion activity of C-cadherin, an important classic cadherin that is expressed in *Xenopus* embryos; this process is required for normal gastrulation in *Xenopus* embryos (Chen and Gumbiner, 2006). Regulation of early morphogenesis through the δ2-group protocadherins has also been reported in zebrafish. In those studies, knockdown of *pcdh18a* by using morpholino oligonucleotides causes delayed epiboly – a cell sheet movement that occurs during gastrulation of embryos – and impaired cell movements (Aamar and Dawid, 2008), and *pcdh19* knockdown results in impaired convergence during neurulation (Emond et al., 2009). These findings suggest that δ2-protocadherins play a role in cell movement and rearrangement during the early morphogenesis of embryos. Notably, however, gene knockout of *Pcdh8* in mice does not lead to any detectable defects in mesodermal morphogenesis (Yamamoto et al., 2000). It is therefore important to further confirm the roles of δ2-protocadherins in embryogenesis using multiple model systems and methods.

Role of δ2-protocadherins in axon growth and patterning

The members of the δ2 subfamily are widely expressed in the nervous system (Hertel et al., 2008; Kim et al., 2007; Kim et al., 2010; Krishna et al., 2011). Pcdh10 (also known as OL-protocadherin) and Pcdh17 are detected along axon fibers (Hayashi et al., 2014; Uemura et al., 2007), suggesting that they are involved in axon development or function. It has, indeed, been reported that gene knockout of *Pcdh10* in mice causes defects in the formation of various axon tracts, such as the thalamocortical and corticospinal tracts that connect the cerebral cortex and other regions of the central nervous system (Uemura et al., 2007). However, analysis of another *Pcdh10*-knockout mouse line, which was generated independently of that used in the study described previously, failed to detect such phenotypes (I. Kahr, F. van Roy and S. Hirano, personal communication). Therefore, the phenotypes of the brains of *Pcdh10*-deficient mice need further examination.

Another report has shown that *Pcdh17* knockout in mice results in defects in the extension of axons from specific subdivisions of the amygdala, a center in the brain that regulates emotions and motivation (Hayashi et al., 2014). This phenotype has been confirmed through observations of impaired axon growth from amygdala fragments that had been explanted *in vitro*, supporting the idea that Pcdh17 mediates axon growth. In zebrafish, knockdown of *pcdh18b* causes defects in axon arborization of primary motoneurons (Biswas et al., 2014). In this case, depletion of Pcdh18b does not affect the growth of motoneurons themselves, but affects the density of filopodia along the shaft of elongating axons. The potential molecular mechanisms underlying Pcdh17-dependent axon growth are discussed in later sections.

Table 1. Overview of the δ2-protocadherin group

Subtype	Other name and/or isoform	Species analyzed	Intracellular binding proteins	Knockdown or knockout phenotypes	References
Pcdh8	PAPC	*Xenopus*	ANR5, Sprouty, Casein kinase 2β	Impaired cell movement in gastrulation	Chen and Gumbiner, 2006; Chung et al., 2007; Kietzmann et al., 2012; Wang et al., 2008
		Mouse		No detectable defects in mesodermal movement	Yamamoto et al., 2000
	Arcadlin	Rat and mouse	TAO2β	Excess synapse number upon stimulation in hippocampal neurons	Yasuda et al., 2007
		Human	WAVE complex	n.d.	Chen et al., 2014*
Pcdh10	OL-pc	Mouse	WAVE complex	Impaired axon growth in ventral telencephalon	Uemura et al., 2007
Pcdh17		Mouse	WAVE complex	Impaired amygdala axon growth	Hayashi et al., 2014
		Mouse		Excess synaptic vesicles in basal ganglia	Hoshina et al., 2013
Pcdh18	Pcdh18a	Zebrafish		Impaired cell movement in epiboly	Aamar and Dawid, 2008
	Pcdh18b	Zebrafish	WAVE complex	Motoaxon arborization defects	Biswas et al., 2014
		Mouse	Dab1	n.d.	Homayouni et al., 2001
Pcdh19		Zebrafish		Impaired cell movement in neurulation	Emond et al., 2009
		Chick	WAVE complex	n.d.	Tai et al., 2010

n.d., not determined.
*This study showed biochemical interactions between the WAVE complex and each of Pcdh8, Pcdh10, Pcdh17, Pcdh18 and Pcdh19.

δ2-protocadherins at synapses

Activity-regulated cadherin-like protein (arcadlin), rat homolog of Pcdh8 (Table 1), was originally identified as a product of the gene of which the expression is rapidly and transiently increased in rat hippocampal granule cells after neuronal stimulation (Yamagata et al., 1999). Upon stimulation, arcadlin mRNA expression increases more than ten-fold, and its protein products are recruited to synaptic puncta in cultured hippocampal neurons. Arcadlin associates with N-cadherin, and its shorter isoform, but not the longer one, also interacts with a spliced form of TAO2 kinase (TAO2β) through the cytoplasmic region of arcadlin (Yasuda et al., 2007). The homophilic interactions between arcadlins result in activation of TAO2β, and the active TAO2β, in turn, activates p38MAK. These signaling events induce the endocytosis of the arcadlin–N-cadherin complex (Fig. 3A). That study also showed that the number of dendritic spines, structures that are involved in excitatory synapse formation, increases in arcadlin-knockout mice. These observations suggest that arcadlin functions to downregulate N-cadherin that is important for synapse stability (Takeichi and Abe, 2005), leading to enhanced spine dynamics.

A recent study has reported that Pcdh17 is localized at perisynaptic regions in both excitatory and inhibitory synapses of the basal ganglia nuclei and that knockout of the gene causes enhanced presynaptic vesicle accumulation in corticobasal ganglia (Hoshina et al., 2013). In that study, however, the molecular mechanisms by which Pcdh17 regulates synapses were not analyzed. Another study has suggested that Pcdh10 is involved in the synapse elimination that is induced through the fragile X mental retardation 1 protein (FMR1)-dependent activation of myocyte enhancer factor 2 (Mef2)-family members (Tsai et al., 2012). In this synapse elimination process, PSD-95, a postsynaptic scaffold protein that has a role in anchoring synaptic proteins, is ubiquitinylated and then degraded by the proteasome. Pcdh10 was found to enhance PSD-95 degradation by mediating the interactions between PSD-95 and the proteasome, although detailed molecular mechanisms of this process remain unknown. Thus, δ2-protocadherins appear to regulate synapse dynamics. However, the studies on this subject are still fragmentary, and more defined roles of δ2-protocadherins in synapse functions need to be clarified.

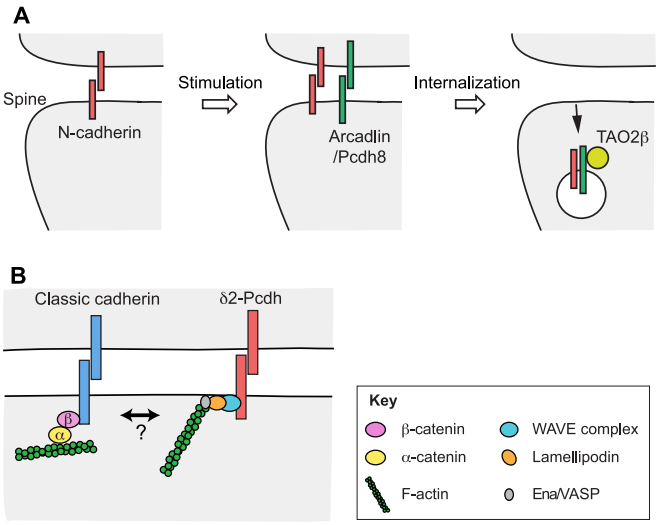

Fig. 3. Potential interactions between δ2-protocadherins and classic cadherins. (A) A model for arcadlin/Pcdh8-mediated internalization of N-cadherin, triggered by neuronal stimulation, which occurs in excitatory synapses of neurons. Arcadlin homophilic interaction induces internalization of N-cadherin through its binding to TAO2β. The diagrams were drawn based on the findings of Yasuda and colleagues (Yasuda et al., 2007). (B) δ2-protocadherins and classic cadherins interact with the actin cytoskeleton through distinct cytoplasmic partners. Only representative cytoplasmic factors are depicted. All δ2-protocadherins interact with the WAVE complex. Lamellipodin and Ena/VASP associate with the Pcdh17-bound WAVE, and Ena/VASP is assumed to regulate actin polymerization (Hayashi et al., 2014). In the classic-cadherin-based adhesion, α-catenin mediates the linkage of cadherin with actomyosin filaments; without this linkage, cadherins are unable to induce firm cell–cell adhesion (Takeichi, 2014). If these two actin-interacting systems are physiologically incompatible at cell junctions, either system might exclude the other.

δ2-protocadherins modulate classic-cadherin-mediated cell–cell adhesion

Similar to the classic cadherins, δ2-protocadherins accumulate at cell–cell contact sites through homophilic interactions (Hayashi et al., 2014; Hirano et al., 1999; Nakao et al., 2008; Tai et al., 2010). So far, it appears that trans-interactions occur only between the same subtypes (Hoshina et al., 2013; Tai et al., 2010) (S.H., unpublished data). Despite their ability to form homophilic interactions, the δ2-protocadherins are, however, less capable of inducing the aggregation of cells in suspension cultures than the classic cadherins (Hirano et al., 1999; Tai et al., 2010), and in the case of PAPC/Pcdh8 and arcadlin/Pcdh8, they do not promote any cell aggregation (Chen and Gumbiner, 2006; Yasuda et al., 2007). These observations imply that the homophilic trans-interactions between δ2-protocadherins regulate cell–cell contacts beyond simple mechanical adhesion between cells, as is the case for the clustered protocadherins. In fact, as mentioned already, arcadlin/Pcdh8 induces the endocytosis of N-cadherin through physically binding to it (Yasuda et al., 2007), and PAPC/Pcdh8 antagonizes C-cadherin-mediated adhesion (Chen and Gumbiner, 2006; Chen et al., 2009b). Another study of PAPC suggests that PAPC and C-cadherin independently bind to the Wnt receptor Frizzled-7, and that the formation of these complexes prevents C-cadherin from clustering in a Wnt-11-dependent manner, leading to the weakening of C-cadherin-mediated adhesion (Kraft et al., 2012). Pcdh19 has also been shown to bind to N-cadherin (Biswas et al., 2010).

These studies suggest that δ2-protocadherins suppress classic-cadherin-mediated adhesion in various ways. As discussed in the following sections, δ2-protocadherins regulate actin polymerization, indicating the presence of another mechanism that affects classic-cadherin-dependent adhesion. The classic cadherins also interact with actin filaments (Takeichi, 2014), but in a manner that is distinct from that used by δ2-protocadherins; this difference might cause functional interference between the two adhesion systems (Fig. 3B).

δ2-protocadherins bind to the WAVE complex and regulate cell motility

Several lines of evidence suggest that one or multiple regions in the cytoplasmic domain of δ2-protocadherins bear adhesion-suppressing functions. For instance, Pcdh17 mutants that lack these cytoplasmic regions induce the lateral clumping of axons when exogenously expressed in neurons of embryonic brains (Hayashi et al., 2014). Similarly, Pcdh19 variants, from which the cytoplasmic region has been deleted, induce the formation of

larger aggregates than full-length Pcdh19 when they are expressed in L cells, a fibroblastic cell line (Tai et al., 2010). Thus, cytoplasmic regions of δ2-protocadherins appear to have an adhesion-inhibiting role.

Efforts to seek molecules that regulate the cytoplasmic function of δ2-protocadherins have identified Nap1 (also known as Nckap1), a component of the WASP family verprolin-homologous protein (WAVE) complex (Krause and Gautreau, 2014), as a binding partner of several members of the δ2 group (Biswas et al., 2014; Hayashi et al., 2014; Nakao et al., 2008; Tai et al., 2010) (Table 1). In addition to Nap1, other components of the WAVE complex also associate with δ2-protocadherins, which suggests that the entire complex interacts with them (Fig. 3B). Recent studies have identified a key motif, termed 'WIRS', within the cytoplasmic region of δ2-protocadherins as being responsible for their binding to the WAVE complex (Chen et al., 2014). This motif recognizes an interaction surface that is formed through the Sra- and Abi-family subunits of the WAVE complex. In the case of Pcdh17, the WAVE complex not only binds to the WIRS-containing region but also to an additional site that is located in the N-terminal half of its cytoplasmic region (Hayashi et al., 2014).

The WAVE complex regulates actin dynamics by activating Arp2/3 (Krause and Gautreau, 2014). Alternatively, it can interact with lamellipodin (Lpd; also known as Raph1) through Abi proteins (Law et al., 2013). Lpd is known to bind to Ena/VASP-family proteins (referred to here as Ena/VASP), other regulators of actin polymerization, and Lpd plays a crucial role in advancing the lamellipodia in migrating cells (Krause et al., 2004). We have found recently that the WAVE complex, bound to Pcdh17, interacts with Lpd and Ena/VASP, rather than Arp2/3 (Hayashi et al., 2014) (Fig. 3B). Experiments in astrocytoma U251 cells, which do not express endogenous δ2-protocadherins, have revealed that the WAVE complex is normally localized at the leading edge or the lamellipodia of cells during their migration but that it disappears from these sites when the lamellipodia have collided with one another (Hayashi et al., 2014) (Fig. 4A). However, when Pcdh17 is exogenously expressed in these cells, the WAVE complex is not removed from the initial cell–cell contact sites, because its binding partner Pcdh17 accumulates there. Shortly after the collision of cells, Pcdh17 and the WAVE complex become concentrated at a peripheral region of cell–cell contacts (Fig. 4B). Remarkably, the peripheral contact region containing Pcdh17 and the WAVE complex actively moves forward (Hayashi et al., 2014). Time-lapse imaging has revealed that the membranes organizing these special contacts behave like the leading edges – displaying ruffling and retrograde movement – suggesting that these contact sites are converted into a leading-edge-like structure. Furthermore, in confluent cultures of U251 cells, expression of Pcdh17 (or Pcdh10) accelerates the migration of individual cells (Hayashi et al., 2014; Nakao et al., 2008). These observations suggest that Pcdh17 facilitates the motility of membranes at cell–cell contact sites by recruiting a specific set of actin polymerization regulators – the WAVE-complex, Lpd and Ena/VASP – to these sites and thereby promotes cell migration.

In general, cell migratory behavior is controlled through contact inhibition of cell locomotion (CIL) (Abercrombie, 1979). When two cells touch one another, their lamellipodia seize, resulting in their migration coming to a halt. The disappearance of the WAVE complex from contact sites in normal U251 cells, therefore, is in accordance with the idea of CIL, whereas Pcdh17-expressing cells appear to violate the CIL rule by maintaining the WAVE complex at cell–cell contacts. As it is thought that the establishment of CIL involves the classic cadherins (Theveneau et al., 2010), Pcdh17 might counteract CIL not only by facilitating membrane motility at cell–cell contacts but also by suppressing the classic cadherins. Consistently, our unpublished observations show that N-cadherin does not colocalize with the Pcdh17–WAVE complex, although it appears to localize with Pcdh17 outside of the WAVE complex (Fig. 4B).

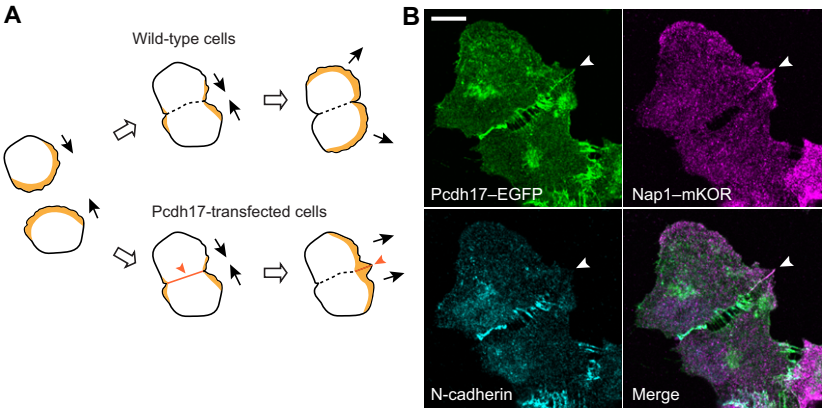

Fig. 4. Pcdh17 regulates cell motility by recruiting the WAVE complex to peripheral cell–cell contacts. (A) The behavior of U251 cells (lacking endogenous Pcdh17) after their collision. When two wild-type cells collide, lamellipodia (orange) and associated proteins are lost from their contact sites. However, when Pcdh17-expressing U251 cells collide, lamellipodial proteins – such as the WAVE complex – remain at their contact sites (red arrowhead, lower middle). Subsequently, these proteins become concentrated only at a peripheral cell–cell contact and induce a protrusion of the in-contact membranes (red arrowhead, lower right), which actively moves forward. The black arrows indicate the direction of cell migration. (B) Fluorescence images of U251 cells co-transfected with enhanced green fluorescent protein (EGFP)-tagged Pcdh17 and monomeric Kusabira Orange (mKOR)-tagged Nap1. EGFP (green), mKOR (purple) and endogenous N-cadherin (cyan) are visualized by using triple immunostaining. Pcdh17 is concentrated throughout cell–cell boundaries, whereas it colocalizes with Nap1 (a WAVE complex subunit) only at a peripheral contact site (arrowhead). N-cadherin is concentrated at large areas of cell–cell boundaries and colocalizes with Pcdh17 at these areas, but not at the site where Pcdh17 colocalizes with Nap1 (S.H., unpublished observation), suggesting that the Pcdh17–WAVE complex might exclude N-cadherin from cell–cell contacts. Scale bar: 10 μm.

Potential mechanisms of δ2-protocadherin-mediated axon extension

Using observations from cell biology experiments of δ2-protocadherin-mediated regulation of cell motility, we can also infer their role in axon extension, which is defective in *Pcdh17*-knockout mice (Hayashi et al., 2014). Growth cones of axons derived from wild-type and Pcdh17-deficient amygdala migrate to equal extents on culture dishes. However, we have found a difference in their behaviors when the growth cones meet other axons (Fig. 5A). Wild-type growth cones continue to migrate along the encountered axons, although some of them simply cross the axons. By contrast, growth cones from *Pcdh17*-knockouts tend to stop moving at the contact points (Hayashi et al., 2014). This cessation of growth cone movement is reminiscent of CIL, leading us to propose that, in normal axons, Pcdh17 counteracts CIL processes, thereby allowing the growth cones to migrate on other axons. Consistent with the observations described above, WAVE complex, Lpd and Mena (an Ena/VASP-family protein that is also known as Enah) are all concentrated at growth cone–axon contact sites in wild-type neurons, but not in *Pcdh17*-null neurons. Thus, the complex comprising Pcdh17, Lpd, Mena and the WAVE complex might sustain growth cone migration by enhancing actin elongation at cell–cell contact sites, as the Lpd–Mena (Ena/VASP) complex has been found to exert this effect at free cell edges, or by destabilizing the adhesion between growth cones and axons, in which the classic cadherins presumably are involved (Fig. 5B). It is also possible that both of these possible mechanisms cooperate. These ideas are consistent with the observation that Pcdh17 mutants that are unable to bind the WAVE complex induce the clumping of axons *in vivo* (Hayashi et al., 2014).

The above models of Pcdh17-dependent axon extension have been proposed based on the observations made using U251 cells. However, the way of contact formation is not identical between U251 cells and growth cones – when two Pcdh17-positive U251 cells meet, they organize a symmetrical Pcdh17-mediated contact and move together (Fig. 4A). However, growth cones generally attach to the stalk of another axon, which is not motile, forming an asymmetrical contact (Fig. 5B). Further investigation is therefore necessary in order to confirm whether the mechanisms disclosed using U251 cells also operate in growth cones.

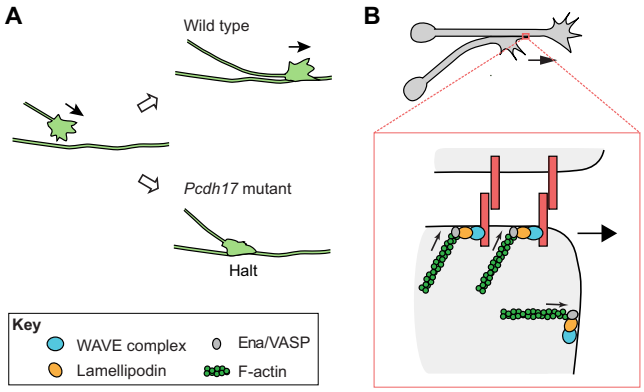

Fig. 5. Pcdh17 regulates growth cone motility. (A) Schematic illustration of growth cone behavior after contact with another axon. When both axons express Pcdh17 (wild type), the growth cones are able to migrate along the axon they encounter, whereas Pcdh17-deficient growth cones become stuck at the contact point with another axon. (B) A model for Pcdh17-mediated axon extension. Pcdh17 recruits the WAVE complex, lamellipodin and Ena/VASP to the contact sites between a growth cone and another axon. This might accelerate the motility of the cell membranes that are in contact by regulating actin polymerization in a way similar to that which occurs at the leading edge of migrating cells. This process might compete with the classic-cadherin-dependent CIL.

Embryonic brains express multiple δ2-protocadherin subtypes, each of which is expressed by distinct neuronal populations (Hertel et al., 2008; Kim et al., 2007; Kim et al., 2010; Krishna et al., 2011). Because of their binding specificities, it is expected that each of the δ2-protocadherins exclusively mediates the interactions between axons expressing the same δ2-protocadherin subtype, and therefore serves to aid the sorting of axons that are derived from different groups of neurons. Consistently, forced expression of Pcdh17 in a group of axons that did not originally express this protocadherin changes their extension path and leads them to intermingle with axons that endogenously express the same δ2-protocadherin (Hayashi et al., 2014), supporting the idea that δ2-protocadherins might be involved in axon sorting. By contrast, despite the broad expression of δ2-protocadherins in the brain, abnormalities in brain morphogenesis have been detected in only restricted regions of the brains of δ2-protocadherin-knockout mice (Hayashi et al., 2014; Uemura et al., 2007). Although the failure to detect phenotypes in knockout mice can be explained by assuming the functional redundancy of the genes or proteins that have been analyzed, it is equally possible that conventional histology does not detect subtle deficiencies occurring in a small population of neuronal cells. To overcome such potential technical problems, the genetic labeling of a subpopulation of cells expressing a particular protocadherin subtype might be an appropriate approach, as has been done for the classic cadherins (Duan et al., 2014).

Protocadherin dysfunction in neurological disorders and cancer

Protocadherin dysfunction has been implicated in neurological disorders, such as epilepsy, autism and schizophrenia (Hirabayashi and Yagi, 2014; Kim et al., 2011). Among the defects in protocadherin genes, mutations of *PCDH19* located on the X-chromosome have been studied most extensively. Clinical evidence indicates that *PCDH19* mutations cause epilepsy and mental retardation restricted to females (EMFR) (Duszyc et al., 2015). EMFR is an early infantile epileptic encephalopathy that phenotypically resembles Dravet syndrome, which is mainly caused by mutations in *SCN1A* – a gene encoding the voltage-dependent Na^+-channel α subunit 1 protein. Mutations in the *PCDH19* gene are now identified as the second most clinically relevant cause of epilepsy, after mutations in *SCN1A* (Duszyc et al., 2015). Mutations in *PCDH19* mainly arise *de novo*, but in the case of families with EMFR, a unique pattern of inheritance is observed – only females carrying heterozygous mutations are affected, whereas males that have the same mutations are not. Despite the increase in clinical evidence for *PCDH19* mutations in EMFR, the molecular mechanisms that cause EMFR are totally unknown.

Protocadherins are also implicated in tumorigenesis (van Roy, 2014). Long-range epigenetic silencing occurs in the chromosomes that contain the clustered protocadherins during colorectal tumorigenesis, as well as in a form of kidney cancer known as Wilms' tumor (Dallosso et al., 2009; Dallosso et al., 2012). Non-clustered protocadherin genes are also subject to epigenetic silencing in tumors or deletion of chromosomes (Kim et al., 2011; van Roy, 2014). Notably, homozygous deletion

of chromosome 13q21, which contains four non-clustered protocadherins (*PCDH8*, *PCDH9*, *PCDH17* and *PCDH20*), often occurs in prostate cancers, as well as in breast cancer cell lines (Cerami et al., 2012; Yu et al., 2008). Based on these findings, protocadherins are thought to be tumor suppressors, although their relevance to tumorigenesis requires further investigation.

Concluding remarks

It is now clear that the biological functions of the clustered and non-clustered protocadherins are quite distinct from those of the classic cadherins. The classic cadherins are essential for stabilizing cell–cell adhesion, protocadherins appear to antagonize or weaken cell–cell adhesion. Clustered protocadherins are important for self-avoidance of neurites, and some of the non-clustered protocadherins promote cell motility. Despite such apparently anti-adhesive functions, many of the protocadherins, although not all of them, do promote cell aggregation under experimental conditions, causing a paradox with regard to their cellular functions. It is highly probable that the homophilic interactions between protocadherins through their extracellular regions are prerequisite to produce cytoplasmic signals, and this initial interaction promotes a mechanical linkage of apposed cell membranes, that is, cell adhesion. However, it can be assumed that such extracellular interactions, in turn, interfere with the classic-cadherin-based adhesion, resulting in a competition between the adhesion-promoting and -inhibiting actions of the cadherins and protocadherins, respectively. In the case of Pcdh17, the binding of the WAVE complex to Pcdh17 occurs only at peripheral sites, probably owing to the presence of active Rac there (Hayashi et al., 2014), even though Pcdh17 is distributed throughout cell–cell contacts (Fig. 4B). This suggests that the ability of Pcdh17 to antagonize cell adhesions, which requires cytoplasmic partners, is elicited only at localized sites of the cells. Therefore, the ability of protocadherins to either promote cell adhesion or inhibit it might be regulated through the physiological cellular contexts.

A number of other problems remain to be clarified. To further understand the *in vivo* functions of the clustered protocadherins, it is important to determine whether Pcdhα and Pcdhβ isoforms also regulate self-avoidance between neurites, and whether the clustered-protocadherins have any other roles. Continued analyses of such knockout mice should be helpful to this end. It is also important to identify cytoplasmic factors that interact with clustered protocadherins in order to investigate the molecular mechanisms of how these protocadherins control neurite patterning, including self-avoidance. Live imaging of neurites undergoing self-avoidance would be helpful in order to elucidate the cellular mechanisms underlying this process, which has been successfully used to analyze the avoidance of Purkinje cell dendrites (Fujishima et al., 2012). It is also crucial for our global understanding of the functions of this molecular group to determine whether and how the Pcdhγ-dependent self-avoidance is related to its reported role in cell survival.

Concerning the non-clustered group, the *in vivo* analysis of δ1-protocadherins using knockout mice has not been reported yet, and therefore this line of analysis is urgent. With regards to molecular mechanisms, it is important to identify the functions of CM1 and CM2 motifs, which are conserved not only in the δ1 but also in the δ2 group. A recent finding that these motifs in PAPC contain GSK3-dependent phosphorylation sites (Kai et al., 2015) provides a clue for this line of studies. As for the reported functions of δ2-protocadherins, our molecular interpretations of how they facilitate cell motility and affect classic-cadherin-mediated adhesion are still incomplete. It is therefore urgent to uncover more detailed mechanisms by which the complex of δ2-protocadherin, WAVE complex, Lpd and Ena/VASP regulates actin dynamics and enhances membrane motility at cell–cell interfaces, and how these processes promote cell migration. In addition, various cytoplasmic factors have been reported as binding partners for different δ2-protocadherin subtypes (Table 1). It is therefore necessary to ascertain whether the subtypes of this molecular group have any subtype-specific functions. Finally, linking the basic studies of protocadherins to protocadherin-related diseases is most important, as it might contribute to the development of therapeutic strategies. Overall, studies of the protocadherins are steadily progressing, deepening our understanding of cell–cell interacting mechanisms, as well as contributing to efforts to treat human brain diseases.

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Competing interests

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