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Writing, Reading, and Translating the Clustered Protocadherin Cell Surface Recognition Code for Neural Circuit Assembly
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Supplemental Text, Section 1

The role of *Drosophila Dscams* in self-avoidance and tiling.

Self-avoidance and tiling are two distinct, but related mechanisms by which axons and dendrites achieve proper spacing between one another to maximize target coverage. Both self-avoidance and tiling depend upon the intrinsic ability of individual neurons to recognize themselves, and avoid crossing over of their neurites through repulsion-based molecular mechanisms. Self-avoidance promotes repulsion between sibling neurites of the same neuron by their ability to distinguish self from non-self. Tiling, on the other hand, is defined as the property of neighboring neurites of different neurons of the same functional cell type to recognize and avoid one another (Dong et al 2015, Grueber & Sagasti 2010, Zipursky & Grueber 2013).

The molecular and cellular mechanisms of self-avoidance and tiling have been revealed by an elegant series of biochemical, structural and genetic studies of Down Syndrome Cell Adhesion Molecules Dscam1 and Dscam2 genes in *Drosophila,* both encoding transmembrane proteins of the immunoglobulin (Ig) family (Hattori et al 2008, Millard & Zipursky 2008, Zipursky & Grueber 2013, Zipursky & Sanes 2010). Stochastic alternative splicing of the *Dscam1* Pre-mRNA generates up to 19,008 extracellular protein isoforms, each with a distinct protein sequence (Schmucker et al 2000). This mechanism allows for the generation of enormous neuronal cell surface diversity, which acts as a cell surface “barcode”, and provides individual neurons with distinct “identities”. On average, an individual neuron is estimated to express a repertoire of 10 to 50 distinct Dscam1 isoforms. Interestingly, this repertoire can switch during the life-time of a neuron (Miura et al 2013).

Dscam1-mediated self-avoidance in *Drosophila* relies on the ability of the *Dscam1* gene to generate enormous protein diversity, and on the ability of Dscam1 proteins to engage in highly specific homophilic interactions between the same isoforms located on opposing surfaces of sister neurites (Wu et al 2012). Such interactions, however, are not adhesive. Rather, they result in neurite repulsion. As in the case of the vertebrate clustered Pcdhs, the mechanisms by which homophilic recognition leads to neurite repulsion remains unknown. While protein isoform diversity by Dscam1 is required in multiple neuronal cell types, the actual number of isoforms necessary to promote self-avoidance varies between different neuronal cell types (Petrovic & Schmucker 2015, Zipursky & Grueber 2013). Reduction of Dscam1 protein diversity leads to excessive Dscam1 mediated homophilic interactions and ectopic repulsion between non-sister neurites, resulting in the disruption of neuronal circuits (Hattori et al 2007). The *Dscam2* gene has been shown to be required for tiling in *Drosophila*, which is necessary to control proper spacing between axonal termini of lamina interneurons during development of the retina (Millard et al 2007). In stark contrast to *Dscam1*, the *Dscam2* gene encodes only 2 protein isoforms generated by alternative pre-mRNA splicing (Lah et al 2014). Owing to the cell type specific splicing of *Dscam2,* neurons of the same type display identical trans-membrane protein isoforms (Lah et al 2014). Similar to Dscam1, Dscam2 proteins also mediate homophilic interactions followed by repulsion (Lah et al 2014). Recent studies have shown that Dscam2 might also play a strictly “adhesive role” in neural circuit assembly, raising the intriguing possibility that Dscam2 might also function in a tiling-independent mechanism (Tadros et al 2016).

Remarkably, although mammals have two Dscam genes (*Dscam* and *Dscaml1*), their genomic organizations are similar to *Drosophila Dscam2* - that is, they do not encode significant protein diversity. (Schmucker & Chen 2009, Zipursky & Grueber 2013). Mammalian Dscam proteins play an important role in neurite spacing, however they are not functional homologs of the *Drosophila* Dscam1 proteins (Schmucker & Chen 2009). Moreover, at a mechanistic level, mammalian Dscam proteins do not mediate neurite repulsion, but rather appear to mask cell-type-specific adhesive interactions between dendrites of the same cell type (Zipursky & Grueber 2013).

Supplemental Text, Section 2

Evolutionary conservation of the clustered *Pcdhs*.

While the clustered Pcdhs are absent from the genomes of the ecdysozoan model organisms, such as *D. melanogaster* and *C. elegans*, non-clustered Pcdhs have been identified in the sea slug *A. californica* and the starlet sea anemone *N. vectensis*; thus, this protein family predates the Bilateria (Hulpiau & van Roy 2011). The existence of a diverse set of clustered Pcdh isoforms has been found in cartilaginous fishes (e.g. elephant shark), which are the oldest living phylogenetic group of jawed vertebrates, bony fishes (e.g. zebra fish and *fugu*) and coelacanth, which is the closest living relative of all tetrapods, as well as in amphibians (e.g. *Xenopus*) (Etlioglu et al 2016, Hulpiau & van Roy 2009, Noonan et al 2004a, Tada et al 2004, Yu et al 2008). However, these isoforms are not orthologous to the mammalian Pcdh isoforms (Noonan et al 2004b). A striking example of Pcdh gene expansion is provided by mollusks (cephalopod) where a dramatic expansion of the number of Pcdh genes is observed between oysters and octopus. This

selective increase in gene number correlates with a major difference in the size and complexity of the nervous system, and sophisticated behavior (Albertin et al 2015). It is interesting to note that in addition to this gene expansion, Octopus displays unprecedented levels of adenine to inosine (A to I) RNA editing in coding regions (Liscovitch-Brauer et al 2017). Thus, the combination of gene expansion and RNA editing could result in high levels of Pcdh diversity in octopus, compared to its immediate ancestor.

Among vertebrates, the diversity and genomic structure of clustered Pcdhs are highly conserved, but individual isoforms are not. Most human Pcdh isoforms are orthologous to the mouse isoforms in the corresponding position (Wu et al 2001). However, in both species certain individual isoforms have been lost or become relics from each cluster, and isoform duplications have occurred in the *Pcdhα* and *Pcdhβ* gene clusters (Noonan et al 2004b, Wu et al 2001). Additionally, all three clusters also show signs of gene conversion between parts of the variable region exons (Noonan et al 2004b). Together, these observations suggest that the clustered *Pcdhs* must have appeared very early in the vertebrate evolution. Their expansion and enrichment in the nervous system in vertebrates and other organisms is an example of convergent evolution at the molecular level.

Given the fact that insects and vertebrates shared a common ancestor about 500 million years ago, the striking functional similarity between *Dscam1* and clustered *Pcdhs* in the nervous system is remarkable, highlighting the necessity of self-avoidance and tiling in the assembly of neural circuits during vertebrate evolution.

Of particular interest to the divergent evolution of *Dscam1* and clustered *Pcdh* genes is the existence of a hybrid gene family that has been recently identified in the subphylum Chelicerata. There, shortened *Dscam* genes (*sDscam*) with high sequence similarity to the 3' region of *Drosophila Dscam1* are arranged in tandem clusters that resemble the clustered *Pcdhs* (Yue et al 2016). The unique genomic organization of *sDscam* genes suggests the existence of a hybrid mechanism to generate cell surface diversity in Chelicerata. That is, the sDscam protein diversity is generated in a similar fashion of clustered Pcdhs – promoter choice of alternate isoforms from a gene cluster, rather than by alternative splicing from a single gene.

Supplemental Text, Section 3

Additional roles of clustered Pcdhs in neuronal development.

One of the initial roles identified for clustered Pcdhs is neuronal survival, which emerged from the analysis of Pcdhγ-deficient mice. Mutant neonates completely lack of coordinated movement, although they display spinal reflexes, and die shortly after birth. In both the spinal cord and retina, elevated apoptosis was observed in specific subtypes of neurons, and the neuronal loss was accompanied by reduced numbers of synapses (Lefebvre et al 2008, Prasad et al 2008, Wang et al 2002b, Weiner et al 2005). Although genetically blocking apoptosis in these mutants rescued the neuronal survival and partially the synaptic defects (Weiner et al 2005, Lefebvre et al 2008), it failed to rescue the neonatal lethality. This observation suggested that the Pcdhγ proteins have additional and perhaps neuron-type dependent roles during postnatal development. Chen et al. demonstrated that the role of the *Pcdhγ* gene cluster in neuronal survival is primarily, if

not entirely, mediated by one or more of the Pcdhγ C-type isoforms (Pcdhγc3, Pcdhγc4 and Pcdhγc5) (Chen et al 2012). In contrast to the *Pcdhγ* full gene cluster knockouts, genetically blocking apoptosis rescued the neonatal lethality of the C-type specific knockouts, which survived up to 6 months despite apparent motor defects (Chen et al 2012). Deletion of the entire Pcdh locus, containing all of the 58 Pcdh genes, led to early embryonic lethality, which was attributed to the loss of *Taf7*, an essential component of the TFIID complex (Hasegawa et al 2016, Mountoufaris et al 2017). Adding back the co-deleted non-*Pcdh* genes and their putative regulatory elements into the *Pcdhαβγ -/-* genetic background rescues embryonic lethality and homozygous mutants were recovered alive at P0. Interestingly, these mutant mice appear to show more compromised neurological functions than those of *Pcdhγ -/-* neonates (Hasegawa et al 2016, Mountoufaris et al 2017). Specifically, the level of neuronal death observed in spinal cord, brainstem and retina increased as more Pcdh gene clusters were deleted (*Pcdhγ -/-* < *Pcdhβγ -/-* < *Pcdhαβγ -/-*) (Hasegawa et al 2016). When the Pcdh*αβγ* -/- neurons were transplanted in a wild type neural network, an increase in apoptotic death was observed (Hasegawa et al 2017). Moreover, the central pattern generator (CPG) locomotor pattern was disrupted in Pcdh-null embryos in which apoptosis was genetically blocked (Hasegawa et al 2017).

Owing to the high levels of neuronal cell death in *Pcdhγ* KO mice, it was difficult to determine whether Pcdhγ proteins have a direct role in neuronal wiring, so conditional KO studies were carried out. When the *Pcdhγ* gene cluster was conditionally deleted in the forebrain, a reduction in dendritic arborization in cortical neurons was observed (Garrett et al 2012). Using loss- and gain-of-function experiments on the *Pcdhγ* gene

cluster in pyramidal neurons and astrocytes in the cortex, Molumby et al reported that Pcdhγ isoforms control dendritic branching and arborization independently of contact mediated repulsion (Molumby et al 2017). Recent evidence indicates that Pcdhγ proteins interact with Neuroligin 1, a synaptic cell adhesion molecule that modulates the assembly and maturation of synapses between neurons (Bemben et al 2015). Such interactions negatively control dendritic spine number and morphology, in order to promote dendritic arborization of cortical neurons (Molumby et al 2017). In addition, Pcdh-γC5 has been implicated in the stabilization and maintenance of some GABAergic synapses (Li et al 2012) and Pcdhγ protein isoform(s) have been reported to modulate dendritic arborization and synaptic density in granule cells of the olfactory bulb (Ledderose et al 2013).

In contrast to *Pcdhγ* deficient mice, which die as neonates, *Pcdhα* knockouts and severe hypomorphs are viable and fertile with no obvious defects (Chen et al 2017, Hasegawa et al 2008, Katori et al 2009, Suo et al 2012, Wu et al 2007). Early studies implicated a role for *Pcdhα* in axonal patterning in the olfactory system, as *Pcdhα* hypomorphic mice displayed subtle abnormalities in axonal projections of olfactory sensory neurons (OSNs) to the olfactory bulb (Hasegawa et al 2008). A small number of satellite glomeruli surrounding the main glomerulus, which are normally eliminated during postnatal development, remained in hypomorphic *Pcdhα* adult mutant animals (Hasegawa et al 2008). Constitutive expression of a single isoforms (*Pcdhα1)* in each OSN was sufficient to “rescue” the satellite glomeruli phenotype of *Pcdhα* mutant mice, arguing for a role for Pcdhα proteins in satellite glomeruli elimination (Hasegawa et al 2008, Hasegawa et al 2012). Yet, further studies are required to determine the relationship between the satellite glomeruli elimination phenotype in *Pcdhα* hypomorphic

mice and role of Pcdh isoforms in axonal arborization of OSNs (or their synaptic partners). Besides the role of *Pcdhα* in olfactory system, the optic nerve fiber projections in the dorsal lateral geniculate nucleus (LGd) and in the primary visual cortex aggregate in *Pcdhα* hypomorphic mutants (Meguro et al 2015). This phenotype is reminiscent of the axonal arborization defects of serotonergic neurons (Chen et al 2017), although it is not known whether this is due to defective tilling. Perhaps not surprisingly, these mutant mice display defects in visual discrimination behaviors that assess visual acuity (Meguro et al 2015). *Pcdhα* genes have also been implicated in dendritic development and spine morphogenesis (Suo et al 2012), and more recently in cortical neuron migration (Fan et al 2018). Together, these findings support additional roles for Pcdh isoforms that are likely independent of self-avoidance. However, the full Pcdh repertoire in many neuronal cell types mentioned above is currently unknown, which complicates some of the interpretations of experiments focused on single *Pcdh* gene cluster analysis. Thus, further analysis will be required in order to understand the intricate relationship between the different reported roles of clustered Pcdhs *in vivo.*

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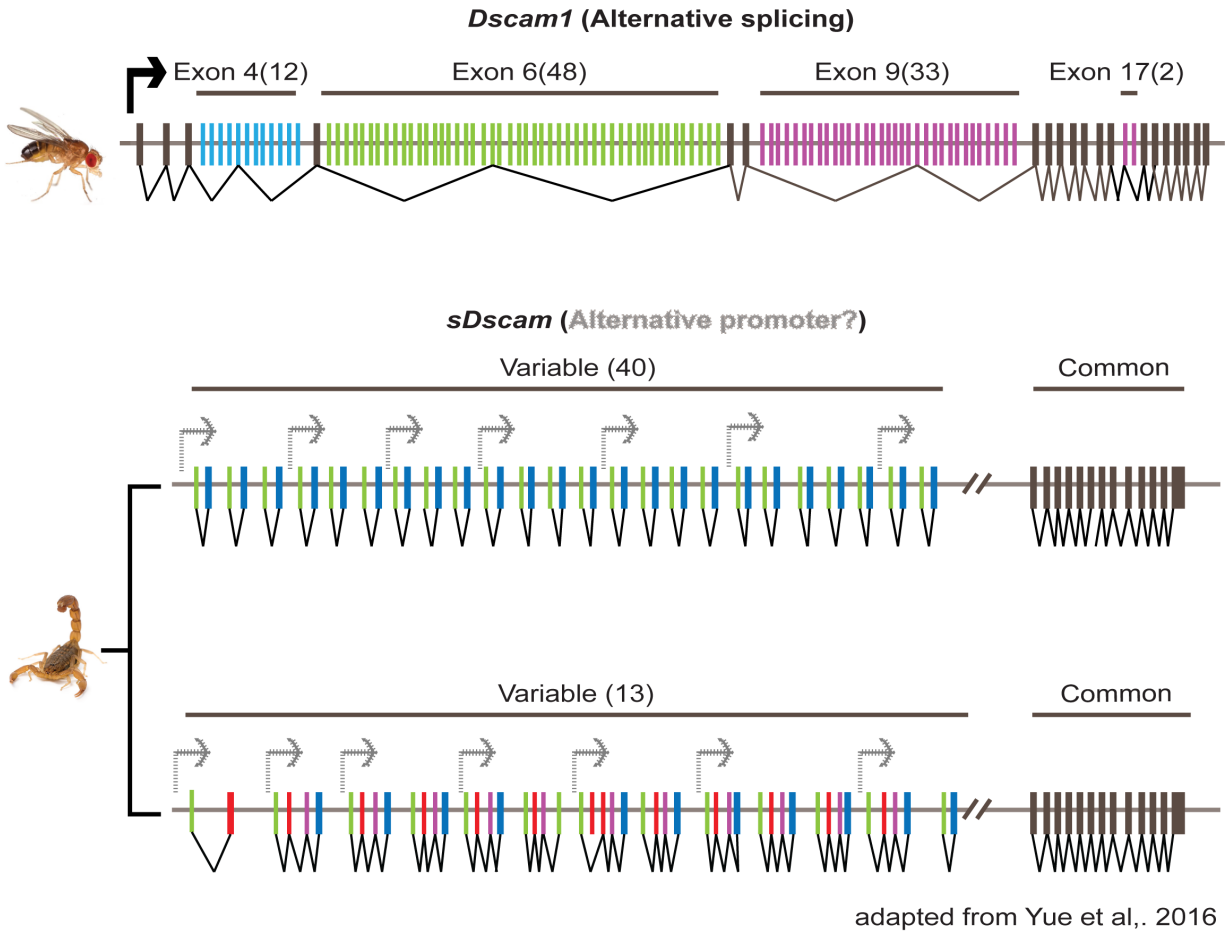

Supplemental Figure 1

Genomic organization and transcription of Dscam genes in *Drosophila melanogaster* and *Mesobuthus martensii.* The tandem array of *Dscam1* and *sDscam* exons are depicted as colored or black boxes. Colored boxes represent variable exons. Black boxes represent constitutive exons. (figure adapted by Yue et al., 2016).