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Review

Cadherin genes and evolutionary novelties in the octopus

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ABSTRACT

All animals with large brains must have molecular mechanisms to regulate neuronal process outgrowth and prevent neurite self-entanglement. In vertebrates, two major gene families implicated in these mechanisms are the clustered protocadherins and the atypical cadherins. However, the molecular mechanisms utilized in complex invertebrate brains, such as those of the cephalopods, remain largely unknown. Recently, we identified protocadherins and atypical cadherins in the octopus. The octopus protocadherin expansion shares features with the mammalian clustered protocadherins, including enrichment in neural tissues, clustered head-to-tail orientations in the genome, and a large first exon encoding all cadherin domains. Other octopus cadherins, including a newly-identified cadherin with 77 extracellular cadherin domains, are elevated in the suckers, a striking cephalopod novelty. Future study of these octopus genes may yield insights into the general functions of protocadherins in neural wiring and cadherin-related proteins in complex morphogenesis.

Contents

1. Evolution of brains & the octopus nervous system

Nervous systems are circuits of cells dedicated to organizing animal behaviors. Though all bilaterian animals have nervous systems, not all bilaterians have brains. Brains arose independently in bilaterians at least four times in evolutionary history, in arthropods, annelids, molluscs, and chordates (Fig. 1) [1]. Other bilaterians, in contrast, feature nervous systems of decentralized nerve nets and nerve rings (sea stars, roundworms) or discrete ganglia (gastropod molluscs) [1]. The evolution of bilaterian nervous systems from ancient nerve nets into complex brains must have required novel molecular mechanisms to facilitate the correct formation of functional neural units.

Soft-bodied or coleoid cephalopods (cuttlefish, squid, and octopus) present a particularly interesting group for comparative molecular research due to their complex behaviors and large brains. Cephalopods belong to the phylum Mollusca, an ancient and successful group that also includes the bivalves (oysters, mussels, clams) and gastropods (slugs, snails, limpets) (Fig. 1) [2]. Cephalopods diverged from bivalves and gastropods ~540 million years ago (mya), at the edge of the Ediacaran and Cambrian Periods [3,4]. Early cephalopods were extremely prolific: the fossil record reveals over ~4000 genera occupying marine habitats worldwide [4]. The coleoids diverged from the hard-shelled nautiloids ~416 mya [5], and subsequently developed many striking morphological features, including their large nervous systems (see Section 6).

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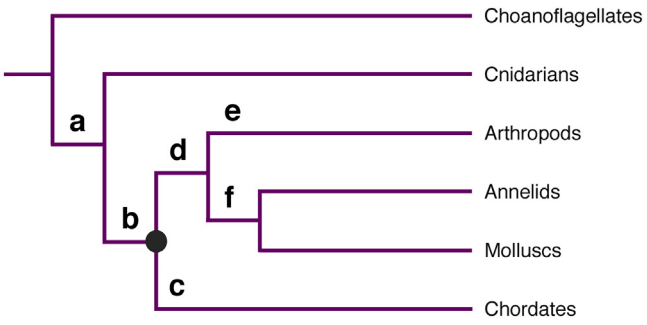

Fig. 1. Simplified Phylogeny of Extant Metazoans. A great diversity of neural characters exists in metazoans (a), but brains are found only in bilaterians (b). Both deuterostomes (c) and protostomes (d) include lineages with brains. Chordate, ecdysozoan (e), and lophotrochozoan (f) brains arose independently from one another. Octopuses belong to the phylum Mollusca. Circle represents last common ancestor of deuterostomes (such as humans) and protostomes (such as octopuses).

The cephalopod body plan, as illustrated for the octopus (Fig. 2a), features 3 major parts: the mantle, the head, and the foot. In the octopus, the foot makes up the eponymous eight arms [2]. Each arm is a muscular hydrostat lined with one or two rows of suckers (see Section 6). The head of the octopus contains its large brain (Fig. 2b), flanked by the two camera-like eyes. The mantle is a muscular cavity filled with the internal organs of the octopus, including two gills, three hearts, the ink sac, digestive tract, and gonads.

The central nervous system of octopuses consists of a central brain mass situated around the esophagus, a pair of optic lobes, and an axial nerve cord running down the length of each arm (Fig. 2b). The octopus nervous system represents a dramatic enlargement of the ancestral nautiloid nervous system: for example, the common octopus, *Octopus vulgaris*, has over half a billion neurons. Only about one third of these neurons reside in the central brain and optic lobes; the remaining neurons are located in the axial nerve cords of the eight arms [6]. The central brain above the esophagus (the supraesophageal mass) contains lobes for higher motor control as well as multiple lobes forming two largely separate learning and memory systems. The lobes of the subesophageal mass serve to control important functions such as adaptive coloration and regulation of vasomotor tone. The optic lobes contain more than twice the number of neurons found in the central brain, reflecting the important role that vision plays in these marine predators. In typical invertebrate fashion, the lobes and axial nerve cords are arranged with neuronal cell bodies in the outer layers of the lobes and the neuropil of neuronal processes in their centers [6].

The extraordinarily elaborate octopus nervous system stands out among invertebrates for both its absolute and relative size. Octopus brain-to-body ratio exceeds that of many fishes and amphibians [7]. Given the great evolutionary distance between vertebrates and octopuses (Fig. 1), the two brains show an incredible degree of convergent evolution [8]. For example, the vertical lobes, the learning and memory centers of the octopus brain, possess the fan-out-fan-in neural organization characteristic of vertebrate cerebellum [9,10]. The cephalopod lineage demonstrates the great complexity that nervous systems gained independently in bilaterian evolution.

2. Challenges to building a complex brain

Though vertebrate and invertebrate nervous systems have different morphologies, brains of the different evolutionary lineages face similar developmental challenges. Developing neurons must discern their own processes from those of other neurons and correctly target their synaptic partners. One solution to this challenge is to generate unique molecular identities at the cell surface so nascent neurons can recognize self from non-self, partner from non-partner before establishing stable connections [11,12]. *Drosophila* and mouse brains have found variations on a very similar molecular scheme to address this problem of self-recognition and proper synapse formation: arthropods generate many protein isoforms of one gene through alternative splicing while chordates use multiple genes within a single superfamily to yield multiple recognition proteins [13–16].

The arthropod *Drosophila melanogaster* utilizes Dscam1, the homolog of human Down Syndrome cell adhesion molecule, to provide neurons with exclusive identities [17]. *Dscam1* is unique for its four alternatively spliced exons, which have 12, 33, 48, and 2 variants, respectively [18,19]. By splicing together variant exons with a set of constant exons, flies can generate tens of thousands of Dscam1 protein isoforms with distinct complements of extracellular IgG domains from a single *Dscam1* gene [17,20]. The majority of Dscam1 protein isoforms form homodimers in *trans*, which then trigger repulsion between cell surfaces [12]. Recent molecular and genetic work has revealed that different *Dscam1* isoform subsets are expressed in a probabilistic fashion in different neurons, thus providing a molecular mechanism whereby processes of the same neuron can grow away from each other after touching, and remain free to form appropriate connections with other neurons [20].

The vertebrate DSCAM protein shows remarkable sequence conservation with *Drosophila* Dscam1 [21]. However, instead of using Dscam isoforms, vertebrates employ alternative splicing of the

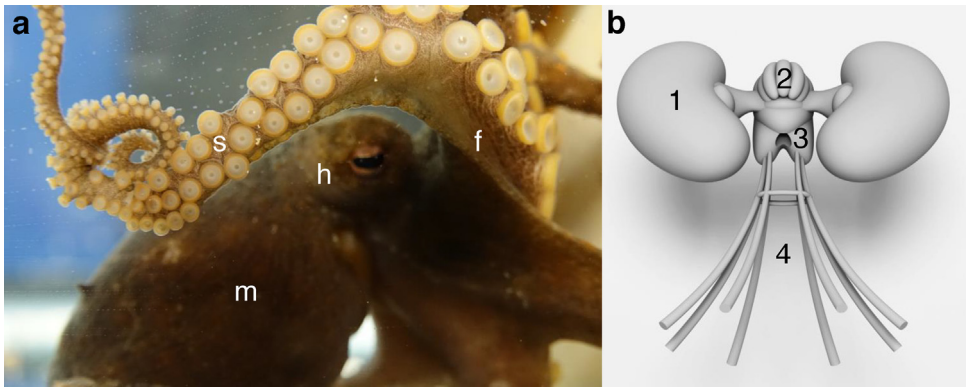

Fig. 2. Octopus Body (a) and Brain (b) Anatomy. Molluscan body plans are made up of three parts: the mantle (m), the head (h), and the foot (f), which corresponds to the arms of the octopus. The head contains the optic lobes (1) and the central brain mass, which is divided by the esophagus into the supra- (2) and subesophageal masses (3). Octopus arms are lined with suckers (s), a novel morphological innovation specific to coleoid cephalopods. An axial nerve cord (4) runs down the length of each arm. Copyrighted image used with permission of MICRO, The Smallest Mollusk Museum: www.smallestmollusk.com.

tandemly duplicated clustered protocadherin (*PCDH*) genes to generate cell-surface identity that mediates self-avoidance between neurons [14,22]. The PCDHs form the largest subfamily of vertebrate cadherins; the human and mouse genomes encode 53 and 58 clustered PCDHs, respectively, each with a distinct complement of six extracellular cadherin (calcium-dependent adhesion) domains [23]. PCDH proteins form multimeric complexes in *cis* that bind homophilically in *trans*, and even a single PCDH isoform mismatch can disrupt homophilic binding [15,24,25]. Through alternate promoter choice and heteromeric assembly, the 53 human *PCDH* genes could produce hundreds of thousands of unique molecular signatures. Homophilic binding is thought to facilitate cellular avoidance [11]. In addition, PCDHs serve as crucial mediators of circuit formation and dendritic patterning in the vertebrate nervous system [23,26–28].

Almost nothing is known about what molecular mechanisms mediate these functions in the annelid and molluscan brains. Indeed, the Annelida and Mollusca are in the superphylum Lophotrochozoa, and evolutionarily well removed from arthropods (Ecdysozoa superphylum) and chordates (Deuterostomia superphylum; Fig. 1). However, the recent sequencing of the *Octopus bimaculoides* genome, yielding discovery of octopus clustered *PCDH*s, suggests the surprising possibility that cephalopods may employ a major chordate molecular strategy for correct wiring of neural circuits.

3. Octopus PCDHs

The massive size and complex organization of the octopus brain necessitates a molecular solution for the proper establishment of neural circuits. The octopus genome revealed candidate gene families for such functions, including, most notably, an expansion of *PCDH* genes. We found 168 octopus *PCDH*s by baiting the octopus genome and tissue transcriptomes with available bilaterian *PCDH* sequences [3]. This was the first report of a *PCDH* expansion in any invertebrate species. This expansion of *PCDH*s in octopus is independent from those described in vertebrate genomes, however, they do resemble the mammalian *PCDH*s in many key ways.

Mammalian PCDHs are enriched in the nervous system, where they are essential for circuit formation [14,29]. Octopus *PCDH*s are also predominantly expressed in the nervous tissues and appear dedicated to nervous system function. Some octopus *PCDH*s are broadly elevated throughout the nervous system, while others show restricted expression to specific neural tissues. In particular, the optic lobes and the axial nerve cord present an impressive enrichment in *PCDH*s [3]. These structures will likely be central to future study of cephalopod PCDH function.

Octopus *PCDH*s cluster together on the genome [3] mirroring the unique organization of mammalian clustered *PCDH*s in which the α, β, and γ clusters are arrayed next to each other along the same chromosome [14]. Individual exons of the α and γ clusters are arranged and transcribed in the same direction. Genes of the β cluster, which contain only a single exon, are also all transcribed in the same direction. The three largest octopus clusters identified have 31, 17, and 10 *PCDH*s, respectively [3]. At least 25 other scaffolds contain two or more *PCDH*s. The clustered octopus *PCDH* genes are also arranged in head-to-tail fashion, indicating uniform, unidirectional transcription of these genes. Future experimental studies will reveal the functional implications of these shared genomic structures, such as, for example, whether the CTCF-mediated mechanism of chromatin looping, exon transcription, and stochastic expression is a shared feature of the vertebrate and octopus clustered protocadherins [30,31].

All six cadherin domains of the human clustered *PCDH*s, as well as the transmembrane domain, are coded by an exceptionally large first exon (>2400 nt). Other cadherins, by contrast, have a more typical exon-intron organization, in which several short exons make up each cadherin domain (Fig. 3a) [32,33]. Octopus clustered *PCDH*s also encode six extracellular cadherin domains along with conserved transmembrane domains [3], all of which are present on a large first exon (Fig. 3b). The conservation of exon-to-protein domain correspondence, coupled with the tandem arrangement of *PCDH* clusters, provide evidence that the fundamental evolutionary unit of both human and octopus clustered *PCDH* genes is a large first exon.

In vertebrates, there are twelve non-clustered protocadherins, which are collectively called δ-PCDHs [34]. δ-PCDHs have 6 or 7 extracellular cadherin domains. In addition, ten of the δ-PCDHs share motifs in their intracellular domains (CM1 and CM2, and sometimes CM3), which frequently appear as alternatively spliced variants [34,35]. We searched for evidence of these motifs among the octopus clustered and non-clustered *PCDH* genes. A motif only distantly related to CM1 was identified in the predicted cytoplasmic domain of four alternatively spliced octopus PCDHs. We conclude that, although some unclustered octopus PCDH genes predict 7 extracellular cadherin domains, the δ-PCDH designation is not particularly informative for octopus PCDH categorization.

The further study of octopus PCDHs is seriously impeded by the absence of crucial experimental techniques, the most critical of which will be gene manipulation and cell culture, and by the absence of any other sequenced cephalopod genomes. This latter limitation will surely be overcome soon as multiple cephalopod genome projects are currently underway [36]. Indeed, initial surveys of assembled transcriptomes reveal a substantial expansion of *PCDH* genes in the longfin inshore squid and the cuttlefish *Sepia officinalis* but not in nautilus, which lacks the elaborated coleoid nervous system [3,37]. Strikingly, the octopus and squid *PCDH*s are significantly enriched in RNA editing sites, but nautilus *PCDH*s are not [37]. With few exceptions, the octopus and squid *PCDH* sequences group separately on the phylogenetic tree. This lineage-specific phylogenetic pattern has also been documented for *PCDH*s of different vertebrate lineages and may be the result of "concerted evolution" [38]. Only extensive RNAseq data and genome assemblies of both closely and distantly related cephalopods can fully clarify the relative contributions of intracluster gene conversion, independent *PCDH* expansions and contractions, and possibly other mechanisms to this striking concerted evolution that is shared between the vertebrate and cephalopod clustered gene families.

4. Lophotrochozoan PCDHs

In recent years, several PCDHs have been identified in many invertebrate species. The genome of the starlet sea anemone *Nematostella vectensis*, a cnidarian with a diffuse nerve net, was found to contain a single *PCDH* gene [39]. By contrast, in the polychaete annelid worms *Capitella teleta* and *Platynereis dumerilii*, we found 18 and 29 *PCDH*s, respectively [3]. The gastropod mollusc *Aplysia californica*, the California sea hare, has 12 *PCDH*s [3], at least one of which has been verified independently [39,40]. The owl limpet *Lottia gigantea*, another gastropod mollusc, and Pacific oyster *Crassostrea gigas*, a bivalve mollusc, were found to have 17 *PCDH*s each [3]. Importantly, these newly identified molluscan *PCDH*s show various degrees of genomic clustering. For example, in *L. gigantea,* 14 *PCDH*s group together on one scaffold while the *C. gigas PCDH*s are arranged in two clusters of four and three doublets [3].

The presence of *PCDH*s in cnidarian, annelid, molluscan, and chordate lineages indicates an ancient origin for these proteins. Phylogenetic analyses of all bilaterian *PCDH*s show that they segregate by lineage: the annelid, molluscan, and mammalian *PCDH*s

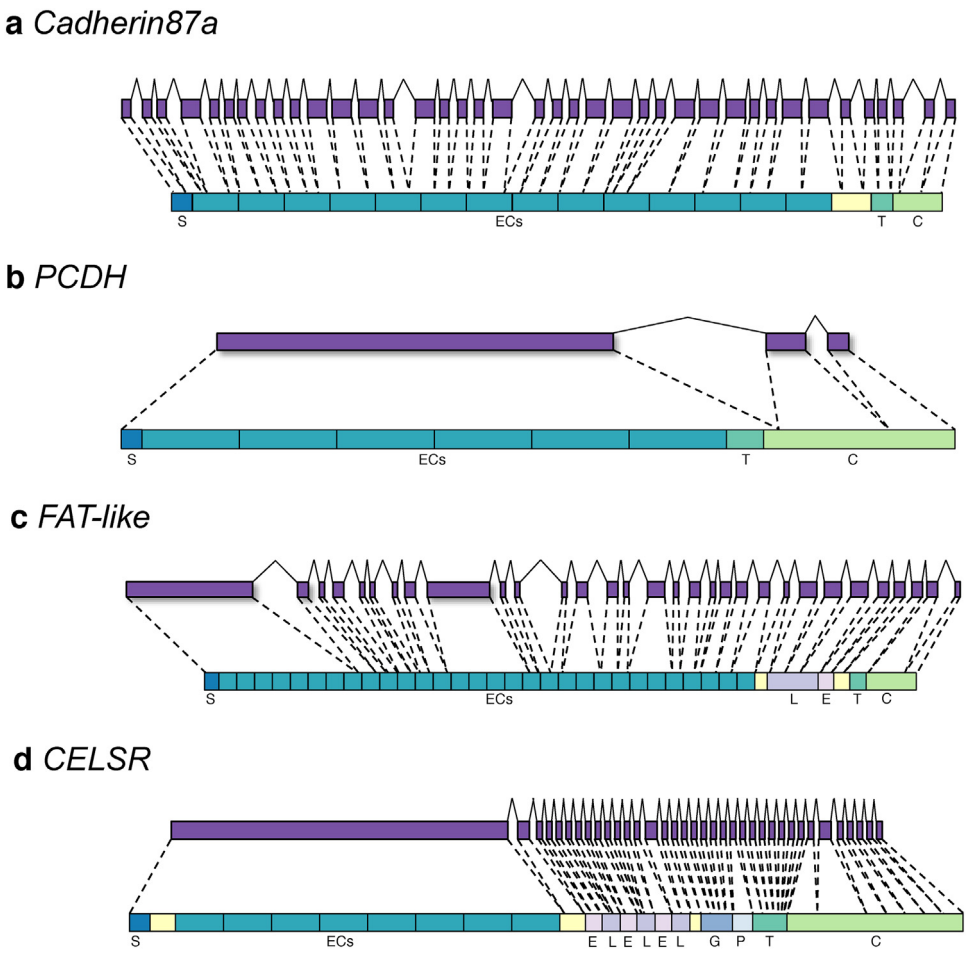

Fig. 3. Conserved Exon-to-Domain Correspondence in Octopus Cadherins. The cadherin family of genes in octopus and humans share exon-intron structure. Coding exons of octopus genes represented in purple. S, signal sequence; ECs, extracellular cadherin domains; T, transmembrane domain; E, EGF-like domain; L, laminin G domain; G, GPCR autoproteolysis inducing (GAIN) domain; P, GPCR proteolysis site. Yellow represents regions with no identified protein domain structure. a. The octopus homolog to *Drosophila* Cadherin87a, like the human classical cadherins, has a highly fractionated genomic structure in which many small exons code for the cadherin domains. b. Octopus clustered protocadherins contain a large first coding exon that accounts for all of the cadherin domains and the transmembrane domain, and a variable number of exons that code for the cytoplasmic region. This unique organization was first discovered in the human protocadherins [31]. c. *FAT* genes encode their cadherin domains through a combination of large and small exons. Octopus and human *FAT* genes have 2 sizable cadherin exons separated by several small exons. d. CELSRs, like PCDHs, have a large first exon that accounts for all of its cadherin domains.

almost all group separately from each other [3]. Thus, at least one *PCDH* gene was most likely present in the last common bilaterian ancestor, secondarily lost in lineages that gave rise to modern arthropods, and elaborated in molluscan, annelid, and chordate clades.

5. Octopus atypical cadherins

The clustered *PCDHs* are by no means the only cadherins implicated in neural process outgrowth and maintenance of the synapse. Recent research in *Drosophila* and mouse models demonstrates that the atypical cadherins are involved in stabilizing cell junctions and dendrite growth [23]. Interestingly, these atypical cadherins, which include the FAT/FAT-like, Dachsous, and the 7TM cadherins, are present in the genomes of *C. elegans* and *D. melanogaster*, which lack the PCDHs altogether [41,42].

We identified four octopus *FAT/FAT-like* genes, which are all elevated in the neural transcriptomes, as well as a *Dachsous* gene. The *FAT* genes encode large cadherins that are implicated in the formation of dendritic arbors through binding of its ligand Dachsous [43,44]. To participate in this signaling, FAT4 must fold its 34 ectodomains to fit in the extracellular space [45]. Three octopus genes have 30–33 cadherin domains, and the partial sequence of the fourth gene reveals 13 cadherin domains. As in humans, the cadherin domains are coded by a combination of large and small exons (Fig. 3c) [33].

The 7TM cadherins are also important for neurite development [46,47]. The human *CELSR* genes are special adhesion GPCRs with cadherin domains at the N-terminal that are encoded by a large first exon [33,48]. We have identified a single *CELSR* gene in the octopus genome, as well as one each in the genomes of other lophotrochozoans, including *C. gigas*, *L. gigantea*, and *C. teleta*. The three mammalian CELSRs have distinct neural expression and developmental roles [46,47]. For example, CELSR2 promotes dendrite outgrowth and branching in cortical pyramidal neurons, but CELSR3, which is central for neuronal motility and axonal development, opposes these actions [47,49,50]. These behaviors depend on a single amino acid difference in the first loop of the transmembrane region: CELSR3 has a histidine residue conserved across the secretin-type GPCRs, but CELSR2 has an arginine substitution [47]. The octopus *CELSR*, which shares gene organization with human *CELSRs* (Fig. 3d), also shows the arginine substitution [33]. This illustrates the great importance of developing cell culture techniques in cephalopod molluscs to examine the functional role of octopus cadherins, including protocadherins, CELSR, and other octopus atypical cadherins (see below).

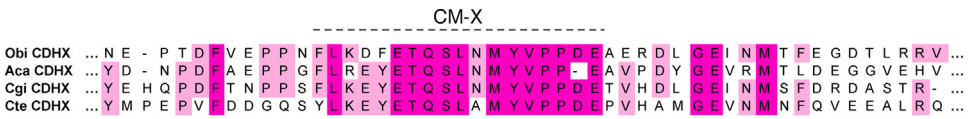

Fig. 4. A Novel Conserved Amino Acid Motif Identified in the Cytoplasmic Domain of CDHX. CDHX is an extraordinarily large member of the cadherin gene family and is found only in lophotrochozoan species. Alignment of the cytoplasmic region reveals a highly conserved motif (CM-X, Conserved Motif of CDHX). Obi, *Octopus bimaculoides*; Aca, *Aplysia californica*; Cgi, *Crassostrea gigas*; Cte, *Capitella teleta*.

6. Cadherins and coleoid innovations

Large brains are but one of the morphological innovations that are found in coleoid cephalopods but not in their ancient cephalopod relatives, the nautiloids [51–53]. One prominent novelty is the suckers of the coleoid arms (Fig. 2a). Suckers provide coleoids with tactile and chemosensory information about their environment that is important for motor actions such as prehensile prey capture [54,55]. It has been proposed that novelties in coleoid body morphology and neural control structures may have been driven by millions of years of an "arms race" against other marine predators, such as fish [7]. Suckers, which are exquisitely sensitive sensorimotor organs, would have provided early soft-bodied cephalopods with a great advantage over both their hard-bodied counterparts and early vertebrates [4].

Despite the importance of suckers, little is known about the genes responsible for their innervation, development, or function. Transcriptome analyses revealed that the octopus suckers are particularly enriched in cadherin gene expression [3]. The most highly expressed cadherins include a new member of the cadherin-related gene family, octopus cadherin-related-3-like, and one of the two octopus homologs to the *Drosophila* gene cadherin 87a.

We have also discovered an extraordinarily large cadherin gene [3]. This octopus cadherin, tentatively named *CDHX*, encodes 77 extracellular cadherin domains and is expressed in the suckers. Using the octopus *CDHX* sequence as bait, we identified homologs of this exceptionally large cadherin in the genomes of two other molluscs, *A. californica* and *C. gigas*, and the annelid worm *C. teleta*. Each member of the *CDHX* gene family has between 77 and 84 extracellular cadherin domains, a single transmembrane domain, and a short cytoplasmic region with a highly conserved motif (Fig. 4).

Homologs of this gene have yet to be found in non-lophotrochozoan animals. Surprisingly, however, extra-large cadherin genes, with 56 extracellular cadherin domains, have been identified in the genomes of choanoflagellates, one of the closest living relatives to metazoans (Fig. 1) [56,57]. Interestingly, both the choanoflagellate cadherin-related genes and octopus CDHX lack some calcium-binding motifs [56,58]. The loss of calcium-binding motifs in some cadherin domain linker regions permits the mammalian FAT4 and Dachsous cadherins to adopt hairpin-like bends among their extracellular domains [45]. If the CDHXs are indeed involved in cell–cell interactions, the ectodomains may also adopt non-linear shapes that permit them to fit in typical intercellular spaces in a manner similar to that of the large mammalian cadherins.

Only future structural and experimental analyses of the extraordinarily large *CDHX* gene will reveal its conformation and possible modes of binding. Because *CDHX*-like genes have not been identified in non-bilaterian metazoan genomes, it is most likely that the lophotrochozoan CDHXs are unrelated to the choanoflagellate proteins, although some similarity in their functions cannot be precluded.

7. Conclusion

Cephalopods genomics, though still in its infancy, has emerged as an outstanding system for the study of brain evolution. The octopus genome has revealed that these invertebrates show remarkable convergent evolution with vertebrates on both the anatomical and molecular levels. A greatly expanded set of clustered *PCDH*s, as well as other cadherins, has been discovered in the octopus genome. Remarkably, the brain and suckers, which are morphological novelties of the soft-bodied cephalopods, show the highest enrichment of cadherin expression, including a novel gene, *CDHX*, with 77 extracellular cadherin domains. As of now, these data reside almost exclusively in the realm of genomics and transcriptomics. The development of molecular and genetic techniques in cephalopods, as well as the availability of other cephalopod genomes, promises to reveal the rich history that PCDHs and cadherins play in the evolution of bilaterian brains and morphological novelty.

Methods: The lophotrochozoan PCDHs and cadherins described in this text, including all members of the octopus cadherin superfamily, were identified according to methods previously detailed in Albertin et al. [3]. In short, we searched the octopus genome and transcriptome assemblies using BLASTP and TBLASTN with annotated sequences from human, mouse, and *D. melanogaster*. Genes identified in the octopus genome were confirmed and extended using the transcriptomes. Multiple gene models that matched the same transcript were combined. We used BLASTP and TBLASTX to search for cadherins and PCDHs in deposited genome and transcriptome databases of other lophotrochozoan species. Candidate PCDHs and cadherins were verified with BLAST, PFAM, and NCBI CDD analyses [59–61]. There currently exists no biochemical evidence that the octopus PCDHs bind calcium. Consequently, all putative cadherin domains mentioned in this paper were recognized through these computational methods. All octopus genome and transcriptome sequence reads are deposited in the SRA as BioProjects PRJNA270931 and PRJNA285380.

To study exon-to-domain correspondences of the octopus genes, we examined the intron-exon boundaries predicted in v2.1 of the gene annotation. The gene annotation was performed according to the methods described in Albertin et al. [3]. A browser of this genome assembly is publically available at http://octopus.metazome.net/. We confirmed the boundaries of transmembrane and cytoplasmic domains with PSIPRED [62].

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