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ORIGINAL ARTICLE

Analysis of the MCTP Amino Acid Sequence Reveals the Conservation of Putative Calcium- and Lipid-Binding Pockets Within the C2 Domains In Silico

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Abstract

MCTPs (Multiple C2 Domains and Transmembrane region Proteins) are evolutionarily and structurally related to other C2 proteins, which are central to exocytosis and membrane trafficking; however, their specific function has been little studied. MCTPs are associated with endosomes and the endoplasmic reticulum and possess three C2 domains (C2A-C2C) and two transmembrane regions (TMRs) well conserved in different species. Here, we generated structural models of the MCTP C2 domains of *C. elegans* and analyzed their putative function by docking, which revealed that these domains possess Ca^{2+}- and lipid-binding pockets, suggesting that MCTPs play a significant, calcium-dependent role in membrane physiology.

Keywords C2 domain · Calcium signaling · Docking · Membrane traffic

Introduction

Cellular signaling via proteins that possess calcium-binding domains is fundamental for multiple processes, such as endocytosis, exocytosis, vesicular trafficking, and phospholipase activities (Corbalan-Garcia and Gómez-Fernández 2014). The C2 domain is present in many proteins involved in neurotransmission, such as synaptotagmins (Bagur and Hajnóczky 2017; Evans et al. 2016; Nalefski and Falke 1996; Shin et al. 2005). This domain was initially described as the second of four calcium-binding domains of the calcium-dependent protein kinase C (PKC) (Nalefski and Falke 1996; Nishizuka 1988). Molecular and functional studies have elucidated the role of proteins that possess C2 domains in different signaling cascades; however, questions remain about their structure and role in cell physiology. C2 proteins involved in membrane trafficking, such as synaptotagmins and ferlins, contain a transmembrane region and at least two C2 domains whose affinity for Ca^{2+} increases upon binding phospholipids (Rizo and Sudhof 1998).

A novel family of C2 proteins known as MCTPs was discovered by in silico genomic sequence analyses (Shin et al. 2005). They contain three C2 domains (C2A, C2B, and C2C) oriented towards the cytoplasm: one to two transmembrane regions that anchor the protein to intracellular membranes and one short C-terminal sequence.

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Mammals have two MCTP coding genes (*MCTP1* and *MCTP2*), while invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have only one MCTP gene (Shin et al. 2005). The cellular distribution of MCTPs has been reported in endosomes and the endoplasmic reticulum, which enables them to sense changes in Ca^{2+} concentration (Shin et al. 2005; Genç et al. 2017; Espino-Saldaña et al. 2020) and promote lipid droplet biogenesis (Joshi et al. 2018, 2021). Assessment with selective antibodies for each isoform has demonstrated that MCTPs are widely expressed in different tissues, especially in striated muscle and heart (Qiu et al. 2015; Shin et al. 2005).

MCTPs seem to be implicated in heart and brain disorders. For example, a deletion of 2.2 Mb in the 15q26.2 chromosome, where the *MCTP*2 gene localizes, causes coarctation of the aorta and hypoplastic heart syndrome (Lalani et al. 2013). Furthermore, the importance of the *MCTP*2 gene for proper cardiogenesis in frogs was tested by knocking down its gene expression with morpholinos, which yielded embryos with a heart condition resembling coarctation of the aorta (Lalani et al. 2013). In other studies, single-nucleotide polymorphisms in the *MCTP2* gene were found to be associated with schizophrenia and bipolar disorders; however, their role in these psychiatric diseases remains elusive (Djurovic et al. 2009).

Studies of MCTP function have also been carried out in invertebrates. Only one gene has been identified in the genome of *D. melanogaster*, and a gene-trap study showed that it is expressed in the accessory cells of the olfactory organ. A P-transposon insertion at the 3'-end of the noncoding region of the MCTP gene is extremely lethal to the fly (Tunstall et al. 2012), while other allelic mutants of the MCTP gene are viable but defective in the presynaptic homeostatic plasticity in motor neurons (Genç et al. 2017). In *C. elegans*, *mctp-1* is regulated by two alternative promoters in the nervous system and spermatheca and gives rise to four alternatively spliced isoforms (Téllez-Arreola et al. 2020). In addition, *mctp-1* mutants exhibited impaired sensory function, locomotion, and hyposensitivity to an acetylcholinesterase blocker, suggesting that MCTP-1 is required for neurotransmitter release in the worm (Téllez-Arreola et al. 2020).

While it has been proposed that MCTPs can modulate the neuronal activity in invertebrates and that mutations in the *mctp* genes are involved in human disease, evidence of such roles has only recently emerged (Genç, et al. 2017, Téllez-Arreola et al. 2020, Liu et al. 2021). To shed some light on the molecular structure and phylogenetic distribution of this protein family, in this study, we present a phylogenetic analysis and a computational structural modeling and docking analysis of the C2 domains of the *C. elegans* MCTP-1 protein, in an effort to understand the structure of this emerging family of calcium sensors.

Materials and Methods

Mapping MCTP Relatives and Their Orthologs

All sequences for MCTP proteins were found using SMARTBLAST and Orthofinder (https://github.com/davidemms/OrthoFinder) (Emms and Kelly 2019; Schultz et al. 1998). To map the MCTP relatives, we employed the https://shoot.bio/ platform, and all domains in life databases were selected; then, after identifying the close relatives, we added two nodes. Finally, we used the *C. elegans* MCTP protein sequence (NP_491908.2) as a template in all cases.

Multiple Sequence Alignment and Phylogenetic Analysis

A circular phylogenetic tree connecting MCTPs with other C2 domain functional protein families was constructed using the https://shoot.bio/ platform, then imported into Newick format and parsed to Rstudio using the ggtree package (Yu 2020). The C2 domain phylogenetic tree was generated using the http://www.phylogeny.fr/index.cgi platform. "A la carte" option was used. "MUSCLE" for alignments, Gblocks default settings for curation, and maximum likelihood method with bootstrapping (100 replicates) were selected (Dereeper et al. 2008). Jalview was used to color sequence alignments derived from MUSCLE results (Waterhouse et al. 2009).

MCTP Computational Secondary Structure Prediction, Structural Modeling, and Docking Analysis

We used PSIPRED software for secondary structure prediction and RaptorX Structure Prediction software to model the tertiary structure of MCTP from *C. elegans* (**NP_491908.2**) using the default parameters of the server (Jones 1999; Källberg et al. 2012). The automatically generated PDB model was manually cropped in Molsoft ICM-Pro v3.8–3, keeping all the predicted domains in a standard configuration. Three Ca^{2+} ions were loaded from ChemSpider into the ICM project (CSID: 4,573,905) and fitted into the MCTP C2A domain, using the structure of the C2A domain of PKC as a comparative guide (PDB: **1DSY**, Verdaguer et al. 1999) and employing the Pocket Finder function of ICM (Dey and Chen 2011). The final model was submitted to the

Yasara energy minimization server (Krieger et al. 2009), and images were generated in PyMOL v2.2.2 Schrodinger LLC (Schrödinger, San Diego, CA).

Results

MCTPs Were Found in Metazoan But Not in Unicellular Eukaryotes

The emergence of MCTP proteins is poorly understood; thus, we first used the *C. elegans* (**NP_491908.2**) protein sequence as a query to search for putative MCTPs and analyzed their evolutionary relationship with other C2 domain proteins (Fig. 1). We found that MCTPs belong to a group of PKC-C2 domain families, the closest relatives of which are PCKs, Need4s, Ferlins, and Copines, all considered Ca^{2+} sensors (Zhang and Aravind 2010) (Fig. 1). We also found that MCTPs are widespread in metazoans, from cnidarians to humans. We did not detect MCTPs in prokaryotic organisms or unicellular eukaryotes. The dendrogram analysis shows the MCTP protein family's divergence into three major classes: MCTP1 and MCTP2 were present in chordates, whereas MCTPi was found only in invertebrates (Fig. 1; see representative species in Table 1). Taking advantage of the 2021 ensemble.org database, we searched for the gene tree of MCTPs. We found gene duplication events in vertebrates for both the MCTP1 and MCTP2 genes (Fig. S1A). MCTP1 is duplicated in perching birds and fish, whereas MCTP2 is duplicated only in fish (Fig. S1A-B). We did not find duplication events for MCTPi (Fig. S1C).

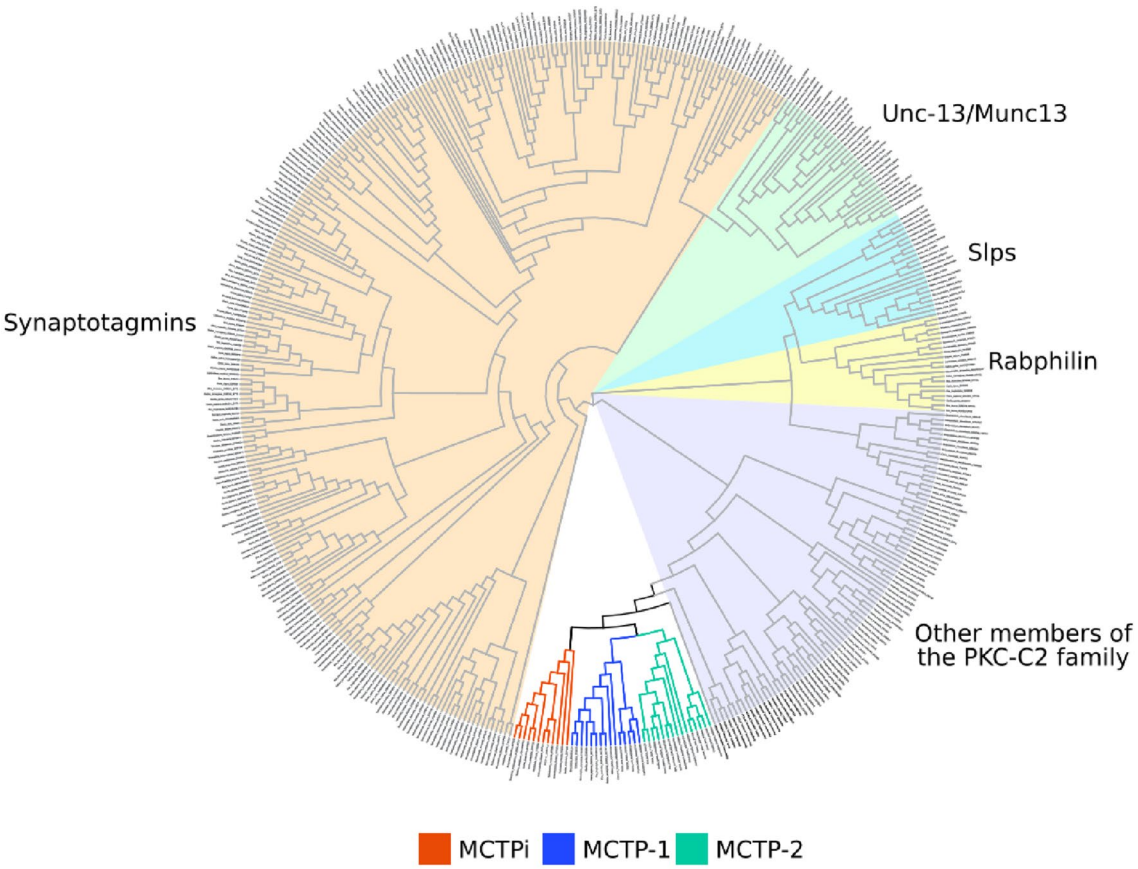


Fig. 1 Phylogenetic relationship between MCTPs and other C2 domain functional protein families. The platform https://shoot.bio/ was employed to identify MCTP orthologs and generate an unrooted Maximum likelihood tree. The orange group represents MCTPi, the blue group represents MCTP1, and the green group represents MCTP2. As stated earlier, MCTPi is specific to invertebrates, while MCTP1 and MCTP2 are found in vertebrates. Other members of the PCK-C2 family group are PKC, cPLA, RIM, Need4, Copine, Ras, Doc2, and Ferlins. Abbreviations: PKC-C2, Protein kinase C family; Slps, synaptotagmin-Like Protein; RIM, Rab3-interacting molecule; DOC2, double C2-like domain-containing protein; cPLA, cytosolic phospholipase; Need, neural precursor cell expressed developmentally down-regulated protein 4. UniProt reference proteomes 2020 and https://ensembl.org databases were used for the analysis. See supporting material for bootstrapping values. (Color figure online)

Table 1 Representative MCTPs orthologs found in https://shoot.bio/

Species	Common name	Uniprot orthologs ID
Thalassiosira pseudonana	Marine diatome	B8CEK8_Copine7
Caenorhabditis elegans	Round worm	E9P851_MCTP1
Nematostella vectensis	Sea anemone	A7RS41_MCTP1
Anopheles gambiae	Mosquito	Q5TX35_MCTP1
Drosophila melanogaster	Fruit fly	A1ZBD6_MCTP1
Helobdella robusta	Leech	T1G9G4_MCTP1
Branchiostoma floridae	Florida lancelet	C3YNP3_MCTP1
Tribolium castaneum	Red flour beetle	A0A139WFN8_MCTP1
Ixodes scapularis	Blacklegged ticks	B7PNA1_MCTP1, B7PV25_MCTP2
Bos taurus	Cattle	A0A3Q1LLI1_MCTP1 G3N1Z4_MCTP2
Canis lupus	Wolf	F1Q107_MCTP1, F1Q381_MCTP2
Monodelphis domestica	Gray short-tailed opossum	F7DQX_MCTP1, F7C9M4_MCTP2
Gorilla gorilla	Gorilla	G3RGR1_MCTP1 A0A2I2ZM06_MCTP2
Homo sapiens	Human	Q6DN14_MCTP1, Q6DN12_MCTP2
Pan troglodytes	Chimpanzee	A0A2I3SFC7_MCTP1 A0A2I3SYS5_MCTP2
Mus musculus	Mouse	E9PV86_MCTP1 Q5RJH2_MCTP2
Rattus norvegicus	Rat	D4ABL6_MCTP1 A0A096MKH3_MCTP2
Gallus gallus	Red junglefowl	A0A3Q2UI49_MCTP1 A0A1D5PZL7_MCTP2
Xenopus tropicalis	Western clawed frog	A0A6I8SNR9, A0A6I8RH94
Danio rerio	Zebra fish	A0A0R4IF57_MCTP1b A0A2R8RKL9_MCTP1a E7F9J5_MCTP2b F1R4M9_MCTP2a
Oryzias latipes	Japanese rice fish	H2L667_MCTP1b A0A3B3I194_MCTP1a A0A3B3I539_MCTP2b H2LWX8_MCTP2a
Lepisosteus oculatus	Spotted gar	W5M089_MCTP1 W5NAS4_MCTP2

Calcium-Binding Residues Are Conserved in C2A and C2C Domains But Not in C2B

The classical C2 domain calcium sensors contain five aspartic acid residues that bind Ca^{2+}. As previously described, MCTP proteins possess three cytosolic C2 domains in tandem (Fig. 2A). First, taking advantage of C2 domain similarity across species, we analyzed the amino acid sequences corresponding to individual C2 domains (C2A, C2B, and C2C) from the different classes of MCTPs from animal models to determine their degree of conservation, including the calcium-binding pocket (Fig. 2B). A comparison of the C2 domains revealed a high degree of conservation in the primary sequence: the C2C domain is the most conserved, followed by C2A and, finally, C2B. The calcium-binding sites are more conserved in C2A and C2C than C2B (Fig. 2B). C2C calcium-binding sites have not been previously reported in other proteins; thus, it may have a specialized function in MCTPs. Calcium-binding residues in the top loop region of C2 domains interconnect β-sheets, leading these domains to interact with Ca^{2+} and membranes. The aspartate residues of C2 domains in MCTPs may bind Ca^{2+} (Shin et al. 2005). We found that five of these residues are partially conserved in the three C2 domains of MCTPs. Aspartate residues 1 through 4 are conserved in the C2C domain, whereas the fifth presents the substitution D → E in invertebrates (Fig. 2B). C2B has several amino acid substitutions, including D → A, E, H, N, or T (Fig. 2B). Other MCTPi and MCTP2 C2Bs from *C. elegans*, *Haemonchus contortus, Xenopus laevis*, *Xenopus tropicalis*, and some isoforms from humans and mice contain only one or two aspartate residues, suggesting that these C2Bs may not bind Ca^{2+} (Fig. 2B). The position of amino acid residues predicted correctly that calcium-binding sites are well conserved in C2A and C2C in all MCTPs analyzed, but remarkably, such is not the case for C2B.

Sequence Analysis Reveals a Conserved Lipid-Binding Motif in MCTP C2A and C2B Domains

The lipid-binding selectivity of C2 domains is dependent on the electrostatic charges formed by the calcium-binding region (Corbalán-García et al. 2003). The ability to bind lipids independently of the calcium-binding sites has been described in C2 domains of PKCs (Zhang and Aravind 2010). Hence, we performed a systematic conservation sequence analysis of lipid-binding sites of MCTP C2 domains.

We found that the C2A and C2B domains of MCTPs possess a lysine-rich motif, termed the polybasic cluster,

between β-sheets three and four (Corbalan-Garcia and Gómez-Fernández 2014; Di Paolo and De Camilli 2006). This polybasic motif is formed by positively charged lysines (K, K, K) flanked by aromatic residues (Y, W) (Figs. 2B, 3) (Guerrero-Valero et al. 2009). The polybasic cluster can bind phosphatidylserine and phosphatidylinositol 4,5-bisphosphate (PIP_2) to calcium-binding domains, such as the C2B domain of synaptotagmin proteins (Honigmann et al. 2013). In MCTPs, the lysine-rich motif is found in the C2A and C2B domains. This creates room for a new working hypothesis that phospholipids may be necessary for binding Ca^{2+}, which contrasts with the initial observation that MCTP function is independent of lipid interactions (Shin et al. 2005). Altogether, we found at least two putative PIP_2-binding sites that resemble other C2 domains known to penetrate the plasma membrane; these PIP_2-binding sites coordinate with the aspartates in C2A, C2B, and C2C and may stabilize Ca^{2+} ions (Fig. 3).

MCTPs Have Two Putative Transmembrane Regions at the C-Terminus

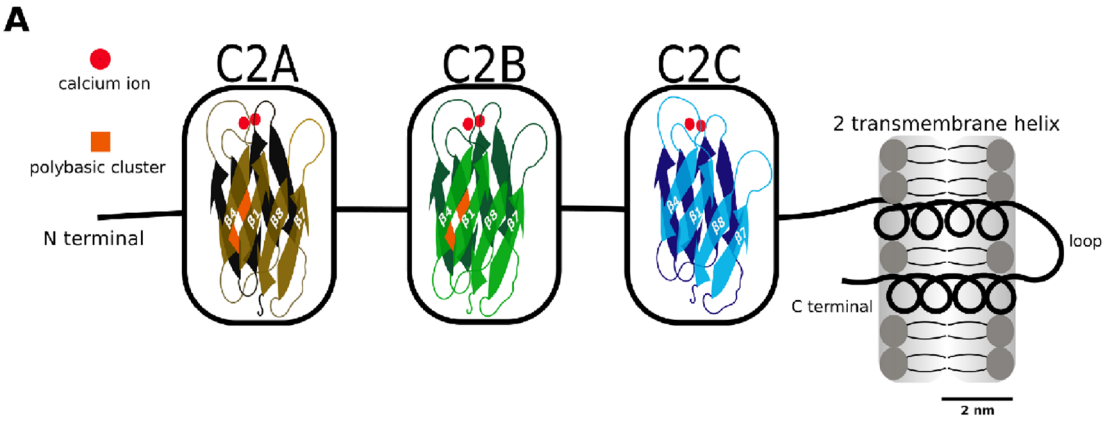
One criterion to establish whether a protein belongs to MCTPs is the presence of two stretches of hydrophobic residues that form the transmembrane regions (TMR), which allows the protein to anchor to cell vesicles (Shin et al. 2005). The presence of transmembrane regions in MCTPs is still debated. Some studies in mammals suggest that this is the only family of calcium sensors to contain two TMRs (Shin et al. 2005), while others report that the C-terminus of MCTP2 is a reticulon domain (ER-shaping RHE) (Joshi et al. 2018). To provide new insights, we analyzed the C-termini of MCTPs from different species. Based on Kyte and Doolittle (not shown) and secondary structure prediction analysis, we found that most MCTPs have two predicted segments, with the exception of the human protein MCTP2–2, which lacks the first TMR due to an alternative splicing event (Fig. 4). It is unknown whether a lack of one TMR misdirects the protein; a single TMR seems to suffice to anchor the protein to intracellular vesicles (Shin et al. 2005). The amino acid sequence of MCTP TMRs is not related to other known C2 proteins involved in membrane trafficking, such as synaptotagmins. In MCTPs, the second TMR is more conserved (70%) in different species, whereas the first TMR sequence is less conserved (10%). Our results are in line with the observation that MCTPs have two TMRs (Shin et al. 2005); however, we do not discard the possibility that the isoforms lacking the TMR1 might be functionally distinct.

The Polybasic Motif Is a Fingerprint Among MCTP C2 Domains

C2 domains are detectors which integrate responses in the presence of Ca^{2+} and lipids (Corbalan-Garcia and Gómez-Fernández 2014; Téllez-Arreola et al. 2020). A wide array of proteins has been identified to contain functional domains that bind Ca^{2+} and lipids; some participate in membrane trafficking, others in signal transduction (Corbalan-Garcia and Gómez-Fernández 2014; Min et al. 2007). However, the well-conserved amino acid sequences that bind Ca^{2+} and lipids seem to be crucial elements among MCTP C2 domains. Thus, we investigated whether these sequences are essential to define the C2 domain class. First, we determined whether the amino acid sequence of C2 domains of MCTPs varies among species. These domains span approximately 125 residues with predicted secondary structures suggesting they fold into eight β-strands. This is a characteristic of the C2 domain conformation Class 2 (type II topology) in which the N- and C-termini are near the bottom (Corbalan-Garcia and Gómez-Fernández 2014). Next, amino acid sequences of all three C2 domains from representative species of each clade were used to generate maximum likelihood trees. We observed two general clades: the first clade includes the C2C domain from all species (*C. elegans*, *H. contortus*, *C. intestinalis*, *X. laevis, X. tropicalis*, *D. rerio*, *M. musculus*, and *H. sapiens*). The second clade includes C2A and C2B, which suggests they may have similar functions as indicated by their primary amino acid sequence and computational analysis. It is worth noting that both C2As and C2Bs possess the lipid-binding residues included within the lysine-rich domain identified by our analysis; in sharp contrast, C2Cs possess calcium-binding sites instead of the lysine-rich cluster, a structural feature which suggests different Ca^{2+} sensitivities and membrane bridging (Fig. 5).

Discussion

In the present study, we provide new insights into the structure and molecular function of the MCTP proteins. MCTPs are composed of a variable N-terminal region, three C2 domains, one or two TMRs, and one short C-terminal sequence (Shin et al. 2005). We used this approach to determine peculiarities in the amino acid sequence of MCTPs to formulate a working hypothesis to understand their functional role. MCTPs belong to the PCK-C2 family of proteins; all members within this family are considered calcium sensors (Zhang and Aravind 2010). Calcium sensor proteins are central molecular components of cell biology; they appeared early in the evolution of eukaryotes, and there is evidence of their presence in more primitive placozoans (Barber et al. 2009; Lek et al. 2012; Washington and Ward 2006). Our


A
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C2A
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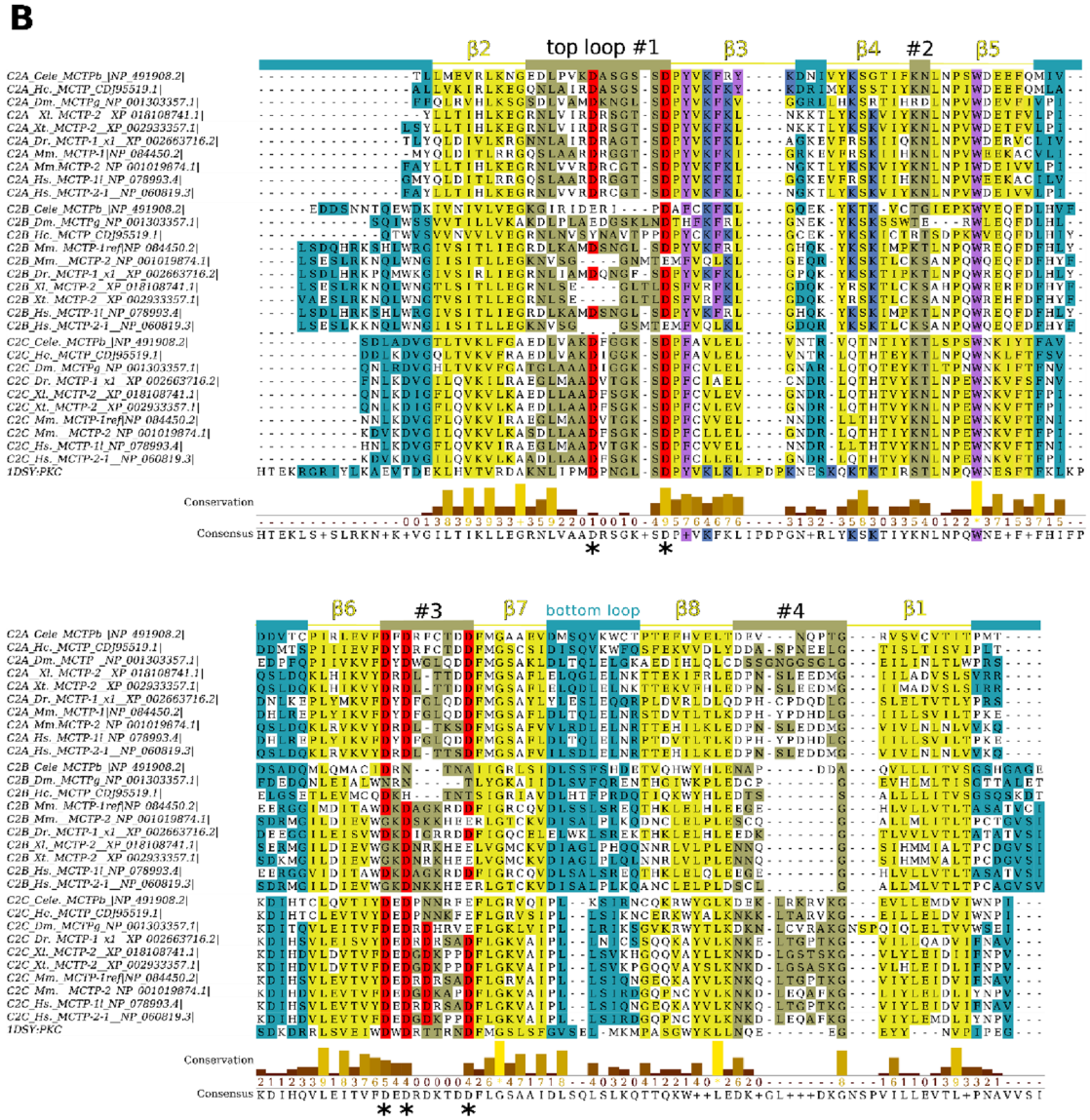
B
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Lysine-rich cluster YxKxn1KxK xn2W(Y/L/C)Xn3Asn

◂**Fig. 2** Multiple sequence alignment of MCTP C2 domains from different biological model organisms reveals the presence of calcium- and lipid-binding motifs. **A** General diagram of MCTPs using the consensus sequence generated from sequence alignment of the model species. Three C2 domains followed by two transmembrane propellers at the C-terminus anchor the protein to the membrane. The putative calcium-binding sites are located in C2A and C2C, and the lipid pocket (polybasic motif) is shown in C2A and C2B. **B** MCTP C2 domain multiple sequence alignment of model organisms. The alignment was colored in JAlVIEW. Secondary structure prediction was performed by PSIPRED. Abbreviations: *Cele., C. elegans; Dm, D. melanogaster; HC, Haemonchus contortus; Mm, Mus musculus; Dr, Danio rerio; Xl, Xenopus laevis; Xt, Xenopus tropicalis; Hs, Homo sapiens; PKCα, protein kinase C- alpha*

results indicate that MCTPs were not present in early eukaryotes distributed widely across this phylum; thus, it may be inferred that MCTPs emerged as an adaptation in metazoans. We identified three subgroups of MCTPs: MCTP1 and MCTP2 are present in vertebrates, whereas MCTPi is observed only in invertebrates. While MCTPs have been documented in plants, there are remarkable structural differences between MCTPs belonging to plants and animals, such as the number of C2 domains. In both cases, however, these MCTPs belong to the KG009 group according to the eggNOG database (see supporting material).

The number of C2 domains in a protein may indicate a wide range of functions, from vesicular transport to signal transduction. Proteins with multiple C2 domains in tandem act primarily as membrane trafficking effectors, while proteins with only one C2 domain are involved in signal transduction. Such is the case for synaptotagmins and PKCs, two of the most well-known proteins to contain C2 domains (Corbalan-Garcia and Gómez-Fernández 2014).

The sensitivity of C2 domains for binding Ca^{2+} and lipids and/or sensing protein interactions is influenced by several factors: (a) the orientation of the C2 domains, (b) the degree of conservation of residues involved in Ca^{2+} binding, and (c) the presence of a polybasic cluster. In some proteins, such as synaptotagmin 1, C2A and C2B bind two or three molecules of Ca^{2+} with low affinity, an interaction which is strengthened in the presence of phospholipids (Bai et al. 2002; Evans et al. 2016; Honigmann et al. 2013; Kojima 1995; Rizo and Sudhof 1998; Sutton et al. 1995). In other cases, such as with synaptotagmin 4, substitutions within the C2A calcium-binding sites impact calcium affinity, whereas

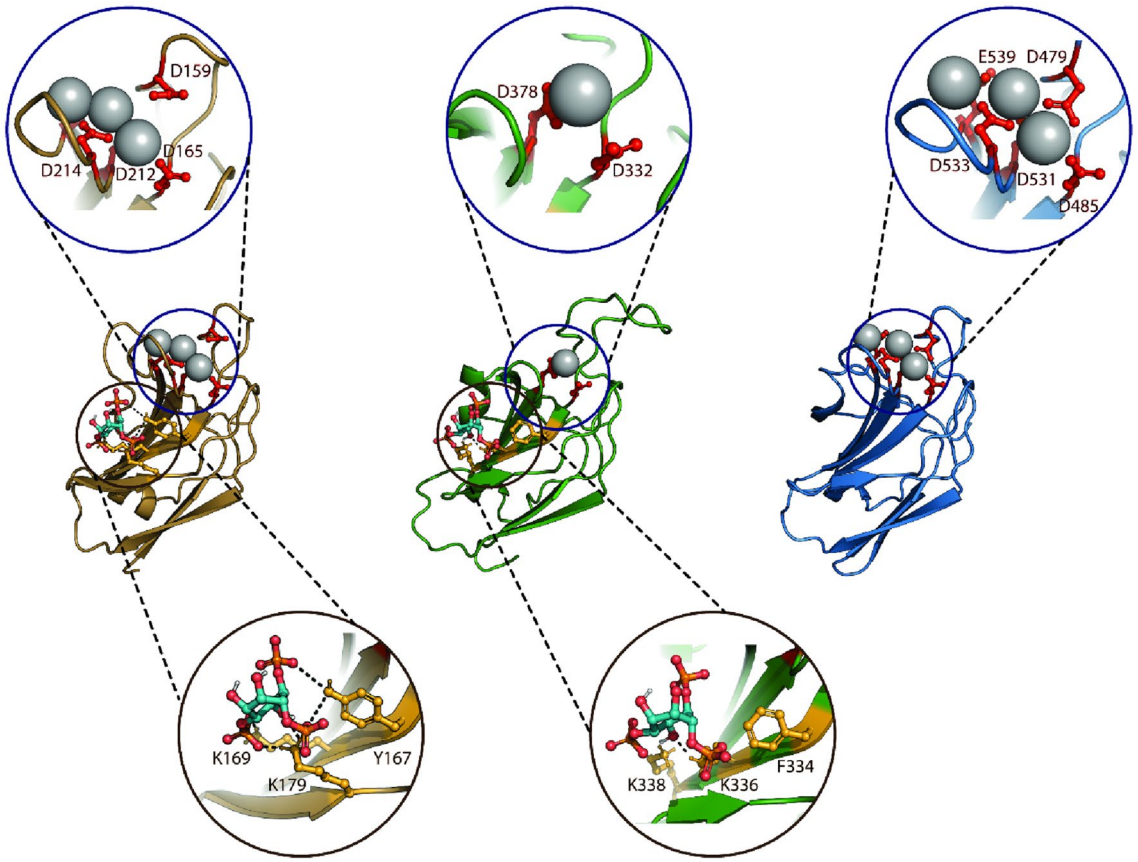

Fig. 3 Model for Ca^{2+} and PIP_2 docking of the MCTP C2 domains. Docking of three *C.elegans* MCTP1 C2 domains: C2A (brown), C2B (green), and C2C (blue) with Ca^{2+} and PIP_2. The closeup images at the top show the aspartate region (red lines) binding Ca^{2+} (gray spheres). The closeup images at the bottom show the predicted polybasic motifs (yellow) and their interaction with PIP_2 (red). Dashed lines represent hydrogen bridges formed by interactions with the phosphate groups of PIP_2 (Color figure online)

substitutions within the C2B domain affect its conformation such that the domain is unable to properly form calcium pockets. However, the latter is not the case for the homologous protein syt4 in *D. melanogaster*, whose function relies on binding calcium (Barber et al. 2009; Dai et al. 2004).

The diversity of C2 domains found in MCTPs suggests that they play a role in both calcium- and lipid-binding responses in different scenarios. The secondary structure of the three MCTP C2 domains is highly conserved in all selected organisms. Our results are consistent with those reported by Shin et al. (2005), who showed that several organisms lack the calcium-binding aspartates in the C2B domain. Interestingly, we also found that *C. elegans*, *H. contortus, X. laevis*, *X. tropicalis*, *M. musculus*, and *H. sapiens* lack calcium-binding sites in the C2B domain, suggesting that this domain does not bind Ca^{2+} in the species listed above. However, we do not discard the possibility of a lower binding affinity to Ca^{2+} as displayed by the C2A synaptotagmin 4 from rats, which despite lacking aspartate residues, demonstrates a lower affinity for Ca^{2+} (Barber et al. 2009; Dai et al. 2004). Another possibility is that the MCTP C2B domain resembles the RIM C2B domain, whose function relies significantly on PIP_2 binding and protein interactions in lieu of binding Ca^{2+} (Betz et al. 2001; de Jong et al. 2018; Guan et al. 2007; Hu et al. 2013; Wang et al. 1997). Our results indicate that the C2A and C2B domains of MCTP contain a well-conserved polybasic cluster, and that these motifs may function similarly to the synaptotagmin 1 C2 domains, which extend to the plasma membrane by interacting with PIP_2. This orientation increases the sensitivity of C2A to Ca^{2+} while facilitating the role of C2B in protein interactions located within PIP-enriched zones. We also do not discard other possible molecular mechanisms, such as one resembling the C2A and C2B C2 domains of Rabphilin, both of which exhibit Ca^{2+}-binding activity facilitated by the interaction of PIP_2; however, they interact differently with other phosphoinositides (Guillen et al. 2013).

Recently, it was discovered that MCTP2 binds in vitro to the charged lipids PI4P; PI4,5P2; PI3,4,5P3; and cardiolipin (Joshi et al. 2021), which supports our observations of lipid-binding in silico; however, the molecular affinity for lipids and responses of each MCTP C2 domain in different biological scenarios are still poorly understood, and further experiments are needed to determine their precise role. The presence of three C2 domains (A-C) opens the door to a variety of scenarios in which MCTP could play a role in membrane biology. For example, the C2C domain of MCTP is similar to the C2C domain in E-Syts and Munc13-1 in that it is an essential component of calcium-independent membrane tethering (Min et al. 2007; Quade et al. 2019). In E-Syts, C2C plays a critical role in ER-PM tethering in E-Syts, while in Munc13-1, it is essential for liposome fusion (Quade et al. 2019). Our findings indicate that the C2C domain of MCTP could be a singular case of calcium-binding pockets, since calcium-binding residues are highly conserved across diverse species. This suggests that the C2C domain is more sensitive to Ca^{2+} in the context of membrane binding. Even though we did not find a polybasic cluster within the structure of the C2C domain, we do not discard a

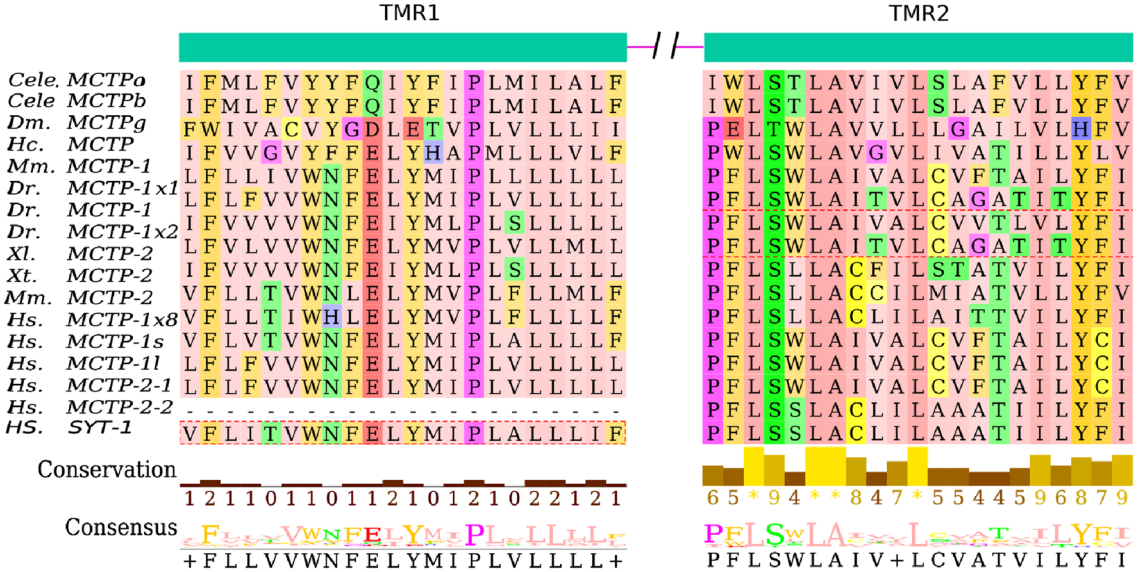

Fig. 4 Multiple sequence alignment of the putative transmembrane helix. Sequence alignment of putative transmembrane regions. Green rectangles at the top represent the transmembrane 1 and 2 helices. The degree of conservation of the sequence and the consensus of each segment are indicated. The secondary structure was modeled using PSIPRED. *Cele., C. elegans; Dm, D. melanogaster; HC, Haemonchus contortus; Mm, Mus musculus; Dr, Danio rerio; Xl, Xenopus laevis Xt, Xenopus tropicalis; Hs, Homo sapiens; SYT-1, synaptotagmin 1* (Color figure online)

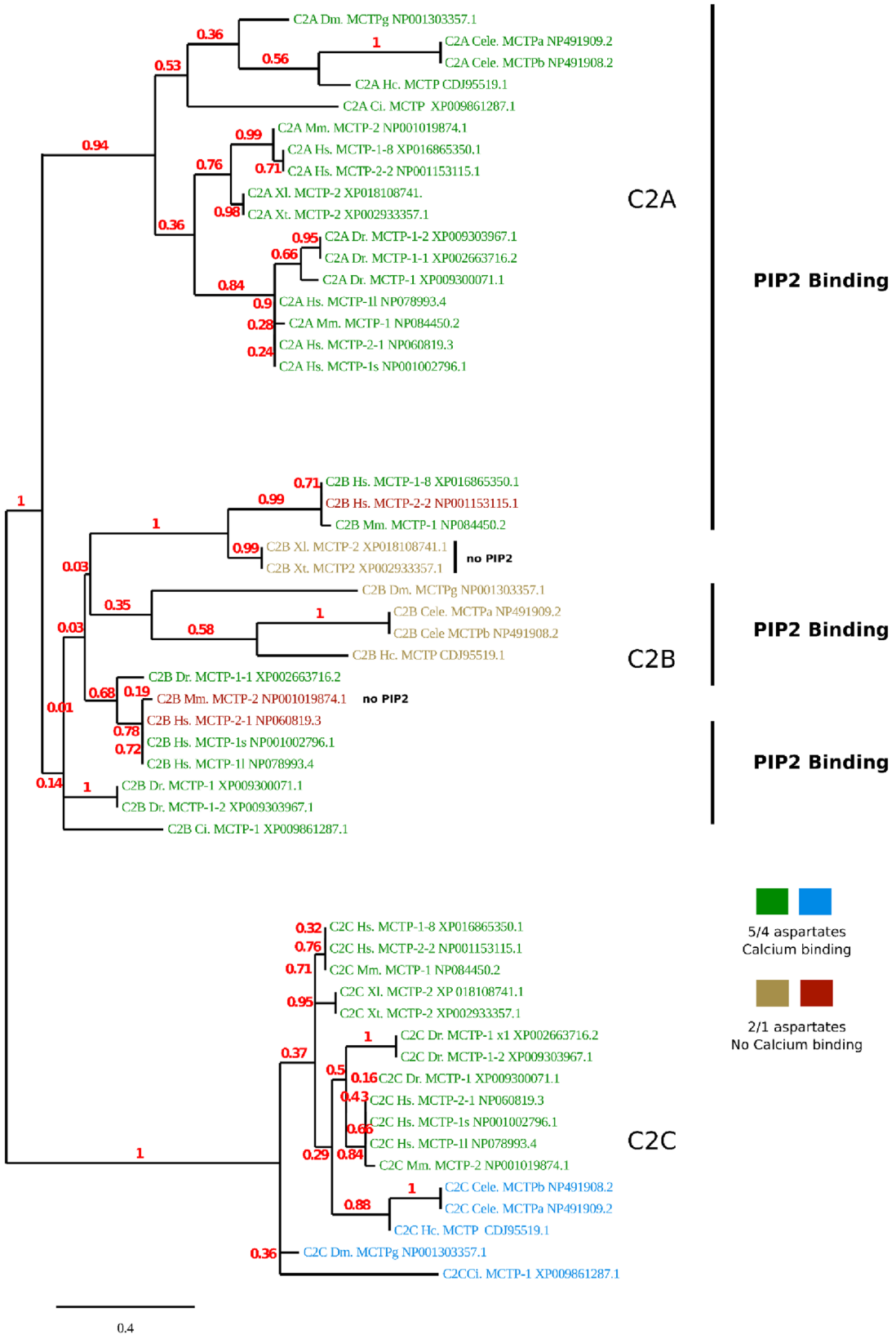

Fig. 5 Maximum likelihood tree of MCTP C2 domain sequences from different biological models. 100 replicates were used for bootstrapping. Bootstrapping values along branches are denoted in red; domains with putative calcium-binding abilities are colored in green and blue. The scale bar represents the tree branch length (Color figure online)

possible lipid association resembling that of the C2C domain in otoferlins (Padmanarayana et al. 2014).

E-Syts and ferlins are two other families of proteins which, like MCTPs, are calcium sensors and have been found to contain more than three C2 domains in tandem. Otoferlin plays an essential role in the exocytosis of neurotransmitters at the ribbon synapse, whereas E-syts function as tethers in ER-plasma membrane contact sites (Giordano et al. 2013; Herdman and Moss 2016; Saheki et al. 2016). In invertebrates such as *C. elegans* and *D. melanogaster*, it has been demonstrated that *mctp*-null mutants display defective neurotransmitter release (Genç et al. 2017; Téllez-Arreola et al. 2020), whereas in plants, MCTPs play a role as tethers in ER-PM contact sites (Brault et al. 2019). The functional differences between MCTP subgroups are not well defined, although they have similar calcium-binding properties and may play similar or complementary roles to isoforms with two TMRs. It is not known which differences are essential for distinguishing between various classes of MCTPs. The N terminus might be related to the specialized function of the C2 domains of each isoform, as is the case for ferlins and E-syts (Barber et al. 2009; Washington and Ward 2006). It is already known that MCTPs are calcium sensors which contain three C2 domains in tandem with two transmembrane regions. We propose additionally that MCTPs are evolutionarily conserved across multicellular organisms but not in prokaryotic or unicellular eukaryotic cells, and that MCTPs may be sensitive to calcium- and/or lipid-binding events due to the functional properties exhibited by their three C2 domains. For example, the MCTP C2C domain may bind Ca^{2+} and membrane lipids in response to changes in Ca^{2+} concentration at the synapse, thus conferring MCTP proteins a unique calcium- and lipid-binding property. Considering the wide variety of scenarios in which MCTPs can bind Ca^{2+}, lipids, or membranes, further experimentation is required to assess the selectivity of the C2 domains and to test whether MCTPs rely on specific membrane interactions to play essential roles in endocytic and exocytic pathways.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

References

Alwarawrah M, Wereszczynski J, Qiu X, Ge J, Gao Y, Teng M, Niu L (2017) Investigation of the effect of bilayer composition on PKCα-C2 domain docking using molecular dynamics simulations. J Phys Chem B 121:78–88

Barber CF, Jorquera RA, Melom JE, Littleton JT (2009) Postsynaptic regulation of synaptic plasticity by synaptotagmin 4 requires both C2 domains. J Cell Biol 187:295–310

Bagur R, Hajnóczky G (2017) Intracellular Ca2+ sensing: its role in calcium homeostasis and signaling. Mol Cell 66:780–788

Bai J, Wang P, Chapman ER (2002) C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I. Proc Natl Acad Sci U S A 99(3):1665–1670. https://doi.org/10.1073/pnas.032541099

Betz A, Thakur P, Junge HJ, Ashery U, Rhee JS, Scheuss V, Rosenmund C, Rettig J, Brose N (2001) Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. Neuron 30:183–196

Bolker JA (2014) Model species in evo-devo: a philosophical perspective. Evol Dev 16:49–56

Brault ML, Petit JD, Immel F, Nicolas WJ, Glavier M, Brocard L, Gaston A, Fouché M, Hawkins TJ, Crowet J, Grison MS, Germain V, Rocher M, Kraner M, Alva V, Claverol S, Paterlini A, Helariutta Y, Deleu M, Lins L, Tilsner J, Bayer EM (2019) Multiple C2 domains and transmembrane region proteins (MCTPs) tether membranes at plasmodesmata. EMBO Rep 20:1–26

Corbalan-Garcia S, Gómez-Fernández JC (2014) Signaling through C2 domains: more than one lipid target. Biochim Biophys Acta Biomembr 1838:1536–1547

Dai H, Shin O-H, Machius M, Tomchick DR, Südhof TC, Rizo J (2004) Structural basis for the evolutionary inactivation of Ca2+ binding to synaptotagmin 4. Nat Struct Mol Biol 11:844–849

de Jong APH, Roggero CM, Ho MR, Wong MY, Brautigam CA, Rizo J, Kaeser PS (2018) RIM C 2 B domains target presynaptic active zone functions to PIP 2 -containing membranes. Neuron 98:335-349.e7

Dereeper A.*, Guignon V.*, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36:W465–W469

Desper R, Gascuel O (2004) Theoretical foundation of the balanced minimum evolution method of phylogenetic inference and its relationship to weighted least-squares tree fitting. Mol Biol Evol 21:587–598

Deutekom ES, Snel B, van Dam TJP (2021) Benchmarking orthology methods using phylogenetic patterns defined at the base of Eukaryotes. Brief Bioinform. https://doi.org/10.1093/bib/bbaa206

Dey R, Chen L (2011) In search of allosteric modulators of a7-nAChR by solvent density guided virtual screening. J Biomol Struct Dyn 28:695–715

Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. Nature 443:651–657

Djurovic S, Le Hellard S, Kähler AK, Jönsson EG, Agartz I, Steen VM, Hall H, Wang AG, Rasmussen HB, Melle I, Werge T, Andreassen OA (2009) Association of MCTP2 gene variants with schizophrenia in three independent samples of Scandinavian origin (SCOPE). Psychiatry Res 168:256–258

Doncheva NT et al (2019) Cytoscape StringApp: network analysis and visualization of proteomics data. J Proteome Res 18(2):623–632

Emms DM, Kelly S (2019) OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol 20(1):238

Espino-Saldaña AE, Durán-Ríos K, Olivares-Hernandez E, Rodríguez-Ortiz R, Arellano-Carbajal F, Martínez-Torres A (2020) Temporal

and spatial expression of zebrafish mctp genes and evaluation of frameshift alleles of mctp2b. Gene 738:144371. https://doi.org/10.1016/j.gene.2020.144371

Evans CS, He Z, Bai H, Lou X, Jeggle P, Sutton RB, Edwardson JM, Chapman ER (2016) Functional analysis of the interface between the tandem C2 domains of synaptotagmin-1. Mol Biol Cell 27:979–989

Fernandez-Busnadiego R, Saheki Y, De Camilli P (2015) Three-dimensional architecture of extended synaptotagmin-mediated endoplasmic reticulum-plasma membrane contact sites. Proc Natl Acad Sci USA. https://doi.org/10.1073/pnas.1503191112

Genç Ö, Dickman DK, Ma W, Tong A, Fetter RD, Davis GW (2017) MCTP is an ER-resident calcium sensor that stabilizes synaptic transmission and homeostatic plasticity. Elife 6:1–23

Giordano F, Saheki Y, Idevall-hagren O, Colombo SF, Pirruccello M, Milosevic I, Gracheva EO, Bagriantsev SN, Borgese N (2013) ER-PM interactions mediated by the extended synaptotagmins. Cell 153:1494–1509

Grishin NV (1995) Estimation of the number of amino acid substitutions per site when the substitution rate varies among sites. J Mol Evol 41:675–679

Guan R, Dai H, Tomchick DR, Dulubova I, Machius M, Südhof TC, Rizo J (2007) Crystal structure of the RIM1α C 2 B domain at 1.7 Å resolution. Biochemistry 46:8988–8998

Guillen J, Ferrer-Orta C, Buxaderas M, Perez-Sanchez D, Guerrero-Valero M, Luengo-Gil G, Pous J, Guerra P, Gomez-Fernandez JC, Verdaguer N, Corbalan-Garcia S (2013) Structural insights into the Ca2+ and PI(4,5)P2 binding modes of the C2 domains of rabphilin 3A and synaptotagmin 1. Proc Natl Acad Sci 110:20503–20508

Hao P, Wang H, Ma L et al (2020) Genome-wide identification and characterization of multiple C2 domains and transmembrane region proteins in *Gossypium hirsutum*. BMC Genomics 21:445

Herdman C, Moss T (2016) Extended-synaptotagmins (E-Syts); the extended story. Pharmacol Res 107:48–56

Honigmann A, van den Bogaart G, Iraheta E, Risselada HJ, Milovanovic D, Mueller V, Müllar S, Diederichsen U, Fasshauer D, Grubmüller H, Hell SW, Eggeling C, Kühnel K, Jahn R (2013) Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. Nat Struct Mol Biol 20:679–686

Howe KL, Achuthan P, Allen J, Allen J, Alvarez-Jarreta J, Ridwan Amode M, Armean IM, Azov AG, Bennett R, Bhai J, Billis K, Boddu S, Charkhchi M, Cummins C, Da Rin Fioretto L, Davidson C, Dodiya K, El Houdaigui B, Fatima R, Gall A, Garcia Giron C, Grego T, Guijarro-Clarke C, Haggerty L, Hemrom A, Hourlier T, Izuogu OG, Juettemann T, Kaikala V, Kay M, Lavidas I, Le T, Lemos D, Gonzalez Martinez J, Carlos Marugán J, Maurel T, McMahon AC, Mohanan S, Moore B, Muffato M, Oheh DN, Paraschas D, Parker A, Parton A, Prosovetskaia I, Sakthivel MP, Abdul Salam AI, Schmitt BM, Schuilenburg H, Sheppard D, Steed E, Szpak M, Szuba M, Taylor K, Thormann A, Threadgold G, Walts B, Winterbottom A, Chakiachvili M, Chaubal A, DeSilva N, Flint B, Frankish A, Hunt SE, IIsley GR, Langridge N, Loveland JE, Martin FJ, Mudge JM, Morales J, Perry E, Ruffier M, Tate J, Thybert D, Trevanion SJ, Cunningham F, Yates AD, Zerbino DR, Flicek P (2021) Ensembl 2021. Nucleic Acids Res 49(1):884–891

Hu Q, Zeng M, Wang M, Huang X, Li J, Feng C, Xuan L, Liu L, Huang G (2021) Family-wide evaluation of multiple C2 domain and transmembrane region protein in *Gossypium hirsutum*. Front Plant Sci 1:2. https://doi.org/10.3389/fpls.2021.767667

Hu Z, Tong XJ, Kaplan JM (2013) UNC-13L, UNC-13S, and Tomosyn form a protein code for fast and slow neurotransmitter release in Caenorhabditis elegans. Elife 2013:1–20

Jones DT (1999) Protein secondary structure prediction based on position-specific scoring matrices. J Mol Biol 292:195–202

Joshi AS, Nebenfuehr B, Choudhary V, Satpute-Krishnan P, Levine TP, Golden A, Prinz WA (2018) Lipid droplet and peroxisome biogenesis occur at the same ER subdomains. Nat Commun 9:2940

Joshi AS, Ragusa JV, Prinz WA, Cohen S (2021) Multiple C2 domain-containing transmembrane proteins promote lipid droplet biogenesis and growth at specialized endoplasmic reticulum subdomains. Mol Biol Cell 32:1147–1157

Källberg M, Wang H, Wang S, Peng J, Wang Z, Lu H, Xu J (2012) Template-based protein structure modeling using the RaptorX web server. Nat Protoc 7:1511–1522

Kojima T (1995) Functional diversity of C2 domains of synaptotagmin family. J Biol Chem 270:26523–26527. https://doi.org/10.1074/jbc.270.44.26523

Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K (2009) Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: four approaches that performed well in CASP8. Proteins 77:114–122

Lalani SR, Ware SM, Wang X, Zapata G, Tian Q, Franco LM, Jiang Z, Bucasas K, Scott DA, Campeau PM, Hanchard N, Umaña L, Cast A, Patel A, Cheung SW, McBride KL, Bray M, Craig Chinault A, Boggs BA, Huang M, Baker MR, Hamilton S, Towbin J, Jefferies JL, Fernbach SD, Potocki L, Belmont JW (2013) MCTP2 is a dosage-sensitive gene required for cardiac outflow tract development. Hum Mol Genet 22:4339–4348

Lázaro-Guevara JM et al (2018) Gene's hubs in retinal diseases: a retinal disease network. Heliyon 4(10):e00867

Lek A, Evesson FJ, Sutton RB, North KN, Cooper ST (2012) Ferlins: regulators of vesicle fusion for auditory neurotransmission, receptor trafficking and membrane repair. Traffic 13:185–194

Lek A, Lek M, North KN, Cooper ST (2010) Phylogenetic analysis of ferlin genes reveals ancient eukaryotic origins. BMC Evol Biol 10:231

Liu L, Li C, Liang Z, Yu H (2017) Characterization of multiple C2 domain and transmembrane region proteins in arabidopsis. Plant Physiol 178:2119–2132

Liu Y-X, Liu L, Dong Y, Zhao M, Sheng Y, Fan L-L (2021) Novel heterozygous mutation of MCTP2 gene in a patient with coarctation of the aorta. QJM An Int J Med 115:157–159

Maeda I, Kohara Y, Yamamoto M, Sugimoto A (2001) Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. Curr Biol 11:171–176

Min SW, Chang WP, Sudhof TC (2007) E-Syts, a family of membranous Ca2+-sensor proteins with multiple C2 domains. Proc Natl Acad Sci USA 104:3823–3828

Nalefski EA, Falke JJ (1996) The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci 5:2375–2390

Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665

Padmanarayana M, Hams N, Speight LC, Petersson EJ, Mehl RA, Johnson CP (2014) Characterization of the lipid binding properties of otoferlin reveals specific interactions between PI(4,5)P2 and the C2C and C2F domains. Biochemistry 53:5023–5033

Papadopoulos JS, Agarwala R (2007) Sequence analysis COBALT: constraint-based alignment tool for multiple protein sequences. Bioinformatics 23:1073–1079

Qiu L, Yu H, Liang F (2015) Multiple C2 domains transmembrane protein 1 is expressed in CNS neurons and possibly regulates cellular vesicle retrieval and oxidative stress. J Neurochem 135:492–507

Quade B, Camacho M, Zhao X, Orlando M, Trimbuch T, Xu J, Li W, Nicastro D, Rosenmund C, Rizo J (2019) Membrane bridging by munc13-1 is crucial for neurotransmitter release. Elife 8:1–30

Rizo J, Sudhof TC (1998) C2-domains, structure and function of a universal Ca2-binding domain. J Biol Chem 273:15879–15882

Saheki Y, Bian X, Schauder CM, Sawaki Y, Surma MA, Klose C, Pincet F, Reinisch KM, De Camilli P (2016) Control of plasma

membrane lipid homeostasis by the extended synaptotagmins. Nat Cell Biol 18:504–515

Saheki Y, De Camilli P (2017) The extended-synaptotagmins. Biochim Biophys Acta Mol Cell Res 1864:1490–1493

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evo 4:406–425

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. Proc Natl Acad Sci 95:5857–5864

Shin OH, Hau W, Wang Y, Sudhof TC (2005) Evolutionarily conserved multiple C2 domain proteins with two transmembrane regions (MCTPs) and unusual Ca2+ binding properties. J Biol Chem 280:1641–1651

Sutton RB, Davletov BA, Berghuis AM, Thomas CS, Sprang SR (1995) Structure of the First C2 domain of synaptotagmin I: a novel Ca2 + / phospholipid-binding fold. Cell 80:929–938

Téllez-Arreola JL, Silva M, Martínez-Torres A (2020) MCTP-1 modulates neurotransmitter release in *C. elegans*. Mol Cell Neurosci 107:103528

Train CM, Glover NM, Gonnet GH, Altenhoff AM, Dessimoz C (2017) Orthologous Matrix (OMA) algorithm 2.0: more robust to asymmetric evolutionary rates and more scalable hierarchical orthologous group inference. Bioinformatics 33:i75–i82

Tunstall NE, Herr A, de Bruyne M, Warr CG (2012) A screen for genes expressed in the olfactory organs of Drosophila melanogaster identifies genes involved in olfactory behaviour. PLoS ONE 7:e35641

Verdaguer N, Corbalan-Garcia S, Ochoa WF, Fita I, Gómez-Fernández JC (1999) Ca(2+) bridges the C2 membrane-binding domain of protein kinase Calpha directly to phosphatidylserine. EMBO J 18:6329–6338

Voleti R, Tomchick DR, Südhof TC, Rizo J (2017) Exceptionally tight membrane-binding may explain the key role of the synaptotagmin-7 C_2A domain in asynchronous neurotransmitter release. Proc Natl Acad Sci USA 114(40):E8518–E8527

Wang Y, Okamoto M, Schmitz F, Hofmann K, Südhof TC (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature 388:593–598

Washington NL, Ward S (2006) FER-1 regulates Ca2+ -mediated membrane fusion during *C. elegans* spermatogenesis. J Cell Sci 119:2552–2562

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview version 2-A multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191

Yu G (2020) Using ggtree to visualize data on tree-like structures. Curr Protoc Bioinform 69(1):e96

Zhang D, Aravind L (2010) Identification of novel families and classification of the C2 domain superfamily elucidate the origin and evolution of membrane targeting activities in eukaryotes. Gene 469:18–30